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DOTTORATO IN MANAGEMENT & INFORMATION TECHNOLOGY



CURRICULUM: INFORMATION SECURITY & INNOVATION SYSTEMS

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Ciclo XVII N.S.

Novel tools for reproducible
Next Generation Sequencing data analysis and integration

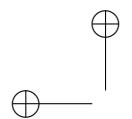
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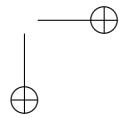
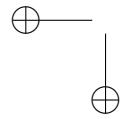
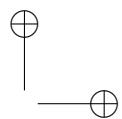
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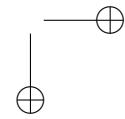
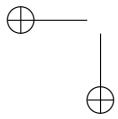
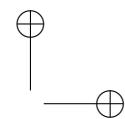
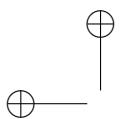
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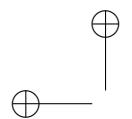


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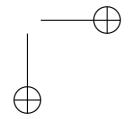
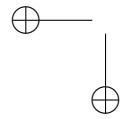
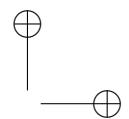
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Chapter **1**

Introduction

1.1 Biological Background

1.2 Sequencing Techniques

1.2.1 RNA-Seq

1.2.2 Atac-Seq

1.3 Computational Aspects

Chapter 2

Time Course RNA-Seq analyzer *ticorser*

This chapter illustrates the features of Time Course RNA-Seq data Analyzer (*ticorser*), a tool developed for analyzing RNA-Seq time course data.

2.1 Introduction

ticorser is an R package for complete and fast analysis of this data type, it helps the analysis of time course RNA-Seq data, offering a vast amount of hypothesis tests, setup for differential expression between two or more conditions. With the help of edgeR, DESeq2 and nextMASigPro the tool offers the possibility to setup 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, starting from the counting 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 also

graphics as KEGG-maps and heatmaps.

2.1.1 Time Course RNA-Seq

2.2 Methods

2.2.1 Time Course Methods

ticorser is a tool totally devoted to the Time Course (TC) RNA-Seq data offering features to inspect data, to normalize them, to capture differential expression of genes at static time point and overall time points, supporting different experimental designs.

Moreover, it’s possible to compare the results of different analysis and to investigate the most influenced biological functions (i.e. Gene Ontology terms and Pathways).

Overall, *ticorser* offers gives the possibility to analyze data using different R packages, to compare the results in order to choose the best combination of tools for the user specific problem. Therefore, *ticorser* offers a vast amount of exploratory and diagnostic interactive plots to explore data not just at pre-processing but also during the post-processing phase.

ticorser automatically implements a set of Reproducible Research functionalities to trace all the analysis steps selected by the user, generating a final report with both executed analysis code chunks and their produced results. Furthermore, *ticorser* has been provided also of a caching system providing, for each analysis step, a caching database file within all the input and output processed data, useful, not only to speed up computations, but also to share data and results through the Internet.

gives the possibility to analyse time course RNA-Seq data starting from *BAM* files. It enables RNA expression quantification with `featureCounts` method producing a count matrix useful for Differentially Expressed Genes (DEGs) detection.

We gave particular attention to the normalization phase, giving the possibility not only to use several traditional normalizations methods, but also the

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possibility to remove batch effect.

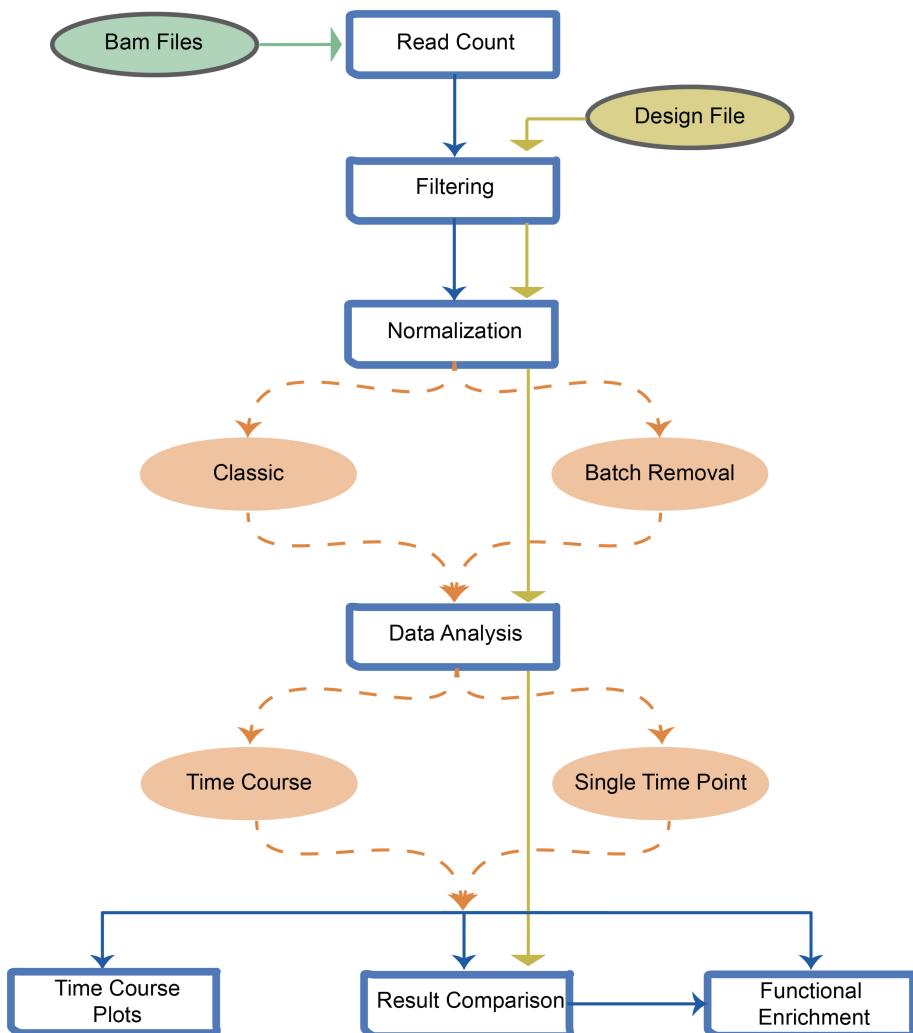


Figure 2.2.1: Main flow of ticorser R package.

ticorser offers four different ways for analyzing time course RNA-Seq data.

Moreover, it offers three different ways for analyzing different biological conditions in a single time point.

2.2.2 Case Study

2.2.3 Conclusions and Future Works

Chapter 3

Differential Enriched Scan 2 DEScan2

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

Some studies [1, 2] demonstrated the importance of genomewide chromatin accessibility of a broad spectrum of chromatin phenomena activation using sequencing techniques as *Atac-Seq*, *Sono-Seq*, etc. Even if there are some methods for the analysis of these omic data types, there still is lack of them, in particular for an emerging omic as *Atac-Seq*.

To address this lack, 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 the RNA-Seq, need the comparison of two or more different biological conditions. Starting from a set of already published [1] scripts, we designed Differential Enriched Scan 2 (DEScan2), a software for helping 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 of them.

3.1 Introduction

The DEScan2 is an R [3] 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 [4] data structures and methods, and it is available through Bioconductor repository since version 3.7.

The tool is organized in 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 on 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's scores and one on the number of samples.

The third step produces a counts 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 so produced counts matrix, as illustrated in the figure 3.2.1, is useful both for doing differential enrichment between multiple conditions and for integrating the epigenomic data with other -omic data types.

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 3.2.4.

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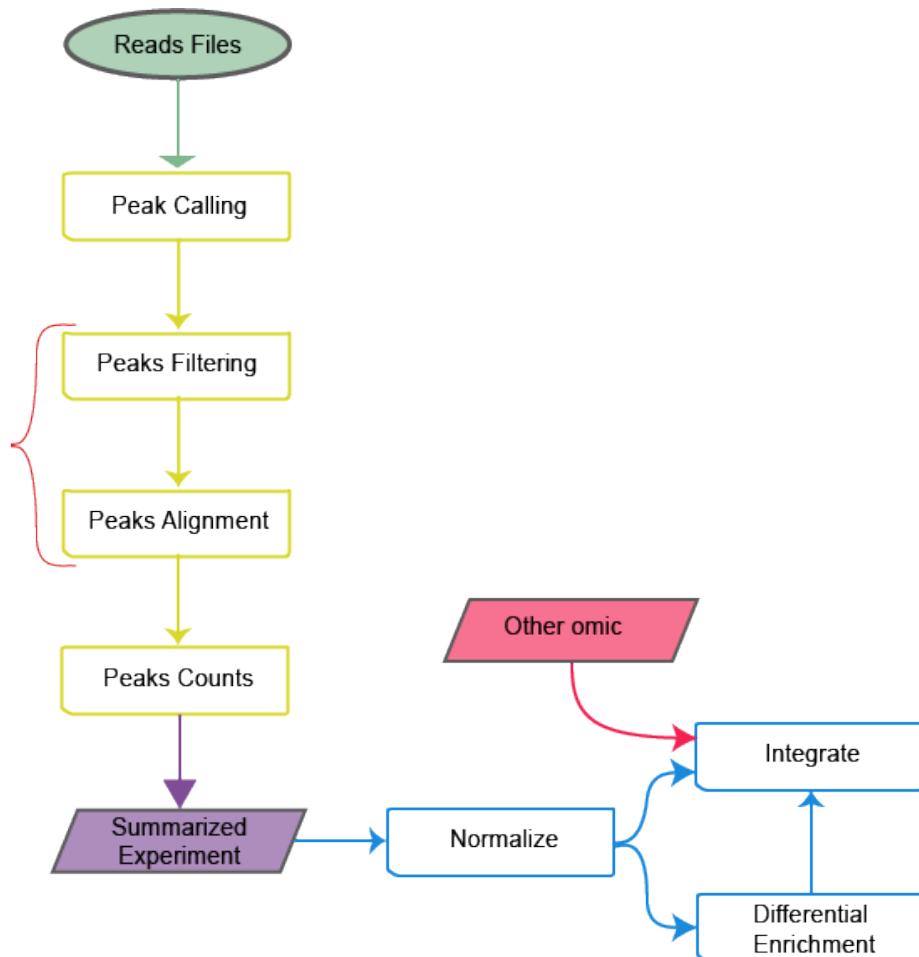


Figure 3.2.1: A differential enrichment flow representation. DEScan2 steps are highlighted in yellow.

3.2.1 Peak Caller

The Peak Caller (defined by the `findPeaks` function) takes as input a set of alignment files (BAM [5] or BED format) with the code identifier of the refer-

3. DIFFERENTIAL ENRICHED SCAN 2

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DESCAN2

ence 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 `GenomicRangesList` [6], 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 as bins of `binSize` parameter length (the default value is 50bp) and then computes the reads coverage on the bins with moving scan windows, spanning from `minWin` to `maxWin` parameters of `binSize` interval.

In order to be able to catch small and spread 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 coverages 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 coverages 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 λ is the Poisson parameter to derive with a MLE, described as the estimator:

$$\lambda_n = \frac{1}{n} \sum_{i=1}^n x_i$$

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

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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 the samples, using two different thresholds. One on the peaks’s 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 [7], as described in section 3.2.4.

Firstly, using the threshold on the peaks’s 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 *ChIPpeakAnno* [8] 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. On our experience, we suggest to set the samples threshold as a mutiple 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 the counts, where the rows and the columns, respectively, represent the regions and the samples.

In order to keep trace of all information associated to the regions, it produces a *SummarizedExperiment* [9] data structure, giving the possibility to retrieve the *GenomicRanges* of associated peaks and the count matrix, respectively, using the `rowData` 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 omic data types, 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 peaks, 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 over the others is `fromSamplesToChrsGRangesList`, which gives the possibility to split a *GenomicRangesList* by the chromosomes. This procedure could be useful for parallelizing the computations on the chromosomes, when common operations on them, between multiple samples, are needed. Assigning a single chromosome to a single computing unit. Taken

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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

Atac-Seq is an emerging evolved technique which enables to investigate the open chromatin regions at whole genome level. It has been demonstrated the adequacy of this technology in the regulation of mouse brain activity under different conditions.

To illustrate the performances of DEScan2 we chose a dataset [10] that describes *in vivo* adult mouse dentate granule neurons before and after synchronous neuronal activation using Atac-Seq and RNA-Seq technologies (see sections 1.2.2 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.

3. DIFFERENTIAL ENRICHED SCAN 2

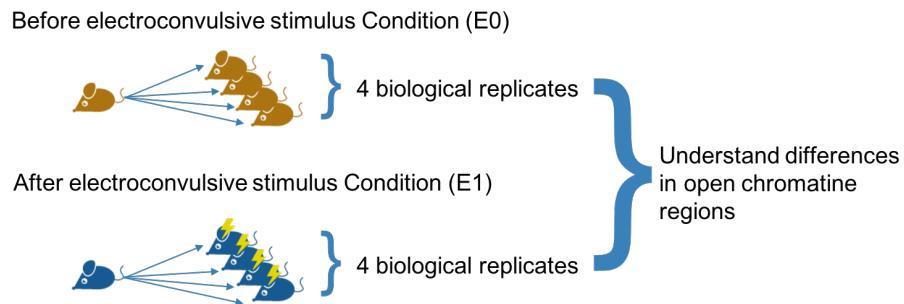
DESCAN2

Figure 3.3.1: An illustration of our extraction of the GSE82015[10] dataset.

We downloaded the data from Gene Expression Omnibus (GEO) database [11, 12] with accession number GSE82015¹ and mapped raw data using *STAR* [13] with default parameter on *Mus Musculus* Genome ver.10 (mm10).

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 such a 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* on the same samples, and (as shown in figure 3.3.2) looking to the numbers DEScan2 always outperforms *MACS2* peaks.

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

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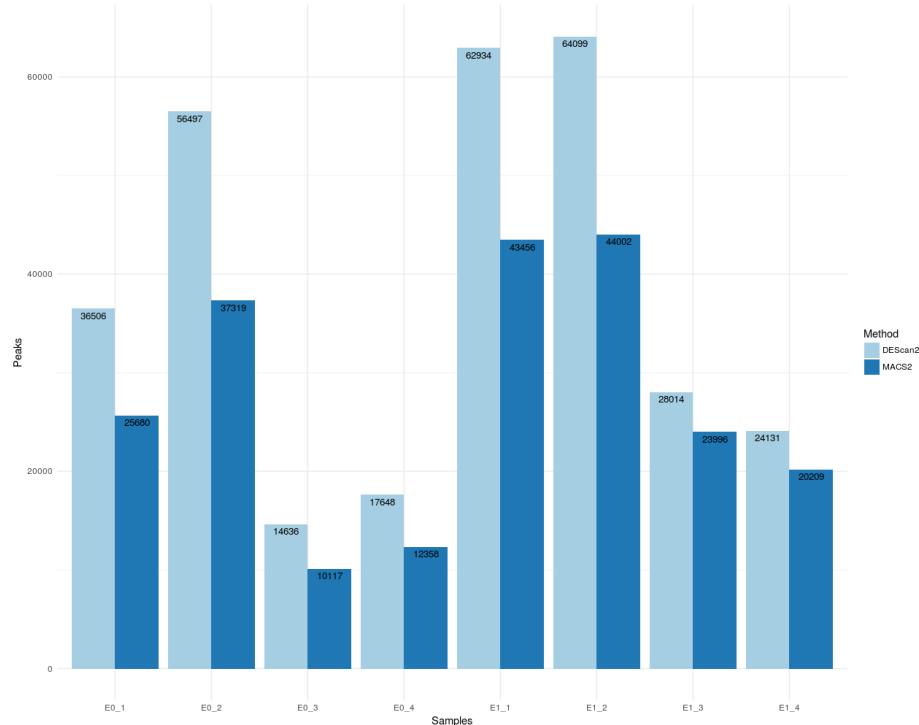


Figure 3.3.2: A comparison of DEScan2 and MACS2 detected peaks for each sample in the dataset.

To be more robust, we compared DEScan2 detected peaks with the same validated regions (*Arc*² and *Gabrr1*³) of the original work [10]. 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 careful in the smaller peaks detection.

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

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

3. DIFFERENTIAL ENRICHED SCAN 2

DESCAN2

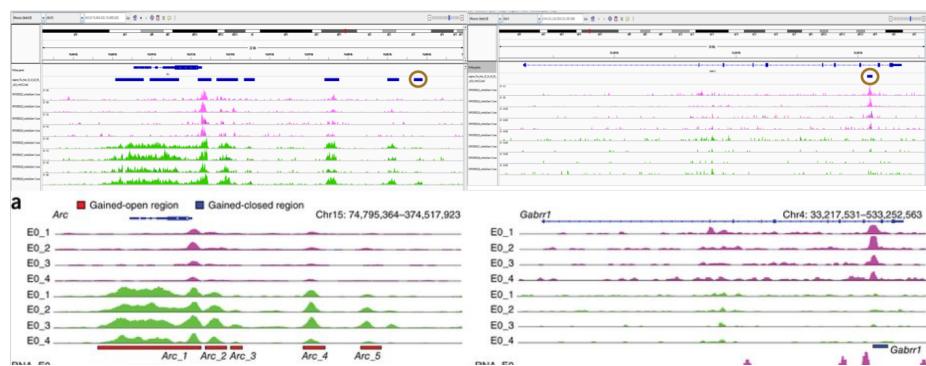


Figure 3.3.3: A comparison of DEScan2 detected peaks with validated peaks in article [10].

While it is very important to detect good peaks with a peak caller, it seems to be more relevant to detect reliable 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 higher are the thresholds on the scores and the number of replicates, lower is the number of the detected peaks. Highlighting a proportional inversion between the number of the peaks and the combination of the number of samples and the detected regions score.

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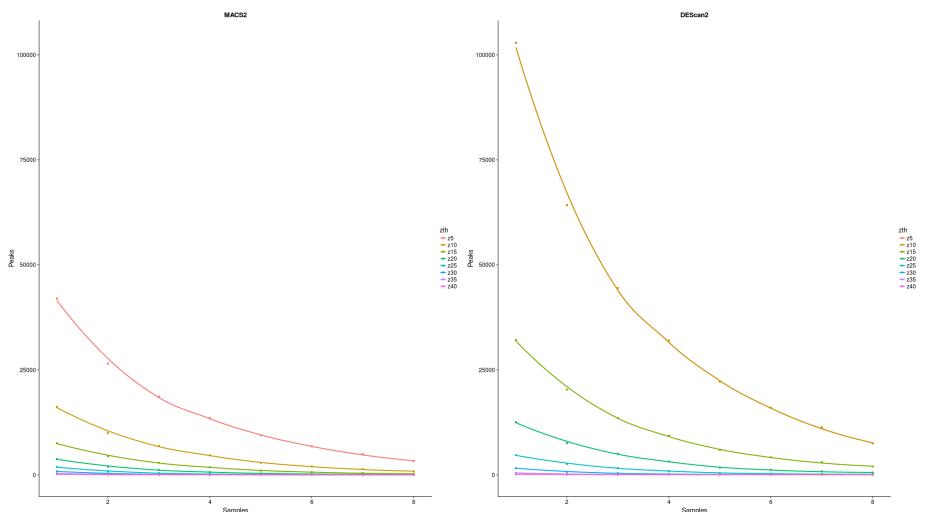


Figure 3.3.4: Filtering the detected regions with different thresholds on peak scores between *MACS2* and *DEScan2*.

Moreover, comparing left and right panels, we notice the high difference in pooling the samples-peaks together with the *DEScan2* filtering/aligning step when using the *MACS2* and the *DEScan2* peaks. Using the *MACS2* peaks the pooling highly reduce the number of detected peaks, even using a low threshold 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.

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 kind of omics, as RNA-Seq.

3. DIFFERENTIAL ENRICHED SCAN 2

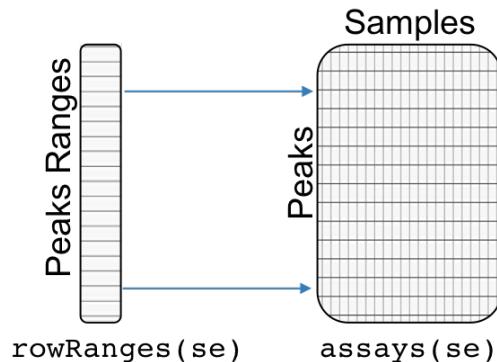
DESCAN2

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.

Before to proceed to detect DERs, it is a good standard to normalize the data, also because without any kind of normalization we are not able to detect any DER. The nature of the data, in count format, makes it possible to apply several well known RNA-Seq normalizations techniques, such as *TMM*, *upper-quartile*, *full-quartile*, *RUV-Seq*, etc [14–16]. In this case, we fixed the peaks's score threshold to 10, in order to have as much signal as possible.

While the *TMM* and *upper-quartile* normalizations modify the data in a way that makes it impossible to detect DERs, other kind of normalizations and combinantions of them give good results.

The figure 3.3.6 sintetizes this concept very well, highlighting a relation between the number of DERs (y-axis) and the minumum number of samples (x-axis) used for filtering the data during the DEScan2 filtering step.

To better compare the normalization effect, we created a *null dataset* of 8 samples, simply doubling the E0 samples and differentially express the signal between E0 (4 samples) and E0-fake (4 samples).

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The right panel of the plot, representing the "full dataset", shows that *upper-quartile*, even if combined with *RUV-Seq* normalization, is not able to linearly detect a good amount of DERs, while *full-quartile*, when combined with *RUV-Seq* seems to affect the data in a way that overdetect the number of DERs. When looking at the *full-quartile* and *RUV-Seq* by themself seem to perform better than the other normalizations. The first one has a downhill almost linear, while the second one has a very fast downhill with a regrowth when the number of samples is higher.

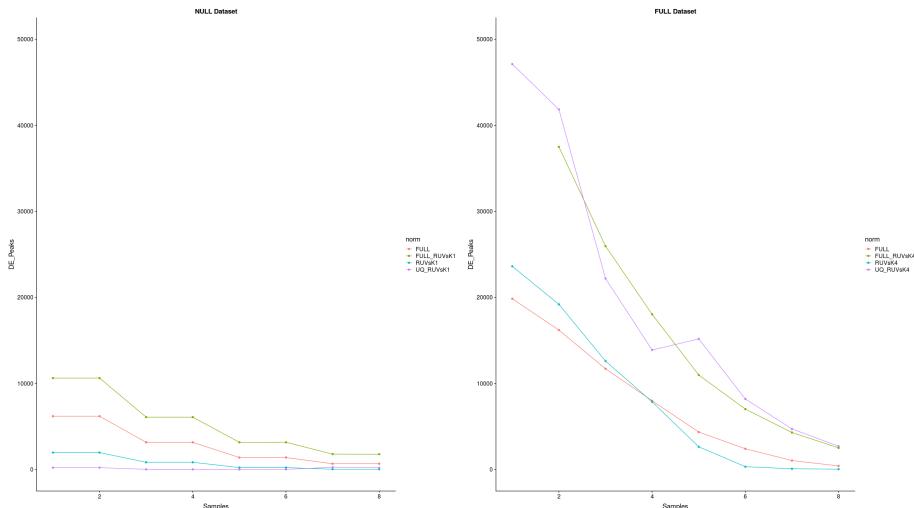


Figure 3.3.6: The figure shows the effects of different normalizations on the epigenomic differentially enriched regions.

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

The left panel represent the "null dataset" highlighting that portion of DERs due to randomness/bias. Indeed, any kind of normalization produces almost the same trend, underlying that *full quartile*, even if combined with *RUV-Seq* still not reduces the bias. While *upper quartile* preserves oscillations when using

7/8 samples. The one which seems to well interpret the data, producing a good compromise between bias and signal, is RUV-Seq. Indeed, it preserves a gradually downhill of the DER without totally flatten the signal.

A note to be accounted is that in the case of the "null dataset" we had to set the RUV-Seq k parameter to 1, otherwhise we were no able to obtain any result.

To estimate the DERs, any of the RNA-Seq methods can be applied, such as *DESeq2*, *edgeR*, *NOISEq*, etc [17–19].

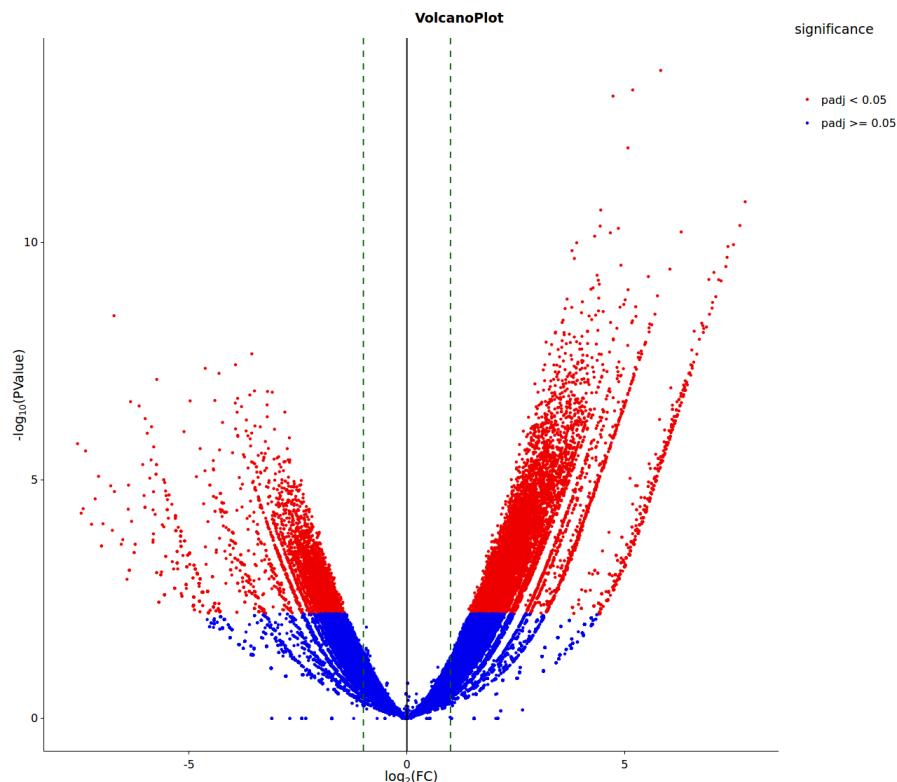


Figure 3.3.7: A volcano plot of Differential Enriched Regions. Blue dots represent the not significant DERs, while the red ones represent the significant DERs.

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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 RUV-Seq normalized counts with k parameter set to 4, we modeled the experimental design with the `model.matrix` function, adding to our model not only the experimental conditions, but also the RUV-Seq estimated weights. Then we used the resulted design to estimate the dispersion and fit a Quasi-Likelihood test, as defined in *edgeR*[17].

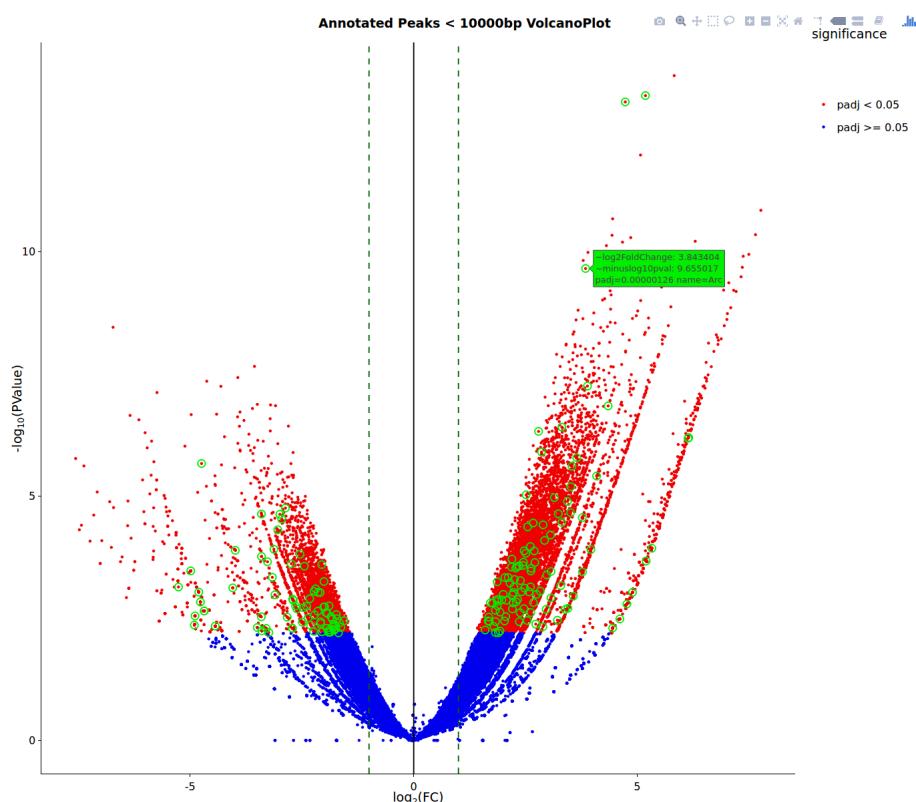


Figure 3.3.8: A volcano plot of DERs. Blue dots represent the not significant DERs, while the red ones represent the significant DERs. Green circles highlights the peaks with a DEG annotated.

The figure 3.3.7 shows a volcano plot of DERs between E0 and E1 conditions. Red dots highlights the regions with a False Discovery Rate (FDR)[20] lower than 0.05, while blue dots highlight not significant regions.

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 firstly quantified the signal with `featureCounts` methods available in the *Rsubread* [21] R/Bioconductor package. Then we filtered lowly expressed genes with the *proportion* test as implemented in *NOISEq* package, and applied the `noisep` 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 *ChIPpeakAnno*. Figure 3.3.8 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 we used the annotated genes to do functional annotation on Gene Ontology (GO) [22, 23] and Reactome pathways, which showed several interesting results for the neuronal regulation.

Insert tables for the functional results, to discuss with Davide/Lucia

3.4 Conclusions and Future Works

In the lack of methodologies for open chromatin region detection and 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, and, because it's newly born, aims to be more powerful and attractive in this field.

We demonstrated to be able to catch not only spread signal, but also small re-

3.4. CONCLUSIONS AND FUTURE WORKS

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gions across the samples. And with our filtering/aligning step we demonstrated to be able to keep relevant signl producing data structures as *SummarizedExperiment* which are candidates to become standards in the biological data analysis. With our 3-steps analysis we puts 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.

In the next future we plan to check if other distributions, as *Negative Binomial*, fits better this data kind and to improve our filtering/aligning step with additional probabilistic methodology.

3. DIFFERENTIAL ENRICHED SCAN 2

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DESCAN2

Chapter **4**

IntegrHO - Integration of High-Throughput Omics data

4.1 Introduction

4.2 Methods

4.2.1 Single Omic Approach

4.2.2 Multi Omic Approach

Low Level Itegration

High Level Itegration

4.3 Implementation Aspects

4.4 Reproducible Computational Research

4.5 Results

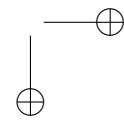
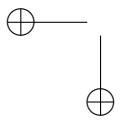
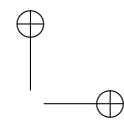
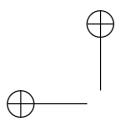
Chapter 5

Conclusions & Future Works

Chapter 6

Reproducible Computational Research

Appendices



.1 R Language

.2 R Markdown Language

Chapter 7

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Acronyms

DEG Differentially Expressed Gene. 27, 29

DER Differentially Enriched genomic Region. 24–29

DEScan2 Differential Enriched Scan 2. 15–17, 20–26, 45

FDR False Discovery Rate. 27

GEO Gene Expression Omnibus. 21

MLE Maximum Likelihood Extimator. 18

mm10 *Mus Musculus* Genome ver.10. 22

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