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RNA-Ribo Explorer: interactive mining and visualisation of Ribosome profiling data.

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Abstract

Context Long, translation was thought as a stable and uniform process by which a ribosome produces a protein encoded by the main Open Reading Frame (ORF) of an mRNA. Recently, growing evidence support uncomplete correlation between RNA and protein levels, the existence of alternative ORFs in numerous mammalian RNAs, and ribosomes' implication in gene expression regulation, thereby challenging previous views of translation. Ribosome profiling (aka Ribo-seq) has revolutionized translation study by enabling the mapping of translating ribosomes on the whole transcriptome using deep-sequencing.

Motivation Despite increasing use of Ribo-seq, recent review articles prompt that flexible, interactive tools for mining such data are missing. As Ribo-seq protocols still evolve, flexibility is highly desirable for the end-user.

Result: Here we describe RNA-Ribo-Explorer (RRE) a stand-alone tool that fills this gap. With RRE, one can explore read-count profiles of the transcriptome obtained after mapping, compare them in many conditions, and visualize the profiles of individual RNAs. Importantly, one can interrogate datasets using queries combining several numerical criteria to detect interesting subsets of RNAs. This feature seems useful for finding candidate RNAs whose translation status or processing has changed across conditions.

Availability RRE is written in Java and can be used on Linux, Mac or Windows platforms; it comes with a manual and is freely available at <http://atgc-montpellier.fr/rre>.

1 Introduction

Translation regulation refers to alterations of ribosome behavior that influence the process of translation and thus impacts the final protein level of a gene. Translation is an intricate process performed by ribosomal complexes. In eukaryotes, the ribosome scans the RNA from the cap, finds a start codon which triggers translation until the end of the Open Reading Frame (ORF) [15]. This scanning model has been challenged by the existence of ORFs located upstream the main ORF, i.e. between the cap and the main ORF start codon. Now thousands of such alternative ORFs located in UTRs have been found in mammalian transcriptomes, and mutations that create or alter such ORFs were proven control the protein synthesis of the main ORF [15]. The well studied case of ATF4 mRNA in Human shows that several upstream ORFs are exquisitely combined to achieve a precise regulation of that gene [17]. Mutations in untranslated region can create, shorten or lengthen upstream ORFs and induce dysregulation of the main ORF, which causes a pathological disorder like in for instance hereditary melanoma [6]. Actually, when in response to a stress, a cell turns down translation of most genes, but increases the peptide synthesis of a subset of "response" genes, it often controls the latter by bypassing upstream ORF [16]. All these phenomenons emphasizes the importance of translational regulation of gene expression. However, the mechanisms by which allow this control require further investigations. The advent of *Ribosome profiling* or *Ribo-seq* has opened new avenues in this direction. A fine understanding of translational regulation may also help improving gene expression for applications in synthetic biology [9].

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Ribo-seq

RNA-seq allows to explore the transcriptome – both qualitatively and quantitatively – and to uncover its astonishing variability, while quantitative proteomics can provide estimates of protein abundance at large scale. However, it is still challenging to predict protein abundance from transcript quantities, for the intermediate step of translation participates in the regulation gene expression more deeply previously thought. Ribosome profiling, also termed Ribosome sequencing or Ribo-seq, is a recent Next Generation Sequencing (NGS) assay that enables us to interrogate the translation process in a transcriptome wide manner. Quoting Kuersten et al., with Ribo-seq "translation gets its omics moment" [5]. Ribo-seq allows to map the positions of translating ribosomes on the entire transcriptomes. Current protocols follow multiple steps, among which are i/ a cell treatment with a drug that inhibits either elongating or initiating ribosomes, ii/ application of nuclease to digest RNA portions not covered by ribosomes and capture of Ribosome Protected Fragments (RPF), iii/ fragment selection on size, rRNA depletion, conversion into DNA, library preparation and deep sequencing [4].

Despite the facts that Ribo-seq remains somehow a challenging technique and suffers from some biases, it has established itself as main molecular assay to study translation at large scale and is increasingly used to answer a variety of questions. Notable aspects of translation studied until now include i/ the quantification of translational control, ii/ the identification of alternative Open Reading Frames (short ORFs, upstream ORF located in 5'UTR, etc) in peptidomics or functional genomics for instance, iii/ the dynamics and mechanics of translation [2]. Research based on Ribo-seq have for instance uncover that translation is more pervasive in mammals than previously thought [4]. Existing Ribo-seq datasets show that Ribo-seq profiles considerably differ from RNA-seq profiles because first in Ribo-seq read accumulation strongly depends on the kinetic of translation, and depth of sequencing is generally higher in RNA-seq. Moreover, Ribo-seq reads are short (usually between 25 and 30 nucleotides), exhibit a trinucleotidic periodicity related to the coding frame, and require filtering of reads coming from ribosomal RNAs. Due to those differences and specificities, most analyses of Ribo-seq data are not performed using software made for RNA-seq.

Tools for Ribo-seq analysis.

Logically, a wide range of computational methods are being developed for analyzing ribosome profiling datasets:

- to preprocess or denoise Ribo-seq reads,
- to collate or view footprint profiles for known RNAs [10], to normalize the read counts [11], or to estimate translation rate with respect to transcript abundance
- to postprocess Ribo-seq profiles to predict distinct features like alternative ORFs [1], ribosome stalling events, or estimate Codon Usage Bias [13].

A nice survey on methods for predicting ORFs was published in October 2017 [2], while a more comprehensive overview of computational resources for all kinds of analyses appeared in January 2019 [18], witnessing a booming interest in ribosome profiling. Available computational methods for Ribo-seq data often concentrate on one precise aspect (like removing of contaminant reads [8], or estimation of the position offset [3]), are implemented in different frameworks (R package, standalone programs, or Galaxy tools), which does not facilitate their combination and inter-operability. Noteworthy, both of the above-mentioned review articles underline the lack of tool for mining Ribo-seq data. As the protocols of Ribo-seq assays are still evolving, their yield, reproducibility, and biases hinder the establishment of standard in computational analysis of Ribo-seq.

We therefore reason that it is crucial for the end user to be able to visualize and mine its Ribo-seq datasets in a simple, flexible and interactive way. Currently, a tool for this task is missing, despite the availability of dedicated read processing pipelines or of visualization solutions based on genome viewers [2],[18]. Hence, we propose RNA-Ribo Explorer, a stand alone software for visualization and mining of Ribo-seq datasets on the whole transcriptome.

2 Material

To demonstrate the functionalities of RNA-Ribo Explorer, we use two publicly available Ribo-seq datasets: 1) one compares human tumoral vs normal cells from a fresh kidney carcinoma [7], 2) the other contrasts human embryonic kidney (HEK) cell lines expressing DDX3 mutant or not in normal and arsenite-induced stress conditions [12]. For simplicity, we will refer to the former as the *carcinoma* dataset, and to the later as the *HEK* dataset. Both data comes from Gene Expression Omnibus repository with accession number GSE70804 and GSE59821, respectively.

3 Method

Overview

RRE is an interactive and flexible tool that enables the user to explore and analyze ribosome profiling data in a dynamic, visual, and easy manner. RRE is intended for the biologist for it allows her/him to design queries exactly matching his/her needs, to obtain the query results as selection or plots, and to examine graphically the results for any desired mRNA. The user can also set key parameters interactively, which will refine the results on-the-fly, giving her/him the possibility to test hypothesis dynamically. Furthermore, RRE does not require know-how in computational biology, nor complex installation procedure, and should be as platform independent as the Java programming language is. RRE provides

- specific features required for analyzing Ribo-seq,
- interactive visualization of Ribo-seq profile for any mRNA,
- data mining capabilities which the user perform by running three major query types.

RRE enables interactive data mining of RNA-seq and Ribo-seq, once the raw reads have been processed using a map-and-count pipeline. RRE takes as input specific files containing read counts, which can be generated from a BAM file using our script (Figure~1).

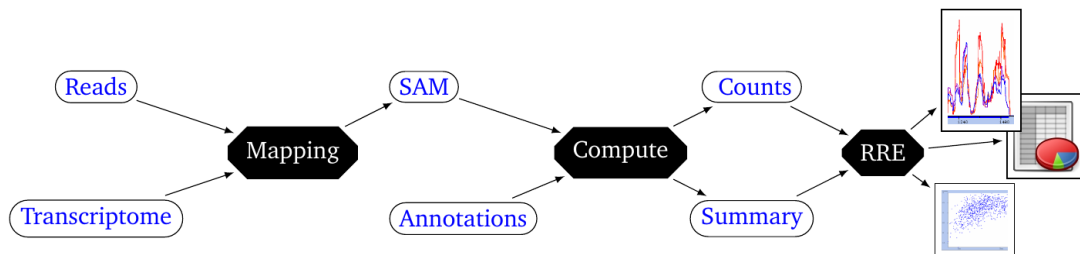


Figure 1: Logical read processing pipeline. RRE is used for interactive data mining in downstream analyses by the end-user.

Ribo-seq specific features.

In Ribo-seq, reads represent RPFs whose expected length is 25-32 nucleotides. As a RPF denotes the presence of a ribosome interacting with an mRNA, the distribution of reads mapped on a transcript should 1/ accumulate at the start codons, 2/ exhibit a trinucleotidic periodicity. RRE provides functionalities to perform classical control quality on each Ribo-seq dataset. The user can plot the distribution of mapped read lengths to check whether they respect the expected distribution, and view the aggregative coverage over all mRNAs (anchored on the start codon) to verify the existence of the start codon peak, as well as the trinucleotidic periodicity (Figure~2). To derive the position of the ribosome P-site (and hence the decoded codon) from the mapping position of a read, one needs to add an offset, which is often set to 12 nucleotides in most studies. However, this offset may depend on the data set, on the protocol and especially of the ribonuclease used. Using the aggregative coverage over all mRNAs for a given dataset, RRE will propose a suitable offset to the user and

redraw the coverage plot taking into account the chosen offset value. The user can try several values and validates the most appropriate one, checking whether or not it improves e.g. the trinucleotide periodicity. Note that such features can each be performed using some existing packages or software, while RRE integrates these functionalities in a single tool, which is more convenient for many end users.

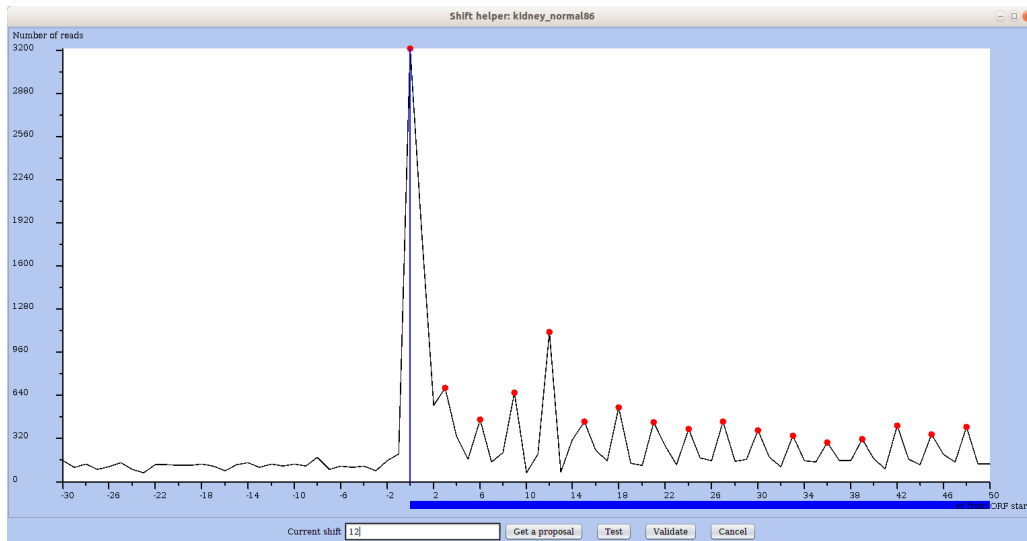


Figure 2: Agregative coverage plot over all mRNAs showing the relative read mapping positions with respect to the start of the annotated main ORF. In the footer, the user can ask RRE to propose an offset/shift value specifically for this dataset or he can provide a value. The offset value is used to determine the P-site from the mapping position of a read (see User's manual).

Interactive visualization of an mRNA profile

Figure~3 shows the profile window for mRNA named P4HB using four selected conditions. The plot displays as many profiles for this mRNA as selected conditions and indicates the reference CDS as a blue bar (below the plot), the 5' and 3' UTR being to the left and right of it. Once the user has selected one mRNA (using a search query or the result of a mining query), RRE let him on-the-fly act on plot parameters, select or deselect conditions, select the range of read lengths considered in the plot, and view the mRNA's rank in the distribution of coverages across all mRNAs. With such a multi-profile plot, the eye easily detects whether peaks and valleys have conserved positions across conditions (as in Figure~3) or not (as in Figure~7). RRE offers the possibility of zooming and moving along the sequence position (with the control panel in lower left corner of the window). Additional biological information can be displayed on demand with a rolling menu. Finally, the user can save the graph as an image (in PNG format) or write down the data of the plot to a tabular file for downstream analyses with R or any script language.

Data mining queries

RRE provides three major types of comparative queries to identify translational changes between conditions.

1. select subsets of mRNAs according to numerical criteria,
2. detect changes in profile comparison at mRNA level for all mRNAs,
3. find mRNAs with coverage changes in user-defined sub-regions.

Let us illustrate each type by giving one example of biological query.

- Select mRNAs with differential profile within UTRs across conditions (Type 1 query) Here, the user wants to automatically select mRNAs whose ref. CDS is translated in normal conditions, while the UTRs become covered RPF in other conditions. Within a dedicated window, the user enters the desired

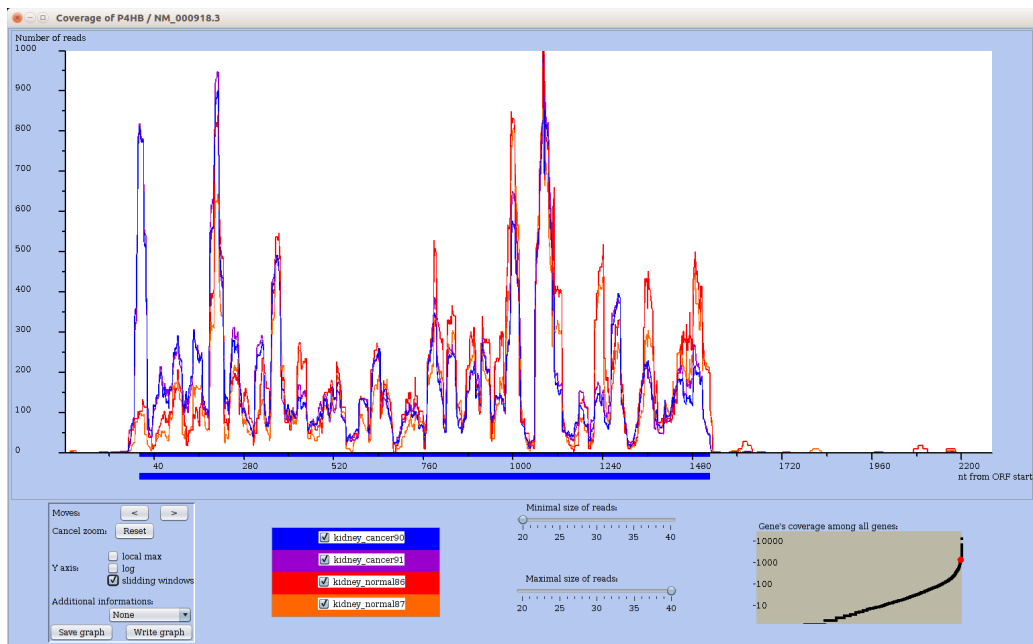


Figure 3: Ribo-seq profiles of a chosen mRNA for multiple conditions. The coding region of the main ORF is materialized by an horizontal blue bar below the profile along the X-axis. The user can interactively change which datasets are plotted, as well as the range of considered read sizes. The plot is updated on-the-fly. Further biological information or annotation, such as measures of codon usage bias, can be displayed on the graph, and the entire graph can be saved as an image file (see User's manual).

conditions that are combined with logical operations, meanwhile RRE determines the selected subset on the fly (see User's manual for a view of the query interface). In our case, the query combines two conditions: (i) a high coverage in the ORF region across all normal datasets (200 reads / along ORF), (ii) a medium coverage on the 5'UTR region in the cancer samples (100 reads in 5' UTR). RRE yields the list of eight selected mRNAs in table and displays the count for each region in each conditions. The table can be exported for further processing with e.g. any statistical analysis software like R or any spreadsheet. Clicking on an mRNA name, the user inspects the profiles in the desired condition for that gene.

- Find mRNAs with distinct profiles in normal vs tumoral conditions (Type 2 query) The user wants to compare for each mRNA its change in profile across two conditions. In the kidney datasets, the query contrasts a normal vs tumoral conditions. For each mRNA it computes using sliding windows a vector that summarizes the mRNA profile for each condition, and calculates the vector correlation[1]. To discard mRNAs having a coverage too low to get a meaningful correlation, the user can subselect genes with a minimal coverage. RRE displays in the correlation plot, the correlation of each mRNA (on y-axis) by a dot in function of its mean of its total coverage in both conditions). Each dot is clickable to provide the detail on the corresponding mRNA and whose nouns can be added on demand. In Figure~4, most mRNAs get a high correlation, meaning profiles that change mostly in the intensity of the peaks. However, some cases like ATP1B1 exhibits a low correlation suggesting that their profile should differ between the two conditions. Figure~5 shows ATP1B1 profile with 5'UTR and CDS start (up to ~180 bp) regions almost free from reads in normal condition, but highly covered in tumoral condition. Note that all selected genes exhibit changes in profile across conditions, but not necessarily the same changes.
- Detect mRNAs with extended potential translation in their 3' UTR only under stress (Type 3 query). In a type 1 query, the user can select genes by setting conditions on the profile of annotated regions (UTR, CDS, ORF). However, because annotations may be uncertain or valid in some cell types only, RRE allows a more flexible inspection on subregions. The user can precisely defines subregions to compare. Here, the query contrasts the profiles of the last 200 nucleotides of CDS vs that of the first 200 nucleotides. of the 3' UTR. RRE plots for each mRNA the subregion count ratios in the two conditions. In Figure~6, a majority of dots lie above the diagonal meaning that the ratio has increased under stress: for those mRNAs the

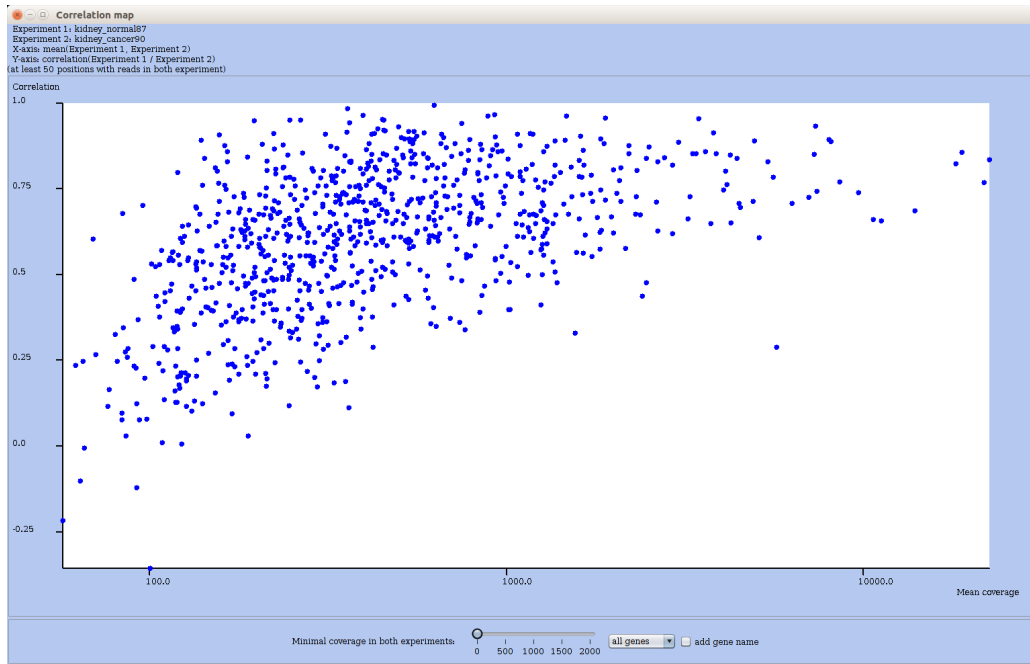


Figure 4: Correlation plot of Ribo-seq profiles of all mRNAs when comparing normal vs tumoral kidney cells. RRE plots for each mRNA the correlation of coverages in both conditions (Y-axis) in function of the mean coverage. Each RNA is represented by a dot, and with a click on that dot, one can get the profile coverage for that mRNA in another window. To ease inspection, the user can further refine the set of plotted RNAs by adjusting the coverage threshold: the plot is then updated immediately.

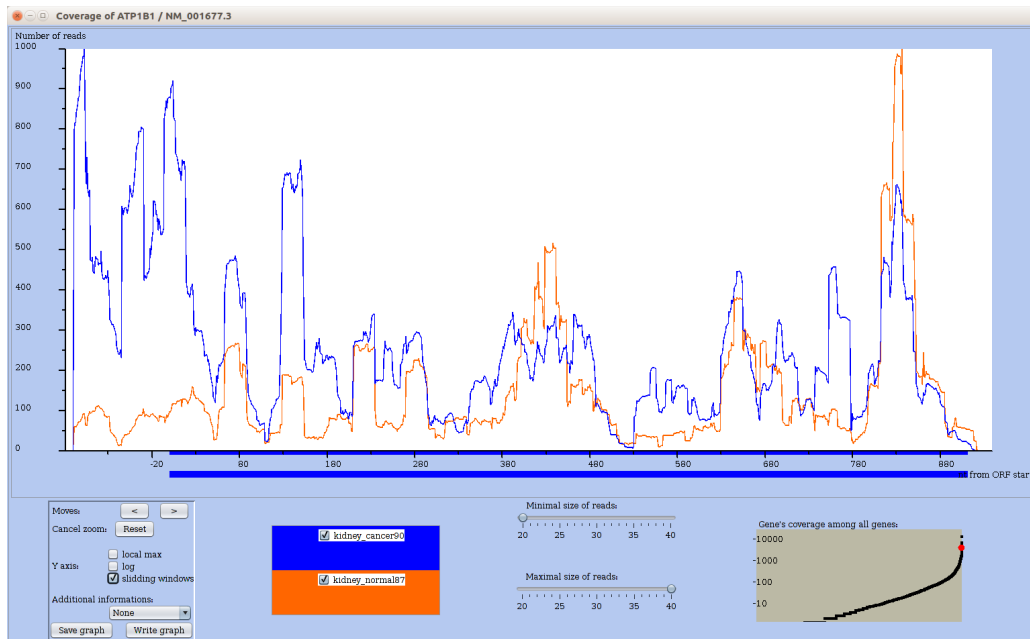


Figure 5: Profile of mRNA ATP1B1, which corresponds to a dot with low correlation between conditions in Figure~4. Sliding window option is on for a better view of the peaks. The normal and cancer conditions differ by the coverage in the 5'UTR of ATP1B1.

relative coverage of 3' UTR / CDS is higher when the cells are treated with arsenite. Viewing the profile plot for COX6B1 (Figure~7) shows an example with large peaks within the 3' UTR only in the arsenite condition. All mRNAs whose dots above the diagonal exhibit an increase coverage in their 3'UTR in the arsenite condition, suggesting an alteration in translation and showing that RRE has automatically extracted mRNAs of interest.

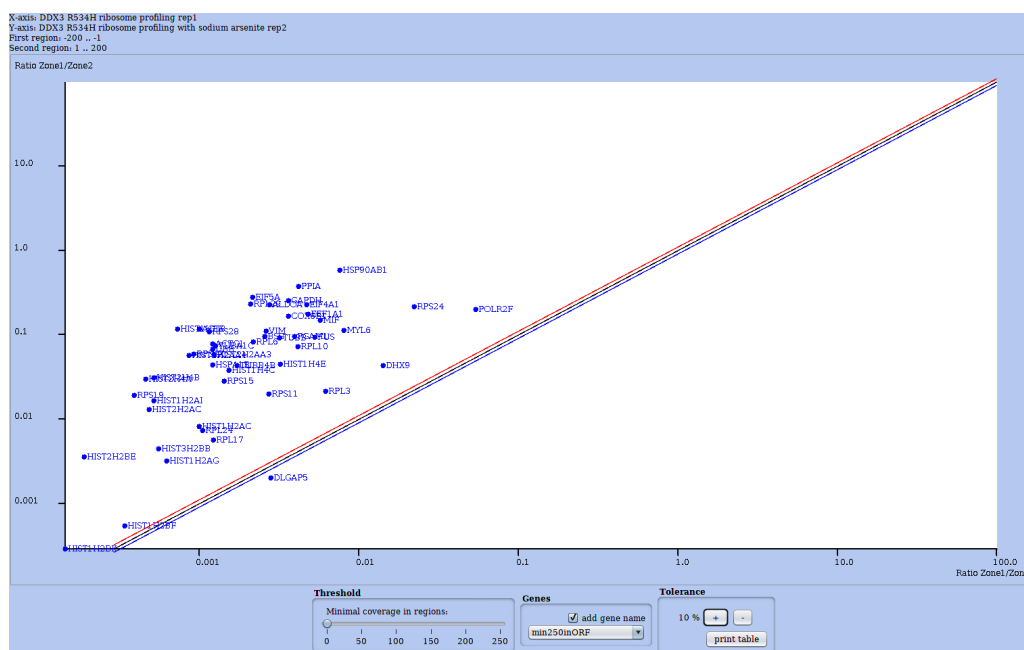


Figure 6: Results of a type 3 query on the DDX3 dataset: Mining mRNAs with coverage in their 3' UTR. An example of selected mRNA is shown in Figure 7. RRE plots the ratio of subregion coverage in one versus in the other dataset. Like in Figure~4 and in all comparative plots, each RNA is represented by dot and clicking on the dot one can get the profile coverage for that gene. The user can further refine the selection by adjusting the coverage threshold. The main diagonal is bounded by two other diagonals showing a deviation from a ratio of 1.

A subregion ratio plot resulting from a type 3 query is restricted to a subset of mRNAs (a selection named "min250inORF") previously selected and obtained by the user with a type 1 query – see Figure~6.

Additional features of RRE

Contrary to other tools, RRE enables the user to restrict on-the-fly the data to a range of read sizes, or to a range of global read coverages, at many steps of the analysis. Using queries the user can save a selection, that is a subset of RNAs of interest, and give it a name. With this selection, general plots illustrated in above Figures or in the manual, can be "restricted" to that subset of RNA interactively. The user can explore its data and save the entire session, which can then be reloaded and continued later. Last, once an analysis has been performed, the user can export graphic figures in PNG format. A comprehensive manual guides the user in all these manipulations with RRE.

4 Conclusion

Despite the publication of numerous methods, programs or R packages for analyzing Ribo-seq [2] [18], a user-friendly, interactive exploration of multiple ribosome profiling dataset remained tedious. Here, we have implemented, made available, and described a standalone tool named RNA-Ribo Explorer (RRE) to offer a solution for mining and visualizing such data to the end user, since Ribo-seq is increasingly used to address a wide variety of questions regarding translational mechanisms and regulation. Our goal was not to re-implement useful R packages or software that perform either read preprocessing (like cleaning and mapping), post-processing

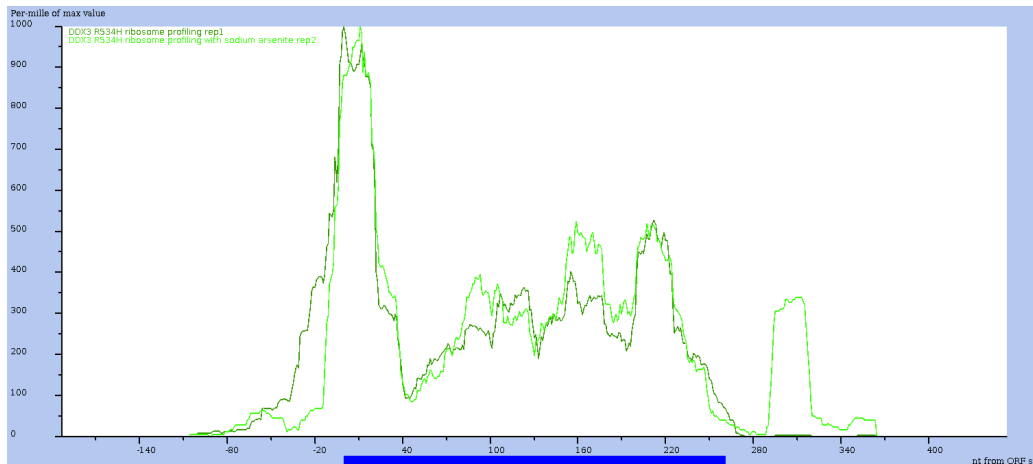


Figure 7: Profile view of mRNA COX6B1, one of the mRNA selected by the type 3 query of Figure~6. Sliding window option is on and the y-axis is relative to the max value in each condition: so the height of the two curves are not comparable (here). One clearly observes a major difference in the 3'UTR: condition with sodium arsenite exhibits a strong relative coverage, while the control condition has almost no reads.

or downstream analyses (like differential expression or Ribosomal stalling events), but to ease the visual inspection of the data – which is important for many practitioners. Instead, we opt for a solution that can read in files containing read counts on a transcriptome (obtained from preprocessing step) and output graphics or subset of data for downstream more specialized analyses. Hence, RRE can be utilized in intermediate steps of the analysis where interactive exploration is needed. Given the absence of standard ways of analyzing such data (which vary according to the protocol), RRE may well prove a timely and suitable solution. The contribution of RRE is to enable the end-user to inspect, compare, and query multiple datasets (either RNA-seq or Ribo-seq) in an easy and flexible manner, and allow adaptable visualization.

Given how RRE Java code is structured, further extensions and developments can be easily envisaged. For instance, complex structural RNA elements could be computed or read from annotations, and either added on profile plots or considered in query conditions. This would allow to check whether regions of altered translation (slowdown, stalling) spatially correspond to locations of such structures. Similarly, annotations of potential upstream ORFs could be obtained from a dedicated tool, read from a file, and manipulated within RRE framework. Of course, information coming from quantitative proteomics or peptidomics would help prioritizing candidate short or alternative ORFs that exhibit coverage in RRE plots/results (an example of confirmation of putative peptide encoding ORFs was published in [14]). Some scores measuring the coding content of a region could be used to locate putative ORFs in mRNAs; in the future, existing measures will be thoroughly evaluated (as suggested in the review [2]), and some score functions will emerge as standard, which would ease the choice for incorporating a new feature in RRE.

Availability: user's manual can be found at <http://atgc-montpellier.fr/rre>. Source code is available on gitlab: <https://gite.lirmm.fr/rivals/RRE>.

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