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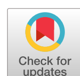

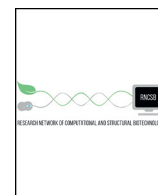

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Mini Review

Following Ribosome Footprints to Understand Translation at a Genome Wide Level

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ABSTRACT

Protein translation is a key step in gene expression. The development of Ribosome Profiling has allowed the global analysis of this process at sub-codon resolution. In the last years the method has been applied to several models ranging from bacteria to mammalian cells yielding a surprising amount of insight on the mechanism and the regulation of translation. In this review we describe the key aspects of the experimental protocol and comment on the main conclusions raised in different models.
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1. Introduction

The decreasing cost of obtaining Next Generation Sequencing (NGS) data [1–3] together with the huge information sets arising from these technologies is revolutionizing several research fields of life sciences (see an example in [4] or in disease biology [5,6]). Ingenuity is continuously leading to the development of new methods, a very interesting

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case is an application named Ribosome Profiling (RP), or Ribo-Seq, developed by Ingolia & Weissman in 2009 [7] where the deep sequencing of mRNA fragments covered by ribosomes during translation yielded an original view of translation at a genome wide scale. The footprints of active ribosomes are obtained using an RNAse protection assay, where controlled digestion generates small mRNA fragments/footprints of approximately 30 nucleotides [8]. Therefore, after data processing, translation can be observed at an unprecedented resolution in a variety of biological settings. Before performing the digestion, ribosomes are halted over the mRNAs using translation inhibitory drugs or by quick deep freezing the sample to avoid ribosome run-off. The resulting fragments, *i.e.* the ribosome footprints, are purified and used to construct sequencing libraries to feed short read sequencers. In this scenario, a transcriptome wide picture of the translating ribosomes location over mRNAs is obtained, together with an estimation of the mRNAs translation rates. These expression levels estimated by RP define what is called *translatome*, in analogy to the term transcriptome. Translatome estimations of gene expression levels correlate better with proteomic data than transcriptome-derived estimations (see below). This increased correlation evidences the existence of mechanisms operating in the control of translation that fine tune the synthesis of cellular proteins.

In the context of the rich data obtained in a RP experiment, an interesting outcome was the definition of two concepts: translational efficiency and periodicity. The first concept refers to how much an mRNA is translated considering the level of its coding mRNA, so it is an important parameter yielding information on translation regulation. Translational efficiency is calculated as the ratio between translation (derived from counts of footprints per mRNA) over transcription (derived from RNA-seq mRNA levels) of particular mRNA. The second, refers to the three bases mapping periodicity observed for the reads derived from footprints as a consequence of ribosome movement along mRNA. Since the ribosome moves codon by codon, the 5′-end of the ribosome footprints tend to map at the same position of each codon throughout the whole coding sequence.

Several aspects concerning protocol have been discussed, revised and modified since the original protocol was established. Some aim to adapt the protocol to different biological models, like eukaryotic or prokaryotic cells, specific tissues, *etc*. Other aspects have been intensely discussed, for example what the appropriate method to stop translation is or how to define the correct translation frame from ribosome footprints. Nevertheless, RP protocol is currently a widely used approach to study gene expression in different biological models from virus and bacteria to complex mammalian tissues (examples in [9–11]). In this mini-review we will discuss the main and critical steps in the RP protocol, its uses and main findings obtained in different biological models and the contributions to our knowledge of cellular and molecular biology.

2. Ribosome Profiling Protocol

2.1. Protocol Description

Ribosome Profiling comprise mainly five steps: sample preparation, RNAse protection assay, isolation of ribosome footprints, high-throughput sequencing and bioinformatic analysis (Fig. 1A) [12]. Sample preparation refers to steps necessary to process the biological sample and obtain a post mitochondrial supernatant where lysis conditions ensure to preserve *in vivo* ribosome positioning and RNA integrity. Among others, alternative inputs could be tissue homogenates, isolated tagged ribosomes or a bacterial cell lysate. Critical aspects concerning this step are: ensuring enough biological material to produce quantifiable ribosome footprints and avoiding ribosome run-off. For the last, either drugs inhibitors of translation or physical methods like flash-freezing using liquid nitrogen and dry ice can be used. Indeed, fast freezing becomes crucial in cases where using translation inhibitors are to be avoided.

The RNAse protection assay, also called nuclease footprinting, is another critical step in RP protocol. Several RNAses had been used, mainly RNAse I and micrococcal nuclease (MNAse) in eukaryotic cell models and bacterial cells, respectively. At this step, controlling factors like reaction time and enzyme concentration are critical to ensure an appropriate mRNA digestion, for example it has been stablished that the ratio between RNA and RNAse controls footprints size [13].

The third step is one of the most laborious in terms of protocol. Different strategies had been used to isolate ribosome protected fragments or ribosome footprints, but all of them imply a ribosome/poly-ribosome purification step. Even though commercial columns are available to purify monosomes, the most used approach is the differential sedimentation of ribosomes through a sucrose cushion during ultracentrifugation. The use of this technique of subcellular fractionation ensures the purification of monosomes with bound ribosome footprints. Once monosomes are purified, a polyacrylamide gel electrophoresis in denaturing conditions is run to separate the complex sample by length. Using appropriate size markers, the gel is cut at the corresponding length of 28-30 nt using a dark field transilluminator, even if footprints are not visible as it is usually the case. After disrupting the gel slices, precipitation and re-purification of ribosome footprints, samples are ready to proceed to library preparation.

Library preparation implies a set of protocol steps common in many high-throughput sequencing experiments like end repair, 3′ adaptor ligation, reverse transcription and PAGE cDNA purification, circularization of cDNA and PCR amplification. After checking length and concentration of the ribosome footprints library, they can be submitted to sequencing according to user-preferred sequencing technologies. Due to footprints small size, neither long reads nor paired-end reads are needed. Nevertheless, due to ribosomal rRNA presence in the footprints fraction purified, depletion of rRNA, coupled with extra sequencing depth are usually needed.

Finally, the bioinformatic analysis of data is the most user-dependent step. A typical analysis would include quality control of raw reads, mapping, count normalization and gene expression levels estimation. It could also include, for example, differential gene expression analysis if two biological conditions are contrasted. Table 1 show a list of some of the software available to perform classical analysis over RP data. Nevertheless, how deeply the data is interrogated is on user's hands, here we will discuss some of these downstream analyses later.

2.2. Protocol Variants, User Decisions

Up to this point we have reviewed the main steps in RP protocol considering the classical approaches most used in literature. Henceforth we will mention some protocol variants and why they could be used if is necessary (Fig. 1B). Considering the chronological order of the protocol, we will start with one of the steps where more variants are described in the literature: how to stop translation at the moment the experimental design requires to do so. Efficient stop of translation avoids ribosome run off, sharpening the picture taken of the translatome at a given time point. In the original protocol, a classical translation inhibitor like cycloheximide was used to specifically target translation elongation. However, as it does not interfere with pre-initiation complex scanning and translation initiation, treatment with cycloheximide causes a significant accumulation of ribosomes at initiation sites of mRNAs actively translated. This could represent a source of bias since a lot of ribosome footprints will be generated by initiating ribosomes while elongation is stopped. This issue was highly covered in the literature, with some authors proposing that this accumulation is actually due to an enrichment of slow codons after the initiation and others are in line with the bias hypothesis that generates a skewed distribution. Alternatively, it is possible to stop translation using liquid nitrogen and dry ice [12]. In this scenario, ribosomes are flash-frozen and stopped just by reducing

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