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Analysis of translation using polysome profiling

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ABSTRACT

During the past decade, there has been growing interest in the role of translational regulation of gene expression in many organisms. Polysome profiling has been developed to infer the translational status of a specific mRNA species or to analyze the translome, i.e. the subset of mRNAs actively translated in a cell. Polysome profiling is especially suitable for emergent model organisms for which genomic data are limited. In this paper, we describe an optimized protocol for the purification of sea urchin polysomes and highlight the critical steps involved in polysome purification. We applied this protocol to obtain experimental results on translational regulation of mRNAs following fertilization. Our protocol should prove useful for integrating the study of the role of translational regulation in gene regulatory networks in any biological model. In addition, we demonstrate how to carry out high-throughput processing of polysome gradient fractions, for the simultaneous screening of multiple biological conditions and large-scale preparation of samples for next-generation sequencing.

INTRODUCTION

Gene expression is regulated at many levels: at the epigenetic level, at the transcriptional level, at the translational level and at the post-translational level. Among these levels, translational regulation governs protein production in response to a number of physiological and pathological situations (1,2). Accordingly, almost half of the variation of protein concentration is due to translational control (3). Studying translation leads to a better understanding of gene expression regulation and can explain the differences between transcriptome and proteome analyses (4–7), because the subset of mRNAs actively engaged in translation (i.e. the translome) reflects the functional readout

of the genome at a given time in a given cell type. There are various techniques for specifically studying translated mRNAs, of which polysome profiling is the most common. Polysome profiling is based on sucrose-gradient separation of translated mRNAs, which are associated with polysomes, from untranslated ones. More recently, other techniques have emerged such as ribosome profiling and translating ribosome affinity purification (TRAP) (reviewed in (8)). Ribosome profiling measures translation by deep-sequencing ribosome-protected mRNA fragments. This technique also determines the position of the ribosome at codon resolution, allowing discoveries of new coding transcripts and protein isoforms as well as accurate measurement of translation rates (9). TRAP is suitable for analyzing translation in a specific cell type. Genetically modified cells or organisms are engineered to express a tagged ribosomal protein *in vivo*, under the control of a tissue-specific promoter. Tagged ribosomes are affinity-purified and associated mRNAs are identified by microarray or deep-sequencing (10).

Among these three main methods for mRNA translation analysis, polysome profiling has several interesting applications. Polysome profiling can be used for targeted analyses on specific mRNAs (11–13) as well as for holistic analyses (14–17). Furthermore, this technique gives access to the full-length translated mRNAs, including the untranslated regions (UTRs), in contrast to ribosome profiling in which the protected fragments map to coding sequences only. UTRs harbor cis-acting elements that regulate selective translation (18). Identification of UTRs and conserved cis-acting elements corresponding to translated mRNA isoforms are essential for a mechanistic analysis of gene expression regulation. In addition, polysome profiling can be combined with immunoblotting and/or proteomics to monitor proteins associated with ribosomes and/or initiation complexes. Lastly, polysome profiling is especially suitable for model organisms for which genomic data and/or tools are scarce, and/or for which genetic manipulation is not possible. However, the broad application of polysome profiling has been slowed by its complexity and the difficulty of

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adapting it to simultaneous analyses of multiple biological conditions.

In this paper, we describe an optimized protocol for purifying high-quality polysomes and used it to study the polysomal recruitment of selected mRNAs triggered by fertilization in sea urchin. In sea urchins, early developmental stages undergo well-described translation regulation, with stored maternal mRNAs being progressively used during the first cell-cycle cleavages, without any significant increase in transcription (19–22). Polysome protocols in sea urchin were described in the 1980–1990s (23,24), but have not been used with the recent molecular biology techniques that allow in-depth investigation of the translational status of specific mRNAs. Here, we optimized each step of the protocol (lysis buffer, lysis conditions, RNA purification, etc.) to obtain reproducible, good quality RNAs from polysome gradients. We also provide advice and tips for the critical steps, including polysome disruption, so that this protocol can be adapted to any—common or emergent—model organism. In addition, we demonstrate how the protocol can be adapted for high-throughput processing of polysome gradient fractions, for the simultaneous screening of multiple biological conditions and large-scale preparation of samples for next-generation sequencing (NGS) applications.

MATERIALS AND METHODS

Overview of the protocol

Polysome profiling separates translated mRNAs on a sucrose gradient according to the number of bound ribosomes. First, cells are lysed and loaded on top of a 15–40% sucrose gradient. After ultracentrifugation, the gradient is monitored at A_{254} using a flow cell coupled to a spectrophotometer and then fractionated into equal fractions: untranslated mRNAs (top fractions) are separated from polysome-associated mRNAs (bottom fractions). Fractions are then processed for RNA extraction, either manually by acid phenol–chloroform extraction or in a 96-well format using an automated RNA purification processor, simultaneously handling several gradients. The translational status of a given mRNA species is analyzed by RT-PCR amplification and its relative quantification in each fraction. Alternatively, the content of the polysomal fractions can be identified using a global analysis (such as high-throughput sequencing), granting access to the cell transcriptome. A schematic overview of the method is presented in Figure 1.

Detailed protocol

Sample collection. Experiments were performed using *Paracentrotus lividus* or *Sphaerechinus granularis* sea urchins; the experiments shown herein correspond to *P. lividus* samples. Sea urchins were collected in the bays of Crozon or Concarneau (Brittany, France). Gametes were obtained by intracoelomic injection of 1 ml 0.1 M acetylcholine. Eggs were collected in filtered seawater (FSW), filtered on hydrophilic gauze and washed twice in FSW by centrifugation for 2 min at 2000 rpm (Heraeus, Labofuge 400 with swinging buckets). Eggs were incubated 40 s in FSW supplemented with 0.7 mM citric acid to remove

the jelly coat, and rinsed again in FSW. For fertilization, eggs were suspended in FSW as a 5% suspension. Sperm was collected in a Petri dish and stored at 4°C until use. Extemporaneous dilution in FSW (10 μ l of sperm in 1 ml of FSW) activates sperm for fertilization and 10 μ l of this diluted sperm was added per ml of egg suspension. Embryos were cultured at 16°C under constant agitation. Only batches of embryos displaying a fertilization and division rate above 95% were used.

Cell lysis. Eggs or embryos were collected in FSW by brief centrifugation (1 min at 1000 rpm, Heraeus, Labofuge 400 with swinging buckets), and the pellet was resuspended in four volumes of cold lysis buffer (10 mM Tris pH 7.4; 250 mM KCl; 10 mM MgCl₂; 25 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'- tetraacetic acid (EGTA); 0.4% Igepal; 5% sucrose; RNase-free water and extemporaneously 1 mM 1,4-Dithiothreitol (DTT); 10 μ g/ml aprotinin; 2 μ g/ml leupeptin; 100 μ M emetine; 40 U/ml RNase inhibitor). Lysis was done in a Dounce homogenizer using 10 strokes of the tight B pestle. All steps were done at 4°C, on ice or in the cold room. The lysate was then centrifuged for 10 min at 13000 rpm in a tabletop centrifuge to remove nuclei and cellular debris. The supernatant was carefully transferred into a new microtube. Samples can be frozen in liquid nitrogen and kept at –80°C until further use for polysome fractionation.

The concentration of nucleic acid in the lysate was measured by absorbance at $A_{260\text{nm}}$ of a 5 μ l sample of lysate diluted in 1 ml of water using a spectrophotometer. Starting from 10⁶ eggs or embryos in a 250 μ l pellet, typical yield for sea urchin samples was usually between 20 and 40 OD_{A260}.

Critical steps for lysate preparation. The protocol described above was defined after optimizing several parameters such as starting material (frozen or fresh eggs), lysis buffer composition and volume and lysis techniques. Optimization of the protocol for sea urchin oocytes and embryos is detailed below, and the quality of the RNA in the lysate was tested for each variant of the protocol using acid phenol–chloroform extraction and electrophoresis. We also provide suggestions for adapting this protocol to other models and organisms.

We observed that RNA quality improved when the lysates were prepared with fresh eggs rather than from a frozen egg pellet kept at –80°C (Figure 2, lanes A and B). Alternatively, freezing and grinding under liquid nitrogen prior to lysis works for organisms with cell walls or for solid tissues (12,25). Lysates can be kept frozen at –80°C until use, without detectable loss of RNA integrity.

The ratio of the volume of the egg pellet to lysis buffer was optimized in our conditions to obtain a lysate concentrated enough for subsequent polysome purification, without compromising RNA quality (Figure 2, lanes B–E). The optimal ratio was one volume of egg pellet to four volumes of lysis buffer (1:4; Figure 2, lane E). The extraneous lower bands observed in lanes A, B, C and D correspond to 18S and 28S ribosomal RNA degradation products.

Two lysis techniques were tested: mechanical shearing through a 25G needle (routinely used for protein lysates in our lab) and Dounce homogenization. In our experiments,

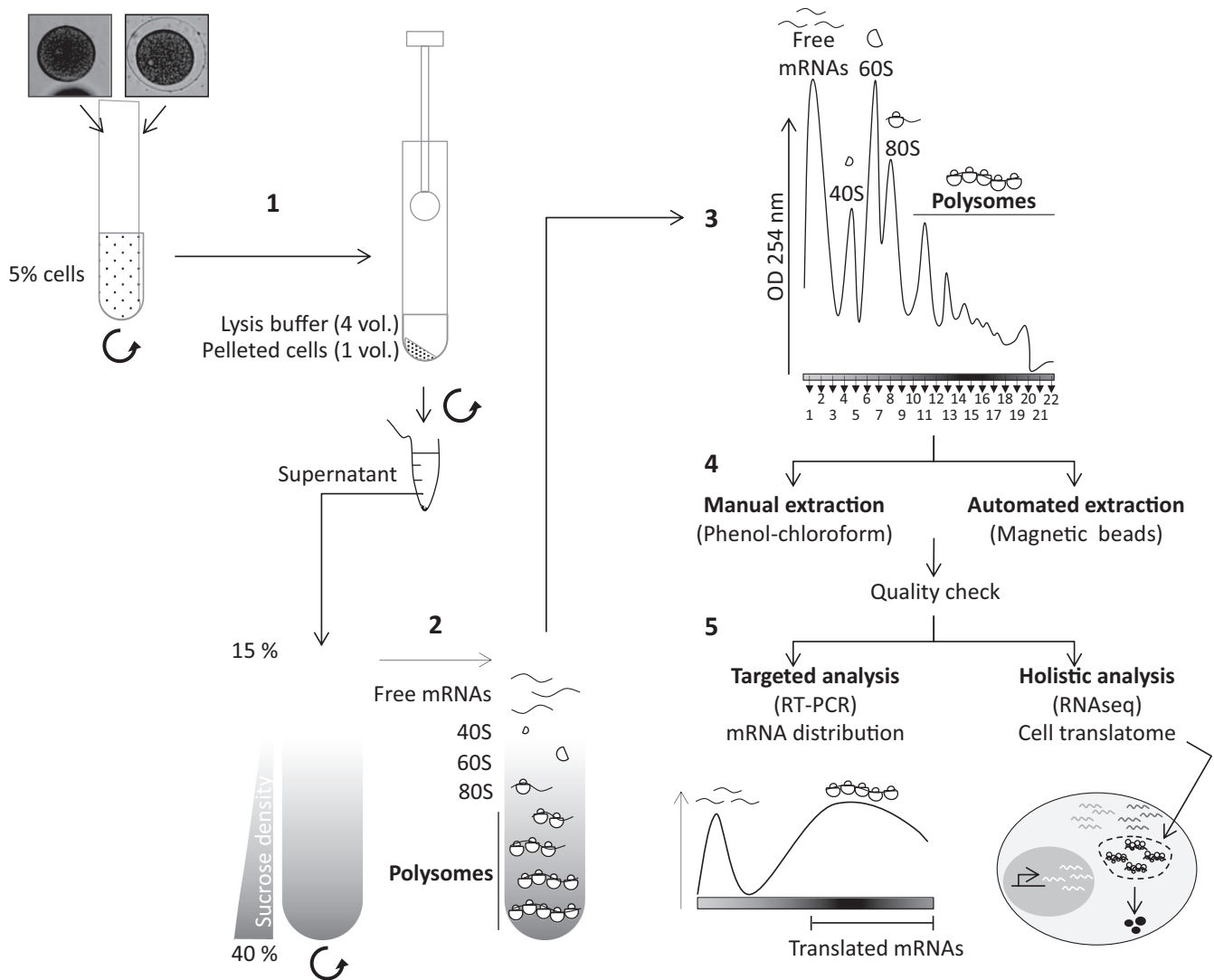


Figure 1. Overview of the polysome profiling protocol to analyze translation activity. The various steps of the protocol involve (1) cell lysis, (2) sucrose-gradient centrifugation and (3) fractionation, (4) RNA extraction and RNA integrity check, (5) analysis of translational status of mRNAs. See the text for details.

the use of a Dounce tissue grinder improved RNA quality (Figure 2, lanes E and F). Using a 7 ml Dounce with the tight B pestle, which leaves a distance of 20–55 μm between pestle and cylinder, we were able to lyse cells without breaking the nucleus to isolate cytoplasmic RNA: the diameter of sea urchin eggs is 100 μm whereas the diameter of the nucleus is less than 20 μm . Proper lysis of the eggs was checked under a light microscope, and 10 strokes were needed to obtain 100% of lysed cells.

Sea urchin oocytes and embryos contain high nuclease activity that depends on Ca^{2+} ions (26,27); a report suggests to rinse them in Ca^{2+} -free seawater prior to the lysis step to improve RNA integrity (27). In our hands and in the urchin species we used, this step did not significantly improve RNA quality (Figure 2, lanes F–H). However, inclusion of EGTA in the lysis buffer (27) improved RNA quality and its reproducibility in the lysates (Figure 2, cf. lanes F, I and J). We also noticed that the high-salt buffer preserved RNA

integrity; therefore 250 mM KCl was also included in our sea urchin lysis buffer.

To generate polysomes that accurately reflect the translational status of the cell, ribosome movement on the mRNA must be minimized during sample preparation to effectively prevent ribosomes from running off the mRNA. We therefore added a translation elongation inhibitor. Cycloheximide is the most commonly used inhibitor in polysome profiling protocols described for mammalian cells (12). In sea urchin embryos, cycloheximide does not work, however emetine is a very efficient protein synthesis inhibitor (28). We therefore included emetine (100 μM) in egg suspensions or embryo cultures 5 min before sample collection, and in the lysis buffer.

A combination of anti-proteases (aprotinin and leupeptin, for example) or a commercially available anti-protease cocktail is necessary to avoid protein degradation and enhance polysome quality. Additional detergents can be included in the lysate buffer, such as Triton X-

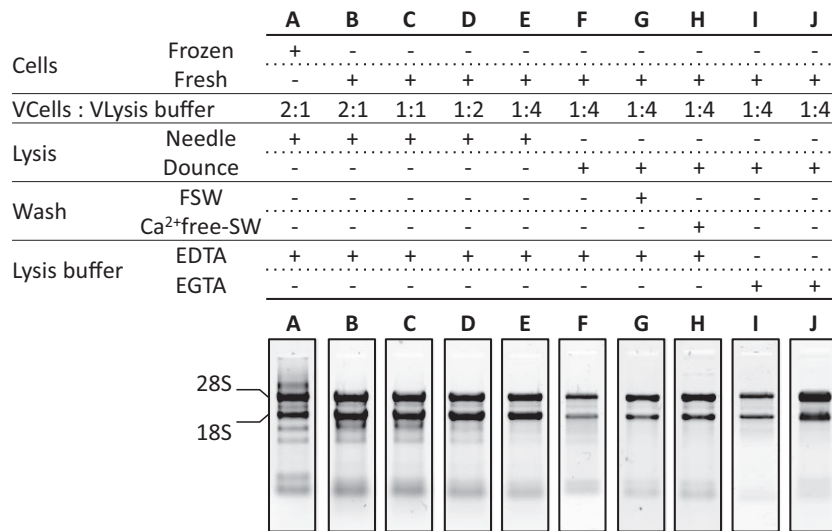


Figure 2. RNA quality with different conditions for lysis of sea urchin eggs. Lysis was done using a 25G needle (A–E) or a Dounce homogenizer (F–J), on frozen eggs (A) or fresh eggs (B–J). The same volume (V) of eggs was lysed in increasing volumes of lysis buffer ranging from 2:1 to 1:4 (B–E). An additional wash with filtered seawater FSW (G) or Ca²⁺-free SW (H) was tested before lysis. Lysis buffer contained either 1 mM EDTA (F) or 25 mM EGTA (I and J). Two RNA quantities prepared using the optimized protocol showed only the 28S and 18S RNA without degradation products (lane I: 250 ng; lane J: 1 µg). RNAs from each lysate were obtained after an acid phenol–chloroform extraction, and separated on a 2% agarose-TBE gel to check for integrity.

100 and sodium deoxycholate to extract membrane-bound polysomes in mammalian tissue or culture cells (12,29) or cetyltrimethylammonium bromide in polysaccharide-containing organisms (30). Several RNase inhibitors can be used in combination to fully inhibit RNase activity, such as heparin or vanadyl ribonucleoside complex (12,29). Heparin, known to be an inhibitor of the RT-PCR reaction, can be removed by treating the RNA samples with heparinase I (31). In our experiments, we only used a commercial RNase inhibitor (from Promega), with satisfactory output of the purified RNA from sea urchin lysates.

We recommend carrying out a cytoplasmic RNA integrity check before proceeding with polysome fractionation. RNA was purified from an aliquot of the lysate by acid phenol–chloroform extraction and resolved on an agarose-Tris/Borate/EDTA (TBE) gel to verify that there was no degradation: ribosomal RNA bands appeared as two sharp bands on an agarose gel (see Figure 2, lanes I and J). Quality can be further determined on a Bioanalyzer. The RNA integrity number, which gives a measurement of the degree of degradation, should ideally be close to 10, corresponding to intact RNA (Supplementary Figure S1).

Polysome fractionation.

Sucrose density gradient formation. Lysates obtained as described above were loaded on a 15–40% linear sucrose density gradient. Two 15 and 40% sucrose buffers were prepared in 10 mM Tris pH 7.4; 10 mM MgCl₂; 250 mM KCl; 25 mM EGTA; 1 mM DTT. To obtain a linear gradient, we used the Gradient Master device (BioComp) equipped with tubes adapted for a SW41 rotor. We used 6 ml of each sucrose buffer in each gradient tube. In the ultracentrifuge tube, the light sucrose solution was underlayered with the heavy sucrose solution using a cannula attached to a 10 ml

syringe. The introduction of air bubbles or mixing of the two solutions should be carefully avoided during this step. The ‘SW41 Short Gradient’ program with the corresponding range of sucrose percentage was run following the manufacturer’s instructions. This program lasts a few minutes (2 min 21 s for a 15–40% gradient) and produces up to six reproducible linear gradients in one run. The gradients should be handled with care to avoid disturbance and stored at 4°C for at least 1 h. The percentage of sucrose used for linear gradients can be adjusted to optimize the separation of polysomal fractions according to the biological sample or experiment.

Sample loading and ultracentrifugation. When comparing two biological conditions, the same amount of lysate (equivalent OD_{A260}) should be loaded on top of each gradient. The maximum volume of lysate that can be deposited on the gradient is 500 µl, with a maximum of 25 OD_{A260}. In our hands, better separation of the 40S, 60S and ribosome peaks was obtained when 10 OD_{A260} was loaded on the gradient, typically corresponding to 350 µl of lysate. Loading was carried out by gently pipetting the lysate onto the top of the gradient. Then, the gradient tubes were carefully balanced before starting the ultracentrifugation run at 38000 rpm for 2.5 h in a Beckman SW41 Ti rotor at 4°C. The acceleration was set to its maximum value, and to avoid any disturbance, the deceleration was set to its minimum value. Once the ultracentrifugation run was finished, the gradients were kept at 4°C for immediate fractionation.

Polysome gradient profile and collection of fractions. During ultracentrifugation, the Isco Density Gradient Fractionation System was set up according to the manufacturer’s recommendations. This density gradient system is equipped with a spectrophotometer with a 254 nm filter

(UA-6 UV/VIS detector, Isco), and produces a continuous absorbance profile as the gradient is collected. Fractionation was performed by piercing the bottom of the centrifuged tube to introduce a dense chase solution and raise the intact gradient by bulk flow. Before each experiment, the entire system was washed first with 0.1 M NaOH, then thoroughly with DEPC-treated water. In our experiments, the peristaltic pump was set at a speed corresponding to 0.9 ml/min.

After centrifugation, the centrifuge tube was connected to a flow cell and the piercing apparatus. The 50% sucrose chase solution was injected by puncturing the tube from the bottom, pushing out the gradient in a continuous manner into the flow cell. Fractions were kept on ice during collection to avoid any RNA degradation. The introduction of air bubbles in the system should be avoided, because bubbles will disturb the gradient. A typical polysome profile (Figure 3A) first showed a peak of A_{254} absorbing material, containing the untranslated mRNAs, then the two peaks of the small and large ribosomal subunits, the monosome peak and finally the polysomal peaks. Polysome profiles can vary according to the general translation activity. For example in unfertilized sea urchin eggs, <2% of the ribosomes are engaged in polysomes and polysome peaks are barely detectable ((19); Figure 3A). After fertilization, protein synthesis activity increases and the proportion of ribosomes in polysomes increases as development proceeds (Figure 3A).

RNA purification and profiles. The RNAs of each fraction were extracted with one volume of acid phenol–chloroform (vol:vol), and precipitated with one volume of isopropanol. Ethanol–sodium acetate precipitation cannot be used, due to the high sucrose concentration in the last fractions. RNAs were pelleted in a tabletop centrifuge at 13000 rpm for 10 min at 4°C, washed with 70% ethanol, pelleted again and air-dried for 20 min. RNAs from each fraction were re-suspended in the same volume of RNase-free water (one-twentieth of the original fraction volume). An aliquot of each fraction was used to check RNA quality on an agarose gel. The polysome profile of fertilized embryos and the corresponding RNA profile of the 15–40% sucrose gradient separated on an agarose gel are shown in Figure 3B. In the first fractions, only low molecular weight RNAs were visible, then fractions containing only 40S subunits were isolated as shown by the presence of the 18S rRNA band, followed by the 60S peak, as shown by the 28S rRNA band. In the middle of the gradient, the bands were more intense, due to the high proportion of monosomes. Starting from fraction 13, 28S and 18S RNAs were present with a 28S:18S ratio equal to 2:1, representing polysomal fractions. On average, starting from 10 OD_{A260} loaded on the gradient, typical RNA yields were 750 ng per polysome fraction.

Protocols often recommend using proteinase K treatment before RNA extraction (10 mM Tris pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate and 200 µg/ml proteinase K, for 25 min at 50°C) to enhance recovery of RNAs from polysome gradients (32). Although it may help for specific samples, in our hands, the proteinase K treatment did not significantly improve RNA quality or quantity (data not shown).

Analysis of the translational status of mRNAs

A critical point of translation analyses is to verify that mRNAs present in polysome fractions are associated with translating polysomes and do not merely co-sediment with them. In the cell, untranslated mRNAs are associated with RNA-binding proteins in so-called messenger ribonucleoparticles (mRNPs) or with stalled polysomes, which may sediment in the same fractions as polysomes when purified on sucrose gradients (19,33). To distinguish between active polysomes and co-sedimenting mRNPs or stalled polysomes, we treated our samples with a polysome disrupter prior to polysome purification. The two commonly used disrupters are EDTA and puromycin. EDTA chelates Mg^{2+} ions and dissociates the subunits of the ribosome. EDTA (30 mM final) was added after lysate preparation, prior to gradient loading. Gradients should also be supplemented with 10 mM EDTA and prepared without Mg^{2+} . Given that EDTA can potentially affect mRNPs, whose formation may depend on Mg^{2+} , the antibiotic puromycin is usually preferred. Puromycin causes the dissociation of the ribosomes in the elongation step of translation, and only affects active polysomes (34). Puromycin can be added *in vivo* prior to lysis (0.6 mM in sea urchin egg suspension or embryo culture) or *in vitro* after lysate preparation (2 mM in lysate), when samples are not easily available for puromycin *in vivo* incubation (23,35). In both cases, the lysate is supplemented with 500 mM KCl, incubated for 15 min at 4°C then for 15 min at 37°C before loading on the sucrose gradient (35).

EDTA treatment disrupted the gradient profile, with a high loss of RNA integrity (Figure 3C). Adding more RNase inhibitor to lysates and in the sucrose gradient improved RNA quality (data not shown). In contrast, the use of puromycin either *in vitro* or *in vivo* did not affect RNA quality after purification. RNAs from polysome fractions shifted toward lighter fractions in puromycin-treated lysates (Figure 3D and E). We therefore used the puromycin treatment for assessing translation of a specific mRNA.

Following polysome profiling and using the puromycin controls in parallel, the translational status of a specific mRNA can be assessed by Northern blot or RT-PCR. The presence of the mRNA can be detected in each fraction, and after quantification of the signal, specific mRNA distribution was expressed as a percentage of the total signal. An illustration of this approach is given below, by analyzing the translational status of mRNAs before and after fertilization in sea urchin.

RESULTS AND DISCUSSION

Translation following fertilization in sea urchin

In this paper, we illustrate the use of polysome profiling in sea urchin to analyze the translational status of specific mRNAs by semi-quantitative RT-PCR analysis on individual fractions of the polysome gradient, as described in (29).

Early embryonic development, especially the oocyte-to-embryo transition, is a particularly interesting stage for the study of translational regulation (e.g. in mouse (14,36) or *Drosophila* (17)). During oocyte maturation, maternal mRNAs are stored in the cell. The early stages of develop-

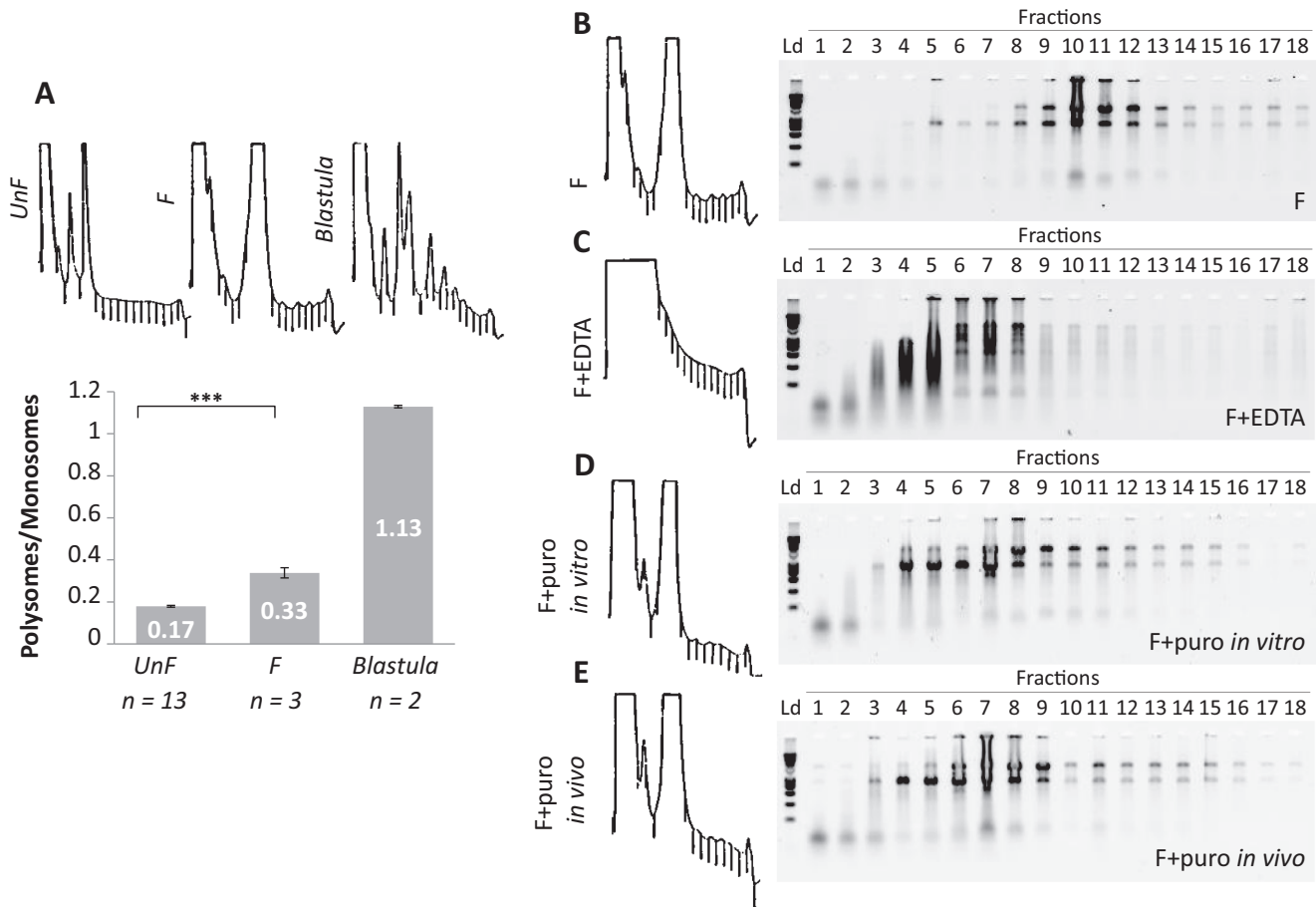


Figure 3. (A) Polysome gradient profile during early development. Optical density profiles (OD_{A254}) of polysome gradient profiles are shown, corresponding to unfertilized eggs (UnF), 1 h post-fertilization embryos (F) and late-blastula stage embryos 30 h post-fertilization (Blastula). The areas under the curve (AUC) of polysomes and monosomes were measured, and the polysome:monosome ratio was then calculated for the three developmental stages; error bars represent SEM and statistics were done using Student's *t*-test (P -value < 0.01). (B–E) Polysome gradient profiles and corresponding RNA profiles after treatment with polysome disrupters. Optical density profiles and extracted RNAs from polysome gradients of fertilized eggs (B), treated with 30 mM EDTA (C), 2 mM puromycin *in vitro* (D) and 0.6 mM puromycin *in vivo* (E) are shown. The RNAs from each fractions of polysome gradient were separated on 2% agarose-TBE gels.

ment following fertilization are mainly based on the translation of these stored maternal mRNAs, independently of new transcription (reviewed in (20)). One of the advantages of the sea urchin model is that oocytes have completed their meiotic maturation and are physiologically blocked in the G1 phase; fertilization triggers the onset of mitotic divisions in a naturally synchronous fashion. Several mRNAs are translated at fertilization: cyclin A, cyclin B and the ribonucleotide reductase small subunit R2 (37–39). We validated our polysome purification protocol by checking the distribution pattern of these mRNAs that have been demonstrated to enter polysomes after fertilization. We also used eIF4A as a negative control, eIF4A being a maternal mRNA that has been shown to remain untranslated shortly after fertilization in mouse (14). The *P. lividus* sequences for the four mRNAs were retrieved from tblastn searches in the *P. lividus* transcriptome databases (<http://octopus.obs-vlfr.fr>) using the homologous human (NCBI) and *Strongylocentrotus purpuratus* (<http://Echinobase.org>; (40)) protein sequences. Primers were designed using Primer3 webtool (Supplementary Figure S2).

Equal RNA volumes (5 μ l) of each polysome gradient fraction were used for reverse transcription using random primers following the protocol recommended by the manufacturer (SuperScriptII, Invitrogen). Semi-quantitative polymerase chain reaction (PCR) was then performed using specific primers, diluting the cDNA in RNase-free water (1 volume RT products:300 volume H₂O) for the PCR reaction using the GoTaq Flexi kit (Promega), so that amplification was in the linear range for 30 cycles of amplification (Supplementary Figure S2). PCRs were carried out as followed: 95°C for 2 min; followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were analyzed on 2% agarose-TBE gels, scanned on a Typhoon Trio (GE Healthcare Life Sciences) and quantified using ImageJ software. Furthermore, we tested a rapid screening method involving the analysis of only every other fraction of the gradients, a quicker assessment that can be used when simultaneously testing different cell treatments or conditions. We showed that this method yielded the same mRNA distribution pattern as every single fraction (Supplementary Figure S3).

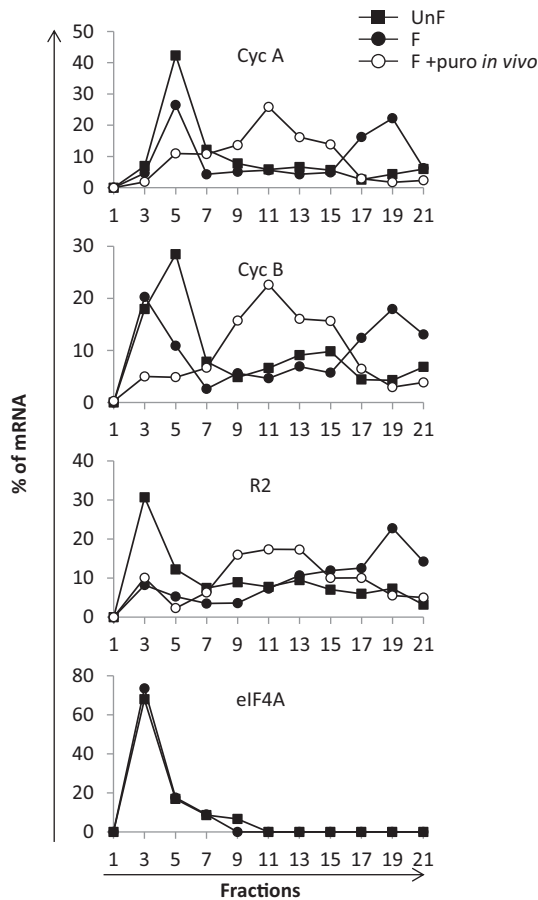


Figure 4. mRNAs coding for cyclin A, cyclin B and the small subunit of ribonucleotide reductase (R2) are actively translated, whereas eIF4A mRNA is not translated after fertilization in sea urchin. mRNAs were detected by RT-PCR amplification in each fraction of the polysome gradient from unfertilized eggs (UnF), 1 h post-fertilization embryos (F) or embryos in presence of puromycin *in vivo* (F+puro *in vivo*). Amplicons were run on agarose gels, quantified using Image J software, distribution is shown along the gradient as a percentage of total mRNA. Fraction #1 corresponds to the top of the gradient (free mRNAs) and #21 corresponds to the bottom of the gradient. Translated mRNAs are associated with the heavy polysomal fractions (from fraction 17 to 21). Figure 4 is a representative result of six independent experiments.

We analyzed the distribution of selected mRNAs along the gradient, in unfertilized and fertilized eggs. In unfertilized eggs, the majority of the mRNAs coding for cyclin A, cyclin B and the ribonucleotide reductase small subunit R2 were present on top of the gradient, suggesting that these mRNAs were not associated with polysomes at this stage (Figure 4). Fertilization induced a change in the distribution of these mRNAs along the sucrose gradient: they shifted to the heaviest polysome fractions in fertilized eggs. In contrast, the distribution of eIF4A mRNA was not modified by fertilization, suggesting that this particular mRNA is not recruited into polysomes (Figure 4). We then studied the distribution of these mRNAs after *in vitro* or *in vivo* puromycin treatment. Puromycin added *in vivo* dissociated active polysomes more efficiently (Supplementary Figure S4). The mRNAs after puromycin treatment concentrated in the middle of the polysome gradient corresponding to the

monosome fractions (Figure 4), in accordance with previous observations (11), indicating that the mRNAs were effectively associated with active translating polysomes.

Altogether, these results show that cyclin A, cyclin B and ribonucleotide reductase small subunit R2 mRNAs are recruited into active polysomes, while eIF4A mRNA remains untranslated following fertilization of sea urchin eggs. Collectively, these data demonstrate the differential behavior of polysomal recruitment of specific mRNAs at fertilization and thus validate our protocol for polysome gradients and translation analysis.

High-throughput processing of polysome fractions RNA purification for holistic approach

Polysome profiling is a labor-intensive procedure, and each sucrose gradient tube yields several fractions, increasing the number of samples to handle, especially for RNA purification. For purification of polysome fractions in a 96-well format and to avoid the use of hazardous substances such as phenol in large quantities, we used automated purification based on paramagnetic particle technology, such as the 'Pure RNA tissue kit' designed for the KingFisher Flex platform (ThermoFisher Scientific). Each fraction was mixed with an equal volume of chaotropic salt-containing solution provided in the kit, and processed as recommended by the manufacturer. A comparison of manual versus automated RNA purification is given in Table 1. One 96-well plate containing up to four gradients (22 fractions each) can be processed in <2 h, and yields RNA ready for subsequent steps. Sucrose gradients processed this way yielded an equivalent amount of final RNA, with quality (Supplementary Figure S5A) comparable to the manual procedure. The RNAs purified using the magnetic bead purification technique (KingFisher) were also analyzed by RT-PCR for cyclin B mRNA distribution patterns along the polysome gradient, and showed an identical distribution on the polysome gradient (Supplementary Figure S5B) compared with the conventional purification protocol (Figure 4).

The major limitation of the polysome analysis by RT-PCR (or northern blots) is that only a modest number of mRNAs can be analyzed simultaneously. A holistic approach is possible—usually for model organisms for which genomic tools are available—using pooled polysomal fractions to identify the translational status of all mRNAs present in the cell by microarrays or deep-sequencing analysis (8,15,16). NGS has become more accessible and less expensive, allowing researchers who work on emerging models or on models with scarce genomic data available to carry out large-scale analysis of gene expression, largely at the transcriptional level (transcriptomes). However, transcriptome data do not accurately reflect the functional subset of mRNAs (16). Polysome profiling and purification of mRNAs associated with polysomes grants access to full-length mRNAs. Therefore, identification of transcripts and, more importantly, of their UTRs is made possible using *de novo* assembly of NGS data. Therefore, it is now possible to employ an 'omics' assessment of translation to dissect translational gene expression programs in development, abiotic and biotic stress or ecological niches for any organism.

Table 1. Comparison of RNA extraction methods following polysome gradient fractionation (run time estimated for processing four gradient tubes with 22 fractions each)

	Phenol–chloroform extraction	Magnetic bead extraction
Hazardous reagents	+++	+
Reproducibility	++	+++
Reagent cost	+	+++
Handling time	4.5 h	0.75 h
Automated processing	/	1 h
RNA precipitation	12 h	/
Total time required	16.5 h	1.75 h

The amount of RNA varies widely among different biological samples; to obtain enough polysomal RNA for library construction, it may be necessary to fractionate and pool several gradient tubes per tested condition. High-throughput processing of RNA purification, as described above, should facilitate holistic approaches that use polysome profiling. Furthermore, deep-sequencing requires high-quality RNA for optimal library construction and downstream sequence assembly. Following gradient fractionation, isolation of high-quality RNA was achieved by double precipitation of RNAs isolated from the polysome fractions, first using isopropanol precipitation and then ethanol–sodium acetate precipitation, which were then used to prepare an RNA-seq library (Illumina RNA library kit, Supplementary Figure S6 and our unpublished data).

CONCLUSION AND PERSPECTIVES

The protocol presented in this study summarizes the critical steps of the polysome profiling technique, and illustrates its successful application to a marine model. Using the protocol presented in this paper, we recently demonstrated that the mTOR signaling pathway controls fertilization-induced cyclin B mRNA translation in two different sea urchin species (13). The number of new organisms studied is currently expanding, spanning all phyla of life, with large sets of associated genomic and transcriptomic data. Of the 31 animal phyla currently described, 12 are strictly marine phyla and represent emergent models in cellular and developmental biology (41,42). Although mRNA transcription is widely studied as the cornerstone of gene expression, the mere presence of an mRNA is not a guarantee that the protein it encodes is translated. Polysome profiling has been proven to be a powerful tool for addressing the translational status of a given mRNA species, at a specific time, in response to biological changes, such as development or adaptation to environment. However, a prerequisite for these translational studies is to preserve the translational status of the cell and polysome integrity. Our protocol highlights the critical steps of polysome purification and provides suggestions for adapting it to other models. Our protocol will be useful for investigators interested in addressing the role of translational regulation in gene regulatory networks, as part of a comprehensive study of a model organism, both in terms of molecular mechanisms and comparative evolution.

The parallel processing of multiple polysome samples described in this study has potential for the analysis of translational regulation during a time course experiment or for testing several environmental or developmental con-

ditions. It also facilitates the design of polysome profiling experiments, often described as time-consuming and complex to implement. This adaptation can be used regardless of organism/sample origin and can be implemented with mammalian polysome gradients. With this technical adaptation for parallel processing of polysome samples, polysome profiling will bring new insights into large-scale analysis of translation regulation and in general translational control, in conventional or emerging model organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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