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In vivo analysis of protein translation activity in sea urchin eggs and embryos

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Abstract

Protein synthesis is a major regulatory step of gene expression in different physiological processes including development. Translation of proteins in sea urchin is stimulated upon fertilization, and is necessary for cell cycle progression and development. Translational control is exerted through multifactorial mechanisms, including mRNA recruitment into polysomes and increased rates of translational activity. In this chapter, we review the methods used in sea urchin eggs and embryos to analyze translation activity *in vivo* both from perspectives of the proteins and of the mRNAs. First, we describe methods to quantify or visualize newly synthesized proteins with radioactive and non-radioactive labeling techniques. Next we present the polysome isolation and profiling on sucrose gradients, allowing the identification of translated mRNAs. Finally, we outline a procedure to follow the translation of a reporter luciferase protein from an mRNA microinjected into the egg.

Keywords: protein synthesis / translation / polysomes / polysome gradient / mRNA / luciferase reporter / microinjection

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Overview

Translation of mRNA is a complex and highly coordinated process, critical for protein production; both global protein synthesis rates and translational efficiencies of specific mRNAs are finely regulated to respond to a number of pathological and physiological situations, such as oocyte-to-embryo transition and early development (Hershey & Merrick, 2000; Horner & Wolfner, 2008). In eukaryotes, protein synthesis is mostly regulated at the initiation step of translation. Initiation predominantly depends on recognition of the mRNA 5' cap by the translation initiation factor 4E (eIF4E), controlled by the master regulator mTOR (mechanistic target of rapamycin) kinase (Jackson, Hellen, & Pestova, 2010). Eukaryotic mRNAs have structural *cis*-acting elements predominately located in 5' and 3' untranslated regions, providing more opportunities for specific regulation by *trans*-acting RNA binding proteins.

The sea urchin embryo is an interesting model organism in that context, since a clear switch of translation regulation occurs at fertilization, from a repressed state in the unfertilized eggs to an increase in protein synthesis activity triggered by fertilization. The protein synthesis activity increase is independent of ribosome biogenesis and transcription, utilizes stored maternal mRNAs, and is absolutely necessary for the entry into cell cycle. Translation inhibitors such as emetine block the cell cycle progression whereas transcription inhibitors have no effects on the first three cell divisions following fertilization (Dube, 1988; Gross & Cousineau, 1963; Wagenaar, 1983). Using [³⁵S]-methionine to label newly synthesized proteins after fertilization, Tim Hunt discovered a protein that was synthesized continuously and destroyed at each cell division (Evans, Rosenthal, Youngblom, Distel, & Hunt, 1983) which turned out to be the mitotic protein cyclin. This discovery led Tim Hunt to share, with Lee Hartwell and Paul Nurse, the Nobel Prize in Physiology and Medicine in 2001 for uncovering cell cycle master regulators.

Translation in eukaryotes is regulated by more than 20 translation factors, all of which are present in the sea urchin genome (Morales et al., 2006). Several translation factors, such as eIF2 (eukaryotic Initiation Factor 2), eIF4E (eukaryotic Initiation Factor 4E) and eEF2 (eukaryotic Elongation Factor 2) are activated following fertilization and participate to the general increase of translation activity triggered by fertilization (review in Cormier, Chassé, Cosson, Mulner-Lorillon, & Morales, 2016). The importance of mTOR-mediated regulation of translation during development has emerged using the sea urchin early development as a model (Cormier et al., 2016). For instance, fertilization triggers rapidly the degradation of 4E-BP, the main inhibitor of cap-dependent translation. The 4E-BP degradation is blocked by rapamycin or PP242 treatment suggesting that the translational switch induced by fertilization is dependent on mTOR activity. This pathway plays major role in the fine-tuning orchestration of cyclin B mRNA translation following fertilization (Chassé et al., 2016, 2018; Salaun et al., 2003).

The sea urchin model was also instrumental in the discovery of translational regulation at the level of mRNA recruitment onto polysomes, with the identification of several mRNAs using polysome gradients following fertilization such as cyclins, ribonucleotide reductase, histones (Alexandraki & Ruderman, 1985; Kelso-Winemiller & Winkler, 1991; Wells, Showman, Klein, & Raff, 1981). The sequencing and annotation of the sea urchin genome (Sodergren et al., 2006) and the deep-sequencing analysis of the polysomal fractions give access to the full set of translated mRNAs. Recently, we have identified the set of mRNA that are specifically translated during the egg-to-embryo transition (Chassé et al., 2018). Functional analysis of these mRNA should help establishing translational regulatory networks that control the first mitotic and early events of the embryonic development.

In this chapter, we review the methods to analyze translation activity *in vivo* in sea urchin eggs and embryos. First, we describe methods to analyze global rates of protein synthesis, to quantify or to visualize newly synthesized proteins by the classical incorporation of radioactive amino acid and by newly developed non-radioactive labeling techniques. Next we present the polysome isolation and profiling on sucrose gradients, allowing the identification of translated mRNAs at specific times of development or in response to signaling pathways activated at fertilization. Finally, we present a procedure to follow the translation of a reporter luciferase protein from an mRNA microinjected into the egg. This method makes possible the investigation of the functional *cis*-acting elements in the untranslated regions of mRNAs responsible for temporally regulated translation.

1- General fertilization and embryo culture

Protocols presented here were developed and used with the European species *Paracentrotus lividus* and *Sphaerechinus granularis*, two species evolutionary separated by 20 million years. Sea urchins, collected in the Brest area in France, were obtained from EMBRC-France at the Roscoff Marine Station. Spawning of gametes is induced by intracoelomic injection of 0.1 M acetylcholine. Eggs are collected in natural filtered seawater (NFSW) and rinsed twice by centrifugation (2 min at 2,000g). Eggs are dejellied by swirling twenty seconds in 0.7 mM citric acid / NSF (pH5) and rinsed three times with fresh NFSW. For fertilization, eggs are suspended in NFSW (5% vol-vol suspension). Diluted sperm is added to the eggs. Cultures are performed at 16°C under constant agitation. For the luciferase-reporter gene assay, the preparation, alignment and fertilization of the eggs are described in the specific section.

2 - Global protein translation analysis

2.1 [³⁵S]-methionine labeling method

Metabolic labeling of eggs and embryos with low-energy β-emitting radioisotopes such as [³⁵S]-methionine is used to follow the biosynthesis of proteins *in vivo*. All labeling reactions are done at 16°C (or at the appropriate culture temperature for a given species).

2.1.1 Pulse labeling

The pulse labeling of eggs or embryos allows analysis of protein synthesis activity at a specific time point.

- Eggs are dejellied and fertilized as described above. Embryos are cultured at a 5% vol-vol suspension in NFSW.
- At the desired time after fertilization, retrieve 500 μl of embryo suspension from the culture to a safe-lock Eppendorf tube
- Add 10 μCi/ml final of [³⁵S]-Methionine
- Incubate 10 to 15 minutes, under constant rotation on a rotating wheel.
- Pellet the eggs or embryos in a microspin centrifuge for 5 s. Discard the supernatant. Samples can be frozen in liquid nitrogen and kept at -20°C at this point until sample processing. Proceed to step 2.1.3 for lysis and radioactivity incorporation measurement.

2.1.2 Continuous labeling

- To measure the fold increase of protein synthesis at fertilization, dejellied eggs are pre-loaded with [³⁵S]-methionine (10 μCi/ml final) for 1 hour with gentle agitation. Let the eggs settle by sedimentation and wash in NFSW. Then fertilize the eggs.
- Alternatively, dejellied eggs are fertilized and [³⁵S]-methionine (10 μCi/ml final) is added to the embryo suspension five minutes after fertilization. Both methods give equivalent results.

- At the desired time after fertilization, retrieve 500μl of embryo suspension from the culture to a safe-lock Eppendorf tube.

- Pellet the eggs or embryos in a microspin centrifuge for 5 s. Discard the supernatant. Samples can be frozen in liquid nitrogen and kept at -20°C at this point until sample processing. Proceed to step 2.1.3.

2.1.3 Quantification of labeled proteins

- Lyse the cell pellets with 300 μl of ice-cold lysis buffer (or your favorite one) by five passages through a 1 ml syringe fitted with a G25 needle. Centrifuge the lysates 10 min at 13,000g in a microfuge at 4°C. Recover the supernatant containing the cytosoluble fractions.
- Spot 10 μl of the supernatant on a Whatman 3M filter (2x1cm), using 4 filters per sample.
- To measure the [³⁵S]-methionine incorporated into proteins, two filters are washed as follows:
 - 10% TCA at 4°C for a minimum of 2 hours up to overnight
 - 5% TCA at 4°C for 15 min
 - 5% TCA at 100°C for 30 min
 - 5% TCA at room temperature for 15 min
- Strain excess liquid from filters. Place each paper in a scintillation vial and add 1 ml scintillant.
- The two untreated filters are used to measure total radioactivity: place each filter in a scintillation vial with 1ml of scintillant.
- Count the radioactivity with a scintillation counter. The results are expressed as a percentage of [³⁵S]-methionine incorporation into proteins over total radioactivity recovered in cells for each time point or condition.
- To identify/visualize the labeled proteins, add SDS-PAGE loading buffer to the supernatant and load 10-15 μl on an SDS-PAGE. The separated proteins are revealed by X-ray film or PhosphorImager autoradiography.

The protein activity can be followed during normal development by continuous labeling for the first 3h of development. If a longer kinetic analysis or a later development time point is needed, pulse labeling is more appropriate. The effect of drugs, inhibitors or chemical agents on translation activity can be studied by this method. Add the reagents to be tested to the eggs or embryos culture at several concentrations, before and/or after fertilization. Use emetine, a translation elongation inhibitor effective in sea urchin, at 100 μ M to block completely the incorporation of methionine into proteins.

Typical labeling experiments at fertilization are shown on figure 1. Incorporation of [³⁵S]-Methionine into proteins is low in unfertilized eggs, as expected. Protein synthesis activity increases after fertilization as shown by continuous (Fig. 1A) and pulse labeling (Fig. 1B). When the embryos are cultured in presence of the mTOR inhibitor PP242, protein synthesis activity was strongly inhibited while some proteins remained translated (Chassé et al., 2016, 2018). The pattern of newly synthesized proteins is presented figure 2A.

Lysis Buffer: 40 mM HEPES pH7.6
 100 mM NaCl
 0.4 mM EDTA
 2 mM DTT
 0.2 mM sodium orthovanadate
 20 mM PPi
 100 mM sodium fluoride
 100 mM β -glycerophosphate
 10 μ g/ml Protease Inhibitor Cocktail (P2714, Sigma Aldrich).

Notes: Radioactive material should be used according to your institution's regulation and safety procedures, in an area designated for radioactive work. Discard liquid and solid radioactive waste in appropriate containers. [³⁵S]-Methionine at 10 mCi/ml is supplied by several companies as a colored solution stored at 4°C, which is easier to use and to detect possible contamination than a frozen, colorless product.

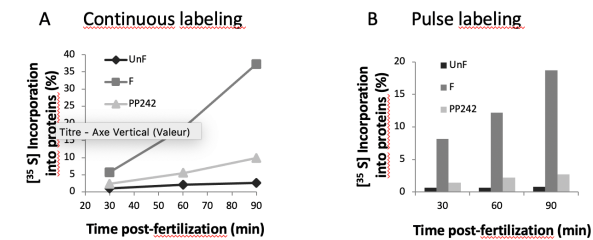


Figure 1: Detection of protein synthesis activity in sea urchin. The rate of *in vivo* protein synthesis was monitored by the kinetics of [³⁵S]-methionine incorporation into proteins after continuous (fig. 1A) or pulse (Fig. 1B) labeling, in unfertilized eggs (UnF), and after fertilization in the absence (F) or presence (PP242) of 10 μ M PP242. Radioactivity incorporation into TCA-precipitated proteins was determined at indicated times.

2.2 Non-radioactive alternative methods

2.2.1 Puromycin labeling

An alternative to classical radioactive labeling methods was recently developed for the study of mRNA translation (Schmidt, Clavarino, Ceppi, & Pierre, 2009), based on the use of puromycin to detect nascent proteins, called SunSET. The antibiotic puromycin is a structural analog of aminoacyl tRNAs that provokes dissociation of the ribosomes in the elongation step

of translation and is incorporated into the newly synthesized protein. When it is used at a concentration that does not block completely translation, puromycin incorporation reflects directly the rate of mRNA translation. Puromycin labeled peptides are detected with an antibody directed against puromycin (clone 12D10; Merck Millipore MABE343)

- Eggs are dejellied and fertilized as described above.

- At the desired time after fertilization, retrieve 200µl of embryo suspension from the culture to an Eppendorf tube.

- Add puromycin at 100 µM final.

- Incubate on a rotating wheel for 10 min.

- Pellet the eggs or embryos by a short spin, resuspend the pellet in 100 µl SDS-FIX.

- Add SDS-PAGE loading buffer to the samples and denature at 95°C for 5 minutes, load 15µl on an SDS-PAGE gel, transfer onto nitrocellulose membrane. Saturate the membrane with TBST-BSA 1% for 1h at room temperature.

- Incubate overnight at 4°C with anti-puromycin antibody diluted at 1:1000 in TBST-BSA1% with gentle agitation.

- After three 10min washes with TBST, add the secondary antibody coupled with HRP (1:5000) for 1h at room temperature. Wash 10 min three times with TBST and detect the puromycin-labeled proteins with chemiluminescent HRP substrate detection kit.

As shown in figure 2, the incorporation of puromycin into proteins as revealed by Western blot analysis, increases after fertilization and is inhibited by the mTOR inhibitor PP242, similarly to the results obtained with the [³⁵S]-methionine radioactive labeling. Note that radioactive labeling allows the observation of full-length newly synthesized proteins, whereas puromycin labels only peptides.

SDS-FIX: 2% sodium dodecyl sulfate (SDS)
10% glycerol
100 mM DTT
62.5 mM Tris pH 6.8

TBST: 20 mM Tris pH7.6
150 mM NaCl
0.1% Tween20

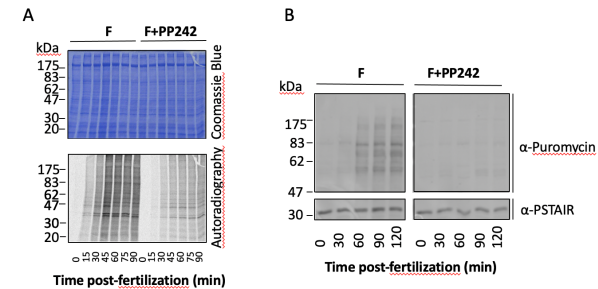


Figure 2: Pattern of newly synthesized proteins detected by [³⁵S]-methionine (A) or puromycin (B) incorporation. A- Control (F) or PP242-treated embryos were incubated with [³⁵S]-methionine by continuous labeling following fertilization. The pattern of proteins obtained is shown after separation on an SDS-PAGE. Total proteins are stained by Coomassie blue, and newly synthesized proteins are detected by autoradiography. B- After puromycin incorporation into control (F) or PP242-treated embryos, total protein extracts are separated on SDS-PAGE and the newly synthesized peptides are analyzed by western blot with an antibody directed against puromycin. CDK1 immunoblot (revealed with the anti-PSTAIR antibody, Sigma) is shown as a loading control.

2.2.2 Click reaction

Another non-radioactive alternative for protein synthesis detection relies on the *in vivo* incorporation of aminoacids or puromycin homologs containing reactive azide or alkyne groups

into proteins followed by chemoselective ligation ("click-it" reaction) of a fluorescent dye or biotin group. Similar to [³⁵S]-methionine, methionine analogs HPG (homopropargylglycine) and AHA (L-azidohomoalanine), or modified translational inhibitor OPP (O-propargyl-puromycin) are added to cultured eggs or embryos and incorporated into proteins during active protein synthesis. Protein synthesis can be detected by performing the click reaction with the corresponding click detection reagent suitable for biochemical analyses by SDS-PAGE, identification by mass spectrometry or for in situ analysis at the cellular level. Using the click-it reaction with HPG and OPP coupled with AlexaFluor 488 detection, Oulhen and coll. recently showed that translation activity is turned off in the primordial germ cells at the blastula stage and is restored to an activity similar to that of neighbor cells at the gastrula stage (Oulhen, Swartz, Laird, Mascaró, & Wessel, 2017). The reader is referred to chapter 11 (this volume) for detailed protocols using the Click-It reaction.

3- Polysome profiling using sucrose gradients

Polysome profiling is the classical standard for translation analysis from the perspective of the mRNA (King & Gerber, 2016). The translation rate is associated with the number of ribosomes translating simultaneously the same mRNA. Translated mRNAs which associate to several ribosomes (polysomes) are separated from untranslated mRNAs on sucrose gradient. A critical point is to verify that mRNAs present in polysome fractions are associated with *bona fide* translating polysomes, and not with co-sedimenting mRNPs (messenger ribonucleoparticles) or stalled polysomes. Before application to the sucrose gradient, samples are treated with puromycin, which only dissociates active polysomes. The mRNAs associated with active translating polysomes shift from the polysome fractions to the middle of the sucrose gradient after puromycin treatment, whereas co-sedimenting untranslated mRNAs are unaffected (Blobel & Sabatini, 1971; Kang & Pomeroy, 2012). The RNA samples can be

used for targeted analyses on a specific mRNA as well as for the identification of the cellular subset of translated mRNAs by deep-sequencing (Chassé, Boulben, Costache, Cormier, & Morales, 2017).

3.1 Cell lysis

- Remove the eggs jelly as described above and fertilize the eggs as a 5% suspension in NFSW.
- Five minutes before sample collection, treat the eggs or embryos with 100 µM emetine. Emetine, a translation elongation inhibitor, is used to effectively prevent ribosomes from running off the mRNA and therefore to accurately reflect the translational status of the cell.
- If a puromycin-treated sample is needed, add 0.6 mM puromycin in sea urchin embryo culture 20 minutes before collection, then proceed as above.
- Collect 5 ml embryos suspension at the required time or condition by brief centrifugation.
- Resuspend the pellet in 1 ml of cold Polysome lysis buffer and transfer to a Dounce homogenizer. Ten strokes of B (tight) pestle are enough to lyse 100% of the cells. All steps should be done at 4°C, on ice or in the cold room.
- Centrifuge the lysate for 10 min at 13,000 rpm in a tabletop centrifuge to remove nuclei and cellular debris. Then transfer the supernatant to a clean microtube.
- 500 µl aliquots are frozen in liquid nitrogen and kept at -80°C until further use for polysome fractionation (step 3.3).
- Measure the absorbance at A_{260nm} of a 5 µl sample of lysate diluted in 1 ml of water using a spectrophotometer. Typical yield for sea urchin samples is usually between 20 and 40 OD_{A260} per ml of lysate.
- Check the quality of your polysome lysate: purify total RNA from a 100 µl aliquot of lysate by phenol-chloroform (pH4) extraction and isopropanol precipitation. RNA pellet is

resuspended in RNase free water and separated on a TBE-agarose gel. The 28S and 18S ribosomal RNAs must be intact.

Polysome lysis buffer: (prepared in RNase-free water)	10 mM Tris pH 7.4 250 mM KCl 10 mM MgCl ₂ 25 mM EGTA 0.4% Igepal 5% sucrose
add extemporaneously:	1 mM DTT 10 µg/ml aprotinin 2 µg/ml leupeptin 100 µM emetine 40 U/ml RNase inhibitor.

3.2 Gradient preparation

To obtain a linear gradient for the polysome fractionation, we use the Gradient Master device (BioComp) and tubes fitted for a Beckman SW41 rotor.

- Prepare two gradient buffers containing 15% or 40% sucrose (make 6 ml of each solution per gradient). The gradients are prepared at room temperature.
- Trace a line on the ultracentrifuge SW41 tube using the marker block (upper edge for short gradient caps). Pour 15% sucrose buffer into the tube up to 2 mm above the line (roughly 6ml). Load the 40% sucrose buffer into a 10 ml syringe with a layering cannula attached, introduce the cannula vertically to the bottom of the tube and slowly layer the heavy sucrose solution (roughly 6 ml) until the interphase is exactly at the level of the line.
- Carefully remove the needle from the tube. Cap the tube with the "short gradient cap", so the excess 15% sucrose and air can go through the cap's escape hole. No air bubble should be left inside the tube. Remove the excess of sucrose buffer in the cap by pipetting it out. Repeat for desired number of tubes (2, 4 or 6 total), and place filled tubes in magnetic tube rack.

- Choose the "SW41 Short Gradient" program with the corresponding range of sucrose percentage following the manufacturer's instructions. The 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.
- Store the gradients at 4°C for at least 1h before spin. The gradients should be handled with care to avoid disturbance. Remove the cap before loading the lysate.
- Prepare 50% sucrose chase solution in RNase-free water, and store at 4°C until use. 100ml of solution is enough for 6 gradients fractionation.

Gradient sucrose buffers: (prepare in RNase-free water)	10 mM Tris pH 7.4 10 mM MgCl ₂ 250 mM KCl 25 mM EGTA 1 mM DTT 15% or 40% (w/v) sucrose.
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3.3 Gradient running and fractionation

- Load the cell lysate prepared in step 3.1 by gently pipetting it carefully on top of the sucrose gradient prepared in step 3.2. Optimal separation of the 40S, 60S and ribosome peaks in sea urchin is obtained with 10 OD_{A260} loaded on the gradient. The maximum volume of lysate that can be deposited on the gradient is 500 µl, with a maximum of 25 OD_{A260}.
- The puromycin-treated lysates are incubated with 0.5 M KCl for 15 min at 4° C then for 15 min at 37° C, before applying them on the sucrose gradient.
- Run the ultracentrifuge at 38,000 rpm for 2.5 h in a Beckman SW41 Ti rotor at 4°C. Set the acceleration to its maximum value, and the deceleration to its minimum value. Keep the gradients at 4°C for immediate fractionation.
- During ultracentrifugation, prepare the ISCO Density Gradient Fractionation System (or similar fractionation system), wash first with 0.1 M NaOH, and then rinse thoroughly with DEPC-treated water. Set the peristaltic pump at a speed corresponding to 0.9 ml/min. Fill the

system with the 50% sucrose chase solution up to the piercing needle, avoiding the introduction of air bubbles, which will disturb the gradient. Connect the centrifuge tube to the piercing apparatus, and injected the 50% sucrose chase solution by puncturing the tube from the bottom, pushing out the gradient in a continuous manner into the flow cell. Collect 21 fractions (600 μ l) on ice to avoid RNA degradation. At this point, proceed to phenol-chloroform extraction or freeze in liquid nitrogen and store at -80°C until use.

- Extract the RNAs of each fraction with one volume of acid phenol-chloroform (pH4, vol:vol), precipitate with 1 μ l of glycogen (20 mg/ml stock) and one volume of isopropanol overnight at -20°C or 2-3 hours at -80°C .

- Pellet the RNAs at 13,000 rpm for 10 min at 4°C in a tabletop centrifuge, wash the pellet with 1 ml of 70% ethanol, pellet again and leave it to air-dry for 20 min. Resuspend the RNA pellet in 30 μ l RNase-free water. The RNAs are stored at -80°C .

- Check the quality of the RNA and the polysome profile on an agarose gel.

Figure 3 outlines the polysome gradient experiment. A typical polysome gradient profile first shows 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 peaks intensity will vary according to the general translation activity, the peaks will be very small shortly after fertilization, and will increase as development proceed. The reader is referred to a recent publication (Chassé et al., 2017) for more details.

3.4 Analysis of mRNAs associated to polysomes

The presence of an mRNA in the polysomal fractions is an indication of its translation. The repartition of an mRNA along the polysome gradient is analyzed by RT-PCR using an equal RNA volume (5 μ l) of each polysome gradient fraction as template. mRNAs are first

reverse transcribed using random primers, and amplified by PCR using specific primers. Dilute the cDNA in RNase-free water for the PCR reaction so that amplification is in the linear range; test RT-PCR must be conducted for each primer pair. PCR products are then analyzed on 2% agarose-TBE gels, scanned on a Typhoon Trio (GE Healthcare Life Sciences), and quantified using ImageJ software. Express the repartition of the mRNA along the gradient as a percentage of mRNA in each fraction over total mRNA.

Furthermore, the polysome gradients allow access to all translated mRNAs by sequencing the mRNAs present in the polysomal fraction compared with the total RNA pool. The pool of translated mRNAs identified across the genome represents the translome. Following gradient fractionation, polysome fractions are pooled and RNAs is precipitated twice first using isopropanol precipitation and then ethanol-sodium acetate precipitation. This pure high-quality RNA is used to prepare the RNA-seq library for deep-sequencing. The translome at the egg-to embryo transition in sea urchin has been described recently (Chassé et al., 2018). The reader is referred to this publication for sequencing data treatment and results from polysome gradient analysis.

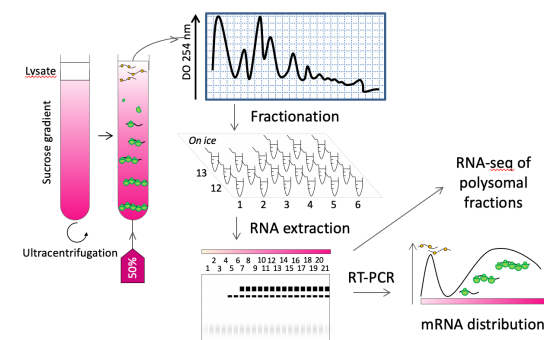


Figure 3: Overview of the polysome isolation and profiling. Lysates from eggs or embryos are

loaded on a linear sucrose gradient and untranslated mRNAs are separated from polysome-associated mRNAs by ultracentrifugation. The gradient, monitored using a flow cell coupled to a spectrophotometer, is fractionated into equal fractions from which RNA is extracted. The presence of a given mRNA species is analyzed by RT-PCR amplification in each fraction, and the distribution of the mRNA along the gradient reflects its translational status. The RNAs present in the polysomes fractions may be sequenced to have access to the full subset of translated mRNAs.

4 - Luciferase reporter genes

General translation can be studied at the cellular level by following the production of an enzyme encoded by a reporter gene, which is introduced as an mRNA after *in vitro* transcription by microinjection into the egg. Luciferase is a popular choice as a reporter because enzyme is functional immediately upon translation and the detection assay is rapid, sensitive and quantitative. Firefly luciferase catalyzes the mono-oxygenation of the substrate luciferin with emission of light, detected with a luminometer. Until now, microinjection represents the only technical means to efficiently deliver an mRNA at a defined concentration into the sea urchin egg. The protocol given below allows the detection of translation activity after microinjection of luciferase reporter mRNAs into only 5 sea urchin eggs, in triplicates.

4.1 Preparation of the mRNA

We use the pT7TS-Fluc plasmid, which contains the firefly luciferase coding region flanked with the 5' and 3' untranslated regions of the *Xenopus* β -globin mRNA, under the T7 promoter (Costache et al., 2012). The plasmid containing the Firefly luciferase is first linearized with BamHI, a restriction enzyme that cuts downstream of the 3'UTR and poly(A) signal, then synthetic mRNA is transcribed *in vitro* with bacteriophage T7 RNA polymerase (please see

chapter 9 vol 150 and chapter 10 this vol), in the presence of 7'-methyl guanosine, necessary for efficient *in vivo* translation. Finally, the mRNA produced is purified to remove unincorporated nucleotides, quantified and quality-checked on an agarose gel.

- Digest 20 μ g of highly pure plasmid with the appropriate enzyme to linearize. Check complete linearization of a 200 ng-aliquot by agarose gel electrophoresis.

- Purify the linearized DNA by phenol-chloroform (pH 8) extraction followed by ethanol precipitation in presence of 100 μ M sodium acetate. Centrifuge the pellet, wash with ice-cold 70% ethanol, dry the pellet for 10 minutes and resuspend in 40 μ l RNase-free water.

- Assemble the 20 μ l *in vitro* transcription reaction using the T7 mMACHINE kit (Ambion AM1344)

linearized DNA template	6 μ l (1 μ g)
2x NTP/CAP mixture	10 μ l
10x reaction buffer	2 μ l
Enzyme Mix	2 μ l

- Incubate 2 h at 37°C. Add 1 μ l (2U) of TurboDNase, to remove DNA template, and incubate 15 min at 37°C.

- Purify the RNA using the MegaClear kit (Ambion AM1908) according to the supplier.

- Quantify 2 μ l of the purified RNA on a NanoDrop, analyze the quality of a 300 ng aliquot on an agarose gel. The RNA should migrate as a sharp band.

- Keep the RNAs at -80°C until use.

- Prepare the injection solution with 150 ng/ μ l RNA and 1mM Alexa Fluor 488-dextran (Molecular Probes) in RNase free water. Centrifuge the mix at 13,000g for 5 minutes before loading in the microinjection pipettes.

4.2 Experimental injection system

The main objective of this step is to be able to measure sufficient translational activity in intensity and reproducibility in a limited number of eggs. For this reason, we have developed a procedure that allows triplicate measurement of translational activity in batches containing 5 unfertilized eggs. Since we want to follow the translational activity in the first minutes after fertilization, the eggs are microinjected just before fertilization.

4.2.1 Glassware preparation for eggs alignment:

In order to easily manipulate a small number of microinjected eggs to measure *in vivo* the translational activity, we have developed glassware assemblies that make it possible to perform experimental points in triplicates (Fig. 4A). Cover slides (24x60 mm) are soaked in a 1% protamine sulphate solution in distilled water for 1 minute (the timing is critical). Following protamine coating, cover slides are washed thoroughly with distilled water and air-dried. Using a diamond glass pen cutter, the cover slides are carefully cut into 2x24 mm pieces. Then 3 coverslip pieces are fixed to a 40 mm long capillary with melted wax. Each coverslip piece needs to be fixed at eight-tenths of one of its end, since the outgoing end of the side attached to the capillary is important for easily handling the glass rectangle with forceps. Finally, the capillary is fixed in a 60 mm Falcon plastic petri dish with melted wax. A mark is made outside the plate using a pen at 5 mm from the end of each coverslip piece to delimit the zone where the eggs will be aligned. Glassware assemblies fixed in petri dishes can be kept several months at room temperature before use.

4.2.2 Preparation of eggs:

Eggs are dejellied by swirling in 10 ml 3.5 mM citric acid / NFSW (pH 5) for 1 minute (the timing is critical) in an agarose-coated petri dish (1% agarose in NFSW). The eggs are

swirled and concentrated in the center of the petri dish and are transferred successively to 2 other agarose-coated petri dishes containing NFSW using a plastic Pasteur pipette. The eggs are allowed to settle again and transferred as above into a third agarose-coated petri dish (90 mm diameter). Dejellied eggs can be stored at 16°C for up to 3 hours without fertilization problem, but in our lab, they are preferentially used in the hour following treatment.

4.2.3 Alignment and microinjection of the eggs.

Before rowing the eggs, petri dishes (one dish by experimental condition required, maximum 5 different conditions) containing glassware assemblies are filled with 10 ml NFSW maintained at 16°C. Using a pulled Pasteur pipet, 5 to 10 dejellied eggs are aligned on the dedicated surface of the protamine sulfate coated coverslip piece. As soon as they are aligned, the unfertilized eggs are microinjected. We use the FemtoJet injection system and we use the free-flow method. This procedure results in the injection of approximately 5% of the volume of each egg. The mRNA solution for injection is prepared as in step 4.1. Fertilization of the eggs is performed rapidly after microinjection procedure. When needed, drugs (emetine, PP242 or other) are added five minutes following fertilization. Fluorescent marker present in the microinjection solution allows standardization of the volume microinjected into the egg (Fig. 4B). For quantification of fluorescent signals, image acquisitions are performed 1 hour and 3 hours after microinjection at low magnification (100x) to maximally capture fluorescent pixels. Eggs that show more than 15 % decrease between the two times of the image acquisition of the fluorescent signal are considered to be lethally affected. The population of lethally affected eggs does not exceed 4%.

4.3 Detection of the luciferase translated *in vivo*

Four hours after fertilization, each coverslip piece is gently manipulated using fine forceps and the opposite end of the rectangle on which are aligned the eggs is immersed in 50 μ l ONE-Glo™ reagent (Promega) in a 2.2 ml dolphin tube (Fig. 4C). After 1 min centrifugation at 13,000g, the 50 μ l solution is transferred in a 96 well microplate and luminescence is measured in a TriStar luminometer (Berthold). The luminescence signal obtained from 5 microinjected eggs is standardized with respect to the fluorescence signal. Results are expressed in relative light unit (RLU) per egg.

As shown in figure 4D, using this experimental set-up we were able to detect the translation from the injected mRNAs following fertilization, that is strongly inhibited by the mTOR inhibitor PP242, reflecting the translation regulation occurring for general mRNAs. The influence of untranslated regions on the translation regulation of a particular mRNA can be now studied by replacing the β -globin untranslated region by the 5' and/or 3' UTR of the mRNA of interest.

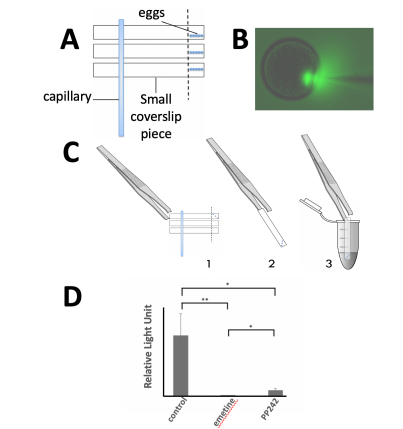


Figure 4: Measurement of *in vivo* translation activity using luciferase reporter gene. A- Glassware assembly for injection of five eggs in triplicates for one experimental condition. B-

Microinjection of egg under fluorescent microscope and imaging of the fluorescent signal. C- Using forceps, the coverslip piece is removed from the capillary (1 and 2) and the end of the rectangle where the eggs are aligned is immersed in ONE-Glo™ reagent (3). D- After standardization with respect to the fluorescence signal, *in vivo* translation activity is expressed in relative light unit (RLU) per egg. Translation measured in fertilized embryos (control) is sensitive to emetine treatment, and is strongly inhibited by the mTOR inhibitor PP242.

5- Conclusion

The sea urchin embryo has been central to several major discoveries for the understanding of protein synthesis regulation (review in (Ernst, 2011)). Classic proven methodologies to analyze global and specific rates of protein synthesis remain widely used, and when they are coupled with deep-sequencing or mass spectrometry, they grant access to the full spectrum of actors involved in translational control. The development of new techniques devoted to the *in situ* analysis of translation will open venues to the single-cell translation approaches. In the emerging picture of gene regulation, translational control appears as paramount process rather than an additional, fine-tuning layer, and the sea urchin model has all the required qualities to still play a major part to uncover new regulatory mechanisms in translation regulation.

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