Video Article

TRAP-rc, Translating Ribosome Affinity Purification from Rare Cell Populations of *Drosophila* Embryos

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Abstract

Measuring levels of mRNAs in the process of translation in individual cells provides information on the proteins involved in cellular functions at a given point in time. The protocol dubbed Translating Ribosome Affinity Purification (TRAP) is able to capture this mRNA translation process in a cell-type-specific manner. Based on the affinity purification of polysomes carrying a tagged ribosomal subunit, TRAP can be applied to translatome analyses in individual cells, making it possible to compare cell types during the course of developmental processes or to track disease development progress and the impact of potential therapies at molecular level. Here we report an optimized version of the TRAP protocol, called TRAP-rc (rare cells), dedicated to identifying engaged-in-translation RNAs from rare cell populations. TRAP-rc was validated using the Gal4/UAS targeting system in a restricted population of muscle cells in *Drosophila* embryos. This novel protocol allows the recovery of cell-type-specific RNA in sufficient quantities for global gene expression analytics such as microarrays or RNA-seq. The robustness of the protocol and the large collections of Gal4 drivers make TRAP-rc a highly versatile approach with potential applications in cell-specific genome-wide studies.

Video Link

The video component of this article can be found at http://www.jove.com/video/52985/

Introduction

Understanding how cells acquire specific properties during development is crucial in order to appreciate the complexity of organs and their potential evolution towards pathological conditions. There is a big research push to decipher the gene regulatory networks that underly cell specification and differentiation by unrevealing global gene expression profiles, Pol II binding status, transcription-factor occupancy on regulatory sequences or post-translational modifications of histones.

To assess gene expression at a global level microarrays and RNA-seq approaches are used. Microarrays allow to detect mRNA abundance, whereas RNA-seq is better geared to informing on the different types of RNAs including coding and noncoding RNAs like microRNAs, lncRNAs or snRNAs. However, these techniques cannot differentiate actively-transcribed, steady-state mRNA, actively-translating mRNA, and mRNA entering the degradation process. Measuring steady-state mRNA levels is known to be a poor estimation of proteome composition[1-3]. In contrary, identifying actively-translating mRNA gives a more accurate picture of protein production.

However, transcriptomic studies have mainly been led at whole-organism level by comparing gain or loss of function of a specific factor to wild-type condition[4-7] or on dissected tissue, making gene expression profiles difficult to interpret. Recent advances in cell targeting tools, affinity purification and sensitivity improvements now allow to perform genome-wide analyses on small cell populations or even single cells in a living organism.

In *Drosophila*, large collections of transgenic lines enable specific temporal and spatial targeting of different tissues and cell subpopulations via the GAL4/UAS system[8-10] yielding more accurate quantification of the molecular mechanisms required for cell function.

Among newly developed approaches polysome profiling by fractionation was described as a way to capture actively-translating RNA[11]. This method based on sucrose gradient centrifugation allows the selection of polysome fraction and determination of mRNA translated genome-wide when analyzed with microarrays or deep sequencing. Polysome isolation can be coupled with nuclease digestion to identify ribosomal footprints corresponding to RNA fragments protected by the ribosomes during the translation process[12]. Ribosomal footprinting increases the accuracy of quantification of RNA translation and RNA sequence-level resolution and ribosome positioning, makes it possible to measure rate of translation. However, to date it has not been applied to cell-specific isolation of translatomes, mainly due to the strong decrease in RNA yield obtained at the end of the ribosome footprinting process. Given that the size selection of RNA may generate potential false-positives from different RNPs that

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could also be protecting RNA in a similar manner, further developments are needed to improve ribosomal footprinting and adapt it to cell-specific approaches.

One of such methods, called the Translating Ribosome Affinity Purification (TRAP) has been described in 2008 by Heiman *et al*. and applied to isolate the translatome from a subset of neurons in mice. By epitope-tagging a ribosomal protein in a cell-type-specific manner, polysomes can be selectively purified without the laborious step of cell dissociation and sorting. In this case, the 60S ribosomal protein L10a at the surface of the ribosome was tagged with eGFP [13]. Since 2008, several studies have used this method in different species, from mice[14-19] to *X. laevis*[20,21], zebrafish[22,23] and Arabidopsis thaliana [24]. The TRAP method has also been adapted to the *Drosophila* model and used to purify cell-type-specific mRNAs from neuronal cells [25] and astrocytes [26]. The versatile binary GAL4/UAS system was used to express GFP-tagged RpL10A in a tissue-specific manner. Heads of adult flies were dissected to perform polysome selection from neuronal cells (targeted by pan-neural Elav-Gal4 driver) and isolated RNAs were sequenced. The large number of up-regulated genes corresponded to those known to be expressed in the nervous system, indicating that the approach has good specificity and prompting us to adapt it to rare cell populations (less than 1% of cells) present in developing *Drosophila* embryos.

Within the *Drosophila* model, the embryonic stage is a model of choice for the study of developmental processes, as the molecular actors and morphogenetic movements are evolutionarily conserved. The only tissue-specific transcriptomic approaches conducted so far in this model have been performed using cell or nuclear sorting and have only been able to study steady-state transcriptome [27-31], creating a need for methods dedicated to tissue/cell-specific translatome profiling in the fly embryo. Here we report the first TRAP protocol dedicated to *Drosophila* embryos. With this method, engaged-in-translation mRNA in a very restricted cell population of around 100 muscle cells per embryo was successfully isolated with high quality and specificity. The binary GAL4/UAS system is used to drive the expression of GFP-tagged RpL10A in subpopulation of muscles without any toxicity, no apparent phenotypes or developmental delay as shown previously[25]. *Drosophila* embryos possess 30 muscles per hemisegment that will give rise to the musculature of the larva. By using a regulatory region discovered in the vicinity of the slouch identity gene, 6 out of the 30 multi-nucleated muscles are targeted. In this assay, ribosomes are immobilized on the mRNA using the translation elongation inhibitor cycloheximide. Cytosolic extracts are then used for affinity purification with GFP antibody-coated magnetic beads. Quality of purified RNA was validated using bioanalyzer. Quantitative reverse transcription PCR (RT-qPCR) was used to determine the specificity and sensitivity of mRNA isolation and demonstrated that our optimized protocol is highly efficient.

Protocol

1. Fly Line Generation

1. Generate two different constructs. In the first construct, RpL10A cDNA (ribosomal protein subunit L10a) is cloned downstream of GFP into pUASP-PL-GFP-Nter plasmid (modified version from[32]). The use of a germline promoter allows a more polyvalent usage of the transgenic line.
   NOTE: Obtain the second construct by cloning the regulatory sequence driving tissue-specific expression of the slouch gene upstream of GAL4 (TF) using pPTGAL4 plasmid.
2. Inject plasmids separately into w^{1118} flies and insert constructs into the fly genome by P-element insertion (according to standard procedure)[33].
3. Select transformants in order to recover fly lines with constructs on two different chromosomes. The final TRAP line is created by combining these two lines (genetic crosses) in order to get one double-transgenic stable line. F0: w-; Cyo, UAS EGFP::RpL10A; MKRS/TM6B x w-;Cyo/Scu;Slouch Gal4/TM6B. F1: w-; Cyo, UAS EGFP::RpL10A; Slouch Gal4/TM6B. Massively amplify this line and collect the desired-staged embryos.

2. Embryo Collection

1. Prepare 8 large cylindrical population cages containing around 40 g of young flies per cage (around 180,000 flies/cage).
2. Proceed with so called pre-lays that allow flies to clear developing embryos from their oviducts. For that, lay the flies for 1 hr on two 11 cm-diameter petri dishes containing a mixture of solidified agar and grape juice and cover ⅓ of the surface with freshly-made yeast paste. After 3 exchanges of grape juice plates collect the real embryos on similar freshly made plates.
   NOTE: Incubation time will vary according to developmental time-window studied.
3. Remove plates from cages and leave them at the same temperature for the time needed to obtain correct developmental stage (*e.g.*, 3 hr of egg-laying + 10 hr of additional incubation gives embryos from stage 10-13 hr After Egg Laying (AEL)). Resuspend the plate content (embryos, yeast paste, dead flies) with 50 ml of water using a brush.
4. Go through a series of sieves (700 µm, 355 µm, 112 µm) to separate embryos from dead flies and remaining body parts and collect embryos retained on the smaller-diameter sieve, then wash briefly in deionized water. Do not use more than 18 plates per collection as it can clog the sieves.
5. Dechorionate embryos in 4.5% bleach in deionized water for 2 min and rinse thoroughly with deionized water for 30-60 sec. Incubate embryos in PBS 0.01% Tween 20, 100 µg/ml cycloheximide, for 5-10 min at RT under agitation.
6. Dry embryos on cellulose absorbent sheets and transfer them to microcentrifuge tubes. Weigh the tubes and flash-freeze the embryos by immersion in liquid nitrogen. At this stage, the dried embryos can be stored for several months at −80 °C.

3. Preparation of Magnetic Beads Coupled to GFP Antibody

1. Thoroughly resuspend the Protein G magnetic beads in the original bottle by gentle agitation.
2. Transfer 90 µl of beads to an RNase-free tube and collect them on a magnet (30–60 sec). 90 µl of beads is necessary to perform immunoprecipitation (IP) with 1 ml of lysate containing 60-80 mg/mL proteins. Respect ratios to prevent bead saturation. Use several tubes according to protein amount.

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3. Wash beads with 1× PBS 0.01% Tween 20 (500 µl) and collect beads on the magnet to discard supernatant. Add 30 µg of GFP antibody in 350 µl of PBS 0.01% Tween 20 to the beads and incubate with slow end over end mixing for 30 min at RT.
4. Collect beads on the magnet (30-60 sec), discard supernatant and rinse in 500 µl of polysome extraction buffer (Hepes 10 mM, KCl 150 mM, $MgCl_2$ 5 mM, DTT 0.5 mM, Triton 1%, Cycloheximide 100 µg/ml, Protease inhibitor 1×, RNase inhibitor 100 U).
5. Collect beads on the magnet and add 500 µl of blocking buffer (BSA 0.1 µg/µl, yeast tRNA 0.1 µg/µl, glycogen 0.1 µg/µl in polysome extraction buffer).
6. Incubate for 30 min at RT and repeat this step once with fresh blocking buffer.
7. Collect beads on the magnet and rinse in 500 µl of polysome extraction buffer, then recollect beads and proceed immediately to the immunopurification step (section 6).

4. Lysate Preparation

1. Before starting, set up the program on the multi-directional fast speed beads grinder: 2×10 sec at 5,000 rpm, 15 sec pause. Transfer dried embryos (1.5 g) to 15 ml tubes prechilled on ice containing polysome extraction buffer, and homogenize immediately. Homogenize 1.5 g of embryos in 4 ml of polysome extraction buffer.
2. Spread homogenized embryos in two prechilled 2 mL tubes and grind the embryos by running the homogenizer program. Transfer the lysate into fresh prechilled microcentrifuge tubes on ice and prepare a postnuclear supernatant by centrifugation at 4 °C for 10 min at 2,000 *g*.
3. Transfer supernatant to a fresh prechilled microcentrifuge tube on ice, add 1/100 sample volume of 10% Nonidet P-40 to the supernatant (final concentration = 0.1%), and mix it gently by inverting the tube.
4. Pulse-centrifuge the sample in a minifuge to collect the liquid at the bottom of the tube, add 1/9 sample volume of 300 mM 1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine (DHPC) (final concentration = 30 mM), mix it gently by inverting the tube, and incubate the mixture on ice for 5 min (mix by inverting several times during incubation).
5. Prepare the post-mitochondrial supernatant by centrifugation at 4 °C for 10 min at 20,000 *g*. Measure protein concentration by the Bradford method: concentration in lysate should be between 60-80 mg/ml. Take the supernatant to a fresh new prechilled microcentrifuge tube and proceed immediately to the pre-absorption step (section 5).

5. Pre-absorption

1. Thoroughly resuspend the magnetic beads by gentle agitation and transfer 30 µl of beads into a microcentrifuge tube at RT (30 µl of beads/1 ml embryonic lysate).
2. Collect beads on the magnet, pipette off the supernatant, and resuspend the beads in polysome extraction buffer (500 µl). Collect beads on the magnet, add embryonic lysate, and incubate for 1 hr at 4 °C on a rotator.
    1. Remove beads and keep supernatant on ice for the immunopurification step. Before immunopurification, keep 100 µl of supernatant (input) to compare global RNA sample with the RNA sample collected after immunopurification, by RT-qPCR for example.

6. Immunopurification

1. Add 1 ml (corresponding to 60-80 mg/ml protein) of pre-absorbed lysate to an RNase-free tube containing 90 µl of blocked beads coupled to GFP antibody. Incubate the samples at 4 °C for 2 hr with gentle end-over-end mixing in a tube rotator. Alternatively, perform the incubation O/N at 4 °C.
2. After incubation, collect beads on the magnet. Use a minifuge to spin down the beads, then resuspend them in 500 µl of wash buffer (Hepes 10mM, KCl 350mM, $MgCl_2$ 5mM, Nonidet P-40 1%, DTT 0.5 mM, cycloheximide 100 µg/ml, RNase inhibitor 100 U) and collect them on the magnet. Repeat this step two more times.
3. Change the beads to a new prechilled RNase-free tube and wash twice. Collect beads on a magnet and proceed to RNA extraction by adding 1 ml TRiZol directly to the beads and follow the manufacturer's instructions.

7. RNA Clean-up and Quality Assessment

1. Use an RNeasy Micro Kit as per the manufacturer's instructions. At completion, elute RNA with (x) µl RNase-free water. Warm for 2 min at 60 °C, and store snap-frozen samples at -80 °C. Check RNA quality using bioanalyzer (follow the manufacturer's instructions).
2. To test the specificity of the TRAPed RNA, perform reverse transcription on 3 ng of purified RNA (input and IP) according to the manufacturer's instructions (superscript III). Use the cDNA obtained for qPCR using gene-specific primer sets.

Representative Results

The *Drosophila* embryonic somatic muscle system is composed of 30 muscles per hemisegment, and each muscle has a specific set of properties: code of identity genes, position, number of fusion events, attachment sites and innervation. Identifying translated mRNA in a specific muscle subset will give information on the proteins required to form these specific muscle characteristics. Using the TRAP-based method, RNA from a subpopulation of muscles expressing the identity gene *slouch* was purified (**Figure 1A-B**). A major concern of this approach is the quality and specificity of the isolated RNA. The optimized protocol reported here enabled systematic recovery of high-quality RNA assessed with bioanalyzer analysis. The profile obtained shows no degradation of RNA (**Figure 1C**).

In other studies developed around the TRAP method, questions were raised over the specificity of the data. Here we improve the method to suppress background linked to non-specific binding of RNA to the beads or the tubes and by the optimization of the wash buffer. In order to assess the background level and efficiency of isolating slouch-expressing cells, we led RT-qPCR trials on 3 replicates of the same embryonic stage. Fold-enrichments of 4 different genes (*mef2, slouch, prospero, soxNeuro*) were calculated compared to input and normalized against the

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RpL32 gene (**Figure 1D**). These results showed 2.3-fold enrichment of pan-muscular gene *mef2* and*5.6-fold enrichment of the slouch* gene. In contrast, the two genes expressed in the neural system were depleted compared to input. Very similar fold change values were observed on 3 biological replicates, demonstrating that the protocol is robust. With Slouch-Gal4 driver and starting with 1.5 g, around 1.5x10^6 embryos were obtained that contain 100 GFP cells/embryo (total of 150×10^6 positive cells).

After running the TRAP experiment, the yield is around 25–45 ng of specific RNA depending on stage-of-interest. This material is enough to run microarray analysis or RNA-seq using an amplification protocol to build an RNA-seq library. Efficiency will depend strongly on the strength of the driver used to express RpL10A-EGFP.

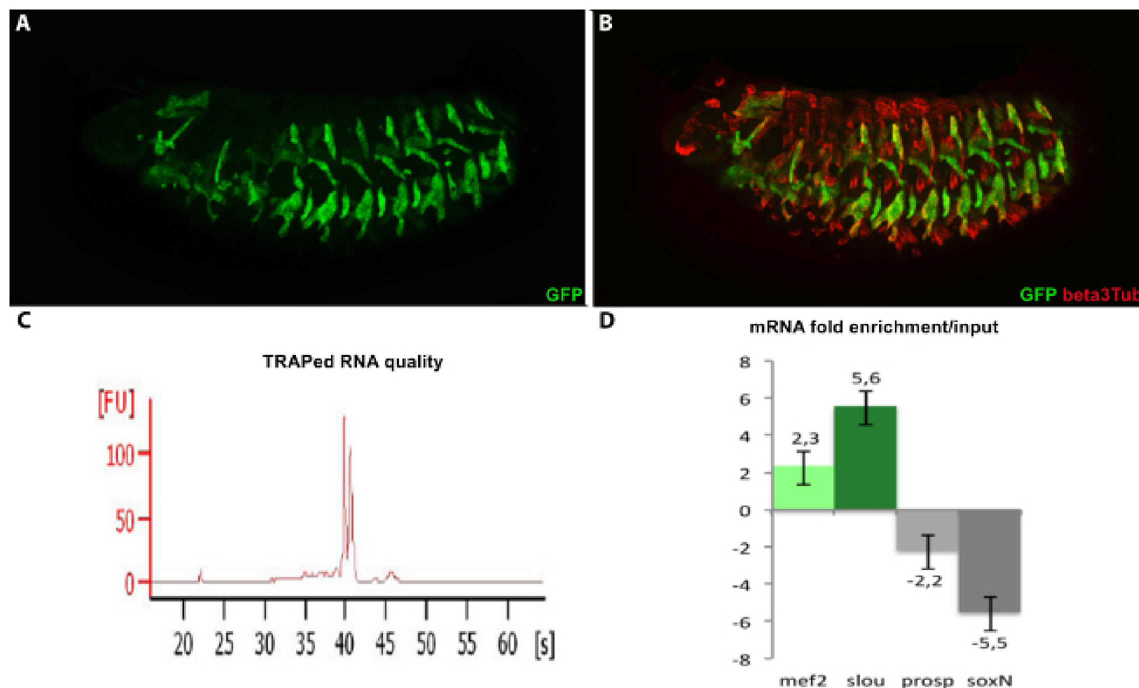

Figure 1. Quality and specificity assessment of TRAP-isolated RNA from Slouch-GAL4>UASRpL10A-EGFP embryos.

(A-B) Confocal images of a stage-16 embryo showing RpL10A-EGFP expression specifically in the six Slouch muscle cells per hemisegment (**A**). Co-localization of RpL10A-EGFP with the general muscle marker Beta3tubulin is observed on merge picture (**B**). (**C**) Quality control of TRAPed RNA ran on bioanalyzer showing perfect integrity of rRNA 18S and 28S. (**D**) RT-qPCR analysis showing high specificity of TRAP-isolated mRNA experiments on 3 biological replicates of stage-16 embryos. Fold change is calculated compared to input and normalized against the *RpL32* gene. *Mef2* transcripts present in all muscle lineages are 2.3-fold-enriched compared to input whereas more restricted *slouch* transcripts are 5.6-fold-enriched. Neural cells expressing *prospero* and *soxN* genes are depleted 2.2-fold and 5.5-fold respectively. Error bars represent standard deviation (n=3). Please click here to view a larger version of this figure.

Discussion

This paper describes a modified Translating Ribosome Affinity Purification protocol (dubbed 'TRAP-rc') dedicated to the study of rare cell populations in *Drosophila* embryos. Information is provided on the key steps for successful isolation of specific RNA with a yield suitable for microarray or RNA-seq analysis, *i.e.*, 1) quantity of biological material required and optimized procedure for embryo lysis and polysome extraction; 2) beads/antibody-to-lysate ratios for optimal immunoprecipitation; 3) steps allowing reduction of background including wash buffer composition so as to run TRAP-rc experiments with high specificity and sensitivity.

This protocol yields reproducible data making it readily applied to any other cell types. This technique makes it possible to analyze differential gene expression at the level of actively-translating mRNA and thus pave the way to understanding protein expression in a specific cell type at a specific time window. Note however that protein abundance will depend on the rate of the translation and degradation processes. A major limitation of the TRAP method is its inability to measure protein content in an accurate quantitative manner or to detect post-translational modification. Improving the quantification by measuring ribosome density along the mRNA body and, in doing so, in a cell-type-specific manner can make TRAP combined with ribosome footprinting a very powerful tool. In a recent paper[34], this combination was performed in human embryonic kidney 293 cells. The authors ran nuclease footprinting followed by affinity purification of an inducible biotinylated form of RpL10A using streptavidin beads. This is a proof of principle that it is possible to perform ribosome footprinting in a whole organism while targeting a specific cell type, the limitation being the amount of biological material required for the purpose.

Performing TRAP experiments in parallel with global transcriptomic analyses will make it possible to track proportions between newly-transcribed and actively- translating RNA. This will be deeply informative of the potential post-transcriptional mechanisms taking place in specific developmental contexts or specific cell populations—by microRNAs for example.

In conclusion, TRAP is a highly efficient, specific and sensitive method for identifying RNAs bound to actively-translating ribosomes in a cell-specific manner. This method can be used in a wide variety of organisms and tissue types. The new TRAP-rc protocol described here was

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optimized for rare cell populations (less than 1% of total cell number) and for a quantity of final material sufficient for subsequent analyses at whole-genome level.

A possible limitation of this method is the requirement of a strong driver to produce tagged ribosomes in sufficient amount to compete with endogenous untagged ones in the targeted cell type. In a recent study[25], authors estimated to 10-30% the ratio of tagged versus untagged ribosomes.

To overcome this problem increasing UAS-RpL10A-EGFP copy number should considerably improve this balance. Alternatively, adding to the described genetic background a UAS-GAL4 transgene will amplify the production of GAL4 protein and indirectly increase expression of RpL10A-EGFP. This should favor a better occupancy of tagged ribosomes on mRNA.

Note that this already efficient approach can be made even more powerful by adapting complementary methods to bring a more quantitative aspect or to discover and unravel molecular mechanisms that are essential for gene expression control.

Disclosures

The authors have nothing to disclose.

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