Chapter 18

Translating Ribosome Affinity Purification (TRAP) for Cell-Specific Translation Profiling in Developing Flowers

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Abstract

The development of a multicellular organism is accompanied by cell differentiation. In fact, many biological processes have cell specificity, such that distinct cell types respond differently to endogenous or environmental cues. To obtain cell-specific gene expression profiles, translating ribosome affinity purification (TRAP) has been developed to label polysomes containing translating mRNAs in genetically defined cell types. Here, we describe the immunopurification of epitope-labeled polysomes and associated RNAs from target cell types. TRAP has the additional advantage of obtaining only translating mRNAs, which are a better proxy to the proteome than a standard mRNA preparation.

Key words mRNA, Cell type, Posttranscriptional regulation, Transcriptome

1 Introduction

Widely used high-throughput transcriptome profiling approaches have been successful in dissecting gene regulatory networks. However, cell specificity in transcriptome profiling is often obscured by the existence of many cell types even within a simple organ. To obtain cell specificity while maintaining genome-wide coverage, several technologies have been developed [1, 2]. Laser Microdissection (LM) uses a laser beam to isolate cells of interest from fixed tissue sections under a microscope. This approach has been used in plants, and isolates cells based exclusively on their morphology and location within tissue sections [3]. Because of its labor-intensive nature, LM can obtain only a limited number of cells and has to be combined with RNA amplification, which usually introduces significant biases. Another approach to isolate specific cell types is Fluorescence Activated Cell Sorting (FACS), in which fluorescent proteins are expressed within cells of interest, and flow cytometry is used to isolate fluorescently labeled cells

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after protoplasting, i.e., cell wall digestion and cellular dissociation [4]. As a result of these treatments, the protoplasts that are purified through FACS undergo cellular stress during the procedure. In addition, the resulting sorted cell population can hardly be free of nonfluorescent cell contaminant. A similar approach is to label and isolate nuclei in cells of interest. Nuclei labeling and purification can be achieved either by expressing a fluorescent protein and flow cytometry [5], or by expressing an epitope-labeled nuclear envelope protein and subsequent affinity purification [6]. It should be noted that nuclear RNA preparations contain many unfinished mRNA molecules without further maturation, such as splicing, and may not reflect the relative abundance of translated mRNAs.

TRAP isolates translating mRNAs from cells of interest, and provides a better proxy to the proteome. Briefly, a ribosomal protein is epitope-labeled and expressed in target cells using cell type-specific promoters in transgenic plants or animals. In *Arabidopsis*, RPL18 protein can be labeled with His and FLAG tags (HF-RPL18) or with FLAG and GFP tags [7–10]. Both GFP-tagged RPL10a and HA-tagged RPL22 have been tested in mice [11, 12], and GFP-tagged RPL10a has been found to work efficiently in *Drosophila* [13]. The epitope-tagged ribosomal proteins are incorporated into ribosomes, and thus into polysomes, which can be affinity purified: total polysomes are isolated from transgenic plants or animals, and epitope tag-labeled polysomes are then purified using corresponding antibody-coated beads. TRAP does not need specialized equipment, and has the advantage of high RNA yield to readily proceed to RNA-seq without further RNA amplification [8]. The entire procedure, from polysome purification to RNA extraction, can be completed within 1 day.

2 Materials

1. Nuclease-free sterile water.
2. Nuclease-free sterile tubes.
3. Polysome extraction buffer: 100 mM Tris–HCl (pH 9.0), 200 mM KCl, 25 mM EGTA (pH 8.0), 36 mM $MgCl_2$, 1 % Brij L23 (Sigma-Aldrich, St. Louis, MO, USA), 1 % Triton X-100, 1 % IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO, USA), 1 % TWEEN 20, 2 % polyoxyethylene (10) tridecyl ether (Sigma-Aldrich, St. Louis, MO, USA), 1 % deoxycholic acid, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 40 U/mL RNase inhibitor (such as RiboLock RNase Inhibitor, Fermentas, Burlington, ON, Canada), 1 mg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA). Prepare the buffer with nuclease-free sterile water and store at 4 °C (*see* **Note 1**).

4. Wash buffer #1: 100 mM Tris–HCl (pH 8.5), 200 mM KCl, 25 mM EGTA (pH 8.0), 36 mM $MgCl_2$. Make up the buffer with nuclease-free sterile water and store at −20 °C.
5. Wash buffer #2: 100 mM Tris–HCl (pH 9.0), 200 mM KCl, 25 mM EGTA (pH 8.0), 36 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 40 U/mL RNase inhibitor. Make up with nuclease-free sterile water and store at 4 °C (*see* **Note 2**).
6. Monoclonal ANTI-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO, USA).
7. 3× FLAG peptide stock solution: dissolve 3× FLAG peptide lyophilized powder (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 M Tris–HCl (pH 7.5) with 1 M NaCl at a concentration of 25 mg/mL. Dilute fivefold with water to prepare a 3× FLAG stock solution containing 5 mg/mL of 3× FLAG peptide.
8. RNeasy mini kit (Qiagen, Hilden, Germany) or an equivalent total RNA isolation kit or reagents.
9. β-mercaptoethanol.
10. 100 % ethanol.

3 Methods

Carry out all procedures on ice or at 4 °C in a cold room unless otherwise specified.

3.1 Polysome Extraction

1. Freeze in liquid nitrogen *Arabidopsis* tissue expressing HF-RPL18 immediately after collection and grind to a find powder under liquid nitrogen using a mortar and pestle (*see* **Note 3**). Transfer the suspension of tissue powder and liquid nitrogen into a liquid-nitrogen-cooled appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw.
2. Add polysome extraction buffer as quickly as possible. Homogenize 1 Vol of pulverized *Arabidopsis* tissue powder with 2 Vol of polysome extraction buffer by gentle shaking. Incubate mixture on ice for 10 min.
3. Transfer mixture to centrifugation tubes and centrifuge at 16,000 × *g* for 10 min at 4 °C.
4. Transfer the supernatant to new centrifugation tubes and centrifuge at 16,000 × *g* for 10 min at 4 °C.
5. Transfer the supernatant to new centrifugation tubes and store at 4 °C for Subheading 3.3.

3.2 Preparation of the Anti-FLAG Agarose Beads

1. Thoroughly resuspend the anti-FLAG M2 affinity agarose gel in the vial to make a uniform suspension of the resin. Transfer 200 μL of suspension to a new centrifugation tube (*see* **Note 4**).
2. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
3. Remove the supernatant with a pipette and add 2 mL wash buffer #1 to the resin.
4. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
5. Remove the supernatant with a pipette and add 2 mL wash buffer #2 to the resin.
6. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
7. Remove the supernatant with a pipette.

3.3 Immunoprecipitation of Polysome

1. Add polysome extract from Subheading 3.1 to prepared anti-FLAG agarose gel from Subheading 3.2.
2. Incubate for 2 h at 4 °C with gentle back-and-forth shaking (*see* **Note 5**).
3. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
4. Transfer the supernatant to new centrifugation tubes, and keep beads at the bottom undisturbed (*see* **Note 6**).
5. Add 4 mL of wash buffer #2 to the beads, mix by gently inverting the tube, and centrifuge at 8,200 × *g* for 30 s at 4 °C.
6. Remove the wash buffer with a pipette and add 3 mL wash buffer #2 to the beads. Incubate tubes with gentle shaking for 10 min at 4 °C.
7. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
8. Remove the wash buffer with a pipette and add 3 mL wash buffer #2 to the beads. Incubate tubes with gentle shaking for 10 min at 4 °C.
9. Centrifuge at 8,200 × *g* for 30 s at 4 °C.
10. Remove the wash buffer with a pipette and add 200 μL wash buffer #2 and 12 μL 3× FLAG peptide stock solution to the beads. Incubate with gentle shaking for 30–60 min at 4 °C (*see* **Note 7**).
11. Centrifuge at 8,200 × *g* for 30 s at 4 °C. Transfer the supernatant to a new tube.
12. Add another 100 μL wash buffer #2 and 6 μL 3× FLAG peptide stock solution to the beads. Incubate with gentle shaking for 30 min at 4 °C.
13. Centrifuge at 8,200 × *g* for 30 s at 4 °C. Combine both supernatant to the same tube.

3.4 RNA Extraction

1. Add 1.6 mL RLT buffer from RNeasy kit and 16 μL *β*-mercaptoethanol to the supernatant and mix well at room temperature.

2. Add 1.1 mL 100 % ethanol and mix by pipetting at room temperature.
3. Apply the sample into an RNeasy mini spin column. Centrifuge at 16,000 × *g* for 15 s at room temperature. Discard the flow through.
4. Add 700 μL RW1 buffer to the spin column and centrifuge at 16,000 × *g* for 15 s at room temperature. Discard the flow through.
5. Add 500 μL RPE buffer to the spin column and centrifuge at 16,000 × *g* for 15 s at room temperature. Discard the flow through. Repeat this step one more time.
6. Transfer the spin column to a new tube and centrifuge at 16,000 × *g* for 2 min at room temperature.
7. Transfer the spin column to a new 1.5 mL reaction tube and add 30 μL of nuclease-free sterile water at 50–55 °C. Incubate for 1 min.
8. Elute RNA by centrifuging at 16,000 × *g* for 1.5 min at room temperature.
9. Repeat the above elution step with another 30 μL nuclease-free sterile water. Combine eluted RNA solution (*see* **Note 8**).

4 Notes

1. Polysome extraction buffer needs to be prepared freshly and can be prepared from stock solutions. Stock solutions of Tris–HCl, KCl, EGTA, and $MgCl_2$ need to be autoclaved.
2. Wash buffer #2 needs to be prepared freshly and can be prepared from stock solutions. Stock solutions for Tris–HCl, KCl, EGTA, and $MgCl_2$ need to be autoclaved.
3. The amount of plant tissue required for TRAP depends on the number of cell that express the tagged construct within the tissue to be collected, and on the expression level of the HF-RPL18 protein in those cells. For flowers expressing *HF-RPL18* under the promoters of *APETALA1*, *APETALA3*, and *AGAMOUS* [14], 2.5–10 mL packed flower buds can be used.
4. The ratio of suspension to packed gel volume is around 2:1, and 200 μL of suspension contains about 100 μL packed gel.
5. Extended overnight incubation at 4 °C may increase the final RNA yield.
6. The supernatant from this step can be used to test immunoprecipitation efficiency with an anti-FLAG antibody by Western blotting.
7. Agarose beads bind RNA molecules nonspecifically. Polysomes must be eluted from agarose beads for RNA extraction.

8. RNA quality can be monitored using a bioanalyzer. Quantification of isolated by using a UV spectrometer is recommended. From the amount of starting floral tissue suggested above, we usually obtain 5–10 μg of total RNA. RNA from TRAP can be used directly for mRNA isolation and RNA-seq (*see* Chapter 23).

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