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Translating Ribosome Affinity Purification (TRAP) of Cell Type-specific mRNA from Mouse Brain Lysates

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Abstract

Mammalian tissues are highly heterogenous and complex, posing a challenge in understanding the molecular mechanisms regulating protein expression within various tissues. Recent studies have shown that translation at the level of the ribosome is highly regulated, and can vary independently of gene expression observed at a transcriptome level, as well as between cell populations, contributing to the diversity of mammalian tissues. Earlier methods that analyzed gene expression at the level of translation, such as polysomal- or ribosomal-profiling, required large amounts of starting material to isolate enough RNA for analysis by microarray or RNA-sequencing. Thus, rare or less abundant cell types within tissues were not able to be properly studied with these methods. Translating ribosome affinity purification (TRAP) utilizes the incorporation of an eGFP-affinity tag on the large ribosome subunit, driven by expression of cell-type specific Cre-lox promoters, to allow for identification and capture of transcripts from actively translating ribosomes in a cell-specific manner. As a result, TRAP offers a unique opportunity to evaluate the entire mRNA translation profile within a specific cell type, and increase our understanding regarding the cellular complexity of mammalian tissues.

Keywords: Protein synthesis, Translating ribosome affinity purification (TRAP), Cell-specific translation

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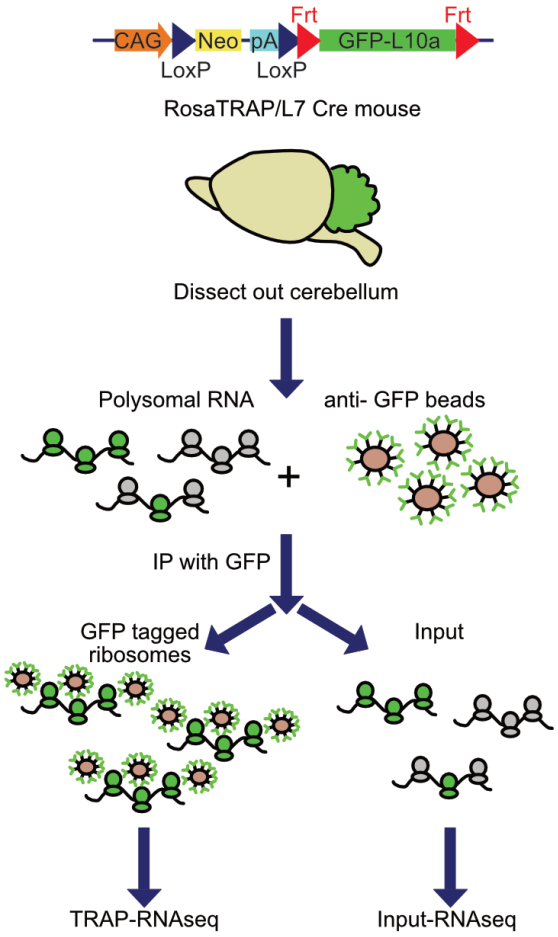
Graphical abstract:



Schematic demonstrating TRAP protocol for identifying ribosome-bound transcripts specifically within cerebellar Purkinje cells.

Background

Over the past two decades, technological advances in the field of 'omics' have revolutionized our understanding of the regulation of gene and protein expression. The advent of microarrays and high-throughput RNA sequencing has allowed for the identification and characterization of mRNA expression profiles (or transcriptome) of entire genomes. However, mammalian tissues are heterogenous and complex, with several layers of post-transcriptional processes playing key roles in the regulation of protein expression. Recent studies have shown that translation at the level of the ribosome is highly regulated, and can vary independently of gene expression observed at a transcriptome level. Therefore, translational regulation can differ between cell populations, contributing to the diversity of mammalian tissues. To understand disease states, one must be able to identify not only the transcriptome, but also the translatome within specific cell populations, to better elucidate the cellular mechanisms in both normal and disease states.

Translation is the process by which mRNA is decoded and proteins are synthesized, and it occurs at the ribosome, which is composed of two subunits, the large (60S) and small (40S) ribosomal subunits (Doudna and Rath, 2002). Translation is comprised of three steps: 1) initiation, the binding of mRNA to the ribosome, 2) elongation of the peptide chain, and 3) termination of the newly synthesized protein from the ribosome (Puria *et al.*, 2021). Initiation

is often thought to be the primary target for translational control, and can be controlled on a global level with various translation initiation factors (eIFs), or at the level of specific mRNAs, by way of intrinsic sequence elements (*i.e.*, IRES or upstream ORFs) or non-coding RNAs, such as microRNAs that can repress translation by targeting the 3'-UTR (Puria *et al.*, 2021). Multiple ribosomes can bind to a single transcript (termed polysomes), as compared to a single ribosome bound to a single transcript (monosome). Previously, it was thought that polysomes were the primary source of protein synthesis (Ostroff *et al.*, 2017, 2018), but this view has been challenged by studies showing that monosomes play an important role in translation in neurons (Biever *et al.*, 2020). Therefore, it is important to be able to understand the regulation of translation, and its impact on disease states.

Polysomal profiling was developed in the 1960s, and served as the gold standard for measuring the translation of mRNA bound by multiple ribosomes. Using a sucrose density gradient, actively translating polysomes are separated from free RNA, the small (40S) and large (60S) ribosomal subunits, and the 80S monosomes (single ribosomes) through centrifugation (Zuccotti and Modelska, 2016). Briefly, transcripts are stalled on ribosomes with cycloheximide, and the cells are then lysed, with the resulting lysate carefully layered onto a sucrose gradient. Ultracentrifugation of the lysate by velocity sedimentation across the sucrose gradient separates out the free RNA, ribosomal subunits, monosomes, and polysomes, based on their molecular weights. Following the fractionation of the lysate, one can analyze the RNA bound to the polysomes by chromosomal microarray, qPCR, and/or RNA sequencing (Zuccotti and Modelska, 2016). Based on the mRNA distribution pattern observed from the polysomal profiling, one can then determine the ribosomal occupancy (the percentage of transcripts associated with ribosomes), as well as the ribosomal density (the number of ribosomes associated with mRNA) to allow for calculation of the translational efficiency for different transcripts (Zuccotti and Modelska, 2016).

While polysomal profiling allows for understanding of changes in translation of individual mRNAs under various conditions, there are also many limitations and/or challenges that can limit its implementation in all research settings. First, polysome profiling requires specialized and often expensive equipment (*i.e.*, ultracentrifuge, fractionator), that may not be available to many members of the research community. Next, the preparation of lysates and sucrose density gradients is both time- and labor-intensive, often limiting the amount of samples one is able to process for a given experiment (Zuccotti and Modelska, 2016). Finally, the polysomal fractions may contain contaminants (*i.e.*, pseudo-polysomes), and/or vary from one preparation to the next, making it imperative that one starts off with a large sample size, such as ten million cells, to obtain enough RNA to proceed with microarray or RNA sequencing analysis (King and Gerber, 2016; Jin and Xiao, 2018).

Ribosomal profiling, also known as ribosomal footprinting, assesses gene expression at the level of translation with nucleotide resolution, by quantifying not only the number of translating ribosomes, but also the location of the ribosomes on the mRNA transcripts, through deep sequencing ribosome-protected RNA fragments (RPFs), or footprints (King and Gerber, 2016; Spealman *et al.*, 2016). Briefly, generation of RPFs (27–30 nucleotides in length) via ribosome profiling requires: 1) the addition of an antibiotic to inhibit translation, such as cycloheximide, which inhibits elongation, or harringtonine, which blocks translation initiation, 2) transcript digestion, 3) transcript purification via a sucrose gradient, and enrichment of desired transcripts by depletion of ribosomal RNA (rRNA, which accounts for 95% of all cellular RNA), 4) sequencing library construction, and 5) RNA sequencing and analysis (Spealman *et al.*, 2016). By determining the location of actively translating ribosomes on mRNA transcripts, compared to total mRNA abundance within a sample, ribosomal profiling has allowed for greater understanding of the mechanisms underlying translation regulation. However, ribosomal profiling is not without its own challenges. Similar to polysomal profiling, ribosomal profiling is a process that is both time and labor intensive, and still requires the use of specialized equipment to isolate the RPFs via a sucrose gradient, as well as a large amount of input sample to ensure purification of enough RNA for analysis (King and Gerber, 2016; Spealman *et al.*, 2016). Additionally, despite rRNA depletion efforts, samples may still be contaminated with rRNA, which results in requiring two times as many reads for RPF libraries, to yield adequate and analyzable data. Finally, ribosomal profiling requires bioinformatics expertise to take the raw RNA-seq and RPF reads and compute the mRNA translation efficiencies, but depending on the antibiotic chosen in the initial step of preparation of the RPFs, there may be a bias in the ribosome loads, evaluation of the translation initiation site, and/or alternative translation initiation sites (Spealman *et al.*, 2016; King and Gerber, 2016).

Translating ribosomal affinity purification (TRAP) is unique, in that it allows for evaluation of gene expression at the level of translation in a cell-specific manner. TRAP requires the use of either transgenic animals, or transfected cells that contain an enhanced GFP (eGFP) affinity tag on the large ribosomal subunit protein L10a (Heiman *et al.*,

2014; King and Gerber, 2016). Cell-specific incorporation of the eGFP-L10a tag directed by Cre-lox promoters allows for affinity tagging of the ribosomes of specific populations of cells (i.e., cerebellar Purkinje cells with L10a-incorporation driven by the L7 Cre promoter), which can then be purified by affinity selection with GFP-coated beads. Isolated RNA from the captured eGFP-tagged ribosomes can be analyzed by qPCR, microarray, and/or RNA sequencing (Heiman *et al.*, 2014; Dalal *et al.*, 2021). In contrast to polysomal and ribosomal profiling, TRAP does not require large amounts of starting material, and is able to evaluate rare populations of cells within an organism. Additionally, in comparison to fluorescent activated cell sorting (FACS), TRAP does not require the fixation or dissociation of tissue to isolate cell-type specific RNA. While TRAP has been used across many tissues and species (Reynoso *et al.*, 2015; Zhou *et al.*, 2013; Tryon *et al.*, 2013), it is particularly suited for the nervous system, due to the ability to focus on homogeneous cell populations within a complex and heterogeneous organ. TRAP also provides the opportunity to evaluate the mRNA translation profile within specific cell types, which more closely reflects the protein content expected to be seen in these cells (Heiman *et al.*, 2014). Despite these advantages, one major limitation of TRAP is the need to generate a transgenic animal line, or specific-RPL10a vector for each cell type one wishes to study.

The objective of this protocol is to allow for the examination of mRNA translation profiles within specific cell types of homogenous, complex tissues to provide important insights into the cellular mechanisms regulating normal and disease states.

Materials and Reagents

1. Microcentrifuge tube
2. Streptavidin MyOne T1 Dynabeads (Thermo Fischer, catalog number: 65602). Store at 4°C
3. Purified Recombinant Biotinylated Protein L (Pierce, catalog number: 29997). Store at 4°C
4. Bovine Serum Albumin (BSA) (IgG-Free, Protease-Free) (Jackson ImmunoResearch, catalog number: 001-000-162). Store at 4°C
5. Cycloheximide (CHX) (Sigma, catalog number: C7698). Store at -20°C
6. 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) (Avanti Polar Lipids, catalog number: 850306P-200mg). Store at -20°C
7. DL-Dithiothreitol (DTT) (Sigma, catalog number: D9779). Store at -20°C
8. Pierce Protease Inhibitor Tablets, EDTA-Free (Pierce, catalog number: A32955). Store at 4°C
9. Recombinant RNasin Ribonuclease Inhibitor (Promega, catalog number N2518). Store at -20°C
10. SUPERase-In (Invitrogen, catalog number: AM2694). Store at -20°C
11. 1 M HEPES solution pH 7.0–7.6, sterile filtered (Sigma, catalog number: H0887). Store at 4 °C.
12. 10× HBSS without calcium chloride, magnesium chloride or magnesium sulfate (Gibco, catalog number: 14185-052). Store at room temperature
13. UltraPure Distilled Water, DNase, RNase Free (Invitrogen, catalog number: 10977-015). Store at room temperature
14. Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, catalog number: 14190-144). Store at 4°C
15. 2 M KCl RNase free (Invitrogen, catalog number: AM9640G). Store at room temperature
16. 1 M $NaHCO_3$ (Sigma, catalog number: S5761-500G), sterile filtered. Store solution at 4°C
17. 1 M $MgCl_2$ RNase free (Invitrogen, catalog number: AM9530G). Store at room temperature.
18. GFP antibodies (Memorial Sloan-Kettering Monoclonal Antibody Facility; clone names: Htz-GFP-19F7 and Htz-GFP-19C8). Store at -80°C
19. Glucose (Sigma, catalog number: G7021). Store at room temperature
20. Sodium Bicarbonate (Sigma, catalog number: S6297). Store at room temperature
21. β-mercaptoethanol (β-ME) (Sigma, catalog number: 63689). Store at room temperature in flammable hood
22. NP40, IGEPAL CA-630 (Sigma, catalog number: 56741). Store at room temperature
23. Ethanol (American Bioanalytical, catalog number: AB00138-01000). Store at room temperature in the flammable hood.
24. ChromoTek TRAP kit (ChromoTek, catalog number: gtmak-20). Store at 4°C
25. Qiagen RNeasy Plus Mini Kit (Qiagen, catalog number: 74134)

26. TRAP transgenic mice, (Gt(ROSA)26Sor$^{tm1(CAG-EGFP/Rpl10a,-birA)Wtp}$, Jax$^{\#022367}$)
27. Dissection Buffer (1×, 150 mL) (see Recipes)
28. Homogenization buffer (see Recipes)
29. Low Salt buffer (see Recipes)
30. 300 mM DHPC (see Recipes)
31. High Salt buffer (see Recipes)

Equipment

1. DynaMag-2 Magnet (Life Technologies, catalog number: 12321D)
2. Teflon-glass homogenizer with serrated PTFE pestle (DWK Life Sciences, catalog number: 8855100020)
3. Refrigerated Centrifuge (Eppendorf, Centrifuge 5430R)
4. End over end microcentrifuge tube rotator
5. Microcentrifuge (Eppendorf, Centrifuge 5415D)
6. Nanodrop One (Thermo Scientific, catalog number: ND-ONE-W)
7. RNase free 1.7mL microcentrifuge tubes (Genesee Scientific, catalog number: 24-282)

Software

1. R v 4.02, https://cran.r-project.org

Procedure

Note: The following protocol was optimized to characterize the mRNA translation within cerebellar Purkinje cells. For each purification, 30 µL of Streptavidin MyOne T1 Dynabeads, 12 µL of biotinylated protein L, and 50 µg of each GFP antibody (100 µg total antibody) were utilized to perform the TRAP experiments. Please include a 0.5–1-fold excess of the reagents, to account for pipetting errors of the matrix components.

Depending on the abundance of your target cell type and the amount of available sample (*i.e.*, volume of cortex versus hippocampus), you may need to adjust the volumes of bead/protein L matrix, while keeping the ratio (2.5:1 beads/protein L) the same to optimize the capture of the translated RNA from the GFP-tagged ribosomes (Heiman *et al.*, 2014). Preliminary pilot experiments are recommended, to confirm that you are using the optimal volume of the bead/protein L matrix to characterize your target cell type, while allowing for differences in translational state and tissue background RNA binding levels. Before performing RNAseq on the TRAP-isolated samples, confirm targeted enrichment by completing qPCR of specific genes known to be enriched in your desired sample.

A. **Bead/Matrix Preparation** (Takes approximately 3 h for preparation)

Note: The matrix (beads/protein L and antibodies) should be prepared as a common stock and then aliquoted into the individual tubes in section C, just prior to the addition of the isolated S20 samples.
We typically prepared the matrix fresh on the day of the experiment, however, if needed, it can be prepared in advance and stored for up to 1–2 weeks at 4°C in the presence of 0.02% sodium azide. If using pre-prepared matrix, wash the matrix stock three times with low salt buffer (see Recipe 3) prior to aliquoting for use in section C (Heiman et al., 2014).

1. Re-suspend Streptavidin MyOne T1 Dynabeads by gentle hand mixing/pipetting (do not vortex). Wash Streptavidin MyOne T1 Dynabeads (30 µL per sample) twice with 1× phosphate buffer solution (PBS) at

room temperature (1 mL for all washes, if using a 1.5 mL microcentrifuge tube). Collect beads on magnet (approximately 30–60 s until the supernatant runs clear), and discard the supernatant.

2. Re-suspend beads in 1× PBS using the appropriate volume (original bead volume minus the volume of biotinylated protein L to be added, still accounting for fold-excess integer to allow for pipetting error).
3. Add Purified Recombinant Biotinylated Protein L (12 µL per sample) to suspended Streptavidin MyOne T1 Dynabeads beads. Rotate end over end at room temperature for 35–45 min.
4. Collect the beads coated with protein L on the magnet. Wash the protein L-coated beads five times with 1× PBS containing 3% (weight/volume) BSA. Collect on magnet with each wash, and discard supernatant.
5. Wash the protein L-coated beads three times with 500 µL of low salt buffer (Recipe 3). Collect on magnet with each wash, and discard supernatant (see Figure 1).
6. Incubate protein L-coated beads with anti-GFP antibodies (50 µg each of HtzFFP-19F7, and HtzGFP-19C8) diluted in 100 µL of low salt buffer end over end at room temperature for at least 1 h.
7. After incubating with anti-GFP antibody mixture, wash the beads three times in low salt buffer. Collect on the magnet with each wash, and discard the supernatant.
8. Re-suspend the antibody-coated beads with protein L in an aliquot of low salt buffer, containing 300 mM DHPC (Recipe 4), to maintain the desired ratio of beads/protein L/GFP antibody just before adding the S20 lysate (Procedure C).

Option 2: Use the ChromoTek TRAP Kit, which provides a pre-prepared GFP-Trap Agarose affinity resin for immunoprecipitation of GFP-fusion proteins. It also contains the wash and elution buffers, as well as a lysis buffer that can be used on mammalian cells, but not for tissue.

1. Resuspend provided beads by gently pipetting up and down, and/or inverting the tube. Do not vortex the beads.
2. Transfer 50 µL of the slurry per sample into a 1.5-mL microcentrifuge tube. Add an extra sample (*i.e.*, 50 µL) to the total, to allot for pipetting error.
3. Add 500 µL of ice-cold dilution buffer (provided in the ChromoTek TRAP kit) to the beads.
4. Separate the beads with the magnet, until the supernatant is clear. Discard the supernatant.
   Note: If you obtain the non-magnetic agarose beads, you can follow the same preparation, but rather than separating the beads out on the magnet, separate the beads by centrifugation at 2,000 × g and 4°C for 3 min.

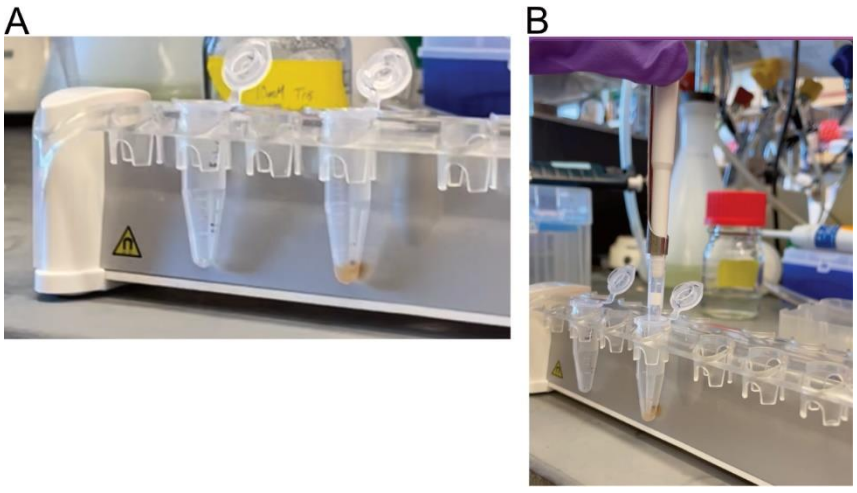


Figure 1. Illustration of how to wash magnetic TRAP beads.
Place Eppendorf tube on magnet (A). Allow supernatant to clear, and then pipet off the supernatant, without dislodging the beads form magnet (B).

B. Dissection of brain tissue

1. Obtain and weigh transgenic animals.
2. Euthanize animals with CO_2, and decapitate. Remove and weigh brain (if desired) prior to dissection.
3. Rinse brain with dissection buffer (Recipe 1), and then rapidly dissect out cerebellum (or area of interest, *i.e.*, cortex, hippocampus) in ice-cold dissection buffer.
4. Place approximately 25–50 mg of dissected tissue in 1 mL of ice-cold homogenization buffer (Recipe 2). Flash freeze the rest of the brain for controls/additional experiments in the future.
   Notes:
   a. *Depending on the abundance of your desired cell type, you may have to decrease or increase the amount of tissue that you place in the homogenization buffer.*
   b. *While you can flash freeze the dissected area of interest of the brain with liquid nitrogen, and store at -80°C prior to homogenization and completion of the protocol, your yield of purified mRNA may not be as high. Thus, for populations of cells with lower abundance, we recommend performing the experiment on fresh lysates. If you do use frozen tissue, do not thaw the tissue, but rather place it directly into the ice-cold homogenization buffer, and proceed with the tissue homogenization (the tissue will thaw during the homogenization).*
5. If desired, save a piece of the animal's tail or ear for re-genotyping, to confirm the experimental genotype.

C. Homogenization/Preparation of lysates

1. Homogenize the cerebellum (~25–50 mg of tissue) in 1 mL of ice-cold homogenization buffer, using 12 motor-driven strokes of Teflon-glass homogenizer.
   Note: Keep the pestle in the liquid to avoid excessive aeration/formation of bubbles, which can result in protein denaturation.
2. Transfer the homogenized lysate to a pre-chilled microcentrifuge tube.
3. Centrifuge at 2,000 × *g* and 4°C for 10 min to remove nuclei.
4. Transfer supernatant to new pre-chilled microcentrifuge tube. Add 1/9 volume of 10% NP-40 (1% final concentration), and invert five times. Then add 1/9 volume of 300 mM DHPC (final concentration 30 mM DHPC), invert 5 times, and incubate on ice for 15 min.
5. Centrifuge at 20,000 × *g* and 4°C for 15 min, to remove mitochondria. The resulting supernatant (S20) contains the polysome fraction. Place the S20 fraction in a new pre-chilled microcentrifuge tube.
6. Save a small aliquot (~50–60 µL) of S20 as an input control. Add low salt buffer to the S20 input sample (to make the final volume up to 250 µL), and incubate at 4°C overnight, along with the immunoprecipitation samples (as in section D). Following incubation overnight, add 750 µL of Trizol, and place at -80°C, or extract RNA via the Qiagen RNAeasy kit described in Procedure D, Option 2.

D. Immunoprecipitation/RNA preparation

1. Add 200 µL of GFP-conjugated beads to the remaining S20 lysate for each sample.
   Note: Remember to gently re-suspend the bead/protein L/GFT antibody matrix, by gentle pipetting immediately prior to aliquoting and adding the S20 lysates.
2. Incubate end-over-end in the cold room (4°C) overnight.
3. Collect the beads on the magnet. Save a small aliquot of the supernatant, which represents the unbound fraction. Wash beads four times in 1 mL of high salt buffer (Recipe 5). Be careful not to introduce bubbles when pipetting during the washes. Collect on the magnet, and discard the supernatant.
4. Re-suspend beads in 250 µL of low salt buffer, and 750 µL of Trizol. Place at -80°C. Prepare RNA as per Trizol protocol when you are ready to proceed.
   Note: You can also use the Qiagen RNAeasy kit as described in Option 2, rather than Trizol.

Option: ChromoTek TRAP Kit

1. Add the entire S20 lysate to 50 µL of equilibrated ChromoTek TRAP beads (pre-equilibrated in Section A).
2. Rotate end-over-end in the cold room (4°C) for 1 h.
3. Separate the beads with a magnet until the supernatant is clear (or centrifuge at 2,000 × *g* and 4°C for 3 min, if using agarose beads). Save 50 µL of supernatant for further analysis of non-bound/flow-through fraction.
4. Discard the rest of the supernatant.
5. Wash beads five times with high salt buffer, and separate beads either on magnet, or via centrifugation (2,000 × *g* at 4°C for 3 min), if using agarose beads.
   Note: You can use the provided wash buffer that comes with the kit, but we tended to have higher yields of RNA using the high salt buffer.
6. After final wash, remove supernatant, and add 350 µL of RLT buffer (Qiagen RNAeasy kit) containing β-mercaptoethanol (β-ME) (10 µL of β-ME for 1 mL of RLT). Incubate at room temperature for 10 min.
7. Depending on your formulation of ChromoTek TRAP beads, either separate beads using the magnet, or centrifuge samples at 2,000 × *g* and 4°C for 3 min. Transfer the supernatant to a new tube.
8. Add 1× volume of 70% ethanol to the tubes, and mix by pipetting.
9. Load 700 µL of the mixture onto the provided Qiagen column. Centrifuge at 8,000 × *g* at room temperature for 15 s. Discard the flow-through.
10. Add 700 µL of RW1 (Qiagen kit) to the column. Centrifuge at 8,000 × *g* at room temperature for 15 s. Discard the flow-through.
11. Add 500 µL of RPE (Qiagen kit) to the column. Centrifuge at 8,000 × *g* at room temperature for 15 s. Discard the flow-through.
12. Add a second 500 µL of RPE (Qiagen kit) to the column. Centrifuge at 8,000 × *g* at room temperature for 2 min. Discard the flow-through.
13. Place the RNeasy spin column in a new 2-mL collection tube, and centrifuge at maximum speed for 1 min, to dry the membrane.
14. Place column in a 1.5-mL microcentrifuge tube, and add 30 µL of RNA-free water directly to the column membrane, to elute the RNA. Centrifuge at 8,000 × *g* at room temperature for 1 min.
15. Take the eluant, and place it back on the column, and spin at 12,000 × *g* for one minute.
16. Determine the concentration of the RNA by Nano-drop.

E. Sequencing

1. cDNA libraries must be constructed prior to sequencing, and there are many commercially available kits for this procedure, as well as many institutions which have core facilities that will perform this service. The main considerations when choosing the kit are mRNA selection method, and starting RNA concentration. Library barcodes can be added during preparation to allow for pooling and multiplexing of samples.
2. Sequence the pooled libraries on an Illumina platform.

Data analysis

A. Quality control of sequencing run

1. Make sure that sequencing run parameters are tolerable (>80% clusters passing filter, >90% Q30). Deviations from these parameters suggest an issue with the sequencing run itself, and can be related to inaccurate quantification of the cDNA library.
2. Run FastQC on each sequencing file.

B. Aligning and quantifying reads

1. Obtain the appropriate version of the genome
   wget https://ftp.ensembl.org/pub/release-105/fasta/mus_musculus/dna/Mus_musculus.GRCm39.dna.primary_assembly.fa.gz
   gunzip Mus_musculus.GRCm39.dna.primary_assembly.fa.gz
   wget https://ftp.ensembl.org/pub/release-105/gtf/mus_musculus/Mus_musculus.GRCm39.105.gtf.gz
   gunzip Mus_musculus.GRCm39.105.gtf.gz
2. Create a STAR index for mapping
   ```
   STAR --runThreadN 8 --runMode genomeGenerate --genomeDir ./GenomeDir --genomeFastaFiles Mus_musculus.GRCm39.dna.primary_assembly.fa --sjdbGTFfile Mus_musculus.GRCm39.105.gtf
   ```
3. Align sequencing files to genome using STAR
   ```
   STAR --runThreadN 8 --genomeDir ./GenomeDir --readFilesIn Sequencing_1.fastq.gz gz --readFilesCommand gunzip -c --sjdbGTFfile Mus_musculus.GRCm39.105.gtf --outFileNamePrefix Sequencing_1 --outSAMtype BAM SortedByCoordinate
   ```
4. Assemble transcriptome using Cufflinks
   ```
   cufflinks -p 8 -g Mus_musculus.GRCm39.105.gtf --library-type fr-firststrand -o ./Sequencing_1 Sequencing_1Aligned.sortedByCoord.out.bam
   ```
5. Merge transcriptomes
   ```
   cuffmerge -p 8 -g Mus_musculus.GRCm39.105.gtf assembly_list.txt
   *assembly_list.txt contains a list of the transcripts.gtf files created by cufflinks
   ```
6. Quantify transcripts
   ```
   cuffquant -p 8 --library-type fr-firststrand -o ./Sequencing_1 ./merged_asm/merged.gtf Sequencing_1Aligned.sortedByCoord.out.bam
   ```
   *Can also use a custom transcript database. For example, we generated the transcriptome using RNA sequencing data from sorted Purkinje Cells, and then we used that .gtf file to annotate transcripts identified in the TRAP data. This greatly facilitated comparison of transcriptome and TRAP data.
7. Create a matrix of gene expression
   ```
   cuffnorm -p 8 --library-type fr-firststrand -o ./output -L Sequencing_1,Sequencing_2,… Sequencing_1/abundances.cxb Sequencing_2/abundances.cxb …
   ```
8. This has created a gene expression matrix across all samples at the gene and transcript level. From here, several analyses are possible, and should be informed by the biological questions posed in the study. One analysis of interest might be to find transcripts that show altered ribosomal binding between conditions. For this analysis, we used LIMMA to identify transcripts showing changes between mutant and control Purkinje Cells. If transcriptome data is available for the sample, then it may be of interest to compare changes at the ribosomal binding level to those at the transcriptome level. In order to do this, we calculated the translation efficiency for each condition, and used the change in translation efficiency to compare between conditions.

Notes

All solutions must be EDTA-free, as EDTA chelates magnesium and will lead to the dissociation of polysomes.

Recipes

1. **Dissection Buffer (1×, 150 mL)**
   10× HBSS, 15 mL Final 1× HBSS
   1 M HEPES, 375 µL Final 2.5 mM HEPES KOH pH 7.4
   1 M Glucose, 5.25 mL Final 35 mM Glucose
   1 M $NaHCO_3$, 600 µL. Final 4 mM $NaHCO_3$
   RNase Free water, 128.775mL
   Add CHX 100 µg/mL (freshly made) to desired volume
   Note: CHX stock is dissolved in methanol for lysates, and DMSO for cultured cells.

2. **Homogenization buffer**
   10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM DTT, 100 µg/mL CHX, 10 µL/mL RNasin, 10 µL/mL SUPERase-In, 1× complete EDTA-free protease inhibitors

3. **Low Salt buffer**
   0.15 M KCl buffer (10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 150 mM KCl, 1% NP40)

4. **300 mM DHPC**
   Adding 1.38 mL 10 mM HEPES pH 7.4 to 200 mg DHPC powder

5. **High Salt buffer**
   0.35 M KCl buffer (20mM HEPES pH 7.4, 5mM $MgCl_2$, 350 mM KCl, 1% NP40, 0.5M DTT, 10 µL/mL RNAsin, 10 µL/mL SUPERase-In, and 100 µg/mL CHX)

Acknowledgments

We would like to acknowledge the original paper from which this protocol was adapted from Heiman *et al*. (2014) “Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP)” (Heiman *et al.*, 2014), as well as our work from which this protocol is derived from Dalal *et al*. (2021; DOI: 10.7554/eLife.67399), “Loss of Tsc1 in cerebellar Purkinje cells induces transcriptional and translation changes in FMRP target transcripts.” (Dalal *et al.*, 2021). The Sahin lab is supported by grants from the NINDS R01NS113591 and from the U.S. Army Medical Research Tuberous Sclerosis Complex Research Program (W81XWH-15-1-0189) and the Boston Children’s Hospital Intellectual and Developmental Disabilities Research Center (BCH IDDRC, P50HD105351). CLS is funded by the CH/BIDMC/Harvard Medical School Neurology Resident Research Education Program NIH (R25NS070682). KDW is funded by a NIH K08 (K08NS112598).

Competing interests

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Ethics

All animal procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals. All the procedures in this protocol were approved by the Animal Care and Use Committee of Boston Children's Hospital (protocol number 18-05-3677R).

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