Cell type–specific mRNA purification by translating ribosome affinity purification (TRAP)

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Cellular diversity and architectural complexity create barriers to understanding the function of the mammalian CNS at a molecular level. To address this problem, we have recently developed a methodology that provides the ability to profile the entire translated mRNA complement of any genetically defined cell population. This methodology, which we termed translating ribosome affinity purification, or TRAP, combines cell type–specific transgene expression with affinity purification of translating ribosomes. TRAP can be used to study the cell type–specific mRNA profiles of any genetically defined cell type, and it has been used in organisms ranging from *Drosophila melanogaster* to mice and human cultured cells. Unlike other methodologies that rely on microdissection, cell panning or cell sorting, the TRAP methodology bypasses the need for tissue fixation or single-cell suspensions (and the potential artifacts that these treatments introduce) and reports on mRNAs in the entire cell body. This protocol provides a step-by-step guide to implement the TRAP methodology, which takes 2 d to complete once all materials are in hand.

INTRODUCTION

The advent of microarray mRNA expression profiling and RNA sequencing has made possible the simultaneous interrogation of the mRNA expression profiles of all genes in a genome. However, these techniques have not been easily applied to the study of cells that reside in complex tissues. In tissues such as the mammalian brain, the high level of cellular heterogeneity, combined with close anatomical intermixing of diverse cell types, complicates the analysis of gene expression data derived from whole tissue samples. Thus, observed gene expression profiles cannot be attributed to any particular cell type, and any changes that are not unidirectional in all cells may not be detected because of averaging of information across different cell types in the target tissue. Conversely, techniques such as *in situ* hybridization and immunohistochemistry afford single-cell resolution, but they cannot be routinely applied at a genome-wide scale. We recently developed a methodology that we termed TRAP, which allows the interrogation of the entire translated mRNA complement of any genetically defined cell type.

Overview of TRAP

TRAP uses both indirect tagging of mRNAs and cell type–specific genetic targeting of transgene expression (**Fig. 1**). Indirect tagging of mRNAs is achieved by the incorporation of an affinity tag, such as enhanced GFP (EGFP), on the large ribosomal subunit protein L10a. EGFP-tagged ribosomes not only assist in cell visualization in tissue, but tagged ribosomes can also be affinity-purified with EGFP antibodies. Cell type–specific expression of EGFP-L10a is accomplished by driving expression of the transgene under regulatory elements known to direct cell type–specific expression. If ribosomes are maintained on the mRNAs that they are translating when tissue is harvested, purification of the cell type–specific tagged ribosomes will also yield cell type–specific translated mRNAs. Translated mRNAs purified in this way can subsequently be analyzed by any of the common methods that are used to study RNA expression, including northern blotting, quantitative PCR (qPCR), microarray or RNA sequencing. We have successfully used TRAP-purified mRNA as input into qPCR, microarray and RNA sequencing reactions (for examples, see refs. 1,2).

Alternative approaches

Several molecular profiling methods have been developed to study the genome-wide expression profiles of cells in complex tissues, including methods that rely on the isolation of cells of interest after tissue dissociation. Such methods include FACS of dissociated neurons[3–9], manual sorting of fluorescent cells[10–12] and immunopanning of dissociated cells with or without FACS[9,13]. Other methods have relied on the identification of cell types of interest in sectioned tissue, without tissue dissociation. These methods include laser-capture microdissection[14,15] and aspiration of patched cells after electrophysiological recording[16,17]. Although each of these methods has unique advantages, they all suffer from the inability to profile cells *in situ*, without the introduction of experimental noise caused by tissue fixation, tissue dissociation, tissue incubation *ex vivo* or high amplification of only single-cell mRNA content. Further, as these methods require dissociation of tissue or extraction of cells from tissue, they produce expression profiles that reflect only the mRNA content of the cell soma, not the entire cell.

Advantages and limitations of TRAP

We have previously described a protocol for the purification of TRAP-tagged ribosome-mRNA complexes from mouse brain tissue[1,18]. As described in these studies, important steps for successful purification of translating ribosomes include the following: immediate homogenization of target tissue; stabilization of translating ribosomes on intact mRNA by inclusion of magnesium, cycloheximide and RNase inhibitors in purification solutions; solubilization of rough endoplasmic reticulum–bound ribosomes under nondenaturing conditions; and the use of an

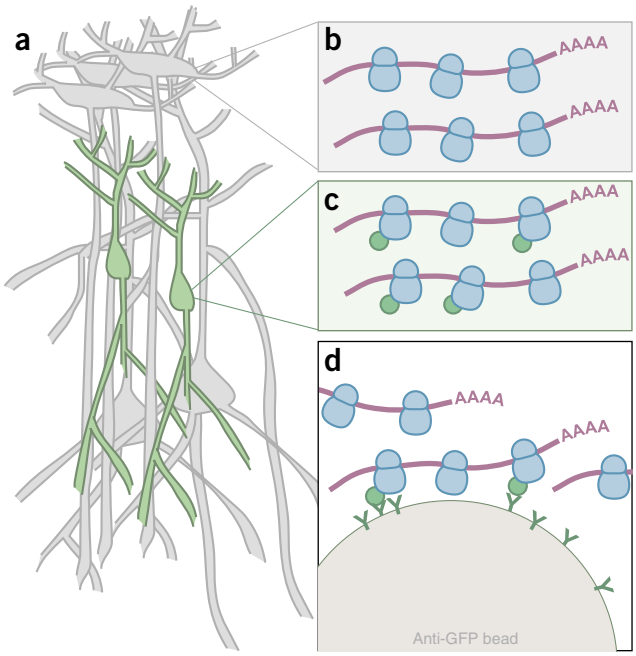

Figure 1 | The translating ribosome affinity purification (TRAP) strategy. (**a**) The cell type of interest is targeted with appropriate genetic elements to express the *EGFP-L10a* transgene. (**b**,**c**) Translating polyribosomes (polysomes) originating from nontargeted cells (gray cells, **b**) do not have an EGFP tag on their ribosomes, whereas those originating from targeted cells (green cells, **c**) have an EGFP tag on their ribosomes. Lysis of all cells releases both tagged and nontagged polysomes. (**d**) Only the tagged polysomes are captured on an anti-GFP affinity matrix, which can be used for purification of the cell type–specific mRNA associated with tagged polysomes.

affinity matrix that has low background RNA binding. A limitation of the TRAP methodology as originally published was the low yield of mRNA, which necessitated the use of a large amount of starting material from transgenic animals. Recent optimizations to the TRAP methodology include an increase in RNA yields, greater signal-to-noise ratios and the ability to freeze tissue before performing TRAP purifications, optimizations that result from a change in the affinity matrix and RNA extraction conditions[2,19–21].

TRAP has the advantage that it does not require the fixation or dissociation of tissue or the extraction of cells from tissue for the capture of cell type–specific mRNA. This allows for the *in situ* profiling of an entire cell's mRNA translation profile. This advantage gives TRAP a higher degree of sensitivity than other methods. Further, the TRAP transgene labels the cell type of interest with EGFP, thus allowing for visualization in immunohistochemical or electrophysiological studies. Another advantage of TRAP versus other gene-expression profiling methodologies is that it reveals the translated mRNA content of a cell, which will more closely match the protein content than will the total RNA gene expression profile. If the true total RNA profile is desired, another methodology may be preferable to TRAP. A limitation of the TRAP methodology as originally published was the need to generate transgenic animal lines for each cell type of interest. However, this potential limitation has been reduced for mouse studies with the recent generation of several conditional TRAP mouse lines[22,23]. Nevertheless, a genetic element is always needed to drive cell type–specific expression of the TRAP transgene in the cell type of interest. In cases where this is not possible (e.g., human brain tissue studies), other methods such as laser capture microdissection may be preferable, even if they have less sensitivity.

Applications

Mouse studies. As originally described, cell type–specific TRAP expression in mice was achieved by the use of cell type–specific genetic targeting driven by bacterial artificial chromosomes (BACs). BACs can carry up to ~200 kb of DNA, which is a large enough segment of DNA to ensure that the regulatory elements of most genes are included with the transgene to be targeted[24–26]. Several options now exist to achieve TRAP expression in mice: an existing BAC-TRAP transgenic mouse line can be obtained[1,18]; a new BAC-TRAP transgenic mouse line can be constructed[27]; viral transduction of a conditional TRAP construct can be used in conjunction with one of many publically available *Cre* driver lines[28,29]; or a conditional TRAP mouse line[22,23] can be used in combination with a *Cre* driver line. In cases where investigators wish to target rare cell populations, BAC-TRAP lines may be of most use, as such lines will drive the highest level of TRAP transgene expression owing to integration of multiple copies of the transgene in the genome (unpublished observations, M.H. and N.H.).

Studies in cultured cells. The TRAP transgene has been successfully used to study translation in cultured, transfected human cells by using a constitutive promoter[18]. We have also succesfully used Amaxa nucleofection, as well as adenoviral and lentiviral transduction, to express the TRAP transgene in primary mouse neurons (M.H., unpublished observations). TRAP could be used to study specific cell types in mixed cultures by placing the TRAP transgene under a compact cell type–specific promoter, or by using cells taken from transgenic, cell type–specific *Cre*–expresing mice (in combination with a conditional TRAP construct).

Studies in other species. TRAP and related ribosome-tagging approaches have now also been successfully used to study cell type–specific gene expression in *Drosophila*[30], plants[31], *Xenopus*[32] and zebrafish[19]. Readers are directed to the appropriate references for details on genetic elements needed to achieve cell type–specific expression in these species. Two important considerations in adapting TRAP to other species is to make sure that codon usage for the EGFP coding sequence is species-optimized and that the sequence encoding ribosomal protein orthologous to mouse large ribosomal protein L10a is used in constructing the TRAP transgene. In addition, the volumes listed in the protocol below were optimized for working with mouse brain tissue (typically 50–200 mg), and thus the number listed may need to be scaled up or down if the amount of starting material is larger or smaller, respectively.

Downstream applications. RNA purified according to the TRAP protocol is of high purity, and it can be used directly for various downstream applications, including northern blotting, qPCR, microarray analysis and RNA sequencing. An important consideration when choosing a downstream application is the TRAP

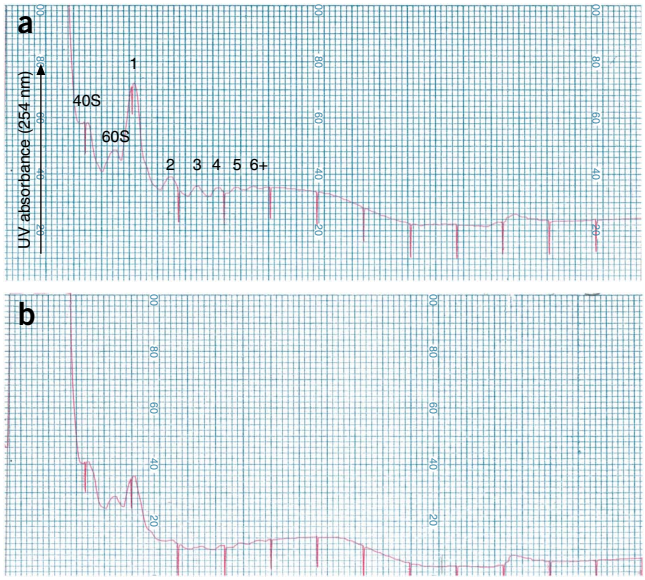

Figure 2 | Representative polyribosome profiles from fresh and frozen striatal tissue. (**a**,**b**) Postmitochondrial supernatants prepared (as described in this protocol) from freshly dissected striatal tissue (**a**) or from tissue that had been flash-frozen and stored for 4 months at −80 °C (**b**) were subjected to sucrose-gradient zonal centrifugation, as previously described[1], and gradient fractions were collected while UV absorbance at 254 nm was monitored. An increase in 254-nm absorbance indicates an increase in the RNA content in the fraction. Both fresh and frozen samples were prepared from young adult TRAP mice. Small ribosomal subunit (40S), large ribosomal subunit (60S), monosome (1) and polysome (2, 3, 4, 5 and 6+) peaks are indicated. Red ticks indicate gradient fractions. Animal experiments were reviewed and approved by the Rockefeller University Institutional Animal Care and Use Committee.

yield of RNA, which will depend on the abundance of the cell type under study.

Experimental design

Polysome stabilization during cell and tissue collection. Stabilization of the polysome complex is essential to collect mRNAs that accurately reflect the translational state of the cell type of interest. Polysomes can be stabilized by the addition of cycloheximide and magnesium[33], and by keeping the samples at 4 °C. Another key step in successfully stabilizing polysomes after cell lysis is to control endogenous RNase activity. Neuronal polysomes have been reported to be particularly sensitive to degradation by RNase[33]. As blood is a major source of RNase contamination[34], a quick wash of the dissected tissue to remove blood is useful to control RNase levels. Keeping the samples at 4 °C, and the addition of recombinant RNase inhibitors to the lysis buffer, will also help minimize RNase activity. High levels of RNase activity that are not controlled by these precautions can be controlled by the addition of 0.75 mg/ml heparin to all solutions[35]. However, we do not routinely use heparin in our preparations because of the subsequent need to perform lithium chloride precipitation of the purified RNA in order to prevent inhibition of downstream enzymes by heparin[36]. If there is doubt regarding the integrity of polysomes in a lysate preparation, polysome profiles should be analyzed after zonal centrifugation, as previously described[1,37] and as shown in **Figure 2**.

Expected RNA integrity and yields. Most of the RNA purified by TRAP will be rRNA, and thus any analysis should show mostly purification of this RNA species, although mRNA (the desired species in most cases) and tRNA are also purified. The integrity (lack of degradation) of mRNA can be indirectly estimated by assessing the quality of the rRNA. By using an Agilent Bioanalyzer, we routinely obtain RNA integrity values of 9.5–10 (out of a maximum 10) from cultured cells and 7.5–10 (out of a maximum of 10) from brain tissue extracted from unperfused, adult mice. Poor RNA integrity is caused by inefficient control of endogenous, or the addition of exogenous, RNase activity. Expected total RNA yields will vary on the basis of the number of cells expressing the TRAP transgene, the expression level of the TRAP transgene, and the size and translational state of the cell types expressing the TRAP transgene. Thus, actively growing, large cells expressing high levels of EGFP-L10a will yield more total RNA than postmitotic, small cells expressing low levels of EGFP-L10a. As a comparison metric, for 0.7×10^6 labeled spiny projection neurons in 15 mg of one mouse striatum, with the transgene driven by a moderate-strength driver (*Drd1a*), we often recover ~50 ng of polysomal RNA[21].

Effects of freezing tissue on polyribosome integrity and mRNA yields. Optimal polysome integrity, and thus TRAP RNA yields, is obtained from fresh tissue that is immediately homogenized in lysis buffer that will stabilize polysomes. Our studies indicate that approximately half of the monosomes, as well as the polysome aggregates, are lost after flash-freezing of tissue in liquid nitrogen, storage at −80 °C and thawing coincident with homogenization (**Fig. 2**). Nevertheless, studies that require the use of large numbers of transgenic animals, or that require animals to be euthanized after defined periods of drug exposure, are impractical to perform with fresh tissue only. On average, our total RNA yields from TRAP frozen tissue samples are approximately half (46.2%) of that normally obtained from fresh tissue, as predicted from the results of the polysome profiles (**Fig. 2**). We suggest that all samples in an experiment be processed in the same way (fresh or frozen), to avoid experimental artifacts; in particular, we predict that the loss of TRAP RNA yield upon freezing may result in a loss of representation of rare messages, when compared with fresh tissue.

Experimental group design. Before starting a TRAP study, investigators should ensure that they have the relevant licenses for animal research, and that their proposed TRAP study has been reviewed and approved by the investigator's Institutional Animal Care and Use Committee (IACUC). Investigators should adhere to the ARRIVE guidelines for reporting animal experiments[38].

- *Feasibility pilot experiment.* To determine whether a particular tissue source is feasible for use in TRAP studies, purifications should be run from TRAP-expressing and control (non–TRAP-expressing) animals to determine the background level of RNA purification from the prospective tissue source. We typically use one TRAP transgenic and one nontransgenic animal each for three different concentrations of affinity matrix (**Box 1**) to

Box 1 | Preparation of the affinity matrix ● TIMING 2–2.5 h

Each purification will require 300 µl of Streptavidin MyOne T1 Dynabeads, 120 µl of biotinylated protein L (1 µg/µl in 1× PBS) and 50 µg each of GFP antibodies 19C8 and 19F7 (100 µg total antibody).

For feasibility pilot experiments (see Experimental Design section), half and double the matrix component amounts, keeping ratios the same, can also be tried. Investigators should keep altering the amounts, keeping ratios the same, until an optimal amount of matrix is found that captures all tagged RNA. For example, if the amounts listed above are optimal for a new cell type, one would expect to see a halving of TRAP yield with half the amount of matrix used in TRAP purifications—and no detectable purification of RNA from the non-TRAP control with either concentration. (We typically use a 1.5-fold excess of optimal concentration in our TRAP experiments, to account for matrix pipetting error.) Such a pilot experiment is recommended because the amount of affinity matrix needed will vary by cell type, as the abundance of the target cell type, its translational state, its size and the local tissue background RNA binding levels are all characteristics that will vary between different investigators' TRAP experiments. The amounts listed above were empirically determined to ensure complete binding of all epitopes in a relatively abundant cell type (e.g., 0.7×10^6 spiny projection neurons in 15 mg of striatal tissue), but rare cells may need a fraction of the amounts listed here. For such rare cell types, it is advisable to use the minimum amount of matrix needed to reduce background RNA binding.

Upon receipt, record the two antibodies' concentrations, as they will vary by batch. If the antibodies arrive unfrozen, mix each tube gently and divide each antibody into single-experiment aliquots (to be used within a week), snap-freeze the aliquots in liquid nitrogen and store the aliquots at –80 °C. If the antibodies arrive frozen, store them immediately at –80 °C; they should be thawed on ice and aliquotted before or at first use. On the day of use, thaw the aliquot to be used that day on ice, spin the tubes at maximum speed (>13,000*g*) in a microcentrifuge for 10 min at 4 °C and transfer the supernatants (antibody) to new tubes. Add sodium azide as needed. The antibody can be kept at 4 °C for a few days. If the interval between IPs is longer than this, divide the antibody into aliquots, snap-freeze them in liquid nitrogen and store the single-use aliquots at –80 °C.

1. Resuspend the Streptavidin MyOne T1 Dynabeads thoroughly in the original bottle by gentle hand mixing.
2. Calculate the amount of Dynabeads required on the basis of the ratios above. Note that the affinity matrix to be used in one particular experiment should be aliquotted to samples from a common source: either prepare all matrix in one larger tube or prepare batches in smaller tubes and combine them into a larger tube for mixing before aliquotting. As recommended by the manufacturer, throughout all manipulations, keep the volume of beads close to the original volume from the source bottle.
3. Transfer the beads to be used to a tube(s) and collect them on a magnet (30–60 s).

? TROUBLESHOOTING

4. Wash the beads with 1× PBS once (1 ml for all washes if a 1.5-ml tube is used).
5. Collect the beads on the magnet and resuspend them in the appropriate volume of 1× PBS (original bead volume minus the volume of biotinylated protein L to be added).
6. Incubate the beads with biotinylated protein L in 1× PBS (aim for a 1-ml total volume if you are using a 1.5-ml tube) for 35 min at room temperature by using gentle end-over-end mixing in a tube rotator.
7. Collect the protein L–coated beads on the magnet.
8. Wash the coated beads 5 times with 1× PBS containing 3% (weight/volume) IgG and protease-free BSA.
9. Proceed to antibody binding in low-salt buffer, by binding 50 µg each of 19C8 and 19F7 (100 µg total, in 1 ml total volume) for 1 h by gentle end-over-end rotation in a tube rotator. Do not vortex affinity matrix after antibody binding.
10. After antibody binding, wash the beads three times with low-salt buffer. After washing, resuspend the beads in a volume of low-salt buffer such that each IP will receive an aliquot of the components listed above—beads/protein L/Ab (the affinity matrix in ratios listed above)—in a 200-µl final aliquot volume.

■ **PAUSE POINT** Once it is prepared, the affinity matrix can be used immediately, or it can be stored for up to 2 weeks at 4 °C with the addition of 0.02% sodium azide. If pre-prepared affinity matrix is stored in sodium azide; it should be washed three times quickly in low-salt buffer before use. Pre-prepared affinity matrix may be difficult to resuspend quickly, and it may be carefully resuspended by gentle agitation overnight on a tube rotator. Do not vortex the affinity matrix after antibody binding.

determine background when using a new tissue source. As long as there is a difference in the amount of RNA purified from these two sources, a TRAP study can be conducted. Brain tissue regions that are very rich in myelin tend to have the highest background RNA levels.

- *Statistical power analysis pilot experiment.* Although the particulars of each investigator's scientific hypothesis will direct the TRAP study design (e.g., sex, drug treatment, genetic perturbation and age of animals), nevertheless a second pilot experiment, with three TRAP-expressing biological replicates collected under the same purification conditions, should be used to determine the sample size that will be needed in the full study. Results from this pilot study will not only inform the investigators as to expected RNA yields, but they should also be used to perform a statistical power analysis based upon the number of genes and hypotheses to be interrogated in downstream applications. It is common for 6–10 biological replicates to be needed for adequate statistical power in genome-wide expression studies.
- *Collecting experimental groups.* As with any experiment that will amplify RNA, it is essential to process paired TRAP samples in downstream amplification steps at the same time to avoid

amplification artifacts (e.g., processing for microarray or RNA sequencing analysis). Optimally, all samples are collected, purified and amplified together. However, in experiments that make use of a large number of experimental groups and biological replicates, this is often impractical. For maximum TRAP sensitivity, each set of samples that will be directly compared at the raw data level (e.g., vehicle and drug treatment) at minimum should be purified and amplified together.

RNase-free technique. RNase contamination on lab equipment can be widespread in a lab not currently conducting RNA work. In addition, RNase is a very stable enzyme. Before starting, it is thus important to set up an RNase-free work zone. Use RNase-decontaminating reagents such as RNase-Zap (Ambion) to decontaminate work surfaces and equipment that may be contaminated with RNase. Certified RNase-free plasticware and reagents, as well as aerosol-resistant tips, should be used whenever possible. Wear and change gloves often, as well as whenever common-use lab equipment that may have RNase contamination is touched.

Material availability. Plasmids for use in making TRAP transgenic mice[27] and published TRAP mouse lines[1,18] are available from The Rockefeller University upon request. Monoclonal GFP antibodies 19C8 and 19F7 are available for purchase from the Memorial Sloan-Kettering Monoclonal Antibody Facility. The TRAP transgene sequence, as used in our studies, is supplied below in the MATERIALS section.

MATERIALS

REAGENTS

- Absolutely RNA Nanoprep kit (Agilent, cat. no. 400753)
- Biotinylated protein L, recombinant, purified (Fisher Scientific, cat. no. PI-29997) ▲ **CRITICAL** Preparation of the affinity matrix by using biotinylated protein L, GFP antibodies and magnetic Dynabeads takes a few hours, and it can be performed up to 2 weeks before the actual experiment (**Box 1**).
- BSA, IgG and protease-free (Jackson ImmunoResearch, cat. no. 001-000-162)
- Cycloheximide (Sigma, cat. no. C7698) ! **CAUTION** Cycloheximide is very toxic, and it is dangerous for the environment. Collect all waste for proper disposal.
- 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC; Avanti Polar Lipids, cat. no. 850306P-200 mg) ▲ **CRITICAL** Order powdered form in the 200-mg size or larger, as smaller sizes often come in nonresealable glass ampules. Store the powder at –20 °C and thaw it to room temperature (24 °C) before opening to reconstitute.
- DL-DTT (Sigma, cat. no. D9779) ! **CAUTION** This is a harmful irritant.
- Ethanol (Sigma, cat. no. E7023)
- GFP antibodies (Memorial Sloan-Kettering Monoclonal Antibody Facility; clone names: Htz-GFP-19F7 and Htz-GFP-19C8, bioreactor supernatant purity) ▲ **CRITICAL** Preparation of the affinity matrix using biotinylated protein L, GFP antibodies and magnetic Dynabeads takes a few hours, and it can be performed up to 2 weeks before the actual experiment (**Box 1**). ▲ **CRITICAL** For TRAP purifications, these monoclonal antibodies are superior to commercially available ones, and thus they should not be substituted. Different lots of antibodies will have different antibody concentrations, but even when normalized for concentration different lots may have slightly different functional antibody concentration if the lots have been freeze-thawed. We recommend ordering a large enough lot to cover most planned purifications, and to save the antibodies in single-experiment aliquots. If additional lots are needed, a side-by-side comparison for efficacy (based on TRAP yield) should be performed with old versus new lots.
- Glucose (Sigma, cat. no. G7528)
- HBSS, 10× (Invitrogen, cat. no. 14065-056)
- HEPES, 1 M, pH 7.3, RNase-free (Affymetrix, cat. no. 16924)
- Magnesium chloride ($MgCl_2$), 1 M, RNase-free (Applied Biosystems, cat. no. AM9530G)
- Methanol (Sigma, cat. no. 322415)
- Nonylphenyl polyethylene glycol (NP-40), 10% (vol/vol), 10 × 5 ml ampules (AG Scientific, cat. no. P1505)
- Nuclease-free water (Applied Biosystems, cat. no. AM9939)
- PBS, 10×, RNase-free (Applied Biosystems, cat. no. AM9625)
- Potassium chloride (KCl), 2 M, RNase-free (Applied Biosystems, cat. no. AM9640G)
- Protease inhibitor tablets, Mini-Complete, EDTA-free (Roche, cat. no. 11836170001) ▲ **CRITICAL** It is essential that no EDTA be used in any of the solutions prepared in this protocol. EDTA will chelate magnesium, leading to the dissociation of polysomes.
- Quant-it RiboGreen assay (Invitrogen, cat. no. R11490)
- RNase Zap Wipes (Applied Biosystems, cat. no. AM9786)
- RNasin, recombinant (Promega, cat. no. N2515)
- Sodium azide (Sigma, cat. no. S2002) ! **CAUTION** Sodium azide is very toxic and dangerous for the environment; contact with acids generates a very toxic gas. Collect and dispose of waste properly.
- Sodium bicarbonate ($NaHCO_3$; Sigma, cat. no. S6297)
- Streptavidin MyOne T1 Dynabeads (Invitrogen, cat. no. 65601) ▲ **CRITICAL** Preparation of the affinity matrix by using biotinylated protein L, GFP antibodies and magnetic Dynabeads takes a few hours, and it can be performed up to 2 weeks before the actual experiment (**Box 1**).
- Sulfolane (Sigma, cat. no. T22209-100G) for use with the Absolutely RNA Nanoprep kit (Agilent, cat. no. 400753)
- Superasin (Applied Biosystems, cat. no. AM2694)
- The TRAP transgene as originally described[1] is a fusion of the EGFP coding sequence (taken from GenBank U57606) to mouse ribosomal protein Rpl10a (GenBank BC083346). This TRAP transgene sequence was assembled as follows: EGFP coding sequence minus its stop codon, linker sequence coding for SGRTQISSSSFEF, followed by the Rpl10a coding sequence minus its first M-encoding codon
- TRAP-expressing cells can be obtained by transfecting or transducing transformed or primary cells with the TRAP transgene sequence under the control of a suitable promoter. Alternatively, TRAP-expressing primary cells can be collected from TRAP transgenic mice (see below) for culture *in vitro*
- TRAP transgenic mice (available from The Rockefeller University by request) ! **CAUTION** Only use mice after receiving proper training by and registration with the IACUC at your institution.

EQUIPMENT

- 2100 Electrophoresis Bioanalyzer with Nanochips and Picochips (Agilent, cat. nos. G2939AA, 5067-1511 and 5067-1513)
- Cell scrapers (Sarstedt, cat. no. 83.1832)
- Homogenizers (Fisher Scientific, cat. no. K8855100020)
- Magnet (will depend on purification scale; samples in 1.5-ml tubes can be concentrated on a DynaMag-2, Invitrogen, cat. no. 123-21D)
- Minicentrifuge (Fisher Scientific, cat. no. 05-090-100, or equivalent)
- NanoDrop 2000C spectrophotometer (Thermo Scientific, cat. no. ND-2000C)
- Refrigerated centrifuge (Eppendorf, cat. no. 5430R, or equivalent, with rotor for 1.5-ml microcentrifuge tubes)
- RNase-free 1.5-ml microcentrifuge tubes (Applied Biosystems, cat. no. AM12450)
- RNase-free 50-ml conical tubes (Applied Biosystems, cat. no. AM12501)
- RNase-free 1,000-µl filter tips (Rainin, cat. no. RT-1000F)
- RNase-free 200-µl filter tips (Rainin, cat. no. RT-200F)
- RNase-free 20-µl filter tips (Rainin, cat. no. RT-20F)
- Rotor for homogenizers (Yamato, cat. no. LT-400D, or equivalent)
- Tube rotator, Labquake brand (Thermo Fisher, cat. no. 13-687-12Q, or equivalent)

REAGENT SETUP

Cell-lysis buffer Mix 20 mM HEPES KOH (pH 7.3), 150 mM KCl, 10 mM $MgCl_2$ and 1% (vol/vol) NP-40 in RNase-free water. Store the buffer at 4 °C

for up to several months. Add EDTA-free protease inhibitors (one mini tablet per 10 ml), 0.5 mM DTT, 100 µg/ml cycloheximide and 10 µl/ml rRNasin and Superasin to an aliquot immediately before use. ▲ CRITICAL Brain ribosomes (monosomes) and polyribosomes (polysomes) require relatively high levels of $MgCl_2$ (10–12 mM) for optimal purification[33].

Cycloheximide In a clean plastic weigh boat, weigh out 100 mg of cycloheximide and resuspend it in 1 ml of methanol. Store it at 4 °C for up to 1 d.

DHPC Reconstitute DHPC in RNase-free water to 300 mM. Warm the DHPC powder to room temperature before reconstitution. Add water and keep at room temperature, with occasional vortexing for ~30 min to produce a solution. Once DHPC is reconstituted in water, use the solution for up to 7 d, and store it at 4 °C in glass. Do not store it in plastic.

Dissection buffer Mix 1× HBSS, 2.5 mM HEPES-KOH (pH 7.3), 35 mM glucose and 4 mM $NaHCO_3$ in RNase-free water. Store the buffer at 4 °C for several months. To an aliquot, add cycloheximide to a final concentration of 100 µg/ml immediately before use.

DTT Reconstitute DTT to 1 M in RNase-free water, filter-sterilize the solution and store it at −20 °C in single-use aliquots.

High-salt buffer Mix 20 mM HEPES KOH (pH 7.3), 350 mM KCl, 10 mM $MgCl_2$ and 1% (vol/vol) NP-40 in RNase-free water. Store it at 4 °C for up to several months. To an aliquot, add DTT to a final concentration of 0.5 mM and cycloheximide to a final concentration of 100 µg/ml immediately before use.

Low-salt buffer See the recipe for cell-lysis buffer above; but there is no need to add RNase inhibitors to the low-salt buffer if it is not used for cell lysis. To an aliquot, add DTT to a final concentration of 0.5 mM and cycloheximide to a final concentration of 100 µg/ml immediately before use.

Protein L Reconstitute protein L as recommended by the manufacturer. Once it is reconstituted, extra protein L should be aliquotted in single-use aliquots and stored at −80 °C.

Tissue-lysis buffer Mix 20 mM HEPES KOH (pH 7.4), 150 mM KCl and 10 mM $MgCl_2$ in RNase-free water. Store it at 4 °C for several months. Add EDTA-free protease inhibitors, 0.5 mM DTT, 100 µg/ml cycloheximide and 10 µl/ml rRNasin and Superasin to an aliquot immediately before use.

PROCEDURE

Lysate preparation ● TIMING 30–60 min for either option

▲ CRITICAL It is important to prepare reagents, including the affinity matrix (**Box 1**), before starting, in order to process cells or tissue as quickly as possible.

1| Lysates can be prepared from either TRAP-expressing cultured cells (option A) or from tissue collected from TRAP transgenic mice (option B). The differences in procedure are driven mainly by the fact that detergent can be used to lyse cells in culture, but it should be avoided in tissue lysis (as we believe that the presence of detergent can cause myelin or fat to aggregate with polysomes).

▲ CRITICAL STEP Always keep samples at 4 °C.

(**A**) **Cell culture lysate preparation**

(i) To the cell culture medium, add 100 µg/ml cycloheximide, and return the plates to the incubator for 15 min.

(ii) Wash the cells three times on ice with 10 ml of 1× PBS containing 100 µg/ml cycloheximide, by aspirating as much PBS as possible after each wash.

(iii) Add 1 ml of ice-cold cell lysis buffer to each 10-cm plate.

(iv) Incubate the plates on ice for 10 min.

(v) Scrape the cells and cell debris from the plate surface with cell scrapers, and transfer them to a prechilled homogenizer.

(vi) Homogenize the samples in a cold room, or at room temperature, by placing the homogenizer in a 50-ml conical tube with ice. Homogenize the samples in a motor-driven Teflon-glass homogenizer, at 900 r.p.m., with 12 full strokes. First insert the Teflon pestle into the glass tube until the solution submerges the entire Teflon pestle, start to stir it at 300 r.p.m. and then raise the speed slowly to 900 r.p.m. Lower the glass tube, but do not let the Teflon pestle rise to the air-solution interface because it will produce substantial aeration, which may lead to protein denaturation. Alternatively, cultured cells can be lysed by vigorously pipetting up and down ten times with a P1000 pipette (without the creation of bubbles), although best lysis is obtained by using a homogenizer.

(vii) Transfer the lysate to a chilled microcentrifuge tube, and centrifuge it at 2,000*g* for 10 min at 4 °C. The lysate volume should be similar to that added to the tissue culture dish.

? TROUBLESHOOTING

(viii) Transfer the supernatants (**S2**) to a new, chilled microcentrifuge tube and add to each sample 1/9 volume (e.g., 111 µl for a 1,000-µl sample) of 300 mM DHPC (DHPC reconstituted in water).

? TROUBLESHOOTING

(ix) Mix by quickly inverting the tube by hand, and incubate the samples on ice for 5 min.

(x) Centrifuge the samples at 20,000*g* for 10 min at 4 °C.

(xi) Transfer the resulting supernatant, **S20**, to new, chilled tubes and proceed to immunopurification (IP; Step 2). Lysates can be stored on ice for several hours while additional samples are collected, before proceeding to Step 2. A small aliquot of **S20** (e.g., 1% total volume) can be saved at this point to compare it with the sample collected after enrichment of transcripts in purified material by downstream assays.

(**B**) **Tissue lysate preparation**

(i) Remove the brain tissue from mice.

▲ CRITICAL STEP Be sure that all procedures for handling mice are registered with your IACUC.

(ii) Dissect the desired tissue region rapidly, and place the tissue into ice-cold dissection buffer quickly to wash it.
! CAUTION Handle liquid nitrogen with proper cryogenic protective equipment and only use tubes that are resistant to liquid nitrogen.
■ PAUSE POINT Dissected tissue can be stored for several years at –80 °C if it is immediately flash-frozen in liquid nitrogen upon dissection. To freeze the tissue, immediately upon dissection wash the tissue in dissection buffer for 2 s; transfer it to a clean, empty microcentrifuge tube; cap the tube and fully immerse the tube immediately into liquid nitrogen; and transfer the frozen tubes to the storage location at –80 °C, without allowing the tubes to warm above –80 °C. When the tissue is ready to use, without allowing the tissue to thaw, use cold forceps to transfer the frozen tissue piece(s) quickly into a cold homogenizer containing prechilled tissue lysis buffer and immediately homogenize it, so that the tissue thaws as it is being homogenized.

(iii) Transfer the tissue to a prechilled homogenizer on ice that contains tissue-lysis buffer. Homogenize ~25–50 mg of tissue per 1 ml of tissue-lysis buffer. The homogenizer listed in the Equipment section is ideal for 1-ml volumes, but similar models of Teflon-glass homogenizers are available for smaller or larger volumes (scale according to a 25–50 mg: 1 ml tissue-lysis buffer ratio).
▲ CRITICAL STEP If more than one dissection is being performed, homogenize each sample as it is dissected.

(iv) Homogenize the samples in a cold room, or at room temperature, by placing the homogenizer in a 50-ml conical tube with ice. Homogenize the samples in a motor-driven Teflon-glass homogenizer, at 900 r.p.m., with 12 full strokes. First insert the Teflon pestle into the glass tube until the solution submerges the entire Teflon pestle, start to stir at 300 r.p.m. and then raise the speed slowly to 900 r.p.m. Lower the glass tube but do not let the Teflon pestle rise to the air-solution interface because it will produce significant aeration, which may lead to protein denaturation.
? TROUBLESHOOTING

(v) Transfer the lysate into a prechilled microcentrifuge tube on ice.

(vi) Prepare a postnuclear supernatant (**S2**) by centrifugation at 4 °C for 10 min at 2,000*g*.

(vii) Transfer **S2** to a new, prechilled microcentrifuge tube on ice.

(viii) Add 1/9 sample volume of 10% NP-40 to **S2** (final concentration = 1%), and mix it gently inverting the tube. Pulse-centrifuge the sample in a minifuge to collect the liquid at the bottom of the tube.

(ix) Add 1/9 sample volume of 300 mM DHPC (final concentration = 30 mM), mix it gently by inverting the tube and incubate the mixture on ice for 5 min.
? TROUBLESHOOTING

(x) Prepare the postmitochondrial supernatant (**S20**) by centrifugation at 4 °C for 10 min at 20,000*g*.

(xi) Take **S20** to a new, prechilled microcentrifuge tube and proceed immediately to IP (Step 2). Lysates can be stored on ice for several hours while additional samples are collected, before proceeding to Step 2. A small aliquot of **S20** (e.g., 1% total volume) can be saved at this point to compare it with the sample collected after enrichment of transcripts in purified material by downstream assays. If so, we recommend incubating this **S20** aliquot at 4 °C for the same length of time as the TRAP IP (16–18 h) before freezing the aliquot at –80 °C, to ensure that all samples are incubated for equal durations.

Immunopurification ● TIMING 18–20 h

2| Thoroughly resuspend the pre-prepared affinity matrix (**Box 1**). If the pre-prepared affinity matrix was stored in sodium azide, wash it three times quickly in low-salt buffer before use.

3| Add 200 µl of freshly resuspended beads to each **S20** sample (~1,000 µl).
▲ CRITICAL STEP Always resuspend the affinity matrix thoroughly by gentle pipetting immediately before use.
? TROUBLESHOOTING

4| Incubate the samples at 4 °C for 16–18 h with gentle end-over-end mixing in a tube rotator. This incubation time is longer than that described in our original method[1], and it reflects recent optimizations to the purification scheme that serve to increase RNA yield[2,19–21] (tenfold for rare cell types).

5| After incubation, collect the beads with a magnet (well-chilled in an ice bucket). Use a minifuge to spin down beads from the caps between washes. The whole fraction, or an aliquot of the unbound fraction, can be saved at –80 °C at this point to compare it with the sample collected after enrichment of transcripts in purified material by downstream assays.

6| Resuspend the beads in 1,000 µl of high-salt buffer, and collect them with a magnet as described above.
▲ CRITICAL STEP All washes should be performed by careful pipetting that avoids the introduction of bubbles. After the beads are visibly resuspended, the beads should be mixed by pipetting at least four more times. High background can result from insufficient bead resuspension during washes.

7| Repeat the wash three times (1,000 µl of high-salt buffer each time, a total four washes).

8| After the fourth wash with high-salt buffer, remove all remaining wash buffer, remove the tubes from the magnet and warm the tubes to room temperature. Resuspend the beads in 100 µl of Nanoprep lysis buffer with beta-mercaptoethanol (β-ME) (use lysis buffer from the Stratagene Absolutely RNA Nanoprep kit or equivalent), vortex the mixture, incubate it for 10 min at room temperature, remove the RNA (now in Nanoprep lysis buffer) from the beads with the magnet and proceed immediately to RNA cleanup, according to the kit manufacturer's instructions.
▲ **CRITICAL STEP** Buffers from other RNA purification kits may be used, but the buffer that is used to release bound RNA from affinity matrix must contain the denaturant guanidine thiocyanate.
▲ **CRITICAL STEP** Guanidine thiocyanate can form crystals at low temperatures. Be sure to extract and clean up RNA at room temperature to avoid crystallization and carry-through to downstream applications.
■ **PAUSE POINT** Eluted RNA (in lysis buffer and removed from beads) can be kept frozen in lysis buffer with β-ME at –80 °C before column cleanup. Warm it to room temperature upon thawing, before resuming purification with the kit.

RNA cleanup and quantification ● TIMING 4–6 h

9| Follow the manufacturer's instructions for the Agilent Nanoprep kit, or a similar kit, to clean up RNA (including the optional DNase digestion and the optional two RNA elutions with elution buffer heated to 60 °C, all optional steps per kit instructions). RNA purified in this manner is of high enough purity for use in most downstream applications, and our studies have indicated that subsequent acidic phenol purification of these samples does not improve the purity of the samples (as judged by spectrophotometer readings and use in downstream amplification reactions, e.g., reverse transcription).
▲ **CRITICAL STEP** After column purification at room temperature, return the samples to ice and keep them on ice at all times; purified RNA should be kept at –80 °C for long-term storage.
▲ **CRITICAL STEP** Perform RNase-free DNase digestion at this step if it is required for downstream applications.
■ **PAUSE POINT** After column purification, RNA can be stored at –80 °C for several years.

10| To assay the integrity of the RNA, analyze 1 µl of each sample on a Bioanalyzer 2100 by using an RNA Pico/Nano chip (follow Agilent's protocol for running chips). The qualitative range of the PicoChip assay is 200–5,000 pg/µl, and the qualitative range of the NanoChip assay is 5–500 ng/µl.
? TROUBLESHOOTING

11| Quantify the samples on a small-volume spectrophotometer (e.g., NanoDrop, Thermo Scientific). For precise quantification of low-concentration samples (<10 ng/µl), run a RiboGreen (or similar) fluorescence-based assay. The use of a spectrophotometer to quantify samples that have been column-purified and that also are of a concentration of less than ~10 ng/µl is not recommended, as debris from the columns leads to inaccurate readings (silica shed from the column scatters light).
■ **PAUSE POINT** After quantification of RNA and after checking its integrity, RNA can be kept at –80 °C for several years.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Box 1, Step 3	Unequal amount of beads appear in equivalent aliquots	Beads are not fully resuspended before being aliquotted	Mix beads well by swirling bottle and and by manual pipetting. Ensure that no bead clumps exist in bottle or are seen on pipette tip during aliquotting step
1A(vii)	Lysate volume is much larger than amount of cell lysis buffer used	Insufficient aspiration of wash solution before cell lysis	Ensure, using a vacuum line, that all wash buffer is removed from the dish surface before lysis buffer is added
1A(viii), 1B(ix)	DHPC is difficult to resuspend	Insufficient time has passed for complete hydration	Reconstitute DHPC with water to 300 mM; it will need to sit at room temperature with occasional vortexing for ~30 min to fully go into solution. Once reconstituted in water, use it up to 7 d later, stored at 4 °C in a glass bottle. Do not store in plastic

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
1B(iv)	Tissue is difficult to homogenize	Too much tissue is used for volume of lysis buffer chosen	Use a Teflon-glass homogenizer and volume of lysis buffer that keeps a ratio of ~25–50 mg tissue: 1 ml tissue lysis buffer
3	Affinity matrix is difficult to resuspend	Affinity matrix has been stored at 4 °C for an extended period of time	On a tube rotator, rotate tube containing matrix gently for several hours until beads are completely resuspended
10	RNA is degraded	RNase contamination	Use only RNase-free reagents, use aerosol-resistant filter pipette tips, change gloves often and keep samples on ice at all times. If working with tissue, homogenize tissue sample immediately after collection and wash tissue before homogenization to remove blood. If working with cultured cells, ensure that no mycoplasma or antiviral response is present. Both of these conditions may elevate endogenous RNase levels markedly in cultured cells
	RNA yields are low	Inefficient immunoaffinity purification caused by RNase contamination Inefficient immunoaffinity purification caused by EDTA present in buffers Inefficient immunoaffinity purification caused by inefficient cell/tissue lysis	Ensure that polysome integrity is maintained by adding cycloheximide, $MgCl_2$ and RNase inhibitors to all solutions, and by keeping samples on ice at all times Ensure that protease inhibitors used do not contain EDTA Ensure that a tight-fitting homogenizer similar to the one listed in the Equipment section is used. Use a volume of lysis buffer that keeps a ratio of about 25–50 mg tissue: 1 ml tissue lysis buffer. Save aliquots of and perform western blots on each step of the purification. Use a non-mouse GFP antibody for western blot analysis: efficiency of purifications can be checked by running western blots (against EGFP) with IP input, unbound and immunopurified samples
	RNA is not of high enough concentration for use in downstream applications	RNA is diluted during cleanup	Concentrate RNA samples using a vacuum concentrator, with no or low heat settings

● TIMING

Step 1, lysate preparation: 30–60 min
Steps 2–8, immunopurification: 18–20 h
Steps 9–11, RNA cleanup and quantification: 4–6 h
Box 1, preparation of the affinity matrix: 2–2.5 h

ANTICIPATED RESULTS

With the stabilization of ribosomes on mRNA and inhibition of RNase activity, all mRNAs associated with EGFP-tagged ribosomes should be recovered. Absolute yields will vary depending on the nature of the target material, as well as on the strength of the regulatory elements that drive the expression of the TRAP transgene. However, for comparison, from 0.7×10^6 labeled spiny projection neurons in 15 mg of one mouse striatum, with the transgene driven by a moderate-strength driver (*Drd1a*), we often recover ~50 ng of polysomal RNA[21]. Integrity of purified RNA from cultured cells is in the 9.5–10 range, and the range is 7.5–10 for RNA purified from brain tissue. These numbers reflect Agilent's Bioanalyzer RNA integrity number scoring system; analysis of samples on agarose gels should reveal sharp, undegraded bands at ~5 kb and 2 kb corresponding to 28S and 18S rRNA, respectively. The TRAP co-purified mRNA usually cannot be visualized directly by these methods because of its small contribution to the total RNA amount. If obtained from the

procedure outlined above, undegraded TRAP RNA that is immunopurified and column-purified is of high enough quality for immediate use in many downstream applications, including qPCR, microarray analysis, RNA sequencing or northern blotting, without the need for additional purification.

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