TechSights

The Expanding Toolkit of Translating Ribosome Affinity Purification

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Translating ribosome affinity purification is a method initially developed for profiling mRNA from genetically defined cell types in complex tissues. It has been applied both to identify target molecules in cell types that are important for controlling a variety of behaviors in the brain, and to understand the molecular consequences on those cells due to experimental manipulations, ranging from drugs of abuse to disease-causing mutations. Since its inception, a variety of methodological advances are opening new avenues of investigation. These advances include a variety of new methods for targeting cells for translating ribosome affinity purification by features such as their projections or activity, additional tags and mouse reagents increasing the flexibility of the system, and new modifications of the method specifically focused on studying the regulation of translation. The latter includes methods to assess cell type-specific regulation of translation in specific subcellular compartments. Here, I provide a summary of these recent advances and resources, highlighting both new experimental opportunities and areas for future technical development.

Key words: **local translation; ribosome profiling; RNASeq; translation regulation; translating ribosome affinity purification**

Introduction

The CNS is composed of hundreds of distinct cell types. Yet, as is clear from the example of Parkinson's disease, disorders of the CNS can be caused by disruptions of just a single one. And because all cell types contain an identical copy of the genome, distinct cell types must be defined by the portions of their genomes that are expressed. However, as these cell types are densely intermingled, dissociating them to assay specific types for gene expression analysis, especially from adult tissues, is challenging. To circumvent this challenge Heiman et al. (2008) developed strategies to purify ribosomes from specific, genetically labeled CNS cell types (Fig. 1*A*). As all protein-coding mRNAs used in a cell are processed by the ribosome, harvesting cell-specific tagged ribosomes allows for quantitative and high throughput analysis of gene expression from the targeted cells.

While translating ribosome affinity purification (TRAP) has clear applications for simply assessing gene expression, it should also be emphasized that ribosome capture allows for study of cell type-specific regulation of translation. Here, I briefly review the original applications of TRAP, primarily for either in-depth molecular characterizations of cell types or assessment of cell type-specific responses to whole-animal experimental manipulations. Then I summarize subsequent innovations enabling wider applications. Finally, I highlight emerging variations of TRAP that focus on the unique opportunities for analyzing translational regulation.

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Initial application

In-depth molecular descriptions of genetically labeled cell types

The most straightforward application of TRAP is for comprehensive profiling of the mRNA usage of the targeted cell type. When TRAP was first applied to survey CNS cell types (Doyle et al., 2008), the diversity in gene expression was remarkable. A comparison of any two cell types via TRAP revealed thousands of differences in transcript abundance. And while neurons and glia clustered separately, there was as much diversity across neuronal types as there was between neurons and glia. While genes classically considered "housekeeping," such as metabolic enzymes or ribosomal proteins, might vary in abundance 2- to 3-fold across cell types, signaling molecules, particularly cell surface proteins, such as receptors and channels, often varied >10-fold. Thus, TRAP as a descriptive method can provide insight into the molecules that define a given cell type.

There were several clear implications of this dramatic divergence of expression across cell types. First, on a practical level, the large-magnitude changes mean that a relatively small number of replicates are sufficient to define transcripts enriched in a particular cell type ("markers"). Even with 1–3 replicates, transcripts with marker-like expression were readily identified. Indeed, estimates using RNAseq power (Hart et al., 2013) indicate that, even with a single replicate sequenced to 25 million reads, one has >80% power to detect a gene with a "marker-like" 5-fold difference between two cells types (and 98% power to detect a 10-fold difference). Second, on a scientific level, the diversity of expression implied that different cell types have markedly different capacities to respond to extracellular ligands, contributing to the

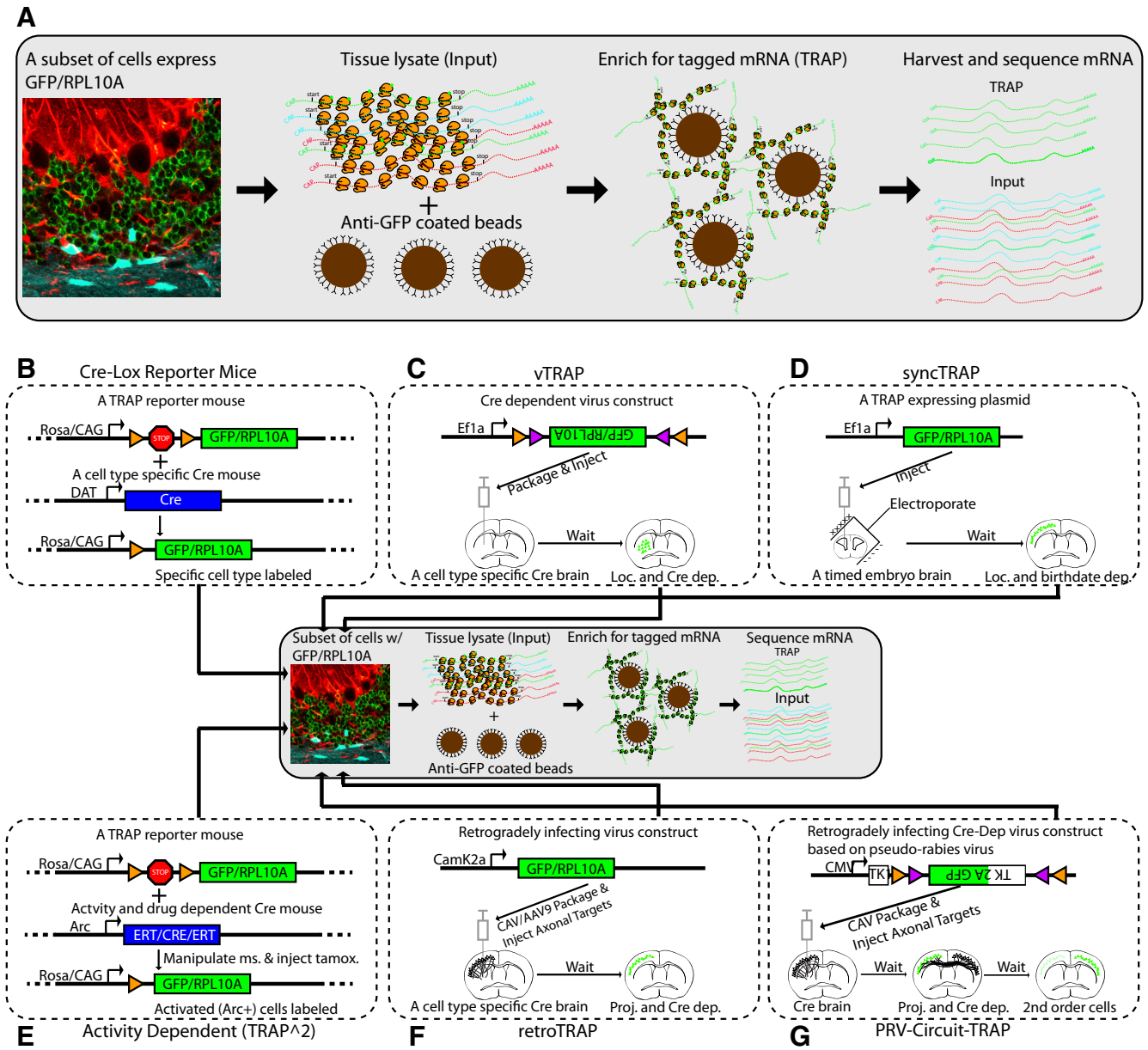

Figure 1. Alternative methods for targeting cell types for TRAP. ***A***, Illustration of standard TRAP work flow. A specific population of cells is driven to express a GFP-ribosomal fusion protein (GFP/RPL10A, green) that allows for immunofluorescence to confirm targeting and provides a tag for affinity purification of ribosomes from the targeted cell types by using anti-GFP magnetic beads. Initially, cells were targeted using bacterial artificial chromosomes with known expression patterns (Table 1). ***B***, A variety of mouse lines (Table 2) now allow GFP/RPL10A or related construct expression in response to the presence of Cre recombinase, which removes a stop cassette flanked by LoxP sites. This allows profiling of the cell types from a large number of existing Cre mouse lines. ***C***, Use of Cre-dependent virus constructs (here illustrated as the double-inverted operon or FLEX cassette, which flips into forward orientation in the presence of Cre), allows access to Cre-expressing cells in specific regions of the adult brain. ***D***, GFP/RPL10A can also be electroporated into the brain at a defined point in development to label a synchronized population of cells born in a particular window. ***E***, Use of a GFP/RPL10A reporter mouse and an activity-dependent inducible Cre allows for profiling of cells activated by a specific behavior. ***F***, Use of a generic neuronal promoter and a retrogradely infecting virus can be used to profile cells based on their projections. ***G***, Coupling with transsynaptic viruses allows for profiling of second-order (or greater) neurons for a given region and/or cell type.

complexity by which different classes of cells might convey information to each other. This diversity of ligand/receptor pairs allows for overlapping information processing networks in the same physical space. Third, it suggested that transcriptomics has sufficient information to define a cellular taxonomy for CNS (Dougherty, 2014), an idea that is being tested now by initiatives in brainwide transcriptome cell atlases. Finally, it suggested that the relationship between gene expression and cell type might inform our understanding of neurogenetics of normal and abnormal behavior, based on the "selective expression" hypothesis (Lage et al., 2008; Xu et al., 2014; Wells et al., 2015) that genes important for a particular phenotype/disease will have enriched expression in the cell types that mediate that phenotype/disease. Studies based on this premise have been applied to better understand both the cells and the genes that matter for particular behaviors.

In one such approach, a "candidate cell type" (a cell type with suspected involvement for the phenotype) is profiled. Enriched transcripts are defined and then manipulated to determine impact on the phenotype. For example, profiling of Hcrt neurons,

implicated in the sleep disorder narcolepsy, identified enriched expression of *Lhx9* in these cells. Subsequently, deletions of *Lhx9* demonstrated a conserved role in modulating Hcrt neuron number and sleep behavior (Dalal et al., 2013; Liu et al., 2015). Numerous similar studies have defined cells and genes that mediate depressive behavior (Schmidt et al., 2012; Shrestha et al., 2015), communicative behavior (Dougherty et al., 2013), nicotinic relapse (Görlich et al., 2013), vulnerability to neurodegeneration (Brichta et al., 2015; McKeever et al., 2017), sociosexual behavior (Nakajima et al., 2014), chromatin modifiers of cerebellar development (Yang et al., 2016; X. Zhu et al., 2016), and glial and lymphoblast genes for development, injury, and repair (Dougherty et al., 2012; Lee et al., 2012; Anderson et al., 2016; Y. Zhu et al., 2017). This approach has even expanded beyond the nervous system to candidate cell types in brown fat (Long et al., 2014) and kidneys (Liu et al., 2014). As TRAP has progressed from microarrays to RNAseq, even particular splice isoforms are being associated with specific cellular phenotypes: the c2 isoform of Pcdha regulates axon tiling of serotonin neurons (W. V. Chen et al., 2017), and differences in Nrxn splicing mediate postsynaptic specializations across hippocampal cell types (Nguyen et al., 2016). Thus, TRAP, through the lens of a candidate cell type framework, can help identify transcripts important for phenotypes of interest.

Assessment of cell type-specific responses to experimental manipulations

The first TRAP experiment highlights the other major application of TRAP, to understand how a targeted cell type responds to an animal-wide manipulation: Heiman et al. (2008) used TRAP to define the responses of two subtypes of striatal neurons to cocaine. Recent studies have taken a similar approach to understanding consequences of L-Dopa (Heiman et al., 2014), stress or LTP in hippocampal neurons (P. B. Chen et al., 2017), or sleep and epilepsy in glia (Bellesi et al., 2015; Clasadonte et al., 2016). Likewise, TRAP from mutant models allowed an understanding of the cellular consequences of disease-causing mutations. These might be particularly informative for mutations in RNA binding proteins (Galloway et al., 2014; Tan et al., 2016) or transcriptional regulators (Rannals et al., 2016; von Schimmelmann et al., 2016), where a robust response might be expected in mRNA.

Two trends are apparent across these studies. First, TRAP has sufficient reproducibility to identify cell type-specific consequences of these manipulations. Second, however, magnitudes of these within-cell type responses are generally substantially smaller than magnitudes of the between-cell type differences. Philosophically, this implies that cell identity is a stronger driver of gene expression than cell state. Practically, this means that these studies need to be well powered to reliably detect these smaller differences. For example, to be 80% powered to detect a twofold difference between conditions within a cell type, one would need at least 5 or 6 samples per condition. Careful balancing of potential confounds, both biological and technical, is essential. Expectations regarding the number and magnitude of transcriptional responses should be moderated.

Despite this and other challenges, TRAP has been widely applied, perhaps because it is more readily scalable than FACS or laser capture microdissection. It will be interesting to see whether newer nuclei-purification based approaches (Kriaucionis and Heintz, 2009; Deal and Henikoff, 2010; Roh et al., 2017) show similar successes, as they also circumvent the challenge dissociating live single cells from complex CNS tissue. Likewise, true single-cell approaches, such as DROPseq (Macosko et al., 2015), appear sufficiently sensitive to identify the large-magnitude differences between cell types ("marker-like" gene expression). However, there are such rapid advances that it is difficult to estimate what the power of the current methods might be for detecting subtle changes within a given cell type in response to manipulations. Power simulations do currently indicate that, across methods as one assesses 100–200 cells for a given cell type, there is also sufficient power to detect the differences between related subtypes of cells (i.e., different types of microglia) (Ziegenhain et al., 2017). Regardless, as single-cell methods improve, they will likely supplant TRAP for some applications. Particularly for counting numbers of given cell types, the ability to conduct DROPseq-like approaches on untagged cells allows substantial experimental scalability. Yet, emerging applications for TRAP are enabling investigation of new experimental questions, particularly around regulation of translation, for which there is not yet a single-cell equivalent. These emerging approaches are highlighted below.

Application

Targeting additional cell types defined by genetics, projections, or activity

The initial bacTRAP mouse lines benefitted from a GENSAT-defined set of bacterial artificial chromosomes to label specific, genetically defined, cell types in the brain. While bacterial artificial chromosomes have advantages and disadvantages (Dougherty, 2014; Ting and Feng, 2014), these reagents enabled profiling many cell types and remain publicly available (Table 1). In addition, Cre-dependent reporter lines (Table 2; Fig. 1*B*) were developed and enable TRAP of Cre-defined cell types. The earliest reporter was the Ribotag mouse (Sanz et al., 2009). It differs from traditional TRAP in that it generates a Cre-dependent HA-tag on the endogenous RPL22 protein (Fig. 2*A*). While both GFP/RPL10A and RPL22/HA tag the large subunit, differences in relative stoichiometry and affinity of ribosomes for tagged proteins, as well as antibody affinities, might lead to differences in performance *in vivo* between the constructs. Likewise, recent work has shown that RPL10A, at least in ES cells, is present at substoichiometric levels, and ribosomes containing RPL10A have altered affinity for ~5% of transcripts (Shi et al., 2017). While this will not likely influence comparisons between cell types all tagged with GFP/RPL10A, as such biases should cancel out; it may lead to interesting differences between the distinct epitope tags. Thus, a direct comparison would be of both scientific and technical interest. A variety of reporter lines expressing GFP/RPL10A in a Cre-dependent (Zhou et al., 2013; Liu et al., 2014) or Tet-dependent (Drane et al., 2014) manner from Rosa26 or alternate loci (Stanley et al., 2013), even with alternate red fluorophores as tags (Hupe et al., 2014) are available.

Also, TRAP constructs are small enough to package into adeno-associated virus (Fig. 1*C*). Coupling a Cre-dependent FLEX design and stereotactic injection allows for expression in spatially, temporally, and genetically restricted cell populations (Nectow et al., 2017). There are real advantages to delivering the TRAP constructs by adeno-associated virus for many cell types. Notably viral expression takes weeks (compared with months for mouse breeding), and may also circumvent well-known problems with transient developmental Cre expression in many mouse lines.

In addition, viral constructs allow for additional innovations in targeting cell types based not only on specific Cre expression, but also potentially by projections, location, and activity (Fig. 1). For example, packaging with capsid proteins that infect axons at their target zones allowed for molecular phenotyping of neurons projecting to particular regions (Cook-Snyder et al., 2015), or

Table 1. List of publicly available bacTRAP lines[a]

Stock #	Name	Targeted cells	Reference
030273	B6;FVB-Tg (Snap25-EGFP/Rpl10a)JD362Htz/J	All neurons	Dougherty et al., 2012
030247	B6;FVB-Tg (Aldh1l1-EGFP/Rpl10a)JD130Htz/J	Astrocytes and other Aldh1L1$^+$ cells	Doyle et al., 2008
030248	B6;FVB-Tg (Aldh1l1-EGFP/Rpl10a)JD133Htz/J	Astrocytes and other Aldh1L1$^+$ cells	Doyle et al., 2008
030271	B6;FVB-Tg (Sept4-EGFP/Rpl10a)DS152Htz/J	Bergmann glia and other Sept4$^+$ cells	Doyle et al., 2008
009159	B6;FVB-Tg (Cnp-EGFP/Rpl10a)JD368Htz/J	Mature oligodendrocytes	Doyle et al., 2008
030268	B6;FVB-Tg (Pdgfra-EGFP/Rpl10a)JD340Htz/J	Oligodendrocyte progenitors and other Pdgfra$^+$ cells	Dougherty et al., 2012
030265	B6;FVB-Tg (Olig2-EGFP/Rpl10a)JD97Htz/J	Oligodendroglia and other Olig2$^+$ cells	Doyle et al., 2008
030251	B6;FVB-Tg (Cmtm5-EGFP/Rpl10a)JD307Htz/J	Weakly positive in oligodendrocytes	Doyle et al., 2008
030249	B6;FVB-Tg (Cck-EGFP/Rpl10a)GM391Htz/J	Cck$^+$ cells	Doyle et al., 2008
030258	B6;FVB-Tg (Grm2-EGFP/Rpl10a)JP77Htz/J	Cerebellar Golgi neurons and other Grm2$^+$ cells	Doyle et al., 2008
030262	B6;FVB-Tg (Neurod1-EGFP/Rpl10a)JP241Htz/J	Cerebellar granule neurons and other Neurod1$^+$ cells	Doyle et al., 2008
030260	B6;FVB-Tg (Lypd6-EGFP/Rpl10a)JP48Htz/J	Cerebellar stellate/basket neurons and other Lypd6$^+$ cells	Doyle et al., 2008
030252	B6;FVB-Tg (Cort-EGFP/Rpl10a)GM130Htz/J	Cortical Cort$^+$ interneurons and other Cort$^+$ cells	Doyle et al., 2008; Nakajima et al., 2014
030261	B6;FVB-Tg (Nek7-EGFP/Rpl10a)MN733Htz/J	Cortical Pvalb$^+$ interneurons and other Nek7$^+$ cells	Nakajima et al., 2014
030253	B6;FVB-Tg (Dlx1-EGFP/Rpl10a)GM520Htz/J	Cortical SST$^+$ and VIP$^+$ interneurons, other Dlx1$^+$ cells	Nakajima et al., 2014
030272	B6;FVB-Tg (Slc6a3-EGFP/Rpl10a)JD1640Htz/J	Dopaminergic neurons	Dougherty, 2017
012365	C57BL/6N-Tg (Slc6a3-EGFP/Rpl10a)I17-11Pggd/J	Dopaminergic neurons	Brichta et al., 2015
030254	B6;FVB-Tg (Drd1a-EGFP/Rpl10a)CP73Htz/J	Drd1 medium spiny neurons and other Drd1$^+$ cells	Heiman et al., 2008
030255	B6;FVB-Tg (Drd2-EGFP/Rpl10a)CP101Htz/J	Drd2 medium spiny neurons and other Drd2$^+$ cells	Heiman et al., 2008
028619	B6;FVB-Tg (Hcrt-EGFP/Rpl10a)JD218Jdd/J	Hypocretin neurons	Dalal et al., 2013
030264	B6;FVB-Tg (Ntsr1-EGFP/Rpl10a)TS16Htz/J	Layer 6 neurons and other Ntsr1$^+$ cells	Doyle et al., 2008
030263	B6.FVB-Tg (Ntf3-EGFP/Rpl10a)PS1046Htz/J	Layer 2/3 neurons and other Ntf3$^+$ cells	Shrestha et al., 2015
030257	B6.FVB (Cg)-Tg (Colgalt2-EGFP/Rpl10a)DU9Htz/J	Layer 5b and other Colgalt2$^+$ (also known as Glt25d2) cells	Doyle et al., 2008
030270	B6;FVB-Tg (S100a10-EGFP/Rpl10a)ES691Htz/J	Layer 5a and other S100a10$^+$ cells	Schmidt et al., 2012
030256	B6;FVB-Tg (Etv1-EGFP/Rpl10a)TS88Htz/J	Mixed projection neurons and immune/microglial cells	Doyle et al., 2008
030250	B6.FVB (Cg)-Tg (Chat-EGFP/Rpl10a,Slc18a3)DW167Htz/J	Motor neurons and other cholinergic cells	Doyle et al., 2008
030269	B6;FVB-Tg (Pnoc-EGFP/Rpl10a)GM64Htz/J	Pnoc$^+$ interneurons and other Pnoc$^+$ cells	Doyle et al., 2008
030266	B6;FVB-Tg (Pcp2-EGFP/Rpl10a)DR166Htz/J	Purkinje neurons	Doyle et al., 2008
030267	B6;FVB-Tg (Pcp2-EGFP/Rpl10a)DR168Htz/J	Purkinje neurons	Doyle et al., 2008
028620	B6;FVB-Tg (Slc6a4-EGFP/Rpl10a)JD60Jdd/J	Serotonergic neurons	Dougherty et al., 2013
030259	B6;FVB-Tg (Grp-EGFP/Rpl10a)JP25Htz/J	Unipolar brush neurons and other Grp$^+$ cells	Doyle et al., 2008
NA	Tg (Tie2-EGFP/Rpl10a)	Endothelial cells	Santhosh and Huang, 2016

[a]Many of the listed lines are available at The Jackson Laboratory (www.jax.org). NA, Not applicable (contact authors). More details on the expression pattern of each line are in the provided references. Many cell lines have been validated deeply for one brain region (e.g., Neurod1 in cerebellar granule cells) but also show expression in discrete populations elsewhere in the brain. Therefore, although TRAP lines were generated independently of GENSAT, a coronal survey of GFP/Rpl10a expression across the entire brain is now also hosted at the GENSAT website http://www.gensat.org/TRAP_listing.jsp.

Table 2. TRAP and TRAP related reporter lines[a]

Availability	Official name	Promoter	Construct notes	Reference
JAX:011029	B6N.129-Rpl22tm1.1Psam/J	Cre-dependent, Rpl22 locus	HA fusion to endogenous Rpl22	Sanz et al., 2009
JAX:022367	B6.129S4-Gt (ROSA)26Sortm1 (CAG-EGFP/Rpl10a,-birA)Wtp/J	Cre-dependent, CAG, Rosa26 locus	eGFP/Rpl10a; also has a Flpe-dependent BirA biotin ligase for protein purification	Zhou et al., 2013
JAX:022386	STOCK Gt (ROSA)26Sortm1.1 (CAG-EGFP/Rpl10a,-birA)Wtp/J	CAG, Rosa26 locus	eGFP/Rpl10a; also has a Flpe-dependent BirA biotin ligase for protein purification	Zhou et al., 2013
JAX:024750	B6;129S4-Gt (ROSA)26Sortm9 (EGFP/Rpl10a)Amc/J	Cre-dependent, CAG, Rosa26 locus	eGFP/Rpl10a	Liu et al., 2014
JAX:024898	C57BL/6J-Tg (tetO-EGFP/Rpl10a)5aReij/J	Tet-dependent, unknown locus	eGFP/Rpl10a	Drane et al., 2014
JAX:029789	B6.Cg-Gt (ROSA)26Sortm1 (CAG-HIST1H2BJ/mCherry,-EGFP/Rpl10a)Evdr/J	Cre-dependent, CAG, Rosa26 locus	Bicistronic: nuclear-mCherry and eGFP/Rpl10a	Roh et al., 2017
JAX:029899	B6.Cg-Gt (ROSA)26Sortm2 (CAG-NuTRAP)Evdr/J	Cre-dependent, CAG, Rosa26 locus	Bicistronic: nuclear biotinylation and eGFP/Rpl10a	Roh et al., 2017
JAX:030305	B6.Cg-Eef1a1tm1Rck/J	Cre-dependent, Eef1a1 locus	eGFP/Rpl10a	Stanley et al., 2013
NA	B6.129-Gt (ROSA)26Sortm1 (CAG-EGFP/Rpl10a)Brsp	Cre-dependent, CAG, Rosa26 locus	eGFP/Rpl10a	Long et al., 2014
NA	B6.FVB-Tg (Syn-NB/RPL10A)#Rck	Syn, unknown locus	Anti-GFP nanobody fused to Rpl10a	Ekstrand et al., 2014
EM:07451	STOCK Gt (ROSA)26Sortm1 (CAG-mCherry/Rpl10a)Sten/Kctt	Cre-dependent, CAG, Rosa26 locus	mCherry serves at the fluorophore/affinity tag on Rpl10a, rather than GFP	Hupe et al., 2014

[a]Summary of currently available lines for purification of ribosomes. JAX, Jackson ImmunoResearch Laboratories; EM, EMMA mouse repository; NA, Not applicable (contact authors).

when coupled with transsynaptic viruses, mapped inputs farther upstream (Ekstrand et al., 2014; Pomeranz et al., 2017). Making expression dependent on activity-dependent promoters allowed for molecular phenotyping of cells activated by particular stimuli (Ye et al., 2016). Yet another approach involves capturing ribosomes with a phosphorylated S6 protein (Fig. 2), a mark that can be deposited on ribosomes by neuronal activity (Knight et al., 2012). These "discovery-driven" studies, based on activity or projection, contrast with the candidate cell type studies where the targeted cell type is selected a priori by marker gene expression. Thus, they require a different perspective on analysis. Cell identity, defined by projection or activity, may not have a one to one correspondence to cell type defined by gene expression. For example, Pomeranz et al. (2017) clearly identified transcripts known to be expressed in multiple disparate cell types based on their projections to the VTA. Approaches leveraging prior molecular descriptions of cell types may help interpret these results (Xu et al., 2014). Also, because TRAP is a measure of the aggregate expres-

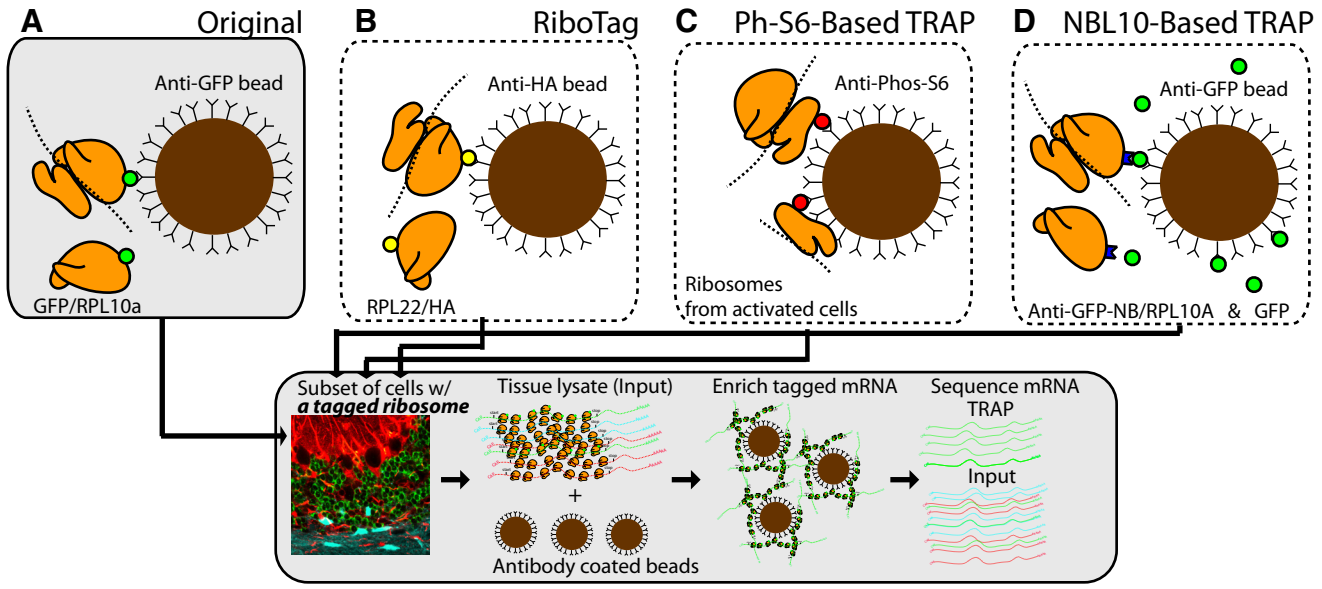

Figure 2. Alternative tags for purifying ribosomes. The standard TRAP protocol (gray box) can be adapted for use with a variety of ribosomal tags. ***A***, The RPL10A/GFP construct tags the large (60S) subunit, which does not engage mRNA until initiation of translation. Thus, it will not capture scanning small subunit (40S) particles before initiation, although it will capture 60S particles unaffiliated with mRNA. ***B***, The HA-tagged L22 protein from the Ribotag mouse also tags the large subunit and should have similar consequences. ***C***, The S6 protein of the small subunit is phosphorylated in response to activity. Thus, capture of phospho-S6 should yield translating as well as potentially scanning or stalled mRNA from activated cells. It is speculated that mRNA capture from each cell will be proportional to the amount of S6 phosphorylation, and thus activity. ***D***, Tagging of RPL10A with a nanobody (NB) against GFP allows the conversion of any GFP expressing virus or mouse line into a TRAP reagent. In this system, soluble GFP in the cell provides a linker for the tagged ribosome and the anti-GFP bead. However, as GFP is soluble *ex vivo*, careful consideration must be given to blocking excess GFP.

sion across all tagged cell types, likely in proportion to their number, amount of ribosomes, and stoichiometry of the tag, from TRAPseq results alone it is impossible to determine whether a transcript is present in a subset or superset of the labeled cells. This level of heterogeneity must be examined with other methods.

In addition to viruses, TRAP constructs can also be delivered by electroporation at specific developmental epochs to target cell types based on birthdate. For example, electroporation of E16 rat cortex allowed for later TRAP analysis of Tcf4 knockdown in layer 2/3 pyramidal neurons (Rannals et al., 2016). Electroporation was also applied to study the synchronized development of granule neurons *in vivo* (Yang et al., 2016) (Fig. 1*D*). Of course, existing TRAP lines can also be used to study specific developmental windows, provided the EGFP/RPL10A transgene is activated early enough (X. Zhu et al., 2016).

Finally, while this review is focused on mammalian nervous systems, TRAP has been adapted for species as varied as zebrafish (Tryon et al., 2013), *Drosophila* (Thomas et al., 2012), *Xenopus* (Watson et al., 2012), and even plants (Juntawong et al., 2015).

Nuclear purification to examine epigenetics and noncoding RNA

Another application of EGFP/RPL10A-expressing mouse lines came from the observation that ribosomes are assembled in a subnuclear structure: the nucleolus. For fluorescently tagged versions, this signal permits FACS of genetically tagged nuclei (Fig. 3*A*), enabling cell type-specific profiling of both chromatin states (Kriaucionis and Heintz, 2009) and direct measures of nuclear RNA, allowing a more direct assessment of transcription of both mRNA and noncoding species of RNA, such as LINCs and circular RNA (Reddy et al., 2017). As ribosomes also coat the nuclear membrane, it is possible that TRAP nuclei may be capturable by anti-GFP, similar to the INTACT method (Deal and Henikoff, 2010). If effective, this combination could allow for direct comparisons from the same mice of cytoplasmic translation and nuclear transcription or chromatin status. Regardless, reporter mice also now exist that separately tag both ribosomes and nuclei, which may further simplify such comparisons (Roh et al., 2017).

Cell type-specific assessment of translation

Although TRAP was used to study gene expression, since it involves capture of the ribosome, it provides a unique opportunity to study the regulation of translation in a cell type-specific manner *in vivo*. The CNS has a remarkable degree of regulation of translation, particularly subcellularly, where local translation of new proteins near synapses is thought to allow alterations of local connections in response to localized activity (Rangaraju et al., 2017). In addition, in parallel to alternative splicing, where multiple mRNA isoforms are made from a single gene, it is now recognized that use of alternative initiation, stop codon readthrough, codon switching, and cryptic open reading frames (ORFs) allows for multiple proteins to be made from the same mRNA isoform (Ingolia, 2016). Furthermore, rates of translation and ribosome occupancy are not equivalent for all transcripts. For example, in cultured neural cells, transcript abundance, as measured by RNAseq only, predicts ~60% of ribosome occupancy (Dalal et al., 2017). The remaining 40% implies a substantial amount of transcript-specific regulation of translation. While to date little is known about how this process varies across cell types or in response to disease, studies below highlight emerging variations of TRAP that advance these investigations.

Translation application

Applications of TRAP to the study of local translation

A specific subset of mRNAs are enriched in dendrites and axons (Rangaraju et al., 2017). Four recent studies adapted TRAP to test the hypothesis that these mRNAs are bound by ribosomes and to identify the mRNAs. First, Kratz et al. (2014) virally expressed an

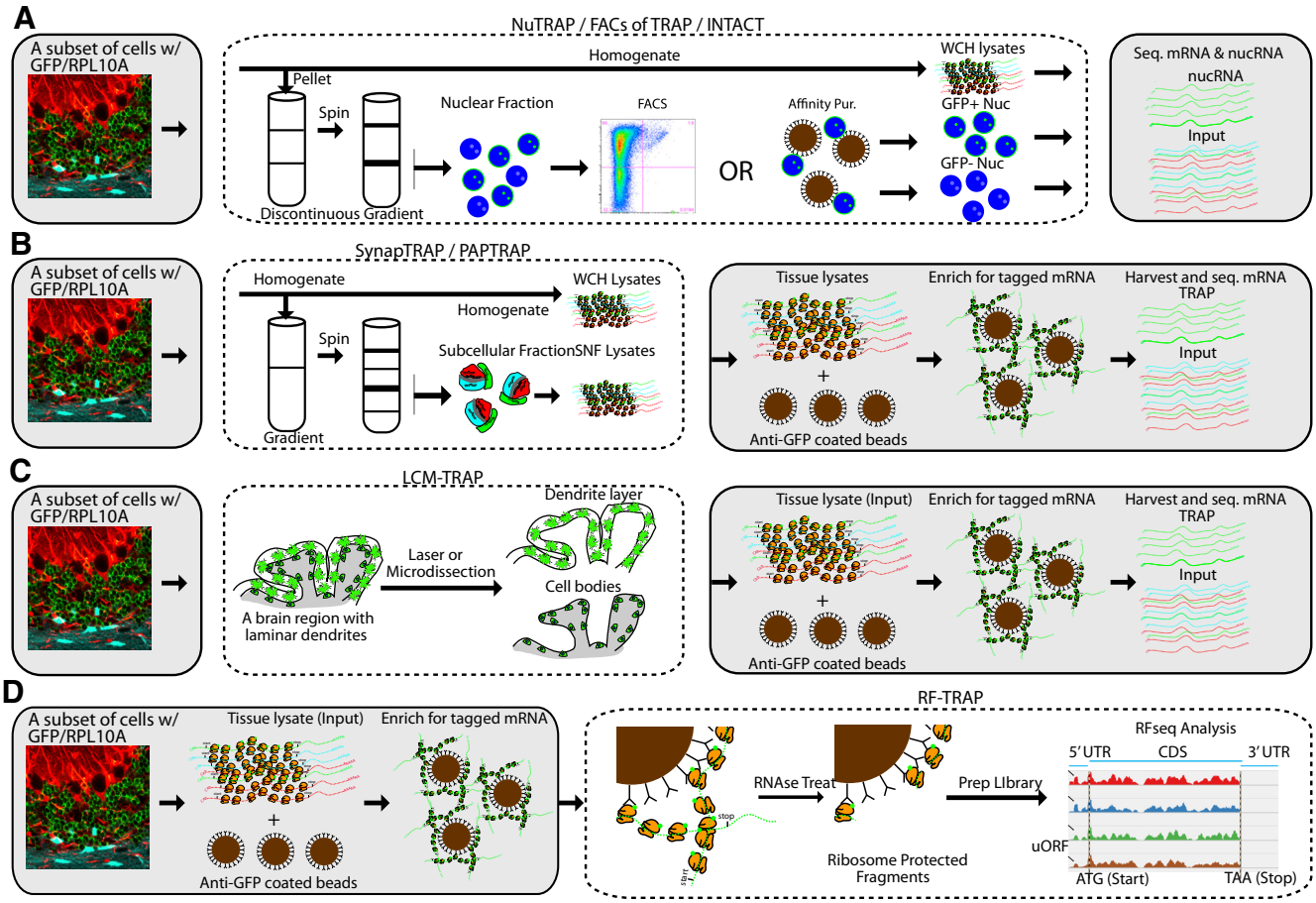

Figure 3. Adaptations of TRAP for studying subcellular RNA localization and regulation of translation. Additional steps can be inserted into the standard TRAP workflow (gray boxes) to enable new investigations. ***A***, GFP/RPL10A is found in the exterior of nuclear membrane (as it is contiguous with the endoplasmic reticulum) and in the nucleoli. Thus, a simple nuclear fraction can be flow-sorted to harvest cell type-specific nuclei for either nuclear RNAseq or epigenetic studies. It is possible that direct affinity purification for GFP might be possible, as has been shown for other nuclear tags in NuTRAP and INTACT. ***B***, Tissue can be prefractionated using classic protocols into specific subcellular compartments based on density to harvest portions of cells enriched in particular membrane-enclosed fragments, such as synaptoneurosome-containing fractions (SNF), which also contain markers of peripheral astrocyte processes. The fractions can then be lysed, and cell type-specific, subcellularly localized ribosomes can be captured with TRAP. ***C***, Likewise, for those cells where dendrites are in lamina that are physically separable from cell bodies, microdissection enables TRAP on each layer independently. ***D***, TRAP'ed ribosomes can be subjected to RNase treatment to leave only those ~30 nucleotide fragments physically protected by the ribosome. Sequencing these "ribosome footprints" (RF) allows for analysis of use of specific ORFs from each mRNA.

EYFP/RPL10A with a Purkinje neuron-selective capsid, then harvested tagged ribosomes from microdissected molecular layers of the cerebellum (Fig. 3*C*), where dendrites of Purkinje neurons are found. They confirmed the translation of numerous mRNAs in Purkinje neuron dendrites, including both rough ER and cytoplasmic transcripts. In a similar approach, Ainsley et al. (2014), using a CamK2a/Tet-dependent mouse line expressing EGFP/RPL10A in CA1 neurons, coupled microdissection of cell bodies and dendritic layers with TRAPseq. They reported alterations in response to fear conditioning in dendritic mRNA bound to the ribosomes (but see also comments in PUBMED commons). Regardless, microdissection-based approaches are limited to regions, such as hippocampus or cerebellum, where cell bodies and dendrites are in separable lamina. In a recent axon-focused study, Shigeoka et al. (2016) used Ribotag with a retinal neuron-specific Cre, followed by dissection of thalamic target regions, to identify mRNAs on ribosomes in developing and mature axons. They noted axonal translation is regulated across development and by splicing-in of specific motifs. They provided clear confirmation to a previously contentious observation that adult axons can indeed contain translating mRNA, although the markedly low yields confirm consensus that ribosomes in axons are much lower in abundance than in other compartments (Rangaraju et al., 2017). Nonetheless, the presence of mRNA across development implies an important role for generation of new protein in axons as well. This approach should be applicable to study additional distally projecting cells.

To access those cell types where axons or dendrites are not dissectible from cell bodies, we developed a complementary approach using biochemical fractionation, rather than dissection. We isolated synaptoneurosome-containing fractions from all cells (Fig. 3*B*) and then conducted TRAP to enrich for mRNAs bound to ribosomes in processes of targeted cell types. The method was developed initially to harvest locally translated mRNA from densely intermingled processes of cortical neurons, identifying numerous enriched and depleted transcripts, likely regulated by splicing and motifs in their 3′UTRs (Ouwenga, et al., 2017). It was also clear that non-neuronal cells contributed to these biochemical fractions as we discovered sequence-specific localized translation in astrocytes as well (Sakers et al., 2017). This method should be applicable to a variety of cell types, in health and disease, and further adaptable to other fractionation methods for additional subcellular compartments.

Nucleotide resolution cell type-specific analysis of translation

A drawback to all TRAP-based studies discussed above is that they do not distinguish which protein isoform is being generated from each mRNA. Coupling of classical ribosome footprinting techniques with modern high throughput RNAseq (RF-seq) allows assessment of ORF usage across the entire transcriptome (Ingolia, 2016). Resulting insights include the unexpected abundance of upstream ORFs in 5′UTRs, alternative initiating events resulting in N-terminal truncations and extensions, and occasional evidence for stop-codon readthrough, allowing for C-terminal protein extensions. Thus, knowledge of a ribosome's precise location on a transcript can predict the protein being synthesized.

The other application of RF-seq is to calculate a ratio of RF-seq to standard RNAseq, termed "translation efficiency," as a measure to identify mRNAs that may produce more protein than predicted from transcript levels alone. This can be calculated both at baseline and in response to manipulation. This identifies transcripts that alter translation rates even when transcription is unaltered, and has been widely applied outside of the CNS (Ingolia, 2014). In the CNS, this measure has been applied to cultured neural cells simulated with KCl and identified hundreds of transcripts that alter translation efficiency independently of transcription alone, adding a substantial new regulatory layer to the classically defined transcriptional response of neural cultures to stimulation (Dalal et al., 2017). However, this analysis could not determine which changes were occurring in neurons and which in glia. Likewise, an RF-seq study conducted in the hippocampus of mice after fear conditioning (Cho et al., 2015) identified numerous changes in ribosome occupancy following stimulation. However, it was not clear which changes occurred in neurons or which were due to variability in dissection (Mathew et al., 2016).

In contrast, Gonzalez et al. (2014) successfully coupled TRAP/Ribotag to RF *in vivo* to identify substantial translational changes in a mouse model of proneural glioma, separating the ribosomes from the tumor imitating cells and their surrounds. This combination of approaches (Fig. 3*D*) opens the possibility of analyzing precise nucleotide positions, ORF usage, and translational regulation in specific cell types of the brain. The authors focused specifically on translation efficiency, defining a decrease in the tumor cells. However, although TRAP provided access to ribosomes, the authors did not have a corresponding cell type-specific total RNA measure, and thus had to rely on a deconvolution algorithm from bulk RNAseq for their assessment of transcription in each cell type, an approach that will be challenging for more rare cell types. However, parallel studies profiling the same mouse lines with TRAP and cell type-specific nuclear FACs/RNAseq (Kriaucionis and Heintz, 2009; Reddy et al., 2017), or new specialized reporter lines (Roh et al., 2017) should simplify these analyses. Thus, the field is on the verge of being able to address, in a cell type-specific manner, regulation of translation *in vivo*.

Thus far, the in-brain RF studies have not taken advantage of the ability to study alternative ORF usage. This is perhaps because upstream ORFs and alternative initiation events normally require for their detection use of inhibitors to stall the ribosomes at initiation. While these inhibitors can work *in vivo*, at least in the liver (Gao et al., 2015), they have not been thoroughly tested for the brain.

Future challenges and opportunities

One of the clearest challenges to TRAP remains the nature of the method as an enrichment, rather than a perfect purification, of the RNA from the targeted cells (Dougherty et al., 2010; Okaty et al., 2011). Analysis of "marker" genes from nontargeted populations (e.g., assessment of the glial GFAP mRNA from a neuronal TRAP sample) typically shows depletion by TRAP, but not a complete absence. Thus, inclusion of comparison groups such as an input RNAseq and negative controls (for example, sequencing TRAP from wild-type mice, as was done by Shigeoka (2016), remains essential to defining transcripts confidently enriched in the targeted cells. However, this requirement increases the number of samples required for sequencing as well as the complexity of the analysis and interpretation. It also means that negative results should be interpreted with caution: a transcript below the level of noise may still be expressed in the cell type, albeit at a much lower level than the surrounding tissue. This nonspecific background could have many sources from the technical (nonspecific association of mRNAs and beads *in vitro*) to the biological (movement of mRNA or ribosomes between cell types *in vivo*, or legitimate but low-level expression of the transgene). It is clear that further development is needed, and direct comparisons of background under different technical conditions would be useful to determine which ribosomal protein, antibody, affinity tag, crosslinking reagent, or transgene expression level might be optimal for improving signal-to-noise.

Nonetheless, TRAP has now been adopted for large and small roles in dozens of studies, and adapted to a variety of new applications outlined here. Straightforward next advances would be combinations of these. As outlined in Figures 1–3, each of the variations impacts different steps of the protocol: thus, combining subsets (e.g., RF-TRAP with SynapTRAP to study alterative translation in neurites) should be feasible. The ability of expanded TRAP methods to enhance the cell specificity of advanced molecular techniques will only continue as new sequencing-based methods of translation are created. This flexibility in the expanding toolkit will enable integration with a wide range of studies, hopefully permitting both a deeper analysis of candidate cell types and a better understanding of the regulation of translation in health and disease.

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