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Rapid Molecular Profiling of Defined Cell Types Using Viral TRAP

Graphical Abstract



Highlights

- vTRAP enables rapid, viral-based tagging of translating ribosomes in defined cells
- vTRAP faithfully recapitulates molecular profiles obtained from bacTRAP
- vTRAP can detect regional, cell-type-specific differences in gene expression
- vTRAP was used for comprehensive molecular profiling of LH MCH neurons

Authors

Alexander R. Nectow, Maria V. Moya, Mats I. Ekstrand, ..., Jeffrey M. Friedman, Nathaniel Heintz, Eric F. Schmidt

Correspondence

anectow@princeton.edu (A.R.N.), heintz@rockefeller.edu (N.H.), eschmidt@rockefeller.edu (E.F.S.)

In Brief

Nectow et al. describe vTRAP, a technology to purify translating mRNAs from genetically defined cell types in a spatiotemporally restricted fashion. Multiplexing vTRAP with other technologies offers a comprehensive strategy to interrogate the precise role of individual, cell-type-specific genes in neural circuit function.

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Rapid Molecular Profiling of Defined Cell Types Using Viral TRAP

Alexander R. Nectow,^{1,2,*} Maria V. Moya,³ Mats I. Ekstrand,¹ Awni Mousa,³ Kelly L. McGuire,³ Caroline E. Sferrazza,³ Bianca C. Field,¹ Gabrielle S. Rabinowitz,⁴ Kirsty Sawicka,⁴ Yupu Liang,⁵ Jeffrey M. Friedman,¹ Nathaniel Heintz,^{3,*} and Eric F. Schmidt^{3,6,*}

¹Laboratory of Molecular Genetics, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

²Princeton Neuroscience Institute, Princeton University, Lot 20 Washington Road, Princeton, NJ 08544, USA

³Laboratory of Molecular Biology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

⁴Laboratory of Molecular Neuro-Oncology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

⁵Hospital Informatics, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA ⁶Lead Contact

*Correspondence: anectow@princeton.edu (A.R.N.), heintz@rockefeller.edu (N.H.), eschmidt@rockefeller.edu (E.F.S.) http://dx.doi.org/10.1016/j.celrep.2017.03.048

SUMMARY

Translational profiling methodologies enable the systematic characterization of cell types in complex tissues, such as the mammalian brain, where neuronal isolation is exceptionally difficult. Here, we report a versatile strategy for profiling CNS cell types in a spatiotemporally restricted fashion by engineering a Cre-dependent adeno-associated virus expressing an EGFP-tagged ribosomal protein (AAV-FLEX-EGFPL10a) to access translating mRNAs by translating ribosome affinity purification (TRAP). We demonstrate the utility of this AAV to target a variety of genetically and anatomically defined neural populations expressing Cre recombinase and illustrate the ability of this viral TRAP (vTRAP) approach to recapitulate the molecular profiles obtained by bacTRAP in corticothalamic neurons across multiple serotypes. Furthermore, spatially restricting adeno-associated virus (AAV) injections enabled the elucidation of regional differences in gene expression within this cell type. Altogether, these results establish the broad applicability of the vTRAP strategy for the molecular dissection of any CNS or peripheral cell type that can be engineered to express Cre.

INTRODUCTION

The characterization of the molecular properties of specific cell types within the brain is essential to better understand CNS function. Differences in the suite of proteins expressed by distinct neuronal populations underlie their unique intrinsic properties, susceptibility to disease, and responses to extrinsic manipulations, such as drugs or trauma. Recent advances in translational profiling methods have provided direct access to the translating mRNAs of genetically defined cell populations in complex tissues. The translating ribosome affinity purification (TRAP) technique and its variants rely on targeted transgene expression (Doyle et al., 2008; Heiman et al., 2008), connectivity (Ekstrand et al., 2014; Nectow et al., 2015), or even synaptic activity (Knight et al., 2012) to access cell types and overcome the challenges posed by the heterogeneous organization of the CNS. By directing the expression of an EGFP-tagged ribosome protein L10a (EGFPL10a) to defined cell populations, TRAP enables the isolation of cell-type-specific polysome-bound mRNAs from whole-tissue homogenates, avoiding potential adaptations and RNA degradation that can result from lengthier cellular isolation methods (Heiman et al., 2014).

The TRAP approach has been employed to profile translation in dozens of cell types in the mouse CNS (Doyle et al., 2008; Mellén et al., 2012; Nakajima et al., 2014; Schmidt et al., 2012; Shrestha et al., 2015) and visceral organs (Grgic et al., 2014; Zhou et al., 2013) and has been valuable in teasing apart neural circuits underlying complex CNS disease. TRAP has been used to identify a single cortical cell type that mediates antidepressant responses (Schmidt et al., 2012), a population of oxytocinreceptor-expressing interneurons in the prefrontal cortex that modulate sociosexual behavior in females (Nakajima et al., 2014), and cortical cells that integrate stress responses in the context of depressive-like behaviors (Shrestha et al., 2015). It was also applied to identify genes altered in specific neuronal populations in the midbrain (Dougherty et al., 2013) and cerebellum (Mellén et al., 2012) in animal models of neurodevelopmental disorders.

Whereas simple and effective, to date, the application of TRAP to study the mammalian CNS has been dependent on engineered mouse strains for EGFPL10a expression. This has been achieved through the generation of a series of novel bacterial artificial chromosome (BAC) transgenic mouse lines in the "bacTRAP" method (Doyle et al., 2008; Nakajima et al., 2014; Schmidt et al., 2012; Shrestha et al., 2015) or conditional



Figure 1. Development and Application of vTRAP Approach

(A) Schematic depicting the design of the Credependent pAAV-FLEX-EGFPL10a vector.

(B) After expression of EGFPL10a transgene in the targeted cell type, EGFP-labeled polysomes are affinity purified to enrich for cell-specific, polysome-bound, translating mRNAs.

(C) Immunofluorescent staining of HEK293T cells co-transfected with pAAV-FLEX-EGFPL10a (green) and pCAG-Cre (red). Nuclei are labeled with DAPI (blue) in right panel. The scale bar represents 20 μm.

(D) Polysome profile plot of sucrose gradient fractions from cortex of Emx1-Cre mice 1 week after AAV-FLEX-EGFPL10a virus injections. Vertical gray lines indicate fraction divisions. Fractions containing the large subunit (60S; fraction no. 4), monosome (m) (no. 5), and polysomes (no. s 6–16) are labeled.

(E) Western blots showing the distribution of the EGFPL10a transgene, endogenous RPL10a, and RPS6 in the sucrose gradient fractions from (D). Short (30 s) and long (5 min) exposures are shown for the anti-GFP blot.

(F) Polysome profile plot of sucrose gradient fractions from cortex of Emx1-Cre mice 4 weeks after AAV-FLEX-EGFPL10a virus injections.

(G) Western blots showing the distribution of the EGFPL10a transgene, endogenous RPL10a, and RPS6 in the sucrose gradient fractions from (F). Anti-GFP blot is from a 30-s exposure. See also Figure S1.

thalamic (CThal) cells, we show that vTRAP was able to capture cell-typespecific translational profiles with high fidelity to the traditional bacTRAP approach. Further, the spatially restricted nature of AAV expression revealed the utility of the vTRAP approach for more localized molecular profiling within neural

expression requiring either Cre recombinase (Liu et al., 2014; Sanz et al., 2009; Stanley et al., 2013; Zhou et al., 2013) or tetracycline-controlled transcription (Drane et al., 2014). To expand the utility of the TRAP approach, we generated Cre-dependent adeno-associated viral (AAV) vectors to express EGFPL10a. These vectors enable rapid access to translating mRNAs from any discrete neural population engineered to express Cre recombinase while avoiding the need to cross multiple mouse strains harboring independent alleles and unwanted recombination during development. This viral TRAP (vTRAP) approach can be immediately applied for use with any of the scores of commercially available rodent Cre-driver strains or in a combinatorial manner in other species using Cre-expressing viruses.

Here, we demonstrate the incorporation of virally expressed EGFPL10a into endogenous polysomes and validate the vTRAP approach in a number of readily available Cre-driver mouse strains, interrogating cell types in the brainstem, hypothalamus, and cortex as a proof of concept. With a focus on layer 6 corticostructures. Taken together, the current work provides a strategy for the rapid and highly efficient targeting of translating ribosomes in a cell-type-specific, anatomically restricted fashion using viral-mediated gene transfer.

RESULTS

Generation and Validation of AAV-FLEX-EGFPL10a

To develop a viral construct that can be used for translational profiling, EGFPL10a was cloned in the reverse orientation into the Cre-dependent pAAV-EF1a-DIO-ChR2-mCherry plasmid, replacing ChR2-mCherry. The flanking loxP and lox2272 sites were left intact, resulting in a virus that had a "double-floxed" inverted open reading frame, or "FLEX switch" (Atasoy et al., 2008), to allow for cell-type-specific expression of the EGFPL10a fusion protein. This construct is hereafter referred to as pAAV-FLEX-EGFPL10a (Figure 1A). In the presence of Cre recombinase, EGFPL10a is reverted back to the "expressed" forward

orientation, allowing for conditional expression of a functional protein. Once incorporated into the large ribosomal subunit, the EGFPL10a protein allows for cell-type-specific polysome immunoprecipitation using the TRAP method (Figure 1B). To confirm Cre-dependent expression of the EGFPL10a construct, we co-transfected HEK293T cells with pAAV-FLEX-EGFPL10a and pCAG-Cre-Myc. Immunofluorescent staining for EGFP and c-Myc revealed that expression of the EGFPL10a transgene was restricted only to cells co-stained with anti-Myc and thus contained the Cre plasmid (Figure 1C).

To demonstrate integration of the EGFPL10a construct into translating ribosomes in vivo, pAAV-FLEX-EGFPL10a was packaged into AAV. The viral vectors were then injected into the cerebral cortex of Emx1-IRES-Cre mice, which express Cre recombinase in most pyramidal cells throughout the neocortex (Gorski et al., 2002). We looked for incorporation of the EGFPL10a transgene into polysomes at 1 week and 4 weeks following virus injections. Polysomes were purified by linear sucrose gradient fractionation of cytosolic lysates from AAV-injected cortex (Figures 1D-1G). Western blots of trichloroacetic acid (TCA)-precipitated sucrose gradient fractions revealed that the distribution of EGFPL10a overlapped significantly with endogenous RPL10a as well as RPS6 ribosome subunits, demonstrating that the transgene was inserted into functional polysomes (Figures 1E, 1G, and S1). Immunoblots using anti-GFP antibody showed that, even after only 1 week, EGFPL10a could be detected in polysome fractions (Figures 1E and S1A). However, levels of EGFPL10a protein were substantially higher 4 weeks after virus injection (Figures 1G and S1B), likely reflecting the timeline needed for maximal AAV expression (Kaspar et al., 2002) and the 2- to 3.5-week turnover rate of ribosomal proteins in the mouse brain (Price et al., 2010). Taken together, these results demonstrate the ability of AAV-FLEX-EGFPL10a to drive Credependent expression of EGFPL10a to label polysomes in vivo.

Using AAV-FLEX-EGFPL10a to Target Genetically Defined Neuronal Populations In Vivo

We next set out to validate functional expression of AAV-FLEX-EGFPL10a in various neural populations throughout the brain. We used existing Cre recombinase transgenic lines to drive expression specifically in dorsal thalamus, cortex, and midbrain (Figures 2A-2C). Anti-GFP immunostaining revealed bright puncta within the nucleolus (Figure 2B), the site of ribosomal biogenesis, and is consistent with transgenic EGFPL10a expression in bacTRAP mice.

To confirm functional integration of EGFPL10a, we performed anti-EGFP immunoprecipitations (IPs) to purify polysomes from specific populations of monoaminergic neurons in the brainstem. We focused on two well-characterized populations each with a set of known cell-specific marker genes: dopaminergic (DA) neurons of the ventral tegmental area (VTA) and serotonergic (5-HT) neurons of the dorsal raphe nucleus (DRN). AAV-FLEX-EGFPL10a was injected into the VTA of DAT-Cre and the DRN of SERT-Cre mice. IPs from each region resulted in substantial RNA yields (average of >9 ng total RNA per IP; data not shown), suggesting that EGFPL10a protein was incorporated into translating ribosomes in each of these cell types. qRT-PCR showed significant specificity for known marker genes for each cell type in the IPs from VTA DA and DRN 5-HT neurons (Figure 2D). Dopaminergic marker genes *Slc6a3* (16.1-fold) and *Th* (25.6-fold) were significantly enriched in DA neurons, whereas serotonergic marker genes *Slc6a4* (267.6-fold) and *Fev* (182.6-fold) were significantly enriched in 5-HT neurons. In contrast, there was no significant difference in *Slc32a1* enrichment, which is known to be depleted from both populations. Lastly, AAV-FLEX-EGFPL10a injection into wild-type mice resulted in negligible RNA yields, demonstrating the dependence of this virus on the presence of Cre for detectable EGFPL10a expression (Figure S2A). Together, these data demonstrate proper conditional expression and functional integration of the AAV-expressed EGFPL10a transgene in vivo.

Molecular Profiling of Lateral Hypothalamic MCH Neurons

To demonstrate the utility of the vTRAP approach, we characterized the molecular properties of a less well-studied population of neurons in the lateral hypothalamus (LH). The LH is known to be a critical node in controlling survival-related behaviors, such as food intake. Melanin-concentrating hormone (MCH) neurons comprise a significant population within the LH and are known to regulate feeding behavior and sucrose preference (Domingos et al., 2013; Shimada et al., 1998). The sparse distribution of MCH neurons in LH has hindered molecular profiling in the past, making them ideal for comprehensive molecular profiling with vTRAP. We injected AAV-FLEX-EGFPL10a into the LH of Pmch-Cre mice and confirmed cell-type-specific expression of EGFPL10a in MCH neurons (Figure 3A). qRT-PCR on vTRAP RNA showed significant enrichment for the cell type marker Pmch (77.7-fold), as well as Cartpt (15.3-fold) and Tacr3 (12.1-fold), two genes co-expressed in MCH neurons (Croizier et al., 2010; Knight et al., 2012; Figure 3B). We also found significant depletion of Agrp, a marker for the arcuate nucleus (2.4-fold; Figure 3B).

High-throughput RNA sequencing (RNA-seq) of vTRAP RNA was performed for comprehensive molecular profiling of MCH neurons (Figure 3C). Differential analysis identified 1,761 genes enriched in the IP over LH input. Notably, the most significantly enriched gene in the entire dataset was Pmch (99-fold), the driver gene for MCH neurons. Additionally, Cartpt (23.7-fold) and Tacr3 (8.2-fold) were among the top 20 most significantly enriched genes (Figure 3D). A direct comparison of vTRAP with TRAP done on LH from Pmch-Cre mice crossed to the Cre-dependent EGFPL10a reporter line, Rosa26^{fsTRAP} (Zhou et al., 2013), showed substantial reproducibility with regard to control genes (Pmch, Cartpt, Tacr3, Agrp, and Anxa1) and MCH markers identified by vTRAP (Crhbp, Dmrtb1, and Mup6; Figures S2B and S2C), further validating the efficacy of the viral approach. An earlier study using the Retro-TRAP approach (Ekstrand et al., 2014) identified a number of genes expressed in hypothalamic cells with projections to the nucleus accumbens (NAc) that included but were not restricted to MCH neurons. Comparative analysis of MCH vTRAP results with NAc Retro-TRAP data (Figure 3E) identified a number of transcripts that were significantly enriched in both datasets, including Crhbp (6.9-fold), Dmrtb1 (12.2-fold), and Mup6 (77.1-fold; Figure 3F). However, we found Anxa1 (p < 0.01; 6.4-fold differential enrichment), a gene specific to midbrain dopaminergic projections to striatum (Ekstrand et al.,



Figure 2. In Vivo Application of vTRAP in Defined Cell Populations

(A) EGFPL10a expression in cortex of TIx3-Cre (top) or thalamus of Slc17a6-Cre (bottom) mice injected with AAV-FLEX-EGFPL10a. Schematics of coronal sections, adapted from Franklin and Paxinos (2008), depicting the relative location of the images (red boxes) are shown on the left, and anti-GFP (green) and NeuN (blue) immunofluorescence from tiled scans of cortex are shown on the right. The scale bars represent 200 μm.

(B) High-magnification image of anti-GFP immunofluorescence from a tiled scan of cortex of the Tlx3-Cre mouse from (A). Arrowheads indicate GFP labeling in the nucleolus. The scale bar represents 50 μ m.

(C) Co-localization between EGFPL10a (EGFP, green) and tyrosine hydroxylase (Th, red) in the VTA of a DAT-Cre mouse (top panels) and EGFPL10a (EGFP, green) and tryptophan hydroxylase 2 (Tph2, red) in the DRN of a SERT-Cre mouse (bottom panels). Schematics at left depict the relative location of the images (red boxes). The scale bars represent 100 μm.

(D) qRT-PCR quantification (mean \pm SEM) of the expression of marker genes for DA (*Slc6a3* and *Th*), 5-HT (*Slc6a4* and *Fev*), or GABA (*Slc32a1*) neurons in VTA (black bars) or DRN (gray bars). ***p < 0.001; ****p < 0.0001.

that coat the outer surface of the virus particles. To test whether we could detect noticeable differences in the ability of vTRAP to enrich for cell-type-specific genes between discrete AAV serotypes, we created pAAV-FLEX-EGFPL10a vectors packaged using three separate capsids commonly used to target neurons in the CNS: AAV2; AAV5; and AAV9 (Aschauer et al., 2013). For these experiments, we examined corticothalamic (CThal) projection neurons that reside in layer 6 of the neocortex because this cell population has been targeted by distinct BAC transgenic lines that express either Cre recombinase (Gong et al., 2007) or EGFPL10a (Doyle et al., 2008). A triple cross of Ntsr1-bacTRAP, Ntsr1-Cre, and Rosa26^{tdTomato} reporter mice revealed a

2014), and *Pmch* (15-fold difference; p < 0.0001) were significantly differentially expressed between these datasets, reflecting the ability of vTRAP to overcome the heterogeneity of projections to the NAc (Figure 3F). These results suggest that the combined use of vTRAP and Retro-TRAP data can allow comprehensive projection- and cell-type-specific profiling of neural circuits.

vTRAP from Multiple AAV Serotypes Enriches for Cell-Specific Markers in Layer 6 Corticothalamic Neurons

The efficacy of infection of recombinant AAV vectors for different classes of cells is dependent on the nature of the capsid proteins

nearly complete overlap of EGFPL10a expression and Cre activity in L6 cells in cortex (Figures S3A and S3B), validating a direct comparison of these lines.

AAV vectors were injected into sensorimotor areas of neocortex of Ntsr1-Cre mice. EGFPL10a expression was detected throughout layer 6 of Ntsr1-bacTRAP mice and in layer 6 at the injection site in the Ntsr1-Cre mice (Figure 4A). Viralmediated expression of EGFPL10a was relatively higher than transgenic expression in the bacTRAP mice, likely reflecting the constitutive nature of the AAV's strong EF1a promoter after Cre-mediated recombination. Remarkably, viral expression in



Figure 3. Molecular Profiling of Lateral Hypothalamic MCH Neurons

(A) Colocalization between EGFP and MCH. Red box indicates region of interest. The scale bar represents 200 µm.

(B) qRT-PCR (mean \pm SEM) of cell type marker genes (*Pmch*, *Cartpt*, and *Tacr3*) and a non-MCH neuron marker gene (*Agrp*) after IPs from the hypothalamus. p < 0.01 for *Pmch* and *Cartpt* and p < 0.05 for *Tacr3* and *Agrp*.

(C) Histogram plotting RNA-seq data from MCH neuron vTRAP, displaying number of genes as a function of fold enrichment.

(D) Scatterplot of RNA-seq results from MCH vTRAP showing normalized counts from IP (x axis) and hypothalamus input (y axis). Gray dots represent individual genes (pink are significantly enriched in the IP). MCH neuron markers (*Pmch*, *Carptpt*, and *Tacr3*) are highlighted. Blue line is the best fit curve for all enriched genes.

(E) Schemata for Retro-TRAP (top) and vTRAP (bottom). For Retro-TRAP, a retrograde tracing virus, CAV-GFP is injected into the NAc of SYN-NBL10 transgenic mice, enabling profiling of NAc inputs from midbrain and hypothalamus. For vTRAP, Cre-dependent AAV-FLEX-EGFPL10a is injected into the LH of Pmch-Cre transgenic mice. Dashed box shows approximate dissection site.

(F) RNA-seq comparison (fragments per kilobase per million mapped reads [FPKM] mean \pm SEM) of genes enriched in both Retro-TRAP (inputs to NAc; data from Ekstrand et al., 2014) and MCH vTRAP (MCH neurons) datasets. *p < 0.05; **p < 0.01; ****p < 0.0001; #p = 0.052. See also Figure S2.

Ntsr1-Cre mice led to clear EGFP signal in distal CThal axons and terminal fields in the thalamus (Figures S3C and S3D). This implies a utility for vTRAP to isolate locally translated transcripts in axons, providing a straightforward strategy for profiling translation from anatomically separated subcellular compartments.

To establish translational profiles of the CThal cells, we isolated polysome-bound mRNA by TRAP from cortex of Ntsr1bacTRAP mice and Ntsr1-Cre mice injected with each of the AAV serotypes. Global differences in gene expression between TRAP IP mRNA (IP) and whole cortex (input) were analyzed by RNA-seq. Despite the inherent variability of virus spread, infection rate, and expression levels between individual virus injections, the biological reproducibility for all vTRAP samples was extremely high ($r^2 \sim 0.97-0.98$ for all groups; Figure S4A). In each case, greater than 600 genes were shown to be enriched in the Ntsr1 cell population (Figures 4B and S4B). Two layer 6 markers, Foxp2 and Syt6, were highly enriched in the sequencing data from the bacTRAP and vTRAP IPs using AAV5 and AAV9 compared to inputs, whereas markers for oligodendrocytes (Mal) and for cortical layer 5 neurons (Tmem200a) were depleted (Figures 4B, 4C, and S4C). qRT-PCR confirmed the RNA-seq results, showing that levels of enrichment of *Foxp2* and *Syt6* in TRAP IP RNA and depletion of *Mal* and *Tmem200a* were similar between the bacTRAP, AAV5 vTRAP, and AAV9 vTRAP samples (Figure 4D). Surprisingly, whereas *Ntsr1, Foxp2*, and *Syt6* were significantly enriched in AAV2 vTRAP IPs, the degree of enrichment was significantly less than the other groups (Figures 4B and 4D).

Translational Profiles from vTRAP Benchmarked against bacTRAP

TRAP-seq data from the MCH LH and CThal neurons clearly demonstrate that the vTRAP approach can enrich for cell-typespecific marker genes similar to transgenic TRAP methods. To ask how well vTRAP translational profiles correlate with those from bacTRAP, we generated a list of genes specific to the CThal population. We applied the "specificity index" (SI) algorithm, a comparative rank-order approach that identifies genes that are unique to each dataset (e.g., cell type) in a given analysis (Dougherty et al., 2010) to bacTRAP data from six neuronal and non-neuronal cortical cell types. In addition to CThal cells, our analysis included layer 5a corticostriatal cells, layer



Figure 4. vTRAP Molecular Phenotyping of Layer 6 CThal Cells Using Three AAV Serotypes

(A) Low- (top) and high (bottom)-magnification images showing EGFPL10a expression restricted to layer 6 in cortex of Ntsr1-bacTRAP (far left) and Ntsr1-Cre mice injected with vTRAP vectors. Anti-EGFP is shown in green, and DAPI is shown in blue. Low-magnification images were taken from composites of tiled scans of coronal brain slices. The scale bars represent 1 mm (top) and 50 μ m (bottom).

(B) Scatterplots of TRAP-seq results showing normalized counts from IP (x axis) and whole-cortex input (y axis) from Ntsr1-bacTRAP (far left) and Ntsr1-Cre mice injected with vTRAP vectors (right). Dots represent individual genes (pink are significantly enriched in IP). The driver gene (*Ntsr1*) and layer 6 markers (*Foxp2* and *Syt6*) are labeled. Blue line is the best fit curve for enriched genes.

(C) Visualization of reads from TRAP-seq datasets shown in (B) mapped to the genomic locus of three cell-type-specific marker genes (*Ntsr1*, *Syt6*, and *Foxp2*), a cortical layer 5 gene (*Tmem200a*), and a glial gene (*Mal*). TRAP IP samples are colored blue, and their corresponding inputs are shown in gray. RefSeq gene structure is illustrated in black at top (exons are vertical bars). The scale of the y axis is shown at bottom right for each gene.

(D) qRT-PCR quantification (mean \pm SEM) of the expression of control genes shown in (C) for bacTRAP and vTRAP samples compared to whole-cortex input. Positive values indicate enrichment in the IP. Dotted lines represent 2-fold enrichment in either direction. *p < 0.05.

See also Figure S3.



Figure 5. The Enrichment of Cell-Type-Specific Genes Is Highly Correlated between vTRAP and bacTRAP Approaches

(A) Schematic depicting the datasets used to target specific cell populations in the cerebral cortex. CPn, corticopontine; CStr, corticostriatal; CThal, corticothalamic.

(B) Heatmap of cell-specific genes (horizontal axis) from bacTRAP lines representing six distinct cell populations and whole tissue from the cerebral cortex. Color is based on the normalized expression for each cell type, and only genes with a p value (pSI) < 0.05 are shown. Yellow box indicates the CThal (Ntsr1-bacTRAP)-specific genes.

(C) Scatterplots comparing the expression of CThal-specific genes from (B) between Ntsr1 bacTRAP (x axis in each) and Ntsr1 vTRAP (y axis) vectors. The linear equation and coefficient of determination (r^2) are shown at the top of each graph, and the best fit curve for each dataset is shown as a solid line.

(D) Heatmap showing enrichment (TRAP IP/input) of marker genes for L6 projection neurons (left) or glial cells (right) identified by a variety of experimental approaches. Rows are individual replicates from bacTRAP ("bac") and vTRAP datasets. The cortical region analyzed in each study is labeled on top, and color-coded bars at bottom indicate the cell-type isolation method. Red box emphasizes genes identified from S1, which overlaps with the vTRAP virus injection site. hsPFC, human prefrontal cortex; S1, mouse primary somatosensory; V1, mouse primary visual. See also Tables S1, S2, and S3 and Figures S4 and S5.

5b corticopontine cells, interneurons, astrocytes, endothelial cells, and whole cortex, which included all other cell types (Figure 5A; Table S1). All bacTRAP mouse strains targeting these cell populations have been described elsewhere (Doyle et al., 2008; Nakajima et al., 2014; Schmidt et al., 2012) except for the endothelial cell-specific Abcb1a-bacTRAP line (see Figures S3E and S3F and Supplemental Information).

The SI algorithm identified 428 genes with pSI < 0.05 for the Ntsr1 CThal population (Figure 5B; Table S2). A direct comparison of sequencing results from vTRAP and bacTRAP revealed an extremely high correlation in the expression of these CThal-specific genes between both methods for each serotype (Figure 5C). The coefficient of determination (r^2) for each comparison was extremely high (\geq 0.940), and the slope of the best fit curves were all close to 1.0 (Figure 5C). There was a slight bias toward the bacTRAP dataset in all three vTRAP conditions (y intercept ranged from -0.49 to -0.87), likely due to vTRAP sampling only a subset of Ntsr1-expressing cells located near the injection site. The levels of expression of IP-enriched genes were also very similar between these methods (Figure S5C). These results demonstrate the ability of the viral approach to obtain cell-specific translational profiles with high fidelity to bacTRAP.

To assess the utility of vTRAP in the context of other celltype-specific profiling methods, we compiled a list of layer 6 projection neuron markers in various regions of cortex identified by sorting retrogradely labeled cells (Galazo et al., 2016), in situ hybridization (ISH) (Sorensen et al., 2015), bacTRAP (Doyle et al., 2008), and single-cell RNA-seq from mouse (Tasic et al., 2016; Zeisel et al., 2015) or human (Lake et al., 2016) cortex (Table S3). The overwhelming majority of these genes were significantly enriched in the vTRAP IPs, including all of the genes identified in studies focused on primary somatosensory cortex (S1), which overlaps with our virus injection site (Figure 4D). In contrast, most astrocyte and oligodendrocyte genes identified by bacTRAP (Doyle et al., 2008), immunopanning (Zhang et al., 2014), or single-cell RNA-seq (Tasic et al., 2016) were depleted in vTRAP IPs (Figure 4D, right). The sensitivity of vTRAP compares favorably to single-cell RNA-seq with over 92% of the top 1,000 genes identified in single-cell datasets from three overlapping populations of L6 cells being detected in Ntsr1 cells by vTRAP (Figures S5A and S5B). Conversely, many low-expressed genes (reads per kilobase per million mapped reads [RPKM] < 10) significantly enriched in vTRAP IPs were not detected in individual single-cell RNA-seq datasets (Figures S5D and S5E).

Direct Comparison of bacTRAP and vTRAP Reveals Regionally Expressed Genes

Because the AAV injections targeted a spatially restricted population of Ntsr1-expressing cells compared to the widely distributed bacTRAP-labeled cells, we asked whether a comparison of these approaches could identify anatomically restricted genes that may be used to define subregions of cortex. We created lists of "bacTRAP-specific" and "vTRAP-specific" genes, defined as the union of those differentially expressed between bacTRAP and vTRAP AAV9 IP samples and enriched in the IP over input for each approach (Figure S6A). Forty-three bacTRAP-specific genes (Figure 6A, blue circles) and 50 vTRAP-specific genes (Figure 6A, orange circles) were identified. These genes accounted for only a minority of the IP-enriched transcripts in each approach (6.7% for bacTRAP and 8.3% for vTRAP), reflecting the high correlation between the techniques.

ISH data from the Allen Mouse Brain Atlas (Lein et al., 2007) were utilized to examine the distribution of bacTRAP-specific or vTRAP-specific genes in the cortex (see Supplemental Experimental Procedures). Whereas about one-third of the genes in each dataset were expressed throughout the cortex (Figure 6B; "widespread"), the expression patterns of 37% (16) of the bacTRAP-specific genes were restricted to regions of the cortex that did not overlap with the site of the vTRAP AAV injections (Figure 6B; "regional"). These included genes such as Col23a1, which was expressed in rostrolateral cortex, and Cbln1, Nnat, and L6g6e, which were found at extreme rostral and caudal cortical sites (Figures 6C, S6C, and S6D). ISH for endogenous Ntsr1 revealed high expression in entorhinal cortex in addition to layer 6 CThal cells, and anti-GFP immunostaining confirmed this pattern was recapitulated in the Ntsr1-bacTRAP mice (Figure 6D). Indeed, entorhinal cortex-specific genes Mum111, Fam70b, Lef1, Cldn1, Cbln4, and Pth2r were not detected by vTRAP but were all present on the bacTRAP-specific list (Figures 6E, S6C, and S6D). Regionally expressed genes accounted for the majority of those with the highest fold change in bacTRAP over vTRAP (red circles in Figure 6A), including pSI-identified CThal genes (Figure S6B). In fact. 31 of the 43 (72%) bacTRAP-specific genes were also significantly enriched when bacTRAP was compared to AAV2 and AAV5 vTRAP data, including 11 of the 16 regional genes, demonstrating remarkable consistency between AAV serotypes. Quantification by qRT-PCR confirmed these differences in expression between bacTRAP and vTRAP with Cbln1, Cbln4, Cldn1, and Nnat, all significantly enriched in bacTRAP IP and significantly depleted in vTRAP IP for each of the AAV vectors used (p < 0.05 for all genes in each group), whereas Pth2r was significant for AAV9, the dataset used to generate the vTRAP-specific list (Figure 6G). Failure to identify vTRAPspecific regional genes may be due to cell-specific genes being enriched in both datasets due to the overlap in expression between bacTRAP and vTRAP, whereas bacTRAP-specific regional genes are depleted from vTRAP data, leading to high differential expression.

DISCUSSION

The TRAP technique has traditionally relied on engineered mouse strains to target the EGFPL10a transgene to cell types of interest. In the current work, we extend the TRAP method to take advantage of viral-mediated gene transfer, enabling rapid and anatomically restricted molecular profiling of any cell type engineered to express Cre recombinase. The broad availability of Cre-driver lines (Gerfen et al., 2013; Gong et al., 2007; Madisen et al., 2010; Taniguchi et al., 2011) allows the viral TRAP strategy to be applied immediately to myriad cell types without the need to generate novel transgenic strains or cross existing lines to Cre-dependent reporters. AAV-mediated overexpression of the TRAP transgene also enables high-quality translational profiling data to be generated as early as 3 weeks following virus injection, allowing for substantial temporal flexibility when designing experiments. Further, viral delivery of the EGFPL10a transgene into adult mouse tissue overcomes the possibility of off-target recombination due to transient expression of Cre in unwanted cell types during development (Lam et al., 2011; Padilla et al., 2010). Cre driver lines that rely on promoters with temporally dynamic expression patterns (Sanz et al., 2009) impose a significant limitation on the utility of Cre-dependent TRAP and RiboTag reporter strains.

The AAV-mediated strategy expands the utility of TRAP to other mammalian species that have historically proven to be genetically intractable. For example, the AAV-FLEX-EGFPL10a vector can immediately be used with the growing number of Cre-expressing rat strains (Witten et al., 2011) or in combination with Cre-expressing viruses in non-transgenic animals, such as monkeys (Oguchi et al., 2015). Projection-specific profiling can also be achieved with vTRAP by combining AAV-FLEX-EGFPL10a with viruses that transduce neurons in a retrograde fashion, such as rabies or canine adenovirus (Schwarz et al., 2015).

Cell-type-specific expression of AAV-FLEX-EGFPL10a in cortex, thalamus, brainstem, and hypothalamus demonstrates the utility of vTRAP to profile a variety of cell types throughout the brain. It also presents a strategy to quickly characterize unknown cell populations labeled with Cre. A simple vTRAP experiment followed by qPCR for selected target genes can easily ascertain neuronal or non-neuronal identity, the neurotransmitter profile (e.g., excitatory, inhibitory, or modulatory), receptor composition, and other properties of the cells without the need for detailed anatomical or electrophysiological investigation. The GENSAT library of Cre-driver mice alone contains a number of strains that express Cre in scattered, morphologically ambiguous cells in areas such as the cortex, hypothalamus, hippocampus, midbrain, and brainstem. These may represent uncharacterized cell populations or known cell types that were not genetically labeled previously.

The observation of EGFPL10a expression in distal axons of projection neurons following AAV-FLEX-EGFPL10a injections presents vTRAP as an intriguing approach to profile axon-specific transcripts in vivo. Translational profiling of axonal mRNA ("axon-TRAP") has previously been achieved in the developing retinotectal system by transplanting transgenic tissue into wild-type animals (Yoon et al., 2012) or relying on very specific transgenic mouse lines with high levels of transgene expression (Shigeoka et al., 2016). The spatial and temporal control of virus injections overcomes these technical hurdles and precludes a reliance on highly restricted Cre driver lines, allowing vTRAP to be exploited for axon profiling of a wider selection of cell types and even across



Figure 6. Differential Expression between vTRAP and bacTRAP Reveals Regionally Expressed Genes

(A) Scatterplot of the expression of genes differentially expressed between bacTRAP (x axis) and vTRAP with the AAV9 vector (y axis) IP samples. Yellow circles are vTRAP-specific genes, and blue circles are bacTRAP specific. Genes with regional expression outside of the site of AAV injection are circled in red.
(B) Pie charts of the quantification of expression pattern scores of genes significantly enriched in vTRAP IP versus input and vTRAP over bacTRAP ("vTRAP specific") or bacTRAP IP versus input and bacTRAP over vTRAP ("bacTRAP specific").

(C) ISH in cortex of selected bacTRAP-specific genes with regional expression. Green ovals specify the relative injection site of vTRAP vectors, and arrowheads identify regions with high expression. Insets show higher magnification of boxed areas 1–6 or the relative AAV injection site (Inj) for each gene.

(D) ISH for endogenous *Ntsr1* (top) and anti-EGFP immunolabeling of Ntsr1-bacTRAP mouse brain (bottom) showing expression in entorhinal cortex (arrowheads). (E) ISH of selected "regional" bacTRAP-specific genes with high expression in the entorhinal cortex (arrowheads). All in situ hybridization images in (C)–(E) are © 2007 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: http://www.mouse.brain-map.org. See Table S4 for datasets used.

(F) qRT-PCR quantification (mean \pm SEM) of the expression (IP/input) of five bacTRAP-enriched genes in bacTRAP (white bars) and three vTRAP serotype (gray bars) samples. Cbln1, Cbln4, Cldn1, and Nnat, p < 0.05 for IP versus input for all groups. **p < 0.01; ***p < 0.001; ****p < 0.0001.

The scale bars represent 1 mm, except injection site insets in (C) (500 μm). See also Figure S6 and Table S4.

species. Such an application of the technique may be used for understanding neuroplasticity, axonopathy, regeneration, and neurodegeneration.

The extremely high correlation between vTRAP and bacTRAP allows for the targeting and profiling of a single genetically determined cell population by either viral or transgenic approaches. This is particularly useful for cases where anatomical distinction is more easily achieved with viral injection rather than physical dissection. For example, vTRAP can be used with the SIc17a6 (Vglut2) Cre-driver mice, in which all projection neurons in thalamus express Cre, to examine discrete thalamic nuclei or with dopamine receptor mice to target dorsal or ventral striatal structures. The localized nature of viral injections also enables the exploration of regional differences in gene expression in genetically similar cells within a single neural structure. This was exemplified in the Ntsr1 cells by the discovery that vTRAP failed to enrich for genes expressed in cortical areas distal to the virus injection, establishing a high level of sensitivity in the approach and its potential to define molecularly distinct subdivisions of anatomical structures. Whereas methods for sequencing the transcriptomes of single cells have greatly improved in recent years, the higher sensitivity of vTRAP for detecting low-expressed cell-type-specific genes provides an advantage for unearthing subtle molecular differences that may result from experimental manipulation, such as behavior or pharmacological treatments.

Different AAV serotypes are known to have tropism for distinct cell types throughout the brain and body. The success of vTRAP experiments using viruses coated with three different capsid proteins implies that vTRAP is not limited by AAV serotype, making this technique amenable to characterizing a wide variety of cell types. However, we found that the AAV2 virus had limited spread in cortex and a number of the layer 6 marker genes were not as highly enriched in the AAV2 dataset when compared to the other serotypes and bacTRAP. This emphasizes the need to carefully test and select AAV serotypes that are most appropriate to target the cell type of interest, especially in less well-characterized brain regions or other tissues throughout the body.

AAVs have become indispensable tools that allow neuroscientists to functionally dissect disparate components of neural circuits in vivo. Vectors that deliver light-activated channels and pumps (channelrhodopsins, halorhodopsins, and archaerhodopsins), designer receptors (DREADDs), genetically encoded calcium indicators (GECIs), and small interfering RNAs (siRNAs) are widely available from academic and commercial sources. In addition, genome editing technologies, such as CRISPR, have advanced to the point where it is possible to acutely manipulate gene expression and epigenetic states using viral-mediated gene transfer (Ran et al., 2015). Multiplexing the vTRAP vectors we report here in tandem with the above approaches offers a comprehensive strategy to interrogate the precise role of individual, cell-type-specific genes in circuit function. These studies could facilitate our understanding of cell-type-specific molecular adaptations underlying changes in neural activity, the impact of targeted genetic alterations on cell function, as well as correlated behavioral alterations observed after such experimental perturbations. Such intersectional strategies will enable the study of neural circuitry at a resolution not previously possible.

This study reports a rapid, spatially restricted tool for translational profiling of cell types that can be applied easily throughout the body. Previous approaches to molecular profiling within complex tissues were restricted to transgenic strategies that took significant amounts of time to generate new and/or cross multiple mouse strains and could potentially result in contamination from off-target cell populations from ontogenetic expression. The vTRAP technology now enables a rapid and highly anatomically restricted methodology, which can elucidate marker genes for specific subregions within a given tissue. The utility of the vTRAP approach for translational profiling arises from its rapidity, ease of use, and broad applicability to systems already utilizing adeno-associated viruses.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving animals were approved by The Rockefeller University Institutional Animal Care and Use Committee and were in accordance with NIH guidelines. Ntsr1-bacTRAP (TS16), Pmch-Cre, and DAT-Cre mice were generated and maintained at The Rockefeller University and described previously (Doyle et al., 2008; Ekstrand et al., 2014; Jego et al., 2013; Knight et al., 2012). Ntsr1-Cre (GN220) and SERT-Cre (ET33) mice were generated by the GENSAT Project (Gong et al., 2007) and were purchased from the Mutant Mouse Regional Resource Center (Stock IDs 017266-UCD and 031028-UCD, respectively). The generation of Abcb1a-bacTRAP mice used for the specificity index is described in Supplemental Experimental Procedures. All mice were bred on a C57BL/6J background and maintained on a 12-hr light-dark cycle. Animals used in the study were male and female. Mice were sacrificed at 10–20 weeks of age within the same circadian period (12:00–16:00).

Generation of AAVs

The plasmid pAAV-FLEX-EGFPL10a was generated by subcloning the EGFPL10a transgene into pAAV-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-HGHpA (Addgene plasmid no. 20297) in the reverse orientation, replacing ChR2-mCherry. Plasmids were then packaged into AAVs at the University of North Carolina Vector Core or the University of Pennsylvania Vector Core. Virus titers were 1.63 × 10¹³ gc/mL for AAV5, 4.7 × 10¹² gc/mL for AAV2, and 7.47 × 10¹² gc/mL for AAV9. See Supplemental Experimental Procedures for more details.

Stereotaxic Surgeries

Bilateral stereotaxic injections of AAV-FLEX-EGFPL10a into adult (8–20 weeks old) Cre mice (male and female) under ketamine/xylazine (100/10 mg/kg) anesthesia were performed as described in Supplemental Experimental Procedures. Animals were sacrificed 1–4 weeks after surgery, and tissue was collected for polysome IPs, histology, or polysome fractionation as described below.

Immunohistochemistry

Immunofluorescent staining was carried out on free-floating brain sections or adherent HEK293T cells and imaged on either a Zeiss LSM780 or LSM700 confocal microscope. For details, see Supplemental Experimental Procedures.

TRAP

Affinity purification of EGFP-tagged polysomes was done 3 or 4 weeks after virus injections. Three biological replicates consisting of tissue pooled from three to five mice (mixed sex) were collected for each condition. IPs and RNA extractions were carried out as previously described (Heiman et al., 2014) and in Supplemental Experimental Procedures. Briefly, brain tissue was homogenized in buffer containing 10 mM HEPES-KOH (pH 7.4),

150 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 100 μg/mL cycloheximide, RNasin (Promega) and SUPERase-InTM (Life Technologies) RNase inhibitors, and Complete-EDTA-free protease inhibitors (Roche) and then cleared by two-step centrifugation to isolate polysome-containing cytoplasmic supernatant. Polysomes were immunoprecipitated using monoclonal anti-EGFP antibodies (clones 19C8 and 19F7; see Heiman et al., 2008) bound to biotinylated-Protein L (Pierce; Thermo Fisher Scientific)-coated streptavidin-conjugated magnetic beads (Life Technologies), and bound RNA was purified using the Absolutely RNA Nanoprep kit (Agilent Technologies). RNA quantity was measured with a Nanodrop 1000 spectrophotometer, and quality was assayed on an Agilent 2100 Bioanalyzer. Only samples with RNA integrity values ≥7.0 were used for RNA-seq and qRT-PCR analyses.

RNA-Seq and qRT-PCR

For cortical samples, TRAP RNA was amplified using the Ovation RNA-Seq System V2 Kit (NuGEN), and RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation Kit v2 (Illumina) following manufacturer's protocols. MCH neuron RNA-seq libraries were prepared using the SMARTer Ultra Low RNA Kit (Clontech Laboratories). Sequencing reactions were done using the Illumina HiSeq 2500 platform with three samples multiplexed per sequencing lane. RNA-seq reads were then aligned to annotated exons using the mm10 mouse reference genome with STAR (Dobin et al., 2013) version 2.0.0e_r291, and quantification of aligned reads was done using HTSeq (Anders et al., 2015) version 0.6.0. Differential expression was calculated by DESeq2 (Love et al., 2014) version 1.4.5, filtering for a false discovery rate (FDR) cutoff of <0.05 and |log2| fold change >1.0. Specificity index analysis was performed using the pSI (specificity index statistic) R-package version 1.1 (Dougherty et al., 2010; Xu et al., 2014). Detailed methods can be found in Supplemental Experimental Procedures.

The Taqman method was used for qRT-PCR analysis on three biological replicates for each condition using either an Applied Biosystems 7500 Fast or StepOnePlus Fast Real-Time PCR System. Data were normalized to *Rpl23* (Figures 2C, 3B, and S2) by standard curve or to *Actb* (Figures 4D and 6G) by the comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

Polysome Fractionation

Polysome fractionations were collected by centrifugation in a sucrose gradient as described previously (Darnell et al., 2011) and in Supplemental Experimental Procedures. Briefly, TCA-precipitated proteins from each fraction were analyzed by western blot, detected by enhanced chemiluminescence (Western Lightning Plus detection kit; PerkinElmer), and quantified by densitometry following film scans (Kodak MR film; ImageJ software).

Statistics

All data analysis was performed in Graphpad Prism, Microsoft Excel, or R (R Core Team, 2013). Student's t test was used to compare differences in qRT-PCR results between IP and input samples in Figures 2D, 3F, 4D, and 6G. Two-way ANOVA on the log2 fold-change values was used to compare each of the vTRAP groups against bacTRAP for the specified genes in Figures 4D and 6G.

ACCESSION NUMBERS

The accession number for the raw RNA-seq datasets reported in this paper is GEO: GSE89737.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.048.

AUTHOR CONTRIBUTIONS

A.R.N. and E.F.S. designed all experiments, and A.R.N., E.F.S., M.V.M., K.L.M., C.E.S., and M.I.E. performed all experiments. G.S.R. and K.S. per-

formed and analyzed polysome fractionations. B.C.F. assisted with IHC. A.R.N., E.F.S., M.V.M., M.I.E., Y.L., A.M., J.M.F., and N.H. analyzed data. A.R.N., N.H., and E.F.S. wrote the paper.

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