Molecular characterization of neuronal cell types based on patterns of projection with Retro-TRAP

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Retro-TRAP (translating ribosome affinity purification) technology enables the synthesis of molecular and neuroanatomical information through the use of transgenic and viral approaches. In contrast to other methods that are used to profile neural circuits such as laser-capture microdissection and FACS, Retro-TRAP is a high-throughput methodology that requires minimal specialized instrumentation. Retro-TRAP uses an anti-GFP ribosomal tag (expressed virally or using transgenesis) to immunoprecipitate translating mRNAs from any population of neurons that express GFP. The protocol detailed here describes the rapid extraction of molecular information from neural circuits in mice using retrograde-tracing GFP-expressing viruses. This approach can be used to identify novel cell types, as well as to molecularly profile cell types for which Cre-driver lines are available, in defined presynaptic loci. The current protocol describes a method for extracting translating mRNA from any neural circuit accessible by stereotaxic injection and manual dissection, and it takes 2–4 weeks. Although it is not described here, this mRNA can then be used in downstream processing applications such as quantitative PCR (qPCR) and high-throughput RNA sequencing to obtain 'molecular connectomic' information.

INTRODUCTION

Molecular profiling of ribosome-bound mRNAs using technologies such as TRAP has enabled the characterization of complex tissues[1,2] at unprecedented resolution[3]. In contrast to total RNA and other polyA-based approaches, TRAP-based technologies offer insights into the actively translated mRNAs in defined cell populations. We recently reported the development of a methodology (termed Retro-TRAP[4]) that enables the high-throughput molecular annotation of neural circuitry in mice through the integration of retrograde-tracing viruses[5], camelid antibodies[6] and TRAP-based technology[7]. When paired with RNA sequencing, Retro-TRAP can be used to identify novel cell types in heterogeneous nuclei of the brain, as well as to translationally profile principal cell types throughout the brain by obtaining high-throughput expression profiles. This protocol focuses on the implementation of Retro-TRAP and Retro-TRAP–based technologies for both of these purposes, using transgenic mice and viruses expressing the two components (GFP and anti-GFP–tagged ribosomal subunit proteins) required for the immunoprecipitation of polysome-bound, translating mRNAs.

Retro-TRAP development

Neural cell types can be classified using a number of criteria such as cellular excitability, morphology, connectivity and principal marker gene expression. Retro-TRAP was originally developed to identify cell types by virtue of both their principal marker gene expression and their projection pattern, and thus it enabled the first high-throughput unification of connectomics and molecular profiling[4]. The original TRAP methodology[1] made use of a direct GFP epitope tag of large ribosomal subunit protein RPL10A (GFPL10). Retro-TRAP is based on a two-component system that utilizes GFP and a heterologously expressed fusion protein consisting of RPL10A N-terminally tagged with an anti-GFP camelid Nanobody (hereafter referred to as NBL10). Expression of this fusion protein confers ribosomes with the capability of binding GFP within the cell. The NBL10 fusion is analogous to the previously reported GFPL10 fusion used to directly access translating mRNAs from molecularly defined cell types via a GFP immunoprecipitation (GFP IP)[1,7]. However, the GFP-NBL10 two-component system, upon which Retro-TRAP is based, allows for substantially increased modularity and flexibility of experimental design. Indeed, this system enables various projection-specific profiling strategies, as well as a number of intersectional genetic approaches that were not previously possible with TRAP-based technology.

We developed two complementary projection-specific profiling strategies to offer different levels of molecular resolution in studying projective cell types. The first approach enabled the identification of principal cell types by virtue of their projection(s) to a defined target region. The second approach then allowed molecular profiling of *a priori* genetically defined subsets of these cell types.

For the first approach to molecular profiling of neurons based on patterns of projection, we generated a transgenic mouse line that expressed the NBL10 fusion protein under control of the human synapsin promoter (SYN-NBL10), which drives the expression of NBL10 in all neuronal cell types. Thus, after an injection of a GFP-expressing retrograde-tracing virus (such as canine adenovirus (CAV)-GFP) into a defined brain region, we could dissect out presynaptic loci and identify the projective cell types giving input to the target region after a GFP IP. The second approach built on this strategy to translationally profile genetically defined subsets of the projective neurons. By combining Cre-driver lines with an adeno-associated virus (AAV) engineered to conditionally express the NBL10 construct in the presence of the enzyme Cre recombinase (AAV-FLEX-NBL10), this approach enabled selective profiling of genetically defined projection neurons. This linear, iterative strategy for refining the molecular identity of myriad neural cell types motivates the current protocol.

A schema for the experimental flow and design of the original Retro-TRAP strategy and the complementary cell type–specific version is presented in **Figure 1**.

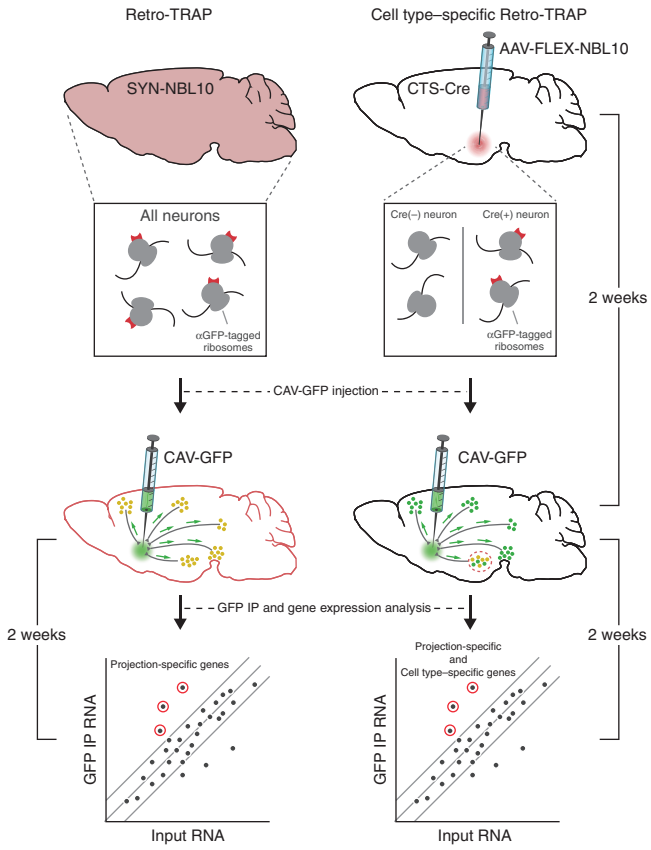

Figure 1 | Retro-TRAP strategy and experimental design. Experimental design for Retro-TRAP and cell type–specific Retro-TRAP proceeds top to bottom, in parallel. Retro-TRAP uses SYN-NBL10 mice, whereas cell type–specific Retro-TRAP uses cell type–specific Cre-driver lines (CTS-Cre). Top, SYN-NBL10 mice constitutively express the NBL10 construct in all neurons. Cell type–specific Retro-TRAP requires the stereotaxic injection of AAV-FLEX-NBL10 into the desired region, followed by a 2-week incubation period allowing for expression and incorporation of the NBL10 fusion protein into ribosomes of Cre-positive neurons. Middle, CAV-GFP injection into a target retrogradely labels all neurons projecting to that region. In Retro-TRAP, all neurons labeled with GFP are accessible to GFP immunoprecipitation (GFP IP). In cell type–specific Retro-TRAP, only the double-positive neurons (expressing both GFP and the NBL10 fusion) are accessible to a GFP IP. A 2-week incubation period enables stable expression of GFP. Bottom, after dissection of the desired brain region, GFP IP and gene expression analysis, projection-specific (Retro-TRAP) or projection- and cell type–specific (cell type–specific Retro-TRAP) marker genes can be successfully identified using high-throughput RNA sequencing.

Alternatives to Retro-TRAP

There exist a number of other techniques that could potentially be used to extract molecular information from neural populations by virtue of their connectivity. These approaches include, but are not limited to, laser-capture microdissection (LCM)[8], FACS[9], Fluidigm[10,11] and manual dissection[12,13], which can be performed after the injection of a retrograde tracer or virus expressing a fluorophore such as GFP. These techniques yield single cells or populations of cells, which enables the extraction of different types of information. Although Retro-TRAP and other TRAP techniques result in a raw output of translating mRNAs, techniques such as LCM and FACS yield all cellular RNAs (including those localized within the nucleus). Thus, Retro-TRAP enables the selective isolation of translating mRNA, whereas the other techniques cannot be used to directly obtain this information. For that they would require multiplexing with another approach such as ribosome profiling[3].

Experimental design

RNA yields from Retro-TRAP studies. To successfully profile cell types, it is necessary to obtain a sufficient amount of RNA, on the order of nanograms. This is essential for the collection of high-quality Retro-TRAP data: there needs to be enough RNA collected to dominate potential background noise introduced from the IPs, and limited amounts of RNA may reduce the robustness of results obtained from high-throughput RNA sequencing (RNA-Seq). We have found that for mesolimbic and nigrostriatal dopaminergic projections, six mice provide sufficient RNA yields (>2 ng RNA per IP replicate). However, these quantities will vary from circuit to circuit because of different factors such as viral tropism, numbers of cells projecting to the target region and efficacy of injections in both presynaptic and postsynaptic loci. Thus, before a large-scale study, smaller pilot studies (low number of animals and small sample size) should be run to ascertain the minimal number of mice required to achieve sufficient RNA yields.

Timing considerations. For cell type–specific Retro-TRAP, sufficient time needs to elapse after injection of AAV-FLEX-NBL10 to ensure that the NBL10 fusion will have successfully integrated into the ribosome, as ribosomal turnover in the brain is relatively slow[14] (~2–3.5 weeks). In our experience, the most effective injection schedule has been AAV injections at T_0, CAV-GFP injections 2 weeks later and GFP IPs 2 weeks after that. This allows sufficient time for the NBL10 fusion to express and integrate into the ribosome, where it can sequester intracellular GFP. Because CAV-GFP infects the presynaptic neurons retrogradely rather rapidly, it is theoretically possible to perform IPs at an earlier time point. However, from initial tests we have found that earlier time points after injection (5–10 d) result in greater experimental variability. Further studies are needed to assess the potential for using earlier time points in Retro-TRAP experiments.

Quality control and confirmation of Nanobody expression. As a built-in control, we have placed an N-terminal hemagglutinin (HA) epitope tag on the NBL10 construct. This enables visualization of the NBL10 fusion protein after successful AAV injection into a Cre-driver mouse via immunohistochemistry. Failure to observe cytoplasmic localization of the NBL10 fusion indicates a potential problem with the injection or Cre-driver line being used, and this is further discussed below in the TROUBLESHOOTING section. The HA tag also enables a critical control study: HA IPs normalized to the input sample should result in cell type–specific marker gene enrichment (independent of the projection target); IPs can be done in parallel with Retro-TRAP studies (after GFP IPs). Failure to enrich for cell type–specific marker genes likely indicates a possible issue with the Cre-driver line (ectopic or limited expression) or viral preparation (lack of specificity in targeting of the NBL10 construct) and a need for further troubleshooting (see TROUBLESHOOTING section for further details). The HA tag can also be used for normalization of the data to the Cre-expressing population of cells that are infected with AAV (for example, if dopamine neurons are infected with the AAV, an HA IP should result in enrichments for dopaminergic marker genes such as *Slc6a3* and *Th*). This normalization can further assist in the identification of transcripts that are truly

specific to projection neurons (and not just representative of the Cre-expressing population as a whole).

Alternative applications of the GFP-Nanobody system. Although we developed the two-component GFP-Nanobody system to molecularly profile neurons based on their connectivity (Retro-TRAP), it can be used for a number of other applications that are involved in bringing GFP to the ribosome, including additional molecular profiling approaches and cell and molecular engineering applications. This system is also flexible and can be extended to other organisms, such as the rat.

Molecular profiling. The GFP-Nanobody system can theoretically be used to profile any cell type in the body that expresses GFP. Within the brain, the remarkable diversity of marker gene expression has been successfully demonstrated through the GENSAT project[15] (http://gensat.org), which established GFP-driver lines for multitudes of genes expressed within the CNS. These GFP-driver mice could be crossed to SYN-NBL10 mice to generate a molecular profile of the neurons expressing the desired marker genes (A.R.N., unpublished data). To profile cell types expressing GFP throughout the remainder of the body (and also within the CNS), pan-cellular NBL10 driver lines or novel AAVs may be developed. Furthermore, because of the two-component nature of the current system, an intersectional genetic approach becomes possible, using Cre-driver lines crossed to GFP-driver lines. In a region where GFP- and Cre-positive cells overlap, AAV-FLEX-NBL10 may be injected to enable profiling of the overlapping (double-positive) cells. This type of approach could also be extended to achieve further granularity in classifying overlapping cell types, as Nanobodies for other fluorescent proteins (such as mCherry) exist and are continually being developed[16]. In addition, to gain further spatial resolution of translation within a defined cell type, future improvements on Retro-TRAP-related techniques may use proximity-specific ribosome profiling strategies that enable the characterization of ribosomes that are actively translating at the endoplasmic reticulum[17] and mitochondrial[18] membranes.

Molecular and cellular engineering. The intracellular environment has a very distinct topographic organization, and the GFP-Nanobody system can also potentially be used for molecular and cellular engineering applications where it might be advantageous to bring a given molecule within close proximity to the active ribosome. Although Retro-TRAP makes use of this two-component system for immunoprecipitation of translating mRNAs, this technology could also be used to recruit various factors to the ribosome to effect and study its function. GFP has recently been functionalized for a number of different applications using the GFP-Nanobody system, such as live-cell antigen targeting[6] and cell type–specific modulation of gene expression[19]. In the context of the ribosome, translational inhibitors or activators could be tagged with GFP to halt or augment translation in a cell type–specific manner, respectively. Furthermore, ribosomal turnover in the brain is relatively slow[14]; thus, this system could potentially be used to stabilize a protein's half-life within the cytoplasm by fusing it to GFP. These applications represent just a few of the myriad possible uses of the GFP-Nanobody system for subcellular localization of proteins.

Advantages and limitations

The GFP-Nanobody system enables molecular profiling of any cell type expressing GFP. This represents a significant advance over previous molecular profiling technologies, as this two-component system can be used in tandem with virally delivered GFP[4], as well as with currently available GFP-driver lines from projects such as GENSAT (http://gensat.org), to make the transcriptome experimentally accessible. Retro-TRAP is also the first high-throughput methodology for profiling cell types based on their connectivity, and it requires minimal specialized instrumentation.

Similarly to bacTRAP, which was developed to molecularly profile cell types based on promoter-specific elements[1,7], Retro-TRAP uses a ribosomal tag/affinity purification platform. Similarly to bacTRAP, Retro-TRAP technology is based on ensemble averages, of both cells and animals. TRAP technologies use pooled samples from multiple animals, which probably obscure the amount of molecular heterogeneity present in a defined population of cell types[20]. In addition, because these techniques rely on an affinity purification of polysome-bound mRNAs, they are unable to purify other RNAs that may have a key role in cellular function but are not necessarily ribosome bound, such as long non-coding RNAs (lncRNAs)[21].

In addition, Retro-TRAP-based approaches rely on a GFP-Nanobody system that is potentially prone to increased background signal because of previously reported GFP spillover[4]. During tissue homogenization, free GFP can promiscuously bind Nanobody-tagged ribosomes, diluting out the enrichment for the proper marker genes; however, in previous work, we demonstrated that this background can be minimized by the addition of a blocking concentration of recombinant Nanobody (rNB). This blocking concentration (200 ng/ml) appears to be invariant with respect to other experimental design parameters tested. An alternative possibility for limiting this issue (and thus the requirement of titrating rNB) would be to increase the relative stoichiometry between NBL10 and GFP (by using either a weaker promoter to drive GFP or a stronger promoter/longer viral incubation period to drive NBL10).

The Retro-TRAP methodology also partially relies on viruses to achieve projection specificity. We identified CAV type 2 as an excellent candidate for molecular profiling on the basis of its low immunogenicity and biocompatibility[5]. However, viruses such as CAV have been shown to induce alterations in host transcriptomic profiles[22], which must be taken into consideration when analyzing Retro-TRAP data. In addition, viral tropism could introduce bias by preferentially targeting certain subsets of projections to a defined target region. To test this possibility, different retrograde tracers could be used in the targeted circuitry.

As previously mentioned, there exist a number of other technologies that may theoretically offer greater resolution in the study of populations of cell types, such as LCM, FACS and manual dissection. However, a number of these technologies currently appear to induce cellular stress that may alter the transcriptome, and could thus interfere with the accurate analysis of a cell type's molecular composition[9]. In direct contrast, TRAP-based methodologies do not require lengthy incubation periods or tissue processing, thus leading to minimal cellular stress and likely a more faithful representation of the cellular state at the time of RNA isolation. Thus, Retro-TRAP and similar technologies used for extracting molecular information from projective neurons currently offer a tradeoff between resolution and sensitivity. As these technologies continue to improve, we will probably see their simultaneous application to a number of important questions where they can offer complementary information.

MATERIALS

REAGENTS

Viral vectors

- NBL10 vectors. For example, AAV-FLEX-NBL10 ▲ CRITICAL pAAV-FLEX-NBL10 can be obtained from the Friedman laboratory directly (see affiliations for contact information; there is no authorship requirement for obtaining pAAV-FLEX-NBL10) and then produced as AAV by vector cores, such as those at the University of North Carolina or the University of Pennsylvania. AAVs should have a titer of at least 10^{12} gc/ml. Upon receipt, AAVs should be thawed, aliquoted into the desired volume on dry ice and placed at −80 °C for long-term storage (years). After initial experimental use, AAVs can be kept at 4 °C for weeks. pAAV-FLEX-NBL10 is in the process of being deposited to Addgene.
- Retrograde-tracing viruses. For example, CAV-GFP ▲ CRITICAL CAV-GFP and other CAV viruses can be obtained from Montpellier Vectorology (PVM, http://www.biocampus.cnrs.fr/index.php/en/plateformes?id=78). For further information, contact PVM directly (CAV.2@biocampus.cnrs.fr). CAV should be used the same day that it is thawed (multiple freeze-thaw cycles significantly reduce titer). CAV-GFP should be stored at −80 °C and is stable for years if stored correctly.

Animals

- For Retro-TRAP. We use 8–14-week-old SYN-NBL10 transgenic mice. These mice can currently be obtained from the Friedman laboratory directly (there is no authorship requirement for obtaining SYN-NBL10 mice). SYN-NBL10 transgenic mice are in the process of being deposited to Jackson Labs ! CAUTION All experiments using animals must be approved by the institutional animal care and use committee (IACUC) at your institution and performed according to institutional and national guidelines.
- For cell type–specific Retro-TRAP. We use 8–14-week-old Cre-driver mice (Cre-driver mice representing many cell types throughout the brain can be obtained from The Jackson Laboratory).

Stereotaxic injection of virus

- Anesthesia: isofluorane (Henry Schein, cat. no. 050033)
- Analgesic: buprenorphine (Sigma-Aldrich, cat. no. B9275)
  ! CAUTION Buprenorphine is a controlled substance, and it must be used according to institutional and governmental guidelines.
- Sterile ophthalmic ointment (Akorn, cat. no. NDC 17478-235-35)
- Ethanol (Sigma-Aldrich, cat. no. 459836)
- Betadine (Fisher Scientific, cat. no. 19-027133)
- 3% hydrogen peroxide (Swan, cat. no. 0869-0871-43)
- Sterile PBS (Life Technologies, cat. no. 10010023)

Manual dissection for brain tissue isolation

- RNaseZap decontamination solution (Life Technologies, cat. no. AM9782)
- 10× HBSS (Life Technologies, cat. no. 14065-056)
- 1 M HEPES, pH 7.3 (Affymetrix, cat. no. 16924)
- Glucose (Sigma-Aldrich, cat. no. G7528)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S6297)
- Nuclease-free water (not DEPC-treated; Life Technologies, cat. no. AM9932)
- Cycloheximide (Sigma-Aldrich, cat. no. C7698) ▲ CRITICAL Freshly prepare cycloheximide on the day of use, and keep it on ice.

GFP IP and RNA preparation

- Recombinant Nanobody GFP-TRAP protein (Chromotek, cat. no. gt-250)
- Kimble-Chase Kontes tubes for Potter-Elvehjem tissue grinders, Size 20 (Fisher Scientific, cat. no. 885512-0020)
- KCl (2 M; Life Technologies, cat. no. AM9640G)
- $MgCl_2$ (1M; Life Technologies, cat. no. AM9530G)
- Recombinant RNasin ribonuclease inhibitor (Promega, cat. no. N2515)
- SUPERase-In RNase inhibitor (Life Technologies, cat. no. AM2696)
- PBS (10×, pH 7.4; Life Technologies, cat. no. AM9624)
- Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche, cat. no. 04693159001)
- BSA (IgG-Free, Protease-Free; Jackson ImmunoResearch, cat. no. 001-000-162)
- Dynabeads MyOne streptavidin T1 (Life Technologies, cat. no. 65602)
- Pierce protein biology products—recombinant protein L, biotinylated (Thermo Scientific, cat. no. 29997)
- DHPC (200 mg; Avanti Polar Lipids, cat. no. 850306P)
- IGEPAL CA-630 (NP-40; Sigma-Aldrich, cat. no. I8896-50ML)
- Sulfolane, 99% (Sigma-Aldrich, cat. no. T22209-100G)
- GFP antibodies HtzGFP-19F7, HtzGFP-19C8 (Monoclonal Antibody Core Facility, Memorial Sloan Kettering Cancer Center)
- Absolutely RNA Nanoprep kit (Agilent, cat. no. 400753)

EQUIPMENT

Stereotactic injection of virus

- Dual small-animal stereotaxic frame (David Kopf, cat. no. 942)
- Tabletop isoflurane delivery system (VetEquip, cat. no. 901806SO)
- Surgical tools: scissors, forceps, skull scraper (Fine Science Tools)
- Cotton-tipped applicators (Fisher Scientific, cat. no. 23-400-101)
- Surgical stereoscope (Leica, cat. no. 10446294)
- Micro-drill (Osada, cat. no. EXL-M40)
- Drill carbide bur (Henry Schein, cat. no. 100-5860)
- Hamilton syringe, 5 µl (VWR, cat. no. 63074-102)
- Hamilton needles, 33 gauge (VWR, cat. no. 82010-234)
- EZ clip wound closure kit (Stoelting, cat. no. 59020)

Manual dissection for brain tissue isolation

- Scissors, forceps (Fine Science Tools)
- Adult mouse brain slicer matrix (Zivic Instruments, cat. no. BSMAS001-1)

GFP IPs

- Variable-speed, reversible homogenizer: 333–4,000 r.p.m. (Glas-Col)

REAGENT SETUP

Preparation for virus injection Attach a 33-gauge Hamilton needle to the 10-µl Hamilton syringe (after removing the original Hamilton needle from the syringe). Fill the syringe with 2 µl of water, followed by 0.5 µl of air. Immediately before injecting virus, load the syringe with the desired amount of virus, plus an additional 0.2 µl. Push out 0.1 µl to confirm that the virus is expelled from the tip of the needle.

Dissection buffer This buffer is similar to one used in a previously published protocol[7]. Make 50 ml of buffer by combining 5 ml of 10× HBSS (1× final concentration), 125 µl of 1 M HEPES-KOH, pH 7.4 (2.5 mM final concentration), 1.75 ml of 1 M glucose (35 mM final concentration), 200 µl of 1 M $NaHCO_3$ (4 mM final concentration), 43 ml of RNase-free water and 50 µl of 100 mg/ml cycloheximide freshly before use (100 µg/ml final concentration). Dissection buffer without cycloheximide can be stored at 4 °C before use. During use, the buffer should be stored on ice.

Homogenization buffer This buffer is similar to one used in a previously published protocol[7]. Make 1.5–2 ml of homogenization buffer per IP. To make 10 ml of buffer, combine 100 µl of 1 M HEPES-KOH (10 mM final concentration), pH 7.4, 750 µl of 2 M KCl (150 mM final concentration), 50 µl of 1 M $MgCl_2$ (5 mM final concentration) and 9.1 ml of RNase-free water, and add the following freshly to the solution: 5 µl of 1 M DTT (0.5 mM final concentration), 1 mini tab protease inhibitors, 20 µl of RNasin RNase inhibitor (2× final concentration), 20 µl of Superasin RNase inhibitor (2× final concentration), 10 µl of 100 mg/ml cycloheximide (100 µg/ml final concentration) and 2 µl of 1 mg/ml rNB (200 ng/ml final concentration). Homogenization buffer without 'fresh' ingredients can be stored at 4 °C for months before use. During use, the buffer should be stored on ice.

Wash buffer #1 This buffer is similar to one used in a previously published protocol[7]. Make 3 ml plus the number of IPs × 200 µl; for example, to perform three IPs, make 3.6 ml buffer. To make 5 ml of buffer, combine 50 µl of 1 M HEPES-KOH, pH 7.4 (10 mM final concentration), 375 µl of 2 M KCl (150 mM final concentration), 25 µl of 1 M $MgCl_2$ (5 mM final concentration), 0.5 ml of 10% (vol/vol) NP-40 (1% final concentration) and 4.05 ml of RNase-free water, and add the following freshly to the solution: 2.5 µl of 1 M DTT (0.5 mM final concentration), 5 µl of RNasin RNase inhibitor and 5 µl of 100 mg/ml cycloheximide (100 µg/ml final concentration). Wash buffer #1 without 'fresh' ingredients can be stored at 4 °C for months before use. During use, the buffer should be stored on ice.

Wash buffer #2 This buffer is similar to one used in a previously published protocol[7]. Make 4 ml per IP. To make 10 ml of buffer, combine 100 µl of 1 M HEPES-KOH, pH 7.4 (10 mM final concentration), 1.75 ml of 2 M KCl (350 mM final concentration), 50 µl of 1 M $MgCl_2$ (5 mM final concentration), 1 ml of 10% (vol/vol) NP-40 (1% final concentration) and 7.1 ml of RNase-free water, and add the following freshly to the solution: 5 µl of 1 M DTT (0.5 mM final concentration), 10 µl of RNasin RNase inhibitor and 10 µl of 100 mg/ml cycloheximide (100 µg/ml final concentration). Wash buffer #2 without 'fresh' ingredients can be stored at 4 °C for months before use. During use, the buffer should be stored on ice.

Sample lysis buffer Approximately 200 µl of sample lysis buffer is required per IP. To make lysis buffer, add 0.7 µl of β-mercaptoethanol per 100 µl of Stratagene Absolutely RNA lysis buffer (for example, add 1.4 µl of β-mercaptoethanol to 200 µl of Absolutely RNA lysis buffer). Sample lysis buffer should be freshly made on the day of experiments. During use, the buffer should be stored on ice.

Anti-GFP magnetic beads Thaw protein L on ice and resuspend it in 1× PBS (1 mg/ml final concentration). Resuspend Dynabeads by hand (do not vortex). Add 300 µl of Dynabeads (per IP) to a microcentrifuge tube. Collect the beads on a magnet and rinse them once with 1 ml of 1× PBS. Collect the beads on the magnet and add 1 ml of 1× PBS plus 120 µl of 1 mg/ml protein L per IP (for three IPs, this would mean resuspending 900 µl of Dynabeads in 1 ml of 1× PBS plus 360 µl of 1 mg/ml protein L). Incubate the beads with rotation at room temperature (20–25 °C) for at least 35 min. Collect the beads on the magnet and wash the beads five times with 1× PBS containing 3% (wt/vol) nuclease-free BSA. Collect the beads on the magnet and add 1 ml of wash buffer #1, and for each IP, add 50 µg each of 19F7 and 19C8 GFP antibodies (100 µg of GFP antibody total per IP). Incubate the beads with rotation at room temperature for at least 1 h. During the fast 20,000*g* spin to clarify the supernatant (see Step 24), wash and prepare the antibody-bound beads for IP. Collect the beads on a magnet and remove the antibody solution. Resuspend the beads in 1 ml of wash buffer #1 and collect the beads on the magnet. Repeat this for a total of three washes. Before removing the last wash buffer, transfer the resuspended beads into separate microcentrifuge tubes for each IP. Remove the wash buffer and resuspend the beads in 180 µl of wash buffer #1 and 20 µl of DHPC. Store the prepared beads on ice until you are ready to proceed with the IP.

PROCEDURE

Stereotactic injection of the virus into the mouse brain ● TIMING 1–2 h (plus virus incubation period)

▲ **CRITICAL** Before Retro-TRAP studies, it is highly recommended that the fidelity of both the Cre-driver line (via a cross to a Rosa reporter mouse) and AAV-FLEX-NBL10/CAV-GFP be tested using immunohistochemical analyses and/or IPs.

▲ **CRITICAL** Mice should be housed according to institutional standards, and surgical tools should be cleaned and sterilized before use.

! **CAUTION** All experiments using animals must be approved by the IACUC at your institution and performed according to all relevant institutional and governmental guidelines.

1| Induce anesthesia in mice using 3% (wt/vol) isoflurane anesthesia, followed by maintenance on 1.5–2% (wt/vol) isoflurane. If you are performing standard Retro-TRAP, use 8–14-week-old SYN-NBL10 transgenic mice. For cell type–specific Retro-TRAP, use 8–14-week-old Cre-driver mice.

▲ **CRITICAL STEP** Test for the absence of the toe-pinch reflex to confirm that the mice are fully anesthetized.

2| Place the animal on a heating pad or hand warmers on a stereotactic frame, and apply ophthalmic ointment.

▲ **CRITICAL STEP** Confirm that the animal's head is aligned properly on the stereotactic frame, as this is essential for accurate injections.

3| Shave and/or clean the mouse's head and wipe it with Betadine solution followed by 70% (vol/vol) ethanol.

4| Make a midline incision and expose the skull.

5| Clean the skull with cotton swabs dipped in hydrogen peroxide, and then dry the skull with clean cotton swabs.

6| Identify bregma under a stereoscope and move to proper coordinates on the skull surface relative to bregma (coordinates can be obtained from the Paxinos Mouse Brain Atlas[23]).

7| Make a burr hole in the target region.

8| Thaw the virus on ice and prepare injection needles, as described in Reagent Setup.

9| Inject an appropriate amount of the appropriate virus (see table below) into the desired region at a rate of 150 nl/min.

Experiment type	Virus	Amount
Standard Retro-TRAP	CAV-GFP	~0.5 µl
Cell type–specific Retro-TRAP: first injection	AAV-FLEX-NBL10	100 nl–1 µl
Cell type–specific Retro-TRAP: second injection	CAV-GFP	~0.5 µl

10| After injection, leave the needle in place for 5–7 min to allow proper diffusion of the virus before withdrawing it slowly.

11| Close the skin wound with a sterile surgical clip.

12| Inject subcutaneous buprenorphine (0.05 mg/kg) after the procedure.

13| Allow mice to recover on a heating pad or hand warmers until they are awake and moving around. After 2 weeks, proceed to Step 14 for cell type–specific Retro-TRAP or Step 15 for noncell type–specific Retro-TRAP.
▲ **CRITICAL STEP** We have found that enrichments are optimal 2 weeks after injection of CAV-GFP.

14| For cell type–specific Retro-TRAP only, 2 weeks after the first injection (of AAV-FLEX-NBL10) repeat Steps 1–13 to inject CAV-GFP into the desired projection target region. Proceed with Step 15 2 weeks after the CAV-GFP injection.
▲ **CRITICAL STEP** Enrichments are optimal for cell type–specific Retro-TRAP when IPs are performed at least 3–4 weeks after AAV injection.
? TROUBLESHOOTING

Brain dissections ● TIMING 30–60 min
▲ **CRITICAL** Before starting dissections, make a fresh stock of 100 mg/ml cycloheximide solution in methanol and the following solutions: dissection buffer, homogenization buffer, wash buffers #1 and #2, anti-GFP magnetic beads and sample lysis buffer (see Reagent Setup).
15| Spray down all tools and surfaces that will come into contact with brain tissue (dissecting block, mouse brain matrix and dissecting instruments) with RNaseZap and let it sit for 20 min.

16| Set the dissecting block on ice and place the mouse brain matrix on top of the dissecting block to maintain it at ice-cold temperature.

17| Place the dissecting buffer on ice.

18| Euthanize the mice and isolate the brains using approved methods, and then place the brains in dissection buffer.
! CAUTION All experiments using animals must be approved by the IACUC at your institution and performed according to all relevant institutional and governmental guidelines.

19| Place the brains on the mouse brain matrix and dissect out the desired region. Place the dissected tissue into fresh aliquots of dissection buffer.
▲ **CRITICAL STEP** It is essential to avoid including the injection site in the dissected tissue. Inclusion of the injection site will dilute out enrichments for true projection-specific marker genes.
▲ **CRITICAL STEP** Do not freeze down the tissue, as this will result in significantly reduced RNA yields (up to ~50%; ref. 1). The earliest time point to freeze down the samples should be after the sample is lysed from the beads, after the IPs have been run (Step 32).
? TROUBLESHOOTING

GFP IPs ● TIMING 4–6 h
▲ **CRITICAL** Steps 20–32 have been adapted from the protocol from Heiman *et al.*[7] for specific application to projection- and cell type–specific translational profiling.
20| Once all dissections have been performed, transfer the brain pieces into glass homogenization tubes, and immediately add 1.5 ml of homogenization buffer to the tubes.
▲ **CRITICAL STEP** Glass homogenization tubes and Teflon homogenizers wrapped in fresh aluminum foil should be kept on ice before use.
▲ **CRITICAL STEP** Make sure that all brain pieces have sunk to the bottom of the glass tubes after the addition of homogenization buffer.

21| In a cold room, homogenize the tissue three times at a slow rotation speed (30–35 setting on Glas-Col), making sure to push the Teflon homogenizer to the bottom of the glass tube; follow this by homogenization ten times at a fast rotation speed (70–80 setting on Glas-Col).
▲ **CRITICAL STEP** If the homogenizer is not pushed all the way to the bottom of the glass tube during the slow homogenization, the brain tissue will not be sufficiently broken up, which ultimately reduces RNA yields. There should be no visible clumps of brain tissue after homogenization.
▲ **CRITICAL STEP** Do not break the surface of the liquid when pulling up the pestle during homogenization, as that will create many bubbles.

Box 1 | Preparation of input/total and unbound samples (15 min)

Input/total and unbound samples will be used later on during qPCR and RNA-Seq analyses to normalize the IPs. This normalization gives the relative enrichment of a given marker gene from Retro-TRAP studies.

1. Remove 50 µl of supernatant and transfer it to a fresh microcentrifuge tube.
2. Add an equal volume of sample lysis buffer to the supernatant (100 µl total volume).
3. Vortex the tube and leave it at room temperature for 10 min.
4. Vortex the tube again and place it on ice until RNA preparation, or place it in a freezer along with the remaining samples after IPs.

22| After tissue homogenization, rapidly transfer the homogenates to individual prechilled 2-ml microcentrifuge tubes and spin them down at 2,000*g* at 4 °C for 10 min.

23| Transfer the supernatant to fresh 2-ml microcentrifuge tubes on ice and discard the pellets.

24| To clarify the supernatant, add a tenth of a volume of 300 mM DHPC (30 mM final concentration), and another tenth of a volume of 10% (vol/vol) NP-40 (1% final concentration). For example, starting with 1.5 ml of supernatant, add 150 µl of DHPC, followed by 165 µl of NP-40. Invert the tube gently to mix, and then spin it down at 20,000*g* at 4 °C for 15 min.
▲ **CRITICAL STEP** To minimize time between tissue lysis and GFP IPs, do not incubate the clarified solution on ice (as is recommended in the protocol by Heiman *et al.*[7]).
▲ **CRITICAL STEP** Anti-GFP magnetic beads can be prepared (as described in Reagent Setup) during this spin.

25| After the spin is complete, save an 'input/total' sample, as described in **Box 1**.

26| Transfer the remaining supernatant to a fresh 2-ml microcentrifuge tube containing 200 µl of anti-GFP magnetic beads for IP. Allow GFP IPs to proceed on a rotator for 40 min at 4 °C.
▲ **CRITICAL STEP** Do not allow IPs to proceed longer than 40 min (as recommended elsewhere[7]), as this can lead to more background because of GFP spillover. In addition, because IPs are only run for 40 min, less RNase inhibitor is required (2× instead of 10×).

27| Collect beads on a magnet for >1 min, and save an 'unbound' sample, as described in **Box 1**.

28| On the magnet, aspirate the remainder of supernatant, and add 1 ml of wash buffer #2 (wash 1). Resuspend the beads thoroughly, and transfer them to a fresh microcentrifuge tube (but save the old tube). Collect the beads on the magnet and aspirate the supernatant.

29| Add 1 ml of wash buffer #2 to the old microcentrifuge tube to collect the remaining beads, and then transfer the solution to the new microcentrifuge tube with the remaining beads from Step 28. Resuspend the beads thoroughly (wash 2).

30| Collect the beads on a magnet and rinse them twice more (washes 3 and 4) by resuspending the beads thoroughly with wash buffer #2. Aspirate the supernatant.

31| Resuspend the beads in 100 µl of sample lysis buffer, vortex the beads and place them at room temperature for 10 min. Vortex the beads again and place them on a magnet for >1 min.

32| Transfer the supernatant (GFP IP) to a fresh microcentrifuge tube.
■ **PAUSE POINT** At this point, one can proceed with RNA purification (Step 33) or the samples can be frozen down at –80 °C (and stably stored for months).

RNA purification ● **TIMING 60–75 min**

33| Add 300 µl of 80% sulfolane (vol/vol) to the collected samples (400 µl total volume) and vortex (if samples were previously frozen, first allow them to thaw on ice).

34| Transfer the samples to RNA spin cups and perform RNA purification according to the manufacturer's protocol (Agilent).
? TROUBLESHOOTING
■ **PAUSE POINT** At this point, RNA can be placed on ice and used for cDNA preparation, or the samples can be frozen at –80 °C (and stably stored for years). Information on downstream analyses for cDNA preparation and qPCR/RNA-Seq can be found elsewhere[4].

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
14	Low NBL10 expression	Viral titer is low	AAV should be concentrated to at least 10^{12} gc/ml
19	Low GFP expression	Poor viral tropism	Test another retrograde-tracing, GFP-expressing virus such as PRV[24] or rabies[25]
34	Low RNA yields	Poor injection of AAV-FLEX-NBL10 and/or CAV-GFP	Confirm substantial numbers of NBL10/GFP double-positive cells
		Insufficient number of pooled animals	Add more mice to each individual replicate
		Insufficient integration of NBL10	Allow for a longer period of time to elapse between AAV injection and IPs, or use a stronger promoter (for example, CAG or CBh) to drive NBL10 expression
		Poor dissection, leading to missed target	Confirm dissection coordinates with the Paxinos Atlas[23]. Use larger input tissue pieces
		Loss of beads during post-IP washes	Collect all beads by rinsing out the old microcentrifuge tube with post-IP washes
	High background (poor enrichment)	Insufficient sequestering of free GFP	Ensure that 200 ng/ml rNB is added to homogenization buffer before homogenization
		Nonspecific expression of the NBL10 fusion	Ensure proper fidelity of the Cre-driver line being used (for example, see specificity of dopamine neuron–specific lines[26]) by crossing to a Rosa reporter mouse. If there is ectopic Cre expression, switch driver lines

● TIMING

Steps 1–14, stereotaxic injection: 1–2 h, plus virus incubation (~2 weeks each)
Steps 15–19, brain dissection: 30–60 min
Steps 20–32, GFP immunoprecipitations: 4–6 h
Steps 33 and 34, RNA purification: 60–75 min
Box 1, Preparation of input/total and unbound samples: 15 min

ANTICIPATED RESULTS

Retro-TRAP studies should result in the acquisition of projection-specific molecular profiling data. RNA yield will vary depending on the neural circuitry targeted. In our experience, for cell type–specific studies (for example, profiling a genetically defined cell type projecting to a defined anatomic output), for six pooled mice, we have observed total RNA yields as low as ~2 ng, and as high as ~50 ng in total. Total RNA yield should be at least 1 ng of total RNA (which is the optimal quantity for RNA-Seq studies). RNA quality, as assessed by an Agilent Bioanalyzer 2100, should have an RNA integrity number (RIN) range of 7–10. RIN values below 7 will probably result in poorer quality data because of RNA degradation. To assess the quality of qPCR data, it is important to confirm enrichment for GFP (note that enrichment is calculated as GFP IP/input), which should be maximally enriched in Retro-TRAP data sets (sometimes upward of 100-fold). If RNA quality and quantity are sufficient and GFP enrichment is high, then it is probable that the acquired data will yield high-quality results for identifying projection-specific marker genes using techniques such as qPCR and high-throughput RNA sequencing.

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