A Flow Cytometry-Based Method for Gene Expression Profiling of CNS Cell Types

by

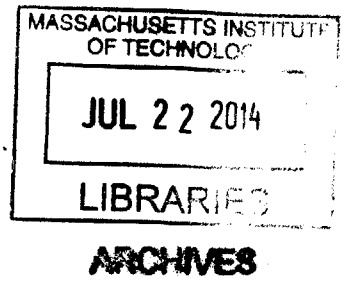
Tewei Luo

B.A. Biology
Pomona College, 2011

Submitted to the Department of Biology in partial fulfillment of the requirements
for the degree of

Master of Science in Biology
at the
Massachusetts Institute of Technology

September 2014



Signature redacted

Signature of Author:_____
Department of Biology
July 21, 2014

Certified by:_____ Signature redacted
Myriam Heiman
Assistant Professor of Neuroscience
Thesis Supervisor

Accepted by:_____ Signature redacted
Michael T. Hemann
Associate Professor of Biology
Co-chair, Biology Graduate Committee

A Flow Cytometry-Based Method for Gene Expression Profiling of CNS Cell Types

by

Tewei Luo

Submitted to the Department of Biology
On July 21, 2014 in partial fulfillment of the
Requirements for the degree of
Master of Science in Biology

ABSTRACT

In this thesis, I present a technique for profiling gene expression of specific cell types in the central nervous system (CNS), called fluorescence activated nuclei sorting (FANS). FANS utilizes flow cytometry to isolate cell nuclei from CNS subtypes and microarray analysis of nuclear mRNA. When compared to an existing technique, translating ribosome affinity purification, it was found that FANS was able to detect differentially expressed genes between two types of medium spiny neurons in the striatum with similar or higher sensitivity, using transgenic mice with fluorescence proteins labeling the desired cell types. Immunofluorescence experiments were also performed to label cell nuclei isolated from wild type mouse CNS for FANS analysis. It was found that our staining method successfully labeled neuronal nuclei using a NeuN antibody, but did not label enriched markers of medium spiny neurons. Further studies are needed to increase the signal-to-background ratio of these stainings, which would allow FANS to be applied to wild type animals.

Thesis Supervisor: Myriam Heiman
Title: Assistant Professor of Neuroscience

Acknowledgements

I would like to thank my advisor, Myriam Heiman, for her support and encouragement throughout this project. I would also like to thank members of the Heiman laboratory, especially Ruth Kulicke, for their advice and help.

Table of Contents

Introduction

The central nervous system (CNS) of higher animals is a highly complex system comprised of specialized types of cells. In order to understand how the CNS carries out its functions, it is important to study the characteristics of individual cell types and how these cells communicate with each other. Neuroscientists have been trying to identify cellular attributes in order to categorize the diversity of neurons for over a century[1]. These attributes include shape, electrophysiological properties, immunomarkers, and genetic markers[2]. Recently modern genetic technologies such as bacterial artificial chromosome (BAC) transgenics[3] enabled in-depth analysis of genetically defined cell types. Genome-wide gene expression profiling technologies have also been developed to uncover the fine-tuned molecular properties of CNS cell types. Here we discuss the biological questions that can be addressed by gene expression profiling of CNS cell types and the current profiling methodologies.

Molecular characterization of CNS cell types

The function of a type of neuron is characterized by many attributes, such as the connection it makes with other neurons, the type of neurotransmitters it secretes and the type of receptors on its cell surface for neuromodulators. In order to describe the identity of a cell type, it is necessary to include as many attributes as possible. Gene expression profiling of CNS cell types provides a powerful means for defining molecular attributes. For example, it was recently discovered that the G-protein couple receptor 6 (Gpr6) is enriched in the dopamine receptor D2 expressing medium spiny neurons (D2 MSNs) in the mouse striatum compared to dopamine receptor D1 expressing MSNs (D1 MSNs)[4]. These two types of MSNs are intermingled and morphological indistinguishable, both GABAergic (gamma-aminobutyric acid), and share many other properties[5]. On the other hand, these two groups of MSNs send their projections to different

structures and express distinct molecular markers, and Gpr6 was identified as a novel marker of the D2 MSNs. Furthermore it was found that lysophospholipid sphingosine 1-phosphate, the endogenous ligand of Gpr6[6], selectively increases dendritic calcium levels in D2 MSNs but not in D1 MSNs. This example demonstrates that gene expression profiling of CNS cell types can lead to the discovery of new functional characterization of neuronal sub-types. Below I list some examples of topics that can benefit from molecular profiling studies, review existing methods for gene expression profiling, and outline the aim of this thesis.

Generation and maintenance of CNS cell types

The development of the CNS and maintenance of cell identity require precisely timed signaling events and gene regulatory programs[7-11]. In order for differentiated neurons to maintain their role in functional circuits, they need to be responsive to internal and external cues such as gene expression programs elicited by growth factors and altered electrical activity[12-14]. Past research has revealed molecular determinants in the delineation of cell fate of many broad categories of cell types, such as dopaminergic neurons[15] and GABAergic neurons[16]. Studying the gene expression landscape of neuronal sub-types can further our understanding of how these cell types are generated and maintained. For example, in a gene expression profiling study it was found that the transcription factor early B-cell factor 1 (*Ebf1*) is enriched D1 MSNs compared to D2 MSNs[17]. It was hypothesized that *Ebf1* plays a role in the development of D1 MSNs and this ideas was tested by analyzing *Ebf1*-null mice and indeed a reduction of D1 MSN markers was detected.

CNS diseases

Some CNS diseases preferentially affect certain cell types. For instance striatal projection neurons are most severely damaged in Huntington's disease (HD)[18], and dopaminergic neurons in the substantia nigra are differentially lost in Parkinson's disease (PD)[19]. Studying gene expression profiles of impacted cell types not only aids in elucidating the molecular mechanism of CNS diseases, but also guides the design of treatment for these diseases. For example, a drug for treating PD, L-Dopa, could induce dyskinesia as a result of long term therapy[20]. This side effect could be attributed to the stimulation of D1 MSNs and not the D2 MSNs[21]. Therefore it is desirable to design new treatments that would mimic the effect of dopamine on D2 MSNs but not D1 MSNs. The genes differentially expressed between the two types of MSNs[4] provide potential targets for implementing this strategy.

Gene expression profiling methodologies

There are several available technologies for gene expression profiling of CNS cell types that are defined genetically or by immunomarkers. Laser-captured microdissection allows the isolation of immunostained samples as small as single cells, but produces RNA samples of low quantity and quality[22]. Fluorescence activated cell sorting (FACS) has been used to isolate enzymatically dissociated neurons from BAC transgenic animals, in which the target cell types express fluorescent proteins[17]. A drawback of this method is that sample preparation causes neuronal processes to be damaged and potentially results in RNA leakage and contamination. More recently a method called translating ribosome affinity purification (TRAP) was developed. TRAP utilizes BAC transgenic mice that express a ribosomal protein RPL10A fused to EGFP in the target cell types, and biochemically isolates translating mRNAs that are bound to the tagged ribosomes.

While the translating mRNA pool is a better representation of the proteome than total RNA, other gene expression features such as epigenetic signatures cannot be studied with TRAP.

Aim of this thesis

In this thesis, we present a new methodology for profiling gene expression of CNS cell types, which is called fluorescence activated nuclei sorting (FANS). Previously studies showed that mRNAs extracted from purified nuclei can be used for gene expression profiling[23-25], but have not been used for differential gene expression analysis in the mammalian CNS. We applied FANS to BAC transgenic animals expressing fluorescent nuclear markers in specific CNS cell types. The nuclei from target cell types were isolated using flow cytometry and mRNAs in these nuclei were extracted for gene expression profiling. We also demonstrate a potential extension of this method where immunostaining is used to mark cell type-specific nuclear epitopes in wild-type animals.

Materials and Methods

Translating ribosome affinity purification

TRAP experiments were performed according to published procedures[4,26]. Minor modifications were included to recover nuclei samples (see next section for details). The bacTRAP transgenic mouse lines CP73 (Drd1a/EGFP-L10a) and CP101 (Drd2/EGFP-L10a) were used in this study. All experimental mice were male, under 5 months old, and animals used in the same experiment were born within 1 week.

Nuclei isolation

Mice were sacrificed in a carbon dioxide chamber. Striata were quickly dissected and transferred to 1mL of lysis buffer (25mM KCl, 5mM $MgCl_2$, 2mM $CaCl_2$, 20mM beta-glycerophosphate, 250mM Sucrose, 0.1% Tween-20) supplemented with 150μM spermine, 500μM spermidine, 1:100 Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA) and 1:500 SUPERase• In (Life Technologies, Carlsbad, CA). Tissue samples were homogenized in 2mL glass Dounce homogenizers (Kimble Chase, Vineland, NJ) with 10 strokes using the type A pestle first, and then 10 strokes using the type B pestle. (If it was desired to recover nuclear sample from a TRAP experiment, tissue lysate was centrifuged at 500xg for 5 minutes at 4°C, and the supernatant was used as the input to subsequent TRAP experiment. Crude nuclear pellet was resuspended in 1mL of lysis buffer and use the suspension as the input for subsequent nuclei isolation.) Lysate or resuspended crude nuclear pellet was mixed with 1mL of 50% iodixanol (Sigma, St. Louis, MO, adjusted to 50% with 6x lysis buffer). The lysate-iodixanol mixture was loaded onto 0.32mL of 29% iodixanol in a 2.2mL ultracentrifuge tube (Beckman Coulter, Pasadena, CA). Samples were centrifuged at 10,000xg for 10 minutes at 4°C using a TLS-55 swinging bucket rotor in a Optima MAX-TL tabletop ultracentrifuge (Beckman Coulter, Pasadena, CA). Supernatant was carefully

removed and the pellet was resuspended in 0.1-0.5mL of lysis buffer to obtain purified nuclei. If it is desired to isolate subpopulations of nuclei using FACS at this stage, refer to the *flow cytometry* section.

Flow cytometry and RNA extraction

Nuclei samples were resuspended in lysis buffer with 15μM of 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA) at a density of 0.5-2 million nuclei/mL, and kept on ice for all subsequent procedures. A FACSAria III cell sorter (BD Biosciehces, Franklin Lakes, NJ) was use to collect fluorescently labeled nuclei with nozzle size of 70μm and drop drive frequency of 85kHz. For each FACS experiment, nuclei isolated from wild-type mice, or unstained controls were also prepared to measure the level of background fluorescence. DAPI signal was examined and only singlet nuclei were isolated. Nuclei were sorted into ice-cold buffer RLT (Qiagen, Hilden, Germany) at 1:1 of volume-to-volume ratio. In order to protect the integrity of RNA in the sorted samples, the collection tubes were kept on ice, and sometimes small amount of dry ice could be supplemented in the collection chamber for long sorting sessions. Upon completion, sorted samples were vortexed briefly and kept on ice. RNA extraction was performed using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) with DNase treatment.

Quality control, quantification, and amplification of RNA samples

The integrity of RNA samples were examined using a Bioanalyzer (Agilent Technologies, Santa Clara, CA) (Figure. 1). RNA samples with typical rRNA profiles and RNA integrity numbers (RIN) higher than 7 were used for further analysis. To quantify the amount of RNA in each sample, Ribogreen assays (Life Technologies, Carlsbad, CA) were performed according to the

manufacturer's instructions, using a GloMax 20/20 Luminometer (Promega, Madison, WI) to read the fluorescence intensities.

RNA amplification and cDNA synthesis were performed using Ovation RNA Amplification System V2 (Nugen, San Carlos, CA) with 10ng of input RNA for each sample. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the cDNA product. Purfied cDNA was fragmented and labeled for microarray analysis using the Encore Biotin Module (Nugen, San Carlos, CA) and then submitted to the BioMicro Center at the Massachusetts Institute of Technology for microarray analysis. Specifically, labeled cDNA was hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions, using the GeneChips Fluidics Station 450 (Affymetrix, Santa Clara, CA). Arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA).

Microarray data analysis

GeneChip CEL files were imported to Genespring GX 12.6 (Agilent Technologies, Santa Clara, CA) and summarized using the GC-RMA method, while probe sets with expression values in the lower 20th percentile were filtered. To determine differentially expressed genes, moderated t-test was performed and p-values are adjusted for multiple testing correction using the Benjamini-Hochberg method. We report a probe set as differentially expressed if the fold difference is greater than 1.5 and the corrected p-value is less than 0.01. Gene ontology analysis was performed with a p-value cut-off of 0.01.

Quantitative PCR

TaqMan (Applied Biosystems, Foster City, CA) pre-designed gene expression assays were performed according to the manufacturer's instructions, with 20ng of amplified cDNA for each

sample and using a LightCycler 480 System (Roche, Basel, Switzerland). The assays include *Eya1*: Mm00438796_m1 and Drd2: Mm00438541_m1, and *Actb*: Hs99999903_m1. Each assay was performed using four technical replicates. Relative expression levels were calculated using the comparative C_t value method (implemented with the Roche Lightcycler 480 Software) with *Actb* as reference amplification.

Immunostaining of isolated nuclei

Lysis buffer was supplemented with 1% (weight/volume) paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) just before use. Tissue was homogenized as described before, and then transferred to a 1.5mL siliconized microcentrifuge tube (Bio Plas, San Rafael, CA). Lysate was incubated at room temperature with end-over-end rotation for 8 minutes. Glycine was added to the lysate to a final concentration of 125mM and incubated for 5 minutes at room temperature with rotation to quench unreacted paraformaldehyde. Cross-linked lysate was immediately placed on ice, and then mixed with equal volume of 50% iodixanol. Nuclei were purified following the same procedures described before, and purified nuclei were resuspended in lysis buffer (without paraformaldehyde) not exceeding 0.3mL of volume.

Methanol was used to permeabilize nuclei samples for better epitope accessibility. Pure methanol (Sigma, St. Louis, MO) was chilled in an ethanol-dry ice bath, and then added to nuclei suspension drop by drop, to a final methanol concentration of 70% (volume/volume). Samples were incubated at 4°C for 10 minutes with end-over-end rotation and then centrifuged at 1000xg for 5 minutes at 4°C. Supernatant was removed such that there was 100μL of buffer remaining. From this point nuclei samples were processed in staining buffer, which consists of lysis buffer supplemented with 1% (weight/volume) bovine serum albumin (Jackson ImmunoResearch, West Grove, PA) and 5% (volume/volume) normal goat serum (Abcam, Cambridge, England). To

rehydrate the nuclei, 40μL, 93μL, and 467μL of staining buffer were added sequentially to the samples, which were allowed to sit on ice for 1 minute between each addition of buffer. Rehydrated samples were centrifuged at 700xg for 5 minutes at 4°C, resuspended in staining buffer, and incubated for 10 minutes at 4°C with rotation to block non-specific interactions.

Primary antibodies were diluted in staining buffer and then added to nuclei samples at desired final concentrations. (Refer to Table1 1 for a list of primary antibodies used in this study.) Normal IgG (Cell Signaling Technology, Inc. Beverly, MA) purified from the same species as primary antibodies was diluted at the same concentrations. Samples were incubated either at room temperature for 1 hour, or at 4°C overnight, both with rotation. (Staining efficacy of these two conditions may vary for different antibodies.) After primary antibody incubation, samples were centrifuged at 700xg for 5 minutes at 4°C, and the pellet was washed once in staining buffer. Fluorescent secondary antibodies (Life Technologies, Carlsbad, CA) were diluted to 2μg/mL in staining buffer, added to nuclei samples, and incubated for 30 minutes at 4°C with rotation. Samples were then centrifuged and washed as above, and resuspended in lysis buffer with 15μM DAPI for FACS.

Immunostaining with tyramide signal amplification (TSA)

In this study, nuclei staining experiments with TSA also included staining for NeuN, which was carried out without amplification. To perform TSA, we followed the same procedures in the previous section to label NeuN with fluorescent secondary antibodies. Next, NeuN stained nuclei were washed once in staining buffer, and then incubated with 100uL of ImmPRESS anti-rabbit IgG peroxidase (Vector Laboratories Ltd, Peterborough, UK) for 30 minutes at 4°C with rotation. After incubation with peroxidase-conjugated secondary antibody, the nuclei were washed once with amplification buffer from TSA Kits (Life Technologies, Carlsbad, CA). A tyramide working

solution was prepared with 0.0015% hydrogen peroxide and tyramide-conjugated Alexa Fluor dye diluted at 1:100 in amplification buffer, and the dye was reconstituted in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. Nuclei were incubated with tyramide working solution at 4°C, and the incubation duration (typically 5-15 minutes) needs to be determined for each primary antibody. Samples were then washed once and resuspended in lysis buffer with 15μM DAPI for FACS analysis.

Results

Quality of FANS-derived RNA

It is important that RNA samples extracted from FANS nuclei retain adequate integrity for gene expression profiling. It was found that by using RNase inhibitors and keeping samples at 4°C as much as possible (see Materials and Methods), RNA samples of good quality can be produced consistently. Specifically, samples were examined using a Bioanalyzer and their RIN was determined (Figure 1). It was found that nuclear RNA samples of RIN greater than 7 could be reliably generated using the procedures outlined in the methods section. Therefore FANS derived RNA samples are suitable for most qPCR, microarray, and RNA-sequencing sample preparation technologies.

TRAP and FANS profiling of D1- and D2-MSN using quantitative PCR

To demonstrate that cell type specific nuclear RNA derived from FANS can be applied to gene expression profiling, we used FANS to study cell types that are well characterized in the literature. In particular, we focused on the D1 and D2 dopamine receptor-expression MSNs in the mouse striatum. These cell types are difficult to separate by mechanical means and special techniques have been developed to characterize the gene expression profiles of them[4,17]. We designed an experiment where TRAP and FANS could be carried out using the same tissue samples. Specifically, the cytosolic fraction of total tissue lysate was subjected to biochemical purifications in TRAP, while the nuclear fraction was further purified for FANS. The transgenic mice used in this experiment express the ribosomal protein RPL10A fused with EGFP in either D1 or D2 MSNs. RPL10A is abundant in the nucleolus where ribosomes are assembled and this results in sufficient fluorescent intensity in the nucleus for isolating EGFP-RPL10A positive nuclei with flow cytometry. Indeed, we were able to detect EGFP-positive populations when analyzing

bacTRAP animal derived nuclei (Figure 2). We used quantitative PCR to measure the abundance of the mRNA of *Eya1* and *Drd2*, which are known to be enriched in D1 and D2 MSNs, respectively[4]. Expression levels of *Eya1* and *Drd2* in FANS RNA extracted from D1 and D2 MSNs agree with their TRAP counterparts and with previous findings (Figure 3). We observed that FANS expression values exhibit smaller variations among biological replicates.

TRAP and FANS profiling of D1- and D2-MSN using microarray

Given results from quantitative PCR experiments described above, we asked whether FANS RNA can be used for unbiased transcriptomic profiling. We used the Affymetrix GeneChip Mouse Genome 430 2.0 technology on both TRAP and FANS RNA samples extracted from D1 and D2 MSNs. Comparative analysis revealed that many previously characterized MSN markers were differentially expression in both TRAP and FANS samples. Some of these genes including D1 MSN markers eyes absent homolog 1 (*Eya1*) (88.5x), Early B-cell factor 1 (*Ebf1*) (29.0x), dynorphin (*Pdny*) (18.6x), ISL LIM homeobox 1 (*Isl1*) (11.7x), dopamine receptor D1A (*Drd1a*) (3.78x), and substance P (*Tac1*) (2.9x), and D2 MSN markers such as dopamine receptor D2(*Drd2*) (35.3x), adenosine 2a receptor (*Adora2a*) (22.6x), enkephalin (*Penk*) (3.41x), LIM homeobox 8 (*Lhx8*) (4.65x), and G protein-coupled receptor 6 (*Gpr6*) (13.9x), where the values in parentheses represent fold difference versus D2 or D1, respectively, using FANS.

Similar to what we observed in the quantitative PCR experiments, expression values measured using FANS RNA exhibit smaller variation and thus lend stronger statistical power in this microarray analysis. In particular, for almost all combinations of p-value and fold-change cutoffs, analysis of FANS RNA yielded more differentially expressed probe sets (Table 2).

We also investigated the difference in transcript composition between TRAP and FANS RNA. Since these RNA samples were collected from distinct cellular compartments, we expected

the difference in composition to be significant. Indeed, over half of all probe sets on the Mouse Genome 430 2.0 array exhibited fold difference greater than 1.5. We also observed that most probe sets with large fold difference are enriched in FANS RNA samples. We performed gene ontology (GO) analysis on probe sets that are enriched in FANS RNA with fold difference greater than 30. We found that the nucleus is among the top three cellular component GO terms, while nucleoplasm, nuclear lumen and nuclear speck were identified as significantly enriched terms as well. Many nucleus associated processes were also found in enriched biological process GO terms, including mRNA processing, RNA splicing, and nuclear export.

Immunostaining of isolated nuclei

The data discussed above demonstrated that FANS could reliably produce gene expression profiling results that agree with published studies. This method could potentially be extended by using immunostaining to label specific cell types derived from wild-type animals, circumventing the process of generating transgenic animals and making this approach accessible to more laboratories that are interested in related studies. This idea was explored in this thesis and preliminary results are presented.

First we developed an antibody staining protocol based on past studies. In particular, we tested different permeabilization reagents, including Tween-20, Triton X-100, methanol, and acetone/methanol (1:1, vol/vol). Some of these reagents can also be used as fixatives and we also tested different fixation conditions, including using PFA with detergent, PFA with methanol, and methanol alone. We found that using methanol to permeabilize nuclear samples resulted in better access to nuclear antigens, and when used with PFA fixation, gave cleaner staining signal. Meanwhile we modified existing methanol permeabilization protocols to reduce aggregation of nuclei in order to improve the yield of singlet nuclei for FACS. We also tested conditions for

antibody incubation and found that for primary antibodies overnight incubation at 4°C and 1-hour incubation at room temperature usually both gave satisfactory staining results. However the optimal condition may depend on specific antibodies. We used this protocol to stain purified nuclei for NeuN, which is a neuron-specific nuclear protein[27]. Our protocol reliably labeled neuronal nuclei with low non-specific staining (Figure 4). Next we selected a number of nuclear proteins (as indicated by their cellular component ontology term from the Gene Ontology Consortium) with D1 or D2 MSN specific expression pattern as identified in microarray analysis using both TRAP and FANS RNA (Table 1). When using normal IgG to measure background staining, we were able to detect staining signal that is significantly above background among neuronal nuclei, which are defined as NeuN immunoreactive nuclei (Figure 5). However the immunofluorescence of the target proteins exhibited a unimodal distribution and therefore we were not able to separate nuclei of D1 MSNs from that of D2 MSNs, or vice versa, in these staining experiments.

One of the possible reasons for the lack of positive staining in the above experiments is that the amount of available epitopes does not yield detectable signal. While there are several signal amplification technologies available, they require that the epitope be in contact with the incubation solution. We reasoned that since the rough endoplasmic reticulum (RER) is continuous with the nuclear envelope[28], proteins that are integral in the RER membrane will also be present on the surface of purified nuclei. Since most plasma membrane proteins are translated on and temporarily anchored to the RER membrane[28], some membrane proteins would be present on purified nuclei in full or partial length. In particular, when anchored to the RER, the cytoplasmic domain of these proteins would be in direct contact with surrounding solution. These proteins could potentially be visualized by the tyramide signal amplification (TSA) strategy, which uses a peroxidase conjugated secondary antibody and deposits dyes in the vicinity of the peroxidases

through oxidization[29]. To test this idea, we applied a commercial TSA kit to amplify signals from primary antibodies targeting epitopes on the cytoplasmic domain of the D1 and D2 dopamine receptors. We varied several parameters in the staining procedure, including the concentration of tyramide and incubation time. Under the conditions tested in this study, we were not able to detect fluorescence signal that is above background for the dopamine receptors. We observed that when primary antibody was absent from the staining procedure, the fluorescence intensity was still comparable to that using primary antibodies. On the other hand, if the secondary antibody was removed from the staining, the fluorescent signal was the same as non-treated nuclei.

Discussion

Effectiveness of FANS in gene expression profiling

In this study we compared FANS with a published method, TRAP, for profiling the same CNS cell types. While the two methods assess different mRNA populations within the target cell types, namely nuclear mRNAs in FANS and translated mRNAs in TRAP, many genes were found to be differentially expressed in both assays. This observation further supports the cell type specific expression pattern of these genes, and the validity of using nuclear mRNA for the purpose of gene expression quantification. Although nuclear mRNA profiling has been applied in *Arabidopsis*[25], *C. elegans*[24], and *Drosophila*[23], our study is the first to establish its utility in studying the mammalian CNS to our knowledge. In addition FANS is relatively simple to implement with the entire procedure being completed in one day, and does not require expensive affinity purification reagents as in TRAP. FANS also provides better yield of RNA per animal, which ranges between 10 to 100ng per mouse striatum, in contrast to the yield of 1-3ng per striatum using FACSarray[17]. The RNA yield of FANS allows using single animals as replicates and effectively reduces the cost of these experiments. Compared to using enzymatic dissociation which often damages neurons and causes RNA leakage and contamination[30], purified nuclei usually retain intact nuclear membrane and reduce the chance of RNA leakage.

Compared to TRAP RNA, FANS RNA is enriched in transcripts encoding proteins with nuclear functions. This observation suggests a mechanism in which some nuclear associated structures facilitate the local translation of these transcripts. Therefore caution should be used when interpreting differential expression of nuclear protein encoding genes with FANS RNA, as difference in expression can be caused by a cellular process that affects the association of these transcripts with the nucleus. On the other hand, FANS collects non-coding RNAs that are not exported for translation while TRAP cannot capture these transcripts. Therefore, depending on the

RNA species to be investigated, the two methods should be chosen accordingly. If both nuclear transcripts and the translatome are of interest, TRAP and FANS can be carried out in parallel as described in this study.

To further test the utility of FANS, we plan to study gene expression changes across different conditions, such as drug treatment and aging. Another potential application of FANS is to study heterogeneity within cell-types or *de novo* cell type identification using single neuron isolation combined with sequencing technologies[31,32].

In summary FANS offers an alternative technique for gene expression profiling of CNS cell types which involves relatively simple procedures, generates satisfactory RNA yield, and has stronger statistical power in comparative expression analyses.

Effectiveness of nuclei immunostaining

We attempted to use immunofluorescence to label cell-type specific nuclei as a natural extension of the FANS experiments using bacTRAP animals. In all staining experiments we were not able to separate the D1 and D2 MSN nuclear populations. We could use several strategies to address this issue in the future. First, the lack of signal could result from non-specific binding of primary antibodies and that the blocking method we are current using is not sufficient. A stronger blocking strategy would be to use normal IgG as blocking reagent, and directly conjugating primary antibodies with secondary antibodies. This method may result in weaker staining signal, but could potentially reduce the level of background significantly. An alternative strategy is to use a combination of antibodies for targeting multiple cell type markers and purify nuclei that are positively stained for all markers.

We have also attempted using TSA to stain membrane proteins that are potentially present on the RER, and did not achieve positive staining signals. We observe that even when primary

antibody incubation is absent from the staining procedure, the fluorescence intensity is comparable to that using primary antibodies. This suggest that better blocking strategy is required. We also found that the duration of incubation with tyramide reagent affects staining intensity and therefore should be tested for any new TSA experiment.

In summary, using immunofluorescence as cell type marker for FANS would enable cell type specific gene expression studies to be conducted without the generation of transgenic animals and the use of postmortem human CNS tissues. However, technical issues need to be addressed for this strategy to work properly.

References

1 Ramón y Cajal, S. *Histology of the nervous system of man and vertebrates*. (Oxford University Press, 1995).

2 Fishell, G. & Heintz, N. The neuron identity problem: form meets function. *Neuron* **80**, 602-612, doi:10.1016/j.neuron.2013.10.035 (2013).

3 Heintz, N. BAC to the future: the use of bac transgenic mice for neuroscience research. *Nature reviews. Neuroscience* **2**, 861-870, doi:10.1038/35104049 (2001).

4 Heiman, M. *et al.* A translational profiling approach for the molecular characterization of CNS cell types. *Cell* **135**, 738-748, doi:10.1016/j.cell.2008.10.028 (2008).

5 Durieux, P. F., Schiffmann, S. N. & de Kerchove d'Exaerde, A. Targeting neuronal populations of the striatum. *Frontiers in neuroanatomy* **5**, 40, doi:10.3389/fnana.2011.00040 (2011).

6 Ignatov, A., Lintzel, J., Kreienkamp, H. J. & Schaller, H. C. Sphingosine-1-phosphate is a high-affinity ligand for the G protein-coupled receptor GPR6 from mouse and induces intracellular Ca2+ release by activating the sphingosine-kinase pathway. *Biochemical and biophysical research communications* **311**, 329-336 (2003).

7 Temple, S. The development of neural stem cells. *Nature* **414**, 112-117, doi:10.1038/35102174 (2001).

8 Maden, M. Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nature reviews. Neuroscience* **8**, 755-765, doi:10.1038/nrn2212 (2007).

9 Lee, K. J. & Jessell, T. M. The specification of dorsal cell fates in the vertebrate central nervous system. *Annual review of neuroscience* **22**, 261-294, doi:10.1146/annurev.neuro.22.1.261 (1999).

10 Ooi, L. & Wood, I. C. Regulation of gene expression in the nervous system. *The Biochemical journal* **414**, 327-341, doi:10.1042/BJ20080963 (2008).

11 Lopes, R., Verhey van Wijk, N., Neves, G. & Pachnis, V. Transcription factor LIM homeobox 7 (Lhx7) maintains subtype identity of cholinergic interneurons in the mammalian striatum. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3119-3124, doi:10.1073/pnas.1109251109 (2012).

12 Curran, T. & Morgan, J. I. Superinduction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepines. *Science* **229**, 1265-1268 (1985).

13 Cohen, S. & Greenberg, M. E. Communication between the synapse and the nucleus in neuronal development, plasticity, and disease. *Annual review of cell and developmental biology* **24**, 183-209, doi:10.1146/annurev.cellbio.24.110707.175235 (2008).

14 Greenberg, M. E., Greene, L. A. & Ziff, E. B. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *The Journal of biological chemistry* **260**, 14101-14110 (1985).

15 Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. & Rosenthal, A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766 (1998).

16 Dullin, J. P. *et al.* Ptf1a triggers GABAergic neuronal cell fates in the retina. *BMC developmental biology* **7**, 110, doi:10.1186/1471-213X-7-110 (2007).

17 Lobo, M. K., Karsten, S. L., Gray, M., Geschwind, D. H. & Yang, X. W. FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains. *Nature neuroscience* **9**, 443-452, doi:10.1038/nn1654 (2006).

18 Han, I., You, Y., Kordower, J. H., Brady, S. T. & Morfini, G. A. Differential vulnerability of neurons in Huntington's disease: the role of cell type-specific features. *Journal of neurochemistry* **113**, 1073-1091, doi:10.1111/j.1471-4159.2010.06672.x (2010).

19 Shulman, J. M., De Jager, P. L. & Feany, M. B. Parkinson's disease: genetics and pathogenesis. *Annual review of pathology* **6**, 193-222, doi:10.1146/annurev-pathol-011110-130242 (2011).

20 Porras, G. *et al.* L-dopa-induced dyskinesia: beyond an excessive dopamine tone in the striatum. *Scientific reports* **4**, 3730, doi:10.1038/srep03730 (2014).

21 Kalia, S. K., Sankar, T. & Lozano, A. M. Deep brain stimulation for Parkinson's disease and other movement disorders. *Current opinion in neurology* **26**, 374-380, doi:10.1097/WCO.0b013e3283632d08 (2013).

22 Luo, L. *et al.* Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nature medicine* **5**, 117-122, doi:10.1038/4806 (1999).

23 Henry, G. L., Davis, F. P., Picard, S. & Eddy, S. R. Cell type-specific genomics of Drosophila neurons. *Nucleic acids research* **40**, 9691-9704, doi:10.1093/nar/gks671 (2012).

24 Steiner, F. A., Talbert, P. B., Kasinathan, S., Deal, R. B. & Henikoff, S. Cell-type-specific nuclei purification from whole animals for genome-wide expression and chromatin profiling. *Genome research* **22**, 766-777, doi:10.1101/gr.131748.111 (2012).

25 Zhang, C., Barthelson, R. A., Lambert, G. M. & Galbraith, D. W. Global characterization of cell-specific gene expression through fluorescence-activated sorting of nuclei. *Plant physiology* **147**, 30-40, doi:10.1104/pp.107.115246 (2008).

26 Heiman, M., Kulicke, R., Fenster, R. J., Greengard, P. & Heintz, N. Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP). *Nature protocols* **9**, 1282-1291, doi:10.1038/nprot.2014.085 (2014).

27 Mullen, R. J., Buck, C. R. & Smith, A. M. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* **116**, 201-211 (1992).

28 Alberts, B., Wilson, J. H. & Hunt, T. *Molecular biology of the cell.* 5th edn, (Garland Science, 2008).

29 Clutter, M. R., Heffner, G. C., Krutzik, P. O., Sachen, K. L. & Nolan, G. P. Tyramide signal amplification for analysis of kinase activity by intracellular flow cytometry. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **77**, 1020-1031, doi:10.1002/cyto.a.20970 (2010).

30 Saxena, A. *et al.* Trehalose-enhanced isolation of neuronal sub-types from adult mouse brain. *BioTechniques* **52**, 381-385, doi:10.2144/0000113878 (2012).

31 Grindberg, R. V. *et al.* RNA-sequencing from single nuclei. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 19802-19807, doi:10.1073/pnas.1319700110 (2013).

32 Jaitin, D. A. *et al.* Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science* **343**, 776-779, doi:10.1126/science.1247651 (2014).

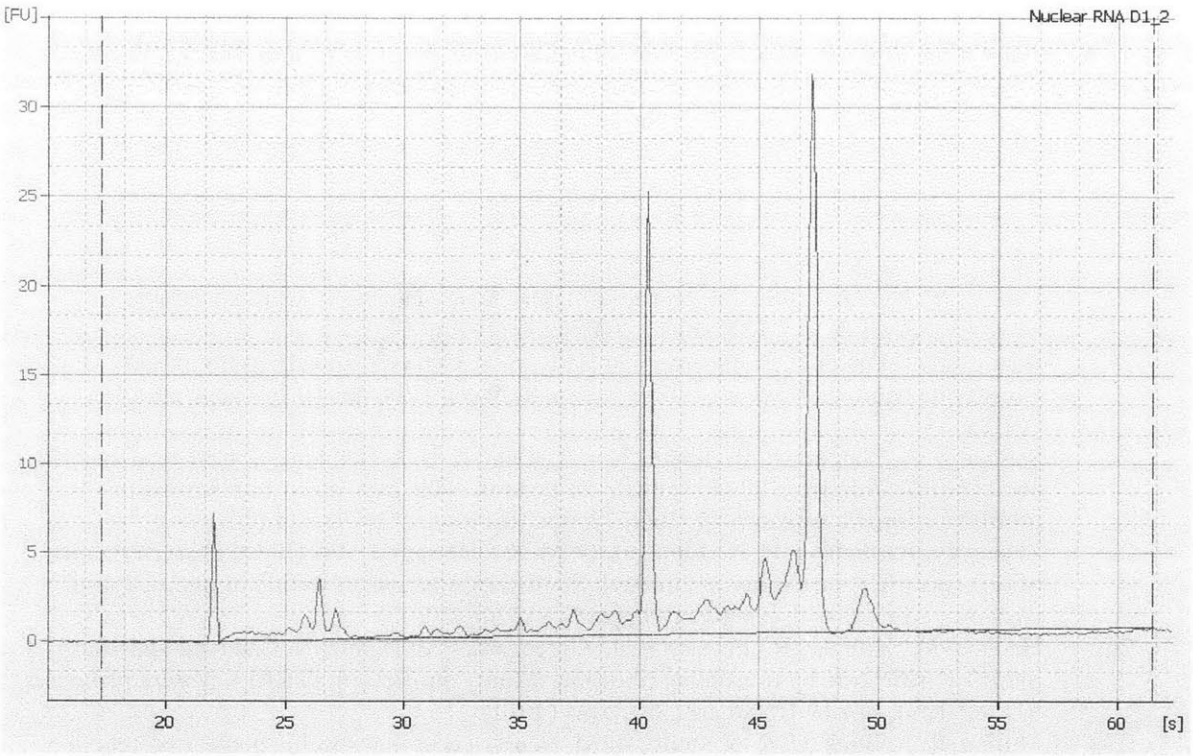
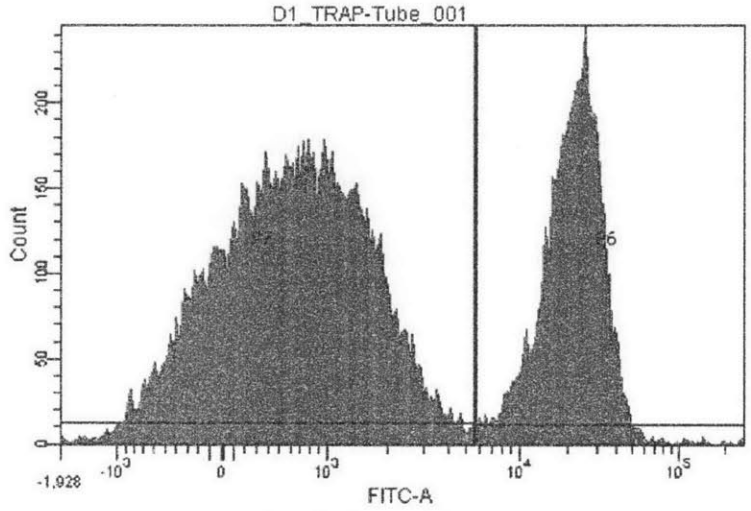
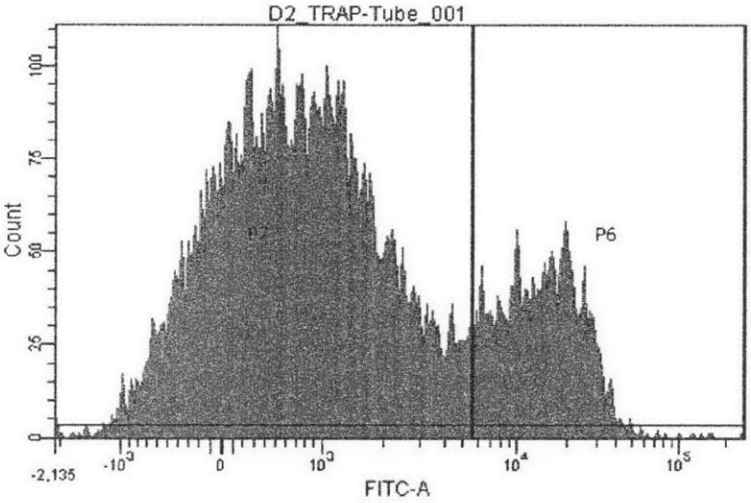


Figure 1 Electropherogram of FANS derived nuclear RNA in an RNA 6000 Pico Kit assay (Agilent Technologies, Santa Clara, CA). The x-axis represents the size of RNA fragments as measured by the run-time compared to a reference probe. The y-axis represents the abundance of RNA species for each size. The peaks at run-times 40sec and 47sec correspond to 18S and 28S ribosomal RNAs, respectively. The RNA integrity number for this sample is 8.4.

A


D1_TRAP-Tube_001
Count
FITC-A
P7
P6
-1,928

B


D2_TRAP-Tube_001
Count
FITC-A
P7
P6
-2,135

C

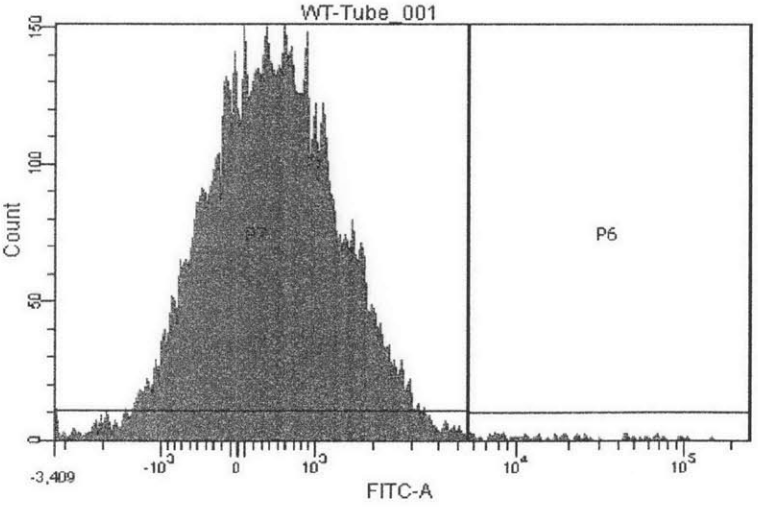

WT-Tube_001
Count
FITC-A
P7
P6
-3,409

Figure 2 FACS separation of EGFP+ nuclei in bacTRAP animals expressing EGFP-L10A in D1 MSNs (A) and D2 MSNs (B). Gating threshold was set using striatal nuclei extracted from wild-type animals (C). Values on the x-axis represent relative fluorescence intensity in the GFP channel, which uses a 488nm laser and a 530/30nm band pass filter. Values on the y-axis represent the number of nuclei corresponding to a particular fluorescence intensity. The vertical bar represents the threshold for gating EGFP+ events. Nuclei with fluorescence greater than this threshold were collected.

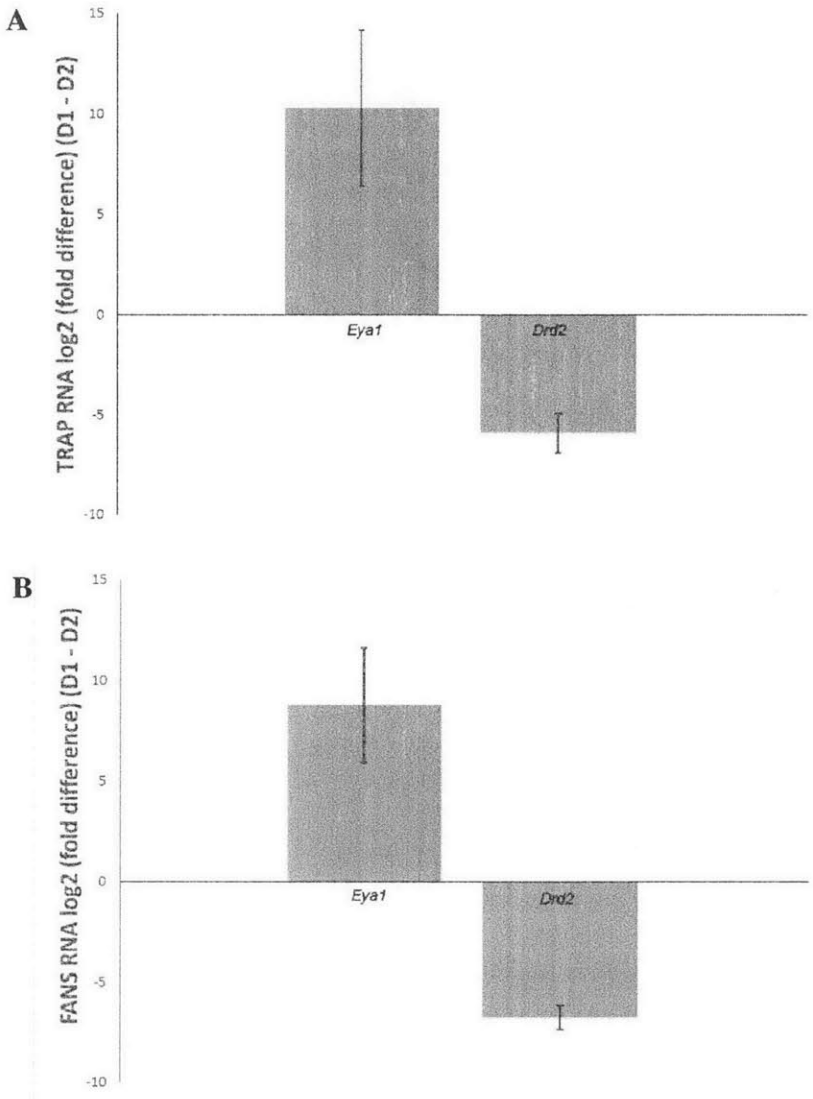


Figure 3 Relative mRNA expression levels using RNAs extracted with TRAP (A) and FANS (B) measured by quantitative PCR. Error bars represent standard deviation (STDEV). STDEV(*Eya1*) = 3.91, STDEV(*Drd2*)= 0.99 in (A). STDEV(*Eya1*) = 2.86, STDEV(*Drd2*)= 0.59 in (B). n=3 animals for each experimental group.

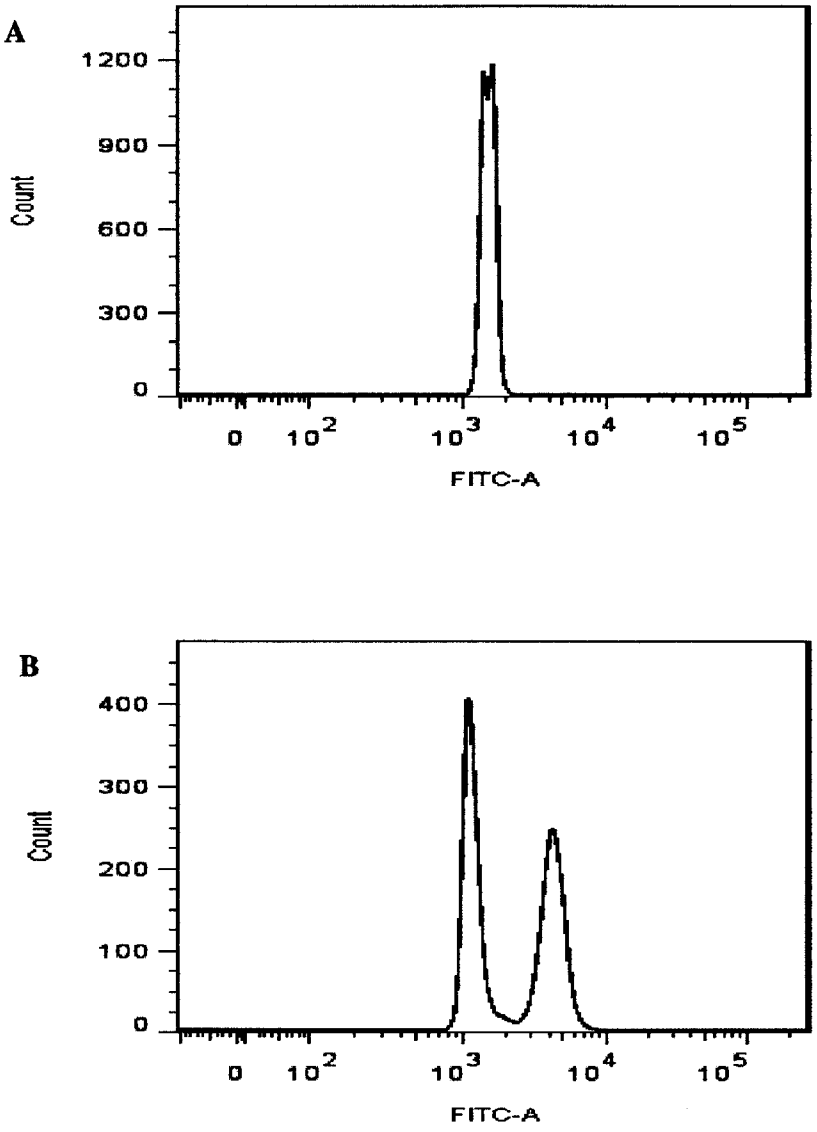


Figure 4 NeuN immunofluorescence of purified striatal nuclei analyzed by FACS. Primary NeuN antibody (Milipore, MAB377) (B) and normal mouse IgG (A) were used. Alexa Fluor 488 conjugated donkey anti-mouse IgG was used as secondary antibody. 6378 events were recorded in both conditions.

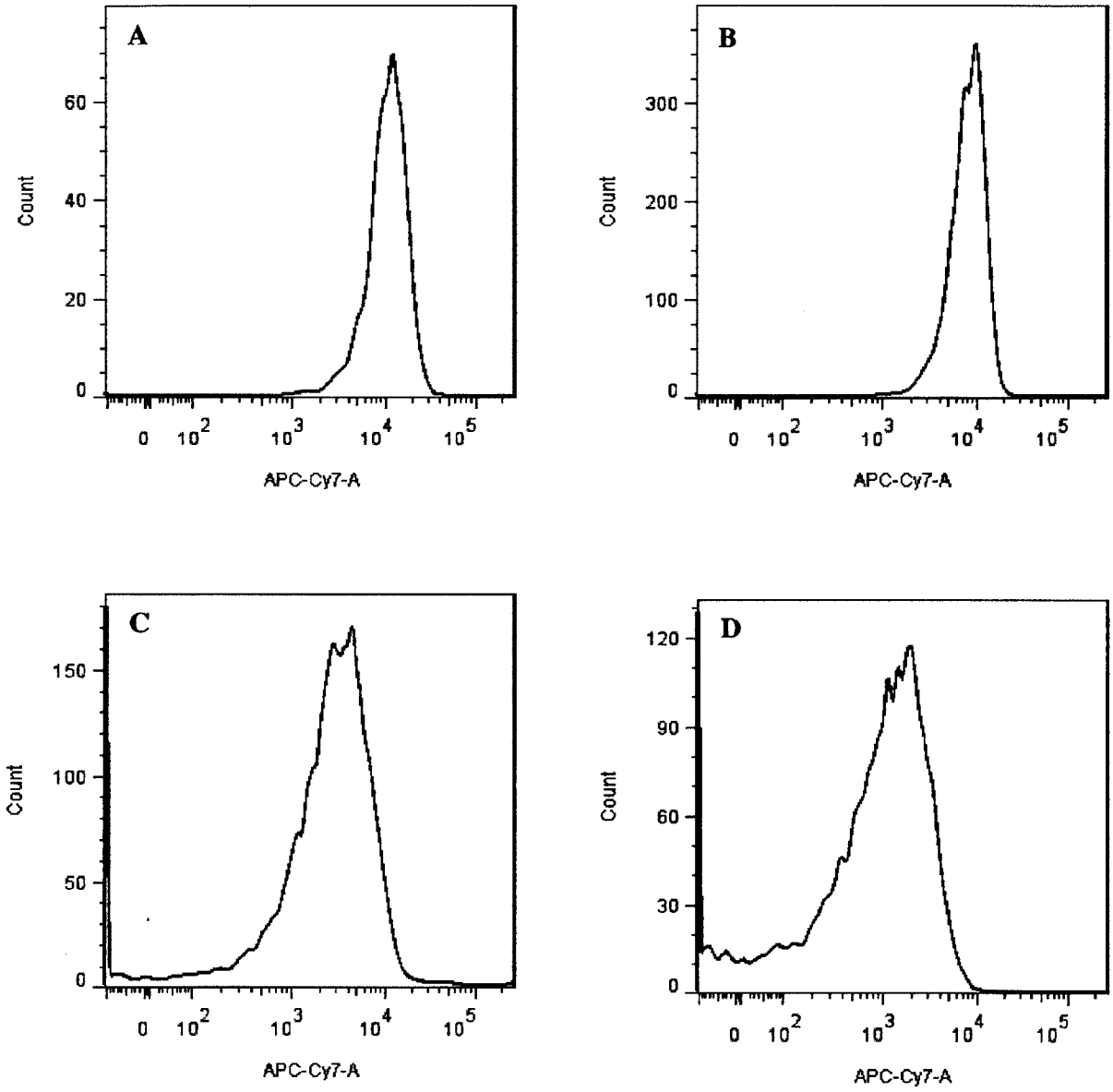


Figure 5 FACS analysis of immunostained striatal neuronal nuclei using antibodies against Eya1(A) and Lhx8(B). Background staining was measured using normal IgG raised in the host species in place of primary antibodies. The lower panels demonstrates background fluorescence in Eya1 staining (C) and Lhx8 staining (D). Staining of other nuclear antigens in Table 1 showed similar results.

Target protein	Type of antibody	Cell type specificity	Manufacturer	Product number	Antigen cellular localization
Eya1	Rabbit polyclonal	D1 MSN	Abcam	ab85009	Nucleus
Isl1	Mouse monoclonal	D1 MSN	Abcam	ab86472	Nucleus
Foxp2	Rabbit monoclonal	D1 MSN	Cell Signaling Technologies	#5337	Nucleus
Nr4a	Mouse monoclonal	D1 MSN	Abcam	ab41917	Nucleus
Lhx8	Rabbit polyclonal	D2 MSN	Abcam	ab41519	Nucleus
Zic1	Rabbit monoclonal	D2 MSN	Abcam	ab134951	Nucleus
Drd1a	Rabbit polyclonal	D1 MSN	Millipore	AB9141	Plasma membrane
Drd2	Rabbit polyclonal	D2 MSN	Millipore	AB1784P	Plasma membrane
NeuN	Mouse monoclonal	Pan-neuronal	Millipore	MAB377	Nucleus
NeuN	Rabbit monoclonal	Pan-neuronal	Cell Signaling Technologies	#12943	Nucleus

Table 1 Primary antibodies used in this study.

A

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.0050	P < 0.0010
FC all	39021	1959	1009	632	436	213
FC > 1.1	18687	1959	1009	632	436	213
FC > 1.5	2303	1082	735	523	391	207
FC > 2.0	550	393	320	263	225	139
FC > 3.0	114	93	87	85	82	64

B

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.0050	P < 0.0010
FC all	38126	2065	1217	862	646	319
FC > 1.1	18582	2065	1217	862	646	319
FC > 1.5	2371	1265	924	724	576	316
FC > 2.0	623	500	437	378	336	236
FC > 3.0	179	170	164	153	144	122

Table 2 Number of probe sets that exhibit differential expression between D1 and D2 MSNs at different statistical significance levels and fold change cutoffs, using TRAP (A) or FANS (B) RNA. The input for this analysis are probe set filtered by expression values so that the lower 20^{th} percentile were removed, and the resulting numbers of input probe sets are 39021 (A) and 38126 (B). The p-values were computed in a moderated T-test with multiple testing correction by the Benjamini Hochberg method.