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APPLICATION NOTE #7

Laser Capture Microdissection (LCM) and Expression Profiling of Long-Range Projection Neurons

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Abstract

Brain tissue is notoriously heterogeneous, and accurate analysis of neural gene expression requires procurement of specific cell types. In this application note a technique is described for using Laser Capture Microdissection (LCM) and in-vivo labeling to obtain purified populations of projection neurons from the discrete brain region HVC of the songbird *Taeniopygia guttata*. HVC contains two types of projection neurons connecting to two distant brain regions, the Robust nucleus of the Archistriatum (nucleus RA) and Area X of the paraolfactory lobe. The cell bodies of these HVC projection neurons sit at the apex of a neural circuit controlling the learning and production of birdsong, offering insight into how a defined neural circuit relates to a learned behavior (Nottebohm et al., 1982). Despite this complex neuronal architecture, in-vivo labeling enabled the laser capture of two distinct interspersed cell types. Differential gene expression between these cell types was then assessed by directly comparing the amplified RNA obtained from these neurons on cDNA microarrays. Genes over-expressed in each cell type were identified on the microarrays, and confirmation of the true expression patterns of these genes was shown by in situ hybridization and Taqman quantitative RT-PCR. Herein we demonstrate how the combination of LCM, RNA amplification and gene expression assays helps to define cell types within identified neural circuits as well as gives molecular information about these circuit elements, both important goals in neuroscience research.

Introduction

The brain is a highly heterogeneous organ, with a wide variety of cell types identifiable by location, morphology, physiology, and connectivity. Connectivity, or a cell's place in a neural circuit, is an important defining trait for neurons, as it is central to the precise circuitry that underlies brain function. Retrograde transport of fluorescent molecules has become a standard method to delineate neural circuits and their component neurons, and new connections between known brain areas continue to be discovered with its use (Kobbert et al., 2000). Here we describe a method that uses fluorescent neuronal tracers as markers for microdissecting cell bodies by LCM (Lombardino et al., 2005). The RNA purified from these captured cells was amplified using RiboAmp® HS and applied to a cDNA microarray made from songbird brain. Differentially expressed genes were discovered from each of two interdigitating projection neuron types, and the results were verified by two other gene expression techniques. This suite of techniques has permitted the molecular characterization of identified neurons as they exist within neural circuits, both in terms of their constitutive gene expression and the regulated transcription of genes induced by behavior.

Equipment and Reagents

I. STEREOTAXIC INJECTION OF TRACERS

- Stereotaxic apparatus for the animal subject (varies depending on species used; e.g. David Kopf Instruments)
- Anesthesia: Ketalar™ (Monarch Pharmaceuticals), Rompun™ (Bayer)
- Cholera toxin conjugated to Alexa-fluor dyes (Molecular Probes, Cat. #C-22841, C-22842)
- Glass micropipettes (Drummond Wiretrol, 1-5 uL Cat. #5-000-1001)
- Microinjection apparatus (Narishige PE-2)

II. FREEZING BRAIN AND CRYOSECTIONING

- Tissue embedding molds (ThermoShandon, Cat. #2219)
- Optimal cutting temperature (OCT) compound (Tissue-Tek, Cat. #4583)
- RNAlater (Sigma cat. #R0901)
- Precleaned glass slides (Becton Dickinson and Co., Cat. #3050)

III. LASER CAPTURE MICRODISSECTION

- Pixcell IIe® with fluorescence (Arcturus, Cat. #LCM1104)
- Capsure® HS LCM caps (Arcturus, Cat. #LCM0214)

IV. RNA PURIFICATION, AMPLIFICATION, ASSESSMENT OF QUALITY/YIELD

- PicoPure® RNA Isolation Kit (Arcturus, Cat. #KIT0204)
- RNase-free DNase Set (Qiagen, Cat. #79254)
- RiboAmp® HS RNA Amplification Kit (Arcturus, Cat. #KIT0205)
- Bioanalyzer 2100 (Agilent Technologies)
- NanoDrop® ND-1000 Spectrophotometer

V. TWO-COLOR cDNA MICROARRAY LABELING, HYBRIDIZATION

- Glass cDNA microarrays for the species of interest (made in-house, printed on Corning slides and hybridized using the Corning Pronto protocol)
- Corning Pronto Plus Microarray System (Promega Cat. #40056)
- All other reagents are identical to Arcturus Protocol #3. Visit www.arctur.com for the complete Protocol #3.

VI. IN SITU HYBRIDIZATION

- Gene-specific probes
- pCRII TOPO cloning kit (Invitrogen Cat. #K460001)
- p^{33}-labeled dUTP (Amersham)
- Cold formaldehyde (Sigma)
- DEPC-treated PBS (Ambion Cat. #9625)
- Triethanolamine (Sigma cat. #T0449)
- Acetic anhydride (Sigma cat. #45830)
- SSC buffer (Sigma cat. #S8015)
- RNase A (Ambion Cat. #2271)
- X-ray film emulsion (Kodak)

VII. REVERSE TRANSCRIPTION AND TAQMAN QUANTITATIVE PCR

- RETROscript® Kit (Ambion, Cat. #1710)
- Gene-specific 6-FAM, MGB-labeled probes and sequence detection primers

(Applied Biosystems, Cat. #4316034 and #4304970)

- Taqman® Master Mix (Applied Biosystems, Cat.#4304437)
- ABI Prism™ Optical Reaction Plate (Applied Biosystems, Cat. #4314320)

Additional lab supplies and equipment needed:

- Surgical instruments
- Disposable gloves
- Ethanol for freezing brain
- Dry ice
- Cryostat
- Thermal cycler
- Microarray Scanner
- Microarray analysis software (e.g. GeneTraffic™ Duo, Affymetrix)
- Darkroom
- Applied Biosystems 7900HT Fast Real-Time PCR System

RNase-free technique

1. All reagents are purchased and stored under RNase-free conditions.
2. Gloves should be frequently changed, and all surfaces of equipment wiped down with RNase AWAY™ prior to performing any procedure.
3. Cryostat-cut sections are kept in a box at -80 until ready for LCM. One or two sections at a time are thawed and dehydrated for microdissection.
4. It is very important to keep the time between thawing of each section to the time the captured cells are in extraction buffer as brief as possible, preferably under 45 minutes. Attempting to capture as many cells as will fit on a cap without respect to time will result in degraded RNA from the earliest captures, and is not recommended.

Method

A. Animals, surgery, and tissue processing

1. 1. Adult zebra finches (*Taeniopygia guttata*) ranging in age from 90 days to 4 years were anesthetized in preparation for surgery, and placed in the stereotaxic apparatus. Fluorescent cholera toxin B (CTB) labeled in red or green was injected into nucleus RA or Area X according to standard stereotaxic coordinates. The anesthetic dosages and stereotaxic coordinates were varied depending on the species of lab animal used. A total of 160 nanoliters of a 2% solution of CTB was injected into each nucleus RA, which has a volume of about 0.25 mm3. A total of 240 nanoliters of CTB was injected into Area X, which has a volume of about 1.5 mm3. These injections were made in even volumes, at four sites within each nucleus, to ensure coverage of all nerve endings projecting from neurons in the HVC to these brain regions.
2. After allowing 3-7 days for retrograde transport of the tracers, birds were sacrificed by decapitation and their brains quickly dissected. RNAlater® was applied to the surface of the brain overlying HVC within 1 minute of decapitation.
   Note: This application of RNAlater® helps ensure the preservation of RNA profiles representative of the in vivo situation. However, this is not possible for structures buried deep within the brain, as RNAlater does not diffuse more than a few millimeters.
3. Each brain was quickly dissected, placed in OCT in a plastic tissue mold, and rapidly frozen by placing the mold in a slurry of ethanol and dry ice.
   NOTE: Be careful not to let any ethanol get into the OCT.
4. Brains were sectioned at 8 µm thickness onto glass slides using a cryostat. The sections were stored in a slide box at -80°C until ready for LCM.

B. Laser capture microdissection

1. Frozen sections were allowed to thaw until frost was seen to retract from the edges of the slide (approximately 30 seconds). Slides were immediately placed in 75% ethanol for 30 seconds for fixation, followed by a 15 second distilled water rinse.
   NOTE: All alcohols and xylenes should be prepared fresh. Steps using 100% EtOH are particularly sensitive, as small amounts of water at this stage may interfere with microdissection. This lab changes the 100% EtOH at least twice each day, and more often if the LCM 'pick-up' is not optimal.
2. Dehydration proceeded as follows:
   75% EtOH-- 30 sec
   95% EtOH-- 30 sec
   100% EtOH-- 30 sec
   Fresh 100% EtOH-- 1 min
   Fresh 100% EtOH-- 3 min
   Xylene-- 2 min
   Fresh Xylene-- 2 min
   Air dry-- 3 min
3. Laser capture of as many neurons labeled with a single color as possible was performed within a time period of 45 minutes from when each slide was thawed. CapSure HS LCM caps were used with Pixcell IIe settings as follows: 450-700 µs duration, 60-85 mW power, 15 µm laser diameter. These settings permitted the capture of single cells with a diameter ranging from 7-12 µm, close to the diameter of HVC projection neuron cell bodies. Microdissection of 1500 neurons projecting to area X and 2000 neurons projecting to nucleus RA from within the boundaries of HVC was performed from each cell type for each animal. This yields approximately equal amounts of amplified RNA for the two cell types.
4. When all desired cells of one color were captured on an HS LCM cap, 15 µL of extraction buffer from the PicoPure RNA isolation kit was added to each cap. Each cap was then incubated at 42°C as per the kit instructions. The tubes containing the extracts were spun down and stored -80°C until ready for purification (no more than 1 week).

C. RNA purification and amplification

1. Samples from a single projection neuron type, from a single animal, were pooled by combining all 15 µL extracts. An equivalent volume of 70% EtOH was added to the pooled sample, and RNA isolation was continued according to the instructions in the PicoPure kit, including the on-column DNase digestion detailed in the user guide Appendix A. Each RNA sample was eluted in 11 µL elution buffer.
2. RNA was amplified through two rounds with the RiboAmp HS kit according to the manufacturer's instructions.
3. aRNA quality was evaluated on the Agilent Bioanalyzer 2100 using the Nanochip, and the aRNA yield was measured on the NanoDrop ND-1000 spectrophotometer. Average yield from the captured songbird bird material was approximately 70-90 µg after two rounds of amplification with the RiboAmp HS kit.
4. aRNA was stored at -80°C until subsequent analyses were performed.

D. Reverse transcription of the amplified RNA

1. Based on the NanoDrop yield measurements, 2.5 µg of aRNA from each type of projection neuron from within the same animal was reverse transcribed in the presence of Cy-dyes using the protocol described in Arcturus Protocol #3. cDNA products were purified as described in Protocol #3 and eluted in 50 µL of nuclease-free water. Samples were then concentrated to 12.5 µL each. The labeled cDNA was then applied to the microarrays as described in Section E below.
2. For Taqman quantitative PCR analyses, an equivalent amount of aRNA from each type of projection neuron (≤ 0.25 µg) and reverse transcribed using the RETROscript® Kit per manufacturer's instructions. Samples were eluted in 30 µL of nuclease-free water. The unlabeled cDNA was diluted 1:10 in water and used in Taqman assays as described in Section G below.

E. Microarray hybridizations, scanning, and analysis

1. 12.5 µL of the concentrated, labeled cDNA from each type of projection neuron was applied to the cDNA microarrays and allowed to incubate overnight according to the Corning Pronto kit instructions.
2. The hybridized microarrays were scanned at each wavelength for Cy3 and Cy5. Software (e.g. GenePix® Pro) was used to obtain the median fluorescent intensity values minus the background for each spot on the microarray.
3. The most differentially expressed genes were identified by either fold-change criteria or statistical analysis using a microarray analysis software package such as GeneTraffic™ Duo.

F. *In situ* hybridization to validate differential gene expression found on the microarrays

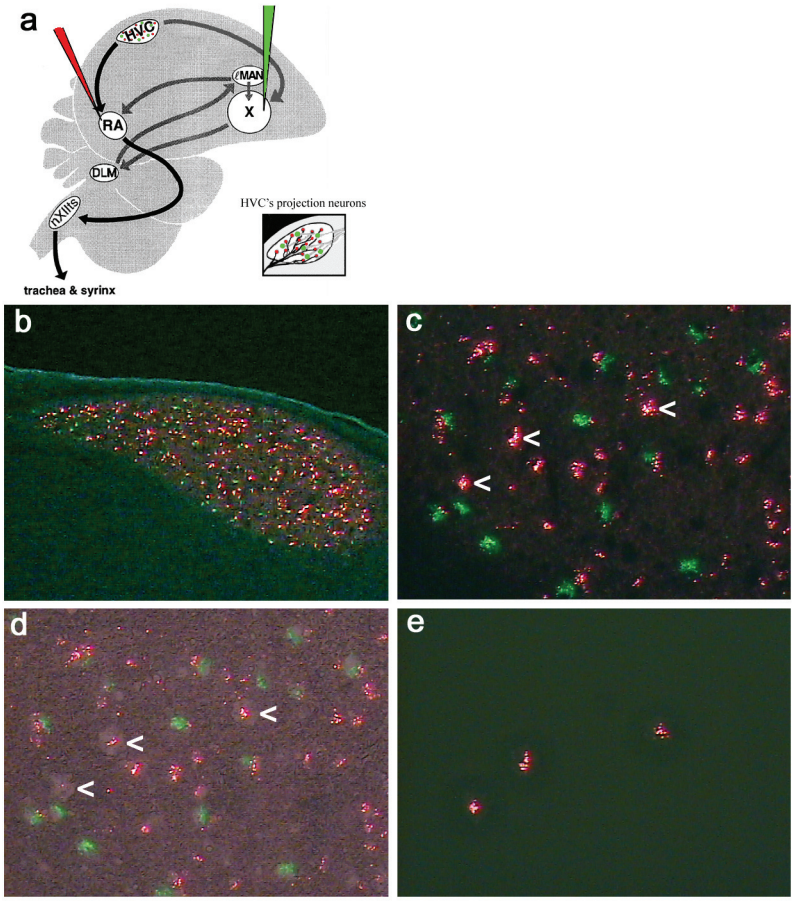
1. Based on the sequence information for the clones identified as differentially expressed on the microarrays, PCR was used to amplify select clones using oligo-dT primed cDNA as template. PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System for 30 cycles as follows: 94°C for 45 seconds, 63°C for 45 seconds, 72°C for 45 seconds.
2. Amplified DNA was purified and cloned into the pCR II-TOPO vector (Invitrogen). The identity of each clone was confirmed by DNA sequencing.
3. Antisense probes were generated by restriction enzyme digestion and transcription from the T7 promoter in the presence of P^{33} UTP.
4. Fresh frozen sections of 6-12 µm in thickness were thawed and fixed in 4% fresh, cold formaldehyde, followed by a wash in DEPC-treated PBS. The sections were aceylated for 5 minutes in 0.1 M triethanolamine with freshly added acetic anhydride, and after a final wash in PBS the sections were dehydrated in graded ethanol solutions: 35%, 50%, 75%, and 100%.
5. P^{33} labeled probe at 1million cpm was added to each section. The sections were coverslipped and submerged overnight in 60°C hot Mineral oil to avoid evaporation. The next day the sections were dipped 10 times in chloroform and de-coverslipped in 50°C warm 2x SSC buffer. After a 1 h wash in 2xSSC, non-specifically bound probe was digested in RNase buffer with 20µg/mL RNase A for 15 min. A final wash was performed for 1h in 0.1x SSC, and the sections were dehydrated in 30%, 75%, and 95% ethanol containing 300mM ammonium acetate.
6. *In situ* hybridizations were visualized on a phosphorimager at low resolution prior to coating with photographic emulsion. The sections were exposed to an X-ray film overnight and then dipped into x-ray film emulsion (Kodak diluted 1:1 with water). The slides were exposed in the dark 2.5-5 weeks (depending on the expected abundance of the gene of interest) and developed as per manufacturer's instructions (Kodak).
7. The clustering of silver grains was analyzed under brightfield conditions and compared to the pattern of fluorescent tracer accumulation under fluorescent microscope conditions.

G. Taqman quantitative PCR to validate differential gene expression on the microarrays

NOTE: Taqman Quantitative PCR was performed with normalized inputs of amplified RNA, based on measurements from the NanoDrop ND-1000 spectrophotometer. It was assumed that the reverse transcription step went to completion.

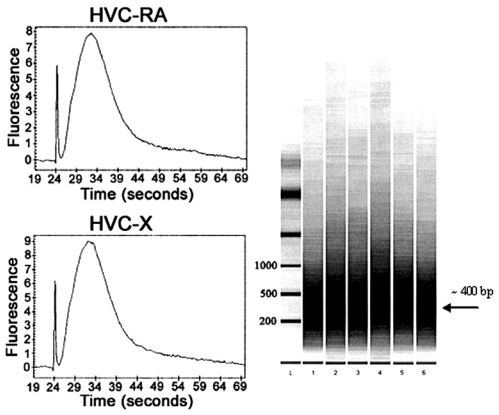
1. Based on the sequence information for the clones identified as differentially expressed on the microarrays, primers and probes were designed using Primer-Express® software (Applied Biosystems). Primers and probes were then ordered with these sequences.
2. Primers and probes were diluted according to manufacturer's instructions (Applied Biosystems) and combined with the appropriate volume of Taqman master mix. Highly reproducible results were obtained using 900 nM of each primer and 250 nM of probe.
3. 22.5 µL of the master mix was loaded for each gene of interest on a 96-well optical reaction plate, and 2.5 µL of cDNA template from each projection neuron sample was added appropriately. The plate was placed into the Applied Biosystems 7900HT Fast Real-Time PCR System and the PCR was run using the following parameters: 95°C denaturation, 60°C annealing/extension for 40 cycles.
4. To generate the standard curve, 2.5 µL of serially diluted standard was added to 22.5 µL of master mix. Copy numbers of aRNA for the sequence of interest were determined by creating standard curves with purified, single-stranded DNA coding for the sequence of interest. This DNA was serially diluted 10-fold five to six times and run in separate q-PCR reactions on the same plate as the unknowns. Copy numbers of the standards were calculated based on the molecular weight of the standard, and a standard curve was generated from the Taqman data by plotting cycle threshold (y-axis) against copy number (x-axis) for the serially diluted standards. Standard curves from this material should have an r^2 of 0.995 or higher. See Bustin, 2000 for more information.

Figure 1. Laser capture microdissection of projection neurons.



a. Schematic of the song system of zebra finches, with the two types of projection neurons in nucleus HVC marked in red (HVC-RA) and green (HVC-X). b. Low-power micrograph of nucleus HVC backfilled with cholera toxin B. c. Higher-power micrograph of individual HVC-RA neurons marked with red CTB and HVC-X neurons marked with green CTB. Three HVC-RA neurons to be captured are identified with white arrows. d. Holes in the tissue section left behind after the three HVC-RA neurons marked in (c) are removed. Note that a small amount of fluorescence remains in the case of the two right-most neurons, indicating less than perfect pick-up; this does not affect the results as we capture 2000 of this type of neuron and pool them for analysis. e. The three microdissected neurons, bearing tracer fluorescence are shown on the LCM cap. Note even details of the pattern of fluorescence from the cells in the tissue are evident in the captured cells, indicating the cleanness of the material that has been microdissected.

Figure 2. Bioanalyzer profiles of the amplified RNA obtained from 2000 HVC-RA neurons and 1500 HVC-X neurons, as run on the Agilent Nanochip.



The curves show a smooth peak of high-quality aRNA that is very similar for the two neuron subtypes. The virtual gel at right shows that the average size of the aRNA obtained is centered around 400 bp in length.

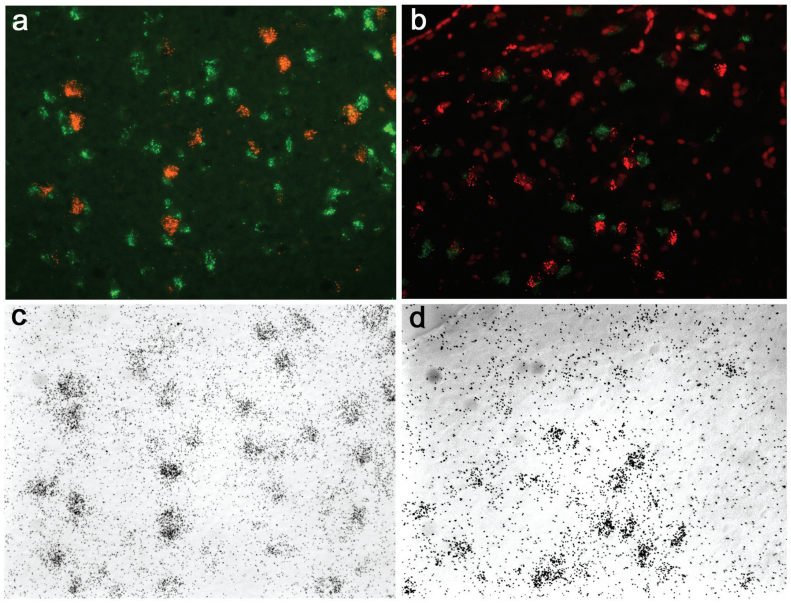
Results

We have marked two types of long-range projection neurons in the zebra finch brain with fluorescent tracers, and then captured 1500-2000 of these neurons one-by-one using a Pixcell IIe (Fig. 1 a-e). RNA from these captures was pooled by neuron type, amplified through two rounds using the RiboAmp HS kit, and then assessed for quality on an Agilent Bioanalyzer. High quality amplified RNA profiles were consistently obtained from each of 14 birds (Fig. 2). The aRNA from each projection neuron type was compared, using a within-animal design, by applying Cy dye-labeled cDNA to cDNA microarrays made from whole zebra finch brain.

More than 98% of the genes were found to be expressed at similar levels in this comparison, as assessed by a cutoff of 2-fold differential expression at least 50% of the time across all birds in the experiment. This arbitrary fold-change cutoff was supplemented with a statistical analysis (Significance Analysis of Microarrays) that also showed more than 90% of the genes in the two neuron types to be expressed at similar levels. We focused on the few genes that showed the most consistently dramatic differences in expression, in an attempt to develop markers of these projection neuron subtypes. Using *in situ* hybridization (Figure 3), we were able to validate 7 of 8 genes meeting a criterion of 2-fold or more differential expression in at least half of the microarray hybridizations. ISH reveals that not every neuron of a given subtype expresses the gene of interest (Fig. 3 b and d), indicating further heterogeneities within a projection neuron class. This is not surprising, given that we pooled hundreds of each type of neuron in our microdissections. Three of these 8 most differentially expressed genes were also validated by Taqman quantitative PCR (Fig. 4), which was performed on the same amplified RNA generated from the original set of birds, as well as on new aRNA from other birds added to the experiment. The results show a significant difference in the expression of these genes by neuron subtype and validate our microarray findings.

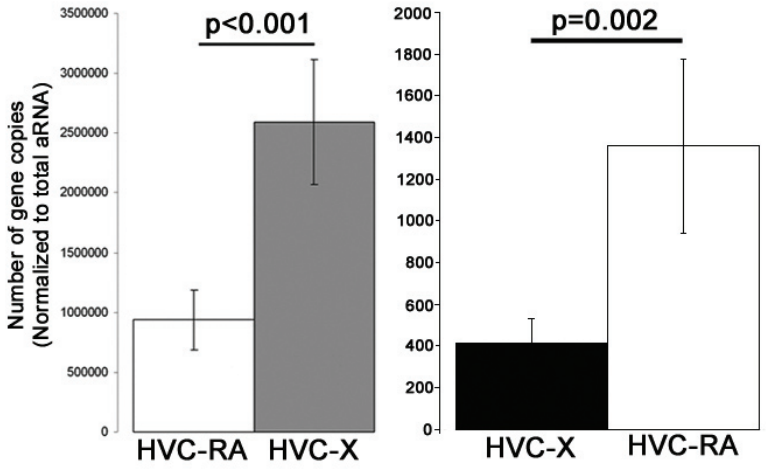
Using cell sorting, only a single report has previously shown that projection neurons can be successfully purified for expression profiling (Arlotta et al., 2005; Dougherty and Geschwind, 2005). The results presented here demonstrate that it is possible to use LCM (Bonner et al., 1996) to microdissect closely apposed projection neurons by guiding the microdissection with fluorescent,

Figure 3. *In situ* hybridization for two genes that show opposite patterns of differential expression between HVC-RA and HVC-X projection neurons.



The top panels show the backfilled neurons from two birds with HVC-RA neurons labeled green in (a) and red in (b). The bottom panels show a gene that is overexpressed in HVC-X neurons (c), and a gene that is overexpressed in HVC-RA neurons (d). Note that the clusters of grains indicating the concentration of mRNA for that gene are centered over the green HVC-X neurons in (c), and over the red HVC-RA neurons in (d). The small, dimmer red cells in panel (b) are blood cells, and should be disregarded.

Figure 4. Taqman quantitative PCR confirmation of the differential gene expression shown in Fig. 3.



The left panel shows higher expression of a gene in HVC-X neurons; this is the same gene for which in situ hybridization data is shown in the left panels of Fig 3. The right panel shows high expression of a gene in HVC-RA neurons; this is the same gene shown in the right panels of Fig. 3. The data are from pooled samples from 14 birds, with the aRNA from each type of projection neuron analyzed separately.

retrogradely transported neuronal tracers. These tracers accumulate outside the nucleus of the cell body, where the pools of mRNA of interest are located. This approach eliminates the need for lengthy tissue dissociation and related steps needed for cell sorting, thus helping to preserve the original, *in vivo* profile of mRNA expression. The *in vivo* labeling approach also minimizes the time needed for tissue preparation for LCM, further preserving RNA integrity. The within-animal design of the microarray experiments makes for highly consistent results, with most genes expressed at similar levels, helping to more easily identify those genes that are most differentially expressed. The generation of a large pool of aRNA with these methods allows for many assessments of gene expression, whether by microarrays for gene discovery or Taqman for quantification of known genes. The combination of these techniques should greatly facilitate the molecular characterization of specific cellular components of neural circuits.

References

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