Video Article

Reliable Identification of Living Dopaminergic Neurons in Midbrain Cultures Using RNA Sequencing and TH-promoter-driven eGFP Expression

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Abstract

In Parkinson's Disease (PD) there is widespread neuronal loss throughout the brain with pronounced degeneration of dopaminergic neurons in the SNc, leading to bradykinesia, rigidity, and tremor. The identification of living dopaminergic neurons in primary Ventral Mesencephalic (VM) cultures using a fluorescent marker provides an alternative way to study the selective vulnerability of these neurons without relying on the immunostaining of fixed cells. Here, we isolate, dissociate, and culture mouse VM neurons for 3 weeks. We then identify dopaminergic neurons in the cultures using eGFP fluorescence (driven by a Tyrosine Hydroxylase (TH) promoter). Individual neurons are harvested into microcentrifuge tubes using glass micropipettes. Next, we lyse the harvested cells, and conduct cDNA synthesis and transposon-mediated "tagmentation" to produce single cell RNA-Seq libraries[1,2,3,4,5]. After passing a quality-control check, single-cell libraries are sequenced and subsequent analysis is carried out to measure gene expression. We report transcriptome results for individual dopaminergic and GABAergic neurons isolated from midbrain cultures. We report that 100% of the live TH-eGFP cells that were harvested and sequenced were dopaminergic neurons. These techniques will have widespread applications in neuroscience and molecular biology.

Video Link

The video component of this article can be found at https://www.jove.com/video/54981/

Introduction

Parkinson's Disease (PD) is an incurable, age-related neurodegenerative disorder. The cause of this relatively common disorder remains poorly understood. There is widespread neuronal loss throughout the brain, with pronounced neuronal degeneration of dopaminergic neurons in the Substantia Nigra (SNc), leading to diagnostic clinical features of bradykinesia, rigidity and tremor.

Primary mixed cultures containing SNc dopaminergic neurons are especially relevant for Parkinson's disease. Ventral Tegmental Area (VTA) dopaminergic neurons have been implicated in reward and addiction. Ventral Mesencephalic (VM) primary mixed embryonic cultures contain both SNc and VTA dopaminergic (DA) neurons and GABAergic neurons. VM primary cultures can be useful for neuroprotection assays and to elucidate the selective vulnerability of dopaminergic neurons. There is no reliable way to identify dopaminergic cells in culture based on morphology. Here we develop techniques to identify and harvest single dopaminergic neurons, and construct single-cell, high-yield RNA sequencing libraries.

We report representative RNA transcriptome data for single dopaminergic and GABAergic neurons isolated from midbrain cultures. This protocol can be used for neuroprotection, neurodegeneration, and pharmacological assays to study the effects of various treatments on the DA/GABA transcriptome. Because dopaminergic neurons represent a small minority of the neurons expressed in primary VM cultures, the ability to reliably identify these neurons in living cultures will enable an enhanced range of single-cell studies. These novel techniques will facilitate advances in understanding the mechanisms taking place at the cellular level and may have applications elsewhere in the fields of neuroscience and molecular biology.

Protocol

NOTE: All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health, and protocols were approved by the Institutional Animal Care and Use Committee at the California Institute of Technology.

1. Derivation of Primary Dopaminergic Cell Cultures from Embryonic Mouse Brain

1. Solutions and Culture Medium

1. Preparation of Ascorbic Acid Stock Solution
    1. Weigh out 352 mg of ascorbic acid. Add sterile H_2O to a final total volume of 20 mL. Place in 37 °C water bath to dissolve. Filter through 0.2 µm syringe tip and store in 500 µL aliquots at -20 °C.

2. Preparation of Papain Stock Solution
    1. Dilute papain to 15 units/mL in 1x Hanks'-balanced Salt solution (HBSS). Pipette up and down 5 times to mix thoroughly. Prepare on the day of dissection.

3. Preparation of DNase Stock Solution
    1. Weigh out 20 mg of DNase. Add sterile H_2O to a total final volume of 20 mL. Sterile filter with 0.2 µm syringe filter tip. Store at -20 °C in 1 mL aliquots.

4. Preparation of Stop Solution
    1. Dilute 5 mL donor equine horse serum to 10% in 45 mL 1x HBSS. Sterile filter with a 0.2 µm syringe filter tip. Store at 4 °C.

5. Preparation of 4% Bovine Serum Albumin (BSA) stock solution
    1. Weigh out 2 g of BSA and add 1x Phosphate-buffered Saline (PBS) to a total final volume of 45 mL. Filter sterilize with 0.2 µm syringe filter tip. Store at 4 °C.

6. Preparation of Plating Medium
    1. Into 494 mL medium, add 1.25 mL culture media that contains L-alanyl-L-glutamine (a stabilized source of L-glutamine) and 5 mL of donor equine serum. Filter sterilize with 0.2 µm syringe filter tip. Store at 4 °C.

7. Preparation of Cell Culture medium
    1. Into 196 mL plating medium, add 4 mL B27 and 200 µL ascorbic acid. Filter sterilize with 0.2 µm filter. Store the solution at 4 °C and use within a week.

8. Preparation of 4% Paraformaldehyde (PFA) Solution
    Caution: General precautions for using paraformaldehyde are as follows: Use a fume hood, avoid skin and eye contact, avoid inhalation of the vapor, keep away from heat or naked flames. Wear appropriate personal protective clothing. PFA can cause sensitization and dermatitis, therefore wash hands thoroughly after handling.
    1. Add 10 mL of 16% PFA to 30 mL of 1x PBS, pH 7.4.

9. Preparation of 0.1% Triton X-100
    1. Pipette 1 mL Triton X-100 into 9 mL 1x PBS to make a 10% solution. Pipette slowly to allow viscous solution to fill pipette tip. Warm in 37 °C water bath to dissolve. Store 10% stock at 4 °C.
    2. Dilute 10% stock to 0.01% (*e.g.*, by performing 2 serial 1:10 dilutions: dilute 1 mL of 10% solution into 9 mL 1x PBS to make 1% solution; 1 mL of 1% solution into 9 mL 1x PBS to make 0.1% solution).

10. Preparation of 10% and 1% donkey serum
    1. Add 1 mL of donkey serum to 9 mL of 1x PBS for a 10% solution. Add 0.5 mL of donkey serum to 49.5 mL of 1x PBS, pH 7.4 for a 1% solution.

11. Preparation of 1x PBS
    1. Dilute 10x PBS to 1x by adding 50 mL into 450 mL of H_2O. Adjust the pH of the solution to pH 7.4 using a pH meter.

2. Coating the Culture Dishes
    1. Poly-L-Ornithine Coating
        1. Coat only the 10 mm diameter glass bottom in the center of all the 35 mm dishes with 120 µL poly-L-Ornithine using sterile technique. Incubate for 1 h at 37 °C. Use a vacuum aspirator to aspirate the poly-L-ornithine. Rinse the dishes twice with sterile H_2O.

    2. Laminin Coating (stock concentration of 1 mg/mL)
        1. Dilute 20 µL laminin with 2 mL of sterile H_2O (final concentration 0.01 mg/mL).
        2. Coat only the 10 mm diameter glass bottom in the center of all the dishes with 120 µL of laminin diluted to 0.01 mg/mL using sterile technique, and incubate for 1 h or O/N at 37 °C before use.

3. Mouse Dissection
    NOTE: The methods in this dissection are designed for minimal exposure of cells, as they are transferred from embryonic sacs to the culture incubator. The viability of the cells is preserved by removing only a portion of the brain for dissection of the midbrain. Isolation of the midbrain proceeds more quickly without the need to dissect the entire brain to access the region. See **Table of Materials** for the mouse strain used in this study.
    1. Euthanize a timed pregnant mouse on gestational day 14 using CO_2.
    2. Spray the abdomen of the euthanized mouse with 70% ethanol. Grasp the skin of the lower abdomen using forceps with 2 x 3 teeth and open the abdominal cavity using blunt blade surgical scissors. Make two cuts moving laterally and proximally on each side. Fold over the abdominal wall using forceps to expose the abdominal cavity.
        NOTE: The uterus is now exposed. Exposing the pleural cavity and creation of pneumothorax also ensures that the animal does not recover.
    3. Cut both ends of the uterine horn to separate the uterus. Place into a Petri dish in a sterile hood.

4. Using straight-tip forceps in each hand open the embryo sac and remove the embryo. Quickly decapitate by pinching head off at the neck with the forceps. Stabilize the head with the forceps firmly placed on the snout so that the dorsal surface is accessible.
5. Using the forceps held in the other hand, pinch the layer of skin and the skull just before the ridge of the mesencephalon. Peel back the skin and the skull caudally along the midline. Place forceps around the ridge with one tip between the cortex and mesencephalon and the other over the cerebellum.
6. Pinch and remove the entire midbrain, leaving behind the forebrain on the rostral side and cerebellum on the caudal side. Place in a Petri dish with cold HBSS under a dissecting microscope.
7. Repeat the procedure for the remaining embryos.
8. Under the dissecting microscope, flip over the brain segment so that the ventral side is now accessible. There are now 4 quadrants visible. Remove all of the meninges and vasculature by gently grasping the meninges and pulling upward away from the brain.
9. Use forceps to stabilize the brain segment while separating the midbrain. Place one tong of forceps into the ventricle approximately at center of the segment. Make the first pinch dorsally, pointing into the dish, then medially on each side. Take the lower two quadrants by making lateral cuts on both sides.
   NOTE: The tissue of interest is in the ventral inferior section, which appears dense. This region contains both the VTA and the SNc.
10. Trim and discard the tissue that is less dense: this includes the superior and inferior colliculi. Place the dissected tissue containing both the VTA and SNc in fresh HBSS on a separate area of the Petri dish.
    NOTE: At this stage of mouse brain development (E14), the region of interest in the ventral midbrain is separated by a ventricle from the zona incerta and other hypothalamic cell groups. The more elongated structure of the developing embryonic mouse brain allows the distinction among these areas, which are located closer together in the adult mouse brain.
11. Repeat for all the remaining brain segments.
12. After all the brain segments have been dissected, use forceps and a No. 11 scalpel to quarter each midbrain section into pieces of approximately equal size.

4. Dissociating the Cells
   1. Enzymatic Treatment of cells
      1. Use a wide-bore P1000 pipette tip to take up all the quartered midbrain segments in the HBSS and place them into a 15 mL conical tube. Let the segments settle to the bottom of the tube and remove HBSS that has been taken up with the quartered pieces.
      2. Add 500 µL of papain solution to the tube and place into a 37 °C water bath for 15 min. Resuspend cells by finger flicking the tube after 7.5 min.
      3. Using a wide-bore P1000 tip, pipette only the midbrain segments into a 1 mL aliquot of deoxyribonuclease I (DNase) solution. Allow the segments to settle to the bottom of the tube.
      4. Using a wide-bore P1000 tip, transfer only the midbrain segments to a 15 mL tube containing 1 mL of cold stop solution to rinse. Let the segments settle to the bottom of the tube and repeat the rinse once more.

   2. Mechanical Trituration of Cell Suspension
      1. Replace the last rinse with 1 mL of fresh stop solution and pipette up and down 7x with a P1000 pipette tip to triturate cells. Avoid excessive trituration to minimize cell lysis.
      2. Underlay the cell suspension by slowly pipetting 200 µL of 4% BSA solution into the bottom of the tube. Carefully withdraw the pipette tip to avoid disrupting the cell suspension layer. After centrifugation at 280 x g for 6 min, aspirate the supernatant with P1000 and resuspend the cells in 1 mL of plating medium.

5. Counting and Plating the Cells
   1. Perform cell count using a hemocytometer. Dilute the cell suspension to 1,000 cells/µL, with plating medium.
   2. Using a vacuum aspirator, aspirate the laminin from the coated dishes, in batches of at most three dishes. Plate 120 µL (1.2×10^5 cells/dish (78.5 mm^2)) of the cell suspension onto the coated surface of the dishes immediately after aspirating each batch. This prevents the dishes from drying out and forming an uneven surface.
   3. After 1 h, carefully add 3 mL of culture medium to an unseeded area of the culture dish to minimize disruption of cells.
   4. Perform a half to full medium change on cell culture dishes at 3 d intervals for 3 weeks.

2. Harvesting Cultured-dopaminergic Neurons for RNA Sequencing

NOTE: An overview of the cell-harvesting and single-cell RNA-Sequencing protocol is given in **Figure 1**.

1. Steps to Perform Before the Day of the Experiment
   1. Clean borosilicate glass capillary tubing by sonicating in 100% ethanol for 15 min and then again in distilled H_2O for 15 min. Drain the tubing and bake overnight in an oven at 200 °C to inactivate RNase.
   2. Use freshly opened RNase-free pipette tips to prepare a stock solution of 10x reaction buffer by mixing 19 µL of 10x lysis buffer from the sequencing kit with 1 µL of the RNase inhibitor solution (20 µL total volume) in a microcentrifuge tube . Spin down the mixture briefly using a micro-centrifuge and keep on ice.
   3. Add 2 µL of the 10x reaction buffer to each of the individual 0.2 mL PCR tubes (used to collect the harvested neurons). Label the tubes with appropriate tags identifying neurons to be harvested and store frozen at 4 °C.

2. Steps to Perform on the Day of the Experiment
   1. Fabrication of the Harvesting Pipettes.
      1. Pull large-tip diameter micropipettes (2 - 8 µm) from the baked capillary tubing using a microelectrode puller and the glass-coated heating filament of a microforge. Set the puller to form micropipettes with a long taper. Use a microforge equipped with a calibrated eyepiece graticule and a high-power objective to break the tip of the micropipette to the desired tip size (10 - 20 µm).

2. Fasten the micropipette into the microforge pipette holder and mount the holder onto the microforge. Bring the tip of the micropipette into focus above the glass-coated heating filament using the mechanical manipulator of the microforge and switch on the current to the filament to heat it. Touch the pipette tip to the heated filament. Switch off the current when the pipette tip has melted to the appropriate diameter.
   NOTE. Shutting off the current will cause the filament to move suddenly downward and detach from the pipette tip, producing a large, open-tipped pipette with the desired diameter.
3. Store the completed micropipettes in a closed box until needed.

2. Harvesting the Neurons.
   NOTE. Cultured neurons were harvested following a modified version of a previously published protocol[6].
   1. Thoroughly clean all surfaces of the cell-harvesting room that come into contact with the cultures dishes and collection tubes using liquid disinfectant and water followed by a water rinse. Wipe down the disinfected surfaces with 80% ethanol and then an RNase-inhibitor-infused wipe.
   2. Fill two insulated containers with ice, one with regular (water) ice and another with dry ice. Place the 0.2 mL PCR collection tubes filled previously with 2 µL of 10x reaction buffer on the regular ice.
   3. Remove the culture dish containing the neurons from the incubator, drain the culture medium from the dish, rinse with 2 mL of RNase-free Dulbecco's PBS (DPBS) and replace with 1 mL of DPBS for harvesting.
   4. Identify fluorescent, TH-positive neurons in the primary midbrain cultures using an inverted, epifluorescence microscope equipped with a 40X phase objective, a Hg lamp, and a GFP filter set.
   5. Position the pipette over the cell soma using a motorized or manual micromanipulator. Aspirate the neuron into the glass micropipette using gentle suction applied by mouth through the plastic tubing attached to the side port of the micropipette holder. Immediately remove the micropipette containing the cell from the bathing solution.
   6. Place the pipette tip inside a 0.2 mL PCR tube collection containing reaction buffer, and break the tip against the side of the tube, near the bottom. Expel the remaining fluid (0.5 - 1.5 µL) from the broken tip of the micropipette by placing a sterile 21 G syringe needle in the back of the micropipette and applying outward pressure.
   7. Centrifuge the collection tube for 5 s using a desktop micro-centrifuge and freeze it in dry ice. Store the collection tubes containing the single cells at -80 °C. Typically, 5 cells are harvested per dish over a 1 h period at ambient temperature.

3. Single-cell RNA-Seq Library Generation

1. Generation of the First strand cDNA
   NOTE: For the following steps use reagents from the commercial sequencing kit.
   1. Bring the below reagents on ice to the PCR cabinet. Prepare a first strand master mix plus 10% by combining the following reagents at room temperature in a PCR cabinet: 2 µL 5x first strand buffer, 0.25 µL DTT (100 mM), 1 µL dNTP mix (10 mM), 1 µL oligonucleotide (12 µM), 0.25 µL RNase Inhibitor, and 1 µL reverse transcriptase (100 units). This is the reverse transcriptase master mix (5.5 µL total volume per reaction).
   2. Take the samples from the -80 ℃ on dry ice and bring to the PCR cabinet. Add the following to the single cell sample: 1 µL 3' primer 1 and 1 µL quantified RNA spikes. Bring samples on a chiller block to a hot-lid thermocycler preset at 72 °C.
   3. Incubate the tubes in the thermocycler for 3 min at 72 °C, and then put the samples on a PCR cooler rack. Add 5.5 µL of the master mix (from step 3.1.1) to each reaction tube and mix by pipetting gently. Spin the 0.2 µL tubes for 5 s and incubate in a thermocycler as follows: 42 °C for 90 min, 70 °C for 10 min. Hold at 4 °C.

2. Purification of Amplified First Strand cDNA.
   NOTE: The PCR-amplified cDNA is purified by immobilization on magnetic beads. Use a magnetic separation device for 0.2 mL tubes.
   1. Aliquot the magnetic beads into 1.5 mL tubes. Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse. Vortex magnetic beads until evenly mixed.
   2. Add 25 µL of magnetic beads to each sample. Mix by pipetting the entire volume up and down at least 10x to mix thoroughly. Incubate at RT for 8 min to let the cDNA bind to the beads.

3. Preparation of PCR Master Mix
   1. Prepare the ds-cDNA amplification PCR master mix for all reactions with an additional 10% by mixing 5 µL 10x PCR buffer, 2 µL dNTP mix (10 mM), 2 µL PCR primer 2 (12 µM), 2 µL 50x 2 polymerase mix, and 39 µL nuclease free-water (50 µL total volume/reaction).

4. Amplification of First Strand
   1. Place the samples and magnetic beads on the magnetic separation device ≥5 min, until the liquid appears completely clear, and there are no beads left in the supernatant.
   2. Keeping the samples on the separation device, pipette the supernatant and discard. Spin the samples for 5 s to collect the liquid from the side of the tube. Place the samples on the magnetic separation device for 30 s, then remove all the remaining supernatant with a P10 pipettor.
   3. Add 50 µL of the PCR master mix to each tube containing DNA bound to the beads from the previous step. Place the tube in a preheated thermal cycler (95 °C) with a heated lid. Commence the thermal cycling using the following program: 95 °C for 1 min, 26 cycles of 95 °C for 15 s, 65 °C for 30 s, 68 °C for 6 min. Then 72 °C 10 min. Hold at 4 °C.
      NOTE: Consult sequencing user manual for more details and to optimize the sample settings.

5. Purification of Amplified Double-Stranded cDNA
   1. Add 90 µL of beads to each sample. Mix by pipetting the entire volume up and down at least 10x to mix thoroughly. Incubate at RT for 8 min to let the cDNA bind to the beads. Place the samples plus beads on the magnetic separation device for ≥5 min, until the liquid appears completely clear, and there are no beads left in the supernatant.

2. While the samples are on the magnetic separation device, remove the supernatant and discard. Keep the samples on the magnetic separation device. Add 140 µL of freshly made 85% ethanol to each sample without disturbing the beads. Wait for 30 s. Pipette off the supernatant. cDNA will remain bound to the beads during the washing process.
3. Repeat the ethanol wash once more.
4. Briefly spin the samples to collect the liquid from the side of the tube. Place the samples on the magnetic separation device for 30 s, then remove all the remaining ethanol with a pipette. Place the samples at room temperature for 2 - 2.5 min until the pellet is dry and no longer shiny (*i.e.* before a crack appears).
   NOTE: Dry the pellet only until it is just dry. The pellet will look matte with no shine. If the pellet is not dry, ethanol will remain in the sample wells, which will reduce the amplified cDNA recovery rate and the yield. If the pellet is over-dry, there will be cracks in the pellet. It will take longer than 2 min to rehydrate and may reduce amplified cDNA recovery and yield.
5. Once the beads are dry, add 22.5 µL of elution buffer to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads. Incubate at RT for 10 min to rehydrate.
6. Spin the samples for 5 s to collect the liquid from the side of the tube. Place the samples back on the magnetic separation device for ≥1 min, until the solution is completely clear.
   NOTE: A small population of beads may not pellet against the magnet during incubation. Pipette these unpelleted beads up and down to resuspend them with the supernatant, and then pipette them toward the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet).
7. Continue the incubation until no beads remain in the supernatant. Transfer clear supernatant containing purified cDNA from each tube to a nuclease-free, low-adhesion tube. Label each tube with sample information and store at -20 °C. The samples may be stored at -20 °C indefinitely.

6. Measurement of DNA Concentration and Fragment size
   1. Conduct a quality control check by quantifying a 1 µL aliquot of the cDNA on a fluorometer. Assess 1 µL (3 ng) of ds-cDNA using an electrophoresis system (as described in[1]).

7. Fragmentation of DNA
   NOTE: Use a DNA Sample preparation kit.
   1. Add 20 µL of cDNA (35 ng total) to a tube. Add 25 µL of Tagment DNA (TD) buffer to the tube containing the cDNA.
   2. Add 5 µL of TDE1 (Tagment DNA Enzyme) to the tube. Pipette up and down 10x to mix. Use a fresh tip for each sample. Centrifuge at 280 x g at 20 °C for 1 min.
   3. Place the samples in a thermocycler with a heated lid and run it at: 55 °C, 5.5 min. Quickly add 50 µL QG solution (gel extraction kit). Pipette up and down 10x to mix. Continue directly to the next step.

8. Purification of Fragmented DNA
   1. Add 170 µL of beads, pipette up and down 10x to mix. Allow it to sit for 10 min. Put tubes on the magnet. Let the tubes sit on the magnet for 10 min. While the samples are on the magnetic separation device, pipette the supernatant and discard.
   2. Leave the tube on the magnet and add 200 µL of 85% ethanol. Let sit for 30 s, then remove ethanol. Repeat the ethanol wash. Spin the tube 1,000 x g at RT.
   3. Put the tube back on the magnet. Pipette off any residual ethanol. Let the samples dry at RT for 15 min.
   4. Add 21 µL of elution buffer from the gel extraction kit. Let the tube sit off the magnet, pipette up and down 10x to mix. Let the tube sit at RT for 15 min.
   5. Return the tube to the magnet for 5 min. Pipette 20 µL of solution (purified DNA) into a new tube.

9. Tagging and PCR Amplification of the Fragmented DNA
   NOTE: In this step the purified DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) as well as common adapters (P5 and P7) (required for cluster generation and sequencing). Refer to the DNA sample preparation guide for more details.
   1. In a new tube add 5 µL of index 2 primers (white cap). Using a new pipette tip add 5 µL of index 1 primer (orange cap). Add 15 µL of PCR master mix to each tube.
   2. Add 5 µl of PCR primer cocktail to each tube. Add 20 µL of the purified DNA to the tube. Gently pipette up and down 3 - 5 times.
   3. Perform PCR using the following program on a thermocycler: 72 °C for 3 min, 98 °C for 30 s, 5 cycles of 98 °C for 10 s, 63 °C for 30 s, 72 °C for 3 min. Hold at 10 °C. Proceed immediately to the PCR clean-up or store the sample at 4 °C for up to 2 d.

10. Purification of Amplified DNA Fragments
    1. Bring the beads to RT. Prepare fresh 85% ethanol. Take samples out of PCR machine and spin down briefly.
    2. Add 30 µL of beads to the tube. Gently pipette mix up and down 10x. Let the tube sit at room temperature for 5 min. Place back on the magnet for 5 min. With the tube on the magnet use a pipette to discard the supernatant.
    3. Add 200 µL of 85% ethanol to each tube. Pipette ethanol off after 30 s. Repeat the 85% ethanol wash. Pipette ethanol off after 30 s.
    4. With the tubes still on the magnet and the caps open, allow the beads to air dry for 15 min. Remove the tubes from the magnet.
    5. Add 32.5 µL of elution buffer. Gently pipette up and down 10x. Incubate off the magnet for 10 - 15 min.
    6. Return the tubes to the magnet for 2 min, or until the supernatant has cleared. Carefully transfer 30 µL of the supernatant to a new tube.

11. Measurement of DNA Concentration and Fragment Size
    1. Conduct a quality control check by quantifying the DNA concentration of 1 µL of the cDNA on a fluorometer. Determine the fragment size of the DNA by electrophoresis. Assess 1 µL (3 ng) of ds-cDNA.

12. Sequencing
    1. Bring the single cell RNA-Seq libraries to a sequencing facility. Generate 100 bp sequencing reads on a sequencer following a previously published protocol[4].

NOTE: Each sequencing library generates >20 million uniquely mapping reads.

Representative Results

Here we report a protocol for culturing ventral midbrain neurons from a mouse expressing eGFP driven by a tyrosine hydroxylase promoter sequence, harvesting individual fluorescent neurons from the cultures, and measuring their RNA transcriptome using RNA-seq (**Figure 1**). The data showed that 100% of the TH-eGFP-positive cells that were harvested and sequenced were dopaminergic neurons, based on the presence of the following three DA-related gene transcripts, TH, dopa decarboxylase (DDC), and the dopamine transporter DAT (slc6a3). All of the TH-eGFP positive cells expressed these three genes as assessed by RNA-Seq. In each single-cell RNA-Seq library 6,000 - 8,000 protein coding genes were detected (**Figure 2**). The dopaminergic neurons robustly express dopamine-related transcripts in addition to several nicotinic acetylcholine receptor (nAChR) subunits (**Table 1**). We observed that not all of the cells that were negative for TH were GABAergic; therefore we defined cells as GABAergic based on the presence of the Gad1 transcript as assessed by RNA-seq. The cells that we defined as GABAergic neurons do not express dopamine-related transcripts but as mentioned do express a major gene for GABA synthesis, Gad1. The single-cell libraries also revealed long non-coding RNA, as well as transcripts encoding channels, receptors, nuclear proteins, histones, organellar proteins, and transcription factors.

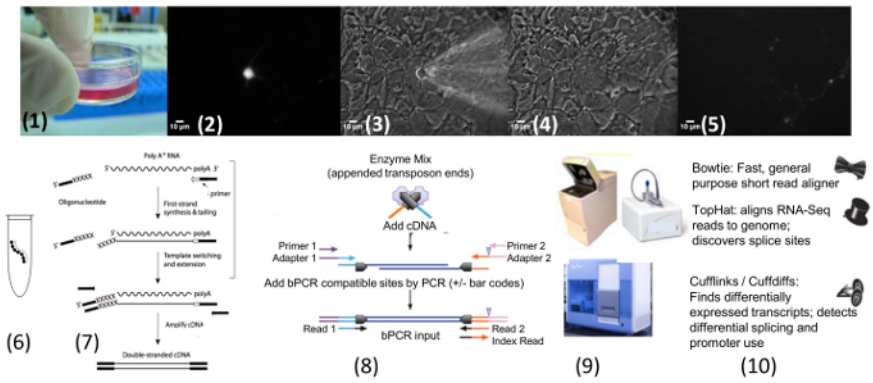

Figure 1. RNA-Seq in Individual Neurons, as Performed in Our Experiments on Primary TH-eGFP Mouse Midbrain Cultures. (**1**) Culture TH-eGFP mouse ventral neurons for 3 weeks. (**2**) Identify fluorescent single neurons by exciting GFP using an Hg light source and FITC/GFP filter. (**3**) With the same 40X objective under phase optics, visualize single neurons and correctly position the pipette. (**4 - 5**) Aspirate the single cell into the glass micropipette. Images show a cell after harvesting: (**4**) Phase 40X; (**5**) eGFP fluorescence. (**6**) Lyse cells with lysis buffer with oligo-dT primer at 72 °C. (**7**) cDNA synthesis; 26 cycles amplification. (**8**) Transposon-mediated "tagmentation" of cDNA. (**9**) cDNA quality control check and multiplexed sequencing. (**10**) Subsequent computational analysis using Tophat, Cufflinks and Cuffdiff[7]. Relative abundance of expressed genes was measured in units of fragments per kilobase of transcript per million mapped reads (FPKM). The goal of RNA-seq is to provide a list of genes expressed in a cell and their relative abundance. Images (**2 - 4**) are from the current work, (**6 - 10**) are modified from a previous report[1]. Please click here to view a larger version of this figure.

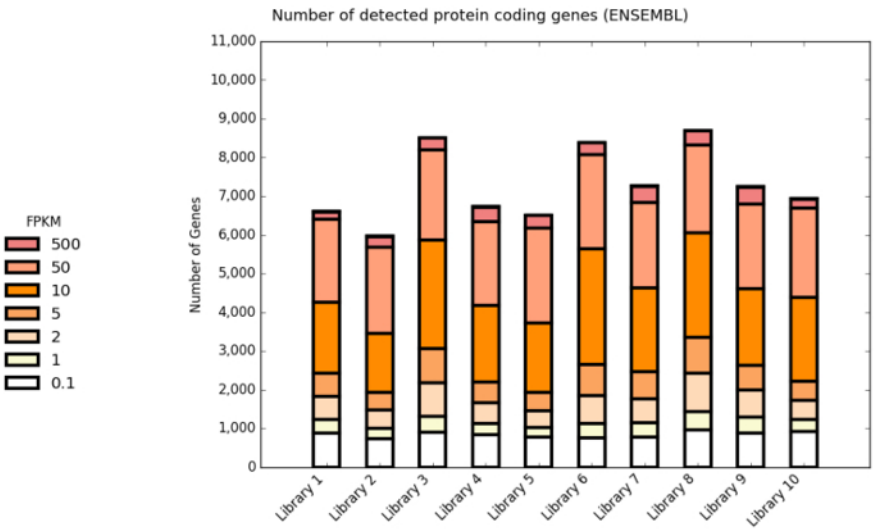

Figure 2. The Number of Protein Coding Genes Detected in the Single Dopaminergic RNA-Seq Libraries Generated from TH-eGFP-positive Cells. 6,000 - 8,000 protein genes were detected in the single cell libraries. FPKM: Fragments per kilobase of transcript per million mapped reads (FPKM). Please click here to view a larger version of this figure.

Genes	DA single cells n = 10	GABAergic single cells n = 7
	FPKM (Mean ±SEM)	
TH	4714 ±764	1.2 ±0.14
DDC	779 ±167	0.8 ±0.1
slc6a3/DAT	506 ±144	0.5 ±0.04
Drd2	14 ±8	0.1 ±0.004
chrna6 (α6)	174 ±57	0.0
chrna4 (α4)	17 ±5	26.4 ±2.3
chrnb2 (β2)	9 ±3	17.3 ±1.2
chrnb3 (β3)	38 ±13	0.0
Gad1	0.0	959.0 ±70.0
Htr3a	0.0	0.6 ±0.01
Htr3b	0.0	0.0

Table 1. Dopamine-related and nAChR Gene Transcripts in Dopaminergic and GABAergic Neurons at Day 21 in VentrMidbrain Cultures. RNA-Seq data for TH-eGFP positive and negative neurons are shown. Single cells were harvested on culture day 21. RNA-Seq data show that TH-eGFP positive neurons had high levels of DA-related gene transcripts, including tyrosine hydroxylase (TH), dopa decarboxylase (DDC), the dopamine transporter DAT (slc6a3), and nicotinic acetylcholine receptor (nAChR) subunits including α6, α4, β2 and β3. However, these cells had low levels of the GABAergic marker gene glutamic acid decarboxylase 1 (Gad1) and the serotonin 5-HT3 receptor A and B subunits (Htr3A and Htr3B). GABAergic neurons have low to no levels of DA-related gene transcripts, chrna6, chrnb3, and Htr3A/B, but have high levels of Gad1. RNA-Seq (Cuffdiff) data are shown for dopamine-related, GABAergic, nicotinic receptor, and serotonin transcripts. FPKM: Fragments per kilobase of transcript per million mapped reads (FPKM).

Discussion

Here we isolate single cells from a heterogeneous population using a fluorescent tag, then study each cell with single-cell RNA-seq. We report that 100% of the live TH-eGFP cells that we harvested and sequenced were indeed dopaminergic neurons, based on the presence of the following three DA-related gene transcripts, TH, DDC and slc6a3. All of the TH-eGFP positive cells expressed these three genes as assessed by RNA-Seq. This was concordant with electrophysiological studies that showed that each TH-eGFP cell also displayed a repertoire of ion channels associated with dopaminergic neurons[8,9]. In this protocol we utilized the GENSAT tyrosine hydroxylase-eGFP mouse strain[10] as a reliable marker for live dopaminergic neurons. In WT cultures there is presently no reliable way to identify live dopaminergic neurons based on their morphology. Despite our extensive experience in identifying and recording from dopaminergic neurons, in the wildtype cultures only three out of ten of the suspected dopaminergic cells harvested were dopaminergic neurons. Additionally, we observed that not all of the cells that were negative for TH were GABAergic. We defined cells as GABAergic based on the presence of the Gad1 transcript and the absence of the DA-related transcripts as assessed by RNA-seq.

Because dopaminergic neurons represent a small minority of the neurons expressed in primary VM cultures, the ability to reliably identify these neurons in living cultures will enhance the range of available single-cell studies. This protocol can be used for neuroprotection, neurodegeneration, and pharmacological assays to study the effects of various treatments on the dopaminergic transcriptome. Additionally, one can use this protocol for immunohistochemical, electrophysiological, biophotonic[11], and in principle, proteomic assays. These assays are especially useful for Parkinson's disease and addiction research. But of course analogous protocols, on GENSAT mice or similarly labeled mice, could be used for other disease states or for cells that are rare. Additionally, this protocol gives an emerging view of the heterogeneity within a given neuronal subtype.

In previous studies, after harvesting the single cell we placed it directly into PBS; however now we place the harvested single cell directly into the reaction buffer. This results in better quality RNA-Seq libraries as assessed by numbers of genes detected in each library. One of the main limitations of the technique is that fluorescently tagged cells are required. As stated earlier, there is no truly reliable way to identify a specific cell type in a mixed culture based on morphology. The large range of cell-specific fluorescent or Cre-expressing mouse lines now provides appropriately marked cells in many cases. However, care should be taken when selecting a suitable fluorescent mouse line, as an extensive study of transgenic mouse lines found that TH-Cre knock-in mouse lines exhibit pronounced transgene expression in nondopaminergic cells[12].

Additionally, another step that may need to be optimized in future experiments is the desired tip size of the micropipette. If future experiments include the use of cell types other than dopaminergic neurons, the micropipette tip size may need to be optimized for the cell of interest.

RNA deep sequencing is a more powerful technique than microarrays. RNA-Seq gives good run-to-run reproducibility. The dynamic range detected is comparable to the actual transcript abundance within cells. One can detect alternative splice forms and novel isoforms. *De novo* analysis of samples without a reference gene is possible; and if a new reference gene is discovered the data can be re-analyzed for the specific gene(s) without having to run the wet-work experiment again[13].

To summarize, we report that 100% of the live TH-eGFP cells that were harvested and sequenced were dopaminergic neurons. This technique extends other reports that identify acutely dissociated or long-term cultured dopaminergic neurons from GENSAT mice,[14,15] and the technique will have widespread applications in neuroscience and molecular biology.

Disclosures

The authors have nothing to disclose.

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