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Application of Cell-Specific Isolation to the Study of Dopamine Signaling in *Drosophila*

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Abstract

Dopamine neurotransmission accounts for a number of important brain functions across species including memory formation, the anticipation of reward, cognitive facilities, and drug addiction. Despite this functional significance, relatively little is known of the cellular pathways associated with drug-induced molecular adaptations within individual neurons. Due to its genetic tractability, simplicity, and economy of scale, *Drosophila melanogaster* has become an important tool in the study of neurological disease states, including drug addiction. To facilitate high-resolution functional analyses of dopamine signaling, it is highly advantageous to obtain genetic material, such as RNA or protein, from a homogeneous cell source. This process can be particularly challenging in most organisms including small model system organisms such as *Drosophila melanogaster*. Magnetic bead-based cell sorting has emerged as a powerful tool that can be used to isolate select populations of cells, from a whole organism or tissue such as the brain, for genomic as well as proteomic expression profiling. Coupled with the temporal and spatial specificity of the *GAL4/UAS* system, we demonstrate the application of magnetic bead-based cell sorting towards the isolation of dopaminergic neurons from the *Drosophila* adult nervous system. RNA derived from these neurons is of high quality and suitable for downstream applications such as microarray expression profiling or quantitative rtPCR. The versatility of this methodology stems from the fact that the cell-specific isolation method employed can be used under a variety of experimental conditions designed to survey molecular adaptations in dopamine signaling neurons including in response to drugs of abuse.

Keywords

Drosophila; Dopamine signaling; *GAL4/UAS*; Magnetic bead cell sorting; Brain; RNA isolation

1. Introduction

Dopamine neurotransmission systems are found in various species where they signal the reinforcing properties of natural rewards such as food, sex, and social interaction (1–4). Genes involved directly or indirectly in the actions of drugs of abuse appear to also be conserved across evolution (5, 6), suggesting a preservation of dopamine mechanisms on addictive behaviors in various species. Due to its genetic tractability, simplicity, and

economy of scale, *Drosophila melanogaster* has become an important tool in the study of neurological disease states, including drug addiction (7). Previous findings indicate that dopamine plays a key role in responses of *Drosophila* to addictive drugs such as ethanol, nicotine, and cocaine (5, 8–11).

Magnetic bead-based cell sorting has emerged as a powerful tool that can be used to isolate select populations of cells, from a whole organism or specific tissue type such as brain, for genomic, as well as proteomic expression profiling (12, 13). Coupled with the temporal and spatial specificity of the *GAL4/UAS* system, we demonstrate the application of magnetic bead-based cell sorting to the specific isolation of genetically labeled, wild-type populations of dopaminergic neurons from the *Drosophila* adult brain. This technique provides a significant advantage over existing methodologies wherein samples derived from the brain may be of heterogeneous cell populations thereby confounding molecular analyses of dopamine signaling in either wild-type or experimentally challenged dopaminergic neurons. RNA derived from these neurons is suitable for downstream applications such as microarray expression profiling or quantitative rtPCR. The versatility of this methodology stems from the fact that cell-specific isolation can be used under a wide variety of experimental conditions designed to survey molecular signaling adaptations in dopaminergic neurons such as in response to drugs of abuse. Moreover, the availability of *Drosophila* strains and commercially available reagents (including antibodies, magnetic beads, and RNA purification kits optimized for recovery from relatively small, pure populations of cells at low elution volumes) provides a unique tool for investigating dopamine signaling at the level of cellular resolution.

2. Materials

2.1. Magnetic Bead Preparation

1. 10× Phosphate-buffered saline (PBS) (MP Bioproducts, Solon, OH) diluted to 1× working solution with RNase-free double-distilled water (ddH₂O) and stored at room temperature (see Note 1).
2. RNase-AWAY (Sigma-Aldrich, St. Louis, MO) stored in a spray-bottle at room temperature for rendering the working surfaces RNase free.
3. Biotinylated Rat anti-Mouse-CD8a antibody (eBioscience, San Diego, CA) 500 µg/mL stored at 4°C.
4. Dynabeads MyOne Streptavidin T1 (Invitrogen, Life Technologies, Grand Island, NY), stored at 4°C. Once coupled to antibody, the magnetic beads are stored at 4°C until needed and maximally up to 1 month. 1 mg of beads can bind up to 20 µg of biotinylated antibody.
5. (8) Rare Earth Magnet Blocks: 2 in. (length) × 0.5 in. (width) × 0.125 in. (thickness) (Magcraft, Vienna, VA). Alternatively DynaMagTM-2 (Invitrogen) may be used.

¹All solutions must be prepared in RNase-free double-distilled water (not DEPC treated). BSA can be prepared prior to each experiment by dissolving the appropriate, pre-weighed quantity of BSA in PBS and vortexing at maximum speed.

6. Tabletop Vortex.
7. Crushed ice for incubating the antibody bead mixture.

2.2. Dissecting and Washing Adult Fly Heads

1. 10× Phosphate-buffered saline (PBS) diluted to 1× working solution in RNase-free ddH₂O and chilled on ice (see Note 2).
2. Transgenic flies expressing *UAS-mCD8::GFP* (Bloomington Stock Center, stock# 30125) under the control of *ple-GAL4* (Bloomington Stock Center, stock# 8848). *ple-GAL4* (also known as *TH-GAL4*) drives the expression of *GAL4* under the control of the *pale* (*ple*) promoter specifically within dopaminergic neuron clusters in the adult *Drosophila* brain which project to specific regions within the mushroom bodies and to the central complex (14) (Fig. 1). The *Drosophila ple* gene encodes the enzyme tyrosine hydroxylase, which is required for dopamine biosynthesis.
3. Glass Pasteur pipettes, fire-polished by quickly rotating the pipette tip over a hot Bunsen burner flame until the pipette edges become smooth.
4. Dissecting tools: two pairs of sharp Dumont No. 5 forceps.

2.3. Dissociating the Tissue into a Single Cell Suspension

1. 10× Phosphate-buffered saline (PBS) diluted to 1× working solution in RNase-free ddH₂O and chilled on ice.
2. Kontes Glass Tissue Grinder, 7 mL working capacity, with large clearance pestle (Kimble Chase, Vineland, NJ) (see Note 3).
3. BSA (Bovine Serum Albumin), Fraction V. Freshly prepare 10–15 mL of 1% BSA solution in 1× PBS and store on ice (see Note 1). This is used for coating the tubes and pipettes to reduce cell loss due to adhesion to glass surfaces.
4. 30 µm cell filters, e.g., MACS Pre-Separation Filter (Miltenyi Biotec, Cambridge, MA).
5. Pipettes (P-1000, P-100, P-10) with disposable, low-retention plastic tips.
6. Pasteur pipettes with fire polished tips of standard diameter, narrowed to ~50% of standard diameter for trituration. Fire polishing and narrowing of Pasteur pipettes is achieved over a Bunsen burner flame by rotating the tip in the flame to polish edges and narrow tip diameter.
7. Fluorescent stereomicroscope equipped with GFP filter-set (a Leica MZ16FA was used in this protocol).
8. Table top micro-centrifuge (1–16,000 × *g*).

²It is important to pre-cool the PBS used for dissection of fly heads. Placing the bottle of PBS covered in crushed ice for an hour before the experiment starts can do this. Alternatively, PBS can be stored at 4°C overnight.

³Pre-cool the tissue grinder/pestle by placing it on ice for a few minutes to prevent cell damage/lysis during tissue homogenization.

9. Tabletop Vortex.
10. Crushed ice.

2.4. Magnetic Bead Cell Sorting

1. 10× Phosphate-buffered saline (PBS) diluted to 1× working solution in RNase-free ddH₂O and chilled on ice.
2. Anti-CD8a antibody-coated magnetic beads from Subheading 3.1, step 4.
3. BSA (Bovine Serum Albumin), Fraction V. Freshly prepare 10–15 mL of 1% BSA solution in 1× PBS and store on ice. This is used for coating the tubes and pipettes to reduce cell loss due to adhesion to glass surfaces.
4. (8) Rare Earth Magnet Blocks: 2 in. (length) × 0.5 in. (width) × 0.125 in. (thickness) (Magcraft). Alternatively DynaMagTM-2 (Invitrogen) may be used.
5. Pipettes (P-1000, P-100, P-10) with disposable, low retention plastic tips.
6. Standard hemocytometer to count the cells and estimate purity.
7. Pasteur pipettes with fire polished tips of standard diameter.
8. Fluorescent stereomicroscope equipped with GFP filter set (a Leica MZ16FA was used in this protocol).
9. Table top micro-centrifuge (1–16,000 × *g*).
10. Tabletop Vortex.
11. Crushed ice.

2.5. RNA Isolation from Magnetic Bead Sorted Cells

1. PicoPure[®] RNA Isolation Kit (Applied Biosystems, Life Technologies).
2. Pipettes (P-1000, P-100, P-10) with disposable, low retention plastic tips.
3. (8) Rare Earth Magnet Blocks: 2 in. (length) × 0.5 in. (width) × 0.125 in. (thickness) (Magcraft). Alternatively DynaMagTM-2 (Invitrogen) may be used.
4. Heating block pre-heated to 42°C stored inside an incubator, pre-set to 42°C.
5. Table top micro-centrifuge (1–16,000 × *g*).
6. Tabletop Vortex.
7. RNase-Free DNase set (Qiagen, Valencia, CA) (Optional step. Needed if performing DNase treatment prior to RNA column purification).

3. Methods

To ensure high-quality RNA isolation from dopaminergic neurons, it is imperative that standard lab procedures for maintaining a clean, RNase-free environment be observed throughout this protocol in order to prevent RNA degradation (see Note 1). Preparation for this method requires isolation of age-matched *Drosophila* adults bearing a *ple-GAL4*

transgene and a *UAS-mCD8::GFP* transgene in order to genetically label dopaminergic neurons for visualization and CD8-mediated cell isolation via magnetic bead cell sorting. Prior to initiating dissection of adult *Drosophila* heads, it is important to prepare magnetic beads in advance for use in cell binding. The antibody-bound magnetic beads are then stored at 4°C until ready for cell isolation. Following dissection of heads from *ple-GAL4,UASmCD8::GFP* adults, the tissue is dissociated and subjected to filtration into single cell suspensions. These single cell suspensions are verified by epi-fluorescent microscopy for the presence of the genetically labeled dopaminergic neurons, which uniquely express green fluorescent protein (GFP). Once verified, antibody-coated magnetic beads are added to the single cell suspensions and allowed to incubate for a limited time to promote high affinity and specificity interactions between the antibody-coated beads and the genetically labeled dopaminergic neurons. Homogenous, cell-specific isolation is achieved by placing the cell suspension in a strong magnetic field to pellet the target cells, followed by rigorous washing to remove any non-specific contaminating cells. The isolated, purified population of cells can be visually verified for specificity by epi-fluorescent assessment of GFP expression in all cells. Ultimately, the purified population of dopaminergic neurons is subjected to RNA isolation for downstream applications. Figure 2 schematically summarizes the procedures involved in this protocol and Table 1 summarizes the timing required for each step of the protocol.

3.1. Preparing Magnetic Beads for Binding Cells: (75–90 min)

This step must be completed prior to the start of the experiment. The prepared beads can be stored at 4°C until needed (see Note 4).

1. Wash 100 µL of Dynabeads StreptavidinT1-coated beads three times in PBS by resuspending the magnetic beads in 1 mL of fresh 1× PBS and pelleting the beads in a strong magnetic field after each wash. Discard the supernatant after each wash step.
2. Resuspend the beads directly in 100 µL of undiluted biotinylated rat anti-mouse-CD8a antibody (antibody concentration is 500 µg/mL).
3. Incubate the mixture for 1 h on ice with occasional mild vortexing to prevent sedimentation [Dynabeads T1 can bind 20 µg/mg of biotinylated Ig].
4. Wash the bead–antibody mixture three times as described in step 1 above to remove any unbound excess antibody. The magnetic beads are now coated with antibody and ready to be used. Store the bead-antibody mixture in 100 µL of 1× PBS at 4°C until use.

3.2. Dissecting and Washing Adult Fly Heads: (10–15 min)

1. Dissect 30–50 age-matched adult *Drosophila* heads from the *ple-GAL4,UAS-mCD8::GFP* strain. Dissection of adult heads is achieved by using two pairs of sharp Dumont No. 5 forceps in cold 1× PBS (see Note 2). One pair of forceps is

⁴The volume of antibody–bead mixture prepared depends on the number of CD8a-positive cells to be isolated given that 1 mg of beads can bind up to 20 mg of biotinylated antibody. Once prepared, it is recommended that the antibody–bead mixture be used within 4 weeks.

used to hold the fly in place at the thorax, while the other pair of forceps is placed around the rear side of the fly head where it meets the thorax. Using the pair of forceps placed behind the fly head, gently separate the head from the body of the fly and transfer the intact head to a 1.6 mL microfuge tube filled with 1–1.2 mL of 1× PBS on ice (see Note 5).

2. Once all fly heads have been dissected and transferred to the microfuge tube, close the tube and vortex it at the maximum setting three times for 1 s each.
3. Using a fire-polished Pasteur pipette discard the supernatant completely. Repeat the wash (Subheading 3.2, step 1) and vortex (Subheading 3.2, step 2) steps 3–4 times until the supernatant is visibly clear of any and debris.

3.3. Dissociating the Tissue Into a Single Cell Suspension: (18–20 min)

1. To avoid the cells from sticking to the glass surface of the pre-chilled 7 mL Kontes tissue grinder and large clearance pestle (see Note 3), pre-coat the tissue grinder and pestle with a 1% BSA in 1× PBS solution and after a brief rinse discard the BSA solution. Subsequently, using a P-1000 pipette with a cut low retention filter plastic filter tip, transfer the heads in 1 mL 1× PBS from step Subheading 3.2, step 3, and add an additional 3–4 mL of 1× PBS to the BSA-coated tissue grinder.
2. Homogenize the tissue with slow and steady strokes, avoiding frothing (approximately 20–30 strokes) (see Note 6).
3. To assess the cell dissociation levels, pipette out a small sample of the solution (up to 50 µL), and observe it under a fluorescent stereomicroscope using the GFP filter set. If one still observes intact or incompletely dissociated tissue, homogenize further.
4. Triturate the solution five times with a fire-polished Pasteur pipette narrowed to approximately 50% of the standard tip diameter (see Note 7).
5. Filter the solution through a 30-Mm cell filter and collect the cell filtrate in a 1.6 mL microfuge tube. The resulting solution should consist of a single cell suspension and is now ready for magnetic cell sorting. Take a small sample of the dissociated cells and observe it under a fluorescent stereomicroscope. If cell-clumps are observed, pass the suspension again slowly through a new 30 µm filter.

⁵This protocol describes a high-throughput method of isolating specific neuronal cells from the *Drosophila* brain beginning with intact heads. We find that this method is highly efficient and isolation of single cell suspensions is not impeded by the head cuticle, which is filtered out when the sample is run through the 30 mm filter. Alternatively, if desired, the fly brains can be dissected according to the method described (15) prior to dissociation, however this will substantially increase the time required during the dissection stage of the protocol.

⁶It is important to homogenize the tissue slowly and steadily without any sudden movements, to avoid unwanted stress and damage to the brain cells.

⁷Trituration must be performed slowly, as forceful trituration may damage cells.

3.4. Magnetic Bead Cell Sorting: (45–75 min, Depending on Antibody Incubation Time)

1. Add 15 μ L of antibody-coated magnetic beads per 1 mL of cell suspension (Subheading 3.3, step 5). The remaining antibody-conjugated magnetic beads can be stored at 4°C until needed for subsequent cell isolations.
2. Incubate the cells with antibody-coated magnetic beads for 30–60 min on ice with occasional hand-mixing via inversion of the microfuge tube (see Note 8).
3. Place the microfuge tube in a strong magnetic field for 2 min. All positively selected cells along with unbound antibody-coated magnetic beads will separate to the side of the tube.
4. Slowly pipette the supernatant, making sure not to disturb the cell pellet and discard.
5. Wash the cells 3–4 times in fresh, ice-cold 1 $\times$ PBS followed by magnet-induced pelleting to remove any remaining nonspecific cells.
6. Resuspend the specifically bound cells coupled to the magnetic beads in 30 μ L of fresh, ice-cold 1 $\times$ PBS.
7. To approximate the purity and yield of cells, pipette 5 μ L of the cell suspension on the polished surface of a hemocytometer and count all the visible fluorescent cells under a fluorescent stereomicroscope equipped with a GFP filter set. Also assess the relative amount of nonfluorescent cells or any other signs of impurities. Typically the sample will be highly enriched for fluorescently labeled, GFP-positive cells (Fig. 3).

3.5. RNA Isolation from Magnetic Bead Sorted Cells: (60–75 min)

Although this protocol is focused on RNA isolation as described here, this step of the overall dopaminergic cell isolation can be replaced by other protein/DNA/chromatin isolation methods depending on nature of the study.

1. After counting, pellet the cells in a magnetic field, discard the supernatant and add 20 μ L of extraction buffer from the PicoPureTM RNA Isolation Kit (Molecular Devices). Depending upon cell number one may need to add a higher volume of extraction buffer.
2. Vortex the tube at maximum speed to enable the mixing of cell pellet with extraction buffer.
3. Incubate the microfuge tube at 42°C for 30 min.
4. To ensure removal of the magnetic beads prior to column purification of the RNA, the tube is briefly centrifuged at 2,000 $\times g$ for 2 min to pellet the magnetic beads. The tube is then placed in a strong magnetic field to retain the pellet, and the supernatant is transferred to a new microfuge tube.

⁸It is important to note that during the incubation of the antibody step, high temperatures or long incubation periods can result in a loss of antibody specificity thereby compromising purity in the isolation procedure.

5. Extract and column purify the RNA according to the PicoPure™ RNA extraction kit manufacturer's instructions. DNase treatment is optional and can be performed on column during the RNA purification according to the analysis requirement. Finally, elute the bound total RNA in a small volume (11–30 µL) of elution buffer and store at –80°C until ready for use. If desired a 1 µL aliquot may be used to assess total RNA quality on a Bioanalyzer 2100 (Agilent Technologies, Inc.) (Fig. 4).

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Fig. 1.

Dopaminergic neuron clusters in adult *Drosophila* brain. Confocal image of whole mount adult *Drosophila* brain from a strain bearing the *ple-GAL4* and *UAS-mCD8::GFP* transgenes. *ple-GAL4* drives expression of *UAS-mCD8::GFP* specifically in dopaminergic neurons of the adult brain.

Fig. 2.

Schematic of procedure for magnetic bead cell sorting of *Drosophila* dopaminergic neurons. (a) Age-matched, intact adult *Drosophila* heads carrying the *ple-GAL4* and *UAS-mCD8::GFP* transgenes are dissected. (b) The heads are transferred to a microfuge tube and washed/vortexed to remove any debris. (c) The heads are then transferred into a 7 mL dounce to homogenize the brain tissue followed by trituration to further dissociate the tissue into a cell suspension. (d, e) The homogenate is then filtered using a 30 µm cell filter to remove any large cellular debris, including the cuticle from the head and filter out the single cell suspension. The filtrate solution contains a single cell suspension of different cell types including neurons and glia. (f) Biotinylated anti-mouse-CD8-antibody-coated magnetic Streptavidin T1 Dynabead are added to the cell suspension, and incubated on ice for 30–60 min. (g) The magnetic beads specifically bind to the dopaminergic neurons that express a mouse CD8-tagged GFP fusion protein under the control of the *ple-GAL4* driver. (h, i) The magnetic bead-coated cells are separated by placing the cell solution in a strong magnetic field. The supernatant is discarded, and the cells are washed three times to remove any nonspecific cells, resulting in (j) highly enrichment of specific dopaminergic neurons. Panels (b–j) are adapted in part (14).

Fig. 3.

Magnetic bead isolated dopaminergic neurons. Representative epi-fluorescent image of a field of positively selected, GFP fluorescent dopaminergic neurons isolated by magnetic bead cell sorting from the adult *Drosophila* brain. The purified cell population of neurons was determined to have little or no contaminating cell impurities. The variation in cell body size reflects natural variation seen in the adult brain among distinct dopaminergic neuron clusters.

Fig. 4.

Bioanalyzer analysis reveals high-quality RNA purification from isolated neurons. Representative Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) electropherogram of total RNA isolated from magnetic bead sorted dopaminergic neurons. The electropherogram reveals excellent RNA quality and integrity suitable for a wide range of downstream applications including microarray analyses and qPCR as indicated by the presence of the 5.8S, 18S, and 28S rRNAs.

Table 1

Summary of experimental work flow and timings

Procedure	Timing
Preparing magnetic beads for binding (prepared prior to cell isolation experiment)	75–90 min
Dissecting and washing adult fly heads	10–15 min
Dissociating the tissue into a single cell suspension	18–20 min
Magnetic bead cell sorting	45–75 min
RNA isolation from magnetic bead sorted cells	60–75 min