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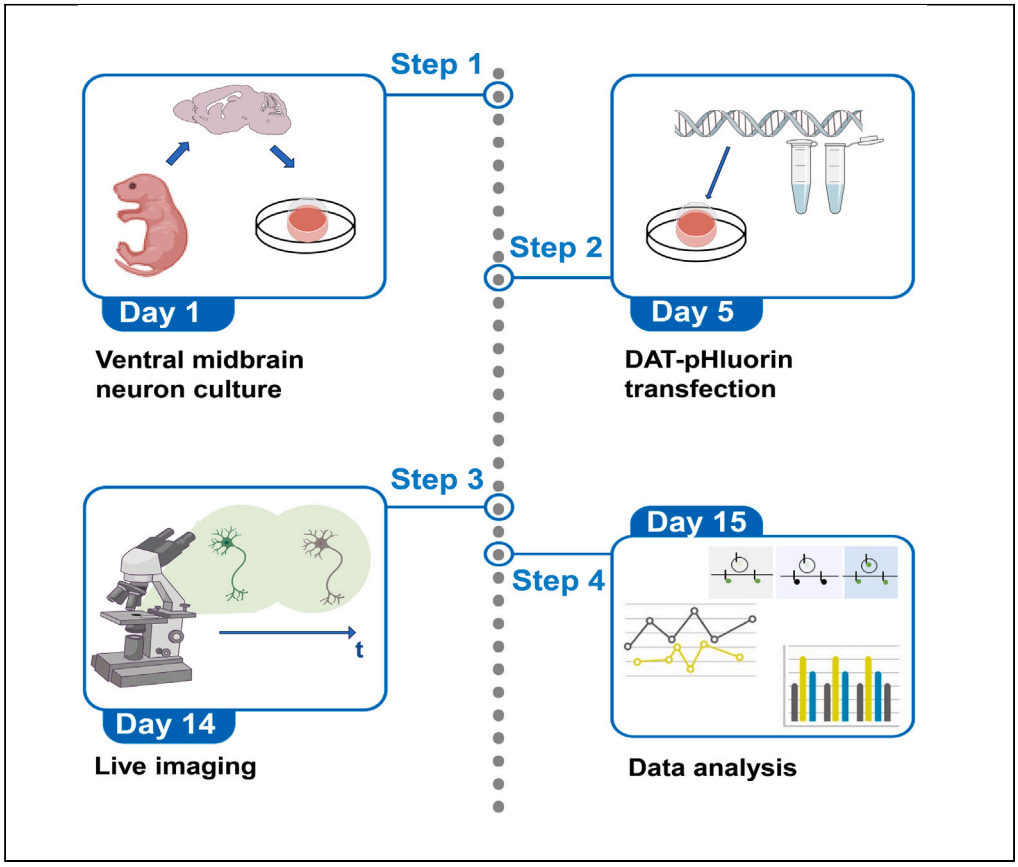
Protocol for live neuron imaging analysis of basal surface fraction and dynamic availability of the dopamine transporter using DAT-pHluorin



Surface availability of the dopamine (DA) transporter (DAT) critically influences DA transmission. Here, we present a protocol that describes the preparation of mouse ventral midbrain neurons, the expression of a new optical sensor, DAT-pHluorin, and the utilization of this sensor to analyze the surface availability of DAT in live neurons via fluorescent microscopy. This approach allows quantitative measures of basal surface DAT fraction under genetic backgrounds of interest and live trafficking of DAT in response to psychoactive substances.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Elnaz Khezerlou, Jacqueline Saenz, Sanjana Surya Prakash, Ping-Yue Pan

pingyue.pan@rutgers.edu

Highlights

Steps for preparing mouse ventral midbrain culture

Steps for quantifying basal surface dopamine transporter fraction using live imaging

Steps for measuring surface dopamine transporter dynamics

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Protocol for live neuron imaging analysis of basal surface fraction and dynamic availability of the dopamine transporter using DAT-pHluorin

Elnaz Khezerlou,[1,4,5] Jacqueline Saenz,[1,2,4,5] Sanjana Surya Prakash,[1,3,5] and Ping-Yue Pan[1,4,6,7,*]

[1]Department of Neuroscience and Cell Biology, Rutgers University Robert Wood Johnson Medical School, 675 Hoes Lane West, Piscataway, NJ 08854, USA

[2]Rutgers School of Graduate Studies, Molecular Biosciences Graduate Program, 25 Bishop Place, New Brunswick, NJ 08901-1178, USA

[3]Rutgers School of Graduate Studies, Biomedical Science Master's Program, 25 Bishop Place, New Brunswick, NJ 08901-1178, USA

[4]Rutgers Addiction Research Center, Rutgers Brain Health Institute, 683 Hoes Lane West, Piscataway, NJ 08854, USA

[5]These authors contributed equally

[6]Technical contact

[7]Lead contact

*Correspondence: pingyue.pan@rutgers.edu
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SUMMARY

Surface availability of the dopamine (DA) transporter (DAT) critically influences DA transmission. Here, we present a protocol that describes the preparation of mouse ventral midbrain neurons, the expression of a new optical sensor, DAT-pHluorin, and the utilization of this sensor to analyze the surface availability of DAT in live neurons via fluorescent microscopy. This approach allows quantitative measures of basal surface DAT fraction under genetic backgrounds of interest and live trafficking of DAT in response to psychoactive substances.

For complete details on the use and execution of this protocol, please refer to Saenz et al.[1]

BEFORE YOU BEGIN

DAT is a 12 transmembrane-spanning protein, specifically expressed in DA neurons. DA signaling in the brain is significantly influenced by DAT not only by its DA reuptake, but also by the Na^+/Cl^- symporter function to regulate membrane excitability.[2] Mounting evidence suggests that DAT undergoes constitutive recycling as well as ligand-induced recycling in a time scale of seconds to minutes. Notably, many drugs of abuse such as cocaine act by directly targeting DAT and inhibiting its reuptake activity to amplify DA signaling.[3–6] There is an abundance of *in vitro* and *in vivo* evidence suggesting that abusive substances like cocaine and amphetamine alter DAT surface availability,[7–9] yet their mechanisms have not been completely elucidated. Abnormal DAT scans have been observed in the early stages of Parkinson's disease (PD) suggesting the loss of DAT maintenance and its potential role in the pathogenesis.[2,10–13] Animal models of PD often exhibit impaired amphetamine-induced locomotor hyperactivity, which is also suggestive of reduced DAT availability.[14] Assays that allow the evaluation of basal DAT surface fraction and the dynamic trafficking of DAT will accelerate our understanding of the disease mechanisms of PD and substance use disorder.

The protocol below describes a detailed methodology that covers the preparation of the ventral midbrain DA neuron culture, optical sensor expression, and live imaging that allows the measurements of 1) basal level DAT surface fraction, 2) the pH of intracellular DAT-containing vesicles and, 3) the dynamics of ligand-induced DAT trafficking in neurites. These measures can be compared across different

genetic backgrounds. This is achieved by using a newly generated genetically encoded DAT reporter, DAT-pHluorin. PHluorin is a pH-sensitive fluorescent protein, also known as superecliptic pHluorin (SEP).[15] We conjugated pHluorin to the 2nd extracellular loop of human DAT cDNA using a pair of linkers.[1] Our previous study demonstrated that DAT-pHluorin exhibits a pKa of 7.0.[1] Due to the neutral pH of the extracellular space and acidic nature of intracellular compartments, DAT-pHluorin is bright on the plasma membrane and becomes dimmer when endocytosed. This biophysical property transforms a submicron scale molecular trafficking event to measurable fluorescence changes that are accessible via conventional microscopy. By expressing DAT-pHluorin in cultured DA neurons, we provide a technology that allows live imaging of the localization of DAT in thin neurites, which is superior in time resolution to conventional methods, such as immunocytochemistry.

Brief overview of the experimental methods

Cultured ventral midbrain neurons are prepared as previously described[16,17] and contain ~10% DA neurons. Tissues from 3–5 pups are pooled to generate a culture of 9–15 dishes. The DAT-pHluorin plasmid is typically transfected between 3 and 5 days *in vitro* (DIV) using calcium phosphate or between 5 and 7 DIV with lipofectamine 2000. The live imaging is performed on DIV 12–17 when the neurons have reached full maturity. Neurons grown on the cover glass are loaded onto a custom-built chamber which allows for the laminar flow of perfusion solutions. The cover glass-chamber apparatus is then transferred to nestle in a Warner PH1 chamber and imaged by a Nikon wide-field fluorescent microscope. The temperature of the perfusion solution is maintained at a range of 30°C–34°C by an objective heater. The microscope body is connected to an Andor Ultra897 EMCCD camera run by the NIS Elements software. Neurons are perfused with a modified Tyrode's solution by gravity flow with timed on/off switch through the ValveLink system. All solutions are removed by an adjustable vacuum line on the other side of the chamber.

To measure the basal level DAT surface fraction, we sequentially perfuse two different pH solutions during live imaging and use the Henderson-Hasselbalch equation during *post hoc* analysis (see step-to-step procedures below). DAT-pHluorin fluorescence is monitored by a GFP excitation/emission cube at 1 Hz. To measure the dynamic availability of DAT to a pharmacological agent, test agent is delivered following a stable baseline recording in normal Tyrode's, typically 30–60 frames at designed frame rate. Imaging data is saved as TIFF files and later analyzed in ImageJ using the "Time Series Analyzer" plug-in. Following the experiment, neurons are fixed in 4% PFA and immunocytochemical analysis of neuronal markers is performed. Some of the worthy markers for midbrain neurons are 1) tyrosine hydroxylase (TH), which is a marker for DA neurons; 2) MAP2 or NeuN, which distinguishes neurons from astrocytes and other glia present in the culture that may also express DAT-pHluorin; 3) Synapsin, which provides discrete staining at presynaptic sites, allowing for the analysis of DAT in synapse versus neurites.

Institutional permission

The protocol described below is approved by the following IACUC protocol: PROTO201800183.

DAT-pHluorin DNA prep

Timing: >1 week before imaging experiment

1. Use QIAGEN endotoxin-free maxi-prep kit (Qiagen, 12362) to prepare the CAGp-DAT-pHluorin plasmid for transfection.

Set up the perfusion system

Timing: >1 week before imaging experiment

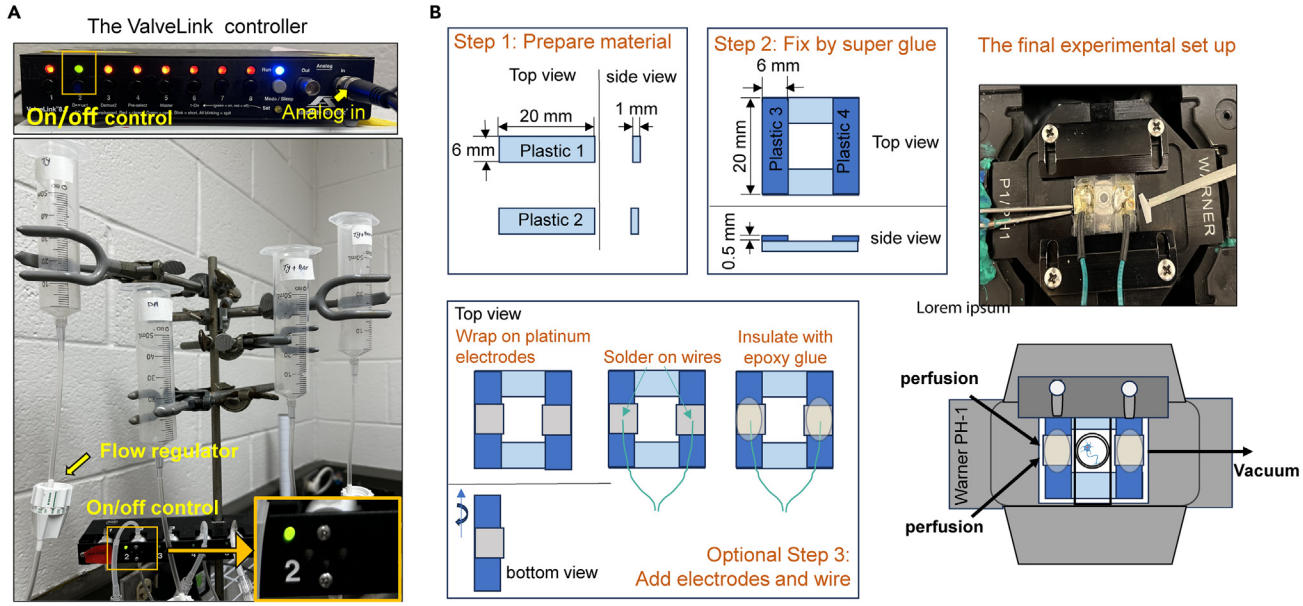

Figure 1. Step-by-step illustration for preparing the perfusion and imaging chamber

(A) Image of the gravity flow perfusion reservoir (syringe), flow regulator, and on/off control, which is synchronized to the ValveLink Controller (top) that takes analogue commands from the Elements software.

(B) Step-by-step illustration of building the imaging chamber with optional steps compatible for electrical stimulation (not included in this protocol).

2. Use a silicone tubing to connect the reservoir (BD 50 mL Luer Lock Syringe) to the perfusion inlet (see Figure 1A) on the ValveLink8.2 controller (Automate Scientific).

   Note: The controller receives input from the NIS-Elements software to achieve timed on/off control of perfusion.

3. Connect a flow rate regulator (Automate Scientific, SKU: 06–21) within the perfusion line to adjust the rate of perfusion.
4. Connect a flat-end needle on the other end of the tubing.
5. Make a custom-built laminar flow imaging chamber to be mounted on a Warner PH1 chamber (See Figure 1B).

   Note: The principle of the design of the imaging chamber is to allow fast and timed exchange of solutions for testing the effects of a reagent.

6. Use non-collapsing thick wall tubing to build the vacuum line. Connect another flow regulator to the vacuum line to adjust the speed of solution removal.

   Note: Glass-bottom imaging dishes or circulating pump may be used as an alternative, however, it should be noted that the speed of a complete solution exchange will occur at a much slower rate.

Set up temperature control

Timing: >1 week before imaging experiment (for steps 7 and 8)

Timing: Any time before imaging experiment (for steps 9 and 10)

7. Install the Tokai Hit heating collar (Part#: tokai-hit-tpie-lh) on the Nikon Plan Apo 60X objective.
8. Measure the flow through of the perfusion media to be at 30°C.

Note: Temperature and CO_2 controlled imaging incubator, thermal plate, or inline heater could be good alternatives if available.

Optional: Obtain controlled substances

9. Purchase D-Amphetamine Hemisulfate Salt (Sigma A5880) from Sigma through authorized institutional distributors.
10. Prepare stock solutions in 10 mM in PBS and used at 1000x dilution or higher.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken anti-GFP	Thermo Fisher Scientific	Cat# A-10262; RRID: AB_2534023 1:1000 dilution
Mouse anti-TH	Sigma	Cat# T2928; RRID: AB_477569 1:1000 dilution
Rabbit anti-TH	Novus Biologicals	Cat# NB300-109; RRID: AB_10077691 1:1000 dilution
Rat anti-DAT	Millipore	Cat# MAB369; RRID: AB_2190413 1:1000 dilution
Chicken anti-MAP2	Novus Biologicals	Cat# NB300-213; RRID: AB_2138178 1:500 dilution
NeuN	Thermo Fisher Scientific	Cat# MA5-33103; RRID: AB_2802653 1:1000 dilution
Chemicals, peptides, and recombinant proteins		
Cytosine β-D-arabinofuranoside hydrochloride (ARA-C)	Sigma-Aldrich	Cat# C6645
GDNF	Sigma-Aldrich	Cat# G1777-10UG
D-amphetamine hemisulfate salt	Sigma-Aldrich	Cat# A5880
Poly-L-Ornithine	Sigma-Aldrich	Cat# P3655
Paraformaldehyde 16%	Electron Microscopy Sciences	Cat# 15710
Experimental models: Organisms/strains		
C57BL/6J mice, postnatal day 0–1, both sexes	IMSR_JAX	Cat# 000664; RRID: IMSR_JAX:000,664
Recombinant DNA		
CAGp-DAT-pHluorin plasmid	N/A	N/A
Software and algorithms		
ImageJ	N/A	https://imagej.net/
OriginPro 2018/2023	Origin Lab	https://www.originlab.com/
Other		
Inverted microscope (wide-field or confocal)	Nikon	Ti-2 or other widefield imaging system or a spinning disk confocal system
High speed camera	Andor	iXon Ultra897 or similar
Perfusion control system	Automate ValveLink 8.2	N/A

MATERIALS AND EQUIPMENT

Cell culture media (Neuro A media) 100 mL		
Reagent	Final concentration	Amount
Neurobasal-A Medium, minus phenol red	60% v/v	60 mL
Basal Medium Eagle (BME)	30% v/v	30 mL
Fetal Bovine Serum - Premium Select, Heat Inactivated	10% v/v	10 mL
B-27 Supplement (50X), serum free	2% v/v	2 mL
GlutaMAX Supplement	1% v/v	1 mL

Papain solution 5 mL		
Reagent	Final concentration	Amount
Papain (Worthington, LK003178)	20 units/mL	1 vial
EBSS (Worthington, LK003188)	100% v/v	5 mL
Kynurenic acid 0.5 M stock	0.5 mM	5 μL
12N HCl	Until Solution turns salmon	0.5–1 μL

⚠ CRITICAL: The HCl amount should not exceed more than 1.5 μL.

Tyrode's Solution (pH = 7.40) 1L		
Reagent	Final concentration	Amount
HEPES	25 mM	5.96 g
NaCl	119 mM	6.96 g
KCl	2.5 mM	0.186 g
D-Glucose	30 mM	5.4 g
$CaCl_2$ 2M stock	2 mM	1 mL
$MgCl_2$ 2M stock	2 mM	1 mL
CNQX 10 mM stock	10 μM	1 mL
APV 25 mM stock	50 μM	2 mL

NH_4Cl Solution (pH = 7.40) 500 mL		
Reagent	Final concentration	Amount
HEPES	25 mM	2.98 g
NaCl	70 mM	2.0475 g
KCl	2.5 mM	0.093125 g
NH_4Cl	50 mM	1.335 g
D-Glucose	30 mM	2.7 g
$CaCl_2$ 2M stock	2 mM	0.5 mL
$MgCl_2$ 2M stock	2 mM	0.5 mL
CNQX 10 mM stock	10 μM	0.5 mL
APV 25 mM stock	50 μM	1 mL

MES Solution (pH = 5.50) 500 mL		
Reagent	Final concentration	Amount
MES	25 mM	2.453
NaCl	119 mM	3.48
KCl	2.5 mM	0.093125
D-Glucose	30 mM	2.7
$CaCl_2$ 2M stock	2 mM	0.5 mL
$MgCl_2$ 2M stock	2 mM	0.5 mL
CNQX 10 mM stock	10 μM	0.5 mL
APV 25 mM stock	50 μM	1 mL

⚠ CRITICAL: Regardless of the variations in the imaging solution recipe, it is important to keep the final adjusted pH at 7.40 for Tyrode's solution, 7.40 for the NH_4Cl solution, and 5.50 for MES solution. The pH of those solutions should be monitored weekly to prevent drifting.

STEP-BY-STEP METHOD DETAILS

Prepare ventral midbrain neuron culture

Timing: 2 weeks before imaging experiment

The following protocol yields neuron/glia mixed culture. It is compatible with culturing on top of a pre-prepared glial bed.

1. Make fresh 100 mL of NeuroA solution as follows (on day of culture).
   a. 60 mL NeurobasalA (Thermo Fisher 12349015).
   b. 30 mL Basal Media Eagle (BME) (Thermo Fisher 21010046).
   c. 10 mL heat-inactivated fetal bovine serum (FBS) (Bio-Techne S11550H).
   d. 2 mL 50X B27 (Thermo Fisher 17504044).
   e. 1 mL Glutamax (Thermo Fisher 35050061).
   f. Sterile vacuum filtration flasks (CellPro V25022).
   g. Keep NeuroA at 20°C–25°C.
2. Autoclave the stir bar (VWR, 58948–309), cloning cylinders (Corning, 3166–6), cover glasses (VWR, 16004–302) and a high vacuum grease pad (Fisher Scientific, 14–635-5D). (on or before day of culture).
3. Set the centrifuge to 17°C (on day of culture, optional).
4. Prepare dishes (immediately before dissection).
   a. Transfer a pre-washed and autoclaved #1.5 coverglass to a 35 mm dish.
   b. Coat the center of the glass with a drop of Poly-L-ornithine (10 μg/mL in sterile filtered dH_2O, Sigma P3655, ~30 μL).
   c. Leave the coated dishes in the cell culture incubator for ~1 h before seeding.
5. Setup for papain digestion (Figure 2): (Prepare before the last 20 min of dissection).
   a. Prepare the Papain solution (see table in materials and equipment).
   b. Sterile filter the solution into a 15 mL conical tube whose cap has been pierced twice with an 18-gauge needle. Connect a STERILE syringe filter on top of the needle.
   c. Fill ~200 mL distilled water in a 400 mL beaker and place on top of a heating plate until the temperature of the water reaches 33°C–37°C.
   d. Place the filtered papain solution (with needle and filter) in the water bath. Use a thermometer to monitor the temperature of the water bath during digestion.
   e. Add ~200 mL distilled water to a vacuum filtering side arm flask.
   f. Insert a hole stopper and a long pipet that should be dipped in water.
   g. Connect one end of the pipet to an oxygen tank (95% oxygen and 5% CO_2) through a tubing.
   h. Connect the side arm to another tubing to oxygenate the papain solution.
   i. Start the oxygen flow at a very low speed (~2–4 bubbles/sec) into the papain 15 min prior to beginning digestion.
6. Tissue dissection and dissociation.
   a. A typical prep requires 3–6 postnatal day 0–1 (P0-1) pups.
   b. Decapitate the pup with a pair of sharp scissors.
   c. Fix the head with a pair of tweezers in one hand and use #11 surgical blade in the other hand to open the skin and skull at the mid-line.

   Note: A "T" cut can be made to fully open the skull. The cut should not penetrate the brain tissue to sever the two hemispheres.

   d. Use a spatula to transfer the brain to a dish with ice cold PBS under a dissection microscope, bottom up (Figure 3A).
   e. Fix the brain by the hemispheres with a pair of tweezers and use #10 surgical blade to make the first cut at the rostral edge of the midbrain (Figure 3B).

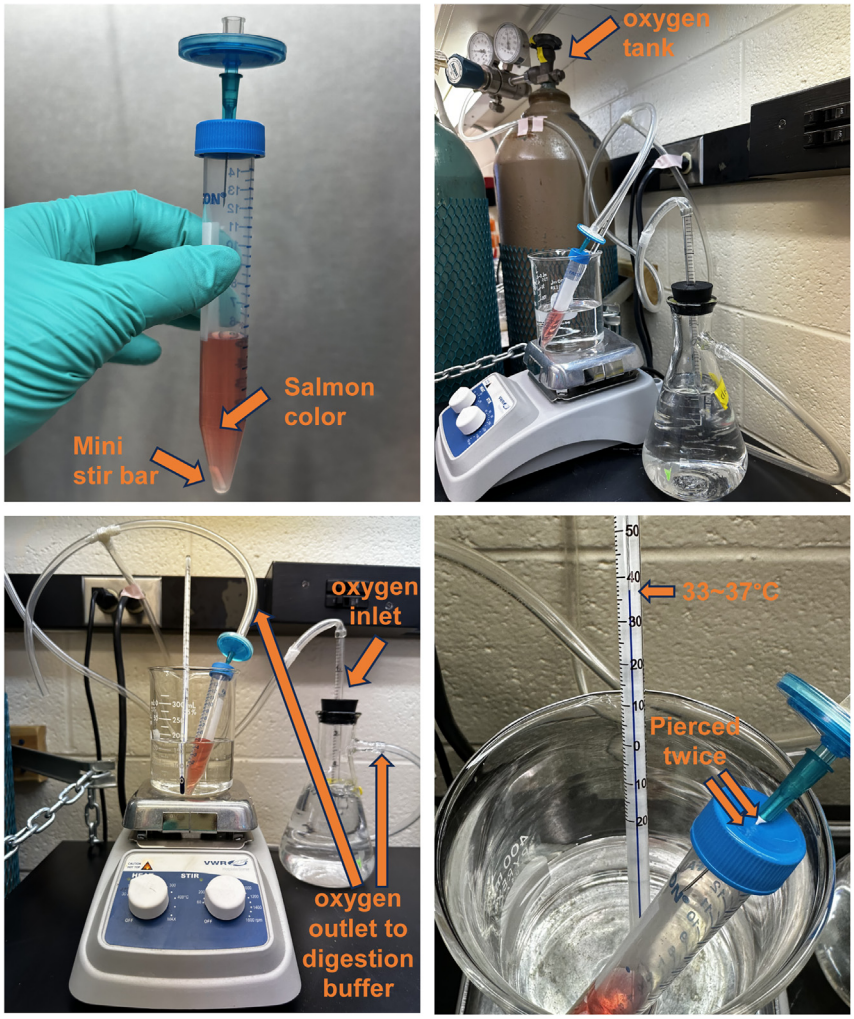

Figure 2. Preparation for papain digestion

Top left: the final papain digestion solution containing a mini stir bar and filter/needle set on the cap for oxygenation. The rest of the three panels are different angles of the digestion setup. It is important to keep the water bath at 33°C–37°C and oxygenate at a very slow speed.

f. The second cut can be made at the edge of the hemisphere to isolate the 2 mm thick midbrain tissue (Figure 3C).

Note: The midbrain tissue slice should contain the 3rd ventricle on the dorsal side (Figure 3D).

g. Fix the dorsal midbrain and use a micro slit knife to cut out the ventral midbrain (Figure 3E).
h. Transfer the ventral midbrain tissue to a collecting dish with PBS and repeat the dissection procedure for the rest of the pups.

Note: The entire dissection procedure should not exceed 40 min and is ideally kept within 30 min (less than 5 min per pup).

i. Transfer all ventral midbrain tissue chunks to the papain tube with a stir bar.
j. Place the papain tube in the beaker and attach the silicone tubing from the humidified oxygen to the syringe end.

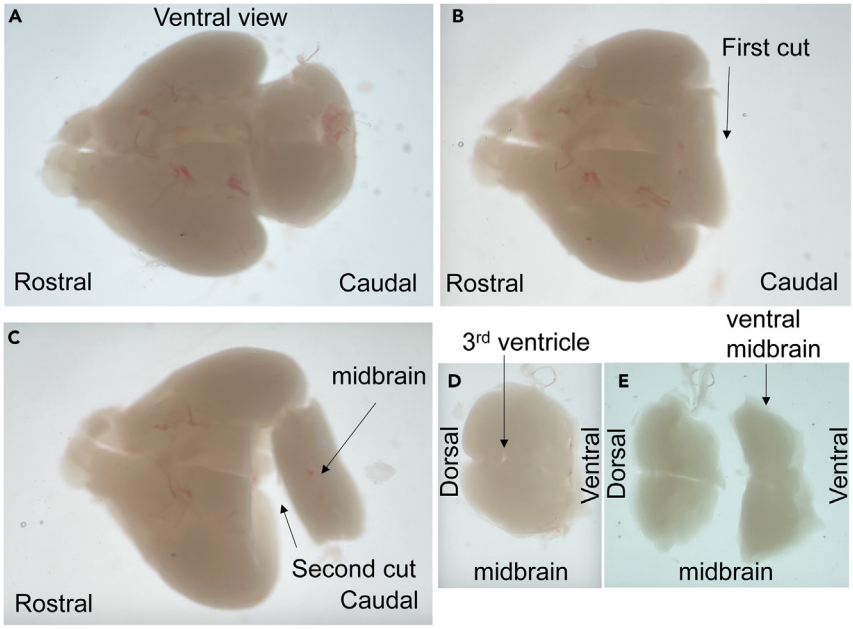

Figure 3. Tissue dissection of postnatal P0-1 pup brains

(A) Bottom/ventral view of the entire mouse brain.

(B) The mouse brain after first cut to remove the cerebellum.

(C) Indication of the second cut to free the entire midbrain slice.

(D and E) Coronal view of the midbrain (D) and where the final cut is made to obtain the ventral midbrain for tissue culture.

k. Turn on stir bar to let rotate VERY SLOWLY, and digest for exactly 12 min.

Note: Temperature MUST be maintained at 33°C–37°C, or the cells will die.

l. Spin down tissue at 0.3 *g* for 10 min at 17°C.
m. Aspirate solution and add 1 mL of NeuroA media. Triturate VERY, VERY GENTLY.
n. Add 4 mL of NeuroA and spin again.
o. Aspirate 4 mL of media.

Note: The first pellet is a loose pellet. Be sure to not aspirate too much.

p. Add 5 mL of NeuroA. Spin, Aspirate.

Note: The second pellet is solid. Aspiration should be more thorough.

q. During the last spin, prepare the dishes. Aspirate the Poly-L-ornithine. Attach a pre-autoclaved cloning cylinder (6 × 8 mm, Corning, 3166–6) to the center of the cover glass (where coated) by high vacuum grease (sterile) and add 100 μL Neuro-A media in each cylinder. Incubate the dishes.

7. Seeding and maintenance.
   a. Add NeuroA (~100 μL per pup) to the pellet, resuspend gently and count cells.
   b. Plate at 30,000 cells per 0.28 cm^2 dish.

⚠ CRITICAL: It is critical to count cells every time because small differences in plating density (too much or too little) can greatly reduce the survival of neurons and quality of the culture.

c. DIV 3: Carefully remove 80% the old media from cylinder and add ~100 μL warm media.
d. DIV5-7: After transfection, the cloning cylinder should be removed and supplement the cultured medium with cytosine arabinoside (Ara-C) (see below in cell culture transfection).
e. DIV13-17: Imaging experiments will take place.
f. DIV15-17: A half medium change would be desirable if culture needs to be grown for an additional week.

Cell culture transfection

Timing: 1 week before imaging experiment

Lipofectamine transfection is carried out on day 5–7 after plating using Lipofectamine 2000 (Thermo Fisher, 11668019) following a company suggested protocol.

8. Make mixtures for each 6 × 8 mm cylinder.

tube A: 50 μL opti-MEM + 0.7 μg DNA (5 min RT incubation).

tube B: 50 μL opti-MEM + 1.5 μL lipo2000 (5 min RT incubation).

9. Combine A and B, incubate at RT for 25 min.
10. Remove the original culture medium in the cylinder and add the 100 μL mixture.
11. Incubate for 45 min (37°C).
12. Remove MEM in the surrounding area and add 3 mL of NeuroA + 10 ng/mL GDNF + 0.2 μg/mL Ara-C (Sigma-Aldrich, C6645).
13. Remove the cylinder.

Live imaging

Timing: 1 day before imaging (for step 14)

Timing: Immediately before imaging (for steps 15–18)

Timing: Ideally, within 1 week after live imaging (for step 19)

14. Prepare the imaging solution.

Note: We used a modified Tyrode's solution (see table in materials and equipment) to mimic physiological conditions. This can be replaced by other routine physiological solutions with different ionic contents and glucose levels.

15. Set up the perfusion lines (Figure 3).

Note: The in-line perfusion system is set up for analyzing the live fluorescence change in response to different pH solutions (such as the NH_4Cl or MES solution) or the live trafficking of DAT-pHluorin when exposed to a ligand (such as dopamine, amphetamine, cocaine, etc.).

a. Cut a strip of the cover glass and mount on top of the custom-made perfusion chamber (Figure 1) using high vacuum grease (Figure 4A).
b. Mount the cells on cover glass UNDER the custom chamber as shown in Figure 4A using high vacuum grease.
c. Transfer the cell-perfusion chamber ensemble to be nestled in the Warner chamber using high vacuum grease (Figure 4B).

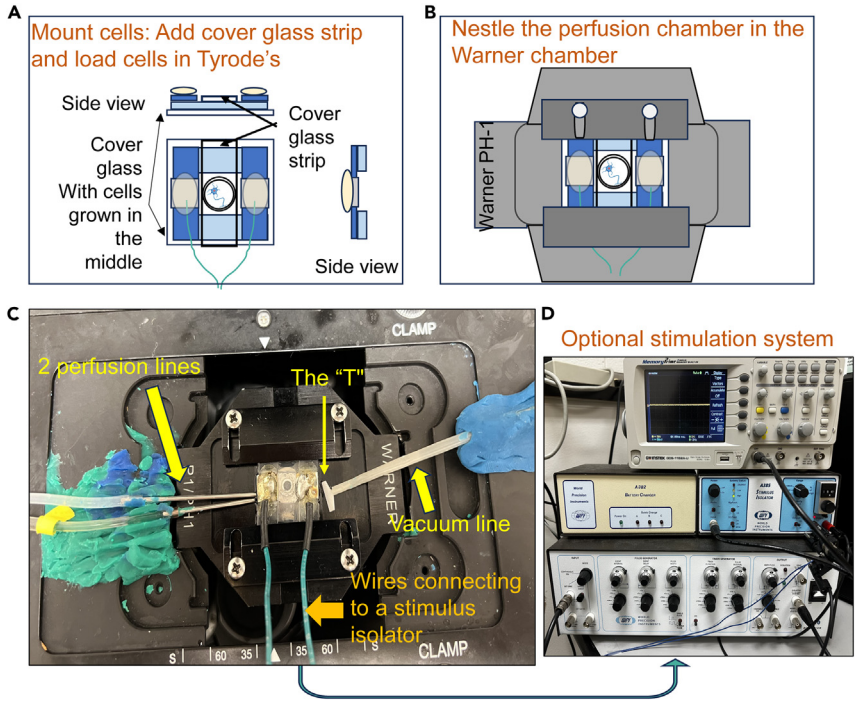

Figure 4. Arrangement of the perfusion system and the optional electrical stimulation system

(A) Illustration for loading the culture on the custom-made chamber.

(B) Illustration for loading the culture/chamber assembly to the Warner PH-1 chamber.

(C) Rea life image of the perfusion setup when the Warner chamber is loaded on top of the objective.

(D) The optional electrical stimulation system with wires connected to the custom-made chamber through gold pin connectors.

d. Load the chamber with live cells to the microscope (Figure 4C).

⚠ CRITICAL: Don't forget to add the glass strip on top of the culture. Without the glass strip, there will not be lamina flow. Make sure the grease forms a tight seal with the Warner chamber so that no solution leaks out onto the objective during perfusion. Also, make sure the cover glass does not break during this assembly.

e. Use the flow regulator and a timer to adjust the perfusion speed at a rate of ~0.2–0.3 mL/min.

⚠ CRITICAL: Setup as many lines as needed and possible, but it's important to keep all lines flowing at the same speed.

f. Cut out a "T" shape from a filter paper for the vacuum line and make sure that the vacuum effectively removes excess Tyrode's solution (Figure 4C).

⚠ CRITICAL: A good balance between the perfusion and the vacuum is the key to a successful live imaging experiment. Use the flow regulator within the vacuum line to adjust suction speed. After all the lines are set, it's highly recommended to pipet a ~0.3 mL Tyrode's to the perfusion side of the chamber and check if the vacuum is effective in removing the excess solutions on the other side.

16. Live imaging: Measure basal surface DAT fraction.
    a. Survey the dish and locate the transfected cell body.

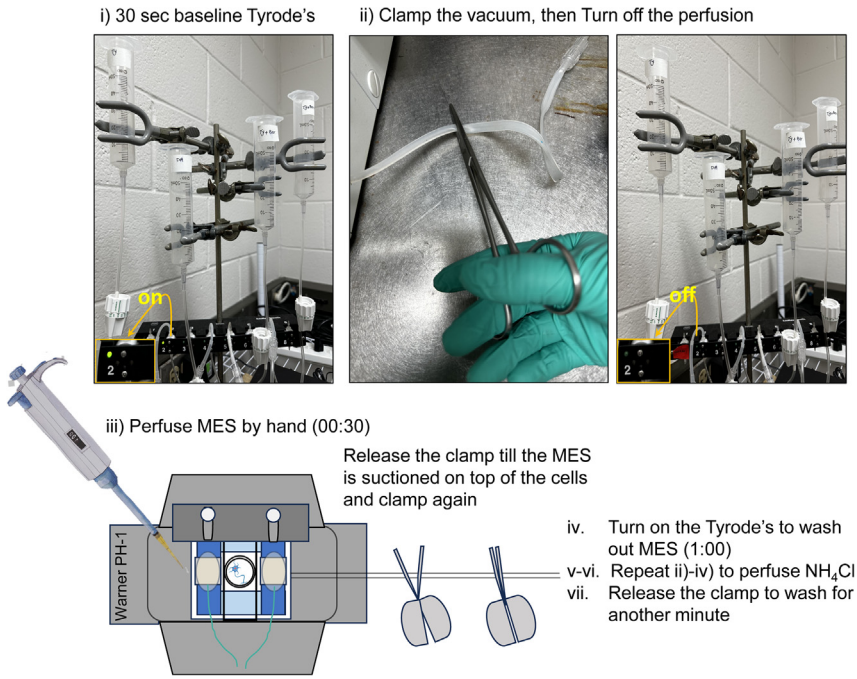

Figure 5. Illustration for the MES-NH_4Cl hand perfusion

Step-by-step (i–vii) illustration of the hand perfusion experiment for live imaging step 16d.

b. Save the mid-left side of the imaging field (where the grease ring is) as position (0, 0) and then save the other relative locations of interest.

Note: This is done in the ND imaging mode/XY tab of the Nikon Elements software and may work with other custom written codes. These stage locations are critical for *post hoc* analysis (see step 19).

c. Set up the imaging program for 1 Hz, 3 min time lapse imaging.
d. MES-NH_4Cl hand perfusion for analysis of basal DAT fraction (Figure 5).
   i. 30 s baseline Tyrode's (00:30).
   ii. Clamp the vacuum line and turn off the Tyrode's perfusion line.
   iii. Pipet ~250 mL of the MES solution using the left hand to the left side of the perfusion chamber and release the clamp on the vacuum line to allow MES solution run through. Clamp again for 30 s (01:00).
   iv. Turn on the Tyrode's line and release the clamp to wash cells with Tyrode's for 30 s (01:30).
   v. Clamp the vacuum line and turn off the Tyrode's perfusion line.
   vi. Perfuse NH_4Cl the same way as MES for 30 s (02:00).
   vii. Turn on the Tyrode's line and release the clamp to wash cells with Tyrode's for 1 min (03:00).

Note: Two or three trials of sequential perfusions can be carried out with 5–10 min spacing for each imaging field. If there is more than one cell of interest in the culture, additional cells can be analyzed as long as the response remains stable.

e. Advance to (18) Wrap up the experiment if no additional experiments are planned.

17. Live imaging: Measure ligand-induced DAT trafficking.

a. Set up the imaging program for 0.2–1 Hz time lapse imaging with 3 phases. Phase 1 = baseline; phase 2 = ligand perfusion; phase 3 = wash.
b. Engage the perfect focus system for better focus stability for long imaging durations. This is particularly important for confocal imaging.
c. Run the protocol.

Note: This experiment could be repeated twice.

18. Wrap up the experiment.
    a. All imaging files are saved/exported as TIFF files.
    b. Stage files are saved for *post hoc* immunofluorescence analysis.
    c. Unload the coverslip and transfer to a 35 mm dish containing Tyrode's.
    d. Use Sharpie to label your favorite corner of the coverslip (i.e., the top left corner). Labeling can be on the cover glass itself or the bottom of the dish.

⚠ CRITICAL: The marked orientation of the culture will be a guide during mounting and *post hoc* imaging. For example, the top left corner of the coverslip should remain the top left corner after mounting, so that the rest of the stage positions can be registered.

    e. Fix the cells with 4% PFA at 20°C–25°C.
19. *Post hoc* immunostaining to determine the identity of the cell.

Note: Immunocytochemistry is performed to determine (1) whether the imaged cell is a TH + DA neuron or a TH- cell, and (2) whether the neurites analyzed were Synapsin-enriched boutons or Synapsin-lacking shafts. This is done following a standard procedure which will not be detailed in this paper. The following antibodies were used for immunofluorescence: chicken anti-GFP (Thermo Fisher, A-10262, 1:1000), rabbit ant-GFP (Thermo Fisher, A-11122, 1:1000). Guinea pig anti-synapsin 1/2 (Synaptic System, 106004, 1:500), mouse anti-TH (Sigma, T2928, 1:1000), rabbit anti-TH (Novus Biologicals, NB300-109, 1:1000).

    a. Load the glass slide on the microscope using the same orientation as live imaging.
    b. Find the mid-left (0,0) position of the culture grease ring.
    c. Uncheck "relative stage" and "move to stage" before opening the saved stage file.
    d. Request the software to perform "offset" to the (0, 0) position, and then restore the "relative stage" function.
    e. Restore the "move to stage" function and use the saved stage locations to find the cell that underwent live imaging guided by the GFP immunofluorescence for DAT-pHluorin.
    f. Image stacks are taken at different focal planes at 0.5 μm interval to include the whole cell and a maximum projection image can be generated for the stack via ImageJ for analysis.
    g. Synapsin 1/2-positive boutons were determined based on the bright punctate staining pattern.

EXPECTED OUTCOMES

The above experiments are expected to generate time-lapse DAT-pHluorin images, whose fluorescence encodes information of DAT localization at designed temporal resolution (see Figure 6 illustrations). Following "Qualification" in the next block of this protocol, we will obtain time traces that can be used to calculate basal surface fraction of DAT, vesicular pH of DAT-vesicles and ligand-induced DAT live trafficking (Figure 6).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Saved TIFF files will be analyzed in ImageJ.

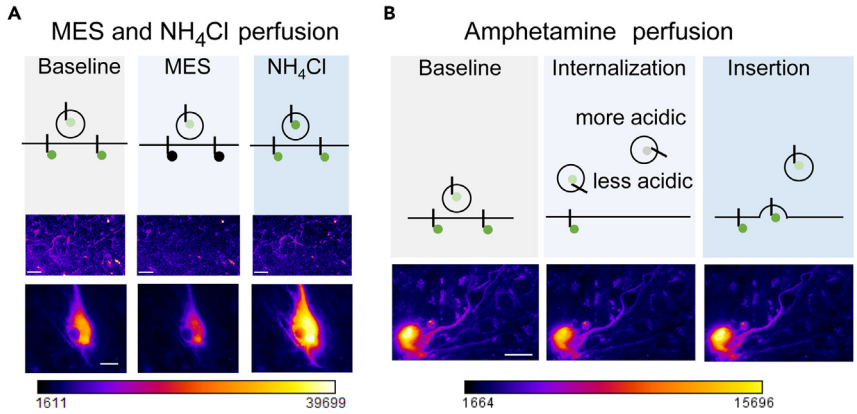

Figure 6. Expected results of DAT-pHluorin imaging

The live imaging experiment yields two types of time-lapse images.

(A) Illustration of the expected change in DAT-pHluorin fluorescence during the MES/NH_4Cl perfusion (top) and representative snapshots of neurite and soma fluorescence during the MES/NH_4Cl perfusion (Bottom).

(B) Illustration of the expected change in DAT-pHluorin fluorescence during the amphetamine perfusion (top) and representative snapshots of DAT-pHluorin fluorescence during 10 μM amphetamine perfusion (Bottom). Scale bar = 20 μm.

2. Install the following plugin: Time series analyzer (https://imagej.net/ij/plugins/time-series.html) and Diffimage1b (Data S1).
3. Select regions of interests (ROIs).
   a. Bring up the Plugins/Diffimage1b function (Figure 7A) and enter the number of frames for baseline, the beginning and the end of the peak fluorescence (Figure 7B).

      Note: This will yield three images, baseline (Prestimulus), peak (Peakstimulus) and the ΔF image (Delta) (Figure 7C).

   b. The baseline image and the ΔF image are used for placing ROIs (Figure 7D).
   c. For soma perfusion responses, use the freehand tool to draw a shape that covers the entire soma (Figure 7E).
   d. Neurite responses are measured using circular ROIs placed through the Time Series Analyzer. Check "add on click" and define the size the ROIs in "AutoROIProperties".

      Note: For neurite, we use 6 × 6 pixels (1.5 × 1.5 μM) circular ROIs (Figure 7D).

   e. Use the "GetAverage" function and the resulting trace is the average perfusion response of all ROIs from one of the above compartments (cell body or neurite).
   f. Define representative background using the freehand tool next to the ROIs (Figure 7F).
   g. Use the "GetAverage" function to obtain a background response.
4. Enter all responses into an OriginPro spreadsheet or another spreadsheet of choice. Cell body or neurite perfusion responses are subtracted from their background responses.
5. The background subtracted response is normalized to its baseline to derive the $\Delta F/F_0$ response trace (Figure 7G).
6. The peaks and troughs of the $\Delta F/F_0$ response for each cell are extracted and used for calculating surface fraction, vesicular pH (detailed below).
7. Calculation for surface fraction and vesicular pH.
   a. Set up the calculation in an excel spreadsheet (Figure 8).
      i. Enter pH_i from 2.0–7.4 in column B.
      ii. Enter the dx equation (see NOTE below) in column C.
      iii. Enter the F(x) equation (see NOTE below) in column D.
      iv. Enter the $\Delta F/F_0$ value from the NH_4Cl perfusion to column D and yield the blue trace.

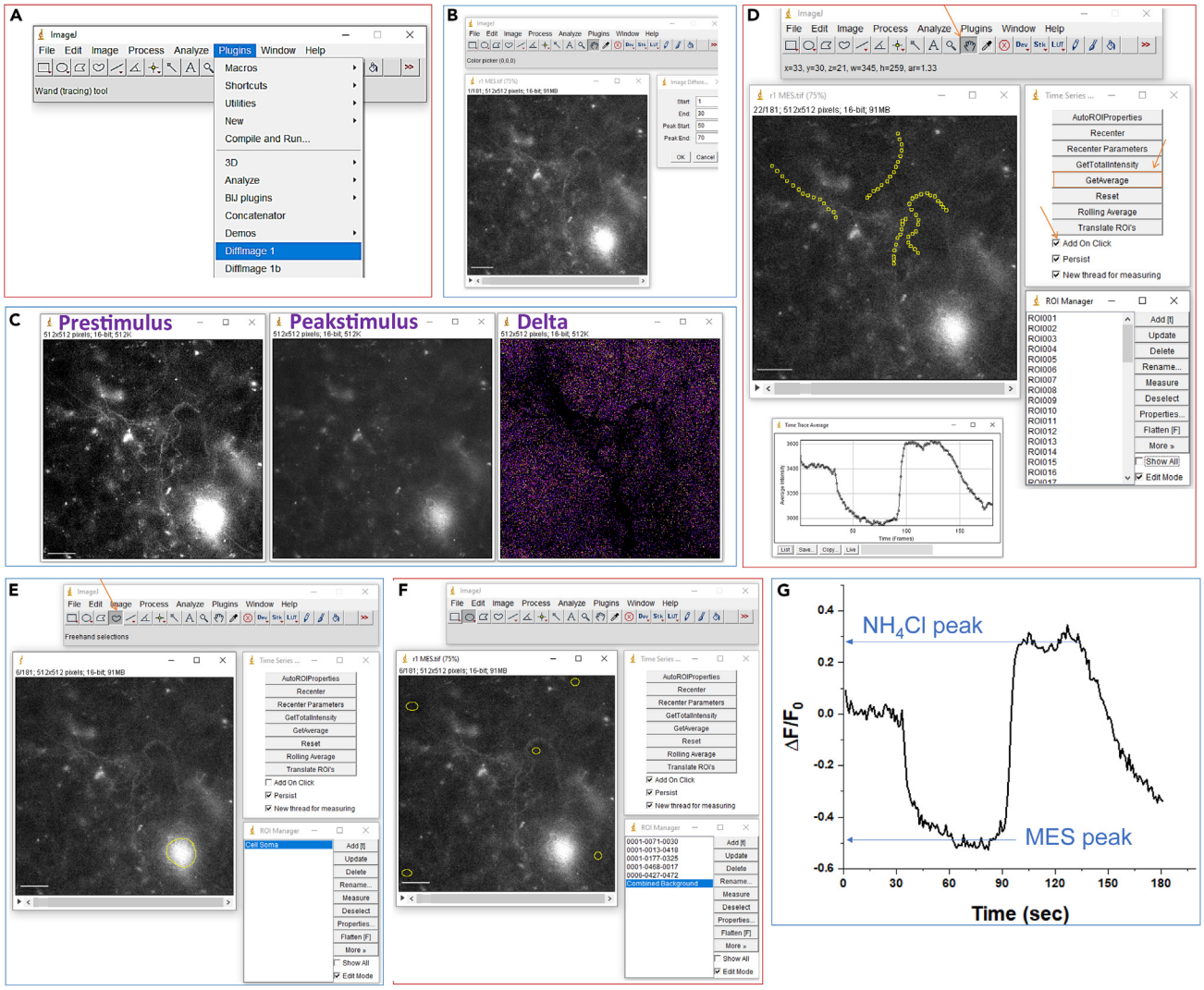

Figure 7. Image Analysis using ImageJ and figure generation in OriginLab

Step-by-step illustration of image processing using image J (A–C), regions of interest selection (D–F), and trace generation in OriginLab (G). Arrows point to MES and NH_4Cl peak values used for calculating surface fraction in later analysis. Scale bar = 20 μm.

Note: Following previous published methods,[18] the fluorescence change during perfusion of a pH 7.4 membrane permeable NH_4Cl solution is determined by the following function assuming pKa = 7.0:

$$dx = \frac{\left(\frac{1}{\left(1+10^{7.0-7.4}\right)} - \frac{1}{\left(1+10^{7.0-\mathrm{pHi}}\right)}\right)}{1/\left(1+10^{7.0-\mathrm{pHi}}\right)}$$

The surface fraction as a function of pH_i is then plotted by.

$$F(x) = \frac{\mathrm{dx} - \frac{\mathrm{dF}}{\mathrm{F0}}\text{of NH4Cl response}}{\mathrm{dx} * \frac{\mathrm{dF}}{\mathrm{F0}}\text{of NH4Cl response+dx}}$$

b. Set up the calculation in an excel spreadsheet (Figure 8).
   i. Enter the F function (see NOTE below) in column F.

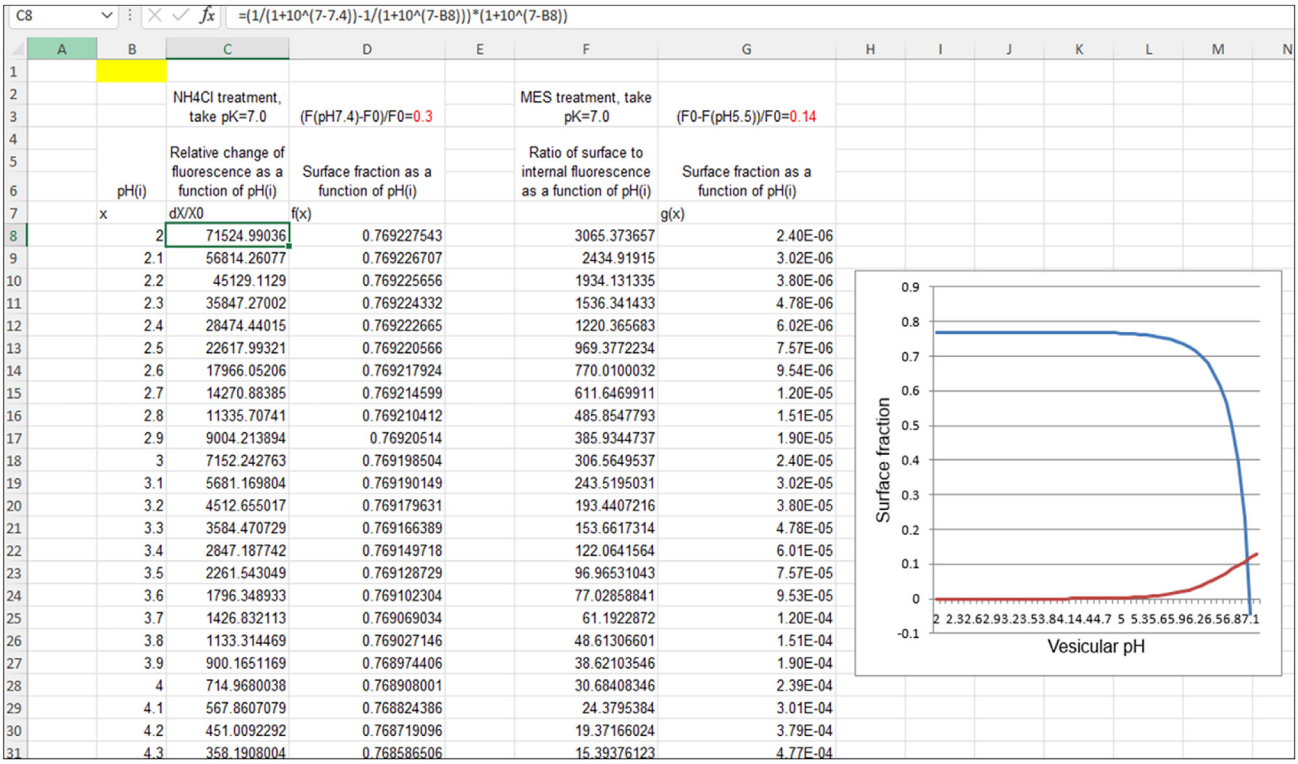

Figure 8. Spreadsheet calculation for surface fraction and vesicular pH

Illustration of the excel spreadsheet function entry and setup for calculating surface fraction and vesicular pH.

ii. Enter the g(x) equation (see NOTE below) in column G.

iii. Enter the $\Delta F/F_0$ value from the MES perfusion to column G and yield the red trace.

Note: During perfusion of a pH 5.5 MES solution, the ratio of surface to internal DAT-pH can be calculated as the following:

$$F = \frac{\frac{1}{(1+10^{7.0-5.5})}}{\frac{1}{(1+10^{7.0-pHi})}}$$

The surface fraction as a function of pH_i is then plotted by.

$$g(x) = \frac{\frac{dF}{F0}\text{of MES response}}{1 - dx + F * \left(1 - \frac{dF}{F0}\text{of MES response}\right)}$$

c. Surface fraction and vesicular pH are determined at the intersection of the red and blue traces.

8. Analysis for amphetamine-induced DAT-pHluorin response.

a. Open the timelapse amphetamine (AMPH)-induced DAT-pHluorin image series in ImageJ.

Note: AMPH-induced DAT internalization will be reflected as a reduction in fluorescence (Figures 9A and 9B).

b. Use the Plugins/Diffimage1b function same as shown in Figure 7 to select ROIs.

c. Use the "GetAverage" function to create a timelapse trace for the AMPH response at selected regions (neurite or soma as shown in Figures 9D and F).

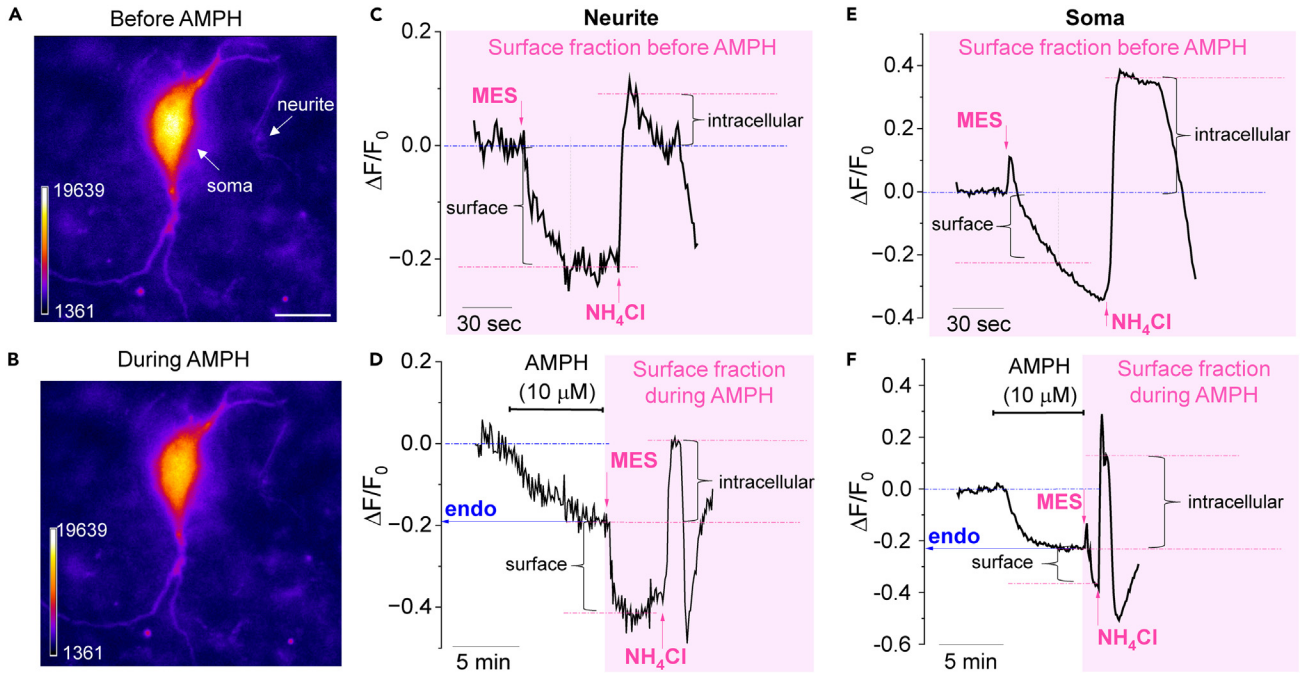

Figure 9. An example of the AMPH perfusion experiment

(A and B) Representative neuron transfected with DAT-pHluorin before (A) and during (B) amphetamine (AMPH).

(C–F) Two rounds of MES/NH_4Cl perfusion were executed before (C and E) and after (D and F) AMPH perfusion. Representative $\Delta F/F_0$ traces of DAT-pHluorin using regions of interests at neurites (C and D) or soma (E and F). Pink shade area represents measurements for surface DAT fraction using MES/NH_4Cl hand perfusion. The AMPH induces a small reduction in fluorescence at both neurites (E) and soma (F), indicating DAT-pHluorin internalization, which is reflected by surface fraction measurements before (C and E) and after (D and F) AMPH perfusion. Scale bar = 20 μm.

d. All traces will be presented after background subtraction and baseline normalization.
e. The total amount of endocytosed DAT-pHluorin will be presented as peak $\Delta F/F_0$ shown by the blue arrows in Figures 9D and F.

Optional: To compare the loss of DAT surface fraction before and after AMPH perfusion, an MES/NH_4Cl perfusion run can be performed before the AMPH run to assess the basal surface fraction (Figures 9C and 9E). Following a 5∼10-min recovery, an AMPH run will be executed, which contains a 2-min baseline, 7-min AMPH perfusion at 10 μM, followed by another round of the MES/NH_4Cl perfusion (Figures 9D and 9F).

Note: For surface fraction following AMPH perfusion, the F_0 needs to be redefined to the peak internalization of the AMPH response (see the new pink baseline in Figures 9D and 9F). The increase in the relative NH_4Cl response (intracellular) to MES (surface) response indicates DAT internalization. The absolute values of surface fraction and DAT vesicular pH should be calculated according to the Henderson-Hasselbalch equation shown in Figure 8.

9. Statistical analysis.

Data type from this experiment include: 1) raw values of DAT-pHluorin surface fraction, 2) DAT-pHluorin vesicular pH, and 3) peak internalization of DAT-pHluorin upon AMPH (or another ligand) perfusion. For datasets that follow normal distribution and exhibit equal variance, Student's t test or ANOVA test are used, followed by Bonferroni correction for post hoc analysis. For datasets that reject normal distribution and exhibit non-homogenous variance, Mann-Whitney test or Kruskal Wallace test should be used, followed by Bonferroni correction for post hoc analysis.

LIMITATIONS

The current sensor is based on human DAT cDNA. The potential difference in trafficking between an hDAT-pHluorin and an mDAT-pHluorin has not been fully understood. This analysis is based on a sensor overexpression strategy. Although our previous study demonstrates lack of DAT overexpression,[1] it remains as a caveat. The CAGp-DAT-pHluorin plasmid expression is non-selective and can be expressed in non-dopaminergic neurons and glial cells in the culture. Without *post hoc* determination of the dopaminergic identity, the analysis may not reflect trafficking of DAT in DA neurons.

Cell body contains heavy fluorescence from DAT-pHluorin in the ER and Golgi complexes. Thus, the calculated fraction of surface DAT does not represent the fraction of surface DAT in the recycling pathway. DAT-pHluorin expression in the neurite is quite weak and sometimes indistinguishable from the heavy glial background. A weak baseline fluorescence can make the F_0 a bit arbitrary and its value may vary based on the selection of the background. This limitation can be circumvented by selecting bright structures for analysis.

MES is not completely membrane impermeable. For ROIs with strong endogenous buffers, such as the neuronal soma, it is challenging to determine an accurate ΔF response if an obvious plateau is not observed. This limitation can be partially alleviated by standardizing the analysis for MES peak response at 30 s following the start of perfusion. According to our experience, neurites that contain negligible amount of endogenous buffer typically reach plateau within 30 s (see gray dotted line in Figures 9C and E). Other membrane impermeable acidic buffers can be used if available.

TROUBLESHOOTING

Problem 1

Lack of DAT-pHluorin expression (live imaging, step 16).

Potential solution

- Check the quality of culture. Typically, a lack of transfection is due to lack of neurons in the ventral midbrain culture. Perform immunocytochemistry to determine whether there are TH + neurons in the culture.
- Check DNA. Sometimes, the DNA concentration can be increased or reduced after freeze-thaw cycles.

Problem 2

Lack of expected response to a ligand (live imaging, step 17).

Potential solution

- Double check the perfusion line. Did the perfused ligand pass through the chamber or did the solution get stuck on one end of the chamber?
- Check if the cell is responding to perfusion of the MES or NH_4Cl solution. If not, the cell might not be alive.

Problem 3

Cells are drying out during experiment (live imaging, steps 16 and 17).

Potential solution

- Be sure all the lines are calibrated to the constant rate of Tyrode's. Tyrode's should always be measured first before setting up the treatment solutions. It is acceptable to have a slightly higher rate in the treatment line. For example, if Tyrode's rate is 250 μL/min, the treatment can be ~275 μL/min to avoid drying out during perfusion line switching.

- If the drying is happening at baseline with constant Tyrode's, the vacuum speed needs to be adjusted accordingly. Position and size of the "T" regardless of rate, can also have an impact on how quickly the Tyrode's solution passes through.

Problem 4

NH_4Cl and MES responses do not intersect on the spreadsheet (quantification and statistical analysis, step 7).

Potential solution

- Double check background selection. Does the background contain a MES or NH_4Cl response element?
- Double check pH of the solutions. Do they need recalibration?

RESOURCE AVAILABILITY

Lead contact

For further information and requests for resources and reagents, please direct email to pingyue.pan@rutgers.edu.

Technical contact

For additional technical assistance and inquiries, please direct email to pingyue.pan@rutgers.edu.

Materials availability

The pCAG-DAT-pHluorin construct will be made available to the research community upon email request.

Data and code availability

All data and code used for analysis are included in this protocol. The software used is listed in the key resources table.

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AUTHOR CONTRIBUTIONS

P.-Y.P. and E.K. developed the methods and made the figures. P.-Y.P., E.K., S.S.P., and J.S. wrote and edited the manuscript. P.-Y.P obtained funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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