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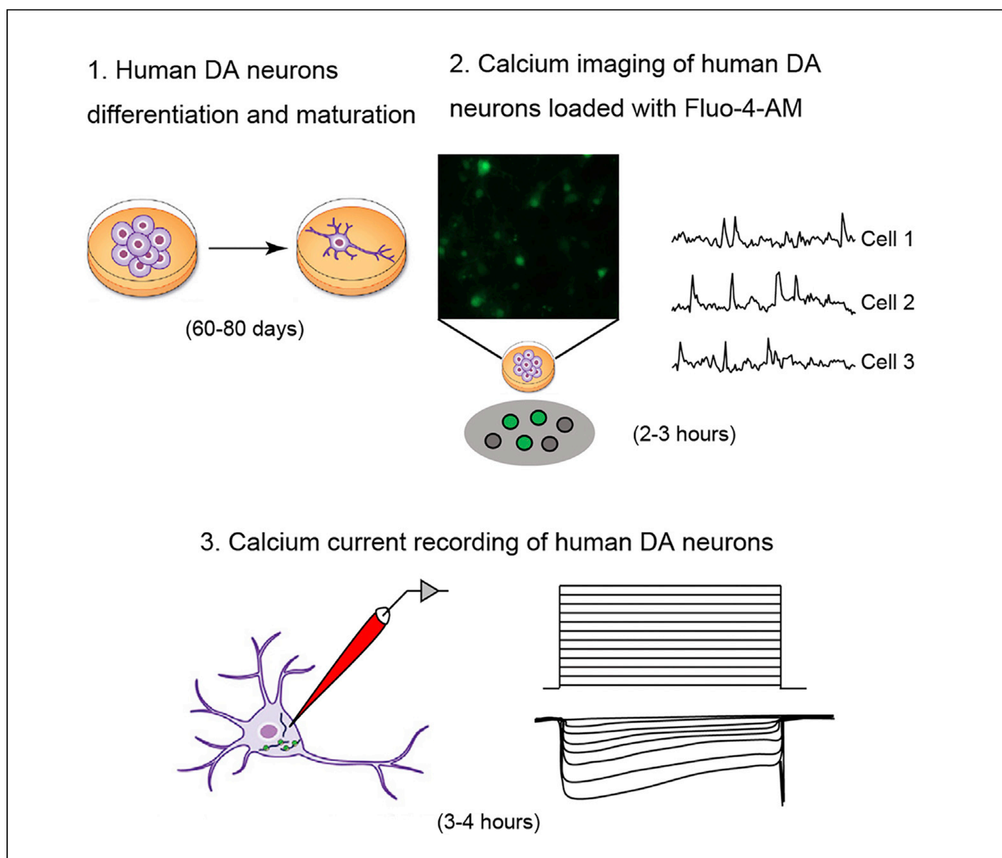
Protocol

Protocol for measurement of calcium dysregulation in human induced pluripotent stem cell-derived dopaminergic neurons



Calcium regulation is a critical process in neurons, and Ca^{2+} signaling is a major contributor to neurological disorders including Parkinson's disease (PD). Here, combining calcium imaging with whole-cell Ca^{2+} current recording, we provide a detailed protocol for measuring Ca^{2+} homeostasis in dopaminergic (DA) neurons derived from human induced pluripotent stem cells (hiPSCs). This approach can be applied to investigate the role of Ca^{2+} homeostasis in neuronal functionality as well as in disease processes.

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HIGHLIGHTS

Calcium imaging of human iPSC-derived DA neurons loaded with Fluo-4-AM

Whole-cell voltage clamping for dye loading and calcium current recording

Detailed guidelines for analyzing calcium dysregulation in human DA neurons

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Protocol for measurement of calcium dysregulation in human induced pluripotent stem cell-derived dopaminergic neurons

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SUMMARY

Calcium regulation is a critical process in neurons, and Ca^{2+} signaling is a major contributor to neurological disorders including Parkinson's disease (PD). Here, combining calcium imaging with whole-cell Ca^{2+} current recording, we provide a detailed protocol for measuring Ca^{2+} homeostasis in dopaminergic (DA) neurons derived from human induced pluripotent stem cells (hiPSCs). This approach can be applied to investigate the role of Ca^{2+} homeostasis in neuronal functionality as well as in disease processes.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2020).

BEFORE YOU BEGIN

Preparation of reagents and solutions

Timing: 1–2 days

Prepare all stock and medium solutions, following the guidelines and storage indications listed in the materials and equipment section. Refer to key resources table for a complete list of reagents and materials.

Culture and maintenance of hiPSCs

Timing: 1–2 weeks

1. Culture hiPSC cells on inactivated mouse embryonic fibroblasts (MEFs) in human embryonic stem cell (ESC) medium containing DMEM/F12, 20% knockout serum replacement (KSR), FGF2

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(4 ng/mL), Glutamax (1 mM), non-essential amino acids (100 μM), and 2-mercaptoethanol (100 μM). Replace medium every day.

Note: Refer to materials and equipment for human ESC medium recipe.

2. Passage hiPSC cells at a ratio of 1:6–1:12 when cells reach 80%–90%.
3. Prepare MEF plates following steps 4–6 one or two days before passaging hiPSC cells.
4. Coat plates with 2% Matrigel (in DMEM/F12) and incubate the plates at 22°C–25°C for at least 1 h.
5. Aspirate Matrigel and plate 3×10^4 cells/cm^2 of mitotically inactivated MEFs in MEF medium.

Note: Refer to materials and equipment for MEF medium recipe.

6. Keep MEF plates in a 37°C, 5% CO_2 incubator.
7. Aspirate the MEF medium and add 37°C water bath pre-warmed human ESC medium 3–4 h before plating hiPSC cells.
8. Check hiPSCs plate under microscope to make sure the cells are ready to passage. Add collagenase (1 mg/mL in DMEM/F12) solution to the plate containing hiPSCs, and stop the incubation when the edges of the colonies start to pull away from the plates.

Note: The volume of the collagenase solution should be enough to cover the whole surface of culture vessels.

9. Remove the collagenase and collect hiPSCs in pre-warmed ESC medium.
10. Centrifuge hiPSCs at 200 × *g* for 5 min, resuspend the hiPSC pellets in ESC medium, and add appropriate volume of cell suspension to each MEF plate.

⚠ CRITICAL: Passage cells at least a week before starting neuronal differentiation to make sure cells grow well and get stabilized before differentiation. Passage number is determined based on researcher's following experiments. We use one 24-well plate for calcium imaging and one 24-well plate for Ca^{2+} current recording. The experiments are repeated three times.

Differentiation of hiPSCs to DA neurons and DA neuron maturation

Timing: 60–80 days

Differentiate hiPSC cells to DA neurons (Figure 1A).

11. At day 0, digest hiPSCs colonies with collagenase (1 mg/mL in DMEM/F12) and plate cells on Matrigel-coated plate at a density of 4×10^4 cells/cm^2 in human ESC medium for 2 days. Change medium every day.
12. From day 2 to day 6, switch to serum replacement medium (SRM medium) containing growth factors and small molecules including FGF8a (100 ng/mL), SHH C25II (100 ng/mL), LDN193189 (100 nM), SB431542 (10 μM), CHIR99021 (3 μM), and purmorphamine (2 μM). Change medium every day.

Note: Refer to materials and equipment for SRM medium recipe.

13. From day 7 to day 12, maintain cells in neurobasal medium containing B-27 supplement minus vitamin A, N-2 supplement along with LDN193189 and CHIR99021. Change medium every day.
14. At day 13, dissociate colonies into single cell suspension and seed cells at density of 4×10^5 cells/cm^2 on poly-ornithine and laminin coated plate in neurobasal medium containing

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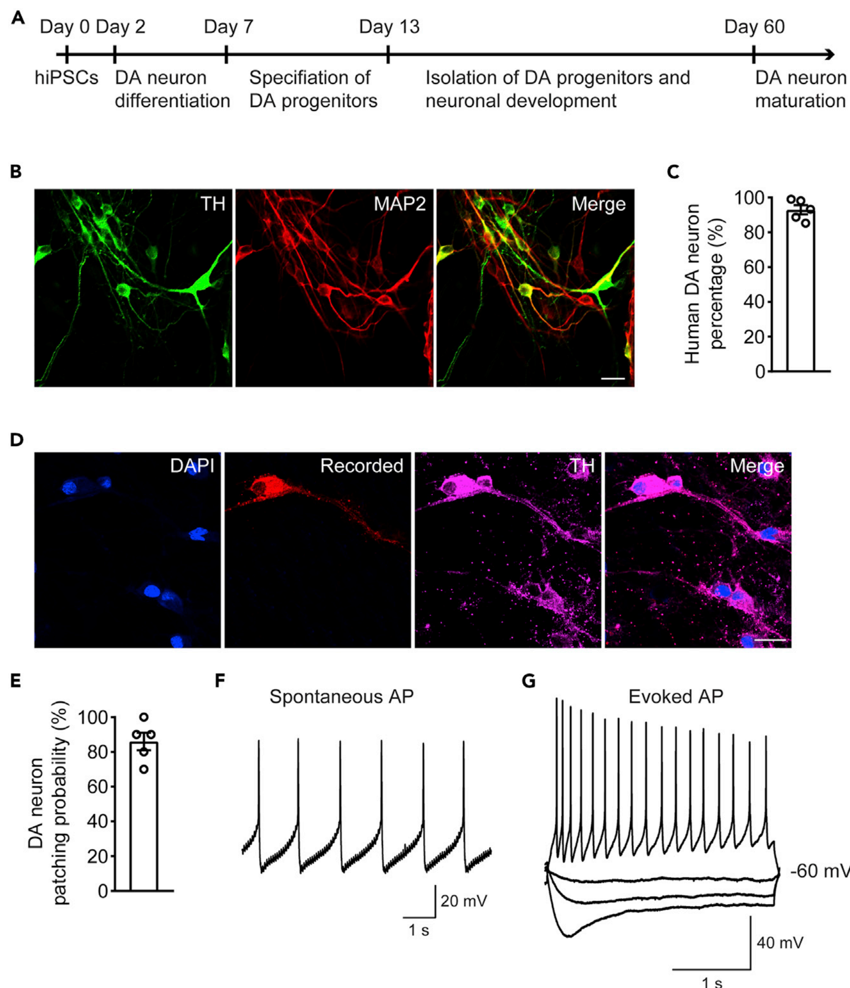

Figure 1. Identification of human DA neurons

(A) Timeline of human DA neuron differentiation and maturation.

(B and C) TH and MAP2 immunostaining showing hiPSCs-derived DA neurons after differentiation. TH is used as a DA neuron marker. Scale bar, 20 μm. Data are presented as mean ± SEM.

(D) Patched DA neuron is labeled with Alex Fluor 555, and further confirmed with immunocytochemistry after recording. Scale bar, 20 μm.

(E) The probability that a DA neuron is patched. Data are presented as mean ± SEM.

(F) Representative action potential (AP) firing pattern in human DA neurons. DA neurons display spontaneous activity at a constant rate of 1.2 ± 0.3 Hz.

(G) Representative current-clamp recordings evoked by series of 1000 ms current pulses.

Figures 1B, 1F, and 1G are reprinted with permission from Kim et al., 2020.

B27 minus vitamin A, BDNF (20 ng/mL), GDNF (20 ng/mL), TGF-β3 (1 ng/mL), ascorbic acid (0.2 mM), dibutyryl-cAMP (0.5 mM) and DAPT (10 μM) until maturation.

Note: To prepare poly-ornithine and laminin coated plates, cover the whole surface of culture plates with poly-ornithine (20 μg/mL) and incubate the plates at 37°C for 2 h. Aspirate poly-ornithine and rinse the plates three times with sterile water. Add laminin solution (5 μg/mL) in the plates and incubate them at 37°C for 2 h. Aspirate laminin solution before plating cells or store plates at 4°C until use.

15. DA neurons are specifically identified by immunostaining and electrophysiology (Figures 1B–1G). For immunostaining, anti-tyrosine hydroxylase (TH) antibody is used as a specific marker

for DA neurons. Electrophysiologically, DA neurons display spontaneous activity at a constant rate of 0.5–3.0 Hz, with wide action potentials (2.5–5.0 ms duration) (Figure 1F). DA neurons are further identified by the presence of a large sag (arrow) in the voltage response to hyperpolarizing current steps (Figure 1G).

Note: After differentiation, it takes approximately 60 days for DA neurons to get mature. So, human DA neurons cultured for > 60 differentiation days (68–75 days after differentiation) are used for calcium imaging and Ca^{2+} current recording.

Prepare equipment for calcium imaging

Refer to materials and equipment.

Setup whole-cell patch-clamp instrument

Refer to materials and equipment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Donkey anti-Mouse IgG - Alexa Fluor 555 (1:500)	Thermo Fisher Scientific	Cat# A31570; RRID: AB_2536180
Donkey anti-Rabbit IgG - Alexa Fluor 488 (1:500)	Thermo Fisher Scientific	Cat# A21206; RRID: AB_2535792
Mouse monoclonal anti-MAP2 (1:1000)	Millipore Sigma	Cat# M9942; RRID: AB_477256
Rabbit polyclonal anti-Tyrosine hydroxylase (1:1000)	Millipore Sigma	Cat#AB152 RRID:AB_390204
Chemicals, peptides, and recombinant proteins		
Ascorbic acid	Millipore Sigma	Cat# A4034
B-27 supplement minus vitamin A	Thermo Fisher Scientific	Cat# 12587010
BDNF	R&D Systems	Cat# 248-BD-025/CF
CHIR99021	Stemgent	Cat# 04-0004
Collagenase	Millipore Sigma	Cat# C9722
DAPT	Stemgent	Cat# 04-0041
Dibutyryl-cAMP	Millipore Sigma	Cat# D0627
Dulbecco's modified Eagle's medium (DMEM)	Thermo Fisher Scientific	Cat# 10569010
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12)	Thermo Fisher Scientific	Cat# 11320033
Fetal Bovine Serum (FBS), ESC-qualified	Thermo Fisher Scientific	Cat# 10439024
FGF2	PeproTech	Cat# 100-18B
FGF8a	R&D Systems	Cat# 4745-F8-050
GDNF	R&D Systems	Cat# 212-GD-010/CF
Glutamax	Thermo Fisher Scientific	Cat# 35050061
Knockout serum replacement (KSR)	Thermo Fisher Scientific	Cat# 10828028
Laminin	Millipore Sigma	Cat# L2020
LDN193189	Stemgent	Cat# 04-0074
Matrigel	Corning	Cat# 356231
N-2 supplement	Thermo Fisher Scientific	Cat# 17502001
Neurobasal medium	Thermo Fisher Scientific	Cat# 21103049
Non-essential amino acids	Thermo Fisher Scientific	Cat# 11140050

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Poly-ornithine	Millipore Sigma	Cat# P4957
Purmorphamine	Millipore Sigma	Cat# SML0868
SB431542	R&D Systems	Cat# 1614/1
SHH (C25II)	R&D Systems	Cat# 464-SH-025
TGF-β3	R&D Systems	Cat# 243-B3-002/CF
2-Mercaptoethanol	Thermo Fisher Scientific	Cat# 21985023
$CaCl_2 \cdot 2H_2O$	Millipore Sigma	Cat# C7902
CsCl	Millipore Sigma	Cat# 203025
CsOH	Millipore Sigma	Cat# 232041
Cs-$MeSO_3$	Millipore Sigma	Cat# C1426
D-glucose	Millipore Sigma	Cat# G6152
EGTA	Millipore Sigma	Cat# 03777
Fluo-4-AM	Thermo Fisher Scientific	Cat# F14201
HEPES	Millipore Sigma	Cat# H6147
KCl	Millipore Sigma	Cat# 31248
K-gluconate	Millipore Sigma	Cat# G4500
MgATP	Millipore Sigma	Cat# A9187
$MgCl_2 \cdot 6H_2O$	Millipore Sigma	Cat# 2393
NaCl	Millipore Sigma	Cat# 71380
Na_3GTP	Millipore Sigma	Cat# G8877
TEA-$MeSO_3$	Millipore Sigma	Cat# 86877
Experimental models: cell lines		
hiPSCs (isogenic)	Gasser Lab (Reinhardt et al., 2013)	N/A
Inactivated mouse embryonic fibroblasts (MEFs)	Gibco	Cat# S1520-100
Critical commercial assays		
CytoTune®-iPS Sendai Reprogramming Kit	Thermo Fisher Scientific	Cat# A13780
Software and algorithms		
Clampfit	Molecular Devices	https://www.moleculardevices.com/products/axon-patch-clamp-system/acquisition-and-analysis-software/pclamp-software-suite
ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov/ij/
PatchMaster	HEKA Elektronik	https://www.heka.com/downloads/downloads_main.html
Prism	GraphPad software	https://www.graphpad.com/scientific-software/prism/
ZEN lite	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
Others		
Ag/AgCl electrode	Warner Instruments	Cat# 64-1310
Borosilicate glass with filament	Sutter Instrument	Cat# BF150-110-10
Centrifuge	Thermo Fisher Scientific	N/A
CO_2 incubator	Thermo Fisher Scientific	N/A
Cover glasses	Fisher Scientific	Cat# 12-545-81
Flaming-Brown micropipette puller	Sutter Instrument	P-1000
HEKA EPC10 USB patch clamp amplifier	HEKA Elektronik	N/A
High-flow peristaltic pump	Instech	Cat# 50-811-69
In-line solution heater	Warner Instruments	Cat# NC9976304
In-line heater holder	Warner Instruments	Cat# NC0912977
Inverted confocal microscope	Zeiss	LSM880
Micro-Osmette Osmometer	Precision Systems	Cat# 5004
Multi-micromanipulator system	Sutter Instrument	Cat# 693185

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nylon syringe filters	Fisher Scientific	Cat# 03-391-3A
Petri dishes	Thermo Fisher Scientific	Cat# 150466
P720 pump tube sets	Instech	Cat# 50-811-81
Temperature controlled microscope stage adaptor	Zeiss	IMA-74-128 × 86 insert
Temperature controller	Warner Instruments	Cat# 642400
Upright microscope with a 40× water immersion objective	Zeiss	AX10
Water bath	Thermo Fisher Scientific	N/A
0.2 μm Syringe filter	Fisher Scientific	Cat# 09-719C
6-Well plates	Thermo Fisher Scientific	Cat# 140675
24-Well plates	Thermo Fisher Scientific	Cat# 142475

MATERIALS AND EQUIPMENT

Equipment for calcium imaging

We use an inverted confocal microscope LSM880 with incubation chamber and perfusion system. Zen software with the module for region of interest (ROI) measurement is required.

Whole-cell patch-clamp setup

A fully equipped whole-cell patch-clamp rig is needed. We use HEKA EPC10 amplifier, microscope setup with 40× water immersion objective by fluorescence and DIC optics, and multi-micromanipulator system. It should have a recording chamber suitable for cover glasses (with a diameter of 12 mm) and a heating and perfusion system to, respectively, heat up and exchange the recording solution continuously.

Pipette puller

A Flaming-Brown micropipette puller (P-1000) is used to pull glass pipettes. Glass capillaries with filament, 100 mm long, with 1.10 mm inner and 1.50 mm outer diameters, are needed for the patch pipettes. The ideal pipette resistance for human DA neuron whole-cell patch-clamp recording is 3–5 MΩ. Further details can be found in P-1000 operation manual [https://www.sutter.com/manuals/P-1000_OpMan.pdf].

Human ESC medium		
Reagent	Final concentration	Amount
DMEM/F12	N/A	400 mL
KSR	20%	100 mL
FGF2	4 ng/mL	2.0 μg
Glutamix	1 mM	500 μL
non-essential amino acids	100 μM	50 μL
2-mercaptoethanol	100 μM	50 μL

⚠ CRITICAL: Human ESC medium can be stored at 2°C–8°C up to 4 weeks.

MEF medium		
Reagent	Final concentration	Amount
DMEM	N/A	450 mL
FBS	10%	50 mL
non-essential amino acids	100 μM	50 μL
2-mercaptoethanol	100 μM	50 μL

⚠ CRITICAL: MEF medium can be stored at 2°C–8°C up to 4 weeks.

SRM medium		
Reagent	Final concentration	Amount
KSR	N/A	500 mL
FGF8a	100 ng/mL	50 μg
SHH (C25II)	100 ng/mL	50 μg
LDN193189	100 nM	0.1 μL
SB431542	10 μM	10 μL
CHIR99021	3 μM	3 μL
Purmorphamine	2 μM	2 μL

⚠ CRITICAL: SRM medium can be stored at 2°C–8°C up to 4 weeks.

External solution (for action potential recording)		
Reagent	Final concentration	Amount
NaCl	137 mM	8.01 g
KCl	5 mM	0.37 g
$CaCl_2 \cdot 2H_2O$	2 mM	0.29 g
$MgCl_2 \cdot 6H_2O$	1 mM	0.20 g
HEPES	10 mM	2.38 g
D-glucose	10 mM	1.80 g
Deionized water	N/A	~1000 mL
Total	N/A	1000 mL

⚠ CRITICAL: Solution should be prepared freshly on the day of the experiment and saturated with 95% O_2/5% CO_2 for at least 15 min before recording.

⚠ CRITICAL: Solution should be adjusted to pH 7.4 with NaOH, and osmolarity is at 300–310 mosM.

Intracellular solution (for action potential recording)		
Reagent	Final concentration	Amount
K-gluconate	126 mM	1.476 g
KCl	8 mM	0.030 g
HEPES	20 mM	0.238 g
EGTA	0.2 mM	0.004 g
NaCl	2 mM	0.006 g
MgATP	3 mM	0.079 g
Na_3GTP	0.5 mM	0.013 g
Deionized water	N/A	~50 mL
Total	N/A	50 mL

⚠ CRITICAL: This intracellular solution is used for spontaneous and evoked action potential (AP) recording of human DA neurons. Solution should be adjusted to pH 7.3 with KOH, and osmolarity is adjusted to 290–300 mosM with sucrose. Keep the intracellular solution in −80°C in aliquots up to 6 months, and avoid repeated freezing and thawing.

Fluo-4-AM stock solution		
Reagent	Final concentration	Amount
Fluo-4-AM	1 mM	50 μg
DMSO	N/A	45.58 μL
Total	N/A	45.58 μL

⚠ CRITICAL: The Fluo-4-AM stock solution can be stored at −20°C for 1–2 months.

HEPES-buffered artificial cerebrospinal fluid (ACSF, for calcium imaging)		
Reagent	Final concentration	Amount
NaCl	125 mM	7.31 g
KCl	5 mM	0.37 g
$CaCl_2 \cdot 2H_2O$	2 mM	0.29 g
$MgCl_2 \cdot 6H_2O$	1 mM	0.20 g
HEPES	10 mM	2.38 g
D-glucose	25 mM	4.50 g
Deionized water	N/A	~1000 mL
Total	N/A	1000 mL

⚠ CRITICAL: ACSF solution should be adjusted to pH 7.4 with NaOH, and osmolarity is at 300–310 mosM.

⚠ CRITICAL: HEPES-buffered ACSF can be stored at 2°C–8°C up to 1 week.

External solution (Ca^{2+} current recording)		
Reagent	Final concentration	Amount
TEA-$MeSO_3$	140 mM	9.34 g
HEPES	10 mM	0.48 g
$CaCl_2 \cdot 2H_2O$	10 mM	0.22 g
Deionized water	N/A	~200 mL
Total	N/A	200 mL

⚠ CRITICAL: Solution should be prepared freshly on the day of the experiment and saturated with 95% O_2/5% CO_2 for at least 15 min before recording.

⚠ CRITICAL: Solution should be adjusted to pH 7.4 with CsOH, and osmolarity is adjusted to 300–310 mosM with glucose.

Intracellular solution (Ca^{2+} current recording)		
Reagent	Final concentration	Amount
$CsMeSO_3$	135 mM	1.539 g
CsCl	5 mM	0.042 g
$MgCl_2 \cdot 6H_2O$	1 mM	0.010 g
MgATP	4 mM	0.105 g
HEPES	5 mM	0.060 g
EGTA	5 mM	0.095 g
Deionized water	N/A	~50 mL
Total	N/A	50 mL

⚠ CRITICAL: No potassium salt or sodium salt is used in the intracellular solution because potassium and sodium channels need to be blocked and stay inactive during Ca^{2+} current recording.

⚠ CRITICAL: This intracellular solution is used for Ca^{2+} current recording of human DA neurons. Solution should be adjusted to pH 7.3 with CsOH, and osmolarity is adjusted to 290–300 mosM with glucose. Keep the intracellular solution in −80°C in aliquots up to 6 months, and avoid repeated freezing and thawing.

⚠ CRITICAL: Fluorescent dye such as Alex Fluor 555 or 647 is added into intracellular solution (1:1000) to label recorded neurons and enable confirmation of the cell type after recording.

STEP-BY-STEP METHOD DETAILS

Calcium imaging

⏲ Timing: 2–3 h

1. Prepare HEPES-buffered ACSF.

Refer to materials and equipment for buffer recipe.

2. Dissolve 1 mM stock solution of Fluo-4-AM with neurobasal medium to a final concentration of 2 μM (1:500 dilution).

⚠ CRITICAL: This Fluo-4-AM working solution is prepared freshly in the hood and protected from light.

3. Load 1 mL of Fluo-4-AM working solution (2 μM) to human DA neurons (at differentiation day 68–75) plated on cover glasses.
4. Incubate neurons in a 37°C, 5% CO_2 incubator for 30 min.
5. Remove Fluo-4-AM working solution from neurons, and wash neurons three times with HEPES-buffered ACSF.
6. Incubate neurons at 22°C–25°C in the dark for additional 20–25 min to allow for complete dye de-esterification.
7. Place neurons in a 30°C–32°C heated adaptor, and live-cell imaging is performed by inverted confocal microscope LSM880 with a 40×/1.35NA oil-immersion objective.

⚠ CRITICAL: Perfuse imaging chamber with warm HEPES-buffered ACSF solution (30°C–32°C). High temperature (>40°C) or low temperature (<22°C) could affect the intracellular calcium level. Cells exposed to temperature range 28°C–40°C essentially maintain stable intracellular calcium level. Basically, temperature at 28°C–37°C is recommended for calcium imaging based on researcher's own choice. In our study, we keep neurons at 30°C–32°C to avoid the temperature effect on intracellular calcium and also to maintain physiological activities of neurons.

⚠ CRITICAL: Keep ACSF perfusion at a low rate of 1 mL/min.

8. Live-cell imaging is conducted with excitation wavelength of 485 nm and emission wavelength of 525 nm. Images are taken every second during the time of experiment.

⚠ CRITICAL: The frequency of acquisition we use is 1.0 Hz. Depending on the exposure time, latency between images can be from 1 to 5 s. More than 5 s is not recommended because fast calcium transitions can be missed.

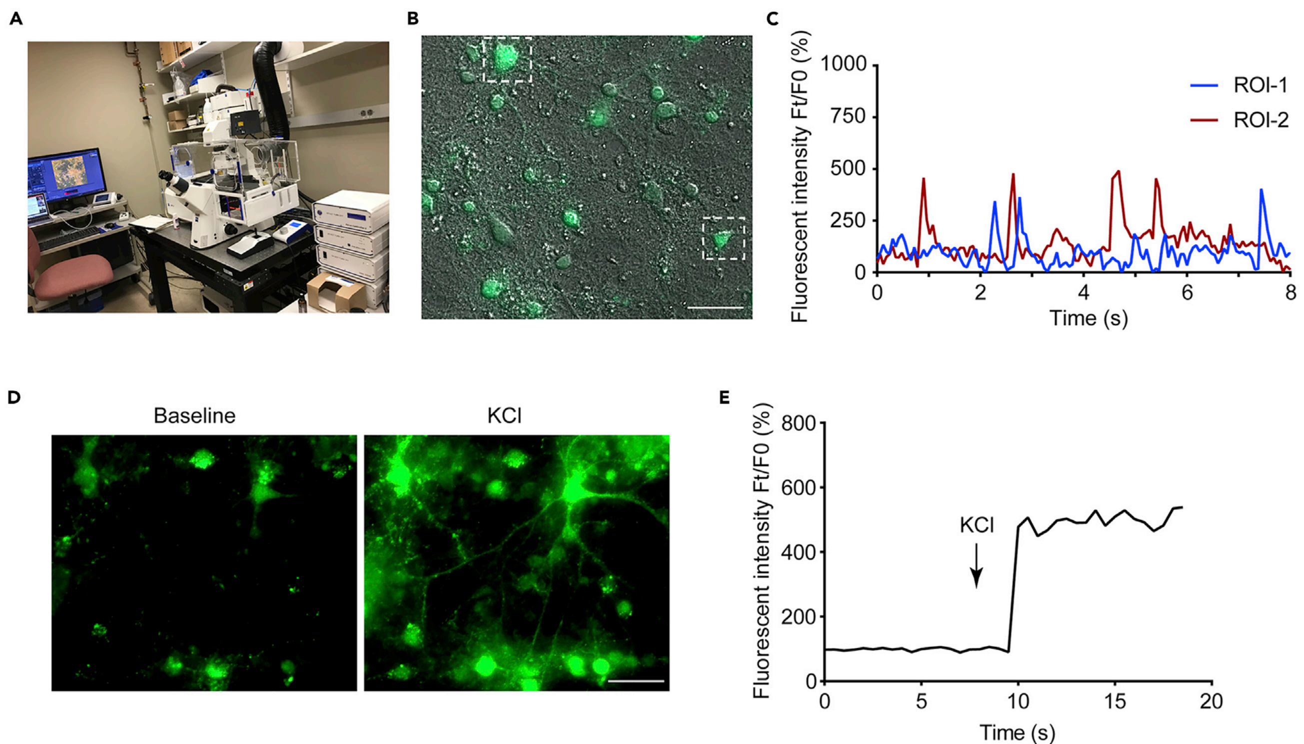


Figure 2. Calcium imaging of human DA neurons

(A) LSM880 setup for calcium imaging.

(B) Human iPSCs-derived DA neurons loaded with Fluo-4-AM. Scale bar, 50 μm.

(C) Recording of somatic calcium level in two neurons (as marked in B) during spontaneous activity.

(D) Changes in intracellular calcium in response to KCl (10 mM) in human DA neurons loaded with Fluo-4-AM. Scale bar, 50 μm.

(E) Changes of fluorescent intensity in human DA neurons induced by KCl (10 mM).

⚠ CRITICAL: For good results, finish live-cell imaging within 2 h after transferring neurons to the microscope. Neurons after calcium imaging are not recommended for further culture.

9. Record basal activity for at least 5 min before applying stimuli.
10. Using Zen software to generate the profile of fluorescence intensity for each cell over time (Figure 2 and Methods video S1).
    a. Open acquired image sequence in Zen software.
    b. Select regions of interest (ROI) by drawing the outline of cell soma. When defining ROIs, it is useful to play the movie to verify that the cells do not move during the course of experiment.
    c. Measure fluorescence intensity values for each time point.
    d. Transfer data to other statistical program (e.g., GraphPad Prism) for further analysis.

Ca^{2+} current recording

⏲ Timing: 3–4 h

11. Prepare external solution on the day of experiment.

Refer to materials and equipment for buffer recipe.

12. Take intracellular solution out from −80°C and thaw the solution on ice.

⚠ CRITICAL: Keep the intracellular solution on ice throughout the recording.

13. Prepare patch-clamp pipettes (3–5 MΩ).

Refer to materials and equipment for more information.

14. Turn on all the equipment and set the pump to perfuse external solution through the recording chamber.

⚠ CRITICAL: The external solution is continuously bubbled with 95% O_2 and 5% CO_2.

⚠ CRITICAL: The speed of external solution should be kept at 1–2 mL/min. A perfusion speed over 2 mL/min might lead to movements of the recording pipette and lifting of the cells from the coverslip.

⚠ CRITICAL: Temperature controller and in-line solution heater are used to keep external solution at 32°C during the recording.

15. Transfer the coverslip with human DA neurons to the recording chamber with the cells facing up.
16. Fill a 1 mL syringe with 200 μL of intracellular solution, connect a 0.2 μm pore filter to the syringe and attach a micro-loader tip to the filter. Use the syringe linked to the filter and micro-loader tip to fill the patch-clamp pipette with intracellular solution.

⚠ CRITICAL: Tap the pipette a few times to eliminate any air bubbles that might present in the tip.

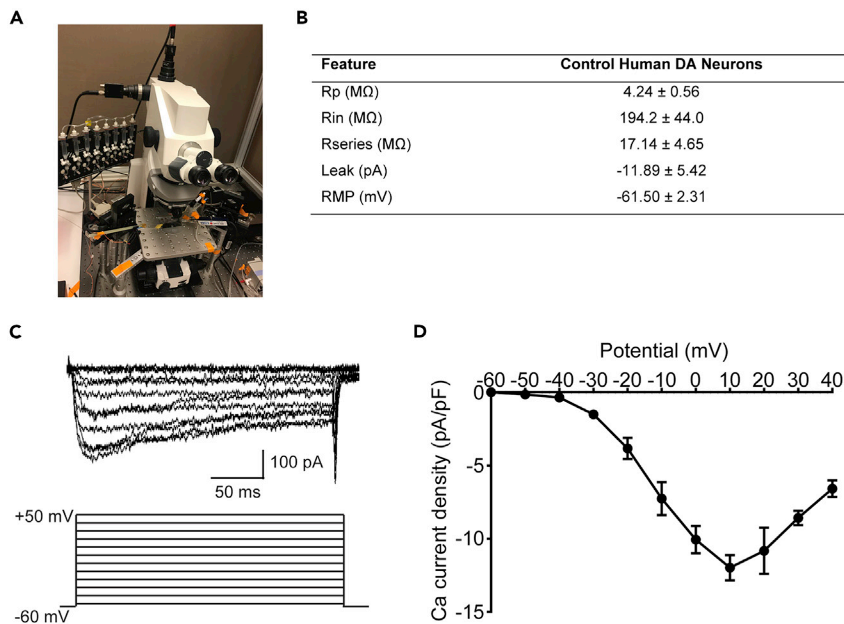
17. Place the pipette in the pipette holder. Put the pipette tip in the bath.
18. Once the pipette is in the bath, apply a very light positive pressure and hold the pressure in the pipette by using a three-way valve.
19. Search and focus on a healthy neuron with 40× water immersion objective.
20. Set the amplifier to voltage-clamp mode and correct the pipette offset so the currents measured at that point are considered as 0 pA.
21. Move the patch-clamp pipette by multi-micromanipulator to carefully approach the selected soma. Once the pipette tip touches the cell, a very small dimple is seen in the cell's membrane.
22. Release the positive pressure and apply a gentle suction to achieve gigaseal formation.
23. Once a gigaseal is obtained, change the voltage clamp to a negative voltage close to the expected resting membrane potential (−60 mV or −70 mV).
24. Rupture the membrane by applying light and short suction pulses using a syringe (or by using the "zap" function on the amplifier).
25. Hold the cell at −60 mV in voltage-clamp configuration. Record Ca^{2+} currents by stepping to various potentials from −60 mV to +50 mV for 250 ms in 10 mV increments.
26. Data are acquired by PatchMaster software, sampled at 10 kHz, and filtered at 2.9 kHz.
27. Once the recording is done, slowly and carefully detach the pipette from the neuron to let the membrane reseal.

Note: Recorded neurons are labeled with fluorescent dye and the cell type will be further validated by immunostaining.

28. Ca^{2+} currents are analyzed using Clampfit 10.5 software (Figure 3).

EXPECTED OUTCOMES

By using calcium imaging, we have measured the intracellular calcium levels in mature human DA neurons (Kim et al., 2020). Fluo-4-AM is excited by 485 nm and emission signals of 525 nm are collected. The brightness of the fluorescent signals represents the relative level of intracellular



Feature	Control Human DA Neurons
Rp (MΩ)	4.24 ± 0.56
Rin (MΩ)	194.2 ± 44.0
Rseries (MΩ)	17.14 ± 4.65
Leak (pA)	-11.89 ± 5.42
RMP (mV)	-61.50 ± 2.31

Figure 3. Calcium current recording of human DA neurons

(A) Whole-cell patch-clamp setup for Ca^{2+} current recording.

(B) Electrophysiological characteristics of hiPSCs-derived DA neurons. Rp, pipette resistance; Rin, input resistance; Rseries, series resistance which is typically 10–30 MΩ; Leak, leak current during recording, RMP, resting membrane potential.

(C) Representative whole-cell Ca^{2+} current traces.

(D) Ca^{2+} current density-voltage relationships. Data are presented as mean ± SEM.

calcium. For recording Ca^{2+} transients/sparks, a line scan mode is normally utilized. Ideally, the detector gain is set at around 600–800 (no digital gain). Line-scan images are acquired at sampling rate of 1.54 or 1.92 ms per line, along the longitudinal axis of the cell. Each line comprises 512 pixels spaced at 0.14 μm intervals. After a sequential scanning, a two-dimensional (2D) image of 512 × 1024 lines will be generated and stored for offline analysis. It is not recommended to scan a cell in the same line region for prolonged time because prolonged scanning in the same region may cause photo-bleaching of fluorescent molecules and experimental artifacts. For analysis, the somas of the cells are defined using the ROI measurement tool from ZEN software. Fluorescence intensity for each cell is generated and normalized as follows: basal fluorescence intensity is used to normalize recording data at each time point. Basal level is considered as the average intensity when cells are not having spontaneous calcium rises during the first minute of recording, before any stimulus is applied. Alternatively, basal level could be considered as the lowest value before addition of stimulus. An increase in the ratio exceeding fluctuation of the basal level (that has to be defined by the researcher based on the profile of each cell) is considered as neuronal spontaneous activity (some types of cells do not display any spontaneous activity). Usually, after a period of baseline time (cells stay in a steady-state condition), stimulus is applied, and an increase in magnitude or frequency is considered as stimulated response.

In our Ca^{2+} current recording procedure, a borosilicate glass pipette filled with intracellular solution is used to patch the human DA neuronal cell in whole-cell configuration. Calcium currents are measured by using Clampfit 10.5 software, and normalized by capacitance (An approximation of the whole cell capacitance can be made by using the formulate: membrane time constant = capacitance × series resistance. Usually, the researcher can set the series resistance and whole-cell capacitance on their setup and read the values off there). Average calcium current density-voltage relationships are calculated and illustrated in Figure 3.

Combining calcium image and Ca^{2+} current recording, this approach enables direct measurement of the dynamic calcium flux within neurons, investigating calcium dysregulation under disease model. This method is also suitable for other type of neurons or glia cells such as astrocytes and oligodendrocytes.

QUANTIFICATION AND STATISTICAL ANALYSIS

Please see a step-by-step example for somatic calcium level in two neurons during spontaneous activity as shown in Figure 2C.

1. Open GraphPad Prism software, click "XY" under "New Data Table and Graph".
2. For "X", choose "Numbers" which represents each time point; For "Y", choose "Enter and plot a single Y value for each point" which represents fluorescent intensity value for each time point.
3. Transfer the data generated from Zen software (Refer to Calcium Imaging: step 10 a–d) to the "New Data Table and Graph". Specifically, copy each time point numbers to "X" column; Copy fluorescent intensity values for each time point for neuron 1 (ROI-1) to the first "Y" column; Copy fluorescent intensity values for each time point for neuron 2 (ROI-2) to the second "Y" column.
4. The graph will be generated correspondingly.

LIMITATIONS

The typical purity of DA neurons, using our differentiation method, is approximately 84.13 $\pm$ 2.21% (confirmed by immunocytochemistry). In our study, we used immunostaining and electrophysiology to validate the properties of DA neurons. Alternatively, by using suitable reporter such as TH-GFP and PITX3-GFP to confirm each cell's identity would be beneficial for other downstream analysis.

Fluo-4-AM is a widely used calcium indicator because of its unique properties that confer a high signal-to-noise ratio, fast kinetics and high sensitivity. Since its introduction, Fluo-4 confocal calcium imaging has made significant contributions to our understanding of dynamics of many elementary process of Ca^{2+} signaling in various cell types. However, the major disadvantage of Fluo-4 dye, is that, upon binding of Ca^{2+}, there is no or little shift in its excitation or emission spectrum, which makes it impossible to perform ratiometric measurements of calcium. Alternative calcium indicators such as Fura-2 could potentially be used for that purpose.

The whole-cell patch-clamp technique is key to successfully achieve Ca^{2+} current recording. It takes practice to be familiar with the membrane rupture procedure and get consistent results. Healthy neurons, ideal patch pipette resistance, and a good gigaseal formation are important steps to ensure that the experiment works efficiently.

TROUBLESHOOTING

Problem 1

iPSC colonies do not attach to the plate after passaging (Culture and Maintenance of hiPSCs: step 10).

Potential solution

Matrigel plates might be old or died out. Ensure to use a Matrigel plate that is no more than 1 week and that every well is properly coated. In addition, some cell line may require a double concentration of Matrigel.

Problem 2

No neuronal spontaneous activity (Ca^{2+} spikes) is observed during baseline recording in calcium imaging (Calcium Imaging: step 9).

Potential solution

Keeping neurons in good condition is the prerequisite for calcium imaging. Make sure neurons are healthy.

Problem 3

No response is detected after stimulus application in calcium imaging (Calcium Imaging: step 9).

Potential solution

Before testing new stimulus, using a positive control to ensure the whole system is working. For example, applying KCl (10 mM) to neurons will induce immediate increase in fluorescence intensity. In addition, the pinhole of the confocal should be higher to increase the amount of the light and reduce the exposure time to avoid photo-bleaching induced damage.

Problem 4

High amount of electrical noise and low signal-to-noise ratio during Ca^{2+} current recording (Ca^{2+} Current Recording: step 18).

Potential solution

Check and make sure the electrophysiological rig is well grounded and separated from other electrical devices. Additionally, to obtain high-quality recordings, it is important to move the patch-clamp pipette very slowly when approaching a neuronal cell.

Problem 5

Difficulties in gigaseal formation and membrane rupture (Ca^{2+} Current Recording: steps 23 and 24).

Potential solution

There are multiple points in the procedure that might contribute to this problem and need to be assessed.

First, neuronal condition affects the quality of Ca^{2+} current recording. Healthy neurons should be selected and used in experiments. Patch another neuron if cells are unhealthy.

Second, preparation of patch-clamp pipette with ideal resistance is a crucial step for high-quality gigaseal formation. Although higher resistance pipettes (6–7 MΩ) are easier to form a gigaseal, they are more difficult to break through the membrane. We choose 3–5 MΩ for human DA neurons, which gives a lower series resistance and is better for reliable voltage-clamp recordings.

Third, it is important to maintain a stable series resistance (< 30 MΩ) throughout the recording because the current amplitudes will be altered if the series resistance changes. Fluctuation of series resistance (>10%) should be rejected from further recording.

Problem 6

No response for Ca^{2+} current (Ca^{2+} Current Recording: step 25).

Potential solution

The pH and osmolarity of external solution and intracellular solution should be accurately adjusted. Notably, the time for intracellular solution preparation should be under 1 h to keep the activity of the solution. Also, keep the intracellular solution on ice during the whole procedure of recording.

In addition, as we know, whole-cell patch-clamp allows sensitive and reliable analysis of the electrical activity of cell membranes at the molecular level. It also allows the use of intra- and extra-cellular recording solutions particularly devised to isolate the ionic membrane conductance of interest. However, one of the major problems is that the rupture of the membrane patch leads to the dialysis

of the intracellular compartment. Therefore, it may wash out some necessary cytoplasmic components that are responsible for the time-dependent changes in electrophysiological responses of cellular responses. The time-dependency decay of physiological cellular responses and membrane ionic currents is termed "run-down". The activity of calcium channels decreases rapidly when contact between membrane and cytosol is disrupted, causing the run-down of calcium channel activity. Ideal pipette resistance, good whole cell configuration, and successful membrane rupture help avoid or minimize the run-down of calcium currents. On the other hand, perforated patch approach can be used instead to preserve cellular integrity. Under perforated patch recording, the membrane patch under the pipet is not disrupted by suction but permeabilized so as to prevent the diffusion of large molecules out of the cell (Akaike and Shirasaki, 1992; Linley, 2013).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Valina L. Dawson, vdawson@jhmi.edu.

Materials availability

Human iPSC lines used in this study are available upon request from the lead contact, Valina L. Dawson, vdawson@jhmi.edu.

Data and code availability

All data needed to evaluate the conclusions in the paper are present in the paper. Additional data and codes related to this paper are available upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100405.

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AUTHOR CONTRIBUTIONS

X.Y. wrote the manuscript and performed the experiments. X.Y. and J.W.K. conceived and developed the protocol. S.L. helped with the experiments. V.L.D. and T.M.D. supervised the experimental study and reviewed the writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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