Application Protocol

RICOH imagine. change.

Ricoh Biosciences, Inc.

Culturing 3D iPSC-Derived Neuronal Spheroids with Primary Astrocytes for Calcium Flux Assay

Introduction

Ricoh Biosciences's proprietary transcription factor-based technology allows rapid and reproducible differentiation of human iPSCs into neurons without sacrificing the purity of the cells. Our excitatory neurons exhibit typical neuronal morphology with outgrowing neurites and express markers characteristic of a variety of neuronal subtypes, including the pan-neuronal marker tubulin beta 3 class III (TUBB3), the glutamatergic neuron marker vesicular glutamate transporter 1 (vGLUT1), and the cholinergic neuron marker choline acetyltransferase (ChAT).

In this Application Protocol, we describe how our human iPSC-derived excitatory neurons can be thawed, plated, and maintained as 3D spheroids utilized in a Calcium flux assay of neuronal activity. Our neurons show spontaneous Ca^{2+} oscillation in approximately 3 weeks and respond to a variety of drugs within 6 weeks after plating when co-cultured with human primary astrocytes.

Required Equipment and Consumables

Item	Vendor	Catalog number
Functional Drug Screening System (FDSS/µCELL)	Hamamatsu Photonics	C13299
Nunclon™ Sphera™ 96-well, Nunclon Sphera Treated, U-Shaped-Bottom Microplate	ThermoFisher	174925
384-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate	Corning	4516
ART™ Wide Bore Filtered Pipette Tips	ThermoFisher	2069GPK
Quick-Neuron™ Excitatory - Human iPSC-derived Neurons	Ricoh Biosciences	EX-SeV-HC-CW50065
Component N	Ricoh Biosciences	COMP-N
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
Glutamax (100x)	ThermoFisher	35050061
DPBS, no calcium, no magnesium	ThermoFisher	14190144
B-27™ Plus Neuronal Culture System Kit	ThermoFisher	N7805200
Ascorbic acid	Sigma-Aldrich	A4544
Neuron Culture Medium	FUJIFILM WAKO Pure Chemical	148-09671
Human Astrocytes	ScienCell Research Laboratories	1800
ScienCell Astrocyte Medium Kit	ScienCell Research Laboratories	1801
Poly-L-lysine solution	Sigma-Aldrich	P4707

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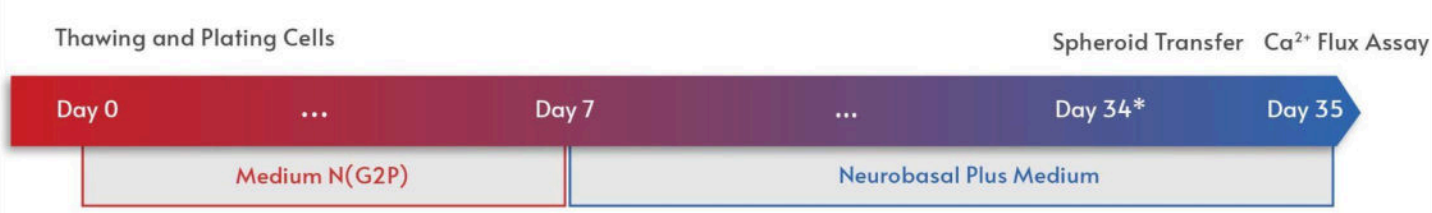
Trypsin-EDTA (0.05%)	ThermoFisher	25300054
Synthetic Trypsin Inhibitor Solution (s-TI)	Cell Science & Technology Institute	1220
Cal-520 AM	AAT Bioquest	21130
Pluronic® F-127 10% solution in water	AAT Bioquest	20053
HBSS (-)	ThermoFisher	14175095
1 M HEPES	ThermoFisher	15630080
Magnesium Chloride Hexahydrate	FUJIFILM WAKO Pure Chemical	133-00161
Calcium Chloride	FUJIFILM WAKO Pure Chemical	038-24985
D(+)-Glucose	FUJIFILM WAKO Pure Chemical	047-31161
Distilled water (cell culture grade)	ThermoFisher	15230170

Workflow



*This should be done 1 day prior to the Ca^{2+} assay. The Ca^{2+} assay can be performed anytime from day 35 until day 42, though we recommend day 35. Cells should continue to be maintained in Neurobasal Plus Medium until the assay.

Experiment Planning

This application protocol gives instructions for making 120 spheroids which fill half of one 384-well plate. Cell numbers and reagents can be multiplied or reduced according to users needs (ie: to make 240 spheroids to fill a full 384-well plate).

Astrocyte Culture

Human primary astrocytes should be thawed according to manufacturer's instructions and maintained using ScienCell Astrocyte Medium as per manufacturer's recommended protocols.

- We recommend establishing astrocyte culture 10 days before proceeding with spheroid formation.
- 1 vial of cryopreserved human primary astrocytes can be thawed, expanded, harvested, and cryopreserved (e.g., 1 x 10^6 cells/vial) until use.
- The instructions below are for using 1 thawed vial of astrocytes although astrocytes may also be used fresh from expansion culture, without prior cryopreservation. Repeat passaging is not recommended.
- 3D spheroid culture with Excitatory neurons requires approximately 1 million astrocytes for half of a 384-well plate. This is usually obtained by using one 100 mm cell culture dish.

Preparation

200 mM Ascorbic Acid

1. Dissolve 352 mg ascorbic acid in 10 ml cell culture-grade water.
2. Sterilize using a 0.22 µm syringe filter.

3. Make aliquots of a convenient volume (e.g., 100 µl).
4. This solution, hereafter referred to as Ascorbic Acid, can be stored at -20°C.

Medium N

1. Prepare Medium N using the reagents listed in the table below.
    - Thaw Component N on ice for 20-30 minutes.
    - Warm all other reagents at room temperature for 20-30 minutes.
    - Tap Component N 3 times and then briefly spin it down before use.
    - Keep Medium N, and any subsequent media made with it, protected from light.
    - Store Medium N for up to 2 weeks at 4°C.
    - Leftover Component N can be discarded or saved at 4°C for up to two weeks.

Reagents	Volume
DMEM/F12	26.2 ml
Neurobasal	26.2 ml
200 mM Glutamax (100x)	275 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	550 µl
Component N	1.7 ml

Medium N(G2P)

1. Prepare Medium N(G2P) using the reagents listed in the table below.
    - Thaw 3 vials of Component G2 on ice and 2 vials of Component P at room temperature for 20-30 minutes.
    - Briefly spin down all Components before use.
    - Medium N(G2P) can be stored at 4°C for up to 2 weeks.

Reagents	Volume
Medium N	44 ml
Component G2	44 µl
Component P	22 µl

Day 0

5-6 hours

Note: We recommend using 4,000-8,000 neurons per spheroid. The recommended ratio of neurons to astrocytes is 1:1-2:1. Following the instructions below, 8,000 neurons and 8,000 astrocytes are used for each spheroid.

Astrocyte suspension preparation

1. Warm Medium N and Medium N(G2P) at room temperature for 20-30 minutes.
2. Take out the vial of frozen cells from the liquid nitrogen storage tank.
3. Incubate the cryovial in a water bath set at 37°C (do not submerge the cap) until most of the content is thawed but a small ice crystal remains (~2 min).
4. Wipe the vial with a dry paper towel. Spray the vial with 70% ethanol and place it inside a biosafety cabinet.
5. Transfer 4.5 ml room temperature Medium N to a new 15 ml conical tube.Set a P1000 pipette to 1 ml but take approximately 500 µl Medium N from the 15 ml conical and add it to the cryovial
6. dropwise at 1 drop per 1-2 seconds.
    - **IMPORTANT!** Use the same pipette tip for Steps 6-10.
7. Gently pipet the cell suspension up and down once.
8. Gently transfer all of the cell suspension to the 15 ml conical tube prepared in Step 5.

9. Take 1 ml of the cell suspension from the conical tube and add it to the original cryovial and pipet up and down 2-3 times and then transfer the entire contents back to the same 15 ml conical tube.
10. Mix the contents in the conical tube by gently pipetting cell suspension up and down 3 times.
11. Remove 200µl of the suspension from the centrifuge tube and count the number of viable cells using a cell counter.
12. Centrifuge the cell suspension in the 15 ml conical tube at 200 x g for 4 minutes.
13. Use an aspirator to remove most of the supernatant from the conical tube, leaving a small volume of the supernatant (<50 µl) to cover the pellet.
14. Tap the side of the conical tube up to 10 times to break up the cell pellet.
15. Add 1 ml room temperature Medium N(G2P) to the conical tube using a P1000 pipettor and pipet up and down no more than 2-3 times.
16. Add additional Medium N(G2P) to reach a concentration of 8 x 10^4 viable cells/ml and pipet to form a cell suspension. A minimum of 13.3 ml is required.
17. Keep the astrocyte cell suspension at room temperature while preparing the neuronal cell suspension.

Neuron suspension preparation

1. Take out the vial of frozen cells from the liquid nitrogen storage tank.
2. Incubate the cryovial in a water bath set at 37°C (do not submerge the cap) until most of the content is thawed but a small ice crystal remains (~2 min).
3. Wipe the vial with a dry paper towel. Spray the vial with 70% ethanol and place it inside a biosafety cabinet.
4. Transfer 4.5 ml room temperature Medium N to a new 15 ml conical tube.
5. Set a P1000 pipette to 1 ml but take approximately 500 µl Medium N from the 15 ml conical and add it to the cryovial dropwise at 1 drop per 1-2 seconds.
    - **IMPORTANT!** Use the same pipette tip for Steps 6-10.
6. Gently pipet the cell suspension up and down once.
7. Gently transfer all of the cell suspension to the 15 ml conical tube prepared in Step 5.
8. Take 1 ml of the cell suspension from the conical tube and add it to the original cryovial and pipet up and down 2-3 times and then transfer the entire contents back to the same 15 ml conical tube.
9. Mix the contents in the conical tube by gently pipetting cell suspension up and down 3 times.
10. Remove 200µl of the suspension from the centrifuge tube and count the number of viable cells using a cell counter.
11. Centrifuge the cell suspension in the 15 ml conical tube at 200 x g for 4 minutes.
12. Use an aspirator to remove most of the supernatant from the conical tube, leaving a small volume of the supernatant (<50 µl) to cover the pellet.
13. Tap the side of the conical tube up to 10 times to break up the cell pellet.
14. Add 1 ml room temperature Medium N(G2P) to the conical tube using a P1000 pipettor and pipet up and down no more than 2-3 times.
15. Add additional Medium N(G2P) to reach a concentration of 8 x 10^4 viable cells/ml and pipet to form a cell suspension. A minimum of 13.3 ml is required.

Plating

1. Mix 13.3 ml astrocyte suspension with 13.3 ml neuronal cell suspension.
2. Distribute 200 µl of cell suspension mix into each of 120 wells of a spheroid plates.A multichannel pipet can be used for this.
3. Incubate at 37°C, 5% CO_2 for 3 days.

Day 3

< 1 hour

Medium Change

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out 100 µl of old medium from each well and add 100 µl of Medium N (G2P) to each well.
3. Incubate at 37°C, 5% CO_2 for 4 days.

Day 7

< 1 hour

Medium Change

1. Prepare Neurobasal Plus Medium using the components listed in the table below.
    - Thaw the B-27™ Plus Supplement (50x) at room temperature for 1 hour (or at 4°C overnight).
    - Store Neurobasal Plus for up to 2 weeks at 4°C.

Reagents	Volume
Neurobasal Plus Medium	48 ml
B-27™ Plus Supplement (50x)	1 ml
200 mM Glutamax (100x)	500 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	500 µl
200 mM ascorbic acid (filter-sterilized)	50 µl
Neuron Culture Medium	5 ml

2. Pipet out 100 µl of old medium from each well and add 100 µl of room temperature Neurobasal Plus Medium to each well.
3. Repeat step 2 approximately every 3 days (twice a week), making more Neurobasal Plus Medium as needed.

Day 34 (the day before Calcium flux assay)

2 hours

Spheroid transfer

Note: While the instructions below are for manual transfer, it is also possible to transfer spheroids using an automated liquid handler such as ASSIST PLUS (INTEGRA Biosciences) and Automated Liquid Handling Platform (Agilent Bravo).

1. Warm Neural Basal Plus Medium at room temperature for 20-30 minutes.
2. Pipet 50 µL of medium into 120 wells of a 384-well assay plate
3. Using a P200 pipette with a bore tip, transfer the spheroids from the 96-well culture plate to the 384-well assay plate.
    - Note: The outermost and second outermost periphery wells should be filled with 100 µl PBS per well. Spheroids can be placed in every other row of the center wells, with unused rows being filled with PBS as well. A multichannel pipette can be used

Preparation

0.1 M $MgCl_2$

1. Dissolve 1.0 g $MgCl_2$ in 50 ml cell culture-grade water.
2. Sterilize using a 0.22 µm syringe filter.
3. This solution, hereafter referred to as 0.1 M $MgCl_2$, can be stored at 4°C.

1 M $CaCl_2$

1. Dissolve 5.5 g $CaCl_2$ in 50 ml cell culture-grade water.
2. Sterilize using a 0.22 µm syringe filter.
3. This solution, hereafter referred to as 1 M $CaCl_2$, can be stored at 4°C.

1 M Glucose

1. Dissolve 9.0 g glucose in 50 ml cell culture-grade water.
2. Sterilize using a 0.22 µm syringe filter.
3. This solution, hereafter referred to as 1 M Glucose, can be stored at 4°C.

Recording medium

1. Prepare Recording Medium using the reagents listed in the table below.
    - Recording Medium can be stored at 4°C.

Reagents	Volume
HBSS (-)	10 ml
1 M HEPES	100 µl
0.1 M $MgCl_2$	10 µl
1 M $CaCl_2$	20 µl
1 M Glucose	44.6 µl

4 mM Cal-520 AM

1. Dissolve 50 µg Cal-520 AM in 11.3 µL DMSO.
2. This solution, hereafter referred to as 4 mM Cal-520, can be stored at -20°C.

Drug preparation (anytime before FDSS assay)

1. Dissolve the compounds in DMSO and prepare 1000-fold the concentration to be used in the assay.
2. Store prepared drugs at -20°C.

0.44% DMSO preparation

1. Add 5.3 µl DMSO to 1194.7 µl PBS.
2. This solution, hereafter referred to as 0.44% DMSO, can be stored at -20°C.

Day 35

5-6 hours

Cal-520 solution preparation

1. Prepare Cal-520 solution using the reagents listed in the table below.
    - Warm the Neurobasal Plus Medium at room temperature 20-30 minutes.

Reagents	Volume
Neurobasal Plus Medium	1.2 ml
4 mM Cal-520	6.6 µl
10% Pluronic	2.4 µl

Calcium flux assay

1. Pipet out 25 µl of old medium from each well and add 25 µl Neurobasal Plus medium.
2. Incubate at 37°C, 5% CO_2 for at least 1 hour.
3. Add 5.0 µL of Cal-520 solution to each well.
4. Incubate at 37°C, 5% CO_2 for 1 hour.
5. Warm Recording Medium at 37°C for 1 hour.
6. Pipet out the Cal-520 containing medium and add 50 µl Recording medium to each well.
7. Incubate at 37°C, 5% CO_2 for 15 minutes.
8. Add 5.0 µL of 0.44 % DMSO to all wells.
9. Incubate at 37°C, 5% CO_2 for 30 minutes.
10. Record Ca^{2+} oscillation at 0.1 sec intervals for 20 minutes to form a baseline prior to drug administration.
11. Add 5 µL of concentration-adjusted drug to each well and incubate at 37°C, 5% CO_2 for 40 minutes.
12. Record Ca^{2+} oscillation at 0.1 second intervals for 20 minutes.

Data Analysis

1. Analyze Ca^{2+} transient waveform using FDSS software and extract the following parameters: Waveform peak number, Peak-to-peak time, Peak luminance value/bottom luminance value ratio, Peak amplitude, Bottom luminance value, Rise and fall slope, Peak pulse width 10 % to 90 %, Peak total area (Area under curve).
2. Perform drug response analysis using relevant parameters of choice.

Appendix

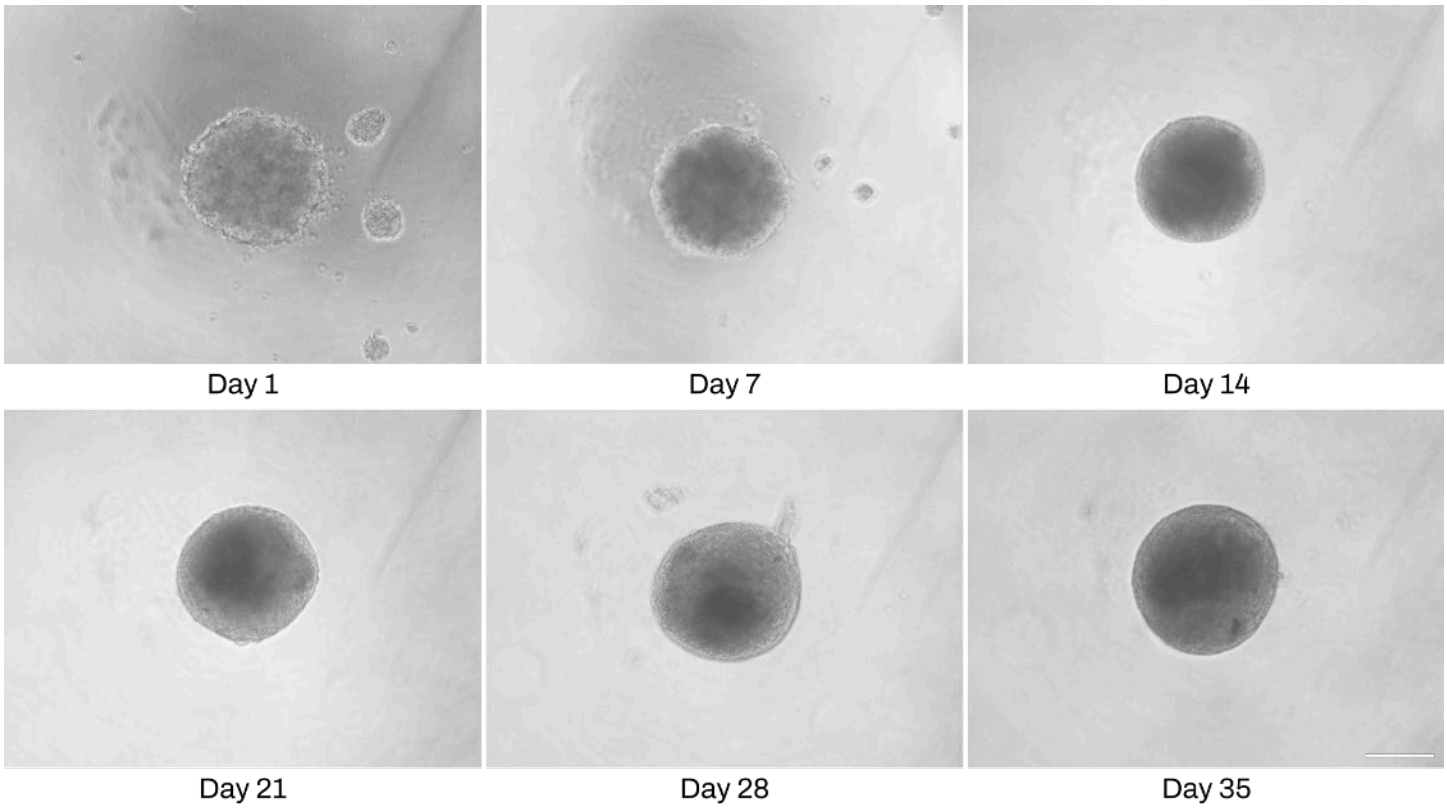


Figure 1. Representative phase contrast images of 3D spheroid cultures on days 1-35 (scale bar = 200 µm).

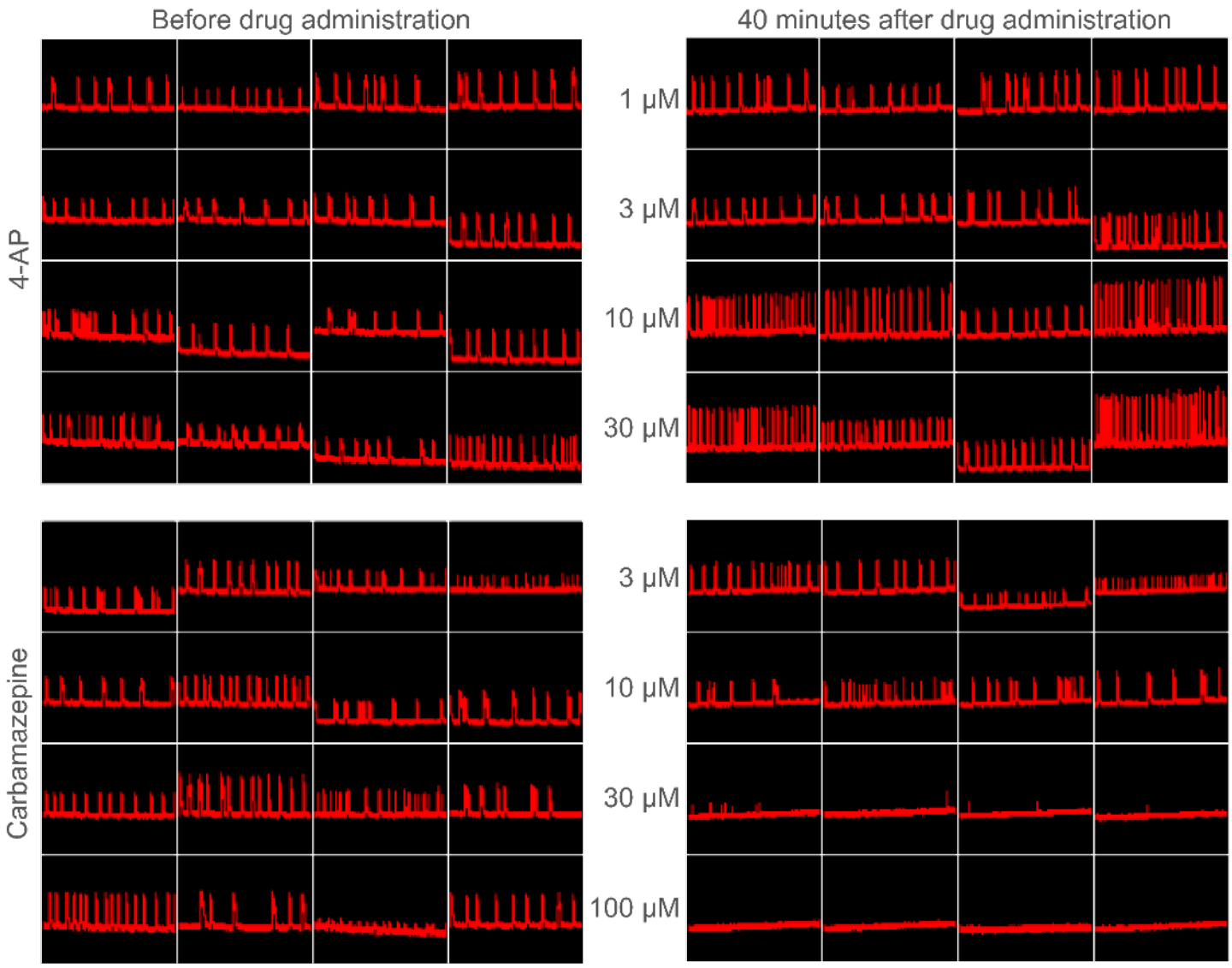


Figure 2. Examples of Ca^{2+}-transient recordings. The effects of 1 to 30 µM of 4-Aminopyridine (4-AP) and 3 to 100 µM of Carbamazepine on hiPSC-derived neurons and human primary astrocytes cultured as 3D spheroids. The traces are at around 40 minutes after the drug administration.

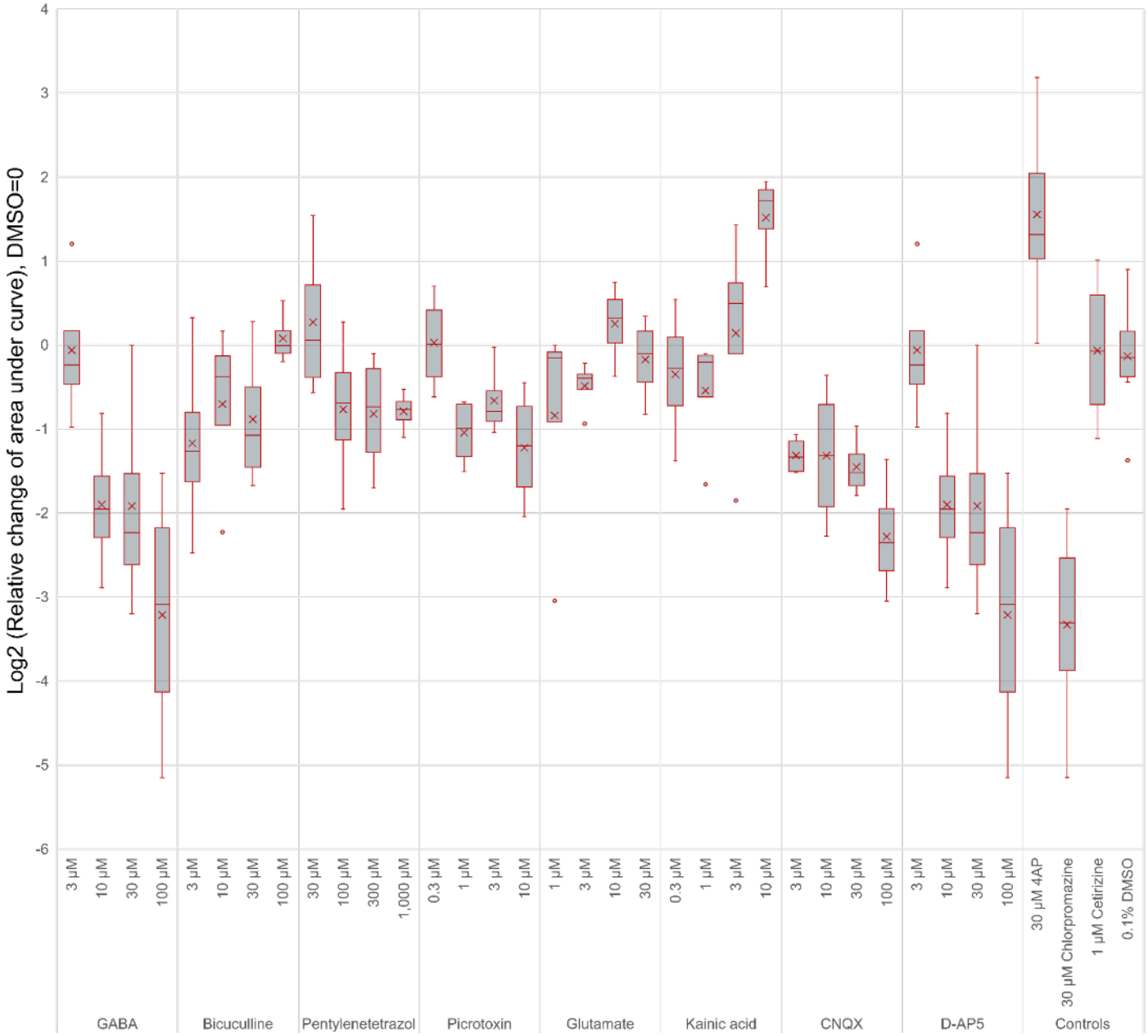


Figure 3. Drug response. iPSC-derived neurons were co-cultured as spheroids with human primary astrocytes in 384-well plates for 5 weeks after differentiation. Ca^{2+} spikes were detected and the area under the curve of the spikes was determined. The ratio of the values before and 40 minutes after the drug administration was calculated.

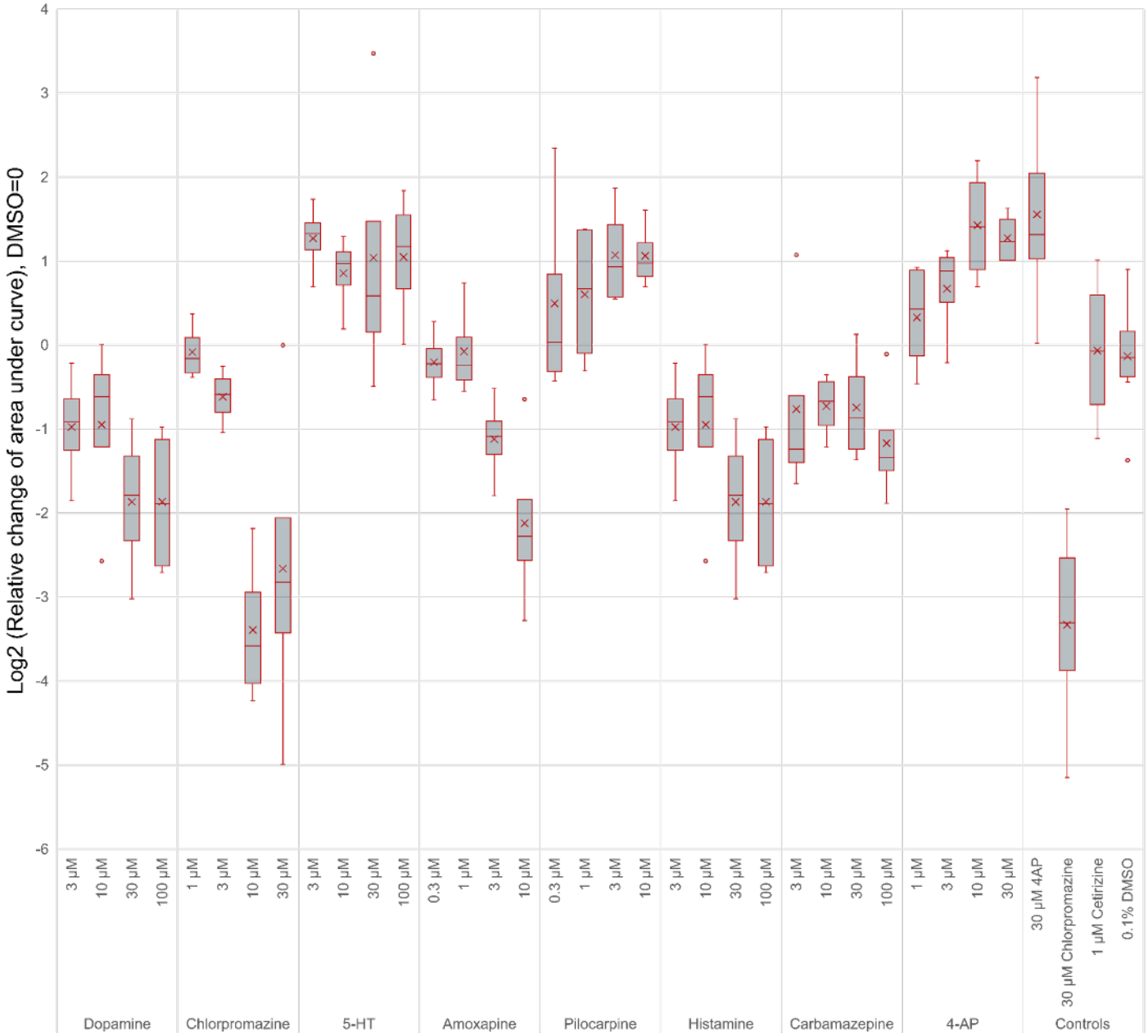


Figure 4. Drug response. iPSC-derived neurons were co-cultured as spheroids with human primary astrocytes in 384-well plates for 5 weeks after differentiation. Ca^{2+} spikes were detected and the area under the curve of the spikes was determined. The ratio of the values before and 40 minutes after the drug administration was calculated.

Acknowledgement

This protocol was optimized and the included data has been generated by the Biomedical Research and Development Department of Ricoh Company Ltd.