CNS-3D Brain Organoids: Calcium Imaging Assay Protocol

Purpose and use

CNS-3D Brain Organoids are compatible with plate-based calcium imaging systems, including:

- Molecular Devices FLIPR platforms (validated settings provided)
- Hamamatsu FDSS platforms
- Curi Bio Nautilai™ and Nautilai Plus™

This protocol covers handling 96-well and 384-well organoid plates, preparing reagents, and performing the calcium imaging assay using the FLIPR® Calcium 6 Assay Kit (Molecular Devices; R8194, R8190, R8191, R8195)[1]. All numeric read parameters in this document are validated for FLIPR platforms. For FDSS or other calcium imaging instruments, contact 28bio for assistance configuring equivalent read modes and methods.

Use: For research use only. Recipients are responsible for safe storage, handling, and use of CNS-3D Brain Organoids. 28bio is not liable for damages or injuries arising from receipt or use of this product.

Step 1 Exchange to PRF medium (3 half-medium changes) → **Step 2** Prepare calcium-6 dye → **Step 3** Add dye to plate 1:1, incubate 2 hrs → **Step 4** Perform calcium read(s) up to 4 hrs

[1] Reference the Molecular Devices FLIPR Assay Kit Manual for which kit is required for assay needs.

Media preparation

IMPORTANT: Prepare medium and establish liquid-handler settings before beginning the assay.

1. Prepare BrainPhys™ Phenol Red Free Complete Medium (PRF Medium) according to the table below.
2. Store prepared medium protected from light at 4°C for up to two weeks.
3. Before use, aliquot the required amount and allow it to warm to room temperature.

Component	Supplier	Catalog #	Amount	Stock Conc.	Final Conc.
BrainPhys™ Without Phenol Red	STEMCELL	05791	500 mL	N/A	1×
NeuroCult™ SM1 Neuronal Supplement	STEMCELL	05711	10 mL	50×	1×
BDNF	STEMCELL	78005	10 µg	100 µg/mL	20 ng/mL
GDNF	STEMCELL	78058	10 µg	100 µg/mL	20 ng/mL
Penicillin-Streptomycin (optional)	HyClone	SV30010	5 mL	100×	1×

Media requirements

- Automated liquid handling requires approximately 10 mL of PRF Medium for priming, plus approximately 30 mL per assay plate for the three PRF Medium exchanges.
- 11 mL of PRF Medium is required per assay plate for C6 dye preparation.
- Additional medium is required if compound dosing is performed with the FLIPR T0 measurement.

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Additional equipment/reagents:

- Automated liquid handler (recommended: Appendix A)
- Multi-channel pipette
- Biosafety cabinet
- 70% ethanol or isopropanol
- 4°C refrigerator

Optional equipment/reagents:

- 384-well FLIPR Pipette Tips (Molecular Devices, 9000-0764)
- 384-well Polypropylene Conical Bottom Microplates (Greiner Bio-One, 781281)

CNS-3D Brain Organoid plate preparation

1. Exchange the culture medium for PRF Medium to remove phenol red before the assay. Perform three half-medium changes with PRF Medium, according to the plate-format parameters below.
2. Remove FLIPR® Calcium 6 Assay Kit Component A (C6 dye) from –20°C and allow it to warm to room temperature before reconstitution. Only Component A is required for calcium imaging.
3. Reconstitute the C6 dye by adding 11 mL of PRF Medium to the vial to form the C6 Dye Solution. Mix the contents thoroughly and transfer to a sterile reservoir.
4. Aspirate half the volume of each well and replace with an equal volume of C6 Dye Solution, according to the plate-format parameters below.
5. Return plate(s) to a cell culture incubator (37°C, 5% CO_2, and 95–98% humidity) and incubate for 2 hours.

NOTE: If using an automated liquid handler, reference Appendix A to establish aspiration protocol.

NOTE: If there is more than one plate for the assay, add dye to each plate at staggered intervals of 10–15 minutes, recording the time of addition for each plate.

Plate-format parameters

Action	96-Well Plate	384-Well Plate
Initial volume	200 µL per well.	50 µL per well.
PRF Medium exchanges	Remove 100 µL, then add 100 µL PRF Medium. Repeat for 3 exchanges total.	Remove 25 µL, then add 25 µL PRF Medium. Repeat for 3 exchanges total.
Aspirate	Remove 100 µL.	Remove 25 µL.
Add C6 Dye Solution	Add 100 µL per well.	Add 25 µL per well.
Final volume	200 µL per well.	50 µL per well.

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FLIPR assay protocol

Instrument setup (30 minutes prior to assay)

1. Power on the camera chilling unit, power on the FLIPR unit, and open ScreenWorks software.
2. Set the stage temperature to 37°C and the camera temperature to −58°C.

Establish the baseline read protocol

3. In ScreenWorks, select File > New and open the green Settings tab. Enter the baseline read parameters in the table below.

Section	Setting	Value
Setup Read Mode	Read Mode Name	Read_Mode_1
	Reading Mode	Fluorescence
	Excitation / Emission wavelength (nm)	470_495 / 515_575
	Excitation intensity (%)	40
	Exposure time (s)	0.4
	Camera gain	40
Plate Assignments	Read Plate	Default 96 or Default 384
	Source Plate 1, 2, 3	NONE
Read Time Interval	Read time interval (s)	0.5
	Number of reads	1200

4. Save protocol using ‘Baseline’ or ‘BL’ in the file name to indicate the protocol is read-only and does not include a liquid transfer step. This protocol will be used for all read-only timepoints.

Reading a plate

5. Remove the plate from the incubator and place it in the READ position within the FLIPR.
6. Click RUN. Indicator lights will change to signal the start of the assay. Progress is shown in the bottom right corner of the user interface.
7. When the read completes (~10 minutes), return the plate to the incubator for 20 minutes. If running multiple plates in succession, begin reading the next plate now.

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Compound dosing

8. If performing manual compound dosing, remove plate from incubator and add 6× concentrated compound according to the plate-format parameters below. Final concentration in the well is 1×.

Action	96-Well Plate	384-Well Plate
Add 6× compound	Add 40 µL per well.	Add 10 µL per well.

9. If dosing using the FLIPR's liquid handling system, proceed to Appendix B.
10. Return the plate to the READ position and repeat steps 6 and 7, ensuring that the plate is maintained for 20 minutes in the incubator in between reads to equilibrate to 37°C, 5% CO_2.

NOTE: Plates may be read out to 4 hours (T240) from compound addition, after which point the C6 dye can begin to affect organoid function.

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Appendix A: Automated liquid handler setup and plate specifications

Establish and verify the aspiration and dispensing protocol before changing medium in CNS-3D Brain Organoid plates.

96-well liquid handler profile

1. Ensure the automated system has the correct plate dimensions for the Corning® Spheroid Microplate (Catalog Number 4520, Figure 1 below).
2. Fill an empty Corning Spheroid Microplate with liquid, such as water.
3. Set the aspiration parameters to leave 100 µL per well, with a low aspiration rate for greater accuracy.
4. Set the dispensing parameters so the tips have a low clearance from the top of the plate and are positioned over the well center. Use a low dispensing rate to avoid turbulence and dispense 100 µL.
5. Test the protocol and confirm 100 µL of fluid is aspirated from and then dispensed into each well.

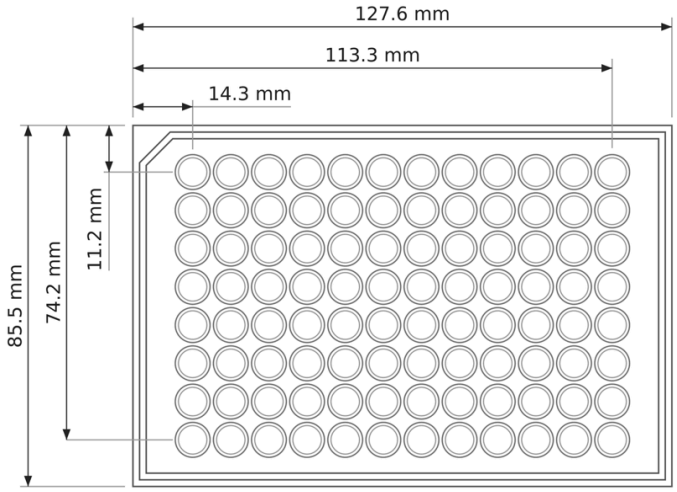


Figure 1. Corning spheroid microplate 4520 overhead dimensions

Key dimensions for Corning Spheroid Microplate 4520 (96-well):

Specification	Value
Total plate height	14.20 mm
Distance from plate bottom to well bottom	1.86 mm
Well bottom thickness	0.09 mm
Well depth	12.10 mm

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384-well liquid handler profile

1. Ensure the automated system has the correct plate dimensions for the Corning® Spheroid Microplate (Catalog Number 4516, Figure 2 below).
2. Fill an empty Corning Spheroid Microplate with liquid, such as water.
3. Set the aspiration parameters to leave 25 µL per well, with a low aspiration rate for greater accuracy.
4. Set the dispensing parameters so the tips have a low clearance from the top of the plate and are positioned over the well center. Use a low dispensing rate to avoid turbulence and dispense 25 µL.
5. Test the protocol and confirm 25 µL of fluid is aspirated from and then dispensed into each well.

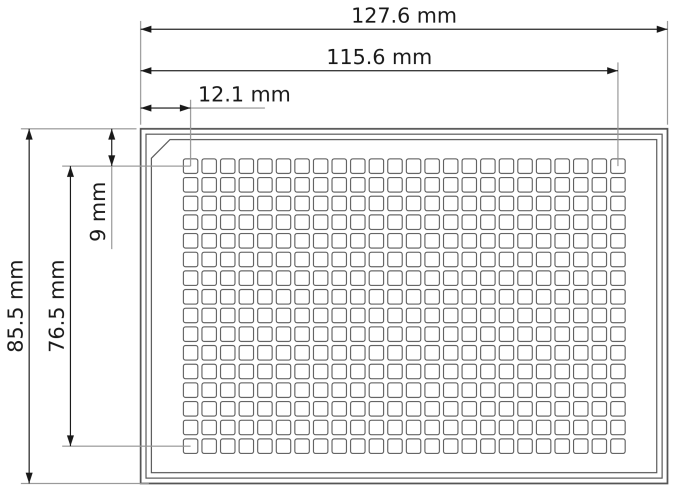


Figure 2. Corning spheroid microplate 4516 overhead dimensions

Key dimensions for Corning Spheroid Microplate 4516 (384-well):

Specification	Value
Total plate height	14.20 mm
Distance from plate bottom to well bottom	1.81 mm
Well bottom thickness	0.09 mm
Well depth	12.30 mm

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Appendix B: Establishing 384-well FLIPR liquid handling protocol

Compound dosing can be performed using the FLIPR liquid handling system in place of manual dosing. This appendix covers 384-well plate dosing only. Dosing 96-well plates requires 96-well-specific consumables and transfer volumes—contact support@28bio.com for support.

1. The following consumables are required:
   - 384-well polypropylene conical bottom microplate as the compound source plate (e.g., Greiner Bio-One 781281).
     a. Add the microplate to the ScreenWorks Plate Library as a Source Plate before first use. Obtain plate specifications from the manufacturer.
     b. Prepare compounds at 6× in the source plate with >40 µL per well to allow adequate dead volume after aspiration.
     c. The same compound plate can dose multiple plates provided >30 µL dead volume remains.
   - 384-well FLIPR pipette tips (Molecular Devices 9000-0764).
     a. Use a new set of tips for each FLIPR read, even when the compound plate is reused.
2. Establish the T0 read protocol. In ScreenWorks, select File > New, navigate to the green Settings tab, and enter the parameters from the table below.

Section	Setting	Value
Setup Read Mode	Read Mode Name	Read_Mode_1
	Reading Mode	Fluorescence
	Excitation / Emission wavelength (nm)	470_495 / 515_575
	Excitation intensity (%)	40
	Exposure time (s)	0.4
	Camera gain	40
Plate Assignments	Read Plate	Default 384
	Source Plate 1	FLIPR Pipette tips (SOURCE 1 TIPS position)
	Source Plate 2	[Compound Plate Name]
	Source Plate 3	NONE
Tips Position	Load tips position	☑
Transfer Fluid: General Settings	Fluid Transfer Type	Single aspirate – single dispense
	Read	☑
Transfer Fluid: Aspirate	Aspirate	☑
	Plate	Source Plate 2
	Volume	10 µL
	Speed	20 µL/s
	Hold volume	0 µL
	Height	5 µL
	Tip up speed	20 mm/s
	Mix fluid before aspirate	☑

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Section	Setting	Value
Transfer Fluid: Dispense	Dispense	☑
	Plate	Read Plate
	Volume	10 µL
	Height	25 µL
	Expel Volume	1 µL
	Pause in well(s)	0
	Speed	10 µL/s
	Removal speed	10 mm/s
	Unload tips after fluid transfer	☑
Mix with Transfer Fluid	Mix plate	Source Plate 2
	Volume	10 µL
	Speed	20 µL/s
	Expel volume	0 µL
	Removal speed	20 mm/s
	Height	10 µL
	Strokes	3
	Pause in well(s)	0
Read with Transfer Fluid: First Interval	Read time interval (s)	0.5
	Number of reads before dispense	120
	Number of reads after dispense	1,300
	Total number of reads	1,420
Read with Transfer Fluid: Second Interval	Read time interval (s)	1
	Number of reads	0

3. Save the protocol with “T0” in the file name to indicate it includes a liquid handling step and reads at T0 of compound addition.
4. Remove CNS-3D Brain Organoids plate(s) from the incubator and place in the READ position within the FLIPR.
5. Place FLIPR pipette tips in SOURCE 1 TIPS position.
6. Place compound plate in SOURCE 2 position.
7. Ensure all plate lids have been removed.
8. Click RUN to start the protocol.
9. After the T0 read completes (~14 minutes), return the plate to 37°C, 5% CO_2, and 95–98% humidity for 20 minutes before proceeding. Dispose of the FLIPR tips. Dispose of the compound plate unless it will be used to dose additional plates.

Product support

For product or shipping support, contact 28bio at support@28bio.com.

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