Protocol: CNS-3D FLIPR Assay

The CNS-3D FLIPR Assay is performed using FLIPR® Calcium 6 Assay Kit (Molecular Devices; R8194, R8190, R8191, R8195)[1]. This document provides all necessary instructions for handling 384-well organoid plates, preparing necessary reagents for the FLIPR assay, and performing the FLIPR assay.

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

Workflow Overview

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

Media Preparation

IMPORTANT: PREPARE MEDIA AND ESTABLISH LIQUID HANDLING SETTINGS PRIOR TO ASSAY

1. Prepare BrainPhys™ Phenol Red Free Complete Medium (PRF media) according to Table 1
2. Store prepared media protected from light at 4°C for up to two weeks.
3. Prior to using the prepared media, aliquot the amount needed and warm to 37°C.

Table 1: Preparation of BrainPhys™ Phenol Red Free Complete Medium

COMPONENT (SUPPLIER)	AMOUNT	FINAL CONCENTRATION	CATALOG #
BrainPhys without Phenol Red (STEMCELL Technologies)	500 mL	1x	05791
SM1 (STEMCELL Technologies)	10 mL	1x	05711
BDNF (STEMCELL Technologies)	10 µg	20 ng/mL	78005
GDNF (STEMCELL Technologies)	10 µg	20 ng/mL	78058
Penicillin-Streptomycin (HyClone) - *optional*	5 mL	1x	SV30010

Media Requirements

- Automated liquid handlers will require approximately 10 mL media for priming plus 30 mL of media per assay plate for the 3x PRF media change.
- 11 mL media is required per assay plate for C6 dye preparation

Additional Equipment/Reagents

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

Optional Reagents

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

CNS-3D Organoid Plate Preparation

4. Prepare 3 half media changes using PRF media to remove phenol red prior to assay, ending with 25 µL per well.
    a. Initial volume per well is 50 µL.
    b. If using an automated liquid handler, reference Appendix A to establish aspiration protocol.
5. Remove FLIPR® Calcium 6 Assay Kit Component A (C6 dye) from -20°C. Allow to warm to room temperature before reconstitution.
    c. Only Component A will be required for the FLIPR assay.
6. Reconstitute the C6 dye by adding 11 mL of PRF media to the vial. Mix the contents thoroughly and transfer to a sterile reservoir.
7. Add 25 µL of C6 dye solution (1:1) of CNS-3D Organoids and return to 37°C, 5% CO_2 incubation for 2 hours.
    d. 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.

FLIPR Assay Protocol

8. Initialize FLIPR unit 30 minutes prior to assay:
    a. Power on chilling unit for camera
    b. Power on FLIPR unit

c. Open ScreenWorks software
d. Stage temperature 37°C
e. Camera temperature -58°C

9. Establish Baseline read protocol. Within ScreenWorks software, select File/New and navigate to the green Settings tab. Add the following parameters:
    a. Setup Read Mode:
        i. Read Mode Name: Read_Mode_1
        ii. Reading Mode: Fluorescence
        iii. Excitation/Emission Wavelength (nm): 470_495 / 515_575
        iv. Excitation Intensity (%): 40
        v. Exposure Time(s): 0.4
        vi. Camera Gain: 40
    b. Assign Plate to the following positions:
        i. Read Plate: default384
        ii. Source Plate 1: NONE
        iii. Source Plate 2: NONE
        iv. Source Plate 3: NONE
    c. Set read time interval:
        i. Read time interval: 0.5
        ii. Number of reads: 1200
10. 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.
11. Remove CNS-3D Organoids from 37°C and place into READ position within the FLIPR.
12. Run the assay protocol by clicking the “RUN” button.
    a. FLIPR indicate lights should change to indicate the beginning of the assay.
    b. Protocol progress can be monitored at the bottom right corner of the user interface.
13. Following the FLIPR read (~10 minutes), return the plate to 37°C, 5%, CO_2 incubation for 20 minutes before proceeding further.
    a. If running multiple plates in succession, begin reading the next plate.
14. If performing manual compound dosing, remove plate from incubator and manually add 10 µL of 6X concentrated compound to each well (final concentration in wells is 1x).
    a. If dosing using the FLIPR’s liquid handling system, proceed to Appendix B.
15. Return the plate to READ position and repeat steps 11 and 12, ensuring that the plate is allowed 20 minutes to equilibrate to 37°C, 5% CO_2 between reads.
    a. Plates may be read out to 4 hours (T240) from compound addition, after which point the C6 dye can begin to affect organoid function.

Appendix A: Automated Liquid Handler Setup Information

Automated Liquid Handler (Recommended Method)

A1. Establish the aspiration protocol before changing media for CNS-3D Organoids.

A2. Ensure the automated system has the correct plate dimensions for the Corning Spheroid Microplate (Catalog Number 4516, Figure 1 below).

A3. Fill an empty Corning Spheroid Microplate with a liquid, such as water, and set the aspiration parameters to leave 25 µL per well. A slower aspiration is preferred for greater accuracy.

A4. Set the dispensing parameters so that the tip(s) has a low clearance from the top of the well/plate and is positioned over the center of the well. Use a low/slow flow rate that avoids turbulence and dispense 25 µL.

A5. Test the protocol and ensure 25 µL of fluid is aspirated then dispensed into each well.

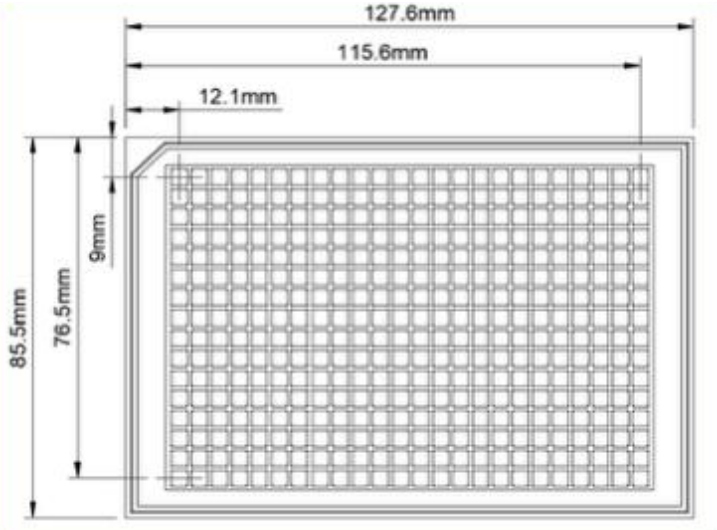

Figure 1. Corning Spheroid Microplate Measurements (Catalog Number 4516). Overhead view showing relevant width and length dimensions.

Key Dimensions for Corning Spheroid Microplate 4516:

- Total plate height = 14.20 mm
- Distance from bottom of plate to bottom of well = 1.81 mm
- Well bottom thickness = 0.09 mm
- Well depth = 12.30 mm

Appendix B: Establishing FLIPR Liquid Handling Protocol

B1. Dosing can be performed using the FLIPR liquid handling system but will require the following:

a. 384-well Polypropylene Conical Bottom Microplates (Ex: Greiner Bio-One, 781281)
   i. The 384-well microplate of your choice must first be added as a Source Plate in the ScreenWorks Plate Library. Plate specifications can be obtained by the manufacturer.
   ii. Compounds will need to be prepared at 6X within the 384-well microplate of your choice with at least 40 µL per well to allow adequate dead volume after aspirating.
   iii. The same compound plate can be used to dose multiple CNS-3D Organoid plates so long as at least 30 µL dead volume is present.

b. 384-well FLIPR Pipette Tips (Molecular Devices, 9000-0764)
   i. A new set of tips must be used for each FLIPR read even if the compound plate is reused.

B2. Establish T0 read protocol. Within ScreenWorks software, select File/New and navigate to the green Settings tab. Add the following parameters:

c. Setup Read Mode:
   i. Read Mode Name: Read_Mode_1
   ii. Reading Mode: Fluorescence
   iii. Excitation/Emission Wavelength (nm): 470_495 . 515_575
   iv. Excitation Intensity (%): 40
   v. Exposure time(s): 0.4
   vi. Camera gain: 40
   vii. Assign Plate to the following positions:
      1. Read Plate: default384
      2. Source Plate 2: (compound plate name)
      3. Source Plate 3: NONE

d. Check Load Tips Position box to select it

e. Transfer Fluid:
   i. Fluid Transfer Type: Single aspirate - Single dispense. Check Read box
   ii. Aspirate
      1. Check Aspirate box
      2. Plate: Source Plate 2
      3. Volume: 10 µL
      4. Speed: 20 µL/s
      5. Hold Volume: 0µL
      6. Height: 5 µL
      7. Tip Up Speed: 20 mm/s
      8. Check Mix Fluid before Aspirate

iii. Dispense
1. Check Dispense box
2. Plate: Read Plate
3. Volume: 10 µL
4. Height: 25 µL
5. ExpelVol: 1
6. Pause in Well(s): 0
7. Speed: 10µL/s
8. Removal Speed: 10 mm/s
9. Check Unload Tips after fluid transfer

e. Mix with Transfer Fluid:
i. Mix plate: Source Plate 2
ii. Volume: 10 µL
iii. Speed 20 µL/s
iv. Expel Volume: 0 µL
v. Removal speed: 20 mm/s
vi. Height: 10 µL
vii. Strokes: 3
viii. Pause in well(s): 0

f. Read with Transfer Fluid
i. First Interval
1. Read time interval: 0.5
2. Number of reads before dispense: 120
3. Number of reads after dispense: 1300
4. Total numbers of reads: 1420

ii. Second Interval
1. Read time interval(s): 1
2. Number of reads: 0

B3. Save protocol using T0 in the file name to indicate the protocol includes a liquid handling step and will read at T0 of compound addition.

B4. Remove organoid plate from 37°C and place into READ position within the FLIPR. Place FLIPR pipette tips in SOURCE 1 TIPS position. Place compound plate in SOURCE 2 position. Ensure all lids have been removed.

B5. Run the assay protocol by clicking the “RUN” button

B6. Following the T0 FLIPR read (~14 minutes), return the plate to 37°C, 5% CO2 incubation for 20 minutes before proceeding further. Dispose of FLIPR tips. Dispose of compound plate unless using for dosing additional plates.