IncuCyte® S3 Spheroid Viability Assay - Fluorescent Label

For the quantification of fluorescently labeled spheroid growth and shrinkage.

This protocol describes a solution for creating single spheroids using a 96- or 384-well round-bottom, ultra-low attachment plate. This method utilizes the IncuCyte® live-cell analysis system for image-based Brightfield and fluorescence within the Brightfield boundary of spheroid area measurements. Cell lines expressing fluorescent protein can be used to monitor spheroid health.

Required materials
- IncuCyte® S3 Spheroid Software Module (Essen Cat # 9600-0019)
- IncuCyte® S3 Spheroid software version 2017B
- Cell fluorescent label reagents and consumables
  - IncuCyte® NucLight Red or Green BacMam 3.0 Reagent for nuclear labeling (Essen Cat # 4621 or 4622)
  - IncuCyte® NucLight Red or Green Lentivirus Reagent (EF-1α, Puro) for nuclear labeling (Essen Cat # 4624 or 4625)
  - IncuCyte® CytoLight Red or Green Lentivirus Reagent (EF-1α, Puro) for cytoplasmic labeling (Essen Cat # 4481 or 4482)
  - Matrigel® (Corning Cat#356234), optional
- 96-well round-bottom, ultra-low attachment plate (e.g., Corning® Cat#7007, S-BIO Cat#MS-9096UZ, BRANDplates® Cat#7816 60, 7819 00, 7819 60)
- 384-well round-bottom, ultra-low attachment plate (e.g., S-BIO Cat#MS-9384UZ)

NOTE: Combination of cells expressing fluorescent proteins with cell health reagents (Cytotox, Annexin V) is NOT recommended.

General Guidelines
- Remove bubbles from all wells by gently squeezing a wash bottle containing 70-100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37 °C for 30 minutes prior to scanning.

Protocol

1. CELL SEEDING (Day 0)
   - Seed cells into 96W or 384W Ultra Low Attachment plate. Centrifuge.

2. SPHEROID FORMATION (Day 0–3)
   - Place plate inside the IncuCyte and scan every six hours.

3. ADD TREATMENTS Day 3
   - Add treatments to plate. Monitor spheroid growth and shrinkage.
Day 0:

1. **Seed cells**
   1.1. Seed cells of interest (100 µL per well for 96-well, 50 µL for 384-well) at an appropriate density into a 96- or 384-well ULA plate such that by day 3, spheroids have formed with the desired size (e.g., 200 – 500 µm after 3 days). Seeding density will need to be optimized for each cell line used, however, we recommend a range of 1,000 – 5,000 cells per well (10,000 – 50,000 cells per mL seeding stock).
   
   **NOTE:** Some cell lines may require the addition of a basement membrane extract, typically 2.5% v/v Matrigel®, to promote tight spheroid formation.
   
   1.2. Centrifuge the ULA plate (125 g, 10 minutes) at room temperature (20-25°C).

Day 0–3:

2. **Spheroid formation**
   2.1. Place the cell plate into the IncuCyte live-cell analysis System and schedule 24 hour repeat scanning:
      a. Objective: 4x or 10x (96-well ULA) or 10x (384-well ULA), 1 image per well
      b. **Channel selection:** Phase Contrast; Brightfield; “Green” or “Red” if fluorescent label OR if a cell health reagent will be added post spheroid formation.
      c. Scan type: Spheroid.
      d. Scan interval: Every 6 hours.

Day 3:

3. **Add treatments**
   3.1. Once spheroids have reached desired size (e.g., 200 – 500 µm), remove the ULA plate from the incubator and carefully add culture media supplemented with cell heath reagent (100 µL per well for 96-well, 25 µL per well for 384-well) containing test material (e.g. small molecules, antibodies; prepared at 2x final assay concentration for 96-well, 3x final assay concentration for 384-well).
   
   **NOTE:** It is not recommended to change media in this assay as it will disrupt spheroids containing necrosing or apoptotic cells.

Analysis Guidelines

**NOTE:** Utilize the IncuCyte® S3 Spheroid Software module in the Brightfield channel to identify spheroid boundaries and analyze fluorescence as needed. See “Guidelines for Analysis,” which can be accessed from the IncuCyte® S3 Technical Notes folder as part of the GUI installer.

1. **For parental (non-transduced) cells** – Brightfield Boundary Measurements
   
   **Result:** Size of spheroid measurement
   
   **Suggested Metric:** Largest Brightfield object (avoid segmentation of small fragments)

2. **For cells expressing fluorescent protein** – Fluorescent and Brightfield Boundary Measurements
   
   **Result:** Size and viability measurements
   
   **Suggested Metric:** Integrated intensity
   
   **Secondary metric:** Mean intensity

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