SARTORIUS

Protocol

Incucyte® Embedded Multi-Spheroid Assay

For Quantifying Growth and Death of Multi-Spheroids Embedded in Matrigel® Label-Free

This protocol describes a solution for monitoring and quantifying the growth and death of multi-spheroids embedded in Matrigel® in 96-well flat bottom plates. The method utilizes the Incucyte® Live-Cell Analysis System and Incucyte® Spheroid Analysis Software Module for image-based brightfield (BF) measurements.

Required Materials

- 96-well flat bottom tissue culture (TC)-treated microplate (Corning Cat. No. 3595)
- Matrigel®, protein concentration ≥ 8 mg/mL (Corning Cat. No. 356234)
- Serum-free cell culture media for Matrigel® dilutions
- Complete culture media for cell culture assay
- Wet ice
- Manual multi-channel pipettes
- Incucyte® Spheroid Analysis Software Module (Cat. No. 9600-0019) including and post version 2021C

Optional Materials

- BioCision® CoolBox™ System for Microplates with CoolSink® (Cat. No. 1500-0078) CoolBox™ 96F System (includes X1 block with gel pack and CoolSink® 96F)
- Tacta® Mechanical Pipette, 12 Channel (Sartorius Cat. No. LH-729230)
- **For CX3:** Incucyte® Cell Health Reagents
  - Incucyte® Caspase 3/7 Dye
  - Incucyte® Annexin V Dye
  - Incucyte® Cytotox Dye
  - Incucyte® Nuclight Lentivirus

Note: Transfect cells with Incucyte® Nuclight Lentivirus prior to performing multi-spheroid experiments by following the protocols supplied with the reagents.

General Guideline

- Review manufacturer's guidelines for thawing and storing 100% Matrigel®. Thaw Matrigel® overnight by submerging the vial in cold water in the rear of a refrigerator (+ 4 °C). Do not allow Matrigel® to warm to room temperature at any time, as this will induce polymerization.
- Following cell seeding or media additions, remove bubbles from wells by gently squeezing a wash bottle containing 70–100% ethanol (inner straw removed) to blow vapor over the surface of each well.
- After cell seeding, place the plate in the Incucyte® Live-Cell Analysis System and allow the plate to warm to 37 °C for 30 minutes prior to scanning.

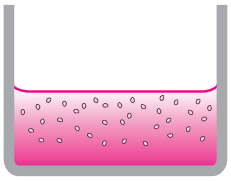
Protocol

Quick Guide

1. Coat plate (Day 0)

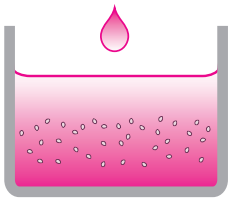
Coat plate (50% Matrigel®, 40 µL/well). Polymerize at 37° C for 30 minutes.

2. Add cells (Day 0)



Add cells (50% Matrigel®) on top of coated wells (30 µL/well). Polymerize at 37° C for 30 minutes.

3. Add media and monitor formation (Day 0–4)



Overlay polymerized Matrigel® with media (100 µL/well). Place inside the Incucyte® to monitor multi-spheroid formation.

Note: Confocal imaging is not recommended during spheroid formation.

4. Add treatments and/or cell health reagents (Day 3-4)

Add treatments at 2X final assay concentration (100 µL/well). Continue to monitor multi-spheroid growth and death.

Important:

1. In advance of experiments, it is important to have:
   a. Thawed Matrigel® Matrix overnight at 4° C. Keep on ice for duration of experiment.
   b. Stored Coolsink 96F accessory at 4 °C for at least 4 hours.
2. Keep all culture-ware and reagents in contact with Matrigel® on ice for the duration of assay setup.
3. Store pipette tips used for dispensing Matrigel® at 4 °C.

Day 0

1. Coat Plate with Matrigel®

1.1 In a cell culture hood, chill plates (10–15 minutes) on a pre-chilled CoolSink® 96F within a CoolBox™ 96F box.

1.2 In a cold polypropylene tube, dilute 100% Matrigel® 1:1 in cold serum-free culture media (keep all Matrigel® solutions on ice).

Note: To prevent incomplete gel formation, we recommend using ≥ 4 mg/mL Matrigel® for coating.

   a. To coat a single 96-well plate, add 2.5 mL of cold serum-free culture media to a pre-chilled polypropylene tube.
   b. Using a cold serological pipette, slowly pipette 2.5 mL of 100% Matrigel® into serum-free media and, taking care to avoid bubbles, slowly mix by pipetting the solution up and down.

1.3 Pour prepared solution into a pre-chilled sterile reagent reservoir (keep on ice).

1.4 Using pre-chilled pipette tips and reverse pipetting technique, coat the pre-chilled 96-well plate by carefully adding 40 µL of diluted Matrigel® into the center of each well.
   a. While the plate is cold and Matrigel® is still liquid, gently rock the plate once within the CoolBox™ to ensure even coating of each well.

Note: To avoid cell penetration to the base of the plate, coat wells with a minimum of 40 µL. Using reverse pipetting technique is important to minimize bubbles.

1.5 Remove any bubbles using a wash bottle containing 70–100% ethanol (inner straw removed) to blow vapor over the surface of each well.

Note: The 40 µL Matrigel® base layer is required for Confocal Multi-plane imaging in the Incucyte® CX3.

1.6 Place the plate in a 37 °C incubator for 30 minutes to polymerize the Matrigel®.

2. Seed Cells

2.1 Harvest, count and resuspend cells of interest in complete culture media.

2.2 In a cold polypropylene tube, dilute 100% Matrigel® 1:1 in cold complete culture media. Keep on ice.

2.3 Chill coated plate for 5 minutes by placing it on a pre-chilled CoolSink® 96F within a CoolBox™ 96F.

2.4 Dilute cells in 50% Matrigel® solution at an appropriate density. Keep on ice.

Note: Seeding density will need to be optimized for each cell type used. For example, we recommend seeding A549, MCF-7 and MDA-MB-231 at 1,000–2,000 cells per well or SKOV-3 at 2,000–4,000 cells per well.

2.5 Pour diluted Matrigel® containing cells into a chilled sterile reagent reservoir (keep on ice).

2.6 Using pre-chilled pipette tips and reverse pipetting technique, seed cells into each well of the coated 96-well plate (30 µL/well).

a. Gently rock the plate once within the CoolBox™ to ensure even Matrigel® distribution within each well.

2.7 Gently remove any bubbles using a wash bottle containing 70–100% ethanol (inner straw removed) to blow vapor over the surface of each well.

2.8 Place the plate in a 37 °C incubator for 30 minutes to polymerize the Matrigel®.

2.9 Overlay the polymerized layer with complete culture media (100 µL).

Note: If no additional treatments or reagents are being added, we recommend adding 170 µL of culture media.

2.10 Place plate in a 37 °C incubator for 30 minutes prior to scanning.

Day 0–3

3. Monitor Multi-Spheroid Formation

3.1 Position the plate within the Incucyte® Live-Cell Analysis System and allow it to reach 37 °C for 30 minutes before beginning the imaging.

3.2 For both S-series & CX3: Set up a 24-hour repeat scanning schedule with the following parameters:

a. Scan type: Spheroid

b. Spheroid type: Embedded Multi-spheroid

c. Image Channels: Phase Contrast + Brightfield

d. Objective: 4X

e. Scan interval: Every 6-8 hours

For CX3: Confocal Multi-plane scanning is not recommended during spheroid formation.

Day 3

4. Add a Cell Health Reagent if Desired

4.1 Once embedded multi-spheroids have reached desired size, prepare Cell Health Reagent at 4X final assay concentration (e.g. 50 µL per well for 96-well).

4.2 Cell Health Reagents suggested final concentrations (optimize for specific conditions)

- Incucyte® Cytotox Green Dye (Cat. No.4633)—25 nM
- Incucyte® Cytotox NIR Dye (Cat. No.4846)—0.6 µM
- Incucyte® Annexin V Green Dye (Cat. No.4642)—1:200 dilution
- Incucyte® Annexin V Orange Dye (Cat. No.4759)—1:200 dilution
- Incucyte® Annexin V NIR Dye (Cat. No.4768)—1:200 dilution
- Incucyte® Caspase 3/7 Green Dye (Cat. No.4440)-1:1000
- Incucyte® Caspase 3/7 Red Dye (Cat. No.4704)-1:200

Note: Annexin V Dye requires solubilization in assay media before use. Centrifuge briefly to collect solid in bottom of vial and add 100 µL assay media and mix thoroughly to prepare your Annexin stock solution.

4.3 Add the 4x Cell Health Reagent solution(s) on top of the cells (50 µL per well).

5. Add Treatments

5.1 For suitable treatments:

a. If no cell health reagent is applied: Use a 2x final assay concentration, with 100 µL per well.

b. If a cell health reagent is applied: Use a 4x final assay concentration, with 50 µL per well.

5.2 Remove any bubbles using a wash bottle containing 70-100% ethanol, with the inner straw removed, to blow vapor over the surface of each well

5.3 Place plate in a 37 °C incubator for 30 minutes prior to scanning.

For S-series:

Continue to monitor multi-spheroid growth using the to settings previously specified (e.g., every 6-8 hours for 7 days).

For CX3:

a. If imaging in confocal mode with multi-plane acquisition is preferred, a new vessel must be created and scheduled to scan every 8 hours for a duration of up to 7 days. Channel selection should include Phase Contrast, Brightfield, and Fluorescence, depending on the reagent used.

b. For wide-field mode with single-plane acquisition, continue imaging the test vessel every 6 to 8 hours for up to 7 days, using the settings previously specified.

Analysis Guidelines

1. Create a New Analysis Definition
   - In the Analysis Wizard window select 'Spheroid' analysis type.
   - Select the image channels Phase and Brightfield. For confocal imaging, you will have the option to choose the fluorescent channel of your interest
   - Select a set of representative images.
   - Adjust the background/cells slider to determine the boundary of the objects.
   - Evaluate the Brightfield mask and refine filter parameters accordingly. 'Preview All' to ensure parameters set appropriately mask all representative images within the collection.
   - In confocal imaging, the segmentation mask for fluorescent channels is automatically set to "Top-Hat No Mask" with a radius of 100 µm.
   - Adjust the edge split slider to delineate between individual objects.
   - Evaluate the Brightfield mask and refine filter parameters accordingly. Select 'Preview All' to ensure the parameters set appropriately mask all representative images within the collection.
   - Once satisfied with all parameters, complete the Launch Wizard analysis by selecting the scan times and wells to be analyzed.

     Note: If your experiment is in progress, you will have an option to check 'Analyze Future Scans' to perform real-time analysis.

2. Data Interpretation

   Once the analysis job is complete the following primary metrics are provided for S-Series and CX3 Brightfield imaging:
   - Brightfield Object Count: This metric represents the number of objects per image (well).
   - Brightfield Object Total or Average Area: This metric represents the total or average area of BF objects within the image (well) and is recommended for tracking multi-spheroid size over time.
   - Brightfield Object Average Eccentricity: This metric represents how round the objects are.

   For CX3 multi-plane confocal imaging:
   - All Brightfield Object Mean Intensity
   - All Brightfield Object Integrated Intensity

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