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)

General Guidelines

- 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.

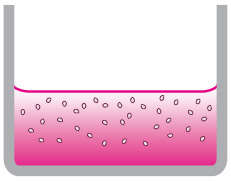
Protocol

Quick Guide

1. Coat plate (Day 0)

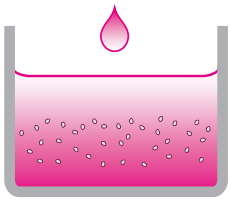
Coat plate (50% Matrigel®, 40 µL/well). Polymerize at 37° C for 30 minutes.

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 0–3)



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

4. Add treatments (Day 3)

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.

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).
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 Place the cell plate into the Incucyte® Live-Cell Analysis System and schedule 24-hour repeat scanning:
   a. Objective: 4X (Corning® 96-well) 1 image per well
   b. Channel selection: Phase Contrast + BF
   c. Scan type: Spheroid, Embedded Multi-spheroid
   d. Scan interval: Every 6 hours

Day 3

4. Add Treatments

4.1 Three days post-seeding, remove the plate from the Incucyte® and carefully add appropriate treatments at 2X final assay concentration (100 µL per well).
4.2 Continue to monitor multi-spheroid growth and death (e.g., every 6 hours for 7 days).

Analysis Guidelines

1. Create a New Analysis Definition
   - In the Analysis Wizard window select 'Spheroid' analysis type.
   - Select a set of representative images.
   - Adjust the background/cells slider to determine the boundary of the objects.
   - Evaluate the BF mask and refine filter parameters accordingly. 'Preview All' to ensure parameters set appropriately mask all representative images within the collection.
   - Adjust the edge split slider to delineate between individual objects.
   - Evaluate the BF 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:
   - BF Object Count: This metric represents the number of objects per image (well).
   - BF 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.
   - BF Object Average Eccentricity: This metric represents how round the objects are.

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