SARTORIUS

Protocol

Incucyte® Organoid Assay for Quantifying the Growth and Death of Organoids Embedded in Matrigel®

Description

This protocol describes a solution for monitoring and quantifying the growth and death of organoids embedded in Matrigel® in 96-well flat bottom plates. The method utilizes the Incucyte® Live-Cell Analysis System and Incucyte® Organoid Analysis Software Module for image-based brightfield measurements. This organoid assay is supported on the Incucyte® S-Series, for Brightfield imaging, and the new Incucyte® CX3, with the added capability for confocal fluorescence imaging and measurements of cell health using supported reagents.

General Guidelines

- For confocal fluorescence multi-plane imaging on the CX3 system, the Embedded No Base protocol (Option 2 shown below) can be utilized with the default Z parameters found in the Organoid Assay scan type. If alternate workflows are desired, the user can modify the Z Parameters (offset, range, and step size) for their desired confocal multi-plane acquisition. For detailed instructions, see the Z Parameters technical guidelines.
- Review manufacturer guidelines for thawing and storing of 100% Matrigel®. Thaw Corning® Matrigel® overnight by submerging the vial in ice 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, media addition or replenishment, remove bubbles from 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 organoid seeding and all media changes, 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.
- For easier handling of Matrigel®, it is recommended to use a Corning® CoolBox System equipped with a Corning® CoolSink

Required Materials

- 96-well flat bottom TC-treated microplate (Corning Cat. No. 3595)

Note: This plate type is required to utilize the Organoid Assay scan type

- Matrigel® Growth Factor Reduced (GFR), Phenol Red-Free (Corning Cat. No. 356231)
- Organoids of interest
- Organoid specific growth medium
- Wet ice
- Manual multi- or single-channel pipettes
- Incucyte® Organoid Analysis Software Module (Sartorius Cat. No. 9600-0034), version 2021A or later for Incucyte® S-series instruments, version 2025C for Incucyte® CX3

Optional Materials

- Tacta® Mechanical Pipette, 12 Channel (Sartorius Cat. No. LH-729230)
- Tacta® Mechanical Pipette, Single Channel (Sartorius Cat. No LH-729050)

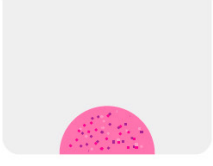
Quick Guide

1 Resuspend organoids in Matrigel® (Day 0)

Harvest and resuspend organoid fragments in 50% Matrigel®

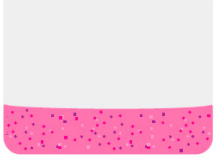
2 Add cells (Day 0)

Matrigel® Dome (Option 1)



Pipette Matrigel® containing organoid fragments in the center of a 96-well plate (10 µL/well). Polymerize at 37°C for 20 minutes.

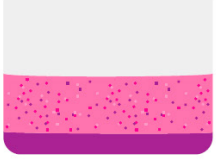
Embedded No Base (Option 2)



Seed organoid fragments into a 96-well plate (50 µL/well). Polymerize at 37°C for 20 minutes.

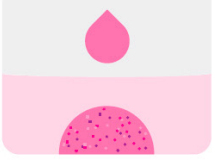
This option is required to utilize the default Z parameters in the CX3 system

Embedded With Base (Option 3)



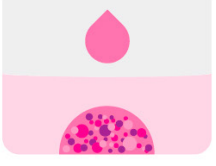
Seed organoid fragments into a pre-coated 96-well plate (50 µL/well). Polymerize at 37°C for 20 minutes.

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



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

4 Add treatments (Day 3)



Remove existing media and add treatments at 1X final assay concentration (100 µL/well). Monitor organoid growth and death.

Protocol

Important:

1. In advance of experiments it is important to have:
   a. Thawed Matrigel® overnight at 4° C. Keep on ice for duration of experiment.
   b. Warmed growth medium to ambient temperature (15–25° C).
   c. Warmed tissue culture treated plates in a 37° C incubator for at least 30 minutes.

      Note: If using CoolBox® system, store CoolSink® accessory at 4° C for at least 4h.

2. Stored pipette tips used for dispensing Matrigel® at 4° C

Day 0:

1. Seed Cells
   1.1 Harvest and dissociate organoids of interest according to model-specific instructions.
   1.2 Aliquot culture media into a polypropylene tube and place on ice.
   1.3 In a separate cold polypropylene tube, dilute 100% Matrigel® 1:1 in cold organoid specific culture media. Keep on ice.
   1.4 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. As an example, and guide, we recommend a range of 500– 2000 cells per well.
   1.5 Pour diluted Matrigel® containing cells into a chilled sterile reagent reservoir (keep on ice).
   1.6 Using pre-chilled pipette tips and reverse pipetting technique seed cells into each well of a 96-well plate. Utilize any of the following assay formats to establish cultures with desired formation, growth, and morphology.
      a. Matrigel® Dome (Option 1)
      - To successfully form a dome, seed cells into the center of each well of a pre-warmed 96-well plate (10 µL per well).

        Tip: For ease, use a manual P100 single-or multi-channel pipette (e.g Sartorius Cat. No. LH-729050, LH-729230 respectively) to spot domes.

        Note: Use reverse pipetting technique to minimize generation of bubbles.

      b. Cells Embedded in Matrigel® (Option 2)
      - Chill plate for 2–5 minutes (Recommended: Use a pre-chilled CoolSink® within a CoolBox® System). Seed cells into each well (50 µL per well).
      - While the plate is cold and Matrigel® is still liquid, gently rock the plate once to ensure even well distribution. If using a CoolBox® system, rock plate once within CoolBox®.

      c. Cells Embedded on Matrigel® Base (Option 3)
      - Place pre-coated plate on a CoolSink® (5 min) and seed cells on top of polymerized Matrigel® base (50 µL per well).
      - See Appendix for plate coating instructions.

   1.7 Gently 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.
   1.8 Place the plate in a 37° C incubator for 20 minutes to polymerize the Matrigel®.
   1.9 Overlay polymerized layer with culture media (100 µL).
   1.10 Place plate in a 37°C incubator for 30 minutes prior to scanning:

Day 0-3:

2. Monitor Organoid Formation
   2.1 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 + Brightfield
      c. Scan type: Organoid, Assay
      d. Scan interval: Every 6 hours
   2.2 For Confocal fluorescence imaging in the CX3 system:
      a. Objective: 4X (Corning® 96-well) 1 image per well
      b. Channel selection: Phase Contrast + Brightfield, and desired confocal fluorescence channels (green, orange, or NIR)
      c. Scan type: Organoid, Assay
      d. Z Parameters: If using Option 2, the default Z parameters can be used. Alternative assay set ups may require modification to the Z parameters (offset, range, or step size). This can be done when setting up the scan.
      e. Recommended scan interval: Every 6 hours

Day 3:

3. Add Cell Health Reagent (if desired)
   3.1 3 days post seeding or once organoids have reached desired size, remove the plate from the Incucyte® and carefully aspirate existing media using a manual multi- channel pipette.

   Note: When removing media, keep the pipette

tip at the edge of the well to avoid disrupting the polymerized Matrigel® layer.

3.2 Cell Health Reagents suggested final concentrations (optimize for specific conditions)
- Incucyte® Cytotox Green Dye (Sartorius Cat. No.4633)—25 nM
- Incucyte® Annexin V Green Dye (Sartorius Cat. No.4642)—1:200 dilution
- Incucyte® Annexin V Orange Dye (Sartorius Cat. No.4759)—1:200 dilution
- Incucyte® Annexin V NIR Dye (Sartorius Cat. No.4768)—1:200 dilution
- Incucyte® Caspase 3/7 Green Dye (Sartorius Cat. No.4440)-1:1000 dilution

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.

3.3 Use 2x Cell Health Reagent solution (100 µL per well) for addition, or 1x Cell Health Reagent if no compound treatment is applied.

4. Add Treatments if desired
   4.1 For suitable treatments:
      a. If no cell health reagent is applied: Gently aspirate the existing media using a manual multi-channel pipette. Use a 1X final assay concentration, with 100 µL per well.
      b. If a cell health reagent is applied: Use a 2X final assay concentration, with 100 µL per well.
   4.2 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
   4.3 Place plate in a 37° C incubator for 30 minutes prior to scanning.
   4.4 Continue imaging organoid growth either by following section 2.1 guidelines or by using confocal fluorescent imaging in the CX3 system as detailed in section 2.2 (e.g., every 6 hours for 5 days).

5. Re-Feed Cultures if desired
   5.1 Maintain cultures by performing 100% media replenishment every 2 days
   5.2 Remove plate from Incucyte®. Carefully remove 100 or 200 µL of media per well and replace with 100 or 200 µL of media containing test agents (1X final assay concentration).

Note: When removing media, keep the pipette tip at the edge of the well to avoid disrupting the polymerized Matrigel® layer.

5.3 Return plate to the Incucyte® and continue to monitor organoid growth and death.

Appendix

6. Coating Plate With Matrigel®
   6.1 Chill plates (10–15 minutes)
   Recommended: Use pre-chilled CoolSink® wtihin a CoolBox® system.
   6.2 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, for coating we recommend using ≥4 mg/mL Matrigel®. Using a cold serological pipette, slowly pipette 100% Matrigel® into serum-free media and taking care to avoid bubbles, slowly mix by pipetting the solution up and down.

   6.3 Pour prepared solution into a pre-chilled sterile reagent reservoir (keep on ice).
   6.4 Using pre-chilled pipette tips and reverse pipetting technique, coat the pre-chilled 96-well plate by carefully adding diluted Matrigel® into the center of each well.
      a. The volume required to coat wells to encourage organoid formation and maturation will need to be optimized for each cell type of interest. We recommend performing a titration using a minimum of 20 µL and maximum of 40 µL Matrigel® per well.
      b. While the plate is cold and Matrigel® is still liquid, gently rock the plate once to ensure even coating of each well.

Note: Use of reverse pipetting technique is important to minimize bubbles.

   6.5 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.
   6.6 Place the plate in a 37° C incubator for 20 minutes to polymerize the Matrigel®.

Analysis Guidelines

1. **Create a New Analysis Definition**
   - In the Analysis Wizard window, select 'Organoid' 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 organoid objects.
   - Evaluate the Brightfield (BF) 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 organoid objects.
   - Evaluate the BF mask and refine filter parameters accordingly. 'Preview All' to ensure 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.

   - For both the Incucyte® S-Series and Incucyte® CX3, you can choose to utilize the 3D Object Classification analysis for data evaluation. This analysis can be configured after the completion of the analysis job. For more information, please refer to the "Introducing the Incucyte® 3D Object Classification Analysis Software Module for Live-Cell Analysis" one sheet.

2. **Data Interpretation**
   - Once the Analysis Job is complete the following primary metrics are provided for S-series and CX3 Brightfield imaging.
   - Organoid Object Count. This metric represents the number of objects per image (well).
   - Organoid Object Total Area. This metric represents the total area of BF objects within the image (well) and is recommended for tracking organoid size over time.
   - Organoid Object Avg. Eccentricity. This metric represents how round the organoids are.
   - Organoid Darkness. This metric is available for tracking changes in organoid brightness over time.
   - For CX3 multi-plane confocal imaging:
     - All Brightfield Object Mean Intensity
     - All Brightfield Object Integrated Intensity
   - For CX3: Confocal multi-plane scans can be analyzed using a Mean or Max Projection image. See the Mean and Max projection technical guidelines for more information.

3. **Spectral Unmixing:**
   - For CX3: Spectral unmixing for cell health reagents is not required.

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