The Next Step in the Evolution of 3D Culture: Utilizing Extracellular Matrix to Enhance Multicellular Tumor Spheroid Models for Proliferation and Invasion

Overview of methodology, tools and reagents for evaluating cell proliferation and invasion using multicellular tumor spheroids.
TOOLS AND REAGENTS
3-D SPHEROID
PROLIFERATION/VIABILITY
Calculate volumes for 10X Spheroid Formation ECM, Complete Growth Medium, and Cells (1 x 10^6 cells/ml):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 Well</th>
<th>3,000 Cells/Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Spheroid Formation ECM (4 °C)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Cells (at 1 x 10^6 cells/ml)</td>
<td>X µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>Complete Growth Medium (4 °C)</td>
<td>45-X µl</td>
<td>42 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
Sample Preparation for Spheroid Formation

- Thaw 10X Spheroid Formation ECM on ice at 4 °C overnight.
- Divide into working aliquots; store at -80 °C for optimal stability, avoid freeze-thaws.
- Keep on ice throughout procedure.
- Dilute with cold (4 °C) complete growth medium, and pipet up and down to mix.
Sample Preparation for Spheroid Formation

- Culture cells to 80% confluence.
- Harvest cells, centrifuge, and aspirate medium.
- Resuspend at $1 \times 10^6$ cells/ml in complete growth medium.
- Add the calculated amount of cells to the tube containing Spheroid Formation ECM.
Sample Preparation for Spheroid Formation

- Add 50 µl of the cells in Spheroid ECM to the 96 Well Spheroid Formation Plate.
- Centrifuge at 200 x g for 3 minutes in a swinging bucket rotor.
- Incubate plate with cells at 37 °C, 5% CO₂ for 72 hours.
Sample Preparation for Spheroid Formation

- Cells assemble into one spheroid/well within 72 hours.
- Prepare solutions containing 2X treatment compounds in complete growth medium.
- Add 2X treatment (50 µl) to each well.
- Incubate at 37 °C, 5% CO2 for desired treatment period.
Image Analysis Evaluation of Spheroid Growth

**STEP**

Resuspend cells in Spheroid Formation ECM and add to 96 Well Spheroid Formation Plate.  

**TIME**

1-2 hours

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Cells assemble into compact spheroids.

3 days

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Add medium containing cell proliferation modulating compounds.

1 hour

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Cells proliferate and spheroids expand over time.

3-6 days

**Total Time:** 6-9 days
3-D Culture Spheroid Proliferation/Viability

MDA-MB-231 3-D Spheroid Growth

- 3-D spheroid cultures are large cell aggregates which exhibit physiological characteristics of small tumors.
- Incorporation of ECM proteins is necessary to promote in vivo cell behavior.
- This assay provides an in vitro model for evaluating tumor growth in response to pharmacological agents.

MDA-MB-231 Breast cancer cells in spheroid culture for 3 days form A) loose colonies in media and B) tight colonies in media + ECM.
Spheroid growth of MDA-MB-231 breast cancer spheroids. Cells were seeded at the corresponding concentrations in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. At that time, 50 µl of complete medium was added to each well, and spheroids were incubated at 37 °C, 5% CO₂. Spheroids were photographed every 24 hours, and images were analyzed using ImageJ software.

Inhibition of MDA-MB-231 cell spheroid viability by Bleomycin. Cells were seeded at 3,000 cells/well in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. Spheroids were then treated with the corresponding doses of Bleomycin and incubated at 37 °C, 5% CO₂ for 96 hours. Spheroids were photographed after 96 hours, and images were analyzed using ImageJ software.
3-D Culture Spheroid Proliferation/Viability Analysis

Fluorometric or Colorimetric Quantitation
Fluorometric Quantitation with Resazurin

**STEP**
Resuspend cells in Spheroid Formation ECM and add to 96 Well Spheroid Formation Plate.

**TIME**
1-2 hours

**STEP**
Cells assemble into compact spheroids.

**TIME**
3 days

**STEP**
Add medium containing cell proliferation modulating compounds.

**TIME**
1 hour

**STEP**
Cells proliferate and spheroids expand over time.

**TIME**
3-6 days

**STEP**
Cells reduce Resazurin is to fluorescent resorufin to quantify cell number.

**TIME**
4 hours

**Total Time:** 6-9 days
Spheroid cell number corresponds to fluorescence output for MDA-MB-231 breast cancer spheroids. Cells were seeded at the corresponding concentrations in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. At that time, 50 ul of complete medium was added to each well, and spheroids were incubated at 37 °C, 5% CO₂ for 96 hours and analyzed using Resazurin.

Inhibition of MDA-MB-231 cell spheroid viability by Bleomycin. Cells were seeded at 3,000 cells/well in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. Spheroids were then treated with the corresponding doses of Bleomycin, incubated at 37 °C, 5% CO₂ for 96 hours, and analyzed using Resazurin.
Colorimetric Quantitation with MTT

**STEP**
Resuspend cells in Spheroid Formation ECM and add to 96 Well Spheroid Formation Plate. 1-2 hours

Cells assemble into compact spheroids. 3 days

Add medium containing cell proliferation modulating compounds. 1 hour

Cells proliferate and spheroids expand over time. 3-6 days

Cells reduce MTT to a purple formazan to quantify cell number. 2 days

Total Time: 8-11 days
Spheroid cell number corresponds to absorbance for MDA-MB-231 breast cancer spheroids. Cells were seeded at the corresponding concentrations in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. At that time, 50 ul of complete medium was added to each well, and spheroids were incubated at 37 °C, 5% CO₂ for 96 hours and analyzed using MTT.

Inhibition of MDA-MB-231 cell spheroid viability by Bleomycin. Cells were seeded at 3,000 cells/well in the presence of spheroid formation ECM and incubated for 72 hours at 37 °C, 5% CO₂ to induce spheroid formation. Spheroids were then treated with the corresponding doses of Bleomycin, incubated at 37 °C, 5% CO₂ for 96 hours, and analyzed using MTT.
3-D SPHEROID INVASION
Sample Preparation for Spheroid Invasion

- Cells assemble into one spheroid/well within 72 hours.
- Chill 96 Well Spheroid Formation Plate on ice at 4 °C for 5 minutes.
- Working on ice, add 50 µl of Invasion Matrix to each well.
- Centrifuge plate at 300 x g for 5 minutes at 4 °C (pre-chilled) in a swinging bucket rotor.
Sample Preparation for Spheroid Invasion

- Transfer the Spheroid Formation Plate to 37 °C, 5% CO$_2$ for one hour to polymerize Invasion Matrix.
- Add 100 µl of complete medium with 2X invasion modulating agents (37 °C) to each well.
- Incubate at 37 °C, 5% CO$_2$ for desired invasion period.
- Analyze plate.
3-D Culture Spheroid Invasion

**DESCRIPTION**

- Resuspend cells in spheroid formation ECM and add to round bottom wells. **TIME:** 1-2 hours
- Cells assemble into compact spheroids. **TIME:** 3 days
- Add Invasion Matrix and medium containing chemoattractants and/or invasion modulating compounds. **TIME:** 1-2 hours
- Cell invade into the surrounding matrix in Response to chemoattractants **TIME:** 2-4 days

**Total Time:** 5-7 days
3-D Culture Spheroid Invasion Analysis
Morphology of 3D cell invasion over a four day period. Cells were seeded at 3,000 cells/well in the presence of Spheroid Formation ECM, and incubated at 37 °C, 5% CO₂ for 72 hours to form spheroids. Spheroids were then embedded in 50 µl Invasion Matrix, and incubated at 37 °C, 5% CO₂ for one hour for polymerization. Then 100 µl of DMEM, 10% FBS was added to each well as a chemoattractant. Non-invasive cells (MCF-7) remain as cell aggregates and do not invade into the surrounding invasion matrix; whereas, invasive cells (MDA-MB-231) invade into the surrounding invasion matrix as spindle-like protrusions.
3-D Culture Spheroid Invasion Analysis

Quantitative analysis of surface area for non-invasive (MCF-7) and invasive (MDA-MB-231) cell lines over a four day period.
Different cell lines exhibit different invasive potentials and morphologies

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>PC-3</td>
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<td><img src="image" alt="PC-3 Day 1" /></td>
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<tr>
<td>MDA-MB-231</td>
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<td><img src="image" alt="HT-1080 Day 4" /></td>
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<td>U-87-MG</td>
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<td><img src="image" alt="U-87-MG Day 3" /></td>
<td><img src="image" alt="U-87-MG Day 4" /></td>
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Invasion of cancer cells out of MCTS and into the surrounding invasion matrix in a time-dependent manner. Cancer cells invade into an invasion matrix composed of 10 mg/ml BME and 1 mg/ml collagen I in response to cell culture medium containing 10% FBS; wells were photographed every 24 hours over a 96 hour period.
MDA-MB-231, human breast cancer cells, were induced to form spheroids (72 hours) and embedded in Invasion Matrix. Serial dilutions of Sulforaphane were added to the DMEM, 10% FBS invasion modulating solution as indicated, and spheroid invasion was conducted over a 96 hour period. Sulforaphane inhibited MDA-MB-231 spheroid Invasion in a dose-dependent manner.
Summary

• 3-D cultures provide suitable environments for cells to self-assemble into organotypic structures mimicking the morphology and function their tissue of origin.

• Spheroids provide ideal tumor models based on morphology, size, physiological gradients, and the formation of heterogeneous cell populations.

• Spheroids exhibit an extracellular matrix which is instrumental in self-assembly of cancer cells.

• The addition of ECM proteins promotes spheroid formation and growth.

• ECM hydrogels provide physiological scaffolds for cancer cells to invade out of tumor spheroids, mimicking early events in metastasis.
## Tools for 3-D Culture Spheroid Proliferation/Viability and Invasion

<table>
<thead>
<tr>
<th>Description</th>
<th>Size</th>
<th>Catalog #</th>
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<tbody>
<tr>
<td>Cultrex® 96 Well 3-D Spheroid BME Cell Invasion Assay</td>
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<td>3500-096-K</td>
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<tr>
<td>Cultrex® 96 Well 3-D Spheroid Fluorometric Proliferation/Viability Assay</td>
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<td>3510-096-K</td>
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<td>Cultrex® 96 Well 3-D Spheroid Colorimetric Proliferation/Viability Assay</td>
<td>96 samples</td>
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References


