FINAL SCRIPT: APPROVED FOR FILMING

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Title: Advancements in the Metabolic Profiling of Three-Dimensional Brain Tumor Spheroids for Drug Screening

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length
Number of Steps: 21
Number of Shots: 41

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Yan Wang:** The research aims to optimize 3D cell spheroid formation for studying energy metabolism using Seahorse technology, focusing on cell behavior, metabolic activity, and drug responses in a clinically relevant model.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figure 1*

How will your findings advance research in your field?

1.2. **Yan Wang:** These findings will enhance drug testing models by providing a more accurate representation of brain tumors, improving therapy evaluation, and metabolic analysis for better treatment strategies.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What new scientific questions have your results paved the way for?

1.3. **Lixiang Xue:** The results pave the way for investigating how energy metabolism alters in 3D spheroid models, how drugs exert effects on metabolic pathways, and the mechanism by which metabolic changes contribute to the development of tumor resistance to therapy. NOTE: The statement has been modified.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. 3D Cell Spheroid Formation

Demonstrator: Xiaojuan Ma

2.1. After resuspending the collected U87 *(U-Eighty-Seven)* cells in DMEM, preheat the unused medium to 37 degrees Celsius **[1]**. Maintain the cell suspensions at room temperature for 30 minutes **[2]**.

2.1.1. WIDE: Talent placing a flask of unused medium into a water bath set to 37 degrees Celsius.

2.1.2. Talent placing tubes of cell suspension on the bench at room temperature.

2.2. Based on the initial cell count, dilute the cell suspension with DMEM containing 10% FBS to a final volume of 20 milliliters, ensuring approximately 200,000 cells **[1]**.

2.2.1. Talent pipetting DMEM containing fetal bovine serum into a tube of cell suspension while monitoring the final volume.

2.3. Mix the final cell suspension thoroughly by repeated inversion **[1]**. Slowly add 200 microliters of the suspension along the edge of each well in a transparent round-bottom ultra-low attachment 96-well plate **[2]**.

2.3.1. Talent inverting the tube several times to mix the final cell suspension.

2.3.2. Talent adding the measured cell suspension along the edge of wells in a transparent round-bottom ultra-low attachment plate.

2.4. Then, seal the plate tightly with parafilm to prevent leakage during centrifugation **[1]**. Place the sealed plate in the centrifuge and run at 200 *g* for 2 minutes to pellet the cells at the bottom of each well **[2]**.

2.4.1. Talent wrapping parafilm securely around the plate.

2.4.2. Talent placing the sealed plate into the centrifuge and initiating the run.

2.5. Remove the plate from the centrifuge **[1]**. Carefully remove the parafilm **[2]** and place the plate in a 37 degrees Celsius incubator with 5 percent carbon dioxide **[3]**.

2.5.1. Talent lifting the plate from the centrifuge.

2.5.2. Talent peeling the parafilm away from the plate without spilling.

2.5.3. Talent placing the plate inside an incubator set to 37 degrees Celsius and 5 percent carbon dioxide.

3. Poly-D-Lysine Coating of the Metabolic Assay Plates

Demonstrator: Lijing Yang

3.1. To prepare a coating solution, dissolve 5 milligrams of poly-D-lysine in 50 milliliters of sterile water **[1]** and mix the solution thoroughly until completely dissolved **[2]**.

3.1.1. WIDE: Talent adding poly-D-lysine powder into a beaker containing sterile water.

3.1.2. Talent mixing the solution with a sterile magnetic stirrer until fully dissolved.

3.2. Add 30 microliters of the coating solution to each well of the metabolic assay plate **[1]**. Ensure the absence of bubbles by gently shaking the plate or aspirating the excess solution **[2]**.

3.2.1. Talent pipetting the coating solution into each well of the plate.

3.2.2. Talent gently tapping or shaking the plate to remove bubbles.

3.3. Incubate the plate with the cover on for 20 minutes at room temperature **[1]**. Then, aspirate the coating solution **[2]** and wash the wells twice with 200 microliters of sterile water **[3]**.

3.3.1. Talent placing the covered plate on the bench for incubation.

3.3.2. Talent aspirating the coating solution from each well.

3.3.3. Talent adding sterile water to the well and removing it.

3.4. Air dry the plate for at least 30 minutes **[1]**. Then, heat the plate at 37 degrees Celsius without carbon dioxide for 30 minutes to stabilize the coating **[2]**.

3.4.1. Talent leaving the plate uncovered on the bench to dry.

3.4.2. Talent placing the plate in an incubator set to 37 degrees Celsius with no carbon dioxide.

3.5. Afterward, add 175 microliters of preheated medium to each well **[1]**. Keep the plate at 37 degrees Celsius in a carbon dioxide-free incubator until it is ready for cell spheroid transfer **[2]**.

3.5.1. Talent pipetting preheated medium into each well of the plate.

3.5.2. Talent placing the plate in an incubator set to 37 degrees Celsius without carbon dioxide.

4. Monitoring Spheroid Growth

Demonstrator: Lijing Yang/Xiaojuan Ma

NOTE: The SCs are filmed by the videographer

4.1. Transfer the spheroid plates to a high-content imaging system after 5 days of culture **[1]**.

4.1.1. WIDE: Talent carrying spheroid plates and placing them into the high-content imaging system.

4.2. To configure the imaging parameters, set the temperature to 37 degrees Celsius and the carbon dioxide level to 5 percent **[1]**. Adjust the plate type, channels, and focus range on the system settings **[2]**.

4.2.1. SCREEN: Show the temperature setting being adjusted to 37 degrees Celsius and carbon dioxide to 5 percent in the imaging software.

4.2.2. SCREEN: Show the selection of the plate type, channels, and adjustment of the focus range in the software interface.

4.3. Next, acquire spheroid images using a confocal microscope with 10 times objectives and the imaging software **[1]**. Then, identify wells containing intact spheroids and record their positions for transfer **[2-TXT]**.

4.3.1. SCREEN: Show the spheroid structure captured at 10 times magnification through the confocal microscope.

4.3.2. LAB MEDIA: Figure 2A-C **TXT: Exclude wells with irregular spheroids**

5. Spheroid Treatment

5.1. Assign the intact spheroids to two groups, a control group with 6 replicate wells and a treatment group with 6 replicate wells for fenofibrate treatment **[1]**.

5.1.1. SCREEN: Show a plate map in the software with wells labeled for control and fenofibrate treatment groups.

5.2. Prepare the fenofibrate working solution in fresh pre-warmed medium at the optimal concentration **[1]**.

5.2.1. Talent pipetting fenofibrate stock into a container of pre-warmed medium and mixing thoroughly.

5.3. Carefully remove the spheroid-containing low-attachment plate from the imaging system **[1]** and place it on a sterile surface inside a biosafety cabinet **[2]**. Position the poly-L-lysine-coated assay plate adjacent to it for transfer **[3]**.

5.3.1. Talent retrieving the spheroid plate from the imaging system.

5.3.2. Talent placing the plate inside the biosafety cabinet.

5.3.3. Talent arranging the poly-L-lysine-coated assay plate next to the spheroid plate.

5.4. Then, trim the tip of a 20-microliter pipette to allow gentle aspiration of the cell spheroids without causing damage **[1]**.

5.4.1. Talent using sterile scissors to trim the end of a 20-microliter pipette tip.

5.5. Using the trimmed pipette tip, gently aspirate each intact spheroid from the bottom of its well **[1]**. Transfer each spheroid to the corresponding well of the coated plate and allow it to settle for 20 seconds **[2]**.

5.5.1. Talent aspirating a spheroid from the bottom of the well using the trimmed pipette tip.

5.5.2. Talent gently releasing the spheroid into the coated well.

5.6. Transfer the 3D spheroid assay plates to a high-content imaging system **[1]**. To analyze the morphological data, apply the **Texture Region** module to coarsely separate 3D spheroids from the background by dividing the image content into three clusters based on texture features **[2]**.

5.6.1. Talent placing the 3D spheroid assay plate into the high-content imaging system.

5.6.2. SCREEN: Show the Texture Region module in Harmony software dividing the image into three distinct clusters based on texture features

5.7. Apply the **Find Image Region** module to refine the separation of pre-isolated 3D spheroids by using volume-based size characteristics, ensuring impurities are removed and intact spheroids are retained **[1]**.

5.7.1. SCREEN: Show the Find Image Region module interface with settings adjusted for volume-based size filtering to isolate intact spheroids.

5.8. Measure the morphological parameters of the 3D spheroids using the **Morphology Properties** module **[1]**. Apply the **Select Population** module to filter and retain only genuine 3D cell spheroids **[2].** Then**,** export the morphological data results **[3].**

5.8.1. SCREEN: Display the Morphology Properties module with calculated parameters such as area, perimeter, and shape index for each spheroid.

5.8.2. SCREEN: Show the Select Population module highlighting the retained spheroids while excluding false positives.

5.8.3. SCREEN: Show the export dialog in the Harmony software with the morphological data table visible, ready for saving.

Results

6. Results

6.1. Spheroids were visibly formed by the 5th day of culture, maintaining a uniform, spherical morphology across all observed samples **[1].** A disintegrated spheroid with visibly disrupted structural integrity was also observed on day 5 **[2].**

6.1.1. LAB MEDIA: Figure 2A–C.

6.1.2. LAB MEDIA: Figure 2D.

6.2. Post-transfer analysis revealed that both control and fenofibrate-treated 3D spheroids remained intact in most cases **[1],** but some fenofibrate-treated spheroids were disintegrated **[2].**

6.2.1. LAB MEDIA: Figure 3A, 3B, 3D, and 3E.

6.2.2. LAB MEDIA: Figure 3C and 3F**.** *Video editor: Highlight the scattered cell debris and red cell segmentation in panels C and F, indicating disintegration*.

6.3. Oxygen consumption rate was significantly reduced in fenofibrate-treated spheroids across all time points compared to the control group **[1].**

6.3.1. LAB MEDIA: Figure 4. *Video editor: Highlight the red line*

1. **DMEM** *(Dulbecco's Modified Eagle Medium)*
   Pronunciation link: https://www.howtopronounce.com/dmem
   IPA (American): /diːˈɛmˌiːˈɛm/
   Phonetic spelling: *DEE-EM-EM*

2. **FBS** *(Fetal Bovine Serum)*
   Pronunciation link: https://www.howtopronounce.com/fbs
   IPA (American): /ɛf.biː.ɛs/
   Phonetic spelling: *EFF-BEE-ESS*

3. **Parafilm**
   Pronunciation link: https://www.howtopronounce.com/parafilm
   IPA (American): /ˈpærəˌfɪlm/
   Phonetic spelling: *PAIR-uh-film*

4. **Incubator**
   Pronunciation link: https://www.merriam-webster.com/dictionary/incubator
   IPA (American): /ˈɪŋkjəˌbeɪtər/
   Phonetic spelling: *ING-kyuh-bay-ter*

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5. **Poly-D-lysine**
   Pronunciation link: https://www.howtopronounce.com/poly-d-lysine
   IPA (American): /ˌpɑːli ˈdiː ˈlaɪˌsiːn/
   Phonetic spelling: *PAH-lee-DEE-LY-seen*

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6. **Spheroid**
   Pronunciation link: https://www.merriam-webster.com/dictionary/spheroid
   IPA (American): /ˈsfiˌrɔɪd/
   Phonetic spelling: *SFEE-royd*

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7. **Fenofibrate**
   Pronunciation link: https://www.merriam-webster.com/dictionary/fenofibrate
   IPA (American): /ˌfɛnoʊˈfaɪˌbreɪt/
   Phonetic spelling: *FEN-oh-FYE-brayt*

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8. **Morphology**
   Pronunciation link: https://www.merriam-webster.com/dictionary/morphology
   IPA (American): /mɔrˈfɑlədʒi/
   Phonetic spelling: *mor-FAH-luh-jee*