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Title: A Three-dimensional Model of Spheroids to Study Colon Cancer Stem Cells

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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 similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

    ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **No**

Protocol Length
Number of Shots: **24**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Michelina Plateroti**: This new method of spheroid culture can be used to study tumor heterogeneity and to analyze different populations of cancer stem cells and can potentially be applied for high throughput drug screening **[1]**.

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

REQUIRED:

1.2. **Maria Virginia Giolito**: This is a robust and reproducible culture system for generating large numbers of homogenously sized spheroids. Moreover, the spheroids can be used to study cancer stem cells **[1]**.

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

OPTIONAL:

1.3. **Maria Virginia Giolito**: This method is set up to study colon cancer biology, but it can be easily used to study other cancers through modification of the 3D culture conditions **[1]**.

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

OPTIONAL:

1.4. **Léo Claret**: Be sure to watch for bubbles in the medium, to not move the plate too much during the spheroid formation, and to be careful when harvesting the spheroids into the strainer **[1]**.

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

AFFILIATION CLARIFICATION:

1.5. This work was completed at the Centre de Recherche en Cancérologie de Lyon-Université Lyon 1, but filmed at the UMR-S1113 – IRFAC, INSERM, Université de Strasbourg.

Protocol

2. Spheroid Culture Preparation

2.1. Begin by pre-treating the wells of a 24-well plate with 500 microliters of anti-adherence rinsing solution per well **[1-TXT]**.

2.1.1. WIDE: Talent adding solution to well(s), with solution container visible in frame *Videographer: Important step* **TEXT: See text for all medium and solution preparation**

2.2. After a few seconds, centrifuge the plate in a swinging bucket rotor **[1-TXT]** and rinse each well with 2 milliliters of warm basal medium **[2]**.

2.2.1. Talent placing plate into centrifuge **TEXT: 5 min, 1200 x g, RT**

2.2.2. Talent rinsing well, with medium container visible in frame

2.3. Observe the plate under the microscope to ensure that the bubbles have been completely removed from the microwells **[1-TXT]**. If no bubbles are observed, rinse the wells again **[2]** before adding 1 milliliter of warm DMEM (D-M-E-M)-basement membrane matrix medium to each well **[3-TXT]**.

2.3.1. Talent at microscope, viewing bubbles **TEXT: If bubbles remain trapped, repeat centrifugation**

2.3.2. Talent adding medium to well(s), with medium container visible in frame **TEXT: Dulbecco's Modified Eagle Medium**

2.3.3. Talent adding BMM medium to each well, with BMM medium container visible in frame

3. Spheroid Generation

3.1. To induce spheroid formation, seed the desired concentration of Caco2 (kay-coh-two) cells in a 2D monolayer in a 10-centimeter dish in DMEM supplemented with 10% FBS (F-B-S) and 1% antibiotics **[1-TXT]** for their culture at 37 degrees Celsius and 5% carbon dioxide in a humidified atmosphere **[2]**.

3.1.1. WIDE: Talent adding cells to dish, with medium container visible in frame **TEXT: FBS: Fetal Bovine Serum**

3.1.2. Talent placing plate into incubator

3.2. When an 80% confluency is reached, wash the cells with 5 milliliters of PBS **[1]** before treating the culture with 2 milliliters of trypsin-EDTA for 2-5 minutes at 37 degrees Celsius and 5% carbon dioxide **[2]**.

3.2.1. Talent washing cells, with PBS container visible in frame

3.2.2. Talent adding trypsin-EDTA to cells, with trypsin-EDTA container visible in frame

3.3. When the cells have detached, neutralize the trypsin with 4 milliliters of complete DMEM **[1]**. After counting, collect the cells by centrifugation **[2-TXT]** and resuspend the pellet in the appropriate volume of DMEM-basement membrane matrix medium to achieve the desired concentration of cells in 1 milliliter of medium per well **[3]**.

3.3.1. Talent adding medium to dish, with medium container visible in frame, and transferring medium to tube. *Videographer: Important/difficult step*

3.3.2. Talent adding tube(s) to centrifuge *Videographer: Important/difficult step* **TEXT: 3 min, 1200 x g, RT**

3.3.3. LAB MEDIA: Table 1 *Video Editor: please emphasize right column*

3.4. Add 1 milliliter of cells to each well of the prepared 24-well plate **[1]** followed by the addition of 1 milliliter of DMEM-basement membrane matrix medium to each well **[2]**.

3.4.1. Talent adding cells to well(s) *Videographer: Important step*

3.4.2. Talent adding DMEM-BMM medium to each well, with medium container visible in frame *Videographer: Important step*

3.5. After seeding, immediately centrifuge the plate **[1-TXT]** and use a light microscope to verify that the cells have been evenly distributed across the microwells **[2]**.

3.5.1. Talent placing plate into centrifuge **TEXT: 5 min, 1200 x g, RT**

3.5.2. Talent checking plate

3.6. Then place the plate in the cell culture incubator for 48 hours without disturbing the cells **[1]**.

3.6.1. Talent placing plate into incubator

4. Spheroid Harvest

4.1. To harvest the spheroids, at the end of the incubation, use a serological pipette and half of the supernatant in each well to gently dislodge the spheroids from their microwells **[1]**.

4.1.1. WIDE: Talent flushing spheroid in well

4.2. When all of the spheroids have been detached, use the pipette to carefully transfer the 3D cultures from each well onto a 37-micron reversible strainer on top of a 15-milliliter conical tube **[1]**.

4.2.1. Talent adding spheroid to strainer *Videographer: Important step*

4.3. Wash each well three times with 1 milliliter of pre-warmed basal medium per wash to collect any remaining spheroids **[1]** and add these additional spheroids to the strainer **[2]**.

4.3.1. Well being washed

4.3.2. Talent adding spheroid to strainer

4.4. After the last wash, check the plate under the microscope to confirm that all of the spheroids have been removed from the microwells **[1-TXT]**.

4.4.1. Talent at microscope, checking plate **TEXT: Repeat wash as necessary**

4.5. Then invert the strainer over a new 15-milliliter tube **[1]** and wash the bottom of the strainer with fresh DMEM-basement membrane matrix medium to collect the spheroids into the tube **[2]**.

4.5.1. Strainer being inverted *Videographer: Important step*

4.5.2. Strainer being washed *Videographer: Important step*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.1., 3.3., 3.4., 4.2., 4.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
3.3. Ensure that you have the correct amount of cells and that you dispense evenly cells in the well (so you are sure that they evenly distributed in each microwell).

Results

5. Results: Representative Colon Cancer Stem Cell Spheroid Formation and Analysis

5.1. Spheroids arising from 500 cells demonstrate the most homogenous increase in size over time **[1]**, with 500 and 600 cells/spheroid determined to be the optimal number of cells under which good growth and low variability can be achieved **[2]**.

5.1.1. LAB MEDIA: Figure 1B *Video Editor: please emphasize 500 graphs*
5.1.2. LAB MEDIA: Figures 2A-2C *Video Editor: please emphasize 500 and 600 images, data bars, and graphs*

5.2. The addition of basement membrane matrix enhances spheroid growth and homogeneity **[1]**.

5.2.1. LAB MEDIA: Figure 2D

5.3. In long-term culture **[1]**, the cells appear as dense multilayers at day 3 **[2]**, while flattened cells arranged in monolayers are clearly visible at day 10 **[3]**.

5.3.1. LAB MEDIA: Figure 3
5.3.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize D3 inset*
5.3.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize D10 inset*

5.4. Interestingly, a lumen appears within the spheroids from day 5 onward **[1]**.

5.4.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize dashed black line in D5, D7, and D10 images*

5.5. In addition **[1]**, a clear increase in proliferating cell nuclear antigen-positive cells is observed at days 5 and 7 **[2]** that declines by day 10 **[3]**.

5.5.1. LAB MEDIA: Figure 4
5.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize red signal in left D5 and D7 images*
5.5.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize red signal in left D10 image*

5.6. Surprisingly, activated caspase 3 is observed in very few cells at days 3 and 5 only **[1]**, while high levels of beta-catenin expression are observed at every time point **[2]**.

5.6.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize green signal in left D3 and D10 images*

5.6.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize red signal in right D3, D5, D7, and D10 images*

5.7. The potential of the spheroids to differentiate into enterocytes is evidenced by their alkaline phosphatase **[1]** and solute carrier family 2A5 (two-A-five) mRNA expression **[2]**.

5.7.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize ALPI graph*

5.7.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize SLC2A5 graph*

5.8. In addition, the spheroids express typical cancer stem cell markers **[1]** and respond to combination chemotherapy treatments routinely administered to colorectal cancer patients **[2]**.

5.8.1. LAB MEDIA: Figure 6 *Video Editor: please emphasize green signal in CD133 column and red signal in CD44 column*

5.8.2. LAB MEDIA: Figures 9A and 9B *Video Editor: please emphasize pink and blue data lines and 72 h FOLFOX and FOLFIRI images*

Conclusion

6. Conclusion Interview Statements

6.1. **Michelina Plateroti**: The spheroids are first composed of different cell populations. They then can be used to study cell-specific responses to stimuli, such as growth factors or drugs **[1]**.

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