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Title: Isolation of Primary Cancer-associated Fibroblasts from a Syngeneic Murine Breast Cancer for the Study of Targeted Nanoparticles

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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? **No**

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?

   ☒Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length
Number of Steps: 23
Number of Shots: 50

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Marta Truffi:** This protocol makes it possible to successfully isolate and culture primary cancer-associated fibroblasts from murine breast cancer to screen novel nanoparticles designed to target the tumor microenvironment **[1]**.

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

1.2. **Leopoldo Sitia:** Primary CAFs represent the most reliable and physiological model for in vitro studies of tumor stroma. In this protocol, we describe how to achieve an optimal yield **[1]**.

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

Videographer's notes:

The first 3 numbers on slate are the reference for script points, the last number is the take.

If a scene is more complex and need to be split in different parts there will be a note under the main numbers on the slate. E.g., 4 | 2 | 1 | 1 / Part 01

The footage has been created using a Sony A7S3 camera with default PP8 profile in SLog3, which has a sharpening setting of -7. The footage however needs to be treated in post-production taking care of:

COLOR: a generic conversion LUT is included with footage. It can help to speed up the color grading, particularly for the interviews and for the shots where skin tones has a significant part. Scene by scene corrections might still be required, but it would take less time starting from the included LUT. It can be ignored by editors with more skills in color grading and experience working on Log footage.

DETAIL: a sharpening filter should be added to increase the detail (with much more control of what could have been done during production). For example in Premiere Pro detail could be increased with the Sharpen slider of Lumetri Color filter and it might be applied on an adjustment layer for the whole timeline. Note: place graphics in a track above the adjustment layer so that it won't be affected by the filter.

Protocol

2. Tumor Dissociation into Single Cells

2.1. To begin, prepare a digestion mix for tumor dissociation **[1-TXT]** and soak the 3 to 5-millimeter tumor fragments in the solution after tightly closing the tube **[2]**.
- 2.1.1. WIDE: Talent with digestion mix solution placed in a laminar flow hood **TEXT: Refer to text for digestion mixture solution**
- 2.1.2. Talent placing the tumor fragments in the solution

2.2. Turn the tube upside down, checking that all the pieces of tumor are at the bottom of the tube toward the cap **[1],** then attach the tube to a mechanical dissociator in the proper housing **[2]** and run a dissociation program specifically designed for tough tumors **[3]**. *Videographer: This step is important*
- 2.2.1. Talent turning the tube upside down
- 2.2.2. Talent attaching the tube to a mechanical dissociator
- 2.2.3. Talent setting a dissociation program to run

2.3. After the run, detach the tube **[1]** and place it upside down to incubate the sample at 37 degrees Celsius for 40 minutes with gentle shaking **[2]**.
- 2.3.1. Talent detaching the tube
- 2.3.2. Talent placing the tube upside down for shaking

2.4. Then, re-attach the tube to the dissociator in the proper housing **[1]** and run the dissociation program for tough tumors twice, ensuring no large tissue pieces remain at the end of the procedure **[2]**.
- 2.4.1. Talent attaching the tube to the dissociator
- 2.4.2. Talent setting the program to dissociate the tumors

2.5. Filter the sample through a 40-micron cell strainer in a 50-milliliter tube **[1]**, then wash the filter with 10 milliliters of RPMI 1640 *(R-P-M-I-Sixteen-Forty)* medium **[2]** and centrifuge **[3-TXT]**.
- 2.5.1. Talent filtering the sample through a strainer
- 2.5.2. Talent washing the filter with medium
- 2.5.3. Talent placing the sample in centrifuge **TEXT: 7 min, 300 × g**

2.6. After centrifugation, resuspend the pellet in PBE buffer composed of PBS, 0.5% BSA, and 2-millimolar EDTA **[1]** and count the cells with trypan blue **[2]**.
- 2.6.1. Talent resuspending the pellet in buffer
- 2.6.2. Talent counting the cells

2.7. Prepare the dead cell removal binding buffer by diluting the 20x binding buffer stock solution with sterile double-distilled water **[1]**, wash the cells with 5 milliliters of 1x binding buffer **[2]**, and centrifuge **[3-TXT]**.
  2.7.1. Talent showing the previously prepared 1x binding buffer
  2.7.2. Talent giving a wash of binding buffer to the cells
  2.7.3. Talent placing the sample in centrifuge **TEXT: 7 min, 300 × g**

2.8. After centrifugation, resuspend the cell pellet with 0.1 milliliter of dead cell removal microbeads for up to 10^7 cells and incubate at room temperature for 15 minutes **[1]**.
  2.8.1. Talent resuspending the pellet in dead cell removal microbeads

2.9. During the incubation, prepare a magnetic stand under the hood **[1]** and hang ferromagnetic separation columns on it with the tips pointing down **[2]**. Equilibrate the columns with 0.5 milliliters of cold binding buffer and wait until the solution has flowed through **[3]**.
  2.9.1. Magnetic stand placed under the hood
  2.9.2. Separation columns with tips pointing down
  2.9.3. Talent adding a binding buffer to the column

2.10. After incubation, add 400 microliters of cold binding buffer to the bead and cell suspension **[1],** load the entire volume onto the column **[2]** and collect the effluent in a 15-milliliter tube **[3]**. *Videographer: This step is important*
  2.10.1. Talent adding a binding buffer to bead/cell suspension
  2.10.2. Talent loading cell suspension to the column
  2.10.3. Talent collecting the effluent

2.11. Wash the column with 0.5 milliliters of binding buffer **[1]** and collect the total effluent in the same tube **[2]**.
  2.11.1. Talent washing the column with buffer
  2.11.2. Talent collecting the effluent in tube

3. Extraction of Cancer-Associated Fibroblasts (CAF's) From Breast Cancer

3.1. For depletion of non-cancer-associated fibroblasts, filter the cell suspension using a 70-micron cell strainer moistened with PBS **[1]** and centrifuge the cells **[2-TXT]**.
  3.1.1. Talent filtering the cell suspension through a strainer
  3.1.2. Talent placing the tube in centrifuge **TEXT: 10 min, 300 × g**

3.2. Remove the supernatant and resuspend the pellet in 80 microliters of cold PBE buffer **[1]**. Add 20 microliters of Non-Tumor-Associated Fibroblast Depletion Cocktail **[2]**, mix well, and incubate the sample at 4 degrees Celsius for 15 minutes in the dark **[3-TXT]**
  3.2.1. Talent resuspending the pellet in buffer

3.2.2. Talent adding cocktail to the tube *Videographer: This step is important*
3.2.3. Talent placing the sample for incubation in the dark **TEXT: Always work with cold buffers**

3.3. During the incubation with beads, prepare ferromagnetic depletion columns on a magnetic stand **[1]**. Equilibrate the columns with 2 milliliters of cold PBE buffer and wait until the solution has flowed through **[2]**.
3.3.1. Separation columns with tips pointing down
3.3.2. Talent equilibrating the columns

3.4. After incubation, add 400 microliters of buffer to the bead and cell suspension **[1]**, load the entire volume onto the column, and collect the effluent in a 15-milliliter tube **[2-TXT]**.
3.4.1. Talent adding a buffer to bead/cell suspension
3.4.2. Talent loading cell suspension to the column and collecting the effluent **TEXT: Precisely follow incubation time** *Videographer: This step is important*

3.5. Wash the column twice with 2 millimeters of PBE **[1]**, collect the total effluent into the same tube **[2]**, and centrifugation the effluent **[3-TXT]**.
3.5.1. Talent giving washes to the column
3.5.2. Talent collecting effluent in tube
3.5.3. Talent placing the tube in centrifuge **TEXT: 10 min, 300 × g**

3.6. For the positive selection of cancer-associated fibroblasts, add 20 microliters of Tumor-Associated Fibroblast Microbeads to 80 microliters of cell suspension **[1]**. Mix well and incubate the sample at 4 degrees Celsius for 15 minutes in the dark **[2]**.
3.6.1. Talent adding fibroblast microbeads to the cell suspension
3.6.2. Talent placing the sample for incubation in the dark

3.7. During incubation, prepare ferromagnetic separation columns on a magnetic stand and equilibrate them with 0.5 milliliters of cold PBE buffer **[1]**.
3.7.1. Talent performing the step as demonstrated

3.8. After incubation, add 400 microliters of PBE to the bead and cell suspension, load the entire volume onto the column and let the unlabeled cells flow through into a 15-milliliter tube **[1]**.
3.8.1. Talent performing the step as demonstrated *Videographer: This step is important*

3.9. Wash the column three times with 0.5 milliliters of PBE buffer and collect the total effluent in the same tube **[1]**.
3.9.1. Talent collecting the effluent in tube

3.10. Remove the column from the magnet and place it in a 1.5-milliliter tube **[1]**. Add 1 milliliter of PBE onto the column **[2]** and immediately push the plunger into the column to flush the cells out **[3]**.
3.10.1. Talent placing the column in the tube
3.10.2. Talent adding a buffer to the column
3.10.3. Talent pushing the plunger into the column *Videographer: This step is important*

3.11. Resuspend the cell pellet in an appropriate volume of DMEM **[1]** and seed the cells in a tissue-culture plate **[2]**.
3.11.1. Talent diluting the cells in medium
3.11.2. Talent seeding the cells in a tissue culture plate

3.12. Check the cell density under a microscope **[1]** and place the plate at 37 degrees Celsius and 5% carbon dioxide to allow the cells to adhere and grow **[2]**.
3.12.1. Talent observing the cells under the microscope
3.12.2. Talent placing the plate in the incubator

Results

4. Results: Isolation and Culture of Primary CAFs from a Syngeneic Murine Breast Cancer

4.1. The injection of 4T1-luc *(Four-T-one-Luciferase)* cells into the mammary fat pad of female mice led to the growth of a detectable tumor mass five days after implantation **[1]**.
- 4.1.1. LAB MEDIA: Figure 1C *Video Editor: please emphasize on Day 5, mice*

4.2. To find a sacrifice window that is adequate for CAF isolation, an optimal compromise was sought between higher tumor size **[1]** and bioluminescence imaging on the one hand **[2]** and an emerging tumor ulceration and necrosis on the other hand **[3]**. Day 20 was set as the time point to optimize cell recovery after the isolation process **[4]**.
- 4.2.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize Day 25 from the graph*
- 4.2.2. LAB MEDIA: Figure 1B *Video Editor: please emphasize on Time (Days): 20 from the graph*
- 4.2.3. LAB MEDIA: Figure 1C *Video Editor: please emphasize on Day 25 and Day 30 mice*
- 4.2.4. LAB MEDIA: Figure 1C *Video Editor: please emphasize on Day 20, mice*

4.3. Two more passages were needed to isolate the population of CAFs from the panel of collected viable cells **[1]**, the depletion of non-tumor-associated fibroblasts **[2]** and the enrichment of tumor-associated fibroblasts **[3]**.
- 4.3.1. LAB MEDIA: Figure 2D
- 4.3.2. LAB MEDIA: Figure 2E
- 4.3.3. LAB MEDIA: Figure 2F *Video Editor: please emphasize on CD90.2$^+$ CD45$^-$ marked with the blue rectangle*

4.4. CAF cells revealed large spindle-shaped morphology typical of fibroblast **[1]** and were different from 4T1 tumor cells **[2]**. FAP expression followed over five passages confirmed that primary CAF culture maintained its original characteristics **[3]**.
- 4.4.1. LAB MEDIA: Figures 3A, B
- 4.4.2. LAB MEDIA: Figure 3D
- 4.4.3. LAB MEDIA: Figure 3C

4.5. When incubation duration and temperature with microbeads were not maintained, higher percentages of undesired cell populations were recovered **[1]**. These contaminants cells were responsible for the presence of small clones with different morphology **[2]** that grew faster and prevailed over the primary CAF culture **[3]**.
- 4.5.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize on contaminant CD90.2$^-$ CD45$^+$ and CD90.2$^-$ CD45$^-$ from the histogram*
- 4.5.2. LAB MEDIA: Figure 4B *Video Editor: please emphasize on black arrowheads*

4.5.3. LAB MEDIA: Figure 4C

4.6. CAFs binding with the recombinant variant of human ferritin heavy chain, HFn (*H-F-N)*-FAP increased at 1:1 **[1]** and 1:5 antibody fragment concentrations by three-fold **[2]** compared to bare HFn **[3]**.

4.6.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize on HFn-FAP 1:1*

4.6.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize on HFn-FAP 1:5*

4.6.3. LAB MEDIA: Figure 5A *Video Editor: please emphasize on HFn*

4.7. On the contrary, for tumor 4T1 cells **[1]**, bare HFn showed higher binding than functionalized HFn **[2]**.

4.7.1. LAB MEDIA: Figure 5B *Video Editor: please emphasize HFn-FAP 1:1 and HFn-FAP 1:5*

4.7.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize on HFn*

Conclusion

5. Conclusion Interview Statements

5.1. **Leopoldo Sitia:** To increase the yield and purity of isolated CAFs, keep buffers cold, respect incubation time with beads, and pool up to four tumors per column before the final enrichment step **[1]**.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.9, 3.2 to 3.10.*

5.2. **Serena Mazzucchelli:** The isolated CAFs can be used to screen several candidate nanodrugs in terms of binding, uptake and pharmacological efficacy, before proceeding with preclinical in vivo experiments **[1]**.

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

5.3. **Marta Truffi:** CAF isolation allowed us to test if nanoparticles can be used to target the tumor microenvironment, thus paving the way to new targeted therapies working in synergy with chemotherapy **[1]**.

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