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Generation and expansion of primary, malignant pleural mesothelioma tumor lines

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Corresponding Author:	Cara Haymaker, PhD University of Texas MD Anderson Cancer Center Houston, TX UNITED STATES
Corresponding Author's Institution:	University of Texas MD Anderson Cancer Center
Corresponding Author E-Mail:	chaymaker@mdanderson.org
Order of Authors:	Tamara Griffiths
	Carlos Ramos
	Cara Haymaker, PhD
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TITLE:

Generation and Expansion of Primary, Malignant Pleural Mesothelioma Tumor Lines

AUTHORS AND AFFILIATIONS:

Tamara M. Griffiths[1], Carlos Ramos[1], Cara L. Haymaker[1]

[1]Department of Translational Molecular Pathology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Email addresses of co-authors:

Tamara M. Griffiths (tgriffiths@mdanderson.org)
Carlos Ramos (cramos2@mdanderson.org)

Corresponding author:

Cara L. Haymaker (chaymaker@mdanderson.org)

KEYWORDS:

Primary tumor cells, pleural mesothelioma, *in vitro* culture, flow cytometry, tumor line expansion

SUMMARY:

The goal of this method paper is to demonstrate a robust and reproducible methodology for the enrichment, generation, and expansion of primary tumor cell lines from surgically resected pleural mesothelioma.

ABSTRACT:

Current methodologies for the expansion of primary tumor cell lines from rare tumor types are lacking. This protocol describes methods to expand primary tumor cells from surgically resected, malignant pleural mesothelioma (MPM) by providing a complete overview of the process from digestion to enrichment, expansion, cryopreservation, and phenotypic characterization. In addition, this protocol introduces concepts for tumor generation that may be useful for multiple tumor types such as differential trypsinization and the impact of dissociation methods on the detection of cell surface markers for phenotypic characterization. The major limitation of this study is the selection of tumor cells that will expand in a two-dimensional (2D) culture system. Variations to this protocol, including three-dimensional (3D) culture systems, media supplements, plate coating to improve adhesion, and alternate disaggregation methods, could improve this technique and the overall success rate of establishing a tumor line. Overall, this protocol provides a base method for establishing and characterizing tumor cells from this rare tumor.

INTRODUCTION:

Malignant pleural mesothelioma (MPM) is a rare tumor highly associated with asbestos exposure. Although immunotherapy-based approaches have shown encouraging results, there is a paucity of treatment options available to patients that develop this disease, and the overall 5-year survival rate is low[1,2]. Efforts are underway at multiple institutions to better understand this

disease and identify novel therapeutic targets that may improve patient outcomes. While there are multiple mesothelioma mouse models, access to primary mesothelioma tumor cells is more limited[3]. *In vitro* expansion of primary mesothelioma tumor cells would provide a valuable model system that can be utilized to study tumor cells directly and their interaction with autologous immune cells such as tumor-infiltrating lymphocytes. While there have been reports on the expansion of primary mesothelioma tumor cells lines, these are few and do not provide a detailed standard operation procedure (SOP). Furthermore, few cell lines are available from commercial sources such as American Type Culture Collection (ATCC). While the availability of primary tumor lines is limited, it has been demonstrated that tumor cells can be expanded from pleural effusions and directly from the tumor tissue[4,5]. In addition, expanded tumor cell lines have been shown to preserve the molecular profile of the original tumor[4-6].

Our laboratory is studying the tumor–immune microenvironment of MPM and has developed a method for expanding primary MPM tumor cells lines from surgically resected cases. This method is adapted from our experience in establishing primary metastatic melanoma tumor cell lines. The goal of this work is to provide a detailed, practical approach to primary mesothelioma tumor line expansion using a 2D model system and subsequent phenotypic profiling. Given the recent success of checkpoint blockade strategies targeting CTLA4 and PD-1 in the first-line setting[2], the ability to generate many primary tumor lines could further the understanding of both tumor intrinsic mechanisms of resistance as well as provide an important model system to assess T-cell recognition, thus deepening our understanding of the immune response in MPM.

The major limitation of this protocol is that every tumor contains a different microenvironment, and there is a high degree of variability of expansion success. In addition, this method selects for tumor cells that can expand in a 2D culture system. Other methods that involve the production of a 3D spheroid or organoid culture could provide an alternative approach that may allow for a higher success rate of expansion or result in the ability to derive cell lines that are unable to expand in a traditional 2D system. Such 3D cultures have been demonstrated to be useful for the generation of tumor types that are difficult to expand, for example, pancreatic cancer[7].

PROTOCOL:

All methods described here have been approved by the Institution Review Board (IRB) of the University of Texas MD Anderson Cancer Center. This pertains to standard-of-care, surgically resected MPM tumors removed following informed consent.

1. Preparation of tumor digestion media and other associated media

1.1. To prepare tumor digestion media, add 5 mL of 100% Pen/Strep (Penicillin/Streptomycin) to 500 mL of sterile Roswell Park Memorial Institute (RPMI) 1640 medium in a laminar flow hood.

1.1.1. Transfer the medium to a 500 mL, 0.22 μm polyethersulfone (PES) sterile filter for vacuum filtration.

NOTE: Filtration and aspiration steps should be performed with a standard vacuum pressure of 75–150 torr.

1.1.2. Label and date the tumor digestion medium and store it at 4 °C.

NOTE: The medium can be stored for 1 month at 4 °C.

1.2. To prepare the tumor-digesting enzymatic cocktail, add 2.5 mL of 3% collagenase type 1, 1 mL of 1.5 mg/mL hyaluronidase, 20 µL of 250,000 units/mL DNAse 1 to 16.5 mL of digestion medium in a 50 mL conical tube in a laminar flow hood.

NOTE: The total volume of the tumor-digesting enzymatic cocktail is 20 mL; however, only 10 mL is needed per tumor sample. As such, the remaining cocktail can be stored at -20 °C until needed. Do not refreeze aliquots.

1.3. To prepare complete tumor medium, add 5 mL of 100% Pen/Strep and 50 mL of fetal bovine serum (FBS) to 500 mL of sterile RPMI 1640 in a laminar flow hood.

1.3.1. Transfer the medium to a 500 mL, 0.22 µm PES sterile filter for vacuum filtration.

1.3.2. Label and date the complete tumor medium and store it at 4 °C.

NOTE: The medium can be stored for 1 month at 4 °C.

1.4. To prepare reduced-serum medium for starvation, add 5 mL of 100% Pen/Strep and 5 mL of FBS to 500 mL of sterile RPMI 1640 in a laminar flow hood.

1.4.1. Transfer the medium to a 500 mL, 0.22 µm PES sterile filter for vacuum filtration.

1.4.2. Label and date the reduced-serum medium and store it at 4 °C.

NOTE: The medium can be stored for 1 month at 4 °C.

1.5. To prepare freeze medium, add 5 mL of dimethyl sulfoxide (DMSO) to 45 mL of FBS in a laminar flow hood.

1.5.1. Transfer the medium to a 50 mL, 0.22 µm PES sterile filter for vacuum filtration.

1.5.2. Label and date five 15 mL conical tubes.

1.5.3. Transfer 10 mL of freeze medium to each tube and store the tubes at -20 °C.

1.6. To prepare antibiotic-free medium for mycoplasma testing, add 50 mL of FBS to 500 mL of sterile RPMI 1640 in a laminar flow hood.

1.6.1. Transfer the medium to a 50 mL, 0.22 µm PES sterile filter for vacuum filtration.

1.6.2. Label and date the antibiotic-free medium and store it at 4 °C.

NOTE: The medium can be stored for 1 month at 4 °C.

1.7. To prepare fluorescence-activated cell sorting (FACS) buffer for phenotypic analysis, add 16.6 mL of sterile bovine serum albumin to 500 mL of sterile 1x phosphate-buffered saline (PBS) in a laminar flow hood.

1.7.1. Label and date the FACS buffer and store it at 4 °C.

NOTE: FACS buffer does not require filter sterilization as long as both components are stored sterile. FACS buffer can be stored prior to opening for 6 months at 4 °C.

2. Digestion of tumor tissue

2.1. Transfer 10 mL of tumor-digesting enzymatic cocktail from step 1.2 to a dissociation tube.

2.2. Transfer the piece of tumor tissue to a sterile 6-well plate lid using a sterile scalpel.

NOTE: The amount of medium that is transferred with the tumor tissue is sufficient for processing.

2.2.1. Remove all fatty tissue, necrotic tissue, and/or blood clots using a new sterile scalpel and forceps.

NOTE: Fatty tissue is normally yellowish in appearance and semisolid. Necrotic tissue is darker than the surrounding tissue in appearance and very soft; both fatty and necrotic tissue will break apart easily. Bloody tissue appears bright red and may release blood into the medium when manipulated. Once removed, the resulting tumor tissue should hold shape and be pale or pink, depending on the tissue type.

2.2.2. Cut the entire tumor tissue into 1 mm^3 small fragments. To ensure the tissue does not dry out, add 500 µL of sterile 1x Hank's Balanced Salt Solution (HBSS) to the tumor tissue.

2.3. Transfer tumor fragments into the dissociation tube containing 10 mL of tumor-digesting enzymatic cocktail (step 2.1). Close the tube tightly, place it cap-side down on the tissue dissociator, and add the heater apparatus by applying it like a sleeve over the dissociation tube and pressing to click it in place.

NOTE: The maximum capacity for each tube is 4 g of tumor and 10 mL volume.

2.4. Select the preprogrammed setting on the dissociator **37C_h_TDK_1**. Check the status after 10 min to ensure there is no clogging error as indicated by the flashing red light.

NOTE: This program processes at 1,865 rpm for **1 h** with **temperature on** at 37 °C.

NOTE: If clogging occurs, remove the dissociation tube and manually swirl it to dislodge any tissue caught in the lid; then replace the tube onto the dissociator and resume the program.

2.5. Following the 1 h digestion, remove the dissociation tube and place it in a laminar flow hood. Filter the digested tumor by placing a 70 µm cell strainer on top of a 50 mL conical tube and pipetting the digested tumor using a 10 mL pipette onto the filter. If the filter clogs, switch to a new filter.

2.5.1. Rinse the dissociation tube with 10 mL of fresh tumor digestion media and pass the wash through the filter.

2.5.2. Remove the 70 µm filter and discard it.

2.5.3. Bring the total volume to 40 mL with tumor digestion medium.

2.6. Centrifuge at 500 × *g* for 5 min at room temperature.

2.7. Using a 2 mL aspirating pipette attached to a vacuum source, aspirate the supernatant without disturbing the pellet, and resuspend the cells using 10 mL of sterile complete tumor medium.

2.8. Repeat step 2.6.

2.9. Aspirate the supernatant as described in step 2.7. Resuspend the cells in 3 mL of sterile complete tumor medium and transfer the cell suspension to one well of a sterile 6-well plate.

2.10. Keep the plate in an incubator at 37 °C with 5% CO_2.

2.11. After 24 h, transfer the used medium from the digested tumor in well 1 to a new well (well 2) in the same 6-well plate. Add 3 mL of sterile complete tumor medium to well 1.

2.12. Transfer the used medium from wells 1 and 2 and combine into well 3 in the same 6-well plate 24 h after step 2.11. Add 3 mL of sterile complete tumor medium into wells 1 and 2.

2.13. Aspirate the medium from all 3 wells and pipette 3 mL of complete tumor medium into each well 48 h after step 2.12.

3. Generation of primary tumor cell line

3.1. Using an inverted phase microscope, determine the percentage of fibroblast contamination in the early passage culture.

NOTE: Fibroblasts are generally long and irregular and have an ill-defined cellular membrane. They appear thin or flat on a Z scale.

3.1.1. If fibroblast contamination is greater than 20% of the cells in the early passage culture, continue culturing after replacing the complete medium with reduced-serum medium.

3.1.2. Repeat the replacement with reduced-serum medium in step 3.1.1 twice per week for 3–4 weeks.

3.1.3. When the culture reaches 80% confluency, passage the cells by splitting 50:50 into 2 new wells of a 6-well plate. Continue passaging 50:50 in reduced-serum media once the cells reach 80% confluency.

3.1.4. Repeat step 3.1.3 for up to 4 weeks, passaging in larger culture flasks as needed by splitting 50:50 once the culture reaches 80% confluency. If the fibroblast population is not reduced to 10% or less, continue to step 3.2 to attempt the differential trypsinization method to remove fibroblast contamination. Otherwise, proceed to step 3.3.

3.2. To enrich for tumor cells, gently rinse the well with 1x PBS to remove the medium containing serum.

3.2.1. Add trypsin as follows: 1 mL per well if in a 6-well plate, 2 mL for a T25 flask, 3 mL for a T75 flask.

3.2.2. Place the 6-well plate or flask in an incubator at 37 °C for 1 min. Remove the plate or flask from the incubator and check the adhesion of the cells using an inverted microscope. If the cells are partially lifting or floating, gently remove the suspended cells and trypsin only. Place the cells in a 15 mL conical tube containing an equal or greater volume of complete tumor medium.

NOTE: This partial collection of cells based on adhesion properties is the first of multiple fractions.

3.2.3. Repeat steps 3.2.1 and 3.2.2 until all the cells are lifted, or there are 4 fractions removed.

3.2.4. Centrifuge all independent fractions at 500 × *g* for 5 min at room temperature.

3.2.5. Aspirate the supernatant as described in step 2.7, and resuspend the cells using 5 mL of sterile, reduced-serum medium.

3.2.6. Plate the cells in a T25 flask for each fraction and place the flasks in an incubator at 37 °C.

3.2.7. Assess the culture after 48 h to determine which fraction is enriched for tumor cells versus

fibroblasts. Discard fibroblast-rich flasks. If fibroblast contamination is <10%, continue expansion in complete tumor medium and proceed to step 4. If fibroblast contamination is 10–30%, repeat steps 3.1.2–3.1.5. If fibroblast contamination is greater than 30%, repeat steps 3.2–3.2.7.

3.3. If few or no fibroblasts are detected within the early passage culture, continue passaging the cells in complete tumor medium by splitting 50:50 once the cells are 80% confluent in their 6-well plate or flask.

4. Expansion of early passage primary tumor cell line

4.1. Once the primary tumor culture contains 10% or less of fibroblast contamination, aspirate the medium and replace it with sterile complete tumor medium twice per week.

NOTE: The optimal medium volume for a T25 is 5 mL; T75 is 12 mL; T150 is 25 mL.

4.1.1. Passage the culture 50:50 into larger flasks as needed once it reaches 80% confluency in the plate or flask.

NOTE: The culture may need to be passaged at a higher ratio depending on its growth. Adjust so the culture reaches 80% confluency every 3–5 days.

4.2. Passage until the culture is at passage 4 or above. Once the culture is 80% confluent in a minimum of two T75 flasks, continue to step 5.

5. Characterization and banking of established primary tumor cell line

5.1. **Cryopreserve** one T75 flask as an early-passage freeze. For mycoplasma testing of the remaining T75 flasks, continue to step 5.2.

5.1.1. For cryopreservation of early-passage cells, thaw 1 tube of aliquoted freeze medium prepared in step 1.5.

5.1.2. Collect the cells by trypsinization by first washing the plate with 1x PBS as described in step 3.2 and adding trypsin as described in step 3.2.1.

5.1.3. Place the flask in an incubator at 37 °C for 3 min. Remove the flask from the incubator and check the adhesion of the cells using an inverted microscope. If the cells are lifting or floating, gently remove the suspended cells and trypsin. Place the cells in a 15 mL conical tube containing an equal or greater volume of complete tumor medium.

NOTE: If the cells remain adherent after 3 min, place the flask back in the incubator for an additional 2 min.

5.1.4. Mix the tube well by pipetting gently and remove 20 µL for counting.

5.1.5. Centrifuge the tube at 500 × *g* for 5 min at room temperature.

5.1.6. While the cells are spinning, count using a lab-specific counting protocol such as trypan blue or acridine orange/propidium iodide (AO/PI).

5.1.7. Resuspend the cells following centrifugation in freeze medium with a minimum of 2×10^6 cells/1 mL freeze medium.

5.1.8. Transfer 1 mL of the cell suspension from step 5.1.7 to prelabeled 1.5 mL cryovials.

5.1.9. Place the cryovials in a controlled-rate freeze chamber and transfer them immediately to -80 °C.

5.1.10. Store the cells at -80 °C for up to 1 week before transferring them to liquid nitrogen for long-term storage.

5.2. Split a T75 flask 50:50 for **mycoplasma testing**. Change the medium to antibiotic-free medium prepared in step 1.6.

5.2.1. After 72 h, thaw the mycoplasma detection reagent, substrate, and controls at room temperature for 15 min before testing.

5.2.2. Collect 1 mL of the supernatant from each culture to be tested and place it in a 15 mL conical tube.

5.2.3. Pellet any suspended cells by centrifuging the supernatant at 500 × *g* for 5 min at room temperature.

5.2.4. Load 100 µL of sample supernatants and controls into a white 96-well plate. Add 100 µL of mycoplasma detection reagent to each sample and control.

5.2.5. Incubate for 5 min at room temperature.

5.2.6. Place the plate in a plate reader and read the luminescence (Reading A, i.e., first Reading).

NOTE: The mycoplasma kit manufacturer's protocol indicates keeping the default settings on multifunctional plate readers. The read is set at **Luminescence endpoint** with an **integration time** of **1 s** and **gain** of 135. The plate reader uses an Auto-scale routine to optimize the gain signal for each experiment. The 1 s integration time is standard default. Both settings are used to adjust the intensity of the luminescent signal interpreted by the plate reader and may need to be adjusted depending on the individual plate reader.

5.2.7. Remove the plate from the plate reader and add 100 µL of the mycoplasma detection

substrate to each sample and the controls.

5.2.8. Incubate for 10 min at room temperature.

5.2.9. Place the plate in the plate reader and read the luminescence (Reading B, i.e., second Reading).

5.2.10. To determine mycoplasma positivity, determine the ratio of Reading B to Reading A.

NOTE: Mycoplasma positivity ratios are described in the mycoplasma kit manufacturer's protocol. Readings A and B are acquired with the same settings and simply reflect the reading order.

5.2.10.1. Consider a ratio < 1 to be mycoplasma-negative.

5.2.10.2. Consider a ratio of 1–1.2 to be inconclusive and repeat step 5.2.

5.2.10.3. Consider a ratio > 1.2 to be mycoplasma-positive and monitor this culture; terminate the culture if necessary.

5.3. Flow cytometry characterization of mycoplasma-negative tumor cells

5.3.1. Dislodge the tumor cells using cell-dissociation buffer.

5.3.2. Count the cells and resuspend the cells at 1×10^6/mL in FACS buffer from step 1.7.

5.3.3. Remove 100 µL of cell suspension into individual tubes for surface staining.

5.3.4. Centrifuge at 500 × *g* for 5 min at room temperature.

5.3.5. Aspirate the supernatant and block Fc receptors by incubating the cells in 500 µL of 5% goat serum in FACS buffer at room temperature for 10 min.

5.3.6. Add 2 mL of FACS buffer and centrifuge the suspension at 500 × *g* for 5 min at room temperature.

5.3.7. Aspirate the supernatant and add the surface stain mix of antibodies (**Table 1**) and FACS buffer so that the final volume is 100 µL. Cover the samples and stain them on ice for 30 min.

5.3.8. Repeat step 5.3.6.

5.3.9. Aspirate the supernatant and fix the cells by resuspending in 200 µL of 1% paraformaldehyde (PFA) plus 0.25% EtOH. Incubate at room temperature for 20 min with the samples protected from light.

5.3.10. Repeat step 5.3.6. Resuspend the cells in 200 µL of FACS buffer for acquisition using an appropriate flow cytometer.

NOTE: Mesothelioma tumor cells are expected to be positive for mesothelin and N-cadherin. Some may also express CD90.

5.4. Expand in complete tumor medium and bank as described in steps 4.1 and 5.1, respectively.

REPRESENTATIVE RESULTS:

To determine fibroblast contamination of early-passage cultures, cells are assessed using an inverted phase microscope to identify the frequency of fibroblasts relative to the other adherent cells present. **Figure 1** shows examples of increasing fibroblast contamination of 80% (**Figure 1A**), 50% (**Figure 1B**), and 30% (**Figure 1C**), compared to a culture with no fibroblast contamination (**Figure 1D**). Based on this visual assessment, adjustments are made to the expansion conditions as described above. Once a mycoplasma-free culture with <10% fibroblast contamination is established, flow cytometry is used to determine the purity of the mesothelioma tumor line.

Figure 2A,B show representative flow cytometry surface staining of two primary mesothelioma tumor lines (MESO171 and MESO176) compared to ATCC-established mesothelioma tumor lines (NCI-H2452 and MSTO-211H) with a melanoma tumor line (MEL526) as a negative control. Mesothelioma tumor cells can express mesothelin (**Figure 2A**) and N-cadherin (**Figure 2B**). Detailed information related to the flow cytometry panel used is shown in **Table 1**. The gating strategy is shown in **Supplemental Figure 1**. Of note, CD90 cannot be used as a fibroblast-specific marker as it can also be expressed by mesothelioma tumor cells. The importance of testing the impact of enzymatic detachment on surface protein marker expression is also shown as both trypsin and the protease–collagenase mixture resulted in the loss of surface expression of N-cadherin (**Figure 2C**) while CD90 was not impacted (**Figure 2D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of increasing frequency of fibroblast contamination in early passage cultures and an established tumor line. (**A**) image of early-passage culture containing 80% fibroblast contamination. (**B**) Image of early-passage culture containing 50% fibroblast contamination. (**C**) Image of early-passage culture containing 30% fibroblast contamination. (**D**) Image of an established primary mesothelioma tumor line. Scale bars = 200 µm.

Figure 2: Representative flow cytometry phenotyping of established mesothelioma tumor cell line. (**A** and **B**) Histograms showing the surface expression pattern of mesothelin and N-cadherin on ATCC mesothelioma cell lines, control melanoma tumor line, and primary mesothelioma tumor lines. (**C** and **D**) Impact of trypsin, protease–collagenase mixture, and cell dissociation buffer on N-cadherin and CD90. Abbreviations: Comp-X-A = compensated area of fluorophore X; PE = phycoerythrin; APC = allophycocyanin.

Table 1: Flow cytometry antibodies used to phenotype tumor lines. Abbreviations: PE =

phycoerythrin; APC = allophycocyanin.

Supplemental Figure S1: Gating strategy for phenotypic analysis of tumor lines. Dot plots are shown for each step in the gating strategy leading up to assessing expression of the markers of interest. Any initial gate using forward scatter and side scatter properties is made to identify the cells of interest, followed by a QC gate based upon the time feature. The cells are then subgated to remove any doublets using forward and side scatter properties. The final QC gate is based upon viability, with the dead cells staining positive for the dye. Following the viability gate, subgating can be performed based on the panel.

DISCUSSION:

While this protocol is straightforward, there are a few critical steps that must be closely followed. The early-passage freeze is important to preserve the ability to repeat the tumor-cell enrichment process if initially unsuccessful. The ability to assess fibroblastic contamination by eye to decide the correct splitting and media starvation technique is vital to preventing fibroblast overgrowth in the culture. In addition, the differential trypsinization method requires careful observation of the cells during incubation. The cells may lift off the plastic plate at different rates, and this will vary from cell line to cell line. While learning this process and refining this decision-making step, a portion of the original culture can be preserved in a 6-well plate using reduced-serum starvation medium (RPMI 1640 with 1% Pen/Strep and 1% FBS) until tumor-cell enrichment can be observed.

One important factor noted in validated phenotypic characterization of the mesothelioma tumor lines was the impact of trypsinization and the protease–collagenase mixture on the expression of the mesothelioma tumor cell surface marker, N-cadherin[8]. We observed a loss of the surface marker if cells were detached using these enzymes, which has been previously described[9]. While it has been shown that most surface proteins are not negatively affected by trypsin, it is important to **test each surface marker during flow panel design**[10]. In addition, it is highly recommended to culture or expand the tumor cells until the cells reach a log phase of expansion and have time to reexpress these markers. Using cell dissociation medium allowed the retention of expression of N-cadherin for detection using flow cytometry. Other types of dissociation media may also allow for marker retention; however, individual labs should carefully test these media prior to establishing a flow cytometry panel.

A major limitation to this study is that the success rate of establishing a cell line is only 50%. This could be due to the heterogeneous nature of the tumor microenvironment. Avenues to improve this process could include the use of 3D culture systems, the addition of media supplements to promote tumor cell expansion, improved disaggregation methods, the use of patient-derived xenografts, or plate coating to improve tumor cell adhesion[11]. Indeed, 3D cultures have been shown to be successful in the generation of challenging tumor cell lines such as pancreatic cancer[7].

One avenue of selection that is not included in this protocol is tumor-cell enrichment by cell sorting based on a mesothelioma tumor marker such as mesothelin. As mesothelioma tumor cells

can express the standard fibroblast marker, CD90, positive selection may be a better alternative. The caveat to this method is the low degree of cellularity in early cultures, which would require sorting into a small volume, multiwell plate such as a 384- or 96-well plate. In addition, as this protocol involves the use of collagenase for tissue digestion, it is not known whether this may also impact the surface expression of N-cadherin or mesothelin. While the mechanism of mesothelin expression is relatively unknown, N-cadherin plays an important role in cellular adhesion, and the removal of this protein could negatively impact the establishment of a tumor line[12]. This is currently being assessed.

This method is significant as it allows the generation of an *in vitro* model system to study this rare tumor type. This can include studies identifying novel surface targets of mesothelioma such as mesothelin with CAR-T cells and uncovering tumor-intrinsic properties of resistance to targeted or immunotherapies. In addition, if these cells can be expanded *in vivo* in mouse models, this would also create a source for testing cellular-based and antibody-based therapeutics as well as small molecules. A future direction for this protocol will be testing the ability to expand other subtypes of MPMs such as the sarcomatoid and biphasic subsets.

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DISCLOSURES:

CH is a member of the SAB for Briacell Therapeutics and the Mesothelioma Applied Research Foundation. All other authors have nothing to disclose.

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Figure 1

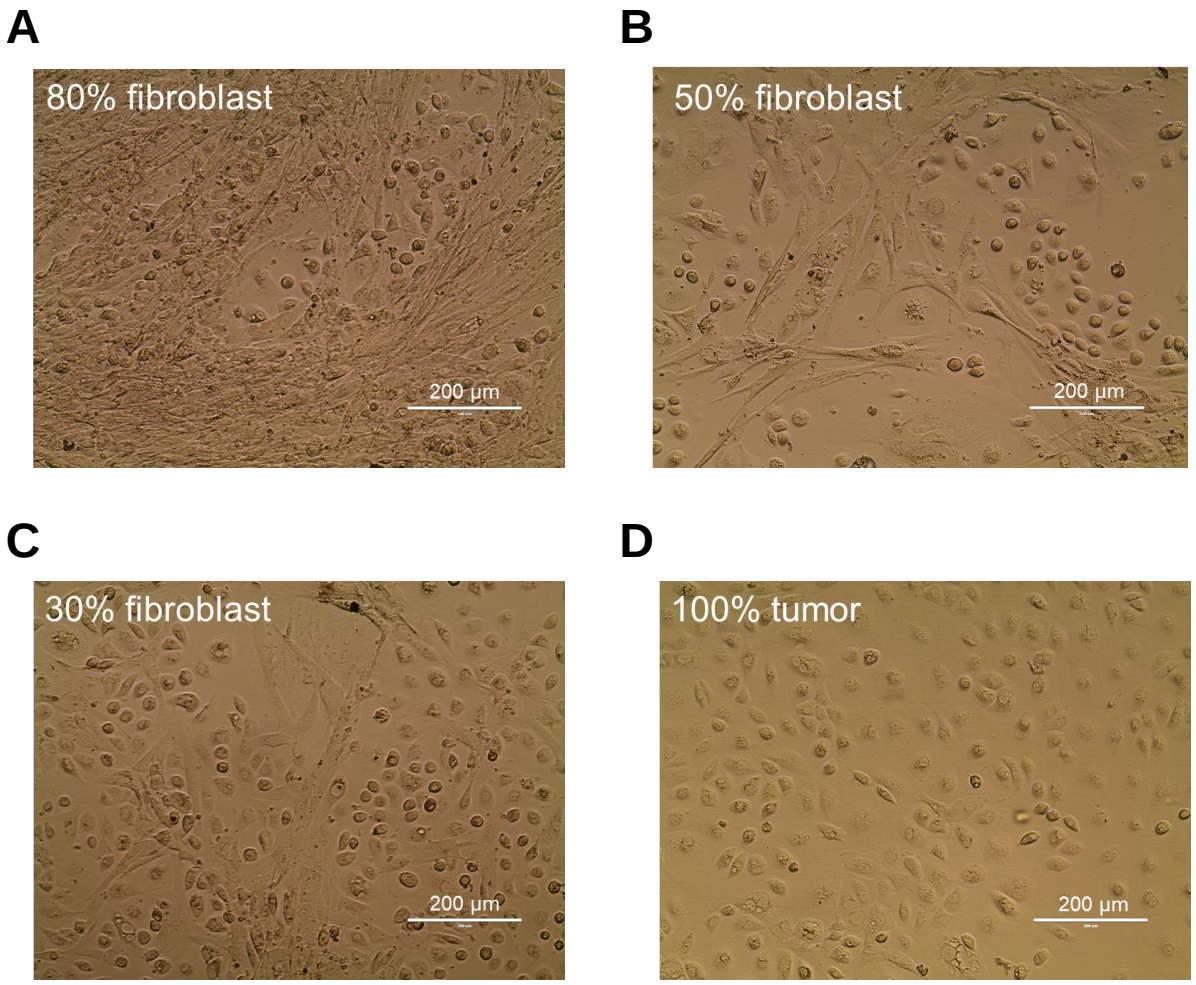

Figure 2

Figure 2

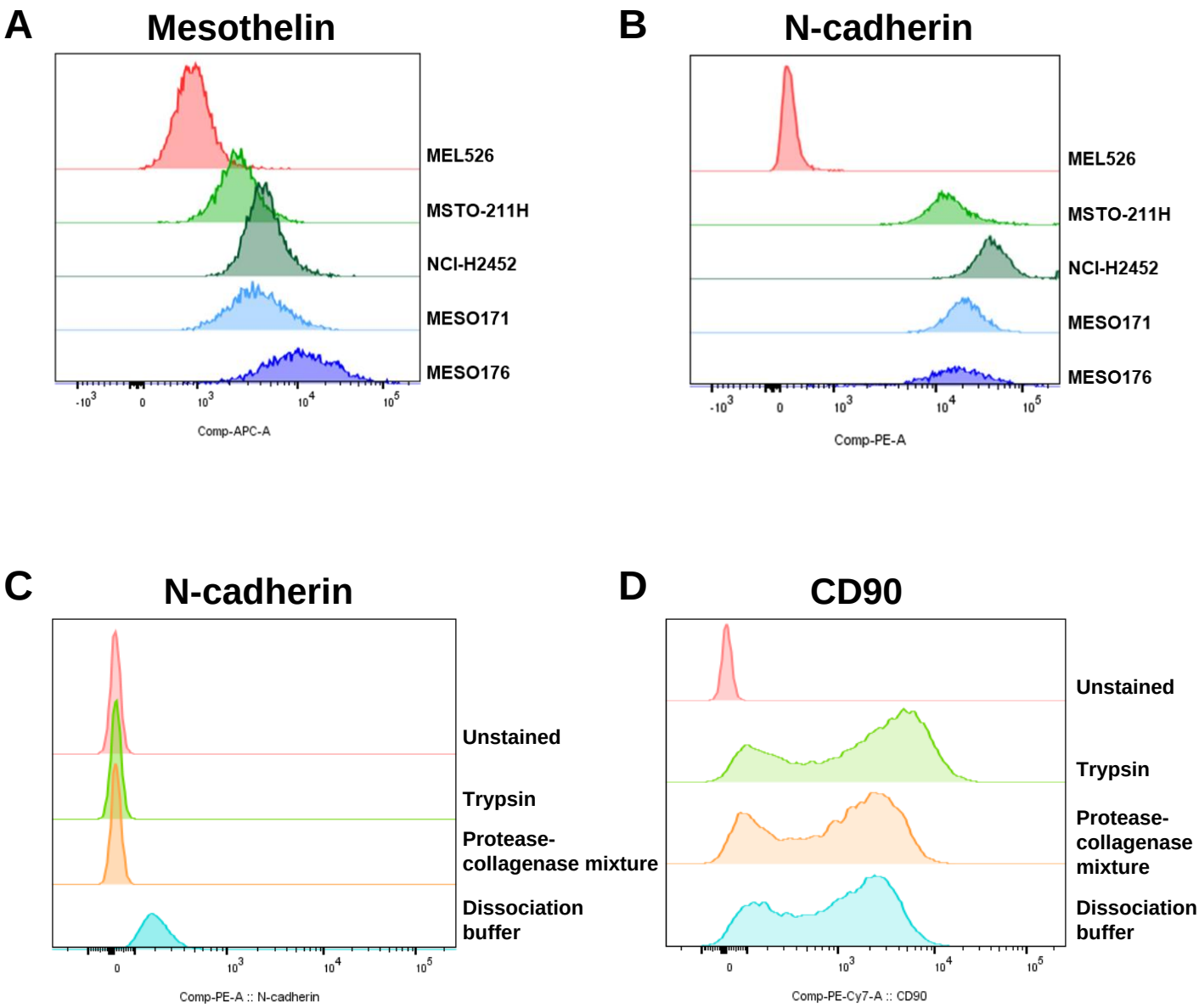

Table 1

Surface Antibody	Fortessa X20 Channel	Laser	Clone	Isotype	Volume/ Sample (µL)
Live/Dead Yellow	BV510	VIOLET	N/A	N/A	1
anti-mesothelin APC	APC	RED	REA1057	IgG1	2
anti-CD325 (N-Cadherin) PE	PE	YG	8C11	IgG1	5
anti-CD90 PE-Cy7	PE-Cy7	YG	5E10	IgG1	5

POINT-BY-POINT RESPONSE

We would like to thank the Editor and Reviewers for the time and feedback. We have addressed the critiques in the point by point below as well as in the manuscript text using track changes. Additions to the Table of Materials have been indicated by yellow highlighting.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed and confirm spelling and grammar issues are fixed if found.

2. Please ensure that abbreviations are defined at first usage.

Thank you. We have reviewed and confirmed.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: MACS C-tube

Thank you for your feedback. We have removed all references to commercial language including trademarks and company names both in the protocol and in Table 1.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Thank you for the feedback. The text has been modified to address the "how".

5. Please add more details to your protocol steps:
Step 1.1.1/1.3.1: Please mention if any particular vacuum pressure was maintained.

This has been updated as a note in the text.

Step 2.2: Please mention from where the tumor tissue was obtained? Collected from patients? Then please mention the exclusion/inclusion criteria of the patients.

Thank you for your comment. This has been clarified in the text to include that type of tissue and that collections are performed following informed consent under a research protocol that is IRB

approved. There is no specific inclusion or exclusion criteria other than surgically resectable cases. This is not a collection as a part of a trial but as a part of standard of care surgery.

Step 2.2.1: How to ensure the complete removal of all fatty tissue, necrotic tissue and/or blood clots?

This has been updated in the text.

Step 2.3: Please include the details of the Heater apparatus in the Table of Materials.

This has been updated in the Table.

Step 2.7/3.2.5: How was the aspiration done? Was a pipette used?

The text has been updated to specify this was performed using an aspirating pipette and the Table of Materials has been updated to include this as well.

Step 3.1.2: Please include the composition of the reduced serum media.

Media composition is described in Step 1.4. We can include a note if needed.

Step 5.2.9: Please mention the instrumental parameters during the luminescence assay.

This information has been added as a Note for the associated step starting the section at 5.2.6.

Step 5.2.10: Please include citations for the microplasma positivity ratios.

A note has been added to this step describing the ratios.

6. Please include a paragraph on the limitations of the method in the Discussion section.

Thank you for the feedback. We have added this text to the Discussion section.

7. Table 1: Please remove the company name and catalog numbers from this table. Please include such details in the Table of Materials.

These details have been removed from Table 1 and included in the Table of Materials

8. Please do not abbreviate the journal names in the References.

We have added the references using Jove's style in Endnote. We are happy to adjust the style if there is a different version that would be preferred.

Reviewers' comments:
Reviewer #1:

Thank you for taking the time to review our protocol and provide the critique below. We hope we have addressed your concerns adequately.

Major Concerns:
1. Referring to the x-axis scale in Figure 2B and 2C, signal intensities for N-cadherin in mesothelioma cells are largely different. Is that due to the different experimental conditions?

The reviewer is correct that the signal intensities shown in the representative data are different. This is indeed due to a different set of experimental conditions. The data shown in 2B was performed just prior to our lab COVID shutdown. The data generated for 2C was post our re-opening and so performed with a different control cell passage, different flow set-up conditions, and antibody lot. We decided to include these data in spite of these differences as it is intended to reflect a different point as it relates to N-cadherin expression and the impact of proteolytic cleavage due to various techniques in cell dissociation/detachment.

2. Experiments for Figure 2C and 2D were done using 100% malignant mesothelioma cells? In this case, is the biphasic distribution of CD90 mean that obtained primary cells comprise two different subpopulations in terms of CD90 expression status?

Thank you for the question. We actually do not yet know if this is due to the multiple subtypes within the culture that have differential expression of CD90. As we build our meso tumor bank, we are moving towards a deeper profiling including single cell RNAseq and single cell ATAC to compare to the original, pre-expansion state of the tumor. For the purposes of this protocol, we have not focused on heterogeneity.

Minor Concerns:
In the "representative results" section, figure numbers mentioned in the text does not match the figures.

Thank you for catching this. We have revised the text accordingly.

Reviewer #2:

Thank you for taking the time to review our protocol.

Supplemental Figure S1 – Flow cytometry gating strategy