**Submission ID #: 68367**
**Scriptwriter Name: Sulakshana Karkala**
**Project Page Link: https://review.jove.com/account/file-uploader?src=20851983**

# Title: Isolation, Culture, and Characterization of Prostate Cancer-Associated Fibroblasts

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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? **Yes, all done**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location: MBC, Via Nizza 52**
Will the filming need to take place in multiple locations? **NO**

**Current Protocol Length**
Number of Steps: 24
Number of Shots: 54

# Introduction

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

1.1. **Valeria Poli:** We aim to understand the mechanisms via which Cancer Associated Fibroblasts contribute to tumor progression in Prostate Cancer, with the goal to neutralize this cross-talk as a therapeutic strategy.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.2*

What are the most recent developments in your field of research?

1.2. **Valeria Poli:** Distinct populations of CAFs with different properties can be assessed in 3-D organoids obtained using tumor and stromal cells, upon various manipulations. Our protocol allows to reliably generate CAFs for these purposes.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.1*

What are the current experimental challenges?

1.3. **Somayeh Mirzaaghaei:** Main challenges are the reliable isolation of tumor and normal cells from the same patient, microenvironment heterogeneity, absence of specific CAF markers, CAFs plasticity, and limited in vitro models recapitulating TME complexity.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.2*

What significant findings have you established in your field?

1.4. **Valeria Poli:** With similar methods we characterized a fundamental pro-tumoral role of the transcription factor STAT3 and its target genes in murine breast cancer CAFs, demonstrating their efficacy as therapeutic targets.

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

What advantage does your protocol offer compared to other techniques?

1.5. **Somayeh Mirzaaghaei:** An optimized isolation of fibroblasts from both the tumor and the adjacent normal regions of radical prostatectomy specimens obtained from prostate cancer (PCa) patients, using small amounts of tissue.

1.5.1. 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.***

**Testimonial Questions (OPTIONAL):**

***Videographer:***

- ***Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.***
- ***Also, kindly note that testimonial statements will be presented live by the authors, offering their spontaneous perspectives.***

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Valeria Poli, Professor, University of Turin** : (authors will present their testimonial statements live)

**Ethics Title Card**

This research has been approved by the Bioethical Committee of the Città della Salute Molinette Hospital

# Protocol

## 2. Isolation of Fibroblasts from Human Prostate Gland

**Demonstrator:** Somayeh Mirzaaghaei

~~2.1. To begin, place the prostate gland under a biosafety hood on a sterile drape **[1].** Orient it using anatomical landmarks **[2].**~~

~~2.1.1. WIDE: Talent placing the prostate gland on a sterile drape under the biosafety hood.~~

~~2.1.2. Talent orienting it anatomically.~~

~~2.2. With a sterile blade, excise tissue slices from areas identified in the preoperative biopsy report as neoplastic or tumor-free **[1].** Prepare 2.5-micrometer-thick cryostatic sections from both tissue samples **[2].** Then stain them with hematoxylin and eosin to confirm the presence or absence of tumor cells **[3].**~~

~~2.2.1. Talent using a sterile blade to excise specific tissue slices from marked regions.~~

~~2.2.2. Shot of prepared thin cryostatic sections.~~

~~2.2.3. Talent applying hematoxylin and eosin stain over the sections.~~

**NOTE: Steps 2.1 and 2.2 were deleted by authors**

2.3. To begin, weigh the tissue samples on a balance on Day 1 for fibroblast isolation **[1]**. Using sterile forceps, transfer the tissue into 6-centimeter dishes **[2].**

2.3.1. Talent placing tissue samples on a digital scale in a 1.5 mL tube and recording the weight.

2.3.2. Shot of tissue being transferred into the PBS containing 6 cm dish.

2.4. Wash the tissue twice in 4 milliliters of ice-cold PBS **[1]** and twice in 4 milliliters of ice-cold complete DMEM **[2-TXT].**

2.4.1. Talent adding ice-cold PBS to the dish with tissue.

2.4.2. Shot of the tissue being transferred into a dish with complete DMEM. **TXT: PBS and DMEM contain 200 U/mL Penicillin, 200 µg/mL Streptomycin (2x)**

2.5. Now transfer the tissue into a 6-centimeter plate on ice **[1].** Add 1 milliliter of antibiotic-supplemented DMEM to the plate **[2-TXT].** Then use scissors or blades to mince the tissue into fragments smaller than 1 square millimeter **[3]**.

2.5.1. Shot of the tissue being transferred into a 6 cm dish placed on ice.

2.5.2. Talent pipettes 1 mL supplemented DMEM. **TXT: DMEM supplemented with 100 U/mL penicillin-G and 100 µg/mL streptomycin**

2.5.3. Talent mincing the tissue into small fragments in cold medium on ice.

2.6. Transfer the minced tissue to a 15-milliliter conical tube containing 5 milliliters of ice-cold complete DMEM **[1].** Then centrifuge the suspension at 754 *g* for 5 minutes at 4 degrees Celsius **[2].**

2.6.1. Talent transferring minced tissue to a conical tube.

2.6.2. Talent placing the tube in a centrifuge.

2.7. Using a 10-milliliter pipette, remove the supernatant **[1].** Resuspend the pellet in 5 milliliters of ice-cold complete DMEM and centrifuge again **[2]**.

2.7.1. Talent pipetting out the supernatant.

2.7.2. Talent adding 5 mL ice-cold complete DMEM to the pellet.

2.8. After removing the supernatant, resuspend the pellet in Collagenase II solution **[1-TXT]**. Transfer the suspension into 1.5-milliliter microtubes **[2]**.

2.8.1. Shot of the pellet being resuspended in Collagenase II solution. **TXT: 1 mL/100 mg of tissue of Collagenase II (1mg/mL in DMEM)**

2.8.2. Talent transferring collagenase-suspended sample into microtubes.

2.9. Seal the microtubes with paraffin film **[1].** Then place them at 37 degrees Celsius for overnight digestion with continuous rocking for 8 to 12 hours **[2]**.

2.9.1. Talent sealing tubes with paraffin film.

2.9.2. Talent placing the sealed tubes in a rocking heat block.

2.10. The next day, transfer the digested samples to 15-milliliter conical tubes **[1].** Pipette 5 milliliters of ice-cold complete DMEM to inactivate collagenase **[2].** Then centrifuge the suspension at 754 *g* for 5 minutes at 4 degrees Celsius **[3]**.

2.10.1. Talent transferring digested samples into 15 mL conical tubes.

2.10.2. Talent pipetting 5 mL ice cold DMEM to the tubes.

2.10.3. Talent placing the tubes in a centrifuge.

2.11. Resuspend the pellet in 1 milliliter of 0.05 percent Trypsin-EDTA after pipetting out the supernatant **[1]**. Incubate for 5 minutes at 37 degrees Celsius with occasional shaking **[2]**.

2.11.1. Talent resuspending the cell pellet in Trypsin-EDTA.

2.11.2. Shot of the tube being placed in an incubator.

2.12. Next, pipette 1 milliliter of freshly prepared DNase I solution to the samples and mix well **[1-TXT]**. After centrifuging and removing the supernatant, resuspend the pellet in 5 milliliters of cold complete DMEM and centrifuge again **[2].**

2.12.1. Talent pipetting DNase I into the sample and mixing thoroughly. **TXT: DNase I:**

**25 mg /mL solution in PBS**

2.12.2. Shot of the pellet being resuspended in cold DMEM.

2.13. Resuspend the resulting cells in complete DMEM supplemented with 20 percent fetal bovine serum **[1]**. Plate the cells and incubate at 37 degrees Celsius with 5 percent carbon dioxide and 95 percent humidity for at least 3 days **[2]**.

2.13.1. Talent adding complete FBS supplemented DMEM to the pellet.

**2.13.2.** Talent plating resuspended cells in dishes and placing them in an incubator.
**AUTHOR'S NOTE: Shot split into 2.**
**2.11.2 A) resuspending and plating**

**2.11.2 B) placing in the incubator**

2.14. After 3 days, examine the cells for morphology and viability **[1-TXT].** Once the cells reach confluency **[2]**, passage them into a 10-centimeter dish containing complete DMEM with 20 percent fetal bovine serum **[3]**.

2.14.1. Talent inspecting cell cultures and replacing part of the medium. **TXT: replace two-thirds of the medium to retain cell-secreted growth factors**

2.14.2. LAB MEDIA: Normal-fibroblasts.tif and Cancer-associated-fibroblats.tif
*Video Editor: Please place both images side by side and label as "Normal Fibroblasts" and " Cancer-associated Fibroblasts", respectively*

2.14.3. Talent transferring confluent fibroblasts into a larger culture dish.

## 3. Anchorage-Independent Growth by Soft Agar Assay

**Demonstrator:** Chiara Verrengia

3.1. Plate cancer-associated fibroblasts or normal fibroblasts at 70 percent confluency in 12-well plates **[1]**.

3.1.1. Talent seeding fibroblasts into 12-well plates.

3.2. On the following day, add 0.45 grams of low melting point agar to 12.5 milliliters of PBS in a 50-milliliter conical tube under sterile conditions **[1-TXT]**. Dissolve the mixture using a microwave oven **[2]**.

3.2.1. Talent weighing agar into a 50-milliliter conical tube and adding phosphate-buffered saline to it inside a biosafety cabinet. **TXT: 4x soft agar stock**
**AUTHOR'S NOTE: Shot split into 2.**
3.2.1A) (weighing),

3.2.1B) (adding PBS)

3.2.2. Talent heating the conical tube in a microwave until agar is completely dissolved.

3.3. Cool the agar solution down to 37 degrees Celsius **[1].** Then dilute it in a one to four ratio with prewarmed complete DMEM to prepare a working solution **[2]**.

3.3.1. Shot of cooled agar solution.

3.3.2. Talent mixing the soft agar solution with warm culture medium to obtain the working concentration.

3.4. Aspirate the medium from the 12-well plate **[1].** Pipette 500 microliters of the working agar solution into each well **[2]**.

3.4.1. Talent removing medium from wells.

3.4.2. Talent carefully adding 500 µL of working agar solution into each well.

3.5. Incubate for 20 minutes at 4 degrees Celsius to allow for agar solidification **[1]**. Meanwhile, keep the remaining agar solution at 37 degrees Celsius **[2]**.

3.5.1. Talent placing the plate in a refrigerator to solidify the bottom agar layer.

3.5.2. Talent storing the remaining agar mixture in a 37 degrees Celsius water bath.

3.6. Trypsinize the tumor cells and prepare a cell suspension of 20000 cells per milliliter.

3.6.1. Talent preparing the tumor cell suspension.

3.7. Mix equal volumes of the tumor cell suspension and the agar solution to obtain a final volume of 7 milliliters, accounting for three technical replicates per each condition **[1].** Pipette up and down multiple times to ensure even mixing **[2].**
**AUTHOR'S NOTE: Both shots have been combined in 1**

3.7.1. Talent combining tumor cells and agar.

3.7.2. Shot of the mixture being pipetted up and down multiple times.

3.8. Then dispense 500 microliters of this mixture, containing approximately 5000 cells, on top of the solidified base layer in each well **[1]**. After letting the agar solidify for 20 minutes at 4 degrees Celsius **[2],** add 1 milliliter of complete DMEM to each well **[3].**

3.8.1. Talent plating 500 µL of the mixture over the base layer.

3.8.2. Shot of solidified agar in the plate.

3.8.3. Talent pipetting 1 mL cDMEM medium into each well.

3.9. Change the medium every other day by aspirating gently from the edge of the well and adding fresh medium to the center **[1]**. Incubate until colonies become easily visible **[2]**.

3.9.1. Talent removing and replacing medium.

3.9.2. SCOPE: 68367_Poli_SCOPE-shot-3.9.2.mp4. 00:00-00:06

3.10. Once colonies are visible, discard the culture medium **[1].** Then stain the colonies with 200 microliters of nitroblue tetrazolium chloride solution**[2-TXT]**. Incubate overnight at 37 degrees Celsius in a humidified incubator **[3]**.

3.10.1. Talent removing old medium from the wells.

3.10.2. Talent adding 200 µL of nitroblue stain to each well. **TXT: Nitroblue tetrazolium chloride solution: 1 mg/mL in PBS**

3.10.3. Talent placing the plate in the incubator.

3.11. The next day, discard the staining solution **[1].** Acquire images of stained colonies using a stereomicroscope **[2]**.

3.11.1. Talent pipetting out the staining solution.

3.11.2. Shot of the plate being placed under a stereomicroscope.

# Results

## 4. Results

4.1. Pure fibroblasts were successfully isolated **[1].** In many cases, particularly at early passages, cancer-associated fibroblasts displayed a more spindle-like morphology compared to normal fibroblasts **[2].**

4.1.1. LAB MEDIA: Figure 2A. *Video Editor: Please highlight the last panel corresponding to CD90*

4.1.2. LAB MEDIA: Figure 2B. *Video Editor: Please highlight the image corresponding to CAF*

4.2. Quantitative analysis confirmed that proliferation of DU145 *(D-U-One-Forty-Five)* cells treated with cancer-associated fibroblast conditioned media was significantly higher over 120 hours **[1]** than those treated with normal fibroblast conditioned media or left untreated **[2]**.

4.2.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the steep curve labeled "CAF conCM".*

*4.2.2.* LAB MEDIA: Figure 3B. *Video editor: Highlight the curve labeled "NF conCM"and "DU145".*

4.3. DU145 cells co-cultured directly with cancer-associated fibroblasts and normal fibroblasts also showed increased proliferation over 96 hours **[1]**, relative to the controls **[2]**.

4.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the yellow signal at Time 96h in the "+CAF" column.*

4.3.2. LAB MEDIA: Figure 3C. Video editor: Highlight the yellow signal in the "+NF" and "DU145" columns at Time 96h.

4.4. Corresponding growth curves demonstrated that co-culture with cancer-associated and normal fibroblasts resulted in a similar increase in DU145 proliferation over time **[1]**.

4.4.1. LAB MEDIA: Figure 3D. *Video editor: Highlight the curves labeled "CAF" and "NF".*

4.5. The effect of conditioned media on DU145 proliferation varied across cancer-associated fibroblast and normal fibroblast pairs, with early passage pairs showing significantly stronger effects than later ones **[1]**.

4.5.1. LAB MEDIA: Figure 3E. *Video editor: Highlight the CAF bars at P9 and P11 are higher than the NF bars.*

4.6. In soft agar colony formation assays, both cancer-associated and normal fibroblasts

increased the number and size of DU145 colonies compared to controls, indicating enhanced anchorage-independent growth **[1]**.

4.6.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the colonies in the "CAF" and "NF"*

AND Figure 4A. *Video editor: Highlight the bars for CAF and NF in both size categories on the graphs. (top panel)*

4.7. Detached fibroblast layers formed due to technical issues prevented colony formation in some replicates **[1]**.

4.7.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the three faded wells sequentially from left to right*

4.8. Conditioned media alone did not promote anchorage-independent colony formation in DU145 cells **[1]**.

4.8.1. LAB MEDIA: Figure 4C.

And Figure 4C. *Video editor: Highlight the bars for all groups in the graphs (bottom panel).*

**Pronunciation Guide:**

**1. fibroblast**
**Pronunciation link:** https://www.merriam-webster.com/dictionary/fibroblast YouTube+6Cambridge Dictionary+6Merriam-Webster+6
**IPA:** /ˈfaɪ-brə-ˌblæst/
**Phonetic Spelling:** FYE-bruh-blast

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**2. prostatectomy**
**Pronunciation link:** https://www.merriam-webster.com/dictionary/prostatectomy Merriam-Webster
**IPA:** /ˌprɑs-tə-ˈtɛk-tə-mi/
**Phonetic Spelling:** pros-tuh-TEK-tuh-mee

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**3. chemo**
**Pronunciation link:** https://www.merriam-webster.com/dictionary/chemo Merriam-Webster
**IPA:** /ˈki-moʊ/
**Phonetic Spelling:** KEE-moh

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**4. informatics**
**Pronunciation link:** https://www.merriam-webster.com/dictionary/informatics Merriam-Webster
**IPA:** /ˌɪn-fər-ˈmæt-ɪks/
**Phonetic Spelling:** in-fer-MAT-iks