

Submission ID #: 69548

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Project Page Link: https://review.jove.com/files_upload.php?src=21212188

Title: Establishment of Tumor Organoids, Carcinoma-Associated Fibroblasts, and Counterpart Fibroblasts from the Same Esophageal Cancer Patient

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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. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 11

Number of Shots: 42

Interviews

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

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Tetsuo Kobayashi**: This research establishes patient-matched ESCC organoids, CAFs, and CFs to study tumor–stromal interactions while eliminating inter-patient variability.

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

Describe the research gap or limitation in existing methods and how this protocol addresses them.

- 1.2. **Motomi Nasu**: Conventional ESCC studies rely on long-cultured cell lines and mismatched CAFs; this protocol overcomes variability using matched patient-derived cells.

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

CONCLUSION:

What does this protocol allow researchers to study or measure?

- 1.3. **Tetsuo Kobayashi**: The protocol enables evaluation of how CAFs influence organoid growth, morphology, and drug responsiveness.

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

Describe the most important consideration or challenge when performing this protocol.

- 1.4. **Tetsuo Kobayashi**: Maintaining tissue freshness and proper mincing is critical for reproducible organoid formation and fibroblast outgrowth in this protocol.

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

How can future studies build on this work? Describe the questions or research directions that can be investigated next.

- 1.5. **Akira Orimo:** Future studies can identify CAF-specific mechanisms, signaling pathways, and therapeutic vulnerabilities using patient-matched ESCC tumor–stroma models.
 - 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.

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) or equivalent body at the Juntendo University Faculty of Medicine

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Passaging Esophageal Squamous Cell Carcinoma (ESCC) Organoids

Demonstrator: Peining Zhang

- 2.1. To begin, obtain established tumor-derived ESCC (*E-S-C-C*) organoids grown in 3D Basement Membrane Matrix domes [1-TXT]. Aspirate the old medium [2]. Use 1 milliliter of PBS (*P-B-S*) to wash each well [3]. Add 0.5 milliliters of pre-warmed trypsin for organoid to each well [4]. Use the edge of a P1000 (*P-1000*) tip to disrupt the Basement Membrane Matrix dome, then pipette vigorously [5].
 - 2.1.1. WIDE: Show the experimental set up and then zoom in to show the plate with the established organoids. **TXT: ESCC: Esophageal squamous cell carcinoma**
 - 2.1.2. Talent aspirating the old medium from each well.
 - 2.1.3. Talent washing the well with phosphate-buffered saline.
 - 2.1.4. Talent adding pre-warmed trypsin for organoid to the well.
 - 2.1.5. Talent disrupting the Basement Membrane Matrix dome with the edge of the P1000 tip and pipetting vigorously.
- 2.2. Incubate for 10 to 15 minutes in a carbon dioxide incubator [1]. Collect the suspension into a low-attachment 15 milliliter tube [2]. Add 0.5 milliliters of DMEM (*D-M-E-M*) to rinse the well 4 times [3]. Transfer all rinses to the same low-attachment tube [4].
 - 2.2.1. Talent placing the sample in the carbon dioxide incubator.
 - 2.2.2. Talent collecting the organoid suspension into a low-attachment 15 milliliter tube.
 - 2.2.3. Talent rinsing the well with DMEM and 10 percent FBS.
 - 2.2.4. Talent transferring all rinses into the same low-attachment 15 milliliter tube.
- 2.3. Centrifuge the tube at 300 *g* for 5 minutes [1]. Remove the supernatant [2]. Resuspend the pellet in minimal DMEM with 10 percent FBS (*F-B-S*) [3]. Adjust the suspension volume to 80 microliters [4].
 - 2.3.1. Talent placing the low-attachment 15 milliliter tube in the centrifuge.
 - 2.3.2. Talent removing the supernatant from the tube.

2.3.3. Talent resuspending the pellet in minimal DMEM and 10 percent FBS.

2.3.4. Talent adjusting the suspension volume to 80 microliters.

2.4. Mix 80 microliters of the organoid suspension with 240 microliters of ice-cold Basement Membrane Matrix at a 1 to 3 ratio [1]. Plate the mixture onto a pre-warmed 6-well plate at 37 degrees Celsius [2]. Dispense 4 to 6 domes per well [3]. Allow the domes to solidify for 15 minutes [4].

2.4.1. Talent mixing the organoid suspension with ice-cold Basement Membrane Matrix.

2.4.2. Talent plating the mixture onto the pre-warmed 6-well plate.

2.4.3. Talent dispensing 4 to 6 domes into the well.

2.4.4. Talent placing the plate down for dome solidification.

2.5. Add 3 milliliters of Eso_v1 (*EE-soh vee-one*) medium per well and keep the plate in a carbon dioxide incubator [1]. Remove the spent medium and replace it with fresh medium every 3 to 5 days [2].

2.5.1. Talent adding Eso_v1 medium to the well.

2.5.2. Shot of the talent removing the spent medium and then adding fresh medium.

3. Cryopreservation of ESCC Organoids

3.1. Collect organoids for cryopreservation when they reach a diameter of 100 to 200 micrometers or when dome crowding exceeds 90 percent [1]. Add 3 milliliters of PBS to wash each well [2]. Add 1.5 milliliters of trypsin to each well [3]. Pipette vigorously [4]. Incubate for 10 minutes in a carbon dioxide incubator [5].

3.1.1. Talent setting the plate containing organoids on a clean sterile surface and preparing for the experiment.

3.1.2. Shot of the talent adding 3ml PBS and washing the well.

3.1.3. Talent adding trypsin for organoid to the well.

3.1.4. Talent pipetting the organoid culture vigorously.

3.1.5. Talent placing the sample for incubation.

3.2. Collect the entire suspension into a low-attachment 15 milliliter tube [1]. Use DMEM supplemented with 10 percent FBS to rinse the well 4 times [2]. Centrifuge the tube at 300 g for 5 minutes [3]. Remove the supernatant [4].

3.2.1. Talent collecting the entire suspension into a low-attachment 15 milliliter tube.

3.2.2. Talent rinsing the well 4 times.

3.2.3. Talent placing the low-attachment 15 milliliter tube in the centrifuge.

3.2.4. Talent removing the supernatant from the tube.

3.3. Resuspend the pellet in 2 milliliters of 90 percent FBS and 10 percent dimethyl sulfoxide [1]. Aliquot 0.5 milliliters into each cryovial [2]. Place the vials in a freezing container and store it at minus 80 degrees Celsius [3]. Transfer the vials to liquid nitrogen for long-term storage [4].

3.3.1. Talent resuspending the pellet in 90% FBS and 10% dimethyl sulfoxide.

3.3.2. Talent aliquoting 0.5 mL suspension into each cryovial.

3.3.3. Talent placing the cryovials in the freezing container and transferring them to a -80 °C freezer.

3.3.4. Talent transferring the cryovials to liquid nitrogen storage.

4. Recovery of Cryopreserved ESCC Organoids

4.1. Thaw the cryovial rapidly in a 37 degrees Celsius water bath [1]. Transfer the contents into 5 milliliters of DMEM and 10 percent FBS in a 15-milliliter tube [2]. Centrifuge the tube at 300 g for 5 minutes [3].

4.1.1. Talent thawing the cryovial in a 37 degrees Celsius water bath.

4.1.2. Talent transferring the thawed contents into a 15-mL tube containing DMEM and 10% FBS.

4.1.3. Talent placing the 15-mL tube in the centrifuge.

4.2. Remove the supernatant [1]. Resuspend the pellet in 40 to 60 microliters of DMEM and 10 percent FBS [2].

4.2.1. Talent removing the supernatant from the tube.

4.2.2. Talent resuspending the pellet in DMEM and 10% FBS.

4.3. Mix the suspension with ice-cold Basement Membrane Matrix at a 1 to 3 ratio [1]. Plate the mixture onto a pre-warmed 6-well plate at 37 degrees Celsius [2]. Dispense 4 to 6 domes per well [3]. Allow the domes to solidify for 15 minutes [4]. Add 3 milliliters of Eso_v1 medium per well [5].

4.3.1. Talent mixing the suspension with ice-cold Basement Membrane Matrix.

4.3.2. Talent plating the mixture onto the pre-warmed 6-well plate.

4.3.3. Talent dispensing 4 to 6 domes into the well.

4.3.4. Talent placing the plate down for dome solidification.

4.3.5. Talent adding Eso_v1 medium to the well.

Results

5. Results

5.1. Spheroid numbers were quantified over time for early-passaged cancer organoids seeded with 1,000 initial cancer cells [1], 5,000 initial cancer cells [2], or 10,000 initial cancer cells [3].

5.1.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the line labelled 1000 cells.*

5.1.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the line labelled 5000 cells.*

5.1.3. LAB MEDIA: Figure 2C. *Video editor: Highlight the line labelled 10000 cells*

5.2. Most cancer organoids maintained a well-defined spherical morphology during growth [1].

5.2.1. LAB MEDIA: Figure 2D. *Video editor: Zoom in on the single round structure in the center of the image*

5.3. After recovery from cryopreserved tubes, numerous small cell aggregates were observed at day 0 [1], and many spheroids grew after 2 days [2].

5.3.1. LAB MEDIA: Figure 2E. *Video editor: Show the panel labeled "Day 0" and emphasize the many small, scattered circles*

5.3.2. LAB MEDIA: Figure 2E. *Video editor: Show the panel labeled "Day 2" and point to the larger round spheroids*

5.4. Prolonged passaging beyond passage 15 resulted in the emergence of morphologically irregular organoids [1].

5.4.1. LAB MEDIA: Figure 2F. *Video editor: Point to the organoids marked by arrows showing irregular shapes*

Pronunciation Guides

1. Esophageal

Pronunciation link: <https://www.merriam-webster.com/dictionary/esophageal>

IPA: /ɪˌsɑːfəˈdʒiːəl/

Phonetic Spelling: ih·saa·fuh·jee·uhl

2. Squamous

- Pronunciation link: <https://www.merriam-webster.com/dictionary/squamous>
IPA: /'skweɪməs/
Phonetic Spelling: skway·muhs
3. Carcinoma
Pronunciation link: <https://www.merriam-webster.com/dictionary/carcinoma>
IPA: /ˌkɑːrsə'noʊmə/
Phonetic Spelling: kar·suh·noh·muh
4. Organoids
Pronunciation link: <https://www.merriam-webster.com/dictionary/organoid>
IPA: /'ɔːrgəˌnoɪd/
Phonetic Spelling: or·guh·noyd
5. Basement
Pronunciation link: <https://www.merriam-webster.com/dictionary/basement>
IPA: /'beɪsmənt/
Phonetic Spelling: bays·muhnt
6. Membrane
Pronunciation link: <https://www.merriam-webster.com/dictionary/membrane>
IPA: /'mɛmˌbreɪn/
Phonetic Spelling: mem·brayn
7. Matrix
Pronunciation link: <https://www.merriam-webster.com/dictionary/matrix>
IPA: /'mɛtrɪks/
Phonetic Spelling: may·triks
8. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /'æspəˌreɪt/
Phonetic Spelling: as·puh·rayt
9. Phosphate-buffered
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>
IPA: /'fɑːsˌfeɪt/
Phonetic Spelling: fos·fayt
10. Saline
Pronunciation link: <https://www.merriam-webster.com/dictionary/saline>
IPA: /'seɪˌliːn/
Phonetic Spelling: say·leen
11. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /'trɪpsɪn/
Phonetic Spelling: trip·sin
12. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪnkjəˌbeɪt/
Phonetic Spelling: in·kyuh·bayt

13. Suspension
Pronunciation link: <https://www.merriam-webster.com/dictionary/suspension>
IPA: /sə'spɛnjən/
Phonetic Spelling: suh·spen·shuhn
14. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sɛntrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
15. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈnɛntənt/
Phonetic Spelling: soo·per·nay·tuhnt
16. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriːsə'spɛnd/
Phonetic Spelling: ree·suh·spend
17. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪkrəʊˌliːtər/
Phonetic Spelling: my·kroh·lee·ter
18. Celsius
Pronunciation link: <https://www.merriam-webster.com/dictionary/Celsius>
IPA: /ˈsɛlsiəs/
Phonetic Spelling: sel·see·uhs
19. Cryopreservation
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryopreservation>
IPA: /ˌkraɪoʊˌprezərˈveɪʃən/
Phonetic Spelling: kry·oh·prez·er·vay·shuhn
20. Diameter
Pronunciation link: <https://www.merriam-webster.com/dictionary/diameter>
IPA: /daɪˈæmətər/
Phonetic Spelling: dy·am·uh·ter
21. Micrometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkraːmətər/
Phonetic Spelling: my·krah·muh·ter
22. Dimethyl sulfoxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>
IPA: /daɪˈmɛθəl sʌlˈfɑːksaɪd/
Phonetic Spelling: dy·meth·uhl sul·fok·syde
23. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

IPA: /'æɪ, kwɑ:t/

Phonetic Spelling: al·ih·kwaht

24. Cryovial

Pronunciation link: No confirmed link found

IPA: /'kraɪəʊ, vaɪəl/

Phonetic Spelling: kry·oh·vy·uhl

25. Nitrogen

Pronunciation link: <https://www.merriam-webster.com/dictionary/nitrogen>

IPA: /'naɪtrədʒən/

Phonetic Spelling: ny·truh·juhn

26. Thaw

Pronunciation link: <https://www.merriam-webster.com/dictionary/thaw>

IPA: /θɔ:/

Phonetic Spelling: thaw

27. Morphology

Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>

IPA: /mɔ:r'fɑ:lədʒi/

Phonetic Spelling: mor·fol·uh·jee

28. Spheroid

Pronunciation link: <https://www.merriam-webster.com/dictionary/spheroid>

IPA: /'sfɪ,rɔɪd/

Phonetic Spelling: sfeer·oyd

29. Quantified

Pronunciation link: <https://www.merriam-webster.com/dictionary/quantify>

IPA: /'kwɑ:ntə,fɑɪd/

Phonetic Spelling: kwahn·tuh·fyde

30. Passaging

Pronunciation link: <https://www.merriam-webster.com/dictionary/passaging>

IPA: /'pæsɪdʒɪŋ/

Phonetic Spelling: pas·ih·jing

31. Emergence

Pronunciation link: <https://www.merriam-webster.com/dictionary/emergence>

IPA: /ɪ'mɜ:dʒəns/

Phonetic Spelling: ih·mur·juhns

32. Morphologically

Pronunciation link: <https://www.merriam-webster.com/dictionary/morphologically>

IPA: /,mɔ:rfə'lɑ:dʒɪkli/

Phonetic Spelling: mor·fuh·loj·ihk·lee