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Title: Standardized Method to Detect Tunneling Nanotubes in Human Skin Cells for Tissue Engineering Applications

Authors and Affiliations:

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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: 15

Number of Shots: 38

Introduction

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

Authors: The answers are slightly edited according to journal guidelines.

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

- 1.1. **Tengfei Feng:** We develop methods to reliably detect and analyze tunneling nanotubes between human skin cells and study their potential role in skin regeneration.

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

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Tengfei Feng:** Our protocol is designed for fixed samples of primary human skin cells. It is useful in research related to skin biology and tissue engineering.

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

CONCLUSION:

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

- 1.3. **Tengfei Feng:** The foremost challenge is preserving the fragile Tunneling Nanotubes. This requires gentle fixation, appropriate timing, and the use of freshly prepared fixatives.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Tengfei Feng:** Using our protocol, the quantity, length, and diameter of the Tunneling Nanotubes can be measured under varying cell conditions to explore their potential functions.

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. **Tengfei Feng:** In the future, we will fluorescently label mitochondria to study their transfer via Tunneling Nanotubes.

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 Ethics Committee at the Ningbo University Affiliated People's Hospital

Protocol

2. Culturing Human Epidermal Keratinocytes and Dermal Fibroblasts

Demonstrator: Tengfei Feng

2.1. To begin, thaw and culture human epidermal keratinocytes and dermal fibroblasts in 60-millimeter dishes containing the appropriate growth medium [1]. Supplement the keratinocyte cultures with Y-27632 to a final concentration of 5 micromolar during resuscitation [2]. Maintain all cultures at 37 degrees Celsius in a humidified incubator until they reach approximately 80 percent confluency [3].

2.1.1. Talent plating cells in two 60 millimeter dishes filled with growth medium.

2.1.2. Talent pipetting Y-27632 into the keratinocyte dish and mixing gently.

2.1.3. Talent placing the cell culture dishes into a humidified incubator set to 37 degrees Celsius.

2.2. Then, remove the medium from the culture dish [1]. Rinse the cells briefly with 1 milliliter of PBS to maintain physiological pH and osmolarity during washing [2]. Aspirate the PBS completely [3].

2.2.1. Talent aspirating the spent medium from the culture dish.

2.2.2. Talent adding 1 milliliter of PBS and gently swirling the dish.

2.2.3. Talent aspirating the PBS from the dish.

2.3. Add 1 milliliter of 0.05 percent trypsin-EDTA and distribute it evenly across the cell surface [1]. Incubate the dish at 37 degrees Celsius for 1.5 to 2.5 minutes [2].

2.3.1. Talent pipetting trypsin-EDTA onto the cells and tilting the dish to spread it evenly.

2.3.2. Talent placing the dish into a 37 degrees Celsius incubator.

2.4. Gently resuspend the cells by pipetting up and down [1]. Add 2 milliliters of growth medium to neutralize the trypsin [2]. Transfer the cell suspension into a 15-milliliter tube [3] and centrifuge at 200 g for 5 minutes at room temperature [4].

2.4.1. Talent pipetting the cells up and down to resuspend.

- 2.4.2. Talent adding 2 milliliters of growth medium into the dish.
- 2.4.3. Talent transferring the cell suspension into a 15 milliliter conical tube.
- 2.4.4. Talent placing the tube in the centrifuge and starting the spin.

- 2.5. After the centrifugation, discard the supernatant from the centrifuged tube [1]. Resuspend the cell pellet in 1 to 2 milliliters of medium [2] and count the cells using an automated counter [3].
 - 2.5.1. Talent pouring off the supernatant from the centrifuged tube.
 - 2.5.2. Talent pipetting medium into the tube to resuspend the cell pellet.
 - 2.5.3. Talent loading the cell suspension into an automated counter and initiating the count.

- 2.6. Next, seed 12,000 dermal fibroblasts or 20,000 keratinocytes in each well of an 8-well chamber slide in a final volume of 400 microliters [1]. After seeding, cross-shake the chamber well to distribute the cells evenly [2] and incubate the cells at 37 degrees Celsius for approximately 24 hours [3].
 - 2.6.1. Talent pipetting either fibroblasts or keratinocytes into each well of the chamber slide.
 - 2.6.2. Talent cross-shaking the chamber slide gently in multiple directions.
 - 2.6.3. Talent placing the chamber slide into the incubator set at 37 degrees Celsius.

- 2.7. Following incubation, confirm if the cell confluency is around 80% under a microscope [1].
 - 2.7.1. Talent placing the sample under the microscope.

3. Fixing the Cells for Staining

- 3.1. When cultures reach 80 percent confluency, gently add several drops of Fixative Solution 1 directly to the chamber well to pre-fix the cells [1]. Incubate the cells at room temperature for 4 minutes, ensuring that the fixative spreads evenly without disturbing the cells [2].
 - 3.1.1. Talent carefully adding drops of Fixative Solution 1 to each chamber well.

- 3.1.2. Talent placing the chamber slide on the bench.
- 3.2. Then, aspirate the pre-fixative from the well [1] and add 140 microliters of fresh Fixative Solution 1, ensuring complete coverage of the well surface [2]. Incubate the slide at 37 degrees Celsius for 15 minutes [3-TXT].
 - 3.2.1. Talent aspirating the pre-fixative from the chamber well using a pipette.
 - 3.2.2. Talent pipetting 140 microliters of fresh Fixative Solution 1 into the well.
 - 3.2.3. Talent placing the chamber slide into an incubator set to 37 degrees Celsius. **TXT: Repeat the fixation steps with Fixative Solution 2**
- 3.3. Next, remove the fixative from the chamber well [1] and add 140 microliters of 100 millimolar ammonium chloride to quench residual aldehydes [2]. Incubate the slide at room temperature for 10 to 30 minutes [3].
 - 3.3.1. Talent aspirating the Fixative Solution 2 from the well.
 - 3.3.2. Talent pipetting 140 microliters of 100 millimolar ammonium chloride into the well.
 - 3.3.3. Talent leaving the slide on the benchtop.
- 3.4. Rinse the samples three times with PBS for 30 seconds each at room temperature [1]. During each rinse, gently pipette the buffer along the chamber wall to avoid washing away cells or damaging tunneling nanotubes [2].
 - 3.4.1. Talent removing the quenching solution.
 - 3.4.2. Talent gently adding PBS along the chamber wall to rinse the cells.

4. Membrane and Cytoskeletal Staining of Fixed Cells

- 4.1. Incubate the cells with 140 microliters of a 1 to 300 volume-to-volume dilution of Wheat Germ Agglutinin–Alexa Fluor conjugate in PBS [1]. Perform the incubation in the dark at room temperature for 20 minutes to label plasma membrane glycoproteins for visualization of membrane-derived tunneling nanotubes [2].
 - 4.1.1. Talent pipetting 140 microliters of the Wheat Germ Agglutinin–Alexa Fluor conjugate solution into each chamber well.

- 4.1.2. Talent covering the slide to protect it from light and placing it on the benchtop.
- 4.2. Then, wash the cells three times with PBS for 30 seconds each [1]. Then, incubate the cells with 140 microliters of phalloidin conjugate diluted 1 to 250 times in 1 percent BSA for 30 minutes in the dark at room temperature [2].
 - 4.2.1. Talent adding the cells with PBS.
 - 4.2.2. Talent placing the foil covered sample on the bench.
- 4.3. After washing the cells three times with PBS, incubate them with 140 microliters of DAPI solution for 5 minutes at room temperature in the dark [1-TXT].
 - 4.3.1. Talent pipetting 140 microliters of DAPI solution into each well and covering the sample with aluminum foil. **TXT: Wash the cells 3x with PBS after 5 min**
- 4.4. Finally, add 2 to 3 drops of mounting medium to each well [1] and incubate the sample at room temperature for 20 minutes [2]. Store the slides at 4 degrees Celsius in the dark for at least 20 minutes until imaging [3].
 - 4.4.1. Talent adding 2 to 3 drops of mounting medium into each well.
 - 4.4.2. Talent placing the sample on the bench.
 - 4.4.3. Talent placing the slide in a storage container and placing it in a dark 4 degrees Celsius refrigerator.

Results

5. Results

5.1. In dermal fibroblast cultures, tunneling nanotubes were observed as long, thin projections connecting adjacent cells, with membranes stained by wheat germ agglutinin and F-actin labeled by phalloidin [1].

5.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight the bottom inset (zoom) panel and focus on thin projections marked with yellow arrowheads in green and red channels.*

5.2. In epidermal keratinocyte cultures, tunneling nanotubes with characteristic F-actin–positive protrusions were also observed bridging neighboring cells [1].

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the zoomed-in region at the bottom and focus the regions marked by yellow arrowheads.*

5.3. In co-cultures of dermal fibroblasts and epidermal keratinocytes, tunneling nanotubes were consistently detected between dermal fibroblasts and epidermal keratinocytes, visualized by membrane and cytokeratin 5 staining [1].

5.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the boxed “inset(zoom)” in panel E and F, showing regions marked with yellow arrowheads.*

5.4. The tunneling nanotubes in the co-cultures exhibited diverse cytoskeletal organization, with examples containing only F-actin [1], both the filaments [2], F-actin with partial alpha-tubulin [3], or with low alpha-tubulin levels [4].

5.4.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the bottom inset zoom panel and focus on regions marked by yellow arrowheads.*

5.4.2. LAB MEDIA: Figure 6B. *Video editor: Highlight the bottom inset zoom panel and focus on regions marked by yellow arrowheads.*

5.4.3. LAB MEDIA: Figure 6C. *Video editor: Highlight the bottom inset zoom panel and focus on regions marked by yellow arrowheads.*

5.4.4. LAB MEDIA: Figure 6D. *Video editor: Highlight the bottom inset zoom panel and focus on regions marked by yellow arrowheads.*

1. Keratinocytes
Pronunciation link: <https://www.merriam-webster.com/dictionary/keratinocyte>
IPA: /kəˈrætɪnəˌsaɪt/
Phonetic Spelling: kuh·ra·tuh·nuh·syɪt
2. Fibroblasts
Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
IPA: /ˈfaɪbroʊˌblæst/
Phonetic Spelling: fy·broh·blast
3. Micromolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/micromolar>
IPA: /ˌmaɪkroʊˈmoʊlər/
Phonetic Spelling: my·kroh·moh·ler
4. Confluency
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluency>
IPA: /kənˈfluːənsi/
Phonetic Spelling: kuhn·floo·uhn·see
5. Humidified
Pronunciation link: <https://www.merriam-webster.com/dictionary/humidified>
IPA: /hjuːˈmɪdɪˌfaɪd/
Phonetic Spelling: hyoo·mi·duh·fyɪd
6. Incubator
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubator>
IPA: /ˈɪŋkjəˌbeɪdər/
Phonetic Spelling: ing·kyuh·bay·der
7. Trypsin-EDTA
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: trip·sin
8. Centrifugation
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>
IPA: /ˌsentrəˈfʃəˌgeɪʃən/
Phonetic Spelling: sen·truh·fyoo·gay·shuhn
9. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·nay·tuhtnt
10. Osmolarity
Pronunciation link: <https://www.merriam-webster.com/dictionary/osmolarity>
IPA: /ˌɑːzmoʊˈlærɪti/
Phonetic Spelling: oz·moh·lar·uh·tee
11. Ammonium chloride
Pronunciation link: <https://www.merriam-webster.com/dictionary/ammonium>
IPA: /əˈmoʊniəm/
Phonetic Spelling: uh·moh·nee·uhm

12. Aldehydes
Pronunciation link: <https://www.merriam-webster.com/dictionary/aldehyde>
IPA: /'ældɪˌhaɪd/
Phonetic Spelling: al·duh·hyd
13. Cytoskeletal
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoskeleton>
IPA: /ˌsaɪtoʊ'skelɪtəl/
Phonetic Spelling: sy·toh·skel·uh·tuhl
14. Agglutinin
Pronunciation link: <https://www.merriam-webster.com/dictionary/agglutinin>
IPA: /ə'gluːtənɪn/
Phonetic Spelling: uh·gloo·tuh·nin
15. Phalloidin
Pronunciation link: <https://www.merriam-webster.com/dictionary/phalloidin>
IPA: /fə'lɔɪdɪn/
Phonetic Spelling: fuh·loy·din
16. Tunneling nanotubes
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanotube>
IPA: /'nænoʊˌtuːb/
Phonetic Spelling: nan·oh·toob
17. Cytokeratin
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytokeratin>
IPA: /ˌsaɪtoʊ'kerətɪn/
Phonetic Spelling: sy·toh·ker·uh·tin
18. Tubulin
Pronunciation link: <https://www.merriam-webster.com/dictionary/tubulin>
IPA: /'tuːbjʊlɪn/
Phonetic Spelling: too·byuh·lin
19. DAPI
Pronunciation link: No confirmed link found
IPA: /'deɪpiːaɪ/
Phonetic Spelling: day·pee·eye
20. Alexa Fluor
Pronunciation link: No confirmed link found
IPA: /ə'leksə'flʊər/
Phonetic Spelling: uh·lek·suh floor