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Title: Generation and Genetic Manipulation of Human Cervical Organoids

Authors and Affiliations:

Yeonhyo Choi¹, Jihyeon Myeong¹, Gun Oh Chong^{2,3,4}, Youngtae Jeong^{1,5,6}

¹Department of New Biology, DGIST

²Department of Obstetrics and Gynecology, Kyungpook National University Chilgok Hospital

³Clinical Omics Institute, Kyungpook National University

⁴Department of Obstetrics and Gynecology, Kyungpook National University School of Medicine

⁵New Biology Research Center, DGIST

⁶Graduate Program in Biomedical Science & Engineering, DGIST

Corresponding Authors:

Youngtae Jeong

jyt@dgist.ac.kr

Jihyeon Myeong

mjh8389@dgist.ac.kr

Email Addresses for All Authors:

Yeonhyo Choi

yeonhyo@dgist.ac.kr

Gun Oh Chong

gochong@knu.ac.kr

Youngtae Jeong

jyt@dgist.ac.kr

Jihyeon Myeong

mjh8389@dgist.ac.kr

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**

If **Yes**, can you record movies/images using your own microscope camera?

No

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

LEICA EZ4

SCOPE shots: 2.3.2, 2.4.1

Videographer: Please film the above-mentioned shots using the scope kit

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: 24

Number of Shots: 56

Introduction

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

INTRODUCTION:

NOTE: There are minor changes in the introduction statements

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

- 1.1. **Youngtae Jeong:** We established a human cervical organoid system to study cervical biology and pathology, including genetically engineered organoids for investigating gene function and disease mechanisms.
 - 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. **Youngtae Jeong:** This protocol can be applied to study human cervical biology, model human cervical diseases, and screen effective drugs. In addition, this protocol can be modified to establish other epithelial organoid systems.
 - 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. **Jihyeon Myeong:** This protocol enables quantitative evaluation of drug and gene function in human cervical stem cells, facilitating mechanistic studies of cervical development and disease.
 - 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. **Jihyeon Myeong:** Peeling or scraping of small pieces of cervical epithelia in the process of single cell dissociation can be technically challenging for beginners.

- 1.4.1. 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?~~

- 1.5. **Jihyeon Myeong**: Histologic analyses such as H&E staining and immunostaining, transcriptomic or proteomic analyses, and functional perturbation assays can be done following this procedure.
 - 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 Internal Review Boards at DGIST and Chilgok
Kyungpook National University Hospital

Protocol

2. Single-Cell Isolation from Ectocervical Tissues

Demonstrator: Yeonhyo Choi

- 2.1. To begin, place the biopsied tissue in a 10-centimeter Petri dish [1-TXT]. Using ice-cold Dulbecco's PBS without calcium and magnesium, wash the tissue once to remove blood and debris [2].
 - 2.1.1. WIDE: Talent placing the biopsied tissue into a 10-centimeter Petri dish on the bench. **TXT: Human ectocervix biopsy sample is used** **NOTE: Please add the onscreen text**
 - 2.1.2. Talent adding ice-cold Dulbecco's PBS to the dish and gently washing the tissue.
- 2.2. Transfer the tissue to a 1.5-milliliter tube containing 1 milliliter of Dispase solution at 2.4 units per milliliter [1]. Place the tube on a tube rotator set to 20 revolutions per minute and incubate for 1 hour at 37 degrees Celsius with gentle agitation [2].
 - 2.2.1. Talent transferring the tissue into a 1.5 milliliter tube containing Dispase solution.
 - 2.2.2. Talent placing the tube onto a tube rotator set to 20 revolutions per minute inside a 37 degrees Celsius incubator.
- 2.3. Then, transfer the sample to a new 10-centimeter Petri dish [1]. Using forceps, carefully separate the white epithelial layer from the rest of the tissue [2].
 - 2.3.1. Talent transferring the sample into a new 10 centimeter Petri dish.
 - 2.3.2. SCOPE: carefully separating the white epithelial layer from the remaining tissue.
- 2.4. Now, with sharp forceps, gently peel off the epithelial surface as an intact sheet [1] and immediately transfer the isolated epithelial layer into a 1.5-milliliter tube kept on ice [2].
 - 2.4.1. SCOPE: peeling the epithelial surface as an intact sheet with forceps.
 - 2.4.2. Talent transferring the isolated epithelial layer into a 1.5-milliliter conical tube placed on ice.
- 2.5. Add 1 milliliter of recombinant trypsin-based dissociation solution to the peeled or scraped sample [1]. Place the 1.5-milliliter conical tube containing cervical epithelium

on a tube rotator set to 20 revolutions per minute [2-TXT].

2.5.1. Talent adding 1 milliliter of recombinant trypsin-based dissociation solution to the sample.

2.5.2. Talent placing the 1.5 liter conical tube onto a tube rotator set to 20 revolutions per minute. **TXT: 5 min; 37 °C; Gentle agitation**

2.6. Add 10 milliliters of freshly prepared ice-cold blocking buffer composed of PBS with 2 percent bovine calf serum to the cell suspension to stop the enzymatic reaction [1]. Place the tube in a centrifuge and spin at 450 g for 5 minutes at 4 degrees Celsius [2].

2.6.1. Talent adding 10 milliliters of ice-cold blocking buffer to the 50 milliliter conical tube containing the cell suspension.

2.6.2. Talent placing the 50 milliliter conical tube into a centrifuge set to 450 g and 4 degrees Celsius.

2.7. After discarding the supernatant, add 1 milliliter of red blood cell lysis buffer to the pellet, followed by 20 microliters of deoxyribonuclease [1-TXT]. Then, resuspend the cell pellet in 200 to 500 microliters of organoid culture medium [2].

2.7.1. Talent adding 1 milliliter of 1 times red blood cell lysis buffer to the cell pellet. **TXT: Incubate at RT (5 min); Wash 2x with cold blocking buffer**

2.7.2. Talent adding organoid culture medium to the pellet and gently pipetting to resuspend the cells.

2.8. Using a hemocytometer and trypan blue solution, count the cell number [1].

2.8.1. Talent mixing the cell suspension with trypan blue and loading it onto a hemocytometer for counting.

3. Ectocervical Organoid Seeding and Culture

3.1. Resuspend 500 to 2,000 viable cells in a final volume of 50 microliters of organoid culture medium in a 1.5-milliliter microcentrifuge tube [1].

3.1.1. Talent pipetting organoid culture medium into a 1.5 milliliter microcentrifuge tube containing the cell pellet and gently resuspending the cells.

- 3.2. Add 50 microliters of basement membrane matrix to the cell suspension [1] and gently mix on ice to avoid bubble formation [2]. Dispense the 100-microliter mixture into the interior of a cell culture insert placed in a 24-well plate [3].
 - 3.2.1. Talent adding 50 microliters of basement membrane matrix to the cell suspension tube kept on ice.
 - 3.2.2. Talent gently mixing the tube on ice, avoiding bubble formation.
 - 3.2.3. Talent dispensing the 100 microliter mixture into the interior of a cell culture insert positioned in a 24 well plate.
- 3.3. Place the plate in a 37 degrees Celsius incubator for 20 to 30 minutes until the basement membrane matrix-cell mixture solidifies [1]. Next, add 400 microliters of organoid culture medium to the well below the insert after the mixture has solidified [2]. Culture the organoids for 1 to 2 weeks until they reach an average diameter of 150 to 250 micrometers [2].
 - 3.3.1. Talent placing the 24 well plate into a 37 degrees Celsius incubator.
 - 3.3.2. Talent pipetting 400 microliters of organoid culture medium into the well beneath the insert.
 - 3.3.3. Shot of the 24 well plate being placed inside the incubator.

4. Organoid Dissociation and Reseeding

- 4.1. Add 1 milliliter of Dispase solution at 2.4 units per milliliter on top of the cell culture insert [1]. Using a pipette, gently dissociate the basement membrane matrix [2]. Transfer the Dispase-basement membrane matrix mixture to a 15-milliliter tube and incubate [3-TXT].
 - 4.1.1. Talent adding 1 milliliter of Dispase solution onto the cell culture insert in the 24 well plate.
 - 4.1.2. Using a pipette, talent gently triturating to dissociate the basement membrane matrix.
 - 4.1.3. Talent transferring the mixture into a 15 milliliter tube. **TXT: 37 °C; 1h**
- 4.2. Now, add 10 milliliters of freshly prepared ice-cold blocking buffer to the cell suspension to stop the enzymatic reaction [1]. Place the tube in a centrifuge and spin at 450 *g* for 5 minutes at 4 degrees Celsius [2].
 - 4.2.1. Talent adding 10 milliliters of ice-cold blocking buffer to the 15 milliliter tube.

- 4.2.2. Talent placing the 15 milliliter tube into a centrifuge set to 450 g and 4 degrees Celsius.
- 4.3. Remove the supernatant [1] and resuspend the pellet in 200 microliters of trypsin [2].
 - 4.3.1. Talent carefully aspirating and discarding the supernatant from the tube.
 - 4.3.2. Talent adding 200 microliters of TrypLE Express to the pellet and gently pipetting to resuspend.
- 4.4. Next, add 1 milliliter of ice-cold blocking buffer and gently mix to neutralize the enzyme [1]. Filter the suspension through a 40-micrometer cell strainer to obtain single cells [2].
 - 4.4.1. Talent adding 1 milliliter of ice-cold blocking buffer to the tube and mixing with pipette.
 - 4.4.2. Talent placing a 40 micrometer cell strainer over a collection tube and passing the cell suspension through the strainer.
- 4.5. Place the tube in a centrifuge and spin at 450 g for 5 minutes at 4 degrees Celsius [1]. After discarding the supernatant, resuspend the cell pellet in 200 to 500 microliters of organoid culture medium [2].
 - 4.5.1. Talent placing the tube into a centrifuge set to 450 g and 4 degrees Celsius.
 - 4.5.2. Talent adding organoid culture medium to the pellet and gently pipetting to resuspend the cells.
- 4.6. Using a hemocytometer and trypan blue solution, count the cell number [1]. Expect a yield between 50,000 and 500,000 cells per well of a 24-well cell culture insert [2].
 - 4.6.1. Talent mixing the cell suspension with trypan blue and loading it onto a hemocytometer.
 - 4.6.2. Close-up of the hemocytometer grid with dye-cell mixture.
- 4.7. Resuspend 500 to 2,000 cells in 50 microliters of organoid culture medium in a microcentrifuge tube [1] and add 50 microliters of basement membrane matrix to the cell suspension [2]. Gently mix to avoid bubble formation [3] and dispense the 100-microliter mixture into the interior of a cell culture insert placed in a 24-well plate [4].
 - 4.7.1. Talent resuspending 500 to 2,000 cells in 50 microliters of organoid culture medium.
 - 4.7.2. Talent adding 50 microliters of basement membrane matrix to the cell

suspension on ice.

4.7.3. Talent gently mixing the suspension without introducing bubbles.

4.7.4. Talent dispensing the 100 microliter mixture into the interior of a cell culture insert in a 24 well plate.

4.8. Place the plate in a 37 degrees Celsius incubator for 20 to 30 minutes until the basement membrane matrix-cell mixture solidifies [1]. Add 400 microliters of organoid culture medium to the well below the insert once the mixture is fully solidified [2]. Next, culture the organoids in organoid culture medium using a 3-dimensional extracellular matrix-embedded method in a humidified incubator [3-TXT].

4.8.1. Talent placing the 24 well plate into a 37 degrees Celsius incubator.

4.8.2. Talent pipetting 400 microliters of organoid culture medium into the well beneath the insert.

4.8.3. Shot of the 24 well plate inside a humidified incubator set to 37 degrees Celsius and 5 percent carbon dioxide. **TXT: 1-2 weeks, 37 °C, 5%CO₂; Replace the medium every 2 - 3 days**

5. Genetic Manipulation of Human Cervical Organoids by Electroporation

Demonstrator: Jihyeon Myeong

5.1. Prepare the Nucleofector Solution and Supplement mixture by combining 16.4 microliters of Nucleofector Solution with 3.6 microliters of Supplement per tube [1]. Resuspend the cell pellet in 20 microliters of the prepared Nucleofector Solution and Supplement mixture from the transfection Kit [2].

5.1.1. Talent pipetting 16.4 microliters of Nucleofector Solution and 3.6 microliters of Supplement into a microcentrifuge tube and mixing.

5.1.2. Talent adding 20 microliters of the prepared mixture to the cell pellet and gently pipetting to resuspend.

5.2. Then, add 300 nanomolar of either control or target small interfering RNA oligo to each tube containing the resuspended cells and mix gently [1]. Carefully transfer 20 to 22 microliters of the nucleofection mixture into the electroporation cuvette vessels from the transfection kit [2].

5.2.1. Talent adding the appropriate volume of control or target small interfering ribonucleic acid oligo to the tube.

- 5.2.2. Talent pipetting the nucleofection mixture into the electroporation cuvette vessel.
- 5.3. Gently tap the Nucleocuvette vessel to ensure the sample covers the bottom of the cuvette [1]. Close the lid of the Nucleocuvette vessel [2] and place the vessel into the retainer of a powered-on 4D-Nucleofector unit [3]. To initiate the nucleofection process, select the P4 primary cell process and set the pulse code to **EA-125** for each cuvette [4].
- 5.3.1. Talent gently tapping the Nucleocuvette vessel on the bench to distribute the sample evenly.
- 5.3.2. Talent closing the lid of the Nucleocuvette vessel.
- 5.3.3. Talent inserting the vessel into the retainer slot of the powered-on 4D-Nucleofector unit.
- 5.3.4. Display the 4D-Nucleofector interface and show selection of the **P4 Primary Cell** process followed by setting the pulse code to **EA-125**, then starting the run.
- 5.4. After completion, carefully remove the Nucleocuvette vessel from the retainer [1] and incubate the Nucleocuvette vessel at room temperature for 10 minutes to allow the cells to stabilize [2]. Next, open the lid of the Nucleocuvette vessel [3] and add 180 microliters of 37 degrees Celsius pre-warmed organoid culture medium to each cuvette [4]. Gently transfer the cell suspension to a new 1.5-milliliter tube for resuspension [5].
- 5.4.1. Talent removing the Nucleocuvette vessel from the 4D-Nucleofector unit
- 5.4.2. Talent placing the Nucleocuvette vessel on the bench at room temperature.
- 5.4.3. Talent opening the lid of the Nucleocuvette vessel.
- 5.4.4. Talent adding 180 microliters of pre-warmed organoid culture medium to the cuvette.
- 5.4.5. Talent gently pipetting the cell suspension and transferring it into a new 1.5 milliliter tube.
- 5.5. Finally, take a volume of the cell suspension containing 500 to 2,000 cells [1] and resuspend in organoid culture medium to a final volume of 50 microliters [2-TXT].
- 5.5.1. Talent calculating and pipetting the appropriate volume of cell suspension corresponding to 500 to 2,000 cells.
- 5.5.2. Talent adding organoid culture medium to reach a final volume of 50 microliters and gently mixing. **TXT: Culture the organoids; Perform RT-PCR experiments**

Results

6. Results

- 6.1. Human cervical organoids grew up to 500 micrometers in diameter from single cells over 2 weeks [1].
 - 6.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the time course images from left to right.*
- 6.2. Hematoxylin and eosin staining clearly revealed the formation of stratified squamous epithelium in the organoids [1].
 - 6.2.1. LAB MEDIA: Figure 2B. *Video editor: Zoom in on the stained tissue section showing the layered epithelial structure.*
- 6.3. Immunofluorescence staining revealed that the basal layer was located in the outer rim of the organoids [1], surrounding the suprabasal layers [2].
 - 6.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the outer GREEN rim signal marked KRT14*
 - 6.3.2. LAB MEDIA: Figure 2C. *Video editor: Highlight RED signal marked KRT1*
- 6.4. Cervical organoids could be passaged at least 10 times over 6 months [1].
 - 6.4.1. LAB MEDIA: Figure 3.
- 6.5. After electroporating the human cervical epithelial cells with siRNA (*S-I-R-N-A*) oligos against AKT, about 97.3 percent of cells were viable [1].
 - 6.5.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the bar siAKT1+2.*
- 6.6. Simultaneous silencing of AKT1 and AKT2 effectively silenced both genes [1] and interfered with cervical organoid formation [2].

- 6.6.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the bars on the left and right labeled siAKT1+2 for AKT1 and AKT2.*
- 6.6.2. LAB MEDIA: Figure 4C and D. *Video editor: Show the bright-field images and bar graph for siAKT1+2_1 and siAKT1+2_2*

1. Ectocervix
Pronunciation link: <https://www.howtopronounce.com/ectocervix>
IPA: /'ektou...səvɪks/
Phonetic Spelling: ektoh-surviks
2. Petri dish
Pronunciation link: <https://www.howtopronounce.com/petri-dish>
IPA: /p'etɹi dɪʃ/
Phonetic Spelling: petree-dish
3. Epithelial
Pronunciation link: <https://www.howtopronounce.com/epithelial>
IPA: /ɛpɪ.θeɪ'lɪəl/
Phonetic Spelling: epi-the-learl
4. Recombinant
Pronunciation link: <https://www.howtopronounce.com/recombinant>
IPA: /rɪ:kɑ':m.bɪnənt/
Phonetic Spelling: ree-kah-m-binuhnt
5. Centrifuge
Pronunciation link: <https://www.howtopronounce.com/centrifuge>
IPA: /s'ɛntɹɪ.fjuːdʒ/
Phonetic Spelling: sentri-fyooj
6. Supernatant
Pronunciation link: <https://www.howtopronounce.com/supernatant>
IPA: /s'u:p.ə.nætənt/
Phonetic Spelling: soo-pur-natuhnt
7. Organoid
Pronunciation link: <https://www.howtopronounce.com/organoid>
IPA: /ɔ:ɹg.æn...o'ɔɪd/
Phonetic Spelling: aw-rgan-o-ueyed
8. Hemocytometer
Pronunciation link: <https://www.howtopronounce.com/hemocytometer>
IPA: /hi:m.ə.sɪ.t'.ɑ:mɪrə/
Phonetic Spelling: hee-muh-si-t-ahmirur
9. Electroporation
Pronunciation link: <https://www.howtopronounce.com/electroporation>
IPA: /ɪ.lɛktɹo...ʊp..ə'ɪɛfən/
Phonetic Spelling: i-lektro-up-ur-raysshuhn

10. Hematoxylin
Pronunciation link: <https://www.howtopronounce.com/hematoxylin>
IPA: /h'ɛmɛtɑːks.ɪlɪn/
Phonetic Spelling: hematah-ksilin
11. Eosin
Pronunciation link: <https://www.howtopronounce.com/eosin>
IPA: /'iː.əsm/
Phonetic Spelling: ee-uhsin
12. Immunofluorescence
Pronunciation link: <https://www.howtopronounce.com/immunofluorescence>
IPA: /ɪ.mjuː.nə.flʊɪɛ'səns/
Phonetic Spelling: i-myoo-nuh-flurre-suhns
13. Squamous
Pronunciation link: <https://www.howtopronounce.com/squamous>
IPA: /skw'ɑː.məs/
Phonetic Spelling: squ-ah-muhs