FINAL SCRIPT: APPROVED FOR FILMING

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Title: In Vitro Time-lapse Live-Cell Imaging to Explore Cell Migration Toward the Organ of Corti

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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**

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

   Yes

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes, walking distance**

Current Protocol Length

Number of Steps: 15
Number of Shots: 34

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sung Kyun Kim:** Existing *in-vitro* methods to investigate migration of MSCs include trans-well or wound healing protocols. The method described here can be used to track migration of MSCs to explant in real time.

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

1.2. **Sung Kyun Kim:** In terms of culture of explant, the advantage of this method compared to others is that it achieves a reduction in time and cost, as well as a simplification of experimental steps.

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

OPTIONAL:

1.3. **Sung Kyun Kim:** This technique can be used to observe stem cell homing to target in real time, leading to development of models with optimized homing efficiency.

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

1.4. **Sung Kyun Kim:** Removal of tectorial membrane is difficult for unexperienced researchers. Depending on the development stage, understanding the right time to remove the tectorial membrane is important for the success of this protocol.

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

Introduction of Demonstrator on Camera

1.5. **Sung Kyun Kim:** Demonstrating the procedure will be Jeong-Eun Park and Su Hoon Lee, research professors from my laboratory.

1.5.1. INTERVIEW: Author saying the above.

1.5.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.6. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Yonsei University at Wonju College of Medicine.

Protocol

2. Cochleae dissection

2.1. Begin by sterilizing the laminar flow tissue culture hood by turning on the ultraviolet light for approximately 30 minutes **[1]**. Spray all surfaces with 70% ethanol prior to use and allow them to dry **[2]**.

2.1.1. WIDE: Establishing shot of talent turning on the UV light in the hood.

2.1.2. Talent spraying the surface with ethanol.

2.2. Place dissection instruments in 70% ethanol for 10 minutes **[1]**, then dry them before using **[2]**. After decapitating a 3 to 4-day old mouse, soak the head in 70% ethanol **[4]** and place it under a stereomicroscope in the laminar flow hood **[3]**.

2.2.1. Talent placing the instruments in ethanol.

2.2.2. Talent drying the instruments.

2.2.3. Talent placing the mouse's head under the microscope. NOTE: switch order of 2.2.3 and 2.2.4

2.2.4. Talent adding ethanol to the head.

2.3. Quickly soak the tissue in tissue dissection solution to remove the ethanol **[1-TXT]**. Cut the centerline of the skull with a surgical blade, then expose the skull by pulling down the skin anteriorly and cutting the external auditory canal of the ear **[2]**.

2.3.1. Talent placing the tissue in the dissection solution. **TEXT: 1x Hank's Balanced Salt Solution, 1 mM HEPES**

2.3.2. SCOPE: Talent cutting the skin and exposing the skull. NOTE: SCOPE shots were not uploaded at postshoot, authors were reminded.

2.4. Cut from the anterior to the posterior part of the cranium across the eye line, then open the skull and remove the forebrain, cerebellum, and brainstem with blunt forceps **[1]**. Using micro forceps, separate the cochlea from the temporal bone **[2]** and transfer it to a Petri dish containing tissue dissection solution **[3]**.

2.4.1. SCOPE: Talent cutting the cranium and removing the forebrain, cerebellum, and brainstem.

2.4.2. SCOPE: Talent separating the cochlea from the temporal bone.

2.4.3. Talent putting the cochlea in a Petri dish.

2.5. Carefully dissect all of the cochlear otic capsule, leaving only the internal cochlear soft tissue **[1]**. Hold the modiolus of the cochleae with forceps and the cochlear duct with another pair of forceps, then slowly separate the two tissues **[2]**. Remove the stria

Commented [AG1]: Authors: Please record all SCOPE shots using the camera on your microscope and upload them to your project page after the shoot: https://www.jove.com/account/file-uploader?src=18893883.

vascularis and tectorial membrane by gently peeling them away **[3]**. *Videographer: This step is difficult!*

2.5.1. SCOPE: Talent dissecting the cochlear otic capsule.

2.5.2. SCOPE: Talent separating the modiolus of the cochleae and the cochlear duct.

2.5.3. SCOPE: Talent peeling the stria vascularis and tectorial membrane.

2.6. Place a sterilized plastic coverslip in new tissue dissection solution **[1]**, then place the organ of Corti on the coverslip, making sure that the basilar membrane faces downward **[2]**.

2.6.1. Talent placing a coverslip in dissection solution.

2.6.2. Talent placing the organ of Corti on the coverslip.

2.7. Immobilize the tissue by pressing the Reissner's membrane and the remaining modiolus tissue onto the coverslip with forceps **[1]**. Transfer the coverslip with the embedded tissue to the center of a 35-millimeter confocal dish **[2]**. *Videographer: This step is important!*

2.7.1. Talent pressing the Reissner's membrane and the remaining modiolus tissue onto the coverslip.

2.7.2. Talent transferring the coverslip to the confocal dish.

2.8. Place the glass cloning cylinder on the dish, with the cochlear explant positioned in the center of the dish **[1]** and add 100 microliters of explant culture medium into the cylinder **[2]**.

2.8.1. Talent placing the cloning cylinder on the dish.

2.8.2. Talent adding explant culture medium to the cylinder.

2.9. Plate 5,000 mouse bone marrow-derived GFP-tagged mesenchymal stem cells, or MSCs, in 2 milliliters of culture medium outside the glass cylinder **[1]** and put the dish in the incubator **[2.10.4]**. *Videographer: This step is important!*

2.9.1. Talent plating the cells outside the cylinder. NOTE: show 2.10.4 after 2.9.1

2.10. After culturing the cells as described in the text manuscript, aspirate all medium inside and outside the cylinder **[1]** and remove the glass cylinder from the confocal dish **[2]**. Add 2 milliliters of fresh culture medium to the dish **[3]**. place it in a humidified incubator until ready for analysis **[4]**. *Videographer: This step is important!*

2.10.1. Talent aspirating the medium.

2.10.2. Talent removing the glass cylinder.

2.10.3. Talent adding culture medium to the dish.

2.10.4. Talent putting the dish in the incubator.

3. Time-lapse imaging

3.1. Use a confocal microscopy system with a stage top incubator **[1]**. Turn on the confocal microscope, fluorescent light, and computer **[2]**. Set the conditions of the stage-top incubator to 37 degrees Celsius and 5% carbon dioxide atmosphere **[3]**.

3.1.1. Confocal microscope setup.

3.1.2. Talent turning on the microscope, fluorescent light, and computer.

3.1.3. Talent setting the incubator conditions.

3.2. Place the sample dish on the dish fixing vessel, cover it with the dish fixing lid **[1]**, and close the chamber with the top heater lid **[2]**. Adjust the zoom and focus to localize the organ of Corti and MSCs in the field of view **[3]**. *Videographer: This step is important!*

3.2.1. Talent placing the sample in the dish vessel and covering it.

3.2.2. Talent closing the chamber.

3.2.3. Talent adjusting the zoom to focus on the organ of Corti and MSCs.

3.3. Open the image processing software. Under the **locate** option, select a **20x Plan-Apochromat** objective, or **numerical aperture 0.8**, and a **0.5x crop area**. Under **Acquisition**, click on **smart setup** and select **EGFP [1]**.

3.3.1. SCREEN: Image video.mp4. 0:44 – 1:05.

3.4. Open the **channel** tab under **Acquisition**, and set the **laser power** to **0.2%**, the **pinhole** to **44** micrometers, the **master gain** to **750** Volts, and the **digital gain** to **1.0**. Click on **ESID** under **imaging setup** and set the **ESID gain** to **4** and the **digital gain** to **7.5**. Click on **Tiles** and **stake** to produce 210 tiles **[1]**.

3.4.1. SCREEN: Image video.mp4. 1:05 – 1:56.

3.5. Open **Focus strategy** and select the **focus mode**. Under **time series**, set the **duration** to **24** hours and the **interval** to **10 minutes**. Under **Acquisition**, set the **frame size** to **512** by **512 pixels**, **scan speed** to **8**, the **direction** to **bidirectional**, the **averaging** to **4x**, and the **bits per pixel** to **16**. Click on **start experiment** to begin the experiment **[1]**.

3.5.1. SCREEN: Image video.mp4. 1:57 – 2:40.

Results

4. Results: Confocal images of the organ of Corti and GFP-labeled MSCs

4.1. The two-dimensional path of mesenchymal stem cells, or MSCs, was tracked in a wound healing method by using a glass cylinder as a barrier between the MSCs and the organ of Corti **[1]**. When the organ of Corti was cultured, fibroblasts grew very quickly outward from the outer hair cells in the outermost layer **[2]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Video 1.

4.2. Most GFP-labeled MSCs were pushed out by fast-growing fibroblasts. Nonetheless, some penetrated the layer of fibroblasts and successfully migrated to the organ of Corti **[1]**.

4.2.1. LAB MEDIA: Video 1.

4.3. The results from 72 hours of incubation showed that MSCs migrated to the organ of Corti and were mainly localized in the modiolus and the area with the cochlear nerve fibers separated from the modiolus. The morphology of the MSCs was altered to a radial shape, similar to that of nerve fibers **[1]**.

4.3.1. LAB MEDIA: Figure 4 B.

4.4. The cells were transformed into a linear shape along the limbus line rather than the hair cell area **[1]**. The MSCs were able to migrate and grow in the nerve fiber collection area by penetrating the various cell layers and physical barriers **[2]**.

4.4.1. LAB MEDIA: Figure 4 E. *Video Editor: Emphasize where the arrow is pointing.*

4.4.2. LAB MEDIA: Figure 4 E.

4.5. When damage to hair cells was induced by a 16-hour treatment with kanamycin and cells were cultured for a further 72 hours after the removal of kanamycin, MSCs were localized not only in the modiolus, but also in the outer hair cells **[1]**.

4.5.1. LAB MEDIA: Figure 4 C and D.

Conclusion

5. Conclusion Interview Statements

5.1. **Sung Kyun Kim:** Immobilizing the tissue on a coverslip is an important step in this protocol. Once the organ of Corti is attached, it does not fall of easily. This is trivial but helpful tip for successful explant culture.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.1.*

5.2. **Sung Kyun Kim:** This method may be adapted to study the efficacy of exosomes secreted from MSCs.

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