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# Long-term time lapse imaging of mouse cochlear explants

--Manuscript Draft--

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| **Abstract:** | Here we present a method for long-term time-lapse imaging of live embryonic mouse cochlear explants. The developmental program responsible for building the highly ordered, complex structure of the mammalian cochlea proceeds for around ten days. In order to study changes in gene expression over this period and their response to pharmaceutical or genetic manipulation, long-term imaging is necessary. Previously, live imaging has typically been limited by the viability of explanted tissue in a humidified chamber atop a standard microscope. Difficulty in maintaining optimal conditions for culture growth with regard to humidity and temperature has placed limits on the length of imaging experiments. A microscope integrated into a modified tissue culture incubator provides an excellent environment for long term-live imaging. In this method we demonstrate how to establish embryonic mouse cochlear explants and how to use an incubator microscope to conduct time lapse imaging using both bright field and fluorescent microscopy to examine the behavior of a typical embryonic day (E) 13 cochlear explant and Sox2, a marker of the prosensory cells of the cochlea, over 5 days. |
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10th of April 2014

Dear Dr Nam,

I am pleased to resubmit a revised version of the manuscript and an accompanying document outlining our response to the reviewers' comments. We feel that the reviewers gave helpful and constructive criticism which we have incorporated into the text and that the resubmitted document marks a significant improvement.

Regards,
Jo Mulvaney

**TITLE:**
Long-term time lapse imaging of mouse cochlear explants

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**KEYWORDS:**
Live-imaging, time lapse, cochlea, ear, reporter mouse, development, incubator microscope, Sox2.

**SHORT ABSTRACT:**
Live imaging of the embryonic mammalian cochlea is challenging because the developmental processes at hand operate on a temporal gradient over ten days. Here we present a method for culturing and then imaging embryonic cochlear explant tissue taken from a fluorescent reporter mouse over five days.

**LONG ABSTRACT:**
Here we present a method for long-term time-lapse imaging of live embryonic mouse cochlear explants. The developmental program responsible for building the highly ordered, complex structure of the mammalian cochlea proceeds for around ten days. In order to study changes in gene expression over this period and their response to pharmaceutical or genetic manipulation, long-term imaging is necessary. Previously, live imaging has typically been limited by the viability of explanted tissue in a humidified chamber atop a standard microscope. Difficulty in maintaining optimal conditions for culture growth with regard to humidity and temperature has placed limits on the length of imaging experiments. A microscope integrated into a modified tissue culture incubator provides an excellent environment for long term-live imaging. In this method we demonstrate how to establish embryonic mouse cochlear explants and how to use an incubator microscope to conduct time lapse imaging using both bright field and fluorescent microscopy to examine the behavior of a typical embryonic day (E) 13 cochlear explant and Sox2, a marker of the

prosensory cells of the cochlea, over 5 days.

**INTRODUCTION:**

The mammalian cochlea is a highly ordered complex organ. In the mouse, between the emergence of the primitive inner ear from the otic vesicle on day E11 and completion of the developmental program at early postnatal stages, multiple waves of cell signaling and coordinated changes in gene expression take place. Running from base to apex of the cochlear duct is the sound detecting sensory epithelium, or organ of Corti. Development of the organ of Corti is exquisitely controlled such that by the end of development, it will consist of a single row of inner hair cells, three rows of outer hair cells interspersed with five rows of supporting cells (two rows of pillar cells, three rows of Deiters' cells) [1]. Deviation from this precise order results in hearing loss, highlighting the importance of study of the genesis and patterning of the sensory epithelium [2].

*In vitro* culturing of the embryonic mouse cochlea is an essential tool in studying the mechanisms of development of the organ of Corti. This technique was established in 1974 and over the last 40 years, has been used to elucidate many of the mechanisms by which the sensory epithelium is specified and the organ of Corti established [3]. The cochlea is a complex organ with dynamic developmental processes; a manipulation may take as long as seven days to manifest [1]. For example, when adding GSK 3β inhibitors to a cochlear explant culture at E13, the optimal incubation period to observe a robust effect of the compound is six days [4].

Live imaging of the developing cochlea allows investigation into changes in the morphology of the organ of Corti; changes in gene expression; tracking of migrating, proliferating or dying cells and it allows real-time observation of the results of pharmaceutical agents and disruption of signaling pathways. Until now, live imaging of the cochlea has mainly been performed using confocal microscopy to image small areas of the organ of Corti over short time periods [5-8], but this technique has limitations due to explant viability. In imaging of the effects of longer-term manipulations on slow developmental processes, the imaging environment is crucial. Typically a confocal live imaging system uses a humidified plastic box that sits on the microscope. Heat and humidity can escape through the gaps in the incubating box where it meets the microscope table; through the access windows; through the hinged openings and through the gaps around various parts of the microscope- such as the objective or the light source. This is not optimal for maintaining healthy explants for more than two or three days.

We define 'incubator microscope' as an inverted microscope sealed inside a standard $CO_2$ incubator, rather than an incubator built around the microscope. An incubator microscope extends the life of the experiment such that rather than imaging over two or three days, samples can be imaged for up to two weeks. An incubator microscope provides an excellent environment for cell growth and differentiation, with minimal disturbance to explant cultures and standard controlled conditions. In studies that take place over multiple days it is common to resort to imaging samples on a daily basis by removing them from the incubator and carrying them to an inverted fluorescent microscope. While this approach can work, removing the dishes from the incubator inflicts stress on the sensitive

developing tissue. Changes in acidity of the culturing medium and fluctuations in temperature due to removal from the incubator can result in suboptimal development and unhealthy tissue. Imaging the same region at the same focal plane and in the same orientation at every time point is extremely challenging. By using an automated system within an incubator, it is possible to maintain healthy tissue, to collect images at more time points and to ensure that the same area is captured in every frame. In recent years several integrated microscope tissue incubators have been developed, these have been useful not only in clinical practice [9] but also in stem cell and cancer research [10,11].

Here we present a protocol for long term live imaging of embryonic mouse cochlear explants. We use an automated microscopy system inside a standard $CO_2$ incubator that has the capability to capture images of multiple samples at set time points. The system consists of an inverted microscope set inside an incubator. Samples are placed in a rotating dais that allows imaging of multiple samples at each time point. Illumination, image capture and rotation of the dais are controlled by an automated system operated through Metamorph software. By setting an imaging routine using the operating software we can set an experiment to run for up to two weeks with minimal human intervention. In this example we use both bright field and fluorescence to show large-scale growth and rearrangement of the cochlea, and specifically, the prosensory region. In this experiment, cochleae will be dissected from *Sox2*$^{EGFP}$ reporter mice on embryonic day E13. *In vitro* cultures will be established and then imaged over five days.

**PROTOCOL:**

Mouse tissue was harvested from *Sox2*$^{EGFP}$-reporter mice [12] maintained and euthanized in accordance with *Canadian* Council on Animal Care guidelines for the care and use of laboratory animals.

**1 Culturing embryonic cochleae**

1.1) Supplement Dulbecco's Modified Eagle Medium (DMEM) by mixing 8.89 ml of DMEM, 1 ml of fetal bovine serum (FBS), 100 µl of 100x N2 supplement, and 10 µl of 10 mg/ml ciprofloxacin. Supplement Hank's Balanced Salt Solution (HBSS) by mixing 495 ml HBSS with 5 ml of 100% HEPES.

1.2) Prepare glass bottom culture dishes.

1.2.1) In a laminar flow hood, resuspend 200 µl basement membrane extract substrate in 5ml DMEM. Prepare 35 mm diameter glass bottom culture dishes with 10 mm wells. Pipette 150 µl substrate/DMEM in to the center of each glass bottomed dish. These dishes can be used after 40 minutes incubation, or can stored in a $CO_2$ incubator at 35 °C for at least a week.

Note: Glass bottomed dishes are used for the following reasons: to ensure that the base of the dish is transparent and suitable for imaging, to create a well in the center of the dish that allows the explants to settle in an easily located area and finally so that after an

experiment the sample can be processed for immunostaining and subsequent analysis.

1.3) Prepare the work-station and tools

1.3.1) Turn on the laminar flow clean bench, and spray down with 70% EtOH to create a clean work space. Soak forceps, spoons and a black 184 silicone elastomer dish (a mix of 184 silicone elastomer (10 parts), curing agent (1 part) and charcoal powder (2.5 g)) in 70% EtOH.

1.4) In the clean work station, harvest embryos for the experiment. Collect embryos of the appropriate gestation in ice cold HBSS supplemented with 1% HEPES.

1.4.1) Determine an external or visible organ that will demonstrate reporter activity and examine the embryos using a fluorescent stereo microscope. Collect the embryos that exhibit reporter activity in ice cold HBSS supplemented with 1% HEPES as these are the subject of the experiment.

1.5) Collect temporal bones.

1.5.1) Working quickly, using a cool light source and fine forceps, collect the heads of the pups. Take care to clip at the cervical vertebrae and below the jaw to avoid damage to the temporal bones.

1.5.2) Carefully open the skull. Remove the brain, trim off the front of the head, and transfer the posterior skull to fresh ice cold HBSS supplemented with 1% HEPES in a clean dish. Carefully dissect out the peanut shaped temporal bones taking care to keep the vestibular system in tact.

1.6) Dissect the cochlea

1.6.1) Transfer the temporal bones to a black silicone elastomer coated dish in ice cold HBSS supplemented with 1% HEPES, pin the vestibular region of the bone. Insert insect pins at an oblique angle in order to stabilize the temporal bone and create room for the forceps. Pinning is a crucial step in the process as if the temporal bone is allowed to move too much it is very difficult to harvest an intact cochlea.

1.6.2) Carefully remove the cartilage surrounding the cochlea. Insert one tine of the forceps into the cartilage at the outer edge of the base of the cochlea and clip a hole into the cartilage.

1.6.3) Clip a flap up the side, insert the tine of the forceps and gently separate the roof of the duct from the cartilage. Clip horizontally across the top and diagonally, and carefully lift off the front section of the capsule. Insert a prong of the forceps in between the remaining cartilage and the duct and gently clip off the last section. The apical surface of the cochlea is now exposed.

1.6.4) Starting at the base, catch the area where the roof of the duct meets the cochlear epithelium and open the cochlear duct. Gently peel off the roof, trimming when necessary until it is completely removed. Trim off any portions of membrane left on the medial side of the duct. Clean the duct of excess mesenchymal tissue and detach the duct from the vestibular system.

1.7) Culture the explants

1.7.1) Place an explant, luminal surface up, in the center of a substrate coated glass bottom culture dish, carefully draw off all of the liquid and leave for two minutes. Add 150 µl supplemented DMEM drop-wise to the explant, taking care not to disturb it. Should the explant float free, reposition with forceps, but take care that the explant settles to the bottom of the dish so that it can attach to the substrate.

1.7.2) Place the glass bottom dishes in a deep 12 cm diameter petri dish, with a small dish of sterile water to maintain humidity. Put the cultures in to a 35 °C incubator overnight in order for the explant to attach to the substrate and flatten.

**2 Live imaging**

2.1) Select explant samples. Use a fluorescent stereo microscope to evaluate the condition of each explanted cochlea. Only select explants where the duct is intact and attached to the glass from base to apex.

2.2) Set the incubator at 35 °C with 5% $CO_2$ to culture cochlear tissue. Put the cultures into the incubator microscope

2.2.1) Gently aspirate off the supplemented DMEM and replace with at least 500 µl fresh media. For imaging up to 6 days, 1-1.5 ml is better. In cases where explants are loosely attached, pipette a ring of media around the edge of the dish. This extra liquid will make a miniature 'humidified chamber' without disturbing the explant while it continues to attach to the matrix.

2.2.1.1) Alternatively, use hinged dish covers to open the lids while the dish stays in its fitting in the microscope if reagents need to be exchanged during intervals between image collections. Hinged dish covers allow the lids to be opened without disturbing the samples or removing the dishes from their settings. This maintains their exact position for subsequent image captures.

2.2.2) Insert the glass bottom dishes containing appropriate samples into the sample dish holder. The microscope in this example has a rotating platform that holds eight 35 mm sample dishes.

2.2.3) Under the laminar flow hood replace the plastic lids with glass lids and insert the dishes into the sample dish holder. Place the sample dish holder inside the incubator taking care not to dislodge the explants from the bottom of the dish or to disturb the media.

2.3) Set up the imaging routine.

2.3.1) Switch on the microscope, UV lamp and camera and open the imaging software.

2.3.2) Locate the samples, pick an imaging area, plane of focus and adjust exposure times for each dish in sequence. Choose the plane of focus depending not only on the view of the explant at the time of starting, but also bearing in mind how the tissue will move and how long the time course will run.

2.3.3) Set a Z stack centering around the selected focal plane in the fluorescence channel. Set the frequency and length of the sampling for the experiment. The frequency of sampling will be limited by the time it takes to collect images, so this should be determined after selecting fields of view and setting a Z stack. In this case sampling takes place every 30 minutes over five days. The duration of the experiment can be up to 14 days.

2.4 ) Generate a movie.

2.4.1) At the end of the time lapse period open the image files. In this case
Metamorph software that opens sequential collection points is used. Frame by frame pick the best focal plane for showing the cell population of interest.

2.4.2) Convert these images to an .avi file, or export them as a montage to generate a set of images that can be opened and analyzed in image processing software or converted to multiple formats using video processing software.

**REPRESENTATIVE RESULTS:**
Here we show a montage (Figure 1A and B) and a movie (Figure 2) demonstrating how a typical organotypic cochlear explant will grow if plated on E13.5. A *Sox2* reporter mouse was used to visualize the prosensory region. The movie illustrates that the cochlea undergoes growth and convergence and extension, the cells in the lateral region of the green *Sox2* domain do not seem to divide as the tissue surrounding it expands. This is a characteristic of the organ of Corti-on E13 the prospective sensory epithelium exits the cell cycle and is subsequently known as the zone of non-proliferation [13]. A second time-lapse experiment centering on the mid base of a different cochlear explant (Figures 3 and 4) demonstrates that as it extends, the prosensory region narrows. Note that after three days in culture the tissue has flattened considerably such that it is possible to visualize individual fluorescing cells, whereas at the beginning there are regions where internal reflection of the light due to tissue thickness make it possible to identify regions of expression but not individual cells.

**Figure 1: Time-lapse images of *Sox2* reporter cochlear tissue collected over five days.**
This montage shows the progress of explant growth starting on day zero and ending on day five, sampling every 30 minutes using a 10X objective. The explant was established on E13 and cultured over night before imaging. As the explant matures it both grows and undergoes convergent extension movements.

A. Sequential images showing the extent of cochlear growth at twelve hour intervals. Visible light channel overlaid with GFP fluorescence generated by the EGFP *Sox2* reporter. B. GFP fluorescence channel only. Scale bar corresponds to 200 μm.

**Figure 2 Time-lapse animation of *Sox2* reporter cochlear tissue collected over five days.** This is the same explant experiment indicated in Figure 1, this time frames are selected at 30 minute intervals over five days and combined as a .avi file to generate a movie.

**Figure 3: Mid base undergoing CE movements and flattening**
Sequential images showing that the prosensory epithelium of the mid base (EGFP) narrows, extends and flattens over the course of five days. Arrows indicate region that narrows. Frames selected from a five day time lapse sequence at 12 hour intervals using a 10X objective. Scale bar corresponds to 200 μm.

**Figure 4: Time-lapse animation of the Mid base undergoing CE movements and flattening.** Animated time-lapse sequence showing convergence and extension of the sensory epithelium shown in figure 3 with frames selected at 30 minute intervals.

**DISCUSSION:**
There are several technical points to consider when cultures are established and in setting up the time-lapse microscope in order for long-term imaging.

We use basement membrane matrix as a substrate for culturing cochlear explants, but the substrate should be matched to the cell type. For example, to image neuronal cultures, it may be better to provide a fibronectin coating. Incubation temperature and gas composition should also be chosen according to tissue type. Choosing the age of the explant is important. We start on E13 at the earliest because on E12 the direction of growth is less predictable. As it is essential that explants are cultured over night to attach to the substrate, experiments should be planned to begin the following day. If an experiment is to start at E15 or older, cultures should be established on E13 as over time the tissue flattens and spreads so the cells are easier to image (see Figure 3).

If the organ of Corti is to be imaged, it is essential that the cochlea is dissected very carefully. Nicks in the lateral edge of the explant break the internal tension of the tissue, hence when the cochlea undergoes morphological rearrangements, cells 'spill' through the tear forming a v shaped protrusion. This protrusion can move the region of interest out of view. Additionally, care must be taken to remove as much of the roof of the duct on the medial side as possible. This tissue continues to proliferate as the explant expands and can grow over the top of the region of interest obscuring it from view.

When setting up the imaging routine, the growth and changes in morphology of the cochlea must be carefully considered. The objective should be chosen based not only on the size of the region to be imaged, but also taking into consideration whether that region will move or expand. Cells in the medial region of the cochlea (Figure 1 and 2) divide and move within a confined area, so a high magnification can be used (20X, 40X, 60X). Cells in the organ of

Corti or the lateral epithelium however, will move as the explant grows; a lower magnification (10X or 20X) is better, as the chances that the cells will remain in the field of view are higher. Imaging of stationary regions of the cochlea is possible at high magnifications. We chose to use a 10X objective in the representative results section because we wished to show the whole explant, and because the tissue movement is dynamic at early stages.

Next to consider is the plane of focus. Given that the explant will grow outwards and flatten, it is likely that the focal plane will change dramatically over the time course. If the region of interest is the organ of Corti or lateral epithelium, anticipating this and focusing on the cells migrating out of the explant can help. This is also an argument for using the lower magnification objectives. It is possible to set Z stacks for both fluorescence and DIC. If the cells of interest are likely to move out of focus, setting up a Z stack of 3-5 µm per step can counteract this, especially if the last step is in the plane of the cover glass. It can be difficult to choose the correct plane when viewing cultures on day one, as the density of packing of the cells and reflected light from surrounding tissue can prevent a clear view. When reviewing the frames to generate a movie or montage later, careful selection of Z-planes also allows tracking of cell movements in three dimensions. The experiment can be paused at any point to adjust the focus settings if the sample moves out of range.

Live imaging of a cochlear explant over a week adds a new dimension to understanding development of the cochlea that will complement confocal live imaging. This is an excellent method to use when organ wide long-term examination is necessary, but will not replace high magnification confocal imaging for short term single cell studies. Choice of technique is dependent on the type of tissue to be imaged and the context of the experiment. This technique will allow investigators to examine spatio-temporal changes in reporter gene expression, cell proliferation and migration in real time and allow study of reporter gene response to pharmaceutical agents and viability of cells in more detail than previously possible.

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Thank you to Willy Sun for technical assistance and Dr. Kris Gellynck for helpful comments and three anonymous reviewers for their constructive advice. This work was funded by the Sunnybrook Hearing Regeneration Initiative

**DISCLOSURES:**
The authors have nothing to disclose.

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Figure
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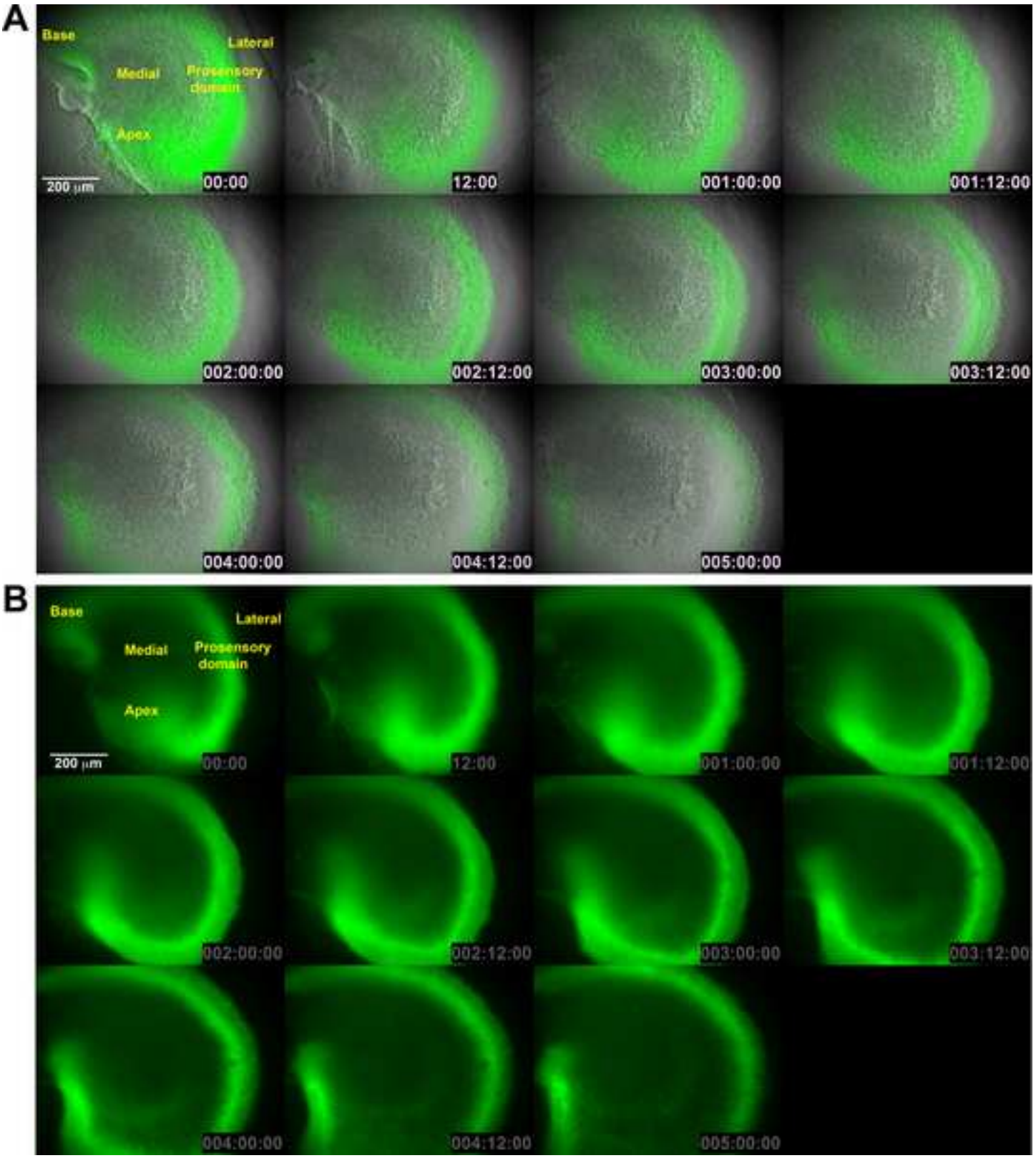

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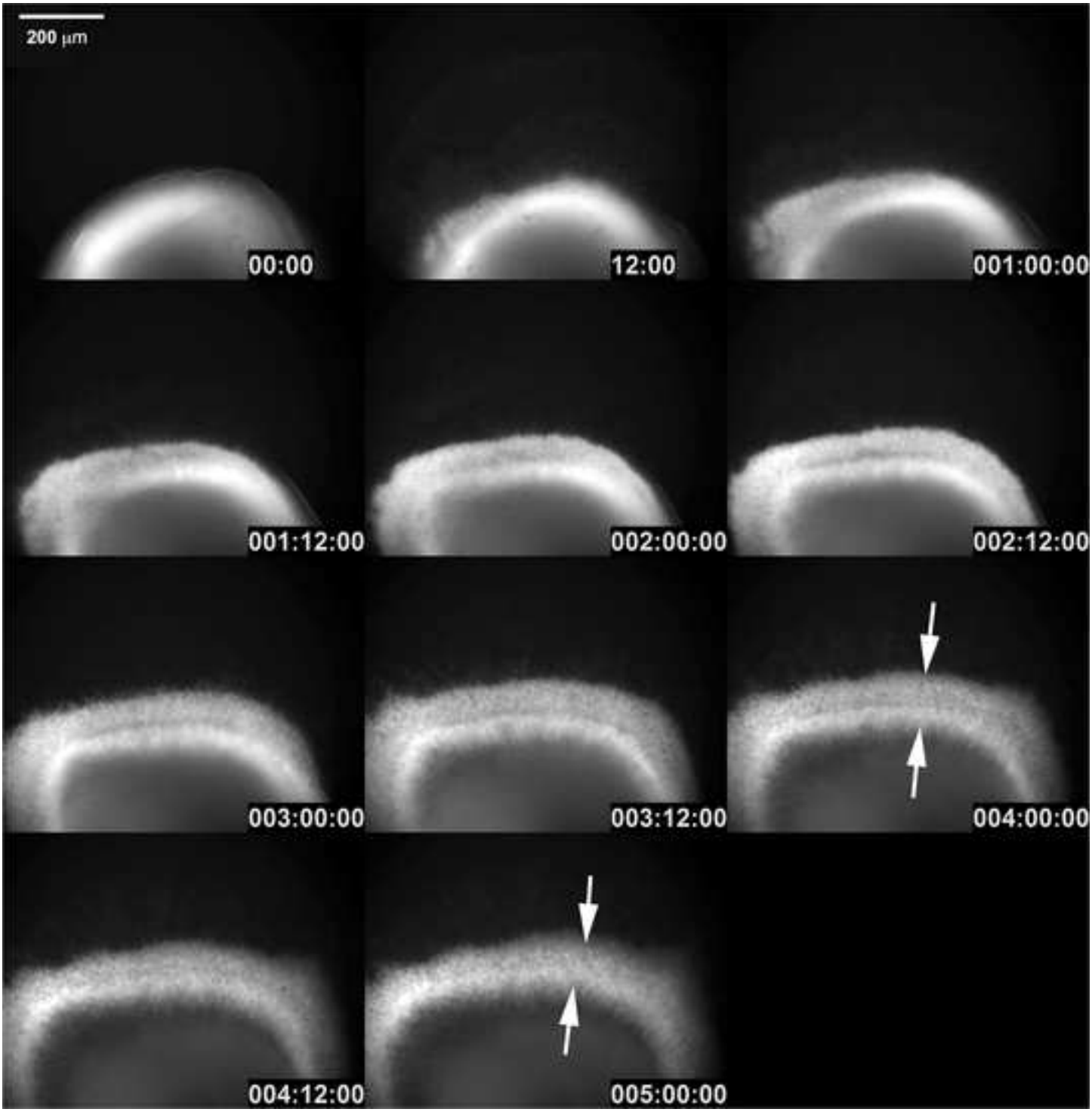

Excel Spreadsheet- Table of Materials/Equipment

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description | Website |
|---|---|---|---|---|
| Dulbecco's Modified Eagle media | Gibco | 12430 | Multiple brands manufactu | http://www.lifetechnologies.com |
| Basement membrane extract | Corning | 354230 | Matrigel. Alternative simila | http://catalog2.corning.com/Life |
| Fetal bovine serum | Gibco | 16000044 | Multiple brands manufactu | http://www.lifetechnologies.com |
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| Ciprofloxacin | Sigma Aldrich | 17850-5G-F | Multiple brands manufactu | http://www.sigmaaldrich.com/ca |
| Hank's balalnced salt solution | Gibco | 14170161 | Multiple brands manufactu | http://www.lifetechnologies.com |
| Fine Forceps | Fine Science tools | 11254-20 | Size number 5 | http://www.finescience.ca/Speci |
| Curette | Fine Science Tools | 10080-05 | size 1 mm | http://www.finescience.ca/Speci |
| Insect Pins | Fine Science Tools | 26001-35 | Must be stainless Steel | http://www.finescience.ca/Speci |
| 50 mm plastic dishes | Corning/Falcon | 351006 | Multiple brands manufacture this | |
| charcoal | Sigma Aldrich | 05105-250G | Multiple brands manufactu | http://www.sigmaaldrich.com/ca |
| 184 silicone elastomer | Dow/Corning | SYLGARD® 184 SILI | Dishes are home made sev | http://www.dowcorning.com/ap |
| Glass bottom dishes | MatTek | P35G-0-10-C | The dimensions of the dish | http://glass-bottom-dishes.com/ |
| Stereomicroscope | Zeiss | 495101-9804-000 | Stemi 2000 model. multipl | http://microscopy.zeiss.com/mic |
| Cold Light Source | Zeiss | 000000-1063-182 | Multiple brands manufactu | http://microscopy.zeiss.com/mic |
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| Incubator Microscope +imaging software | Olympus | Contact Olympus | Inverted microcope sealed | http://olympuscanada.com/seg_ |
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| CO2 incubator | Thermo Scientific | 3310 | Multiple brands manufactu | http://www.thermoscientific.com |

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Article Title: LONG-TERM ~~IMAGING OF~~ TIME LAPSE IMAGING OF MOUSE COCHLEAR EXPLANTS

Signature: JF Mulvaney Date: 11th of March 2014

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**Response to reviewers' comments**

Editorial comments

1. "Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version."
   As requested, we have carefully copy edited the manuscript, see track changes document.

2. "In the Short Abstract, "operate" should be "operates""
   We use the plural, 'processes', in this case the correct verb form is "operate".

Reviewer 1

"The introduction and discussion are poorly written. The grammar and punctuation need to be carefully re-worked. Check especially for comma usage (or omissions) as well as places where the paper needs a semicolon (any sentence that contains two independent clauses requires a semicolon, not a comma). Examples of problems include (but are not limited to) lines 246-249, 269, 277, 281, 288-289, period at the end of line 180, etc. Also check carefully the use (or omission) of hyphens."
We have carefully edited the punctuation and rewritten some sentences to improve them. Please see the track changes document.

1. "The Abstract and Introduction contain a number of references to time that seem conflicting and should be clarified. For example, they indicate that development takes place "over several days" (line 29), "over a week" (line 36), "over 10 days" (line 51). Also please indicate when is "the end of development" (line 55). I have a similar concern about the length of time described for the imaging experiments themselves (several days, a few days, over a week, 5 days - all of these appear in the text)."
The reviewer is correct in pointing out that this may have been confusing, we have clarified the period of development throughout the text as shown in the track changes document.

2. Line 77: What is meant by the statement that a typical live imaging setup is "unsealed"? Does this mean that heat and humidity may escape through the area around the objective?
As the reviewer suggests, there are areas in the conventional live imaging set up that are not sealed to prevent heat and humidity from escaping, we have included a better description to explain in more detail what we mean by this. "Typically a confocal live imaging system uses a humidified plastic box that sits on the microscope. Heat and humidity can escape through the gaps in the incubating box where it meets the microscope table; through the access windows; through the hinged openings and through the gaps around various parts of the microscope- such as the objective or the light source. This is not optimal for maintaining healthy explants for more than two or three days. "

3. Line 82: Please remove the word "slew" and indicate that "several" or "a `number of" microscope tissue incubators have been developed.
We have updated the text from “slew” to “several”.

4. Section 1.1 Methods: I am surprised to see a recommendation to use HEPES, since HEPES is known to contribute to free radical generation in cultures. Since the culture is in an incubator, one can use bicarbonate buffering. Also, I assume the DMEM is phenol free (since phenol red also contributes to free radicals in imaging experiments). This should be added to section 1.1.
This is an interesting point, and an issue that we have not experienced in our work yet. We have not noticed that use of HEPES has detrimentally affected our explants but we will take this into consideration for the future. In writing this protocol it is necessary to give an accurate account of the experimental conditions that we use in order for people to replicate our methods; so here, this will not be changed. We have not seen a negative effect using phenol red either, but we appreciate these helpful technical suggestions and will consider them in the future.

5. Section 1.2.1: When discussing coating glass-bottomed dishes with basement membrane extract, the Authors indicate that the dishes can be incubated at 35 degrees "for up to 3 days". Is there a minimum time necessary for the extract to adhere?
We agree this is a little confusing the text has been clarified to contain the following: “These dishes can be used after 40 minutes incubation, or can be stored in a $CO_2$ incubator at 35 °C for at least a week.”

6. Section 1.3.1: When discussing the components required to make the silicone elastomer dishes (elastomer, curing agent, and charcoal powder), it is unclear if the authors makes these dishes from the individual components. If yes, the perhaps a series of sub-steps describing how to make the dishes is necessary. Please add the charcoal powder to the materials list.
We have included the components for the black sylgard dishes in the excel file submitted with the main document, along with notes explaining that these should be made several weeks in advance. The recipe is literally as written in the text, it is very simple so it may appear at first glance as if it is lacking steps.

7. Section 1.7.1: When drawing off the liquid, it would be helpful to describe how much liquid should remain. Draw off all the liquid? When noting that if the explant does not adhere, the instruction is to reposition with forceps- should one also draw off all the liquid again?
This is now clarified in the text. This should also be shown in the accompanying film of the process.

8. Section 1.7.2: Why are the cultures are kept at 2 degrees below body temperature?
We have found that in our hands, the explants do better at 35 ˚C.

9.. Section 2.2.1: When the explants are loosely attached, how does the "ring of media around the edge of the dish" help the cultures? Also, why is a "hinged dish cover" superior if media exchange is required?
We have clarified these points in the text “In cases where explants are loosely attached, pipette a ring of media around the edge of the dish. This extra liquid will make a miniature ‘humidified chamber’ without disturbing the explant while it continues to attach to the matrix.” “Hinged dish covers allow the lids to be opened without disturbing the samples or removing the dishes from their settings. This maintains their exact position for subsequent image captures.”

10. Section 2.2.2: Why are glass-bottomed dishes used if the imaging takes place through the upper lid?
We have clarified our reasoning for using glass bottomed dishes “Glass bottomed dishes are used for the following reasons: to ensure that the base of the dish is transparent and suitable for imaging, to create a well in the center of the dish that allows the explants to settle in an easily located area and finally so that after an experiment the sample can be processed for immunostaining and subsequent analysis.”

11. Section 2.2.3: Are the plastic lids exchanged for glass lids to aid in the imaging?
This is a point that will be much clearer when the protocol is filmed. Some of these points are specific to our system. The dais that holds the samples is designed to fit bevelled dish lids of a particular size, the lids are supplied with the microscope.

12. Line 230: "as it extends, the prosensory region narrows." It would be helpful to add arrows to the still images that show this narrowing. It is not clear in Figure 3.
We have modified Figure 3 to reflect this request.

Figures: Why is Figure 2 pseudocolored green, but not Figure 4?
Figure 2 is DIC and GFP channels combined. Figure 4 is the GFP channel only. The animation is in black and white because it is easier to see cell shapes with high contrast.

13. Line 288: What objective is used for the illustrated experiments (discussion of the pros and cons of different objectives)?
The text has been updated to state that we used a 10X objective.

14. Line 314: In summing up the advantages of a microscope incubator over confocal imaging, it is stated that "This technique will allow…study of reporter gene response to pharmaceutical agents and viability of cells at a finer resolution than previously possible." While there may be advantages of the microscope incubator system over a confocal system, it seems that the resolution is not an advantage over confocal microscopy. This statement should be revised accordingly.
As requested, this has been changed to “in more detail”.

**Reviewer #2:**

General comments

This paper describes a technique for long term time-lapse imaging of the developing organ of Corti. It is difficult to know if this technique has an interest for the scientific community since it has never been published before. The technique is tricky to develop and the quality of the images may be improved and therefore, the question is which type of project will be able to use this technique?

The reviewer raises an important question. As we show in the representative data, it will allow analysis of changes in reporter gene expression in real time in development or response to manipulations. In addition, it will complement BrdU experiments as timings of cell divisions and the number of times a specific cell divides can now be examined in a faster more accurate manner. Rather than doing multiple BrdU pulses and using single cell fate mapping, we can simply image a set of explants at higher magnification over two weeks and follow the progress of both individual cells and groups of cells. Furthermore, this would be a tremendous improvement to the currently used technique of culturing cochlear explants and only analyzing the experimental results several days later at the end point post fixation. The method is actually quite straightforward; the accompanying film will illustrate this.

Is there any live imaging in one of the paper cited by the authors a a reference for using this technique (Jacques et al., development, 2012)?

The protocol we provide here demonstrates a novel imaging approach for inner ear research. This paper will be the first demonstration that it is possible to image an entire explant developing over the course of five days. The reference refers to the use of explant cultures and GSK3 inhibitors.

The authors should really explain the advantage of this new type of microscope (and give the name of different microscopes, not only Olympus one) over other types of live imaging which used spinning disc or confocal microscopy.

We have updated our explanation as to why this technique is worth pursuing when conventional live imaging is not suitable for the question to be answered. We offer this approach not as a replacement for confocal/spinning disk microscopy, but rather as an extension of the experimental tool kit available. We give the name of the Olympus microscope because that is what we use, the materials lists asks for specific models of items that are not generic. The papers referenced in the introduction provide examples of other systems that have been tested on stem cells and embryos.

Specific technical points :

1.- At which magnification the images have been taken and how the images look like at higher magnification? In other words, would it be possible with this technique to study cellular phenomenon instead of whole tissue changes?

Yes, higher magnifications can be used; however, we are describing imaging growing tissue that undergoes morphological changes.

The choice of objective is dependent on the experiment, we are imaging whole organs so we used a 10X objective. This is clarified in the text "Imaging of stationary regions of the cochlea is possible at high magnifications. We chose to use a 10X objective in the representative results section because we wished to show the whole explant, and because the tissue movement is dynamic at early stages."

2.- What is the ciprofloxacine for? Isn't it toxic for cochlear cells?
We use this to prevent infection. It is not toxic at the dose we use.

3.What type of microscope is used: upright or inverted? and could both of those be used?
The system we use has an inverted microscope.

4. For how long the explant is cultured before imaging?
We culture the explants overnight; we have clarified this in the text.

5.What are "hinged dish covers"? (page 5)
We have amended the text to explain this better, "Hinged dish covers allow the lids to be opened without disturbing the samples or removing the dishes from their settings. This maintains their exact position for subsequent image captures."

Reviewer 3
This submission describes the long-term culture of the organ of Corti using an incubator microscope, so that time-lapse images can be obtained frequently. Since the organ of Corti undergoes dramatic growth and patterning in the time period described (E13-birth), this technique will be especially useful in visualizing those events. Although a number of studies have examined cochlear development, many aspects of its growth and differentiation are still not entirely understood. This technique has the potential to reveal novel aspects of cochlear growth and patterning, due to the ability to image more frequently. As the microscope and the imaging software are the novel features of this technique, extra time/space should be spent describing these elements. For example it was not clear whether the time-lapse imaging was an automated feature or was done manually. Also, other than ease of use (which is important) and ability to image more frequently (also very important), are there any other reasons that this method is superior to manually imaging a culture that is maintained in a regular incubator and imaged at regular intervals? For example, do artifacts arise in the culture due to temperature, pH etc shifts when shifting between incubator and microscope? Is it difficult to align the cochleae to get a time-lapse video when done manually?
These are very helpful comments and we have updated the text to take them into account. The introduction has been amended to include the suggestions:
"We define 'incubator microscope' as an inverted microscope sealed inside a standard $CO_2$ incubator, rather than an incubator built around the microscope. An incubator microscope extends the life of the experiment such that rather than imaging over two or three days, samples can be imaged for up to two weeks. An incubator microscope provides an excellent environment for cell growth and

differentiation, with minimal disturbance to explant cultures and standard controlled conditions. In studies that take place over multiple days it is common to resort to imaging samples on a daily basis by removing them from the incubator and carrying them to an inverted fluorescent microscope. While this approach can work, removing the dishes from the incubator inflicts stress on the sensitive developing tissue. Changes in acidity of the culturing medium and fluctuations in temperature due to removal from the incubator can result in suboptimal development and unhealthy tissue. Imaging the same region at the same focal plane and in the same orientation at every time point is extremely challenging. By using an automated system within an incubator, it is possible to maintain healthy tissue, to collect images at more time points and to ensure that the same area is captured in every frame. In recent years several integrated microscope tissue incubators have been developed, these have been useful not only in clinical practice [9] but also in stem cell and cancer research [10,11].

Here we present a protocol for long term live imaging of embryonic mouse cochlear explants. We use an automated microscopy system inside a standard $CO_2$ incubator that has the capability to capture images of multiple samples at set time points. The system consists of an inverted microscope set inside an incubator. Samples are placed in a rotating dais that allows imaging of multiple samples at each time point. Illumination, image capture and rotation of the dais are controlled by an automated system operated through Metamorph software. By setting an imaging routine using the operating software we can set an experiment to run for up to two weeks with minimal human intervention. In this example we use both bright field and fluorescence to show large-scale growth and rearrangement of the cochlea, and specifically, the prosensory region. In this experiment, cochleae will be dissected from *Sox2*$^{EGFP}$ reporter mice on embryonic day E13. *In vitro* cultures will be established and then imaged over five days."

Specific comments:

Line 52-53: The cochlear cells are not fully differentiated a few days after birth—this sentence should be revised.

We have revised this statement to "and completion of the developmental program".

Line 56-57: The hair cells do not sit atop the supporting cells—the nuclei do, but not the cells themselves as it's a pseudostratified epithelium

This has been corrected to "interspersed".

Line 47 vs Line 90—how many days were the organs cultured for? In some cases it was stated 5, then other places it was stated 6 days, please clarify.

This has been clarified to 5 days throughout.