**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.

1. “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 CO2 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 CO2 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](#_ENREF_9) but also in stem cell and cancer research [10](#_ENREF_10),[11](#_ENREF_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 CO2 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 *Sox2EGFP* 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.