We thank you very much for the interest you gave to our manuscript and for the interesting comments you provided. We have answered all the comments; the modifications in the text are highlighted in yellow.

The important sections for the video are in red.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the following lines to avoid previously published work: 35-37, 44-45, 137-141. The sections were reviewed and I added the references.
3. Please provide an institutional email address for each author. Please revise the title to make it concise without any punctuation marks. We have adjusted the title accordingly
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). all the personal pronouns were removed from the manuscript
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. I removed the commercial symbols from the text
For example: Zeiss, Eppendorf, Arsenazo-III, POINT SCIENTIFIC, etc. Arsenazo is a common name for a chemical and not commercial
6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. I added a statement about the ethic before starting the description of the protocol
7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. I adjusted the numbering as required
8. Line 57-61: Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. (Examples: What is the volume or concentration of isoflurane used. Is the animal revived after the surgery? If not, why is the animal anesthetized and not euthanized?) I added Figure 1 with image description for valve dissection. In the main manuscript more details were added and they are highlighted in yellow.
9. Line 68/76/81: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2 (Line: 68,76,81,109, 108,110, 123, 142, etc.). I corrected the unites as described
10. Line 81/101: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks. The units were corrected as required
11. Line 101-104/114: Please provide all the details necessary to replicate the experiment. What is the volume of PBS used for washing? I added the washing volume in each step.
12. Line 113/115: Please specify the volume of the primary and the secondary antibodies used. I used 50 ul of diluted antibody per sample. The modification is in the text as well.
13. Line 136-141: Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use

of hoods, etc.
14. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. The important steps for the video are highlighted in red.
15. Please move the Figure Legends section to the end of the Representative Results Section. Please upload each figure individually to your editorial manager account. Modifications were performed
16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. I changed the structure of the bibliography to meet the requirement of eh journal.
17. Figure 2: Please revise the figure to include figure labels to make the figure more informative. Please ensure that the description in the figure legend is based on the figure label. Please include scale bars in all the figures of the panel. Please define the scale bar and provide the details of the magnification in Figure Legends. Scale bar is 200 um. I added the details in the legend
18. Figure 3: Does the Y-axis in Figure 2A require units? If so, please include the units within the parenthesis. Please capitalize the Figure labels. There is no unit, because it is a ratio of mg/ml of calcium by mg of proteins
19. Please sort the Table of Materials in alphabetical order.

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Reviewers' comments:

Reviewer #1:

We thank you for the comments that will help the reader to better understand the protocol.

Manuscript Summary:
The authors describe a method to isolate murine valvular interstitial cells, propagate them under cell culture conditions, and characterize them by immunocytochemistry and in an in vitro calcification assay.

Major Concerns:
1. Please start section A of the Protocol part (line 56) with a list of solutions that should be prepared before the procedure starts. Specify the precise composition of all ingredients (as absolute concentration or percentage) and the volume needed for the entire procedure as well as the use of the solutions (wash, culture, etc.). Define a name for each solution and use the name throughout the manuscript. Indeed, I added a section with the name of the solutions and I highlighted them in yellow. This will improve the precision of the technical language and help to make the protocol easier to understand. Do you use HEPES as 1 M solution as stated in the materials list on the last page of the manuscript or at a dilution? I thank you for this important question; yes I forgot to put the dilution. Now I corrected. The HEPES should be diluted to have a final concentration of 10mM

2. Lines 70-73: This step is critical for the procedure. It would be helpful to prepare a set of graphical delineations as Figure 1B (with the present Figure 1 of the workflow as subfigure 1A) to explain where and in which order the cuts of the heart should be set. Give approximate dimensions on the delineations. Thank for the interesting comment, I added new figure 1 with valve dissection details.
3. Line 76: It is not clear how the collagenase solutions at different concentrations should be prepared. "1 mg/ml and 4.5 mg/ml in 5 ml DMEM and 5 ml HEPES" could mean a total volume of 5 ml each in the respective solvents or a total volume of 10 ml each in a 1:1 mix. See comments under point 1. Also, the Notes in lines 77-78, lines 83-84, and lines 98-99 can be avoided with a proper materials list. Each solution is a mixture of 2.5 ml of HEPES and 2.5ml of DMEM. More details were added to main manuscript.
4. Lines 85-86: Does this step require one wash only or one wash, centrifugation, a second wash, and another centrifugation? Please specify what to expect in the solution and pellet, respectively (tissue/endothelial cells). It's only one wash, please see the main manuscript with the required modifications.
5. Lines 87-88: Please specify the time of incubation. What do you mean by "under continuous agitation"? Please specify the shaker and the detailed settings used. Is agitation also needed during the first collagenase digest (lines81-82)? Is a centrifugation step needed at the end, as the next step starts with a re-suspension. The agitation is needed during the incubation of both collagenase solutions. Since this article will be accompanied with a video, the reader will better understand this step.
6. Line 91: Please specify whether the pellet should contain pieces of tissue or cells. at this stage the pellet will have both tissue and cells
7. Instead of using weak phrases like "good number of cells" (line 57) or "Murine aortic valve are tiny" (line 155), please use precise technical language and specify the approximate size of a murine aortic valve cusp. Also, please specify the approximate number of cells that you usually obtain after the 2-day incubation (line 93) and at 70% confluency (line 96). This will help users to troubleshoot if problems may occur. We adjusted the number of cells. However, it's just indicative because it may vary from a user to another one and it is also dependent on the number of mice used for the cell culture.
8. Line 100: Please specify that you used immunocytochemistry as a method to characterize the cells. Also, as compared to e.g. flow cytometry, this method is not so well suited to characterize the "purity" (lines 100, 105) of the cells but rather characterizes the morphology and identity of the cells. we thank you for the comment, in this paper I described mainly cell isolation protocol. Aortic valve tissue is mainly endothelial layer and interstitial cells. We use both western blotting and immunocytochemistry to identify the cells. However, the FACS could be used to purify a specific cells.
9. Lines 105-106: Do you use antibody against CD34 as stated or against CD31 as described in the materials list on the last page of the manuscript? Please specify at which concentration you used the respective antibodies. CD31 was the antibody used.
10. The calcification assay is a very basic method used in the field of aortic valve research. In section C of the Protocol part (line 122) you describe in detail the individual steps of a calcification assay from a specific company (Point Scientific). Similar calcium assays are available from other companies (e.g. Calcium Colorimetric Assay from Sigma Aldrich, #MAK022-1KT). Therefore, I don't see the rationale as to why promoting this specific product. Furthermore, a wide array of calcification assays has been described by now using different detection methods (vis microscopy, absorbance, fluorescence, near infrared fluorescence, etc.) and target analytes (free calcium vs. hydroxyapatite).

It would be more beneficial for this manuscript and helpful for the field to give here an overview of the different assay variants with the respective references and advantages/disadvantages of the respective methods. This may be provided instead of or in addition to the method and results described. We wanted just to clarify that we don't have any specific preference for the company. The Arsenazo III could be prepared in the lab. We are interested to the calcification of the cell membrane as a mimic to the in vivo calcification of the artic valve. As described in the protocol, this reagent doesn't interfere with magnesium. We have tried several method and we figure out that Arsenazo is the most accurate method.

11. In the Discussion part of the manuscript you should elaborate more on species specific characteristics of VICs (as already started with lines 191-194), stressing in particular the advantages and disadvantages of murine VICs in the study of human disease. Furthermore, why do you use and describe here a tissue culture and "out-growth" method instead of a full tissue digestion and straining of cells as used e.g. for the isolation of human VICs? Mouse cells are more sensitive to the collagenase; longer exposer might affect the cells. After 35 minutes we will have isolated cells, we keep the issue to ensure isolating all the cells.

Reviewer #2:

Manuscript Summary:

This method is of potential used by interested audience and worth publishing.

Major Concerns:

As the tile states, the method should focused on the isolation of mouse interstitial valve cells. We thank you for you interest to our paper and for all your comments. We have corrected the title accordingly

Minor Concerns:

The late steps of the isolation of interstitial valve cells need more detailed information such as the culture volume, anticipated initial cell density in 35 mm dish and expansion ratio, etc.

Figure 1 should eliminate the pictures of instruments and instead make a detailed flowchart for the steps of isolation. Adding photos and microscopic images of the final dissection of valve tissue and low magnification images of good quality fresh isolated cells and after passage will be helpful.

We have added a new figure 1 with valve dissection details. In addition, we will record a video of all the steps of the isolation.