Reviewer #1:

Manuscript Summary:

In this study entitled "Isolation of human ventricular cardiomyocytes from vibratome-cut myocardial slices" the authors describe an efficient method to isolate functional cardiomyocytes (CM) from human and animal myocardial slices. The authors compare this approach with currently gold standard methods such as coronary perfusion and tissue-chunk digestion.

The results show that myocardial slices are indeed a valuable and useful alternative for CM isolation and that CM isolation from myocardial slices is more efficient than isolation from tissue-chunks. The protocol is well written and easy to follow and it will indeed help other groups to replicate this approach and expand the utilization of this method.

We thank the reviewer for the positive comments on our manuscript.

Major Concerns:

I do not have any major concern

Minor Concerns:

Below is a list of minor changes that could further improve the manuscript:

1. The vibratome settings for the z-vibration are not indicated (in other paper it is indicated <1µm).

With the mentioned infrared-assisted device to measure z-deflection, we minimized the value to below 0.1 µm. The cutting angle of the blade was 15° We added these details to the protocol.

2. Paragraph 339-346 some more info related to Ca2+ transients and action potentials should be included (time to peak, t50, t90, decay rate…)

We performed additional analyses determining the time to peak, the time to 50% and 90% repolarization of action potentials (APD_{50}, APD_{90}) recorded from human cardiomyocytes. In addition, we determined the maximal amplitude of the calcium transient F/F_0 of human cardiomyocytes and the relative shortening. The respective values are now given in the text of the results part.

3. Fig 1 please better indicate the re-equilibration steps with the actual steps indicated in the manuscript

The individual steps are now numbered according to the protocol. We have also added more detail on the re-addition of calcium (Step 7).

4. Fig 2 the graph could show the individual values for the reader to appreciate the variability between samples.

The graph was changed, now showing the individual datapoints of the experiments.

5. Discussion 432-439: in order to isolate CM with coronary perfusion a Langendorff system is often required. This is an expensive and complex system. Myocardial slices (as indicated in the limitations) is also time consuming and requires specific skill and access to a vibratome, still it should be emphasised that new and simpler methods for human specimens are needed.

The requirement of new methods for human cardiomyocyte isolation is now emphasized at the beginning of the discussion.

6. Line 448 Watson et al (Nat Prot 2017) have shown that in myocardial slices cell damage is limited to the surface of the preparation. This supports the author's justification for improved CM number. This paper should also be acknowledged as the only detailed protocol for myocardial slice preparation available in the literature.

We thank the reviewer for pointing out the importance of the reference. We now reference the publication of Watson et al 2017 and emphasized its importance as it provides a very nice and detailed protocol for myocardial slice preparation.

7. Line 524 long term systems for myocardial slice in vitro culture are briefly mentioned. The bibliography however is quite old. In 2019 three papers have shown novel biomimetic culture systems able to better recapitulate tissue physiology and limit in vitro tissue remodelling (PMID:31092830, PMID:30671746 and PMID:21972180). These are superior to previous methods and they should be cited.

We added the corresponding references, referring to the recent improvements and optimizations of the culture conditions for myocardial slices.

8. In the protocol mechanical sheer stress from the pipette is proclaimed harmful. The introduction of a step to pull apart the slice with a forceps, could also lead to harmful mechanical stress. This could be made an optional step.

We agree that every mechanical intervention may cause shear stress and, consequently, cardiomyocyte damage. However, from our experience a dissociation of the tissue is a prerequisite for sufficient liberation of cardiomyocytes and should not be indicated as an optional step. Nevertheless, we acknowledge, that dissociation by forceps should be done gently and with great care. Therefore, we now explained this issue in more detail in the “Note”.

9. Point 8.4 the minimum time for CM to sediment is indicated. It would be informative to also have the max time for the CM to left in the tube.

We now provide a maximum time value of 30 min for sedimentation, which should not be exceeded due to limited oxygen supply.

10. In the discussion the advantage of cell isolation is clearly described. It would also be interesting to mention the possibility to isolate other cell types such as cardiac fibroblasts, endothelial cells etc. (with an adapted protocol). This will further expand the utilization of this approach.

We agree that this could be an additional application of our protocol. We now mention that, in principle, and with adequate modifications, the protocol could be adapted to isolate cardiac

fibroblasts, endothelial cells or macrophages from myocardial slices. For protocol modifications and cultivation of isolated cells we refer to an earlier publication (Eghbali-Webb, M. & Agocha, A. E. Novel Methods in Molecular and Cellular Biochemistry of Muscle (eds. Pierce, G. N. & Claycomb, W. C.) 195–198 (Springer US, 1997). doi:10.1007/978-1-4615-6353-2_19.)

Reviewer #2:

Manuscript Summary:

The study from Fiegle et al presents a myocardial slice-based method for the isolation of ventricular cardiomyocytes from rats and humans. The method appears promising, but some additional characterization on the quality and function of the isolated cells is required to properly assess the utility of the method.

The rationale for this study is sound and presented clearly; indeed, human cardiomyocyte isolations are challenging, particularly for groups without easy access to cardiac surgical facilities or to very large pieces of myocardium that can be perfused through the coronary vasculature. Improved methodologies for small chunks of human tissue are needed (and the "tissue chunk method" attempted by many in the past is very limited, as the authors confirm here).

The paper is well written - the methods are detailed, user friendly, and seem reasonable. The comparisons that the authors do make are clear and analyzed appropriately. However, the very limited functional characterization of the slice-isolated cells restricts the ability of the reader to discern just how useful this approach may be, particularly when compared to the gold standard of isolation via tissue perfusion.

Major Concerns:

1. The flow cytometry-based quantification of percentage of rod-shaped myocytes in figure 2 is useful and clearly demonstrates the utility of perfusion vs. slice vs. chunk approaches in rats. The quantification of cell yields and viability for slice vs. chunk methods for human isolations in figure 3 is also useful. However, cell viability does not necessarily ensure cell quality for functional assays or cell culture, and the structural/functional characterizations in figures 4 and 5 are limited to single representative examples that are not particularly informative. Using the rat as an easily accessible benchmarking tool, the authors should compare other key metrics of cell morphology and function between perfusion and slice isolated cardiomyocytes. This is partly motivated by the apparent difference in cell quality suggested from the images in Figure 2A. These metrics should include at least cell length, width, resting sarcomere length, percent excitable cells (rod shaped cells that respond to field stimulation) and fractional shortening.

We recognize that Figure 2A was somewhat misleading because the images for “Perfusion” and “Myocardial slice” were acquired at different magnification. This might have contributed to the perception of differences in myocyte quality. For this reason, we acquired new photographs providing more representative example images. These were recorded with the same lens and magnification (see Figure 2B). In addition to the representative examples shown in Figure 4 and Figure 5, we now also provide statistical data recorded from human cardiomyocytes from action potential measurements (TTP: 59.18 ± 7.07 ms, APD50: 400.58 ± 41.06 ms, APD90: 748.87 ± 56.62 ms n =10 cells) and calcium transients (maximal F/F_0 2.9 ± 0.5 AU, relative shortening 4.6 ± 0.8 %, n=31 cells, respectively) in the results part.

To address the quality of the isolated rat cardiomyocytes, we added an additional figure (new Supplemental Figure 2) where we show cardiomyocyte metrics (length and width), assessed by microscopy of fixed cells, isolated either via perfusion or from myocardial slices. Length and width did not differ significantly (length: 110.0 ± 5.4 µm vs 99.4 ± 3.2 µm, p=0.08, width: 33.9 ± 1.9 µm vs 30.6 ± 1.2 µm, p=0.08, for n=19 and 21 cells, respectively). In addition, we analyzed the sarcomere length in alpha-actinin stained cardiomyocytes by Fourier analysis and found no significant difference

(1.62 ± 0.04 µm vs 1.68 ± 0.04 µm, p=0.28, n = 24 and 23 cells for perfusion vs myocardial slice respectively). Furthermore, we compare the mean activation time, maximal signal of the calcium transient (F/F_0), and fractional shortening. These parameters were assessed by confocal calcium imaging after Fluo-4 loading as described previously (Seidel et al. 2019, BRIC, PMID 31673803).

For the experiments, addressing the fraction of contractile cardiomyocytes please see the response to point 2. below.

2. Ideally, these assessments would also be carried out in 48hr cultured cells to demonstrate that slice-isolated cells are amenable to cell culture. Perfusion-isolated rat cardiomyocytes hold up very well in short term culture, importantly allowing high quality gene transfer studies followed by functional assessments. As an example for why such a demonstration is needed, viability from mouse cell isolations can be quite high (>80%) and remain high after 24-48 hrs in cell culture, but these cells are notoriously (and mysteriously) difficult to field stimulate after time in culture (although a few can always be found), and are thus typically not considered acceptable for gene transfer studies. Additional data showing that the slice-isolated cells are healthy, functional, and can (or cannot) maintain their function in culture would greatly strengthen the appeal of this approach and add clarify its usefulness.

We performed an additional experiment using rat hearts and compared the fraction of rod-shaped cardiomyocytes that respond to electrical field stimulation directly after isolation and after 48 hours of cultivation. From the new Supplemental Figure 2, G, H it can be appreciated that 62.1 ± 2.9 % of the cardiomyocytes isolated from myocardial slices are responding to electrical excitation with contraction. After 48 hours of cultivation 31.5 ± 2.3 % of the cardiomyocytes still responded to stimulation. Although, these values were higher in myocytes isolated by perfusion (fresh: 74.3 ± 4.2 %, and 48 hours: 41.9 ± 3.6 %, $p<0.05$), the difference of approx. 10% seems to be well acceptable. In conclusion, the contractility and survival of cardiomyocytes isolated from tissue slices is sufficient for downstream functional experiments and cultivation. We would also like to refer to our recent publication (Seidel et al. 2019, BRIC, PMID 31673803), where we kept human cardiomyocytes isolated with the described protocol in culture for up to three days.

Minor Concerns:

1. I could not find any patient details on the human tissue utilized for these studies, other than to say they were from LVAD cores or explanted hearts. Were the explants non-failing or failing hearts? Did the success of isolation (the authors mention that 21 out of 27 were successful, what is the benchmark of success?) correlate with etiology? Others have found that LVAD cores are more difficult to isolate from then LV free wall, did their success with isolation depend on the tissue location? These additional details will help the user. Also, LVAD cores have been a challenge for many groups, and thus demonstrating convincingly that their slice method can yield high quality cells from LVAD cores will benefit the field and should be robustly demonstrated and highlighted.

We now provide a table listing the total number human specimens used in this study. As stated in “Table 3: Patient data”, we successfully isolated cardiomyocytes from 8 of 8 LVAD cores. In addition, there was no prominent difference of isolation success when regarding disease etiologies. However, we found that transportation times above 36 hours generally reduce the probability of a successful outcome of the experiment. An experiment was defined as successful, when the isolation yielded sufficient viable cardiomyocytes to perform the mentioned downstream experiments (more than

approx. 100 rod-shaped myocytes per dish). To emphasize the possibility to isolate cardiomyocytes from LVAD cores we mentioned the origin of human specimens again in the discussion.

Reviewer#3:

Manuscript Summary:

In the present study Dr. Fiegle et al. explain a protocol to isolate human (and rat) ventricular myocytes from vibratome-cut slices in order to perform calcium handling experiments with freshly isolated myocytes. Human myocytes isolation is always an interesting technique, specially if the myocytes can be used for electrophysiological experiments. The protocol is well described and it allows to obtain good quality cells. However, I have some major concerns:

Major Concerns:

- the authors mention that digestion time is quite variable and recommend to increase time of digestion of enzymes concentrations. This is really unprecise. If all these conditions must be tested in "each laboratory" as the authors say and with each human tissue samples (because its variavility from patient to patient is very high) it seems unlikely to be able to reproduce the protocol just by reading this paper. What are the specific timings for every step of the protocol? How many tests are needed to find the right conditions for every tissue in different laboratories? Also, what happens with small biopsies? Although in the picture of Fig 1 there are 2 cubes of 1x1x1 cm many biopsies would be by large much smaller and that means perhaps just 1 test per tissue…

We thank the reviewer for his/her positive feedback. As we mention in the protocol Collagenase digestion can be quite variable, which does not allow for a general statement for a definite digestion time. However, we used different batches of collagenases and repeatedly observed that a digestion time between 30-40 min gave the best results. Furthermore, human myocardial tissue itself is quite variable. We noticed that fibrotic samples from old patients tended to require longer digestion times (40 min), whereas samples from children required less (30 min). Rat myocardial slices, which show much less variability, usually required 30 min. Following the steps provided in the protocol should lead to a successful outcome. Variation of the digestion time between 30-40 min could however slightly increase the yields of viable cardiomyocytes. If enough sample material is present, it is feasible to perform multiple experiments in parallel to quickly approach the best conditions. Indeed, for very small biopsies, as shown in Supplemental Figure 4, this is impossible as only a small number of slices can be generated. We now describe, that a regular checkup of the tissue texture during the digestion is also recommended. This makes it possible to determine the optimal digestion time also when only little sample material is available.

- What is the advantage of the agarose inclusion and posterior vibratome-cut other than the transport? So, what is the advantage of the proposed agarose inclusion method for the isolation it self? Because if it is just the transport it seems a very complicated method to allow transport of tissues which could be transported in cold cardioplegic solution as for example custodiol

Embedding the cardiac tissue into agarose is only required for cutting on a vibratome. It improves the mechanical stability of the tissue block and ensures myocardial slices of uniform thickness (We refer to recent articles, which describe the generation of myocardial slices in detail accordingly (Fischer, C. et al. Nature Communications **10**, (2019), PMID: 30631059; Watson, S. A. et al. Nature Protocols **12**, 2623–2639 (2017), PMID: 29189769.
However, we do not recommend to embed the tissue in agarose for transportation because the agarose might compromise diffusion of oxygen and nutrients. Transportation should be carried out with the tissue being submerged in cold cutting solution immediately after excision from the heart.

We added a more detailed description in the paragraph “Transportation of cardiac tissue” to clarify this issue.