**Editor comments**

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors. done

• After the Discussion, please add an “Acknowledgments” section. Please list acknowledgments and all funding sources for your work in this section. done

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. done  
  
• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.).  
  
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution. done  
2) 2.1: How is the heart removed? Is it simply pulled? How is it handled? Using forceps?” done

We obtained approximately 5x105 cells per heart.” should likely appear later after cell dissociation. We changed the sentence from 2.1 to the technical comment 2.6   
3) 2.4: How? Is any centrifugation involved? Please mention centrifugation settings (in x g), duration and temperature requirements. done  
4) 2.7: Should this be “conical” tube?done

What medium are cells resuspended in following centrifugation?done.

We described the preparation of medium in 1.2 and termed it “cell culture medium”. For clarity this term is now used throughout the whole protocol.   
5) 3.1: What is the composition of the cell culture medium? done. Please see point 4)  
6) 3.4: Please mention trypsin concentration and duration. Is the trypsin neutralized later? done  
7) 3.5: The cells are first resuspended, counted, and then buffer is used to adjust the cell concentration, correct? Please revise this for clarity. done

The cell number is determined before centrifugation. This allows to adjust the appropriate cell concentration for transfection right after centrifugation using the electroporation buffer.   
8) 3.6 What volume do you load? done  
9) 3.7: How? When is the cuvette loaded? done

What is the voltage/electric field strength used? What is the duration of electroporation?

To perform electroporation, we have used the Amaxa nucleofector II device using the pre-installed program G-009. Since we did not adjust the electroporation parameters by our own, we cannot provide detailed information about voltage and/or duration of the electroporation.

We have mentioned the transfection device and the used program in the materials list/protocol.

10) 3.8: How many cells are seeded? Is this then incubated? If so, please mention incubation environmental conditions? done  
11) 4.3: Please mention magnification and NA. Used objective is not mentioned in 4.4  
12) 4.3, 4.4, 4.5: Please expand significantly and provide detailed instructions for each step. For software steps, please mention what button is clicked on in the software or which menu items need to be selected to do each action. All steps were performed using Zeiss Zen2011 software. We have included detailed information for each step, including the menu items required to adjust FRAP settings.13) 5.1: please mention what button is clicked on in the software or which menu items need to be selected to do each action. done  
14) 5.2: please mention what button is clicked on in the software or which menu items need to be selected to do each action. done

15) 5.3: please mention what button is clicked on in the software or which menu items need to be selected to do each action. Background and reference cell-based corrections (5.2) as well as normalization (5.3) were done using Microsoft Excel. We have now mentioned that FRAP data acquired with Zeiss Zen Software is imported into one Excel file for calculation and described the calculation process in more detail.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step. done  
  
• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps that match the title and abstract. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.  
3) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.  
4) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
5) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length (2.75 pages or less). Please see JoVE’s instructions for authors for more information.  
6) Notes cannot be filmed and should be excluded from highlighting.  
7) Please bear in mind that software steps without a graphical user interface cannot be filmed. done  
  
• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. The discussion part was rearranged and information about modifications of the protocol has been added.  
  
• **Figure/Table Legends:**:  
1) Figure 2: Please define the scale bar. done  
2) Figure 3: Please define the scale bar. done  
  
• **References:**Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]  
1) Please abbreviate all journal titles. done  
2) Please include volume, issue numbers, and DOIs for all references. done  
  
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2) Please remove the registered trademark symbols TM/R from the table of reagents/materials. done  
  
• **Table of Materials:**Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as microscopes, equipment, animal strains, etc. done. Changes are marked in red color.  
  
• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit. done  
  
• Please minimize use of the pronouns “we” and “our” throughout the manuscript. done  
  
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**Reviewer comments**

**Reviewer#1:**

1. *In the Protocol section 2.5, total incubation time should also be mentioned.*

We agree with the reviewer that the time of enzyme digestion is important for the isolation process. Information about incubation time for enzyme digestion has been added (page, 4, protocol step 2.5, line 138).

1. *In Fig 2. In Connexin 43 knockdown experiments along with 3D- FRAP analysis, additional data showing RNA or Protein levels of connexin 43 would complement the study.*

We thank the reviewer for his advice. According to the reviewers suggestion we provide new data about the effect of Cx43 knockdown on the protein level, which is depicted in Figure 3, new subfigure C. Both knockdown and scrambled transfected cells were subjected to immunostaining with anti-Cx43 antibody. Confocal microscopy clearly demonstrated the efficient reduction of protein expression upon Cx43 knockdown compared to control cells treated with scrambled siRNA.

**Reviewer#2:**

1. *There is a possibility, even it may be rare, that fluorescent-tag was lost from miRNA. So, the measured FRAP actually reflects the passage of fluorescent-tag rather than miRNAs. It may be necessary to carry out one control experiment to measure FRAP with the same fluorescence dye without miRNAs. They may have different dynamics because there are different sizes between fluorescent-tag and fluorescent labeled miRNAs.*

We agree with the reviewer that there is a small possibility that the fluorescent-tag detaches from the miRNA. Therefore, we performed FRAP experiments with calcein, a commonly used dye to analyze intercellular communication *(Maeda et al., 2015; Karpinich et al, 2015; Kim et al., 2014*). Once inside the cell, calcein can only diffuse via gap junctions. The molecular weight of calcein is similar to the molecular weight of Dy547 (calcein: ~780g/mol; Dy547: ~640g/mol) and thus will give comparable results in terms of diffusion dynamics.

Since calcein has a smaller size than labelled miRNA molecules, the latter one should demonstrate a slower gap junctional transfer. Indeed, we found that the intercellular exchange of calcein is significantly increased compared to labelled miRNA (Figure 3, new subfigure D). The data indicate that the FRAP data acquired for labelled miRNA actually reflects the intercellular exchange of miRNA molecules.

1. *Although the manuscript was well-wrote, some important references are missing, for example: Zong L, Zhu Y, Liang R, Zhao HB. (2016) Gap junction mediated miRNA intercellular transfer and gene regulation: A novel mechanism for intercellular genetic communication. Sci Rep. 6:19884. Please add such references in the appropriate places, e.g., page 2, line 73-74, line 85-86, and page 7, line 271-272.*

We thank the reviewer for his suggestion and included the reference in the manuscript (page 3, Introduction, line 94)

References

[1] E. Maeda, T. Ohashi, Mechano-regulation of gap junction communications between tendon cells is dependent on the magnitude of tensile strain, Biochem. Biophys. Res. Commun. 465 (2015) 6–11. doi:10.1016/j.bbrc.2015.08.021.

[2] N.O. Karpinich, K.M. Caron, Gap junction coupling is required for tumor cell migration through lymphatic endothelium, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 1147–1155. doi:10.1161/ATVBAHA.114.304752.

[3] Y.J. Kim, J. Kim, C. Tian, H.J. Lim, Y.S. Kim, J.H. Chung, et al., Prevention of cisplatin-induced ototoxicity by the inhibition of gap junctional intercellular communication in auditory cells., Cell. Mol. Life Sci. 71 (2014) 3859–3871. doi:10.1007/s00018-014-1594-3.

**Reviewer#3:**

1. *Overall, the experimental descriptions are adequate and straight-forward; however, the purity of the cultures should be described because there will be contaminating fibroblasts - even with the pre-plating steps.*

We agree with the reviewer that purity of isolated CMs is an important aspect in this protocol. To address this question we performed labeling of isolated cardiomycoytes with an anti-α-actinin, following microscopic quantification. We found that the isolated cell fraction contains 20.38±2.75 % non-cardiomyocytes (α-actinin negative cells), while ~79.62 % demonstrated the expression of α-actinin (n=320 cells; Figure 1, new subfigure B).

Furthermore, we have previously shown that the non-cardiomyocyte fraction demonstrates no coupling activity (*Lemcke et al.,2016*, Figure 6). Calcein based FRAP analysis showed no fluorescence recovery in non-cardiomyocytes (fluorescence recovery at t=13 min: 3.3 %). Moreover, non-cardiomyocytes lack the expression of Cx43 protein (*Lemcke et al,2016*, Figure 6), indicating that contaminating fibroblasts do not interfere with the FRAP data for cardiomyocytes.

1. *At the same time, the authors should include some information regarding the survival of the cells following detachment from culture plates, electroporation, and re-attachment for FRAP.*

We thank the reviewer for his suggestion. To acquire data about the viability cells were prepared according to the FRAP protocol (cardiomyocyte isolation, electroporation and re-attachment) and subjected to Live/Dead analysis using flow cytometry. A representative flow cytometry plot is presented in a new Figure 2 (Figure 2B), showing that the amount of dead cells is about 3.12 % on the day of FRAP experiment.

1. *There should also be some more information regarding the technical aspects of these procedures (e.g. what kind of culture chambers are used for re-attachment, what are is the density of cardiomyocytes, what is the transfection efficiency).*

We appreciate this advice of the reviewer and we have included additional information about used chamber slides in the manuscript (page 5, protocol step 3.9, table of materials). Moreover, we provide new flow cytometry data, showing the transfection efficiency for miR547 (44.72 %, Figure 2, A). These results are comparable to our previously published data, were electroporation of neonatal mice cardiomyocytes with labelled siRNA led to a similar transfection efficiency (*Lemcke et al, 2016*; Figure 2D, 40.08±1.67 %).

To clarify used cell density for FRAP experiments, we included a new microscopic image and provided information about the density of cardiomyocytes in the manuscript (Figure 2C, page 5, protocol step 3.9).

1. *In addition, because the investigators used a super-resolution microscope for these studies that is capable of making optical sections of 84 nm and illuminating the samples with 5 structured illumination grid patterns, they need to more fully describe the acquisition and image reconstruction parameters. The authors should also be more transparent about the time required to process these images. This technique is definitely useful; however, it does not provide 'real-time' information and the processing parameters need to be carefully controlled to avoid false positives.*

We thank the reviewer for his remark and we agree that structured illumination microscopy is not suitable to investigate fast cellular processes in live cells. In our case, structured illumination was only used to acquire high resolution images of isolated cardiomyocytes (Figure 1, A) and not for FRAP microscopy. Therefore, we believe that a very detailed description of super resolution imaging is not required to apply the FRAP protocol described in our manuscript. However, we have referred to our previous publication, which summarized the application of structured illumination, including used processing parameters (Figure 1, Figure legend).

References

[1] Lemcke, H., Peukert, J., Voronina, N., Skorska, A., Steinhoff, G. & David, R. Applying 3D-FRAP microscopy to analyse gap junction-dependent shuttling of small antisense RNAs between cardiomyocytes. J. Mol. Cell. Cardiol. 98, 117–127, doi:10.1016/j.yjmcc.2016.07.008 (2016).