Dear Dr. Iyer,

we would like to thank you and the referees for your careful consideration of our manuscript "Advanced Cardiac Rhythm Management Applying Optogenetic Multi-Site Photostimulation in Murine Hearts" (JoVe 62335) for publication. Based on the insightful comments and assessment of the editorial and reviewing members, we have now made significant changes to our original manuscript. In the text below we provide a detailed point-by-point rebuttal of the editorial and referee comments.

Yours sincerely,

Claudia Richter

– Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide the complete addresses of the affiliations.
The addresses were added as requested

3. Please provide an institutional email address for each author.
Email Addresses were added for every author

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Thank you for the suggestion, we have removed the personal pronouns and in some cases the wording "The authors..." is used

5. JoVE cannot publish manuscripts containing commercial language. 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: e.g., LZ4-40B208-0000, OSRAM, ET470/40x, Chroma, M625L3, Thorlabs, Semrock, Navitar, Photometrics, BS4 73-0200, Hugo Sachs Elektronik, AD MP150, Biopac Systems, Agilent, Cree Europe, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
All commercial names were deleted and documented in the Table of materials. We try to explain the steps of the protocol as detailed as possible with generic terms

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. 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.
We have taken this into account and tried to improve all the sentences in the protocol. Notes were also used when needed

7. The Protocol should contain only action items that direct the reader to do something. Please

move the discussion about the protocol to the Discussion.

We have carefully checked the protocol and have moved the superfluous text into another position or have added a Note wherever needed

8. Line 105/182: Please use standard abbreviations for time units preceded by a numeral. Examples: 3 s, 5 min, 4 h, 2 days.

Thank you very much for this indication. We have modified the text as advised.

9. Line 108: Please specify how are the solutions deaerated. What is the volume of solutions used?

To clarify this point we have added the following point to the protocol: 1.1.3 Fill 500 mL of each Tyrode solution in the corresponding reservoir and deaerate the tubes as well as the bubble trap by running Tyrode solution through the perfusion system until no more trapped air bubbles are seen in the tubes or in the bubble trap.

10. 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 mikroL, 7 cm2 (Lines: 176, 182, 185, 188, etc.)

The SI Units have been revised and changed when needed

11. Line 191/194: Please ensure that the corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) for in-text formatting.

We have thoroughly checked the text and have changed all references in the text to superscripts

12. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

We have changed the legends so that the nonessential text is now mentioned in the discussion or in the representative results as necessary

13. Figure 4: Please label the panels as per the details provided in the Figure Legends. Ensure that all the labels mentioned in the Figure Legends appear in the Figure.

Thank you for pointing this out. We have added the Letters as corresponding to Figure 4

We have tried to address all concerns raised by the referee and have incorporated the suggestions wherever possible, hoping that these adjustments will increase the clarity of the language and presentation of our results throughout the manuscript.

– Comments to Referee 1:

Summary: *This is a manuscript from Claudia Richter's lab who is one of the world's leading experts in developing innovative methods in cardiac optogenetics. Here they use a matrix of 3 x 3 micro-LEDs in combination with an optical mapping system to test different defibrillation strategies based on photostimulation at different frequencies. I found the paper well written and properly organized. The presented data fully confirm the innovation and applicability of the system. I have just few comments to improve the readability of the manuscript. In order of appearance:*

i) I found the abstract too generic. A description of the reported measurements should be included.

Thanks for pointing this out. The abstract has been modified by adding a short description of the measurements. More precisely, we added the following content to the abstract:
The obtained results show that the efficacy of defibrillation is strongly dependent on the parameters chosen for photostimulation during a cardiac arrhythmia. It will be demonstrated that the illuminated area of the heart plays a crucial role for termination success as well as how the targeted control of cardiac activity during illumination for modifying arrhythmia patterns can be achieved.

ii) It is not described which transgenic line has been used and the age of the animals.

The corresponding section in the manuscript was extended. In summary, for the described experiment we used a transgenic alpha-MHC-ChR2 mouse line. This line was established within the scope of the European Community's Seventh Framework Programme FP7/2007-2013 (HEALTH-F2-2009-241526) and kindly provided by Prof. S. E. Lehnart. In general, transgenic adult male C57B6/J, expressing Cre-recombinase under control of alpha-MHC were paired to mate with female B6.Cg-Gt(ROSA)26Sortm27.1(CAG-COP4*H134R/tdTomato)Hye/J. Since the cardiac STOP cassette was deleted in the second-generation, the offspring showed a stable alpha-MHC-ChR2 expression and was used to maintain cardiac photosensitive colonies. All experiments were done with adult mice of both genders at an age of 36 - 48 weeks.

iii) Not clear to me how the authors can reach an optical stimulation frequency of 25 - 35 Hz using Chr2. Several studies (for example Zaglia et al PNAS 2015, Crocini et al Sci Rep 2016) have reported a maximum stimulation frequency of the order of 15 Hz.

Thank you very much for highlighting this point. It is true that the physiological pacing rate of mice hearts lies within the range of 15 Hz. What we tried to explain was the evocation of ventricular arrhythmia for which we applied rapid optical pacing with a frequency of $f_{stim} = 25 - 35\,Hz$.
In order to clarify our aim the Representative Results part has been completed with following text:
"Please notice that the aim of such rapid light pulses is not to capture the cardiac rhythm but it intends more the unbalancing of the cardiac activity such that erratic electrical waves can be generated which then facilitate an arrhythmia."

iv) Discussion could be improved by comparing the defibrillation energy and strategy used in this work with respect to previous work: for example Bruegmann, JCI 2016; Crocini, Sci rep 2016; Emile, Eur Heart J. 2017.

Thank you very much for the suggestion. In order to improve the Discussion of our text, we have added following text:
"The presented results suggest that increasing the irradiated surface recruited a critical number of cardiomyocytes extinguishing the chaotic activity by conduction block as also shown in22. Nevertheless, due to the restricted covered heart surface, the total energy required in this study to successfully photodefibrillate E = 353.15 mJ (using nine micro-LED and pulses with Wdef = 20 ms) is higher as earlier reported in22, 24 where E22 = 228.8 mJ and E24 = 153.6 mJ. While it is true that the necessary energy for termination can be diminished when illuminating a bigger area22 or even the whole heart24, the mentioned approaches do not consider the complexity of the underlying arrhythmia. Another study35 has shown that the measurement of the spatial-temporal

dynamics of an arrhythmia, and therefore the application of targeted patterned photostimulation which is directly related to the ongoing cardiac activity, leads to a reduction of the required area and total energy applied, which is shown to be E35 = 1.8 mJ. In this light the presented work can be further improved by considering feedback-control, which responds with a different pattern of micro-LED illumination depending on the current state of the heart. Moreover, it was also demonstrated that although arrhythmias cannot always be terminated with the current method, the intrinsic complex dynamics can be disturbed during photostimulation leading to a more ordered temporal state. As shown in36, the termination rate is significantly different when addressing monomorphic (more ordered) and polymorphic (less ordered) arrhythmias. Hence the logical step towards a better defibrillation rate might be to influence the cardiac dynamics during a VF episode, turn the arrhythmia into a less complex pattern and terminate with another set of pulses, building in this way a two-step photostimulation approach."

– Comments to Referee 2:

Summary: *Diaz-Maue et al. describe in their manuscript "Advanced Cardiac Rhythm Management Applying Optogenetic Multi-Site Photostimulation in Murine Hearts" a method for optogenetic perturbation of murine hearts ex-vivo, using a Langendorff retrograde perfusion system. With their method, the authors could pace the heart, induce arrhythmia, and terminate arrhythmia with different modes of stimulation. Their stimulation methods included an array of micro light-emitting diodes (micro-LED) and a diffuse illumination setup comprised of two high power LEDs. For electrophysiological monitoring of the heart, the authors used an ECG device and/or an optical mapping system (a LED for excitation of hearts that were preloaded with the voltage sensitive dye Di4-ANBDQPQ, and an EMCCD camera with a high frame rate). With these techniques, the authors could analyze the success rate of different anti-arrhythmic optogenetic interventions, analyze the dominant frequency in the ECG traces, and produce fluorescence intensity maps of the whole heart. The significant contribution of this manuscript is the description of the preparation of light-sensitive heart and the use of micro-LED array for optogenetic stimulation. Nevertheless, certain aspects of the manuscript lack satisfactory details that may make some of these crucial techniques hard to repeat.*

Major Concerns:

1. The micro-LED array produces a relatively localized stimulation, given its small dimensions (1.1 mm2). Consequentially, does increasing the number of activated LEDs within the Micro-LED array really increases the illuminated area or may be it functions by augmenting light intensity. The authors should comment and quantify the area illuminated with different illumination patterns as well as measure light intensity on the surface of the heart. If this is the case, then consider changing the X-axis of the graphs in Figure 3.

In order to give a better explanation for this concern. We have modified the manuscript in the discussion part as follows: "Since the light intensity LIμLED was kept constant during every photostimulation pulse, as mentioned in Step 2.4.6, and the success rate of three micro-LEDs against nine is notably lower, the presented results suggest that the area covered on the heart, the number of micro-LEDs, and thus the total radiant flux applied are crucial factors in achieving defibrillation. Considering that every micro-LED on the array is a Lambertian light source and that they are positioned directly onto the surface of the heart so that the approximate distance to the tissue is zero, it can be assumed that the irradiance contour of the illuminated area on the heart when

using a single micro-LED is equivalent to AμLED = 0.059 mm, as also shown in25 for flat rectangular LEDs. Furthermore, although some photons might leave the micro-LED laterally from the edges, the contribution of those to the total light intensity is considered so small that their effect can be neglected. To quantify the irradiated light of the array, the authors measured the radiant flux from the micro-LED array with a commercial power meter and calculated the light intensity which reaches the heart as shown in Table 1. From Table 1 it can also be read that the radiant flux increases with the number of used micro-LEDs but the light intensity remains constant due to the illumination profile implications mentioned before."
We argument herewith that the light intensity remains constant (as shown in measurements of Table 1) and only the illuminated area of the heart is changed between attempts. Therefore we would like to keep the X-axis of Figure 3 as they are.

2. There is no details at all regarding the mice used and how ChR2 is expressed. Importantly, please describe how the hearts were engineered to express ChR2. Did you use genetically-modified mice or did you use gene delivery to the heart to express the transgene?

For the described experiment we used a transgenic alpha-MHC-ChR2 mouse line. This line was established within the scope of the European Community's Seventh Framework Programme FP7/2007-2013 (HEALTH-F2-2009-241526) and kindly provided by Prof. S. E. Lehnart. In general, transgenic adult male C57B6/J, expressing Cre-recombinase under control of alpha-MHC were paired to mate with female B6.Cg-Gt(ROSA)26Sortm27.1(CAG-COP4*H134R/tdTomato)Hye/J. Since the cardiac STOP cassette was deleted in the second-generation, the offspring showed a stable alpha-MHC-ChR2 expression and was used to maintain cardiac photosensitive colonies.

3. Regarding of the use and assembly of the micro-LED array: the description does not allow readers to repeat the process (lines 70, 154). For a methods paper, the text should explain all the steps instead of referring the reader to other publications.

Thank you very much for indicating this. We have added a new section in the protocol "1.3 microLED array" in which the complete fabrication of the arrays is described in detail.

4. Describing different components or software as "custom-made", again does not allow the readers to repeat the process. We suggest including explanations that are more exhaustive or mentioning commercial alternatives (lines 146; 154-162; 211; 305).

To address this concern alternative components as well as in the text as in the Table of Materials have been suggested. Specifically:

- Custom-built ECG amplifier: An alternative ECG has been added to the Table of materials.
- Custom-built multi-Channel LED driver has been modified to: "1.4.3 Use a multi-channel LED driver to control the current flowing through the micro-LED array. An AFG with multiple outputs is as well suitable for this task." Furthermore an example of AFG (Arbitrary function generator) has been added the the Table of Materials.
- Custom-Software Line 156 has been changed. Since an AFG has been suggested as a driver for the microLED array. It is not necessary to write software. However following suggestion has been added as a Note: "If necessary, the AFG or any other LED driver might be connected to a computer to control the microLED settings remotely. If this is the case connect the LED driver to the computer with the communication protocol of your choice e.g. General Purpose Interface Bus (GPIB) or a serial connection. "

- Custom-Software Line 305 has been replaced with: "the obtained videos were post-processed using the programming language python"

Minor Concerns:

1. Are the DI-4-ANBDQPQ and Blebbistatin prepared in the same 1 ml volume? Please refer to the text in lines 176, 245 and 249.

With the intention to clarify this point we have changed the protocols Steps as follows:

2.1.4 Prepare 1 mL 50 μM DI-4-ANBDQPQ with regular Tyrode solution. Protect the dye from light to prevent photobleaching.

2.1.5 A 10 mM stock solution of Blebbistatin is required. For optical mapping, mix Blebbistatin with the 100 mM Pinacidil-Tyrode-solution (Step 2.1.3) to obtain a 5 μM solution. Wear protective laboratory gloves when handling Blebbistatin.

NOTE: Keep both the dye and the Blebbistatin solution aside until optical mapping begins.

...

2.5.1 Perfuse the heart with the Blebbistatin solution prepared in Step 2.1.5, and wait until mechanical uncoupling occurs. This is accomplished when the heart stops beating, but an ECG signal is still measurable.

NOTE: Mixing the blebbistatin solution to the mentioned concentration and keeping the heart perfused with this solution maintains the cardiac mechanical activity uncoupled from the electrical activity during the whole experiment.

2.5.2. Give the 1 mL voltage dye DI-4-ANBDQPQ (prepared in Step 2.1.4) as a bolus in the bubble trap of the Langendorff perfusion. Wait for 5 to 10 min to allow the dye to perfuse the heart uniformly.

2. Line 245: how exactly is the blebbistatin used? What is the volume of the Tyrode's solution that the blebbistatin is added to? This is not mentioned in step 2.1.4.

We added a point to the protocol and therefore the step mentioned in this concern has moved to 2.1.5. The perfusion system includes two perfusate reservoirs, whereby one is reserved for perfusate including Blebbistatin. In order to get an experimental end concentration of 5 μM of Blebbistatin, we have to add 250 μL of our stock solution ($c = 50\,\mu$M) to 500 mL of perfusate. This approach has the advantage that Blebbistatin-perfusate of given concentrations will constantly perfuse the heart.

3. During the experiment, is there a need for additional boluses of blebbistatin or is it used only as a bolus in the beginning of the experiment?

In fact we only add Blebbistatin whenever we start optical mapping and the uncoupling effect remains for the whole duration of an experiment (around 4 hours). To indicate this we have added following note: "NOTE: Mixing the blebbistatin solution to the mentioned concentration and keeping the heart perfused with this solution maintains the cardiac mechanical activity uncoupled from the electrical activity during the whole experiment."

4. What is the perfusion rate of the heart in your system? Please mention it in the text.

We have added the flow rate in the experiment in line 111 as follows: In the presented work the heart is perfused using a constant pressure setup achieved by elevating the perfusate reservoirs to 1 m height, equivalent to 73.2 mmHg, which yields to a flow rate of 2.633 ± 0.583 mL/min.

5. Please add the hexagonal water bath to the equipment chart.

The list of materials has been updated

6. It is not clear whether the carbogen is used also during the experiment, or only during the

preparation of the heart (lines 104-106).

Thank you very much for pointing this out. We intend to clarify the usage of carbogen in Step 1.1.4 as follows: ”2.2.4 Continue aerating the tyrode solutions during the whole experiment in the reservoirs with Carbogen to ensure that the pH of the perfusate remains stable later during perfusion.”

7. Please consider using terms like ”epicardium” instead of ”pericardium” in line 125.

Thank you for noticing this, the wording has been changed in Line 138

8. Line 127: is the description correct? It says ”628 $\pm$ 40 nm” but might refer to 628 $\pm$ 20 nm.

This concern is correct the 40 nm refers to the bandwidth of the Filter therefore Line 140 is now corrected as earlier suggested

9. Does 28 mW mm-2 refers to a sum off all the micro-LEDs together? Please note it in the text, as the paragraph mentions the possibility to activate 3/6/9 LEDs at a time. Please specify the intensity of the illumination that is produced by 3 and 6 LED.

We try to explain this by adding the measurements of Table 1. Assuming that the illuminated heart area is equal to the area of one micro-LED (as also explained in Major Concerns Point 1). The the light intensity applied with 3/6/9 microLEDs remains constant while the radiant flux increases proportionally to the number of LEDs. Furthermore we have noticed a small error during the calculation of the light intensity and corrected it to 33mW mm-2

10. Is not clear the specific dose according to the followed explanation ”1 ml of 1 ml/ml isoflurane” (line 188).

We have to apologize for the confusing declaration. Meant was that we realized short-time anesthesia of the mice by applying a saturated Isofluran environment to the animal for 2 min and immediately sacrifice via cervical dislocation afterwards. We changed the text passage accordingly.

11. What is the benefit of comparing the frequency of the signal to the frequency of the ECG? Is it just to verify that the recorded signal is not pure noise?

Yes and for clarifying this we have modified the text as follows: ”2.4.4 Turn off the laboratory lights and start recording. Make sure that an optical signal is being acquired by comparing the frequency of the obtained signal to the frequency of the recorded ECG. This ensures that the obtained optical signal is purely related to the electrical activity of the heart.”

12. Please specify what is DM, the acronym is not explained (line 324).

The abbreviation DM refers to a Dichroic Mirror and it was declared the first time in Line 140

13. Figure 1b, please add titles to the y-axes (for both the ECG trace and optical stimulation protocol).

Y axis titles have been added as suggested.

14. ”Abandonment of certain chemicals”, please clarify, the concept is not clear (line 389).

Thank you for the suggestion. We have changed the text to ”Since several chemical compounds are well known for changing the physiological response of the heart, this should be taken into account when analyzing and interpreting the results.”

15. Figure 3 - How do you explain the variable responses to an increase in LED number, pulse

duration, and pulse frequency on the successful defibrillation rate.

We try to explain the results in the discussion with following text: ”It could be shown that cardiac arrhythmias can successfully be terminated with different success rates depending on the parameters which are chosen for photostimulation, as for example the illuminated area on the heart. The presented results indicate that increasing the irradiated surface recruited a critical number of cardiomyocytes extinguishing the chaotic activity by conduction block as also shown in (Bruegmann 2016). ”

16. Line 349. Please correct the figure legend 4. There is a mismatched with the letters a to d.

Thank you for highlighting this, the corresponding letters have been added to Figure 4.

17. Figure 4- What does PDS stand for (right panels). Please add title to the y-axes (for both the ECG trace and optical stimulation protocol) (left panels).

Thank you for pointing this out. PSD stands for Power Spectrum Density and the abbreviation explanation is now written in the corresponding Figure Legend as follows ”Figure 4. Manipulation of the cardiac rhythm by means of photostimulation a) Segment of an ECG recording of a non-terminated arrhythmia. b) Spectrogram of the ECG shown in a) the power spectral density (PSD) of Segment (1) shows an arrhythmia with a dominant frequency of 24 Hz. Segment (2)...”