Dear Editor,

Please find below our response to the concerns/remarks of the three reviewers. Please note that in this document we focus only on major corrections, the remarks have been taken into account and inserted directly in the document using the track change option of MS Word. A few of them were already included in the submission uploaded after the first round of editorial comments, which does not seem to be the version received by the reviewers.

As requested, step 1.7.3 and 2.3.2 have been implemented.

Answers to Reviewer #1:

Major Concerns:
The first main issue is the poor description of the harmonic nanoparticles used in this work, even though the protocol to further use them with cells is clearly given. Thus, the introduction (with adequate references about the synthesis and the first use of similar particles in life sciences) and the protocols need to be completed.

A thorough description of the nanoparticles synthesis is beyond the scope of this video article. The JoVE editors have initially contacted us for the application of this approach for regenerative medicine and for time/space constrains we are bound to limit our work to these aspects. However we have inserted a clearer indication to papers where such information can be found.

Moreover, the author says that it is advantageous to work in infrared region with HNP. They should argue and explain why infrared is advantageous (for example, deeper transmission and less scattering...) and cite more appropriate papers (Webb's work about two-photon microscopy: Denk, W.; Strickler, J. H.; Webb, W. W. 2-Photon Laser Scanning Fluorescence Microscopy. Science 1990, 248, 73-76., Campagnola's work about SHG microscopy: Campagnola, P. J.; Loew, L. M. Second-harmonic Imaging Microscopy for Visualizing Biomolecular Arrays in Cells, Tissues and Organisms. Nature Biotechnology 2003, 21, 1356-1360.).

According to the reviewer's suggestion, we have more explicitly indicated the advantages of working in the infrared spectral region and included the important references indicated.

The second main issue is the use of the figures in the main text. Not all figure panels are well cited and described in the main text, which makes the paper weaker than it really is. Please use the figures better in the text. Especially Fig. 4 is not clear to understand although it is the most important result of the paper. Thus, the use of the figures need to be addressed in the main text and by improving their captions too. A conclusion is needed too, for instance to emphasize the main result and outlook of this technique.

We thank the reviewer for the important remark about Fig. 4. For a mistake during the submission the figure uploaded was not the final one (a panel was missing). We have better addressed the description of the (new) figure in the revised version of the paper.

Changes performed as suggested in questions (*):

**Protocol: 1. Culture and expansion of human ESC: What is FGF?*

Abbreviation added to the text.

**Protocol: 3. Air-liquid 3D cultures of beating clusters and labeling with HNPs: Author should give more motivation on choosing KNbO3 nanoparticles.*

See previous answer.

**Please provide also the PEG protocol for the modification of the surface, which is not in the paper.*

The ACS nano paper cited several times in the text contains the NP preparation procedure.

**Protocol 3: why do you change the medium after day 30 ? Please clarify this.*

The use of Hyclone serum (EU-approved) has revealed critical for the first 4 weeks of the differentiation process. We have observed that after this period, the use of regular FBS (much less expensive) is equally effective in maintain cell survival, in particular that of cardiac cells.

**Protocol: 4. Nonlinear optical imaging: The authors say that they process the pulse to maximize SHG signal. The author should give more information what exactly was changed and how these changes influenced the SHG.*

Explanation added in the text.

**Where is measured the laser power of 50 mW ? at the sample position ?*

*Explanation of the procedure used added in the text *Caption of Figure 1: The author says that HNP are used for 2-photon imaging. You may work with a 2-photon fluorescence system, but you are doing SHG imaging ? Please be consistent in describing your experiment, otherwise you are confusing the reader.*

The text has been clarified in this sense.

**Do you know the laser spot size ? This is important to estimate the cell damage threshold. Please add the peak power and compare it to the cell damage threshold given in this publication: König, K.; So, P. T. C.; Mantulin, W. W.; Gratton, E. Cellular Response to Near-infrared Femtosecond Laser Pulses in Two-photon Microscopes. 1997, 22, 135-136.*

A comparison to this values of max intensity is now given in the text.

**In fig. 3, why don't you keep the autofluorescence the same color for all figures ? It is confusing since it should be consistent in all the figures.*

Explanation of the color codes added in the figure's caption.

**You are comparing the sparse density you are using with other techniques as quantum dots and up-conversion nanoparticles without any reference to other work. Please add references.*

**Moreover, please provide a more quantitative value of the density of your nanoparticles. Or at least an estimation of the particles in a certain volume. The authors states that HNP enables better optical contrast. The author should give estimated values for the HNP SNR and other illumination techniques mentioned in the paper.*

Estimation inserted.

**About Fig.4 : from which panel can we see that the frequency is constant and the amount of displacements. This figure is really confusing and need some more explanation in the caption and in the main text since it is the main results of your paper.*

**In the discussion, you state 'a long term recording', please specify this time.*

Minor concerns: the suggested corrections have been performed.

Reviewer #2:

Figure 4 is actually not that informative, is it possible to also include a couple of images of the EB during this contraction? I assume that panel A is the output of the Matlab routine to track particles? That is not completely clear at the moment.

This has been corrected, as also explained in the answer to reviewer 1. We considered the written document only a support to the final video, which should contain clear explanations of all the plots.

Do the particles partition in the membrane of the cells or are they taken up by the cells as well. In the latter case, does mobility in the cytosol not give a false impression of contractility? Also, it seems that the authors could use concentrations far lower than the 1 mg/ml. Would that not be on the one hand healthier for the cells and on the other hand helpful to distinguish between individual particles during the analysis?

We have ascertained that the particles are localized on the membranes, as no particles have been observed within the cytosol during confocal imaging.

The contraction pattern is associated to cardiac clusters exclusively (appearing at a defined moment in time during cell differentiation). Such contractile movements have not been seen in other structures (i.e. neural rosettes), indicative of the specificity in movement tracking. Moreover, cytosol mobility would not yield an oscillatory movement tracing the same trajectory several consecutive times.

L123: The probe itself will obviously live longer than its host, in case truly used for regenerative medicine. Do the authors have an idea what happens to the probe after months / years of being in an organism? Will the probe be expelled by the cells? Or is this part of future and ongoing research?

Concerning the long-term effect of SHG-NPs on cardiac cell physiology, the longest period of observation has been one week after NP labelling. Although we did not assess the impact of $KNbO_3$ – based NPs beyond this time frame, the reviewer's comment of assessing NP fate over very long periods both *in vitro* and *in vivo* is pertinent for regenerative purposes and this point should be investigated in future studies.

Editorial adjustments:
Abstract has been revised accordingly and indicated modifications throughout the text have been performed.

All Figure citations have been added.

Reviewer #3:

Manuscript Summary:
Nanosized KNbO3 is ferroelectric at room temperature (T. V. Murzina et al. Appl. Phys. Lett. 89, 062907 (2006); http://dx.doi.org/10.1063/1.2336743). The second generation efficiency is sensitive to the orientation of the nanocrystal towards the light field. In plane rotation or angular movement of the nanoparticles can cause reduction/enhancement of the SHG efficiency. According to the protocol it will attributed to the axial displacement (see Fig.4). Have authors provided estimation of the mentioned effect contribution?

It is indeed an interesting point and we are aware of this effect (see for example Bonacina et al. Applied Physics B 2007). However, in the present case the polarization detection is not analyzed, rendering this effect less prominent. Moreover, a misattribution of the intensity oscillations to axial movement instead of crystal orientation during in plane movement is highly unlikely to our opinion. In the latter case we should observe also an in-plane displacement of the particle varying its intensity, which is not seen.