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Harmonic nanoparticles for regenerative research

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Abstract:	In this visualized experiment, protocol details are provided for in vitro labeling of human embryonic stem cells (hESC) with second generation harmonic generation nanoparticles (SGHSHG-NPs). The latter are a new family of probes recently introduced for labeling biological samples for multi-photon imaging. SGHSHG-NPs are capable of doubling the frequency of excitation light by the nonlinear optical process of second harmonic generation with no restriction on the excitation wavelength. Multi-photon based methodologies for hESC investigation by multi-photon microscopy, their differentiation into cardiac clusters (maintained as long term air-liquid cultures) are presented in detail. In particular, evidence on how to maximize the intense second harmonic (SH) emission of isolated SGHSHG-NPs while during 3D monitoring of

	beating cardiac tissue in 3D is shown. The analysis of the resulting images to retrieve three dimensional3D displacement patterns is also detailed.
Author Comments:	An original video animation is included in the submission. It can be modified to be inserted in the final video.
Additional Information:	
Question	**Response**

UNIVERSITÉ DE GENÈVE
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Geneva, June 24th, 2013

Dear Editor,

Please find enclosed our manuscript entitled *"Harmonic nanoparticles for regenerative research"*.

Since a few years we witness a steady increase in the number of multi-photon imaging platforms in universities, hospitals, and industrial R&D departments. This rising interest for nonlinear imaging is motivated by several factors, both scientific and commercial. The former include the inherent three dimensional sectioning capabilities and high spatial resolution of nonlinear approaches, accompanied by the possibility to work in the infrared spectral region, increasing penetration depth and minimizing sample absorption and degradation. The commercial reasons, on the other hand, comprise the increased availability of cost-effective ultrafast lasers and the fact that, in 2009, the patent on 2-photon microscopy ran expired, favoring the opening of this market to several manufacturers. These facts have motivated ours and a few other research groups worldwide to work on the development of "harmonic nanoparticles", a novel family of *inherently nonlinear* microscopy probes. The optical contrast mechanism they exert, contrary to quantum dots and up-converting nanoparticles, is not based on fluorescence but on second harmonic generation. This physical mechanism enables a series of attracting optical properties including excitation-wavelength tunability, complete absence of bleaching and blinking, and narrow (tunable) emission bands. These characteristics, in turn, make these nanosystems amenable for long-term tracking and deep tissue imaging. A context where these properties might be particularly favorable is that of regenerative medicine. In fact, despite the increasing success of stem cells based approaches, a complete understanding of the integration mechanism of stem cells or derivative into a native tissue after transplantation is still missing. This lack of knowledge descends primarily from the difficulty to track in a continuous fashion and for extended time periods (days to weeks) transplanted cells within tissues at sub-cellular spatial resolution. With our video protocol we show for the first time that harmonic nanoparticles can be used to monitor the evolution of embryonic stem cells, follow their differentiation, and even provide three-dimensional information about the beating pattern of cardiomyocites.

We believe that this contribution is particularly suited for JoVE, as it combines different state-of-the-art approaches (non linear nanoparticles, harmonic microscopy, embryonic stem cells) providing sound results and data analysis.

Staying at your disposal for any further information, I remain

Yours sincerely,

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KEYWORDS:

Multi-photon imaging, human embryonic stem cells (ESC), nanoparticles, embryoid bodies (EBs), cardiomyocyte differentiation, cardiac contraction, air-liquid cultures.

SHORT ABSTRACT:

Protocol details are provided for *in vitro* labeling human embryonic stem cells with second harmonic generating nanoparticles. Methodologies for hESC investigation by multi-photon microscopy and their differentiation into cardiac clusters are also presented.

LONG ABSTRACT:

In this visualized experiment, protocol details are provided for *in vitro* labeling of human embryonic stem cells (hESC) with second harmonic generation nanoparticles (SHG-NPs). The latter are a new family of probes recently introduced for labeling biological samples for multi-photon imaging. SHG-NPs are capable of doubling the frequency of excitation light by the nonlinear optical process of second harmonic generation with no restriction on the excitation wavelength.
Multi-photon based methodologies for hESC differentiation into cardiac clusters (maintained as long term *air-liquid cultures*) are presented in detail. In particular, evidence on how to maximize the intense second harmonic (SH) emission of isolated SHG-NPs during 3D monitoring of beating cardiac tissue in 3D is shown. The analysis of the resulting images to retrieve 3D displacement patterns is also detailed.

INTRODUCTION:

Nonlinear microscopy systems, thanks to their inherent three dimensional sectioning capabilities, have increasingly triggered the demand for photo-stable fluorophores with two-photon absorption bands in the near infrared. Only in the last couple of years, to complement the development of fluorescence-based labels (dyes, quantum dots, up-converting nanoparticles), a different imaging methodology has been exploiting the use of a novel family of inherently nonlinear nanoparticles as labels, i.e. harmonic nanoparticles (SHG-NPs) which have been specifically developed for multi-photon microscopy. These labels, based on inorganic noncentrosymmetric crystals, exert optical contrast generating the SH of the excitation frequency: for example by converting a fraction of near infrared pulsed excitation light (λ = 800 nm) into visible blue light (λ /2 = 400 nm). Several authors in the recent past have tested different materials, including iron iodate ($Fe(IO_3)_3$[1], potassium niobate ($KNbO_3$)[2], lithium niobate ($LiNbO_3$)[3], barium titanate ($BaTiO_3$)[4,5], potassium titanyl phosphate (KTiOPO4, KTP)[6-8], and zinc oxide (ZnO)[5,9,10]. Compared to fluorescent probes, SHG-NPs possess a series of attractive properties, such as complete absence of bleaching and blinking, narrow emission bands, excitation-wavelength tunability (from ultraviolet to infrared), orientation retrieval capability, and coherent optical response. These unique properties have been recently explained in two comprehensive review papers[11,12]. The possibility of working in the infrared spectral region, which increases imaging depth by minimizing scattering and absorption, also drastically limits sample photodegradation[13,14]. Moreover, the infinitely photo-stable signal guaranteed by SHG-NPs makes them ideal probes for long-term cell tracking, which is particularly appealing for regenerative medicine applications[15].
In this visualized experiment, protocol details are provided for *in vitro* labeling of human embryonic stem cells (hESC) with unfunctionalized SHG-NPs. The synthesis and preparation of colloidal suspensions is detailed in a previous publication and in references therein[16] and is beyond the scope of this work. Methodologies for hESC investigation by multi-photon microscopy and their differentiation into cardiac clusters (maintained as long term *air-liquid cultures*) are presented. Human ESC can be let to differentiate within so called embryoid bodies (EBs) in two different ways either by EB formation of colony fragments in suspension or, alternatively, forced aggregation of single cells into EBs using the Aggrewell plate, as illustrated in Figure 1A. Culturing beating clusters of cardiac cells on

polytetrafluoroethylene (PTFE) porous filters facilitates their long-term maintenance for further studies (for example electrophysiological measurements of action potentials).

The excitation source of the scanning microscope should be able to deliver ultrashort pulses (with a pulse duration smaller than 300 fs at the sample) in order to reach the peak power needed to perform second harmonic imaging of SHG-NPs. For instance, the most common fs-source used for imaging are tunable Ti:Sapphire lasers. Alternatively, other ultrafast lasers can be employed, for instance Erbium ion[17], Chrome Forsterite[18] or Ti:sapphire pumped infrared optical parametric oscillators. The microscope can be equipped with an objective with preferably a rather high numerical aperture. Very importantly, prior to measurements, and each time the objective is replaced, it is mandatory to minimize the dispersion present in the set-up (lenses) by optimizing the settings of the laser pulse pre-compressor at the working wavelength of choice. This procedure, detailed in the protocol, ensures that the laser pulse is as close as possible to the transform limited duration (i.e. shortest as possible) at the focal plane and maximizes the sample nonlinear response.
The goal of the image analysis described at the end of the protocol is to identify and track in 3D SHG-NPs movements associated with the rhythmic contractions of beating cardiac clusters. Tracking nanoparticles in the image plane is simply realized by identifying their positions in successive movie frames. To extract information on axial movement, a prior calibration of the nonlinear intensity response as a function of axial displacement is mandatory. Note that for long-term measurements, an active interferometric control of sample axial position is required to maintain the validity of the calibration curve in the presence of thermal and/or mechanical drifts.
The SHG-NPs used here to trace beating cells within aggregates are based on potassium niobate oxide ($KNbO_3$), but other available nonlinear nanomaterials are reviewed in detail in the work of Staedler et al.
The nonlinear optical efficiencies of most of the nanomaterials investigated so far are very comparable. The choice for $KNbO_3$ was essentially motivated by the good stability of the colloidal solution and its good biocompatibility, tested on several human cell lines even at fairly high concentration and long exposition times.[16]

Given the novelty of the nanomaterial employed for this work, the main characteristics of SHG-NPs as compared to fluorescent/luminescent bio-markers are shown in a short original computer video animation realized by the authors.

PROTOCOL:

1. Culture and expansion of human ESC

1.1. Prepare the cell propagation medium (called PM) containing Knockout DMEM supplemented with 20 % Knockout Serum, 1 % MEM Non-Essential Amino Acids, 1 % L-glutamine 200 mM, 1 % penicillin-streptomycin, 3.5 µl β-mercaptoethanol.

1.2. Thaw human ESC (hESC) in 8 ml PM medium and centrifuge them 5 min at 115 x g to discard DMSO-supplemented freezing medium.

1.3. Add 1 ml of PM medium containing 10 μM Rock inhibitor to prevent apoptosis and improve cell survival upon thawing (24 h incubation only).

1.4. Add basic fibroblast growth factor (bFGF) to the PM medium at the desired concentration (4-10 ng/ml) for maintenance of pluripotency.

1.5. Plate the hESC on irradiated human foreskin feeder cells (γHFF) (Fig 1A), previously plated at a concentration of $2x10^4$ cells/cm^2.

1.6. Change medium daily and, when necessary, remove portions of colonies starting to differentiate by aspiration using an elongated sharply-cut Pasteur pipette.

1.7. Once a week, passage colonies enzymatically:

1.7.1 Remove medium and wash colonies with 2 ml PBS.

1.7.2 Remove PBS and incubate them with 0.5 ml per well of warm collagenase IV at 1 mg/ml for 10 min at 37 °C, 5 % CO_2.

1.7.3 Collect colony fragments in 2 ml new PM medium and centrifuge at 115 x g for 3 min.

1.8 Alternatively, colonies can be passaged mechanically by scraping:

1.8.1 Remove medium and wash colonies with 2 ml PBS.

1.8.2 Manually scrape colonies using the StemPro EZPassage roller scraper tool (Fig 1B).

1.8.3 Collect colony fragments and centrifuge then for 3 min at 115 x g to remove the supernatant.

1.8.4 Plate fragments on γHFF or process them for differentiation into embryoid bodies (EBs).

2. Human ESC differentiation protocol

2.1. Prepare the differentiation medium (DM) using Knockout DMEM supplemented with 20 % Hyclone serum, 1.0 % MEM Non-Essential Amino Acids, 1.0 % L-glutamine 200 mM, 1 % penicillin-streptomycin, 3.5 μl β-mercaptoethanol.

2.2. Remove medium and wash colonies with 2 ml PBS.

2.3. **EB formation of colony fragments in suspension.**

2.3.1. Remove PBS and incubate them with 0.5 ml per well of warm collagenase IV at 1 mg/ml for 10 min at 37 °C, 5 % CO_2.

2.3.2. Collect colony fragments in 2 ml new DM medium and centrifuge at 115 x g for 3

min.

2.3.3. Culture them in suspension in ultra-low attachment 6 well-plates (2 ml per well) for 4 days (Fig 1C).

2.3.4. Collect and plate the newly formed EBs on 0.1 % gelatin-coated 24-well plates (about 3 EBs/well) (Fig 1D).

2.4. **Alternatively, forced aggregation of single cells into EBs using the Aggrewell plate.**

2.4.1. Remove PBS and dissociate colonies into single cells using accutase (0.5 ml/well).

2.4.2. Centrifuge and resuspend cells in DM medium at 1.2×10^6/ml.

2.4.3. Add 2 ml per aggrewell chamber for 1 day (Fig 1B').

2.4.4. Collect newly formed EBs (Fig 1C') and plate them on 0.1 % gelatin-coated 24-well plates (about 3 EBs/well).

2.5. Change DM medium every 2-3 days until the appearance of beating clusters of cardiomyocytes (15-30 days).

3. Air-liquid 3D cultures of beating clusters and labeling with SHG-NPs

3.1. Identify and manually dissect clusters of beating cardiomyocytes (Fig. 1D), normally appearing after 2-4 weeks of culture, using an elongated sharply-cut Pasteur pipette.

3.2. Deposit 4 PTFE filters per insert that will be placed on a 6well-plate (Fig. 1F).

3.3. Add 1 ml of DM medium on top of each well.

3.4. Add one dissected beating cluster on each PTFE filter (Fig 1E), (maximum 4/well, i.e. 24 aggregates per plate,Fig 1F').

3.5. Change DM medium every 2-3 days.

3.6. To label the clusters, remove the PTFE filter containing the beating cluster and put it on 3.5 cm glass bottom dish (170 µm thick).

3.7. Add 1 ml of SHG-NP at a concentration of 50 µg/ml for 30 min.

4. Non-linear Optical Imaging

4.1. **Sample preparation.** After SHG-NP labeling (see point 3.6), transfer either whole differentiating early hEBs or cardiac beating clusters (dissected upon contraction appearance) on PTFE filters over a 170 µm thick glass-bottom dish compatible with the working distance of high numerical aperture objectives. Alternatively, apply a

direct coating of beating clusters to the glass-bottom dish pre-coated with 0.1 % gelatin.

4.2. Transfer samples to an all-in-one microscope equipped with incubating chamber ensuring stable temperature, humidity and CO_2 control over extended periods of time.

4.3. After initial inspection, image the sample via wide-field imaging under white light illumination to identify structures of interest within differentiated EBs or active beating clusters that will be selected for further investigations.

4.4. If cell autofluorescence from NADH and SH from SHG-NPs have to be simultaneously acquired, set a shorter laser excitation wavelength (i.e. 720 nm) to match the molecular absorption. Use one narrow band-pass spectral filter centered at half the excitation wavelength (i.e., $\lambda = 360 \pm 10$ nm) to detect SH, and another one to detect autofluorescence.

4.5. Firstly perform a fast, low resolution, three-dimensional scan to rebuild the overall morphology of the cardiac cluster and select an image plane within the cluster volume with several visible SHG-NPs.

4.6. If necessary, change the excitation wavelength freely to best match the sample's optical properties or to avoid photobleaching, photodamage and to image deeper into tissues with an IR wavelength (i.e., 790 nm) and replace the second harmonic filter accordingly (i.e., 395 nm). Optimize the settings of the laser pulse pre-compressor at the working wavelength of choice by maximizing the second-harmonic signal of SHG-NPs deposited on the sample.

4.7. To monitor in real time the cardiac cluster contractions, set the microscope optical scanner in the fastest mode such as resonant mode, allowing high speed acquisition of two-dimensional images.

4.8. Choose the settings as best compromise between image contrast and acquisition speed, such as:

- Scan averaging: 4,
- Pixel dwell time: 0.1 µs,
- Image rate: 3.75 frames per second (fps)

4.9. Laser power is adjusted according to focal spot size and scanning speed. 50 mW (measured at the entrance of the objective) is a typical value for 0.1 µs pixel dwell time and Plan APO 20x N.A. 0.75 objective persevering cell viability.

5. Image analysis

5.1 **Axial calibration procedure.** Select a single SHG-NP deposited on a bare substrate. Adjust the focus (i.e. the axial position of the nanoparticle) for maximizing the SH

intensity. Vary in a controlled way the axial position of the microscope stage to obtain the calibration curve relating SH intensity as a function of the SHG-NP offset from the focal plane. The output of this calibration procedure for the Plan APO 20x N.A. 0.75 objective is reported in Fig. 4 C.

5.2 Select SHG-NPs present in all video frames and determine their periodic movements. This procedure can be easily automated, for example by a custom code seeking for signal maximum intensity spots in a defined region of interest and connecting them among different frames (an example code written in Matlab R2009 b using the function *regionprops* of the Image Processing Toolbox is presented).

5.3 Assess the quantification of the out-of-plane displacements, associated with non-continuously traceable SHG-NPs moving along the optical axis, by converting their intensity modulations throughout different frames into axial displacement using the calibration curve established at point 5.1. Note that this procedure gives access to axial movements but cannot be used to determine the absolute direction (upwards or downwards).

REPRESENTATIVE RESULTS:

Prior to assess the beating activity by confocal imaging, a careful characterization of the nonlinear optical response of the PTFE filters was performed, either alone or in the presence of SHG-NPs at high concentration (1 mg/ml). It was ensured that: i) the bare substrate two-photon excited fluorescence is very weak and cannot prevent measuring the relevant biological samples, and ii) the SH emission from isolated SHG-NPs can be easily acquired by imaging through the substrate in epi-detection mode (Fig. 2). The aim was to have reference controls for the quantification of 3D displacement.

In Figure 3, SHG-NP-labeled cardiac structures and EBs are displayed. The red (Panel A), yellow (Panel B and C) and green (Panel D) colors correspond to NADH autofluorescence, while the intense blue SH spots stem from isolated SHG-NP. It is interesting to point out the very good optical contrast achievable by this technique and the relatively sparse labeling as compared to other nanoparticles-based methods, like quantum dots or up-conversion nanoparticles. In the present study, having very intense isolated labels was advantageous for tracking several independent individual particle movements and reconstructing collective movement of the stem cell-derived structures.

Figure 3B illustrates a slice view of a SHG-NP-labeled cardiac beating cluster. Such 3D structures can then be recorded at high speed to monitor the contraction pattern in the cell-SHG-NP aggregate as reported in Movie 1. In this case, to maintain high acquisition speed rate for resolving the NP motion, the overall acquisition sensitivity was not sufficient for recording cell autofluorescence along with SH emission from SHG-NPs.

The application of image analysis described in sect. 5 of the protocol applied to the ensemble of movie frames enables to extract information about individual SHG-NP motion (direction, in-plane and out-of-plane displacement, frequency). The result of this analysis (Fig. 4) indicates that, within the same cardiac cluster, the frequency is constant for in- and out-of-plane motion (see Fourier transform in panel B) and displacements are of the order

of a few micrometers (the lengths of the arrows indicating the exemplary displacements of five NPs in panel A correspond to the maximal elongation of the oscillations).

TABLES AND FIGURE LEGENDS

Figure 1.

Differentiation of hESC into cardiac beating clusters and labeling with SHG-NPs nanoparticles for second-harmonic imaging microscopy. Undifferentiated hESC colonies (A) were either 1) cut down into smaller pieces using the StemPro EZPassage tool (B) and let in suspension to form irregular EBs (C), or 2) dissociated into single cells and re-clustered using the Aggrewell system (B') to form regular EBs (C'). Both types of EBs (C or C') were cultured for 2 days in suspension and then adhered to gelatin-coated dishes. Beating clusters of cardiomyocytes (D) were then manually dissected using a scalpel and cultured on PTFE filters (E) deposited on inserts to allow air-liquid 3D cultures (F, F'). (scale bars for panels A, B, B', C and C': 100 µm; for panels D and E: 250 µm).

Figure 2.

PTFE substrate optical characterization. Panel A: bright field image of the PTFE filter alone. Panel B: Two-photon image of the PTFE filter, which displays a weak fluorescence compared to the rather high laser intensity applied for this control measurement (13 mW). Panels C and D: after adding to the bare substrate a water drop containing SHG-NPs at 1 mg/mL concentration, their SH emission can be easily acquired through the filter at comparatively low laser intensity (2 mW). In Panel D, a 3D image of the nanoparticles spread on the filter is shown. (scale bars: 50 µm).

Figure 3.

Multiphoton imaging of SHG-NP labeled cardiac structures and EBs. Panel A: The two-photon fluorescence signal of a beating cluster is displayed in red and the SH signal from the SHG-NPs is shown in blue. The colors correspond to the intensity measured by the four non descanned photomultipliers equipped with different spectral filters. (Blue 395 ± 11 nm, green 485 ± 20 nm, yellow 531 ± 40 nm, red 607 ± 70 nm). This cluster was imaged directly through the PTFE porous filter with a 10× objective with an excitation wavelength of 720 nm and a mean power of 8.8 mW. Panel B is a slice view of a SHG-NP labeled cardiac structure extracted from a z-stack. Panel C is an EB imaged through the PTFE (scale bars for panels A, B and C: 100 µm). Panel D is a 3D image of an EB labeled with SHG-NPs, reconstructed from z-scans using an apochromatic 40x N.A 1.25 water immersion objective (scale bar: 50 µm). The SHG-NPs are well spread around the whole EB and the cardiac cluster, allowing the movement analysis.

Figure 4.

Panel A: Individual frame of the movie of the beating cluster and vectorial analysis of in-plane SHG-NPs displacements (SH signal shown in black). The lengths of the arrows

correspond to the maximal elongation of the oscillations. Panel: Fourier transform of NPs oscillations showing that within the same cardiac cluster, the frequency is constant for in-(black line) and out-of-plane motion (orange dotted line) and corresponds to 0.4 Hz. Panel C: calibration curve used to convert SHG-NP intensity into axial displacement (See section 5 of the protocol). Circles: experimental datapoints.

DISCUSSION:

The application of nanotechnology to stem cell research is a relatively new but rapidly expanding field. As pointed out by various review articles on the subject, the use of nanoparticles can be applied to accomplish different research tasks, ranging from cell tracking (both *in vitro* and *in vivo*), to intracellular delivery of proteins and genes, not least the creation of biomimetic cellular environments for preferential stimulation/inhibition of specific differentiation pathways.[19,20] The approach described in this protocol is limited to optical tracking and, although the possibility of using infrared excitation leads to increased penetration depth with respect to fluorescence based techniques; optical imaging cannot be extended to whole body and therefore should be complemented by magnetic detection.[21] Previous published work showed several approaches to assess structure and movement of cardiac cell clusters, including labeling by fluorescent dyes[22], quantum dots[23,24], super-paramagnetic iron oxide nanoparticles[25], and up-conversion fluorescent particles.[26,27,28] The advantages of SHG-NPs with respect to these nanoprobes are associated with absence of bleaching over extended periods of time, increased imaging penetration, wavelength tunability for increased contrast with respect to autofluorescence. The peculiar sparse cell labeling (i.e. in Fig. 3-D we count approximately 160 NPs in a 100 μm side cube) that is obtained by this approach turns out to be very well suited to trace cell contractions in 3D human ESC-derived cardiac clusters at high spatial resolution.

The choice of the nanomaterial is not limited to PEG-coated $KNbO_3$, but can encompass a rich family of nanoparticles displaying non-centrosymmetric crystal structures, which can possibly incorporate other functionalities (magnetic, radioactive) allowing multi-modal detection. Moreover, such PEG-coated SHG-NPs can further be functionalized to be specifically directed against epitopes or binding proteins in order to selectively target specific cell subpopulations. On the other hand, the imaging approach proposed here requires a scanning microscope equipped with an ultrafast laser source. A natural follow up that can be envisaged from this work is monitoring the integration of labeled cells into 3D biomaterials and scaffolding structures *in vitro* and further into native tissues, to follow the incorporation and the functionality of transplanted cells over extended periods.

An advantage of using PTFE filters bearing 3D c aggregates, as in our case, is the possibility to perform multiple measurements over days and weeks, as filters can be restored back on their insert supports without risks of contaminations. More importantly, PTFE filters also enable the repetitive assessment of action potentials using multi-electrode arrays.

Cell damage threshold for multi-photon excitation in the near infrared has been previously determined to less than 1 TW/cm^2 by König *et al.* [29]. In our study, using a 0.75 NA 20x objective, the applied intensity remains under the TW/cm^2 (0.26 TW/cm^2) and the short pixel dwell time (0.1 μs) as compared to König's work (80 μs) ensures the possibility to preserve the sample alive and differentiating.

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DISCLOSURES:

The authors have nothing to disclose.

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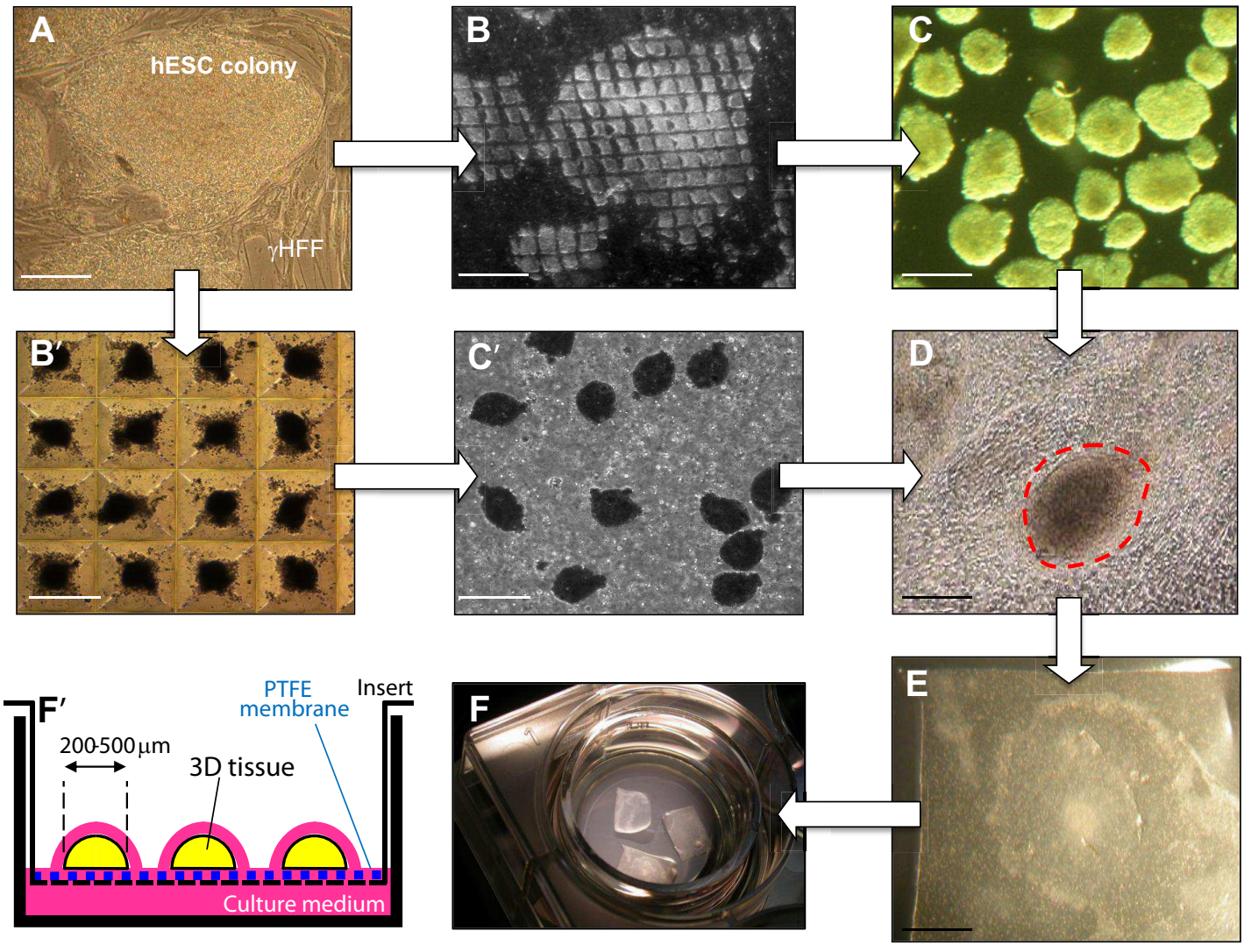

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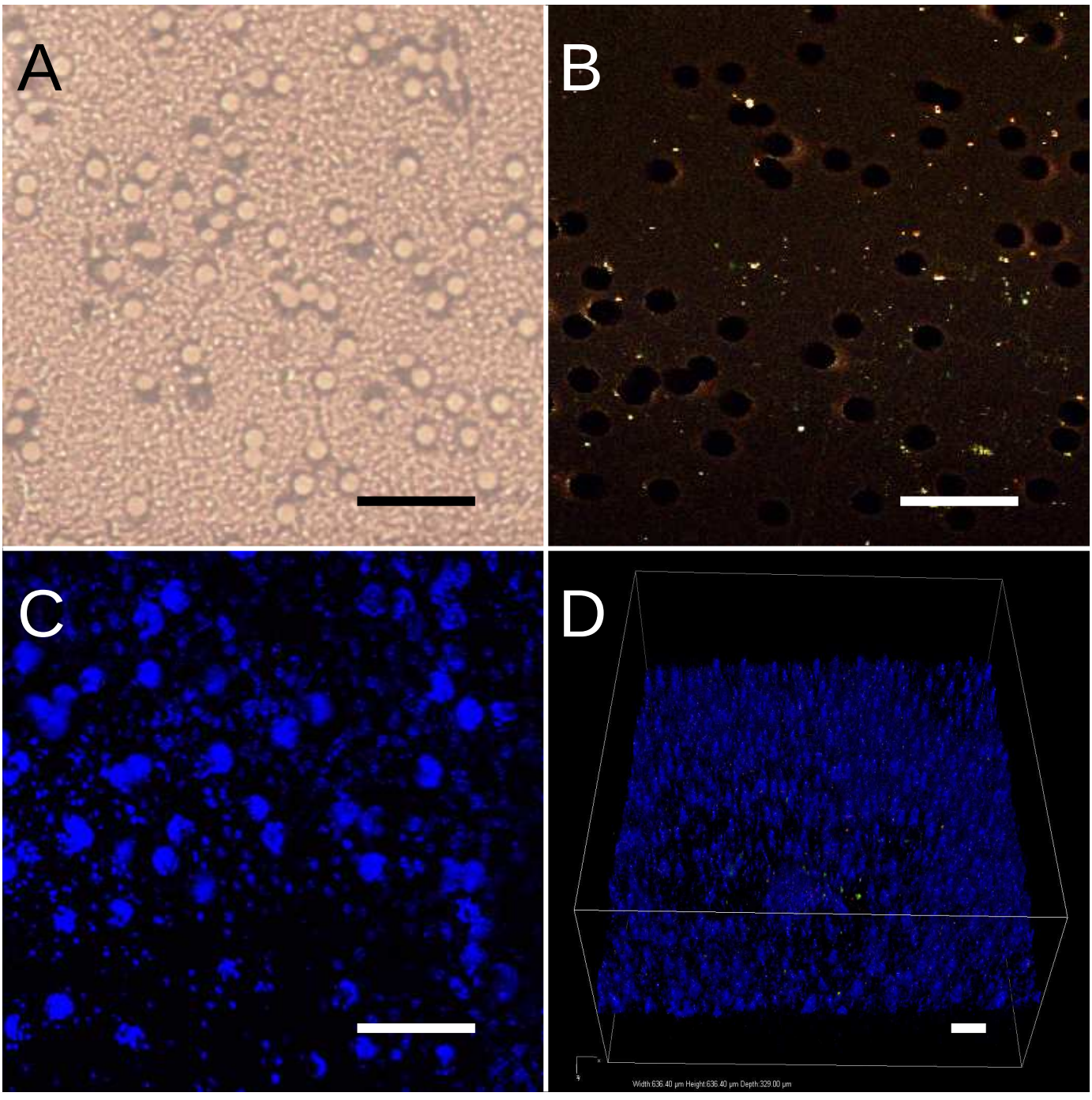

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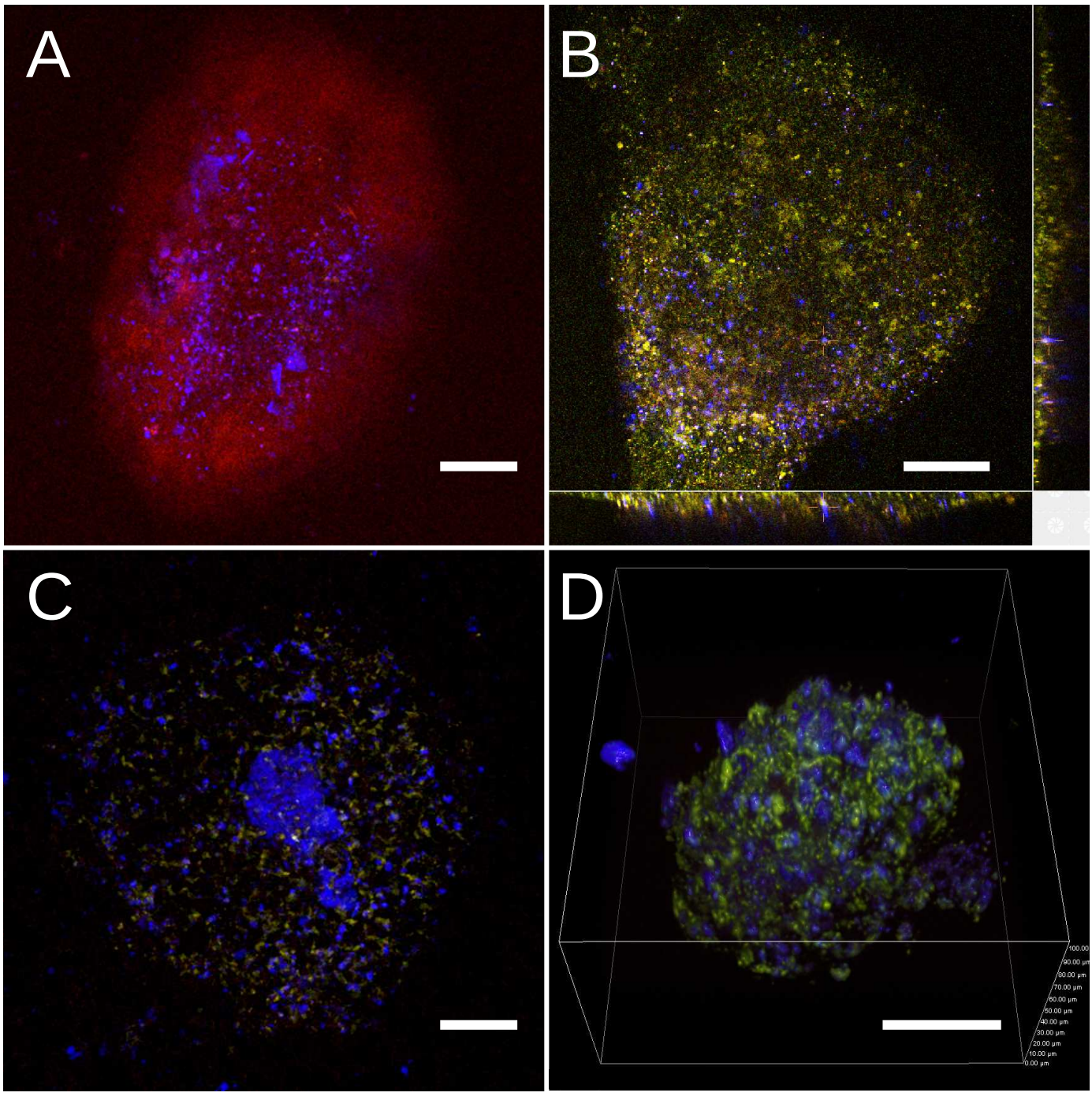

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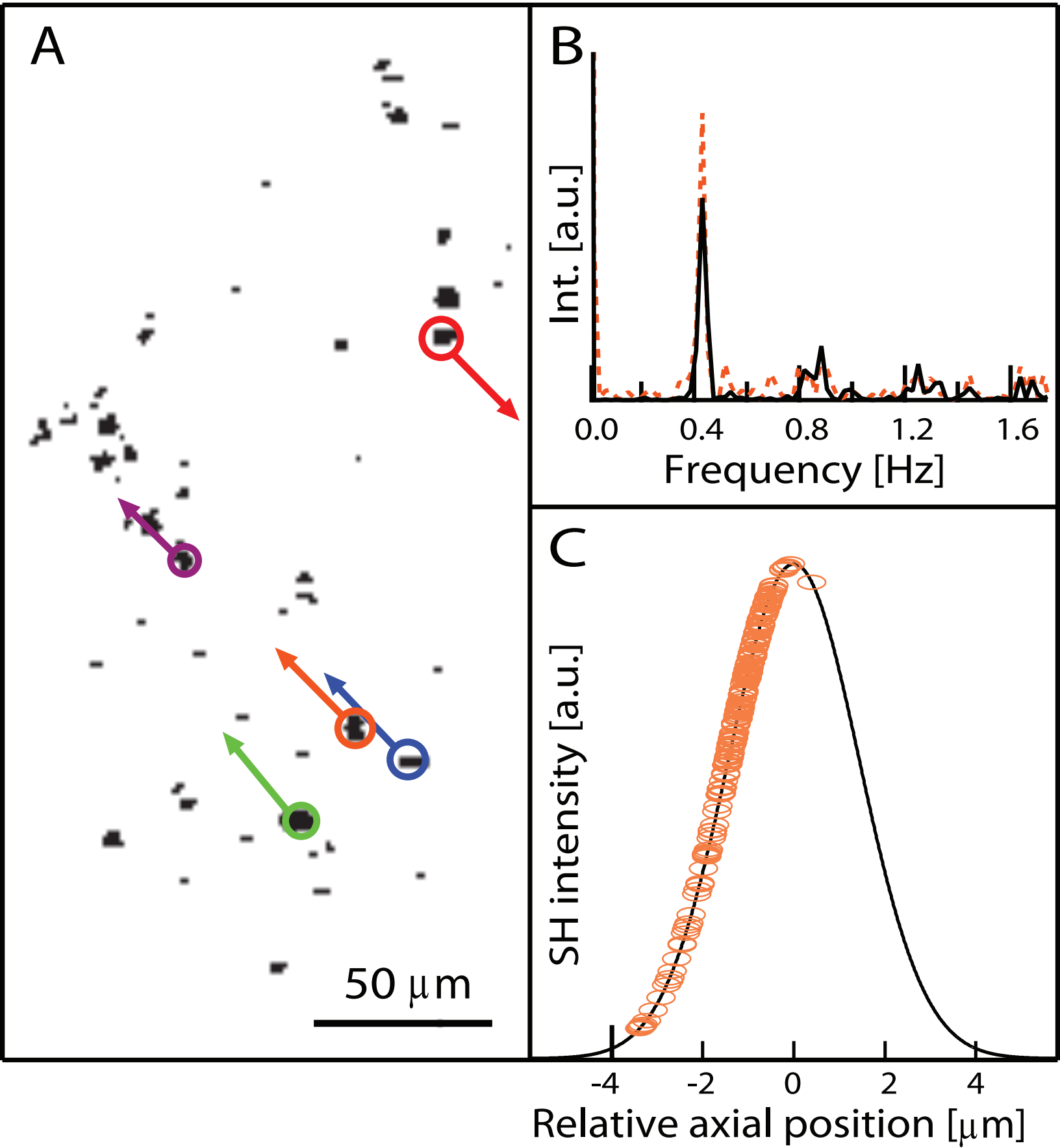

*Excel Spreadsheet- Table of Materials/Equipment

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Material Name	Company
Microscope Incubator	OKO LAB
Multiphoton microscope	Nikon
Filters SHG and autofluorescence	Semrock
Microscope objectives	Nikon
Rhock inhibitor	Sigma
Knockout DMEM	Invitrogen
Knockout Serum	Invitrogen
MEM Non-Essential Amino Acids	Invitrogen
L-glutamine 200mM	Invitrogen
Penicillin-streptomycin	Invitrogen
β-mercaptoethanol	Sigma
Collagenase IV	Gibco
Roller scraper tool	StemPro EZPassage, Invitrogen
StemPro Accutase	Gibco
Aggrewell system	StemCell Technologies
Hyclone serum	Thermo Scientific
Gelatin	Sigma
6-well plates	Falcon
24-well plates	Nunclon
Polytetrafluoroethylene (PTFE) filters	Millipore
Inserts	Millipore

Catalogue Number	Comments (optional)
UNO package (top stage)	37°C, 5% CO2, moisturized
AR1-MP	Fast scanning, four non photomultiplier descanned detectors
FF01-360/12-25 FF01-395/11-25 FF02-485/20-25	
CFI Plan Fluor 10x CFI Plan Apo 20x CFI Apo 40x	NA 0.30, WD 16 mm NA 0.75, WD 1.0 mm, VC NA 1.25, WD 0.18mm λS, Nano-Crystal Coat
Y-27632	
10829	
10828	
1140	
25030	
15140	
M7522	
17104-019	
23181-010	
S11105-01	
27845	
SH30070.03	
G9391	
353046	
142475	
NA76/25	
PICM03050	

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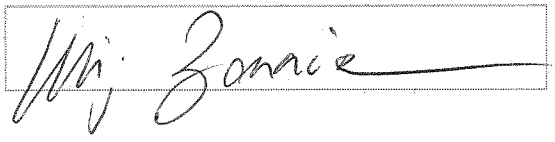
Signature:  Date: 24/06/2013

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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.

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