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Gold nanorod-assisted optical stimulation of neuronal cells

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Abstract:	Recent studies have demonstrated that nerves can be stimulated in a variety of ways by the transient heating associated with the absorption of infrared light by water in neuronal tissue. This technique holds great potential for replacing or complementing standard stimulation techniques, due to the potential for increased localization of the stimulus and minimization of mechanical contact with the tissue. However, optical approaches are limited by the inability of visible light to penetrate deep into tissues. Moreover, thermal modelling suggests that cumulative heating effects might be potentially hazardous when multiple stimulus sites or high laser repetition rates are used. The protocol outlined below describes an enhanced approach to the infrared stimulation of neuronal cells. The underlying mechanism is based on the transient heating associated with the optical absorption of gold nanorods, which can cause triggering of neuronal cell differentiation and increased levels of intracellular calcium activity. These results demonstrate that nanoparticle absorbers can enhance and/or replace the process of infrared neural stimulation based on water absorption, with potential for future applications in neural prostheses and cell therapies.
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gold nanorods, external absorber, neural stimulation, laser excitation, optical stimulation, neuronal cells, *in vitro* models.

SHORT ABSTRACT:
This protocol outlines how to use the transient heating associated with the optical absorption of gold nanorods to stimulate differentiation and intracellular calcium activity in neuronal cells. These results potentially open up new applications in neural prostheses and fundamental studies in neuroscience.

LONG ABSTRACT:
Recent studies have demonstrated that nerves can be stimulated in a variety of ways by the transient heating associated with the absorption of infrared light by water in neuronal tissue.

This technique holds great potential for replacing or complementing standard stimulation techniques, due to the potential for increased localization of the stimulus and minimization of mechanical contact with the tissue. However, optical approaches are limited by the inability of visible light to penetrate deep into tissues. Moreover, thermal modelling suggests that cumulative heating effects might be potentially hazardous when multiple stimulus sites or high laser repetition rates are used. The protocol outlined below describes an enhanced approach to the infrared stimulation of neuronal cells. The underlying mechanism is based on the transient heating associated with the optical absorption of gold nanorods, which can cause triggering of neuronal cell differentiation and increased levels of intracellular calcium activity. These results demonstrate that nanoparticle absorbers can enhance and/or replace the process of infrared neural stimulation based on water absorption, with potential for future applications in neural prostheses and cell therapies.

INTRODUCTION:

Recent studies have demonstrated that the transient heating associated with the absorption of infrared light by water (wavelength > 1400 nm) can be used to induce action potentials in nerve tissue[1] and intracellular calcium transients in cardiomyocytes[2]. The use of infrared light has raised great interest for applications in neural prostheses, due to the potential finer spatial resolution, lack of direct contact with the tissue, minimization of stimulation artifacts, and removal of the need to genetically modify the cells prior to stimulation (as required in optogenetics)[1]. Despite all of these benefits, recently developed thermal models suggested that the target tissue/cells may be affected by cumulative heating effects, when multiple stimulus sites and/or high repetition rates are used[3,4].

In response to these challenges, researchers have recognized the potential to use extrinsic absorbers for nerve stimulation to produce more localized heating effects in the tissue. Huang *et al.* demonstrated this principle by using superparamagnetic ferrite nanoparticles to remotely activate the temperature-sensitive TRPV1 channels in HEK 293 cells with a radio-frequency magnetic field[5]. Although this technique may allow for deeper penetration (magnetic fields interact relatively weakly with tissue), the responses were only recorded over periods of seconds, rather than the millisecond durations required in bionic devices[5]. Similarly, Farah *et al.* demonstrated electrical stimulation of rat cortical neurons with black micro-particles *in vitro*. They showed cell-level precision in stimulation using pulse durations on the order of hundreds of μs and energies in the range of μJ, potentially allowing for faster repetition rates[6].

The use of extrinsic absorbers has also been applied to induce morphological changes *in vitro*. Ciofani *et al.* showed a ~ 40% increase in neuronal cell outgrowth using piezoelectric boron nitride nanotubes excited by ultrasound[7]. Similarly, endocytosed iron oxide nanoparticles in PC12 cells have been reported to enhance neurite differentiation in a dose-dependent manner, due to the activation of cell adhesion molecules with the iron oxide[8].

Recently, the interest in extrinsic absorbers to assist neural stimulation has also focused on the use of gold nanoparticles (Au NPs). Au NPs have the ability to efficiently absorb laser light at the plasmonic peak and to dissipate it into the surrounding environment in the form of heat[9].

Amongst all of the available particle shapes, the optical absorption of gold nanorods (Au NRs) conveniently matches the therapeutic window of biological tissues (near infrared - NIR, wavelength between 750 – 1400 nm)[10]. Moreover, in the context of neural stimulation, the use of Au NRs provides relatively favorable biocompatibility and a wide range of surface functionalization options[11]. Recent studies have shown that a stimulatory effect on differentiation can be induced after continuous laser exposures of Au NRs in NG108-15 neuronal cells[12]. Similarly, intracellular calcium transients were recorded in neuronal cells cultured with Au NRs after laser irradiation modulated with variable frequencies and pulse lengths[13]. Cell membrane depolarization was also recorded after NIR laser illumination of Au NRs in primary cultures of spiral ganglion neurons[14]. The first *in vivo* application with irradiated Au NRs has been demonstrated just recently. Eom and coworkers exposed Au NRs at their plasmonic peak and recorded a six-fold increase in the amplitude of compound nerve action potentials (CNAPs) and a three-fold decrease of the stimulation threshold in rat sciatic nerves. The enhanced response was attributed to local heating effects resulting from the excitation of the NR plasmonic peak[15].

In the present paper, protocols for investigating the effects of laser stimulation in NG108-15 neuronal cells cultured with Au NRs are specified. These methods provide a simple, yet powerful, way to irradiate cell populations *in vitro* using standard biological techniques and materials. The protocol is based on a fiber-coupled laser diode (LD) that allows safe operation and repeatable alignment. The Au NR sample preparation and laser irradiation methods can be further extended to different particle shapes and neuronal cell cultures, providing that the specific synthesis and culture protocols are known, respectively.

PROCEDURE:

1) Au NRs preparation

Note: Au NRs can be synthesized by a number of recipes[16], or purchased from commercial vendors.

1.1) Measure the initial optical density (OD) of the Au NR solution via UV-Vis spectroscopy, by recording the absorption values from 300 nm to 1000 nm with a resolution between 0.5 - 2 nm. Vary the volume of the solution to be used with the available cuvette.

1.2) Evaluate the initial NP molar concentration with a suitable technique[17] (e.g. UV-Vis spectroscopy, single particle inductively coupled plasma mass spectrometry, transmission electron microscopy) or use the concentration values provided by the vendor.

1.3) Prepare a 5 ml stock solution by diluting the initial Au NR sample to reach an OD = 1. For the best repeatability, keep the OD constant for all of the tested samples. Follow the commercial vendor's protocol for the composition of the diluting solvent. If unsure, use deionized water.

1.4) Centrifuge 1 ml of the Au NR solution twice for 15 min at 7800 x g to remove any chemical excess from the solution. Centrifugation cycles can vary in force and time (e.g. 20 min × 5600 g)[18].

1.5) Remove the supernatants and re-suspend the NRs in deionized water. As re-suspended particles might form aggregates in solution, prepare them daily to have the best results. Alternatively, store in a fridge for no longer than 1 week. Do not freeze.

1.6) Before use under cell culture conditions, sonicate the Au NR solution for 5 minutes and then sterilize with UV light for 30 min (UV radiation intensity not less than 400 $mW \cdot m^{-2}$ at 254 nm).

2) NG108-15 neuronal cell line culture and differentiation

2.1) For the cell culture medium, prepare 500 ml of sterile Dulbecco's modified Eagle medium (DMEM) containing 10% (w/v) fetal calf serum (FCS), 1% (w/v) L-glutamine, 1% (w/v) penicillin/streptomycin and 0.5% (w/v) amphotericin B.

Note: Supplements can be aliquoted, stored at -20 °C and added to the media on the day required. Cell culture medium can be refrigerated in a sterile condition for a maximum of 1 month.

2.2) For the cell differentiation medium, prepare 50 ml of sterile DMEM containing 1% (w/v) L-glutamine, 1% (w/v) penicillin/streptomycin and 0.5% (w/v) amphotericin B.

2.3) Grow NG108-15 neuronal cells in 10 ml of cell culture medium on T75 flasks made of polystyrene in an incubator with humidified atmosphere (5% CO_2 at 37 °C). Normally, seed 1.5 - 2 × 10^5 cells on each flask to be ready in 3 - 4 days. Change cell culture medium every two days.

Note: To prevent genetic drifts or variation, do not use cells older than passage 21 for experiments.

2.4) When 70% - 80% confluent in culture, change the medium with warm fresh cell culture medium. Mechanically detach the cells by gently knocking the bottom of the confluent flask. Do not use trypsin.

2.5) Centrifuge the cell suspension for 5 minutes at 600 x g and re-suspend the cell pellet in 2 ml of warm cell differentiation medium.

2.6) Seed 2 × 10^4 cells/cm^2 in a tissue culture polystyrene 96 well plate with 200 µl of cell differentiation medium. Incubate the experiment for 1 day at 5% CO_2/37 °C.

2.7) Add between 3.2×10^9 – 4.2×10^{10} particles/ml of Au NR solution on day 2 and incubate it for additional 24 hours. Do not add the particles for the control experiments.

Note: As an alternative control, Au NPs with a well-differentiated peak absorption wavelength can be used for comparison purposes[14]. For a more consistent cell behavior, keep the cell density constant and do not modify the well surface prior to cell seeding.

3) Neurite outgrowth enhancement

3.1) Couple the laser with a single mode optical fiber (numerical aperture = 0.13) and terminate it with a fiber connector (FC connectors are convenient and commonly available). Measure the output laser power with a standard power meter. To obtain the most effective results, match the peak wavelength of the laser to the plasmon resonance peak of the NRs.

3.2) On day 3 following NR incubation, fix the FC connector to the well. Irradiate samples and controls at room temperature for 1 minute in continuous wave, for different laser powers. Allow the culture to proceed for 3 additional days at 5% CO_2/37 °C. Repeat the laser irradiation for a minimum of 3 independent measurements.

Note: Different irradiation times and pulse frequencies may be selected, depending on the application.

3.3) Characterize the laser in terms of beam diameter and laser irradiance ($W \cdot cm^{-2}$).

3.3.1) For a standard single mode fiber, use $NA = n \cdot \sin\vartheta$, where n is the refractive index of the medium in use and ϑ is the half-angle of the cone of light exiting the fiber (see Figure 1A). From trigonometry, $r = \tan\vartheta \cdot d$, where r is the beam radius and d is the distance between the fiber and the sample. The FC connector matches the diameter of the well, therefore illuminating the sample at a distance $d = 2.70 \pm 0.20$ mm. Light exits the fiber in water ($n = 1.33$), giving $r = \tan(\sin\text{-}1(NA/n)) \cdot d$.

3.3.2) Using the latter equation, calculate the laser beam radius, the corresponding beam area and the average laser irradiance (laser power divided by beam area) at the target (see the example graph in Figure 1B). These values represent the average irradiance over the illuminated area.

3.3.3) Evaluate the errors for the measurements with the general theory of error propagation.

Note: The distance between the FC connector and the sample can be measured by photographs of the experimental arrangement and post-processing of the image using appropriate software (e.g. ImageJ).

3.4) At day 5, remove cell differentiation medium from the experiments and fix the samples with 3.7% (v/v) formaldehyde solution for 10 min, then permeabilize the cells with 0.1% (v/v) Triton X-100 for 20 min.

3.5) Add 3% (w/v) bovine serum albumin (BSA) to the samples for 60 min to block the unreacted protein binding sites. Label the samples for anti-βIII-tubulin overnight (5 µg/ml in PBS supplemented with 1% of BSA) at 4 °C.

3.6) Incubate the cells for 90 min in the dark with an appropriate secondary-antibody (e.g. TRITC-conjugated anti-mouse IgG antibody) using a concentration of 0.4 – 2 µg/ml in 1% BSA in PBS. Label the cell nuclei with DAPI (0.1 µg/ml in de-ionized water) for 10 min.

Note: Antibody conjugation and concentration might vary according to the company protocol. Wash samples with PBS twice for 5 minutes after each staining stage.

[Insert Figure 1 here.]

3.7) Image samples by epifluorescence or confocal microscopy using at least a 20× objective. Choose the microscope filters accordingly to the secondary antibody. Select a DAPI filter (λ_{EX} = 358 nm; λ_{EM} = 488 nm) to visualize the cell nuclei.

3.8) Analyze the pictures by assessing: i) the maximum neurite length (record the length from the tip of the neurite to the beginning of the cell body), ii) the number of neurites per neuronal cell (sum up all of the neurites per cell) and iii) the percentage of cells with neurites (divide the total number of cells expressing βIII-tubulin by the total number of cells with a positively stained nucleus)[19].

4) Laser-induced intracellular Ca^{2+} imaging

4.1) Prepare a 20% (w/v) stock solution of Pluronic F-127 by dissolving 2 g of solute in 10 ml of anhydrous dimethyl sulfoxide (DMSO). Heat the solution of Pluronic F-127 at 40 °C for about 20 minutes to increase the solubility. Prepare it in advance if stored at room temperature.

4.2) On day 3 following NR incubation, prepare a balanced salt solution (BSS; 135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4) and supplemented it with 5 µM of Fluo-4 AM in DMSO and 0.1% (w/v) of Pluronic F-127 solution.

Note: BSS solution can be prepared in advance and refrigerated for a maximum of 1 month. Add the supplements (Fluo-4 AM and Pluronic F-127) on the day of the experiment.

4.3) Remove cell differentiation medium and replace it with the supplemented BSS solution. To obtain the best results with an inverted confocal microscope, incubate the cells in a micro-slide well.

4.4) Load NG108-15 cells with Fluo-4 AM for 30 minutes in the dark at 5% CO_2/37 °C. Following the incubation time, wash samples twice with BSS to remove any extracellular unloaded fluorophore.

4.5) Couple the laser with a single mode optical fiber and cleave the tip using standard techniques. Observe the resulting tip under an optical microscope, to ensure a high quality surface (i.e., the tip should be perpendicular to the fiber axis and flat upon microscopy inspection).

4.5.1) Measure the output laser power with a standard power meter. Insert the light delivery fiber into an optical fiber holder and affix it to a micropositioner. Refer to Brown et al.[20] for more details on optical fiber preparation and positioning.

Caution: Follow the general rules for laser safety during the measurement of the laser power (e.g., don't look directly into laser beam, wear laser safety goggles during handling, prevent stray light exposure to other lab users).

4.6) Connect an oscilloscope to the portable laser to monitor the optical modulation. Use a binary signal with variable frequencies (0.5 - 2 Hz) and pulse lengths (20 - 100 ms). Connect the laser using the modulation signal as transistor-transistor logic (TTL) input for the microscope, following the setup shown in Paviolo et al.[13].

4.7) Place the cells under an inverted confocal microscope and position the light delivery fiber 250 ± 50 µm away from the target cell in transmission illumination mode. At this distance, use the equations presented in 3.3.1 to calculate the beam radius at the target.

4.7.1) Use an argon-ion laser (488 nm) to excite the internalized Fluo-4 AM dye (λ_{EX} = 494 nm; λ_{EM} = 516 nm) and the synchronized LD for the excitation of the endocytosed NRs. Perform the imaging at room temperature of samples and controls using at least a 40× objective by collecting time-series scans with 256 × 256 pixels/frame resolution in roundtrip mode with a frequency of at least 4 Hz. Perform a recording without any LD excitation to identify any argon-ion laser interference (baseline noise).

4.8) Remove the background from the data using adaptively weighted penalized least-squares algorithms[21]. Plot the induced Ca^{2+} variations as a function of time. Analyze the transient peaks in the fluorescence intensity using image post-processing software by thresholding at a level three times the standard deviation of the baseline noise (3σ).

REPRESENTATIVE RESULTS:

By using Protocols 1, 2, and 3 described here, a stimulatory effect on differentiation was observed in NG108-15 neuronal cells cultured with Au NPs (Au NRs, poly(styrenesulfonate)-coated Au NRs and silica-coated Au NRs) after laser exposures between 1.25 and 7.5 $W \cdot cm^{-2}$. Confocal images of rhodamineB-labelled Au NRs demonstrated that the particles were internalized from day 1 of incubation[12]. The localization was predominantly observed in the cell cytoplasm, indicating that the preferred mechanism of uptake was via the cell body membrane[12]. The main morphological changes detected after inducing differentiation in NG108-15 neuronal cells were the arrest of proliferation, the expression of βIII-tubulin protein

and the outgrowth of neurites, which were analyzed in terms of maximum length and number[22].

Samples cultured with NRs showed a neurite length increase at the laser irradiance levels measured here (between 1.25 and 7.5 $W \cdot cm^{-2}$). Control samples (cells cultured without NPs and irradiated with the same laser power) showed no significant change in length. Using an irradiation dose of 7.5 $W \cdot cm^{-2}$, the final neurite length of NG108-15 cultured with Au NRs was roughly 36% higher ($p < 0.01$) than the non- laser-irradiated samples. This behavior was not specifically linked to the surface chemistry of the NRs. These values were almost 20% greater than the neurites developed by NG108 -15 alone and exposed to the same value of laser exposure ($p < 0.05$)[12]. These results are in line with previously published studies on PC12 neuronal cells cultured with piezoelectric nanotubes and irradiated with ultrasound radiation[7].

Control experiments without Au NRs also showed some stimulatory effects of the 780 nm light in terms of percentage of neurons with neurites and number of neurites per neuron. This stimulation was more effective at lower laser powers (1.25 $W \cdot cm^{-2}$) with a subsequent decrease at the highest laser energy (7.5 $W \cdot cm^{-2}$)[12]. A moderate stimulation caused by the NPs without any laser irradiation (poly(styrenesulfonate)-coated and silica-coated only) was detected in the percentage of neurons with neurites[12]. These results are in line with recently published observations that gold nanoparticles can increase neuronal activity *in vitro*[23,24]. Figure 2 shows an example of epifluorescence images of differentiated NG108-15 neuronal cells cultured alone (A) or with Au NRs (B) and irradiated with different laser powers (indicated in figure).
The potential for photo-generated intracellular Ca^{2+} release was assessed using pulsed NIR light in accordance with Protocols 1, 2, and 4. Calcium ions play an important role in different cellular activities, such as mitosis, muscle contraction and neurite extension[25,26]. In response to a stimulus, Ca^{2+} increases, oscillates and decreases, leading to the activation, modulation or termination of a specific cell function. Recently, Ca^{2+} transients have also been observed as a consequence of IR laser exposure in cardiomyocytes. In that work, the Ca^{2+} responses evoked after IR exposure exhibited lower amplitudes and faster recovery times than the spontaneous Ca^{2+} transients[2]. Figure 3 shows an example of NG108-15 neuronal cells loaded with Fluo-4 AM and imaged with a confocal microscope. Fluo-4 AM was observed to enter the cell membrane in a non-disruptive manner, resulting in a generally uniform distribution of the indicator across the cell cytoplasm. Only minor nuclear or cytoplasmic organelle staining was detected. As previously indicated, NRs were expected to be located intracellularly[12]. NG108-15 neuronal cells alone or cultured with Au NRs and poly(styrenesulfonate)-coated-Au NRs were exposed thereafter to laser irradiances between 0.07 $J \cdot cm^{-2}$and 370 $J \cdot cm^{-2}$, with the laser frequency modulated between 0.5-2 Hz.

Figure 4 shows representative examples of how the amplitude of the responses was mapped as a function of time. The amplitude of the Ca^{2+} response varied with the radiant exposure (Figure 4A-B-C) and was observed not to be consistently triggered by the laser pulses (Figure 4C)[13]. The most likely explanations were the transient depletion of intracellular Ca^{2+} stores attributed to incomplete Ca^{2+} loading[2] or the different efficiency of NR internalization in the neuronal cells. When NRs were not used in culture (control experiments, Figure 4D), a stimulatory effect of the

780 nm light was also observed. However, this produced lower fluorescence amplitude peaks and lower probability of activation (detected in only 16% of the analyzed samples). Overall, a 48% probability of NR laser-induced cell activation was achieved and despite the background events due to the 780 nm light, exposure of NR-treated cells demonstrated higher stimulation efficiency with lower laser energy and higher peaks of response[13]. In fact, the calcium response was found to peak at 0.33 J·cm^{-2}in the NR-treated cells. This was attributed to thermal inhibition[13]. During the experiments, no evidence of blebbing or any other form of cell membrane disruption was detected, which is consistent with the results of Huang *et al.* that reported cellular photodestruction with a relatively high power density of 19 W·cm^{-2}applied for 4 minutes[27]. No spontaneous activity in the NR-treated cells was recorded without laser exposure.

Figure 1: Optical fiber experimental setup (A) and average laser irradiances as a function of the laser power for a laser beam of area equals to 0.4 mm^2 (B). Beam parameters are (A): the half-angle of the maximum cone of light exiting the fiber (ϑ), the beam radius (r) and the distance between the optical fiber and the sample (d).

Figure 2: Examples of epifluorescence images of differentiated NG108-15 neuronal cells cultured alone (A) or with Au NRs (B) and irradiated with different laser powers (indicated in figure). Samples were incubated for one day before laser irradiation. Cells were fixed and labelled for anti-β-III tubulin (red) and DAPI (blue) three days after laser irradiation. Scale bars are 100 µm.

Figure 3: Example of differentiated NG108-15 neuronal cells loaded with Fluo4-AM Ca^{2+} indicator. The image was taken using an inverted confocal microscope with a 40× oil-immersion objective.

Figure 4:
Representative examples of laser-induced Ca^{2+} variations as a function of time in NG108-15 neuronal cells cultured in serum-free conditions for three days with (A) poly(styrenesulfonate)-Au NRs, (B,C) Au NRs, and (D) without NRs (control sample). These results were obtained with laser pulses of 100 ms (A-C-D) and 50 ms (B). The frequencies used for the experiments (dashed vertical lines) were 1 Hz (A-B-C) and 0.5 Hz (D). The calculated radiant exposures were 69.4 J·cm^{-2} (A), 34.7 J·cm^{-2} (B), 0.37 J·cm^{-2} (C) and 138.87 J·cm^{-2} (D). F_{max} /F_0 indicates the maximum fluorescence increase detected in NG108 -15 neuronal cells, from calibration with ionomycin (reproduced with permission[13]).

DISCUSSION:
The protocols outlined in this presentation describe how to culture, differentiate and optically stimulate neuronal cells using extrinsic absorbers. The NR characteristics (e.g., dimensions, shape, plasmon resonance wavelength and surface chemistry) and the laser stimulation parameters (such as wavelength, pulse length, repetition rate, etc.) can be varied to match different experimental needs. The effects on cell behavior can be monitored using standard biological assays and materials. Overall the approach provides a simple, yet powerful, way to

irradiate cell populations *in vitro* and could be extended to primary cells, tissue samples and *in vivo* studies.

The main requirements that Au NRs need to satisfy to be used for biological applications are stability (both chemical and physical) and biocompatibility. The latter is particularly critical, due to the presence of a cationic surfactant (commonly CTAB) on the surface of the Au. CTAB is known to induce cytotoxicity both *in vitro*[28] and *in vivo*[29] and it is commonly used during the synthesis process to drive the NR-shape formation[30]. Stability and biocompatibility are often improved by depositing additional coatings on the Au NR surface (e.g. polyethylene glycol, silica)[31]. To assess biocompatibility, different assays can be used *in vitro* (e.g. live/dead, MTS, MTT, etc.)while histological analysis is often performed during tissue experiments[7,8,12,15].

Another challenge to face when working with nanomaterials is the difficulty of correctly determining their molar concentration. The huge variety of NPs in terms of shape, size and chemical properties makes the techniques currently available suitable for only certain classes of particles. For example, the standard dynamic light scattering method assumes that NPs have a spherical shape and scatter light isotropically[17]. Therefore, application of this method to Au NRs results in discrepancies between the measured concentration and the real one. The issue of NP concentration is particularly problematic if related to the nanomedicine field, where the concentration administered needs to be precisely controlled in order to maximize the efficacy of the process (e.g. for drug delivery applications) and minimize the toxicity of the nanomaterials[17]. In the studies reported here, the optical density used gave the good results in terms of cell viability and particle number.

Due to their intrinsic absorption properties, Au NPs are often used in combination with a laser source. During the exposure, absorption and scattering are the two main processes likely to occur at the surface of the NPs. If the laser wavelength matches the plasmon resonance wavelength, absorption often prevails over scattering, exciting the conduction electrons at the NP surface. These form an electron gas that moves away from its equilibrium position and creates a resonant coherent oscillation called the localized surface plasmon resonance (LSPR). The energy is then transferred to the NP crystal lattice as heat, which is thereafter dissipated rapidly into the surrounding environment[32]. Since heat is the main effect observed after the excitation of the NR LSPR, it is advisable to monitor cell viability after laser exposure.

It has been hypothesized that the effects observed on the neurite outgrowth and the intracellular Ca^{2+} pathway were due to the transient heating arising from excitation of the LSPR. This hypothesis is in line with the activation of the temperature-sensitive TRPV1 channel after magnetic exposure of ferrite NPs[5]. This process is also consistent with observations that thermally sensitive TRPV4 channels play an important role in infrared nerve stimulation[33]. At a molecular level, it has been recently shown that TRPV4 is thermosensitive only after the interaction of phosphoinositide -4,5 -biphosphate (PIP_2) with the channel[34]. Therefore it is possible that the transient heating arising after NR excitation could serve to accelerate and/or induce the opening of the TRPV4 channels. This hypothesis can be confirmed with future Ca^{2+} experiments by using Ca^{2+}- free medium and/or molecular controls over the PIP_2 depletion.

Different groups have also demonstrated that small temperature gradients over the physiological temperature range can be used to induce other responses, such as neuronal growth cone guiding[25] or depolarizing currents in human embryonic kidney cells[35]. Yong and coworkers recorded a significant increase in electrical signal activity in primary auditory neurons cultured with silica-coated Au NRs after laser irradiation of the NRs at the LSPR. The heating produced by the laser-irradiated particles was measured using patch-clamp techniques[14]. More recently, Eom *et al.* recorded an increase in the amplitude of CNAPs after laser exposure of 3.4×10^9 Au NRs when continuously perfused in the vicinity of the sheath of the nerve bundle of a rat sciatic nerve[15].

The stimulatory effect of the 780 nm laser diode observed during the experiments was not linked to heat generated by water absorption, as this is known to be negligible at 780 nm[36]. Previously published results have also reported stimulatory effects of 780 nm light on neuronal tissue *in vivo*[37,38]. *In vitro* studies have demonstrated the involvement of reactive oxygen species in the process[39]. However, the detailed mechanisms by which NIR stimulation affects gene transcription and other cellular activities are currently unresolved. Current data suggests the interplay of different effects in the stimulation, including photon absorption within cromophores in the mitochondria (almost 50% of the energy at 780 nm might be absorbed by cytochrome *c* oxidase)[37,39-41], changes in membrane permeability to calcium[37] and inhibition of inflammatory activity in the cells[37].

The results presented in this manuscript demonstrate that nanoparticle absorbers hold great promise for future applications in optical stimulation of neuronal cells. The main advantage lies in the ability of NIR light to penetrate deep into tissues (therapeutic window). These results also show that nanoparticle absorbers can enhance and/or replace the process of infrared neural stimulation based on water absorption. For future applications in neural prostheses it would be of interest to investigate different surface functionalizations with chemical affinity for neuronal axons, which are the main targets in many optical stimulation applications.

DISCLOSURES:
The authors have no competing interests to disclose.

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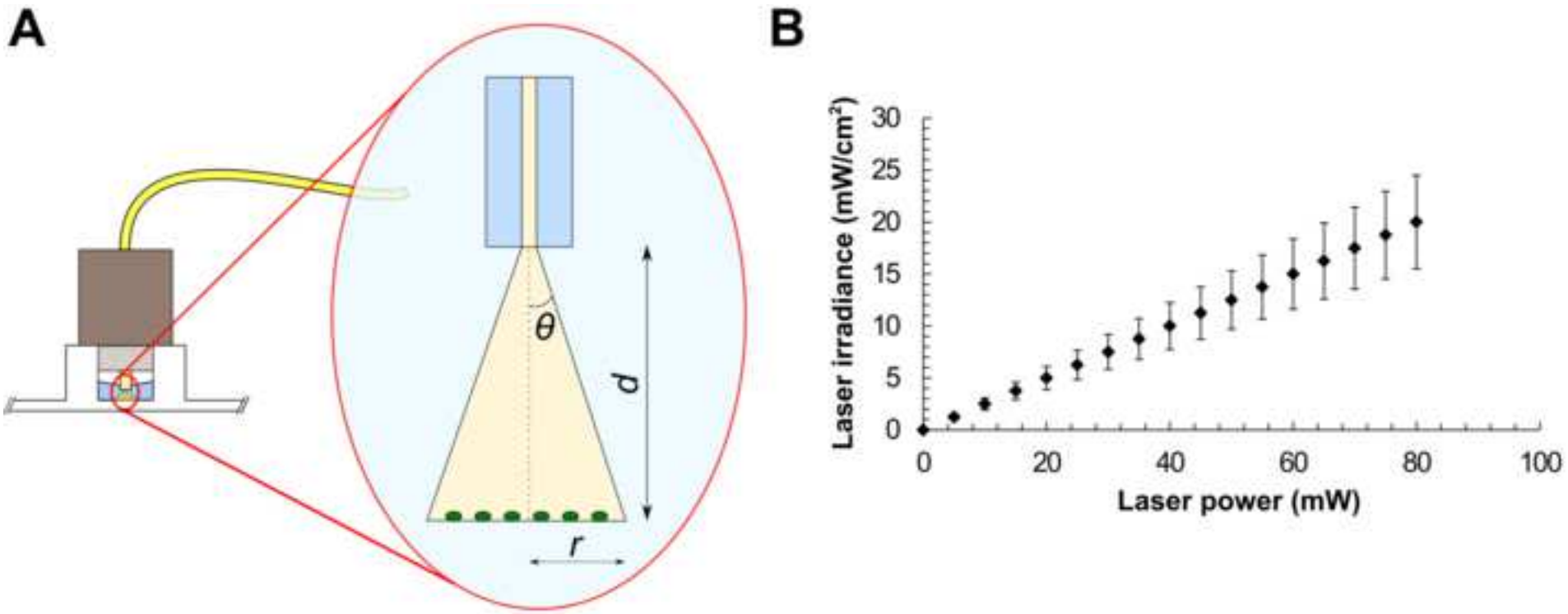

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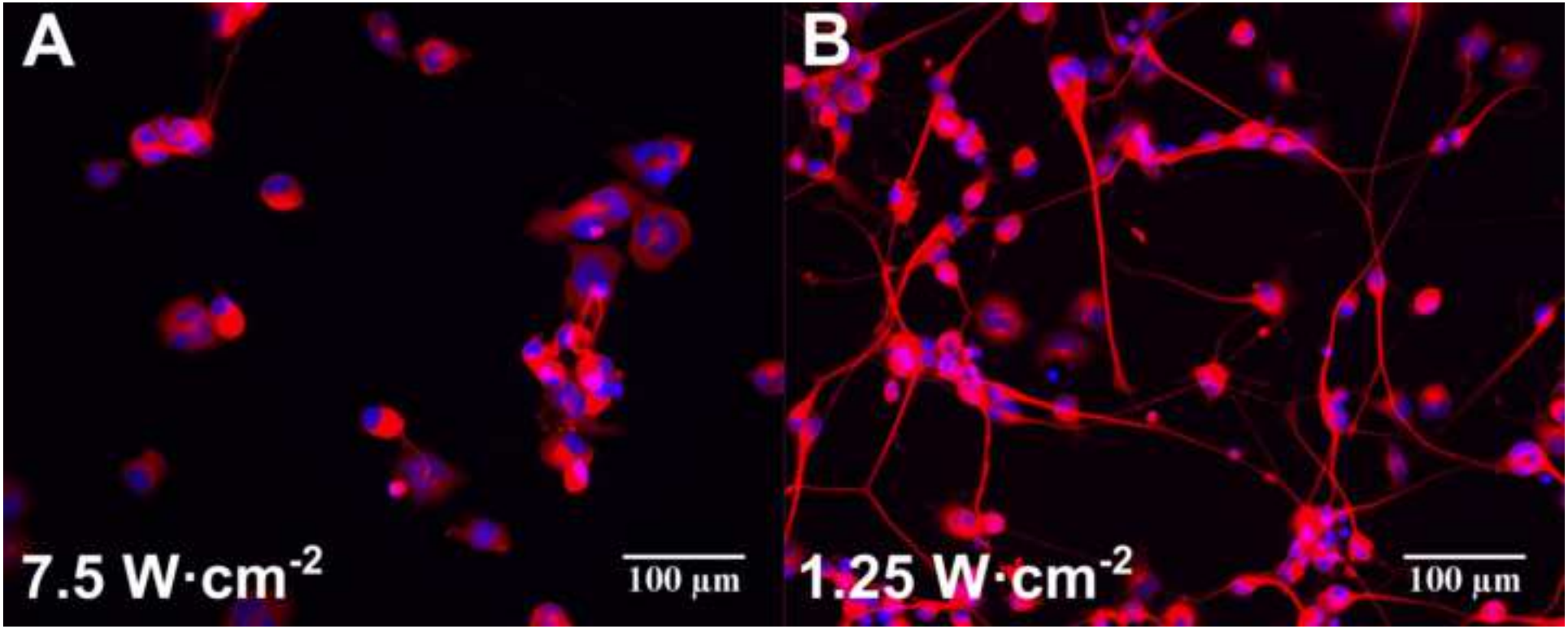

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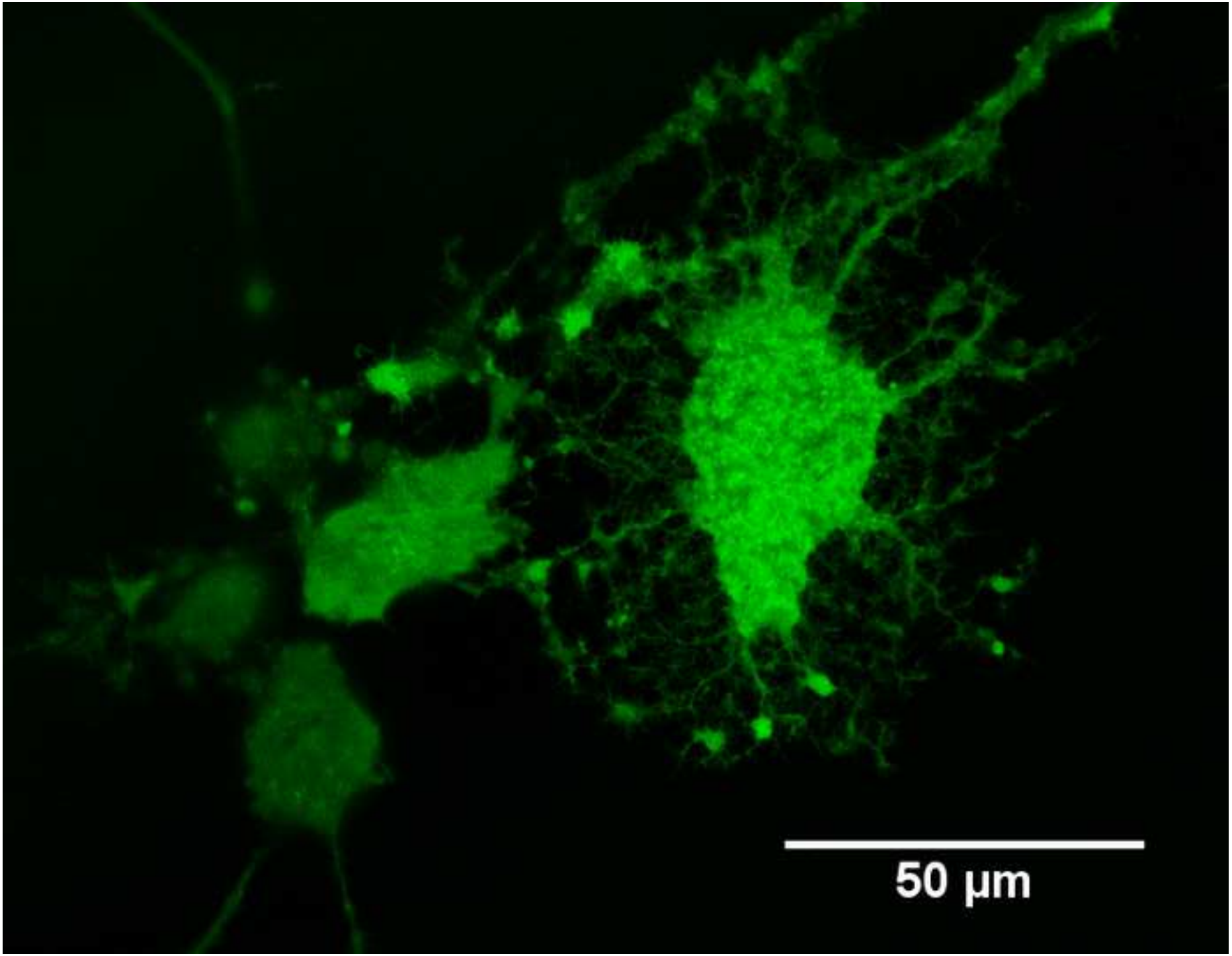

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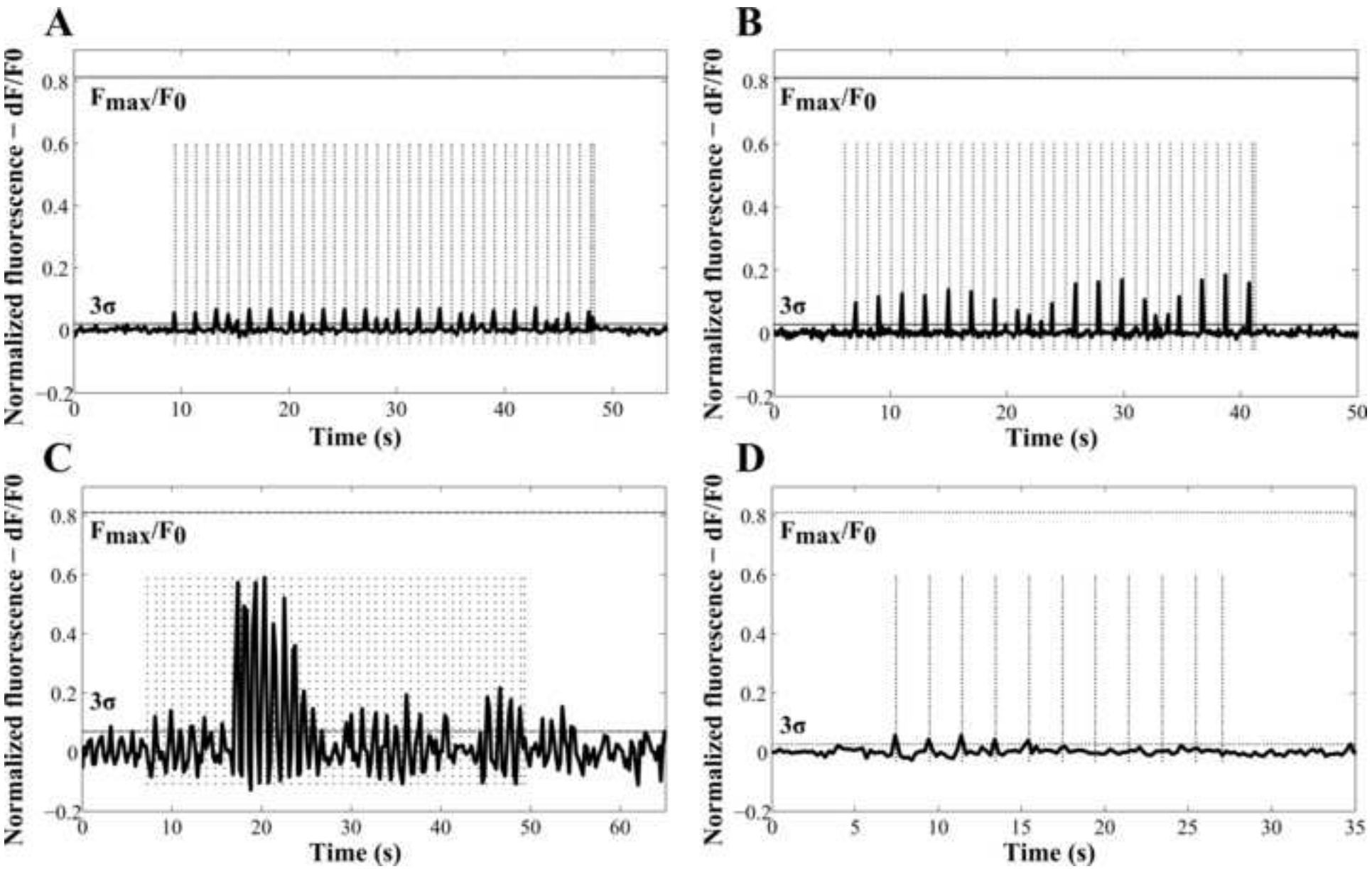

Excel Spreadsheet- Table of Materials/Equipment

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Name of the Reagent	Company
Au NR	Sigma Aldrich
NG108-15	Sigma Aldrich
DMEM	Sigma Aldrich
FCS	Life Technologies
L-glutamine	Sigma Aldrich
Penicillin/streptomycin	Life Technologies
Amphotericin B	Life Technologies
Formaldehyde	Sigma Aldrich
Triton X-100	BDH
BSA	Sigma Aldrich
Anti-βIII-tubulin	Promega
TRITC-conjugated anti-mouse IgG antibody	Sigma Aldrich
DAPI	Invitrogen
Fluo-4 AM	Invitrogen
DMSO	Sigma Aldrich
Pluronic F-127	Invitrogen

Equipment name	Company
UV-Vis spectrometer	Varian Medical Systems Inc.
Mini centrifuge	Eppendorf
Sonic bath	Unisonics Australia
Cell culture incubator	Kendro
Cell culture centrifuge	Hettich
Laser diode	Optotech
Optical fiber	Thorlabs
Power meter	Coherent
ImageJ	http://rsb.info.nih.gov/ij/index.html
Epifluorescent microscope	Axon Instruments
μ-slide well	Ibidi
Inverted confocal microscope	Carl Zeiss Microscopy Ltd.
Oscilloscope	Tektronix

Catalogue Number
716812
8811230
D6546
10100147
G7513
15140122
15290018
F8775
T8532
A2058
G7121
T5393
D1306
F14201
472301
P6867

Catalogue Number
Cary 50 Bio
Mini Spin
FPX 10D
Hera Cell 150
Rotofix 32A
780 nm single mode fibre - coupled LD
780 HP
Laser Check

ImageX-press 5000A
80826
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31 October 2014

Dr. Moshe Pritsker
Chief Executive Officer
Journal of Visualized Experiments

Dear Dr. Pritsker,

Re: Revised manuscript for JoVE

Attached please find the revised manuscript entitled "Gold nanoparticle-assisted optical stimulation of neuronal cells", by C. Paviolo, S. L. McArthur and P. R. Stoddart, for your kind consideration for publication in **Journal of Visualized Experiments.** The specific changes that have been made in response to the Science Editor and reviewer comments are listed below.

In Response to the Science Editor:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

The authors proofread the manuscript and some minor spelling and grammar issues were corrected.

2. Minor question: What is the wavelength of the UV light used in step 1.5?

The value of the wavelength has been added in point 1.6 (Procedure Section).

In response to Reviewer 1:

Manuscript Summary:

This manuscript presents a protocol to prepare gold nanorods to be used with neuronal-type cells for photo-induced stimulation.

The protocol part of the paper is clear and would be of use for the community. However, the representative results require some improvement.

As suggested, the Representative Results Section has been improved by modifying Figure 4.

Major Concerns:

1) Explain how to measure the nanoparticle concentration (Section 2.4.3).

The authors thank the reviewer for this thoughtful comment. Because of the great diversity of NP shapes and chemical properties, currently there is not a broadly applicable method for NP concentration measurements. However, several techniques can be applied to evaluate the molar concentration of a NP solution. Section 1.2 has been added to the text together with a review article showing the state-of-the-art technologies for measuring nanoparticle molar

concentration. This issue has also been presented in a paragraph in the Discussion Section.

2) Measure effect of gold nanorods without any stimulation. There is recent evidence that gold nanoparticles could increase neuronal excitability:

Jung S, Bang M, Kim BS, Lee S, Kotov NA, Kim B, Jeon D: Intracellular gold nanoparticles increase neuronal excitability and aggravate seizure activity in the mouse brain. PLoS One 2014, 9:e91360

Transient extracellular application of gold nanostars increases hippocampal neuronal activity. Salinas K, Kereselidze Z, DeLuna F, Peralta XG, Santamaria F. J Nanobiotechnology. 2014 Aug 20;12(1):31. [Epub ahead of print] PMID: 25135485

The reviewer makes an excellent point. In our previous work [Ref. 11], we observed that the NPs (poly(styrenesulfonate)-coated and silica-coated only) were associated with a moderate increase in the percentage of neurons with neurites, without any laser irradiation. A comment to this effect has been added in the Results Section together with the suggested references. Some other control experiments were mentioned in point 2.7 (Method Section).

In the representative results the authors report a 36% increase in neurite length between the irradiated and non irradiated samples. Please include the difference between incubated and non-incubated samples too.

This point has been clarified in the Results Section.

Another factor in the failure to elicit a calcium response for every laser stimulation pulse could be the efficiency of internalization of nanoparticles by the cells. Could the authors please add some information about what was the percentage of cells that contained nanoparticles?

The reviewer makes an interesting point. Particle endocytosis was checked with rhodamine-B-labelled nanoparticles, showing internalization from day 1 of incubation (comment added in the Result Section).

Can the authors indicate where the nanoparticles are localized in Figure 3?

The localization was predominantly observed in the cell cytoplasm; the nucleus exhibits little or no nanoparticles uptake, while no particles could be observed in the neurites. A comment has been added in the Results Section.

Minor Concerns:

I am sure the excitation wavelength of DAPI is a typo (Section 3.5)

This typo has been corrected in the text.

In response to Reviewer 2:

Manuscript Summary:

This article reports a simple technique to apply heat through Au NR using NIR laser. The technique is used for neuronal cell lines and has a limited application in neural engineering.

Major Concerns:

This work uses neuronal cell line which is different from primary neuronal cell cultures. Thus, the method and data could be quite limited to the cell lines and should discuss. For example, the incubation time of Au-NRs with cells are very long (24 hr) compared to the time used for primary neuronal cultures (Ref. Yoo et al., "Photothermal Inhibition of Neural Activity with Near-Infrared-Sensitive Nanotransducers," ACS Nano, Jul 21 2014). Authors should discuss the limitation when extending the similar method to other neuronal cell preparations.

The authors thank the reviewer for this thoughtful comment. The incubation time of Au NRs in the suggested reference (9 hours) is shorter than the one proposed in this method (24 hours) because of the different purpose of the study. Specifically, Yoo *et al.* functionalized the particles with amine groups to achieve cell membrane attachment, while we recorded internalization. We agree that this point could have raised confusion in the original manuscript, therefore we have clarified it by adding few comments in the Results Section. Please note that a similar work on primary auditory neurons (Ref. Yong *et al.*, "Gold-nanorod-assisted near-infrared stimulation of primary auditory neurons", Adv. Healthcare Mater. 2014) uses a similar incubation time to the one proposed here (15-17 hours).

The motivation of the work came from the infrared optical stimulation technique that was developed for control neural activity such as action potentials. However, the experiment and results are not directly related with the control of action potentials. It is not clear whether the presented method can be effective in neural stimulation of primary neuronal cultures. So, I suggest to modify title and abstract accordingly.

The authors agree with the reviewer that the word "stimulation" can possibly cause some confusion in the reader. However, "stimulation" is generally used to describe a process of evoking or inducing a specific activity and can also refer to how organisms, tissue or cells perceive incoming stimuli.

In the abstract of the manuscript, "stimulation" refers to "*The underlying mechanism associated with the optical absorption of gold nanorods, which can cause triggering of neuronal cell differentiation and increased levels of intracellular calcium activity*". Since the definition is explicitly given, the authors don't see the necessity of changing the title, but we have modified the Long Abstract and Introduction to avoid the impression that we are only referring to electrical stimulation.

Figure 4 should be replaced with more convincing data, as it is difficult to be convinced whether there was a real effect in calcium signals.

The authors thank the reviewer for this comment. Figure 4 has been changed accordingly.

Minor Concerns:

1. **line 114 ~ 137: what kind of Au-NR used? bare? PSS coated? or silica coated?**

The protocol was kept general to be extended to different particle shapes and surface chemistries. Therefore the types of NRs used in the experiments were only mentioned in the Representative Results Section.

2. **line 160: what was used to detach mechanically?**

The required information is included in point 2.4. The wording has been modified to clarify the process: "*Mechanically detach the cells by gently tapping knocking them off from the bottom of the confluent flask. Do not use trypsin.*"

3. **line 178: specify the laser source, optical finer, finer connector**.

The required information is included in point 3.1 "*Couple the laser with a single mode optical fiber (numerical aperture = 0.13) and terminate it with a fiber connector (FC connectors are convenient and commonly available). Measure the output laser power with a standard power meter. To obtain the most effective results, match the peak wavelength of the laser to the plasmon resonance peak of the NRs.*"

4. **line 231 ~ 235, 286 ~ 289: what program was used to analyse the images?**

As already mentioned, the protocol was kept general to match different user needs/materials availability. The program used to analyse the data was added as an example in Section 3.3.3.

5. **Figure 1B: why is the error bar getting bigger as the power increase?**

The errors in Figure 1B were calculated using the general theory of error propagation (see point 3.3.3 in the Method Section). This theory involves the partial derivatives of the measurement and multiplies it by the absolute error. Therefore, if δy is the absolute error, the resulting error δx in the measurement x is given by:

$$\delta x = \frac{\Delta x}{\Delta y}\delta y$$

First of all we evaluated the values of laser irradiance [laser power (x) divided by beam area (y) – see point 3.3.2] and calculate the partial derivative of it ($\Delta x/\Delta y$). Then we multiplied this value by the absolute error, which correspond to the error of the beam area. Given that the beam area and its error were constant, the only variable in the equation was the laser power. Therefore, the error of the measurement also increased with the laser power.

6. Figure 2: there should be figures for line 289 ~ 299. the following conditions: (without AuNR, without laser), (with AuNR, without laser), (without AuNR, with laser), (with AuNR, with laser). Quantitative data should be much helpful.

The authors understand that the addition of quantitative data would be helpful for the reader. Given that this work focusses on the Methods, we have therefore referred to our published papers to provide further clarification on the data.

7. line 322: typo (when NRs when not use …)

This typo has been corrected in the manuscript.

8. Experimental setup figure for Laser induced intracellular calcium imaging is needed

The reference for the experimental setup for the intracellular calcium experiments has been given in the text (end of Section 4.6).

9. Figure 4:

- add the citation if this is from the reference.

- Calcium data should be shown as (delta F/F) (%).

- It is difficult to interpret the stimulation effect. Please show the control signals. Assuming that the dotted line is the light stimuli, there seems to be no correlation between the Ca level and the stimuli.

As mentioned elsewhere, Figure 4 has been changed to address these concerns.

In response to Reviewer 3:

Manuscript Summary:

The manuscript provides a protocol on how to stimulate NG108-15 neuronal cells using gold nanorods heated by an IR laser and attached to the surface of NG108-15 neuronal cells. The manuscript solidly described the protocol and method, but limits the discussion of mechanisms, some challenges and controls.

The authors thank the reviewer for this comment and confirm that the discussion and result parts have been expanded accordingly. However, it is worth noting that JoVE is primarily a scientific methods journal.

Major Concerns:

The description of figure 3 says that "NG108-15 neuronal cells alone or cultured with Au NRs and poly(styrenesulfonate)-coated-Au NRs were exposed thereafter to radiant exposures between 0.07 J/cm2 and 370 J/cm2, with the laser frequency modulated between 0.5-2 Hz", yet figure 4 only shows one case. Figure 4 should include also overlaid traces of the control conditions, such as cells without Au NR but illuminated with IR and/or traces with different IR power to confirm the causality of IR plus Au-rods for the calcium signal.

The authors thank the reviewer for this comment and confirm that Figure 4 has been changed in the manuscript.

In response to Reviewer 4:

Manuscript Summary:

This manuscript proposes a protocol to obtain focused infrared optical stimulation of neuronal cells.

This method is alternative to electrical stimulation with the main advantages of a better spatial resolution and the possibility of stimulating tissue without direct contact. Interesting potential applications are stimulation of motor nerves and sensory system (e.g. cochlea) in the field of neural prostheses.

In this manuscript the authors propose the use of gold nanorods (NRs) as extrinsic absorbers to optimize infrared optical stimulation minimizing cumulative heating effects and consequent thermal damage of the targeted tissue.

Although the topic is of interest for future implementation of bionic devices requiring efficient interfaces with live tissue, the manuscript has some points not clear, some questions in my opinion not properly addressed and it is weak in the discussion and interpretation of neurophysiological data.

Major Concerns:

1) What is exactly meant by "stimulation" in the paper? This term is used in a biologically very undefined way.

The word stimulation was already clarified in the abstract of the manuscript. Specifically, "stimulation" refers to "*The underlying mechanism associated with the optical absorption of gold nanorods, which can cause triggering of neuronal cell differentiation and increased levels of intracellular calcium activity*". However, we have modified the Long Abstract and Introduction to avoid the impression that we are only referring to electrical stimulation.

The authors observe both increase of growth after infrared optical stimulation and "Ca2+ activity" during such stimulation but biological considerations on this respect are very limited, especially when concerning the nature of Ca2+ increases.

A paragraph discussing a possible bio-molecular mechanism for the observed stimulations has been added in the Discussion Section. However, it should be noted that the purpose of this paper is to complement a video presentation of the methodology used to stimulate neuronal cells with gold nanoparticles, which is why the biological considerations have been kept limited. We have referred the interested reader to our previously published reports for further elucidation on the mechanisms.

2) The authors want to demonstrate that use of irradiated NRs stimulates growth at lower power exposures, but the observations reported are confusing.

First it is stated that (line 292-295)

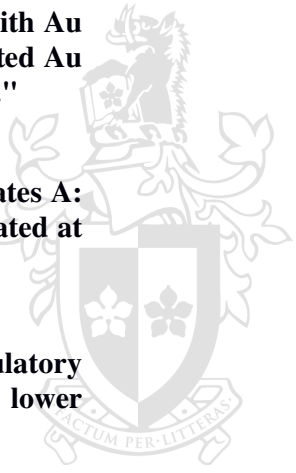
"By using protocols 1, 2, and 3 described here, a stimulatory effect on differentiation was observed in NG108-15 neuronal cells cultured with Au NPs (Au NRs, poly(styrenesulfonate)- coated Au NRs and silica-coated Au NRs) after laser exposures between 1.25 and 7.5 W*cm−2 (Figure 2)."

In respect to which control?

Then legend of Figure 2 is not coherent with the previous text. It states A: culture alone irradiated at 7.5 W cm-2 . B: culture with NRs irradiated at 1.25 W cm-2.

And a few lines below (line 302-304)

"Control experiments without Au NRs also showed some stimulatory effects of the 780 nm light. This stimulation was more effective at lower



laser powers (1.25 W/cm2) with a subsequent decrease at the highest laser energy (7.5 W/cm2) ."

So the absence of growth in fig.2 A is due to the absence of NRs or too strong irradiation power per se? One parameter at a time should be changed. Fig.2 should show responses in growth with or without NRs at the same irradiation power.

Please double check text and Fig.2 legend for these incoherence.

Moreover, any statistical significance over these statements?

The authors apologize for the confusion in the presented data (Figure 2). Several comments and clarifications (including the control samples used, significances, legend etc.) have been added in the Results Section.

3) What it should be reasonable to test, for the purpose of the paper, is the ability of evoking neuronal firing of action potentials by IR stimulation. This should be ideally done by patch clamping or more indirectly by Ca2+ imaging. In this respect NG108-15 is a cell line that possess electrophysiological properties only partially resembling those of real neurons. As far as I know action potentials can be recorded in these cells only after 20 days from differentiation (Liu et al. BMC Neuroscience 2012). I wonder which kind of electrical activity can be stimulated after 3 days from differentiation in these cells. Did the authors make any electrophysiological characterization on their model providing evidence that these cells are capable to fire?

If so, please mention in the text.

The reviewer is correct to highlight this limitation, but the purpose of this methodology was not to describe patch clamp stimulation of neuroblastoma cells. The reviewer might want to refer to Yong *et al.* (Ref. "Gold-nanorod-assisted near-infrared stimulation of primary auditory neurons", *Adv. Healthcare Mater.* 2014) that proved the onset of action potential with irradiated gold nanorods in primary auditory nerve cells.

With respect to the electrophysiological characterization, we haven't done any on the NG108-15 cell model. However, we are aware that electrophysiological recordings were made in NG108-15 after 2-3 days from induction of differentiation (Ref. Yang *et al.* "Neural differentiation and the attenuated heat shock response", Brain Res. 2008).

Moreover when monitoring electrical stimulation of neuronal cells with Ca2+ imaging, the action potential induced Ca2+ signals should be characterized by fast rising phase followed by slow return to the baseline in the order of seconds. The imaging recordings shown in the manuscript do not show at all this typical behaviour and look very unusual. A better characterization of these evoked signals should be done, at least a test with perfusion of TTX (quickly blocking any firing activity in neuronal cells) to demonstrate that the signals are related to membrane depolarization.

The authors thank the reviewer for this comment. This experiment is certainly of interest in future work, but is unfortunately outside the scope of this methods paper. Again, the reviewer might be interested in Yong *et al.* (Ref. "Gold-nanorod-assisted near-infrared stimulation of primary auditory neurons", *Adv.*

Healthcare Mater. 2014) that shows action potentials after irradiation of gold nanorods in primary auditory nerve cells.

With regard to the duration of the Ca^{2+} events, Dittami *et al.* ("Intracellular calcium transients evoked by pulsed infrared radiation in neonatal cardiomyocytes", *J. Physiol.* 2011) shows a smaller amplitude and shorter time course for infra-red evoked Ca^{2+} transients (on the order of 670 ms). A reference to that work has been added in the Results Section of the manuscript.

Minor concerns:

Line 123-126: why the final Au NR sample must be at OD=1?

OD=1 was used throughout the experiments and it was found to give a better compromise between cell viability and particle concentration. A comment on this has been added in the Discussion Section.

Line 229: DAPI λEX= 40 nm ????

This typo has been corrected in the text.

Line 258-261 (4.5) consider to add a picture in the movie.

The movie will show the optical fibre cleaving process, giving a clearer picture to the reader.

Line 280-284: (4.7.1) add the sampling frequency used for Ca2+ imaging.

This information has been added in the manuscript in the Procedure Section (point 4.7.1).

Figure 4. Is this figure as panel C-fig.2 of the paper: Paviolo et al., Laser exposure of gold nanorods can induce intracellular calcium transients (2013)? Is figure 4 expressing fluo intensity in arbitrary units or normalized fluorescence?

Figure 4 has been changed accordingly.

The authors confirm that the work contained within this manuscript is original, unpublished, and not being considered for publication elsewhere.

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Yours sincerely,

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