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)

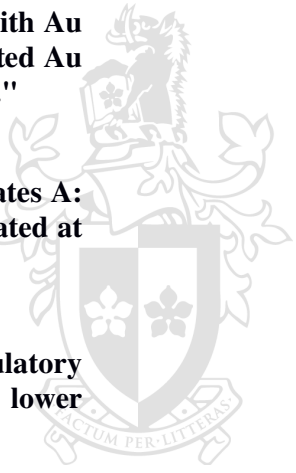
"By using protocols 1, 2, and 3 described here, a stimulatory effect on differentiation was observed in NG108-15 neuronal cells cultured with Au NPs (Au NRs, poly(styrenesulfonate)- coated Au NRs and silica-coated Au NRs) after laser exposures between 1.25 and 7.5 W*cm−2 (Figure 2)."

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.

Any further correspondence regarding this paper should be directed to Paul R. Stoddart at the following address:

A/Prof Paul Stoddart
Faculty of Science, Engineering and Technology
Swinburne University of Technology
Ph +61 3 9214 5839
E-mail: pstoddart@swin.edu.au

Yours sincerely,

Paul Stoddart