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Article

PRUSSIAN BLUE NANOPARTICLE-MEDIATED SCALABLE THERMAL STIMULATION FOR NEURONAL DIFFERENTIATION

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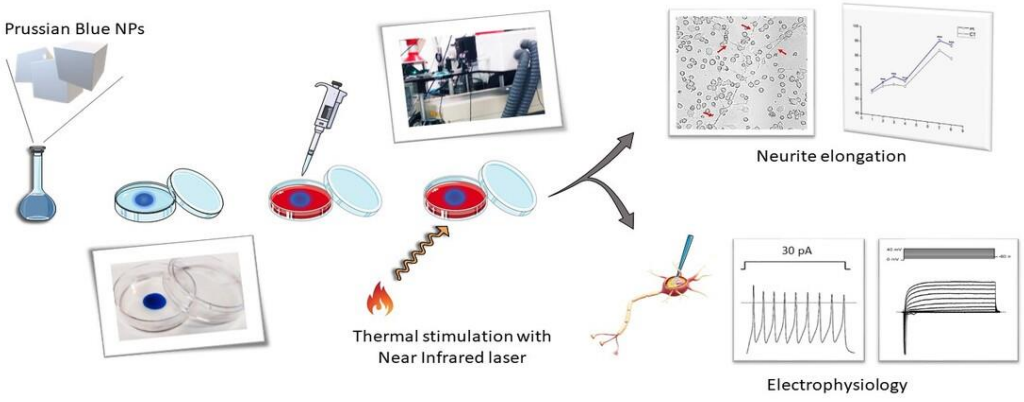
Abstract: Heating has been recently used as an alternative application to electrical stimulation to modulate excitability and to induce neuritogenesis and the expression of neuronal markers, but a long-term functional differentiation has not been described so far. Here we present the results obtained by a new approach for scalable thermal stimulation on the behavior of a model of dorsal root ganglion neurons, the F-11 cell line. Initially, we performed experiments of bulk stimulation in incubator for different time intervals and temperatures, and significant differences in neurite elongation and in electrophysiological properties were observed in cultures exposed at 41,5°C for 30 minutes. Thus, we exposed the cultures to the same temperature increase by irradiating, with a near infrared laser, a disc of Prussian Blue nanoparticles and poly-vinyl alcohol, that we stuck on the outer surface of the petri dish. In irradiated cells neurites were significantly longer and the electrophysiological properties (action potential firing frequency and spontaneous activity) were significantly increased compared to the control. These results suggest that a targeted thermal stimulation could be a promising technique to induce differentiation and support the future application of this method as a strategy to modify neuronal behavior *in vivo*.

Keywords: nanoparticles; thermal stimulation; neuronal differentiation; neurite outgrowth; electrical activity; electrophysiology.



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1. Introduction

Heating has been the subject of increasing attention in the last years for its ability to modify cell behavior and modulate the electrical activity of excitable tissues. Previous studies showed that different combinations of time and temperature, from milliseconds at high temperature to several hours/days at mild temperature, could elicit depolarizing currents or promote neuritogenesis and induce the expression of neuronal markers [1,2,3,4]. These effects are probably due to changes in cell membrane capacitance and in ion channel properties [1,5], and to the activation of differentiation pathways [3], but the underlying mechanism by which these changes occur remains so far unknown. In many studies, infrared neuronal stimulation (INS) has been applied mainly by exploiting the absorption properties of water in the infrared (IR) region [1]. Recently, the combination of near infrared (NIR) with photo-thermally active nanomaterials has been employed in order to scale the excitation wavelength to the interval 700-1000 nm, which lies in the transparent optical windows for tissues [2,6,7,8]. IR photo-thermally active nanomaterials are explored as mediators to convert light, as primary stimulus, to a secondary stimulus, such as heat, which can be localized to stimulate neurons [9,10,11]. These photothermal nanoparticles, alone or together with probes and conjugates, can penetrate biological tissues and can be used for hyperthermic treatments arising from either localized surface plasmon resonances or charge transfer transitions [12]. Nanoparticles are also applied in photothermal (PTT) and photobiomodulation (PBT) therapy, which have become two of the most common therapies used in the last few years [13]. However, for the opto-thermal stimulation, internalization of nanoparticles may cause spatial distribution variability, instability, and cytotoxicity. Nanoparticles could undergo biodegradation in cell environment and damage cell components such as plasma membrane and intracellular organelles. They could compromise cell membrane integrity through lipid peroxidation, generate oxidative stress or inflammation, and they could have a genotoxic potential for cells. For this reason, it is important to study the cytotoxic effect of nanoparticles in order to choose the safest type to use for cell treatments [14].

Prussian Blue nanoparticles (PBNPs) have been approved in 2003 by the U.S. Food and Drug Administration (FDA) as a safe and non-toxic compound available in commerce [15]. They consist of a coordination polymer containing Fe^{3+} hexa-coordinated by the N atoms of $[Fe(CN)_6]^{2+}$ and they can be soluble or insoluble, depending on the exact formulation, namely $Fe_4[Fe(CN)_6]_3 \cdot xH2O$ (x = 14-16, "insoluble" PBNPs) and $KFe[Fe(CN)_6] \cdot xH_2O$ (x = 1-5, "soluble" PBNPs). Soluble PBNPs show lower dimensioned crystals that reach the size typical of the mesophase, forming clear deep-blue colored colloidal solutions, whereas insoluble PBNPs form larger crystals that easily aggregate and give a precipitate. However, all these formulas correspond to the same crystal and molecular structure by X-ray diffraction [16]. PBNPs are used in several applications such as antimicrobial therapy [17] or cancer treatment, often combined with a NIR laser [18]. They can convert light into heat thanks to a metal-to-metal charge transfer between Fe^{2+} and Fe^{3+} through a cyanide bridge, resulting in an intense, large absorption band with maximum at ~ 700 nm; the light irradiation in the 700-750 nm region results in a thermal relaxation.

In this study, we took advantage of using PBNPs, dispersed in a polymeric matrix and not in direct contact with cells, to obtain a scalable and controlled thermal stimulation to

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modulate neuronal behavior and properties of an *in vitro* model of dorsal root ganglion (DRG) neurons, the neuroblastoma F-11 cell line. The effects of this stimulation on cell morphology and electrophysiological properties enabled us to establish a reproducible protocol for optical stimulation, showing for the first time a maturation and a long-time maintained neuronal differentiation.

2. Materials and Methods

2.1. Cell cultures

F-11 cells (mouse neuroblastoma N18TG-2 x rat DRG, ECACC Cat#08062601 RRID: CVCL_H605; [19]) were seeded at 50,000 cells/35 mm dish (Corning®; Sigma-Aldrich, St. Louis, MO, USA) and maintained in a serum-deprived medium to reduce proliferation. The complete composition of the medium was as previously described [20]: Dulbecco's modified Eagle's medium (Cat#D6546; Sigma-Aldrich, St. Louis, MO, USA), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% fetal bovine serum (FBS, Cat# F2442; Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptomycin (10000 U/ml, lot#753901 ref#15140; Gibco™, Waltham, MA, USA). 24 hours after seeding, the cells were thermally stimulated. The stimulation was repeated the following day, with the same parameters of duration and temperature. After each stimulation the cells were replaced in the incubator at 37°C, in a humidified atmosphere with 5% CO_2. Medium was replaced with fresh medium twice per week to prevent cell starvation. Cells maintained at 37°C in incubator were used as control. Morphological and functional analysis were performed for 8 days after seeding for both stimulated and control samples.

2.2. Bulk heating protocol

To verify if heating could effectively induce cell differentiation, experiments were performed in bulk configuration to find out the best protocol to be applied in irradiation experiments. In particular, the temperature of the incubator (Jouan IGO150 CELLife CO_2 Incubator, Thermo Fisher Scientific, Rodano, MI, Italy) was increased from the standard 37°C to higher temperatures for 10 minutes/day for two consecutive days. The temperatures chosen were 39°C, 41,5°C or 43°C. Another series of experiments consisted in heating the cells for 10, 20, 30, 45 or 60 minutes/day for two consecutive days in order to find the best time duration of the heating protocol. Each experiment was performed on two or three independent cultures. After heating, cells were maintained in an incubator at 37°C.

2.3. Prussian Blue nanoparticle preparation

PBNPs were prepared according to literature [16] but increasing from 1 mM to 10 mM the concentration of the reagents; 100 ml of a 10 mM $FeCl_3$ solution were mixed with 10 mM $K_4[Fe(CN)_6]$ in 0.025 M citric acid and heated at 60°C under stirring. After 1 minute stirring at 60°C, the solution was cooled at room temperature. The solution was centrifuged for 25 minutes at 13,000 rpm in 10 ml test tubes for purification. The centrifuged PBNP pellet was resuspended in half the original volume. The absorbance peak of the PBNP aqueous solution was evaluated with Jasco, V-570 spectrophotometer.

2.4. PBNP-PVA layer preparation

A solution containing 7% of poly vinyl-alcohol (PVA, average molecular weight 72000 g mol^{-1}, degree of hydrolysis 98%, Sigma-Aldrich, St. Louis, MO, USA) and 27-30% of 10

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mM PBNPs was made. The PVA powder was dissolved in water and was maintained in oven at 70°C for at least one hour. Then the PBNP solution was added and the mixture was achieved by 1 hour under continuous stirring. 70 µl of the final solution was dropped on the outer surface of the petri dishes and dried in an oven at 70°C for at least 1 hour. The PBNP-PVA patch (blue colored) covered approximately a circular area of 1 cm^2. A circle of the same area was drawn on the control petri dishes to compare similar size regions.

2.5. Irradiation protocol by heating nanoparticle layers

A Ti:Sa laser (Mai-Tai DeepSea Ti:Sapphire®, Spectra Physics®, Santa Clara, CA, USA), tunable between 690 nm and 1100 nm, was used to increase the temperature of the medium into the petri dish in correspondence of the PVA-PBNP layer. By exploiting the 720 nm wavelength, the power was chosen to reach the desired temperatures, which were defined according to the results obtained by bulk heating experiments. Temperature calibrations were made either on dry petri dishes, to check the reproducibility of the layers, and on petri dishes with 2 ml of culture medium, in order to determine the power needed for temperature increase. Accurate temperature measurements were performed by a thermocamera (FLIR E40, FLIR Systems Inc., OR, USA) and by a needle thermocouple (Omega Engineering Ltd., Stamford, CT). The laser spot size has also been accurately calibrated in order to match the beam size with the photothermally active area. To this aim, a beam expander has been placed on the beam path and the spot size has been measured by recording the power after passing through a variable diameter iris. The final spot size was obtained by fitting the curve of power versus the iris diameter. The petri dish was placed on the sample holder and the laser beam irradiated it from below. During the irradiation, the petri dishes were maintained at 37°C by a home-made chamber whose temperature was controlled by The Cube (Life Imaging Services, Basel, CH).

2.6. Morphological analysis

Morphology was determined by imaging the cells for 8 days from seeding. Cells were seeded on day 0, and images were taken in transmitted light mode on day 1; after a recovery of at least 2 hours, they were heated for the first time. The same procedure was repeated on day 2. Electrophysiological recordings were performed on days 7 and 8. Transmitted images were acquired on a Leica SP5 microscope (Leica Microsystems, Wetzlar, D) with an air objective (20X HCX PL Fluoter, Leica Microsystems, Wetzlar, D). Six tiles mode images were acquired to fully cover the irradiated area, where the PBNP-PVA disc was present, or an equivalent one for control and bulk heating samples, in order to acquire images of the same region for the 8 days of the experiment, and a comparative statistical analysis was achieved among different experiments. The images were processed by FIJI ImageJ, version 2.0.0, Opensource code [21]. Neurites were manually traced, then a homemade macro, which subtracts each traced image from the raw one, was run. The new image, on which only the traced neurites were visible, was binary converted and skeletonized. The characteristics of the traced neurites were eventually extracted in a .txt file and statistical analysis were performed.

2.7. Electrophysiological analysis

The functional characterization of the electrophysiological properties of F-11 cells was performed by the patch-clamp technique in the whole-cell configuration at room temperature. Before recording, culture medium was replaced by a standard extracellular solution which contained (mM): NaCl 135, KCl 2, $CaCl_2$ 2, $MgCl_2$ 2, hepes 10, glucose 5, pH 7.4. The standard pipette solution contained (mM): potassium aspartate 130, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 1.3, EGTA 10, hepes 10, pH 7.3. Recordings were acquired by the

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pClamp8.2 software (pClamp, RRID:SCR_011323) and the MultiClamp 700A amplifier (Axon Instruments; Molecular Devices, LLC., San Jose, CA, USA). Resting membrane potential and action potentials were monitored in the current-clamp mode. In the voltage-clamp mode, the resistance error was compensated up to 50-70%. Sodium (INa) and potassium (IK) currents were recorded by applying a standard voltage protocol, which started from a holding potential of −60 mV, conditioned cells at −90 mV for 500 ms, and successively clamped the membrane at depolarizing test potentials in 10 mV-increments, from −80 to +40 mV. Both round-shaped cells and cells with neurite-like processes were tested for this characterization.

2.8. Lactate-Dehydrogenase (LDH) assay

In order to verify if heating induced cell stress, we tested cell viability by measuring the lactate-dehydrogenase (LDH) activity on both stimulated and control samples. LDH is an ubiquitous enzyme usually localized in the cytosol and is released into the medium by damaged cells. The samples used for the assay were maintained at -20°C. According to the protocol, they were defrosted in ice, centrifuged at 1000 rpm for 4 minutes and the supernatant was collected for the assay. The total solution volume of 1 ml was made, which contained (µl): K-phosphate Buffer, 850; NADH, 20, and stimulated or control sample, 70. The reaction started by adding 60 µl of Pyruvate. The rate of the absorbance decrease over time was measured and the ratio of LDH activity (U/ml) into the cell culture medium was calculated by using the standard formula.

2.9. Statistical analysis

For the data analysis, Origin 9 (OriginPro, Version 2019, OriginLab corporation, Northampton, MA, USA) and Excel (Microsoft, Redmond, WA, USA) were used. Data are presented as mean ± S.E. Mean comparisons were obtained using the parametric One-Way ANOVA test or the non-parametric Mann-Whitney test. Percentages of cells with spontaneous electrical activity were compared using the $\chi 2$ test. The significance level was set for $p \leq 0,05$.

3. Results

3.1. Effects of bulk heating: morphological and functional characterization

Experiments in bulk heating were performed to evaluate the eventual effects on the morphology and the electrical activity of F-11 cells. This is a neuroblastoma cell line, which expresses functional properties of mature sensory neurons under appropriate culture conditions [20] or when seeded on matrices mimicking the extracellular environment [22]. Temperatures ranging from 39°C and 43°C and exposure timing from 10 to 60 minutes were tested. Concerning the morphological properties, the most efficient time/temperature combination was 30 minutes at 41,5°C (Figure 1 and Figure S1), which induced significant elongation in neurites (about 27% of increase on day 8). On the contrary, the lowest temperature, 39°C, induced no effect on cell morphology, whereas the highest temperature, 43°C, seemed to determine cell suffering which became particularly evident after the fourth day. Since a visible decrease in neurite length and an increase in cell stress/mortality was seen in samples maintained at ≥43°C, chosen temperatures did not exceed 42°C.

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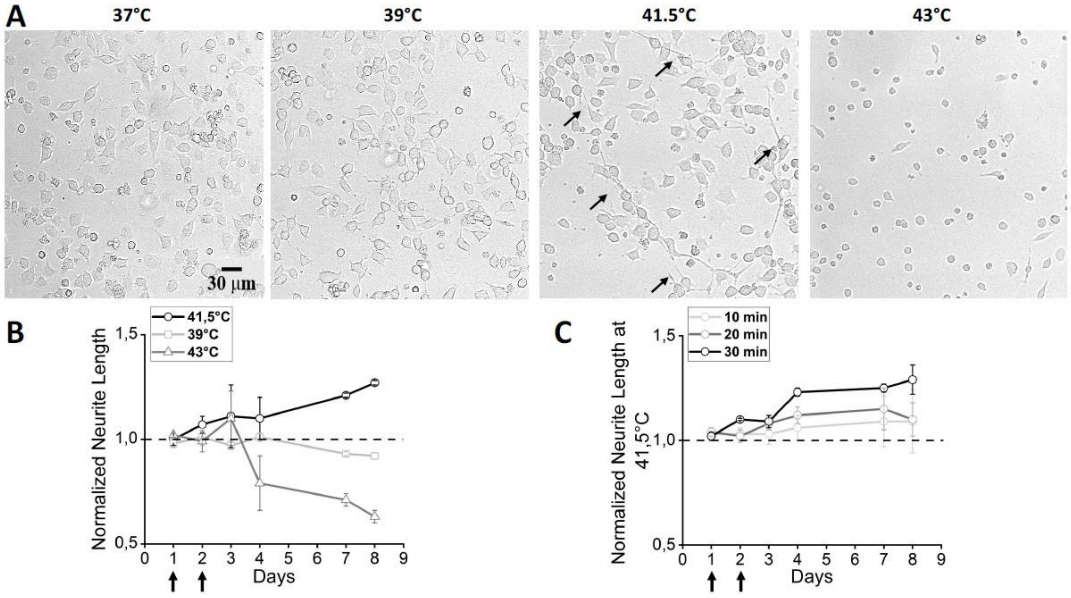

Figure 1: Morphological properties of F-11 cells exposed to bulk heating. (**A**) Representative images of cells at day 8, after exposure to bulk heating for 30 minutes at different temperatures. At 39°C, cells did not show differences with control cells (37°C), whereas, at 41,5 °C, an increase in neurite length (black arrows) was evident. Temperatures higher than 41,5°C (43°C) had detrimental effect on cell viability. (**B**) Neurite length normalized to the value of the control group on one day after seeding. The different symbols refer to the different temperatures applied for 30 minutes on day 1 and day 2 after seeding (indicated by black arrows). The temperature of 39°C (squares) did not induce any effect on cell morphology compared to the control. In 41,5°C samples (circles) neurites were longer versus the control, starting from day 3. The temperature of 43°C (triangles) seemed to induce cell stress particularly from day 4. (**C**) Normalized neurite length of heated (41,5°C) F-11 cells at different times of exposure. Neurite of cells maintained at 41,5°C increased in length with longer exposure times.

Moreover, cells at 41,5°C showed the tendency to sprout a higher number of processes than cells in control condition (Figure 2A). According to the results obtained by morphological analysis, an electrophysiological investigation was performed by the patch-clamp technique, both on cells with neuronal morphology and cells with round shape, to verify if the established protocol could also induce functional differentiation. The electrophysiological parameters investigated were the resting membrane potential, the electrical activity and sodium and potassium current densities. The resting membrane potential was more hyperpolarized in cells exposed at 41,5°C compared to control cells (-42 mV ± 1 mV versus -32 mV ± 2 mV, $p<0,001$, Mann-Whitney Test). Moreover, in heated cultures a significantly higher percentage of cells with spontaneous activity was present (67% versus 25%, $p<0,01$, Chi-Square Test) and a higher action potential firing frequency was measured compared to control (4,6 ± 0,7 Hz versus 2,6 ± 0,9 Hz, $p<0,05$, Mann-Whitney Test) (Figure 2B, C).

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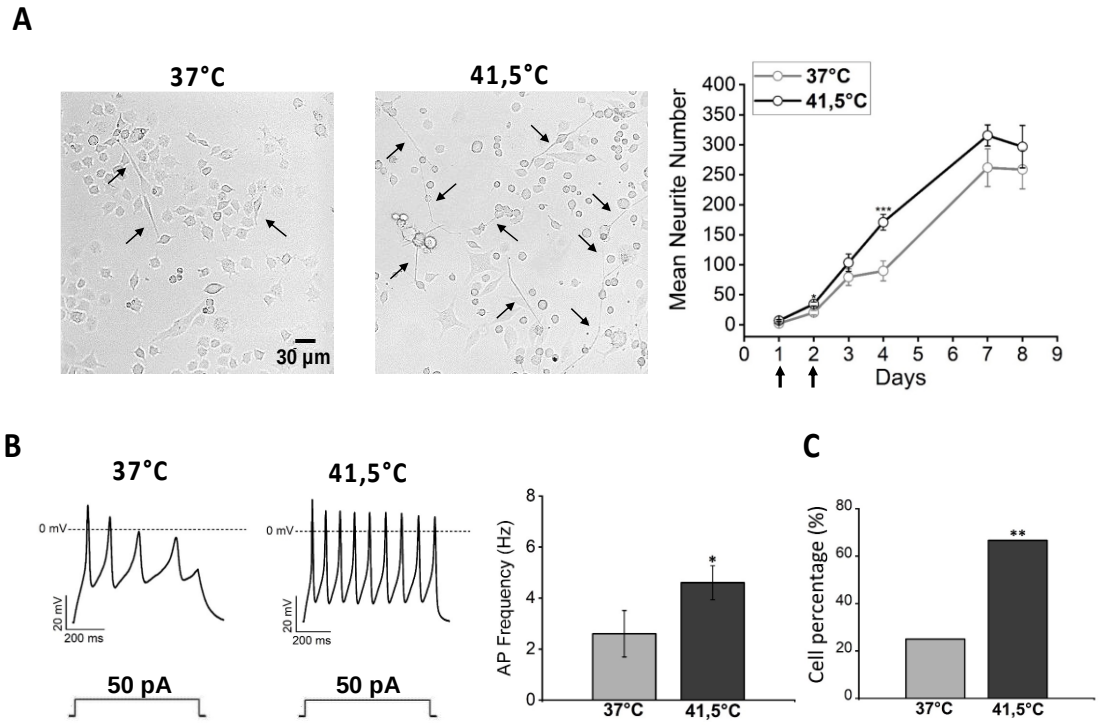

Figure 2: Morphological and electrophysiological properties of F-11 cells thermally stimulated at 41,5°C for 30 minutes. (**A**) Cells heated at 41,5°C on day 1 and day 2 after seeding showed more numerous and longer neurites (black arrows in the picture) compared to control cells maintained at 37°C, and (**B**) showed the ability to discharge spontaneous or induced action potentials at a higher frequency compared to cells maintained at 37°C (for 41,5°C samples: 4,6 ± 0,7 Hz, n=22; for 37°C samples: 2,6 ± 0,9 Hz, n=15; p<0,05, Mann-Whitney Test). (**C**) Moreover, 41,5°C samples had a higher percentage of cells with spontaneous activity compared to the control, indicating that thermally stimulated cultures had higher probability to develop small neuronal networks (for 41,5°C samples: 67%, n=16/24; for 37°C samples: 25%, n=4/16, p<0,001, Chi-Square Test).

Consistently with the electrical activity, heated cells had a trend to express higher sodium and potassium current densities compared to cells maintained at 37°C (Figure 3). Since these parameters are the hallmarks of neuronal maturation, these results indicate that bulk heating could also induce functional differentiation in the F-11 cell line.

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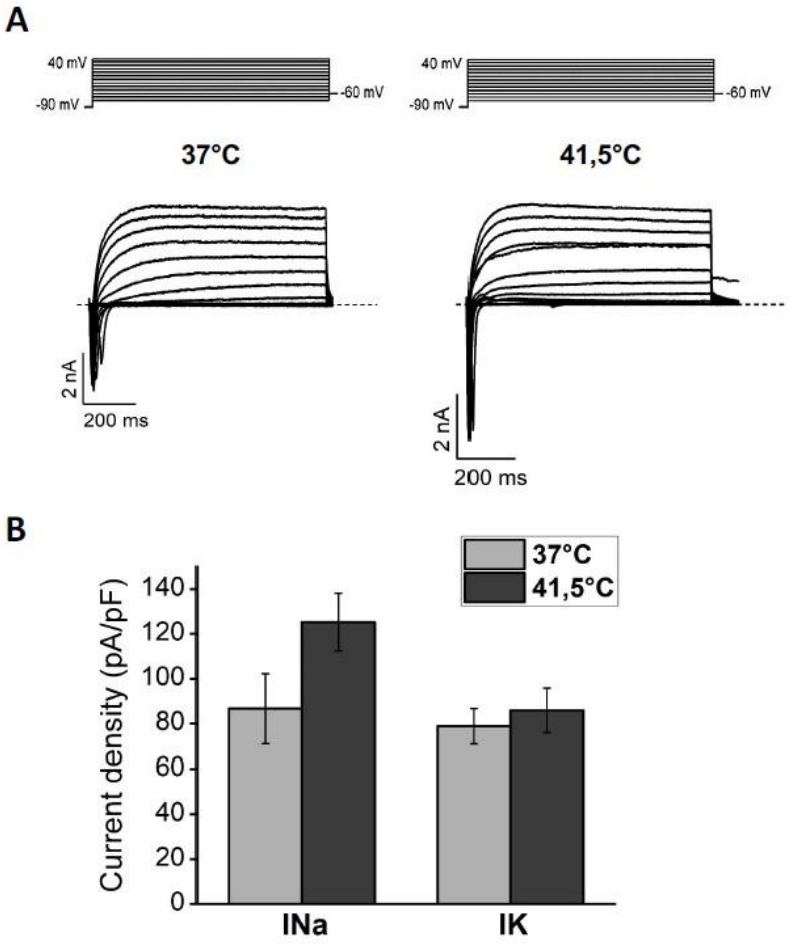

Figure 3: Functional analysis of F-11 cell electrophysiological properties by the patch-clamp technique on days 7 and 8 showed a more differentiated profile for heated cells versus 37°C cells. (**A**) Representative sodium and potassium current traces obtained by the protocol indicated above and described in the Material and Methods section. (**B**) Current density bar graphs; heated cultures showed a trend to express higher sodium and potassium current densities compared to the control (for 41,5 °C samples: INa, 125 ± 13 pA/pF and IK, 86 ± 10 pA/pF, n=23; for 37°C samples: INa, 87 ± 16 pA/pF and IK, 79 ± 8 pA/pF, n=15; for INa p>0,05, Mann-Whitney Test, for IK p>0,05, Mann-Whitney Test).

To exclude that this approach could induce cell stress, a lactate-dehydrogenase (LDH) assay was performed. The results, shown in Table 1, suggested that heated cells released in the medium levels of LDH equivalent to control cells (p>0,05, One-Way ANOVA Test, n=8 samples for each condition), indicating that the treatment was not detrimental to F-11 cell survival.

	Bulk Heating			
	37°C	S.E.	41,5°C	S.E.
Day 7-8	$4{,}98\times10^{-8}$	$1{,}28\times10^{-8}$	$4{,}86\times10^{-8}$	$1{,}16\times10^{-8}$

Table 1: Lactate-dehydrogenase activity measured on bulk heated cells versus control samples. Results showed that this treatment had no detrimental effects on heated samples (p>0,05, One-Way ANOVA Test, n=8 for each condition).

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3.2. Smart Petri dish characterization

Considering the efficacy of bulk heating on cell differentiation, the combination of 41,5°C for 30 minutes of exposure was chosen also for inducing a localized and selective stimulation by means of a NIR laser irradiation of PBNP-based polymer layer, applied on the bottom of the petri dishes (smart petri dishes). PBNPs were evenly dispersed in the hosting polymer matrix as it has been verified by imaging the smart layer on the petri dish by means of an optical confocal microscope in reflection mode (Figure 4). A z-scan performed on the overall extension of the layer revealed a thickness of 90 ±10 μm in agreement with the estimate obtained with a gauge.

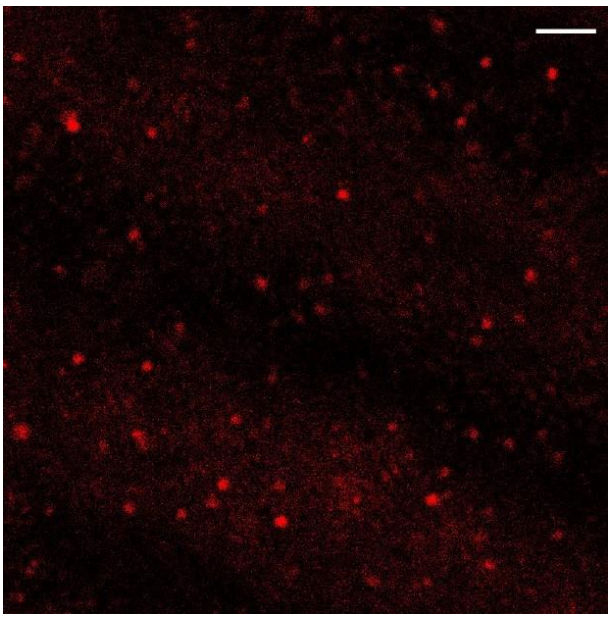

Figure 4. A 30 μm x 30 μm field of view of a selected plane in a z-scan acquisition of the PBNP-PVA smart layer (the bar corresponds to 3 μm). The red spots represent the reflection signal of the PBNPs upon 633 nm excitation.

In order to measure accurately the temperature reached within the medium, the exact conditions used in the NIR laser irradiation experiments were reproduced, and a needle thermocouple was mounted and fixed inside the chamber and fed inside the 2 ml solution in the petri dish by a custom drilled lid. The temperature increase was recorded versus time at different powers in order to select the proper power value necessary to obtain the requested temperature increase. Two examples of the curves obtained are shown in Figure 5. As can be inferred from the figure, after 2 minutes an equilibrium temperature was reached and it was maintained for all the irradiation time. The plateau value depends on the irradiation intensity and the nanoparticle concentration.

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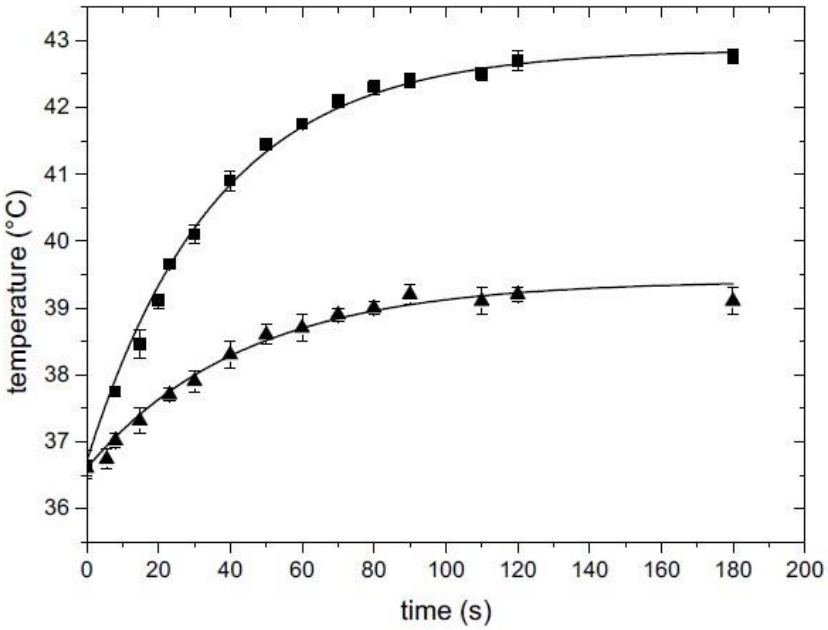

Figure 5. Temperature increases versus irradiation time measured by a needle thermocouple directly inserted in the Petri dish with 2 ml of solution. Squares I=0.15 W/cm^2, triangles I=0.07 W/cm^2.

3.3 Effects of thermal increase by PBNP irradiation

During the irradiation procedure, petri dishes were maintained in a box at 37°C. The temperature of 41,5°C was reached only on the PBNP-PVA disc by using the laser beam. Cultures maintained at 37°C in incubator were used as control. The morphological characterization showed that irradiated cells had longer neurites compared to control especially on day 7 and 8 (Figure 6A, B) as already found in bulk heating experiments. Moreover, stimulated cells showed an increase in mean neurite number starting from day 2 and especially on day 2 and 3 compared to the control (Figure 6C), suggesting that this method of thermal stimulation could induce neuronal differentiation.

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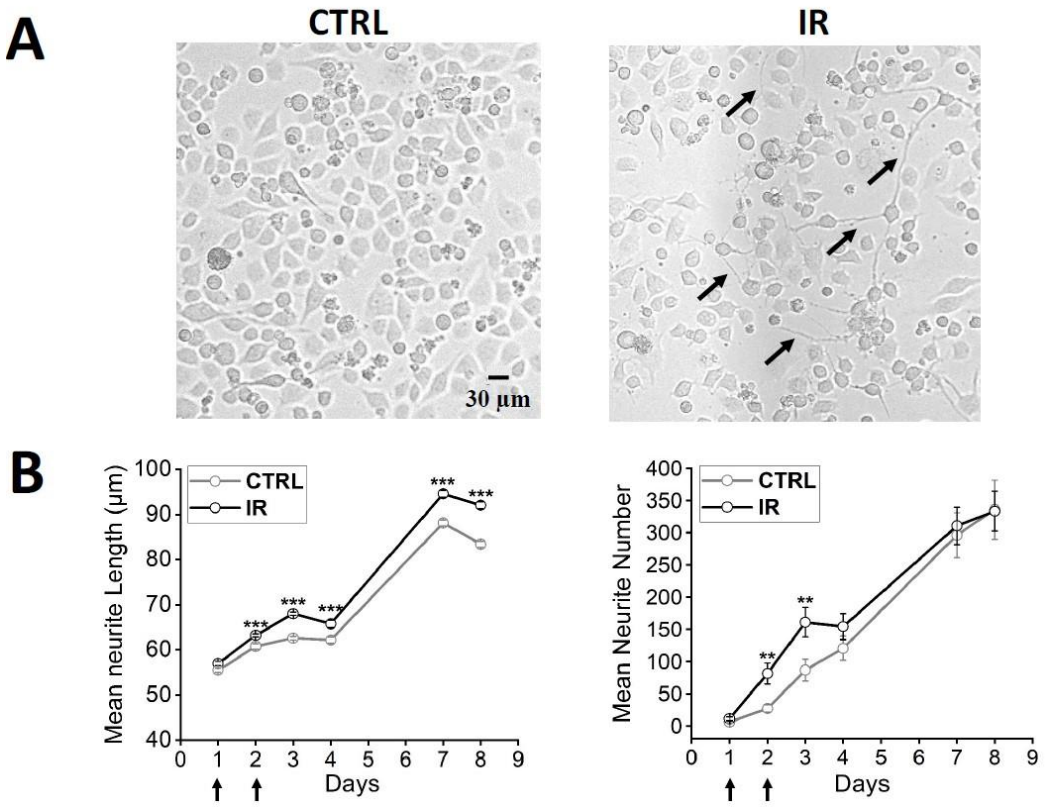

Figure 6: Morphological properties of irradiated F-11 cells. (**A**) Representative images show longer and more numerous neurites in cultures irradiated at 41,5°C (IR) than in cultures maintained at 37°C (CTRL). (**B**) Neurite length of cells irradiated at 41,5°C on day 1 and day 2 after seeding (indicated by black arrows), analyzed from day 1 to day 8, was significantly longer than the control starting from day 2 ($p<0,001$, Mann-Whitney Test). Moreover, in the irradiated cultures the number of neurites increased starting from day 2, with a significant difference at day 2 and 3 ($p<0,01$ and $p\leq0,01$, respectively, Mann-Whitney Test).

A control experiment in which cells were irradiated without the support of PBNPs was performed to verify if the use of NIR laser could induce differentiation by itself. Cells irradiated without PBNPs had a trend comparable to the control on all days of the experiment; moreover, they had shorter neurites compared to cultures irradiated with PBNPs, suggesting that the single NIR laser did not induce differentiation of F-11 cells (Figure S2A).

Furthermore, in order to verify the highest temperature the cells could tolerate, we irradiated samples at 43°C. After being irradiated at this temperature, cells showed shorter neurites compared to the control (Figure S2B) for all the examined time points. Interestingly, although the number of cells seeded on day 0 was the same in each petri dish ($5x10^4$ cells), in 43°C-treated samples, starting from day 4, cells attached in the middle of the irradiated PBNP-PVA disc were fewer, suggesting that this temperature caused excessive cellular stress.

Irradiated cells at 41,5°C had a typical neuronal morphology and were able to form several small neuronal networks. In order to verify the eventual development of the typical properties of electrically mature neurons, we performed an electrophysiological investigation on day 7. Irradiated cells had a resting membrane potential more hyperpolarized compared to the control (-39 ± 1 mV versus -30 ± 2 mV, $p<0,001$, Mann-Whitney Test) and a higher action potential firing frequency (5,9 ± 0,5 Hz versus 2,5 ± 0,5 Hz, $p<0,001$,

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Mann-Whitney Test, Figure 7A) compared to control cells. Moreover, in irradiated cultures a significantly higher percentage of cells with spontaneous activity was found (68% versus 19%, p<0,001, Chi-Square Test, Figure 7A). Cells exposed to the thermal increase showed a higher sodium current density (110 ± 11 pA/pF versus 80 ± 11 pA/pF, p≤0,05, One-Way ANOVA Test) and a trend to a higher potassium current density compared to control cells (Figure 7B). These results confirm that thermal increase by irradiated PBNPs could induce a functional differentiation in F-11 cell line.

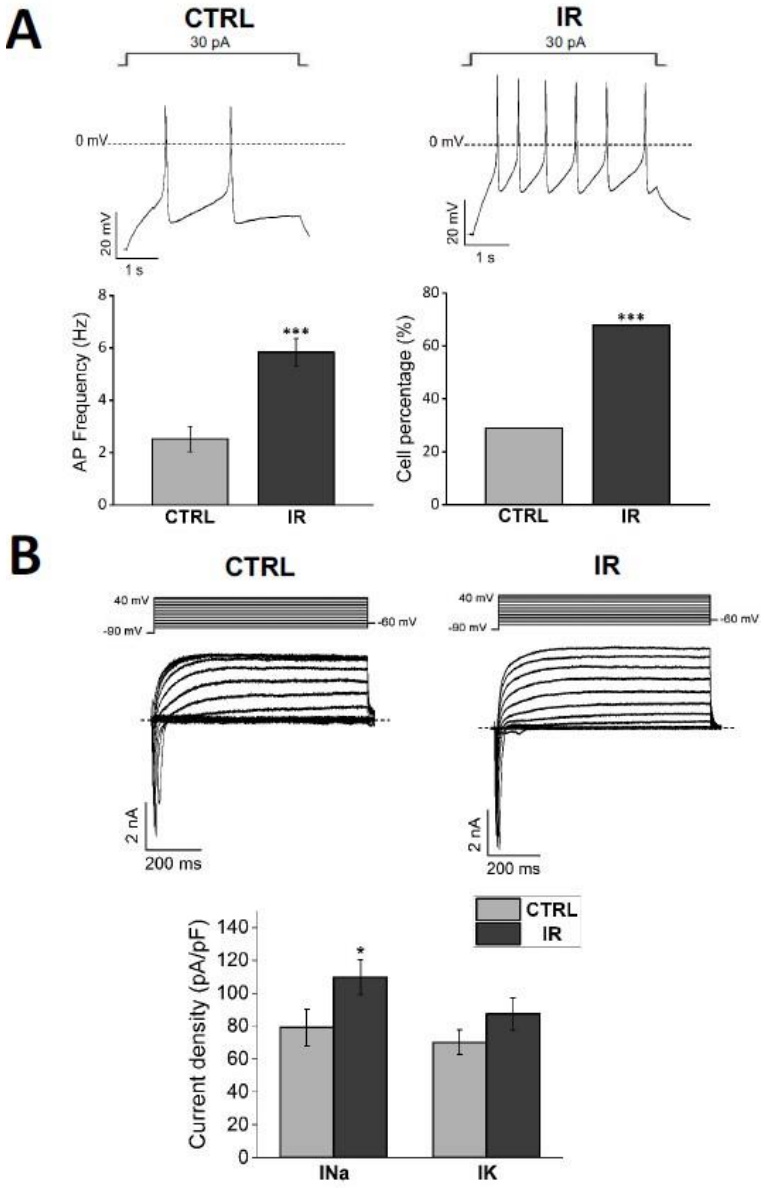

Figure 7: Functional analysis of F-11 cell electrophysiological properties by the patch-clamp technique on days 7 and 8 confirmed a more differentiated profile for stimulated cells (IR) versus 37°C (CTRL). (**A**) Irradiated cultures had higher action potential firing frequency (for irradiated samples: 5,9 ± 0,5 Hz, n=36; for control samples: 2,5 ± 0,5 Hz, n=36; p<0,001, Mann-Whitney Test) and a higher percentage of cells with spontaneous activity compared to the control (for irradiated samples: 68%, n=25/37; for control samples: 19%, n=7/36; p<0,001, Chi-Square Test). (**B**) Stimulated cultures showed a higher sodium current density and a trend to had higher potassium current density compared to the control (for irradiated samples: INa, 110 ± 11 pA/pF, and IK, 87 ± 10 pA/pF, n=32; for control samples: INa, 80 ± 11 pA/pF and IK, 70 ± 7 pA/pF, n=31; for INa p≤0,05, One-Way ANOVA Test, for IK p>0,05, Mann-Whitney Test).

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As for the cultures exposed to bulk heating, the LDH assay was performed both on irradiated and control samples on day 7 and 8. Data in Table 2 show that LDH levels in the medium of thermally stimulated cultures were not significantly different from the control, indicating that this treatment did not impair cell viability ($p>0,05$, Mann-Whitney Test, n=6 samples for each condition).

	Thermal Stimulation			
	CTRL	S.E.	IR	S.E.
Day 7-8	$9x10^{-8}$	$1,2x10^{-8}$	$15x10^{-8}$	$2,3x10^{-8}$

Table 2: Lactate-dehydrogenase activity measured on thermally stimulated cells versus control samples. Results showed that thermal stimulation did not impair cell viability in 41,5°C cells ($p>0,05$, Mann-Whitney Test, n=6 for each condition).

4. Discussion

In this paper, we show the effect of a new approach for *in vitro* neuronal scalable thermal stimulation, constituted by irradiating PBNPs by a NIR laser. PBNPs were embedded in a PVA disc stuck on the outer surface of the petri dish, in which cells to be stimulated were maintained in culture. By this approach we demonstrated that a temperature increase at 41,5°C for 30 minutes, repeated for two days, was efficient to induce neuronal differentiation of F-11 cells, an *in vitro* model of DRG neurons. Neuronal differentiation was investigated and demonstrated by both morphological and functional analysis.

Cell imaging for 8 days after seeding showed that neurites had a trend to increase in number and were significantly longer in thermally stimulated cultures compared to the control. Moreover, electrical properties (resting membrane potential, Na^+ and K^+ current densities, action potential firing frequency and spontaneous activity), recorded on days 7 and 8, also reached values characteristic of mature neurons, confirming that thermally stimulated cultures acquired a significant functional differentiation compared to control cultures. These results indicate that the new approach of thermal stimulation we propose could induce long-term modifications (maintained for at least 8 days) of neuronal properties without the support of genetics or chemical compounds.

Since brain is one of the most temperature-sensitive organs, infrared laser has already been used to stimulate neurons, alone or in combination with nanoparticles [10,28]. However, in several previous studies, the effects obtained on neurons consisted in transient variations of cell properties (membrane depolarization, action potential firing modulation). Some articles showed significant changes in the morphology, which are maintained for few days, but no electrophysiological analysis has been performed to demonstrate functional modifications. Moreover, in articles showing the capability of thermal stimulation to induce neuronal differentiation, cell culture media were enriched with differentiating components or factors, preventing the isolation of the real efficacy of temperature increase [29].

PBNPs are versatile tools endowed with photothermal effect and are excellent candidates for *in vivo* treatments due to their biocompatibility and biodegradability. PBNPs have been explored so far for imaging and chemotherapy, especially for their cancer cell-killing ability [30], and their biostability and capability to convert light into heat allow their employment in the field of the regenerative medicine.

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In this work, our decision to develop a polymer matrix, which embed the photothermal nanoparticles thereby obtaining a "smart" petri dish supporting cell culturing, has the large advantage to avoid any contact with the cells, and also to allow the design of a precise and required geometry of the active heating area, thereby inducing differentiation on a selected region of the cell culture. Moreover, with this approach, the irradiation temperature can be finely tuned and selected by a calibration of the laser spot size and power on one hand, and the photothermal particle concentration in the smart region on the other.

The ability to induce neuronal differentiation by means of a scalable heating opens up new possibilities to treat peripheral nerve injuries and/or neurodegeneration. Among the techniques available, surgery, cell-based therapy and optogenetics are the most used so far. Surgery is the most common therapy, but in general the functional recovery does not exceed 50% of patients and the intervention could lead to neuronal atrophy [31]. Cell-based therapy is a promising approach, but is an invasive process, and safety cell preparations are time-consuming and expensive [31]. Optogenetics, in which neurons are genetically modified with light-sensitive ion channels, has gained great interest in the last years, but it requires gene transfection into neuronal cells which has several limits: the expression efficiency is spatially heterogeneous, the high expression rate could lead to toxic accumulation of protein within the tissue, and the light could be refracted or absorbed by the multiple tissue layers [32]. The approach described in this paper has several advantages: it has no direct contact with cells, the preparation is quite fast and economic and the ability of the nanoparticles to convert the light into heat allows to use the NIR laser at a low potency compared to the laser alone. In the perspective of the clinical translatability of this approach and its potential application in the biomedical field, the molecular and physiological mechanisms underlying the obtained results should be assessed, and other cellular models should be considered to verify the reproducibility of the effects described in this paper. Moreover, *in vitro* models of nerve injuries already available for studying peripheral nerve regeneration, such as DRG/Schwann co-coltures, embryonic spinal cord motor neurons, pluripotent stem cells or organotypic models [33], would provide a reliable confirmation of the efficacy of the approach and would permit the investigation of the molecular mechanisms underlying the neuronal differentiation induced by this scalable thermal stimulation technique.

5. Conclusions

In this paper, we show for the first time a novel method to induce neuronal differentiation by using a combination of light sensitive nanoparticles and NIR laser that we applied with success on a neuroblastoma cell line, maintained in culture without chemical differentiating agents or genetic techniques and without any contamination with the photothermal material. The smart matrix delivering heat can be tuned to the temperature/shape needed for the region to be stimulated. Results showed that targeted heating could be a promising approach for *in vivo* therapy to induce neurite outgrowth and neuronal behavior modifications.

6. Patents

Stefania Blasa, Mykola Borzenkov, Piersandro Pallavicini, Maddalena Collini, Giuseppe Chirico and Marzia Lecchi are inventors of the nanoparticles-near infrared laser technology that induces cell differentiation mentioned in this publication. The patent application was filed by Botti & Ferrari S.p.A.

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Conflicts of Interest: Stefania Blasa, Mykola Borzenkov, Piersandro Pallavicini, Maddalena Collini, Giuseppe Chirico and Marzia Lecchi are inventors of the nanoparticles-near infrared laser technology that induces cell differentiation mentioned in this publication. The patent application was filed by Botti & Ferrari S.p.A. in accordance with the conflict-of-interest policy. The other authors, Valentina Pastori and Lavinia Doveri, do not have any competing interests.

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