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Promotion of neural sprouting using low-level green light-emitting diode phototherapy

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Abstract. We irradiated neuroblastoma SH-SY5Y cell line with low-level light-emitting diode (LED) illumination at a visible wavelength of 520 nm (green) and intensity of 100 mW/cm². We captured and analyzed the cell morphology before LED treatment, immediately after, and 12 and 24 h after treatment. Our study demonstrated that LED illumination increases the amount of sprouting dendrites in comparison to the control untreated cells. This treatment also resulted in more elongated cells after treatment in comparison to the control cells and higher levels of expression of a differentiation related gene. This result is a good indication that the proposed method could serve in phototherapy treatment for increasing sprouting and enhancing neural network formation. © 2015 Society of Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.20.2.020502]

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Phototherapy is the treatment of damaged cells via light illumination. It was first reported as a method for treating infants in order to reduce their serum bilirubin levels.^{1,2} Since then, it was found that the mechanism underlying this therapeutic effect is the interaction between light and tissue.^{3–7} In the last decade, phototherapy was suggested for enhancing peripheral nerve repair.^{8–10} It was argued that neurons show a higher rate of metabolism and better myelin production following laser treatment.¹¹

Phototherapy was tested as an *in vivo* method for cell repair, for internal delicate organs such as the brain, by using optical fibers to lead the illumination to the target location. The small dimensions of optical fibers (several microns) make this method minimally invasive, with a short recovery period and with minor complications.

Most of the phototherapy treatments that have been demonstrated as useful have been performed via low-power laser irradiation (LLI), at wavelengths ranging from the visible to the near-infrared (NIR), and emission time ranging from a few

seconds to more than an hour. Treatment protocols usually include repeated treatments over a period of a few days, or a single treatment, depending on the type of tissues or cells.^{11,12} Murayama et al. have demonstrated that a single treatment of 808-nm LLI for 20, 40, or 60 min significantly decreased the proliferation rate of human glioblastoma cells.¹³ Ang et al. have further studied the effect of a single LLI treatment on glioblastoma cells. They treated the cells with a 405-nm (green illumination) wavelength for similar irradiation times and found that cell proliferation was significantly suppressed following an irradiation at this wavelength as well.¹⁴

However, due to the progress in light-emitting diode (LED) technology, the use of LEDs for phototherapy is advantageous and has been found to be effective as well. Several studies have demonstrated beneficial effects of LED phototherapy for skin treatment.^{15–17} NIR LED-based phototherapy treatments have been proven useful for pain relief¹⁸ and also enhanced morpho-functional recovery and nerve regeneration.¹⁹ Recent studies have reported that visible light LED treatment promoted neurites' outgrowth and synaptogenesis, and protected damaged neurons in animal and in *in vitro* stroke models.^{20,21} Visible light LED irradiation at several wavelengths has been found to affect the outgrowth of PC12 cells.²² Laser and LED illuminations differ in their spatial and temporal coherence.

Here we experimentally test the effect of low-level green LED illumination on the growth of neuroblastoma SH-SY5Y cells cultured with retinoic acid (RA). Neuroblastoma cells were treated with one 30-min dose of green (520-nm) LED illumination. The effect of LED illumination on the cells' morphology was examined. We found that the treatment encouraged multiple neurites' sprouting and promoted the differentiation process.

SH-SY5Y human neuroblastoma cells were cultured in a humidified incubator at 37°C containing 5% CO₂ and then were routinely grown in 10 ml of Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 5% penicillin-streptomycin, 1% amphotericin, and 10% heat-inactivated fetal bovine serum. The medium was replaced every two days and the cells were split by adding 5 ml trypsin when reaching a confluence of ~80%. 10 × 10⁴ cells were plated in a plastic Petri dish a day before the applied irradiation treatment.

In order to examine the effect of irradiation on cells at early stages of differentiation, 3 × 10² cells per dish were seeded in 60-mm plastic Petri dishes. RA was added a day after plating at a final concentration of 10 μM in complete medium. The cells were maintained under these conditions for five days with a medium change every two days and then treated with irradiation. In order to evaluate the level of differentiation, total RNA was extracted from both populations, treated and untreated, 24 h after the irradiation, using the RNeasy kit (Qiagen, Germany). RNA was reverse-transcribed to cDNA (Thermo Scientific, USA), using DreamTaq DNA polymerase (Thermo Scientific, USA), and was followed by polymerase chain reaction amplification. Two genes were examined, β3-tubulin and β-actin, a differentiation marker and a control, respectively (as in Ref. 23).

In order to both treat and image the SH-SY5Y cells, a green LED (UHP-Mic-LED-520, Prizmatix LTD, Israel) was mounted on the second illumination port (not through the magnification system) of an Olympus BX51 microscope (see Fig. 1). The light

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intensity was measured using a photodiode power sensor (NOVA II METER, Ophir PD300-3W, Israel).

The treated dishes were exposed to 30-min LED illumination at a 520-nm (green) wavelength. The irradiated area was 600 to 700 mm². Before the treatment and immediately after the treatment (within 5 min after treatment), images of the exposed areas were acquired, and the dishes were placed back in the incubator. Again, 12 and 24 h following the illumination, the dishes were inspected in the same way. The control group contained dishes with approximately the same number of cells, developing under the same conditions but without LED illumination.

Thirty areas (10 areas of three dishes), consisting of ~1000 cells, were inspected before treatment, immediately following 30 min of illumination treatment, and 12 and 24 h after treatment. The 30 areas were randomly selected, imaged, and analyzed in order to give a representative sampling of cells. The images were processed using MATLAB® program and the cell morphology was quantified.

The SH-SY5Y cells are able to sprout neurites, which over time create a neural network. In previous neural studies, different parameters were tested to evaluate neural functionality, including neurite length, number of large cells, and dendrite split angle.^{11,24} In our work, we focused on the number of neurites originating from the soma per cell, since neurite formation is a primary morphological event in neuronal differentiation and plays an important role in neural repair. Another morphological parameter that was measured is the length/width ratio of the cells per frame (as shown in Fig. 1, right zoom). A cell that its longer dimension (length) was at list four times longer than its short dimension (width) was considered an elongated cell.

Dividing cells were excluded from image analyses since they stop growing and were undergoing mitosis to divide into two daughter cells. These cells have a distinct round morphology, as opposed to growing cells that are spread on the dish surface and develop neurites, as shown in Fig. 2.

As can be seen in Fig. 3, immediately after the LED illumination treatment, there was an average of 1.75 neurites per cell with extensions in the phototreated dish compared to 1.26 in the control dish. This increase of ~40% in the number of neurites indicates an increased neural sprouting. However, this difference vanished 12 h after treatment.

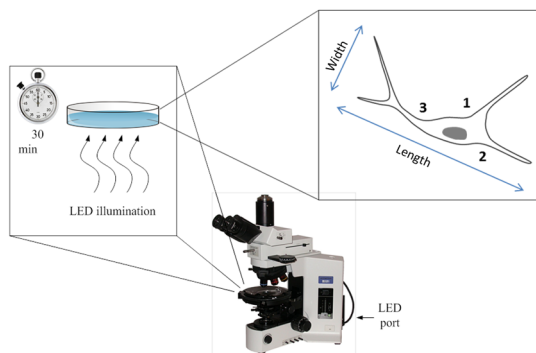


Fig. 1 Experimental setup. Petri dishes with SH-SY5Y cells were placed in the microscope and illuminated for 30 min with 520 nm [green light-emitting diode (LED)] and intensity of 100 mW/cm² that was mounted on the second illumination port of the microscope. Right zoom: a schematic description of the analyzed cellular parameters. The numbers mark the neurites that initiate out of the soma. The arrows designate the length and width of the cell.

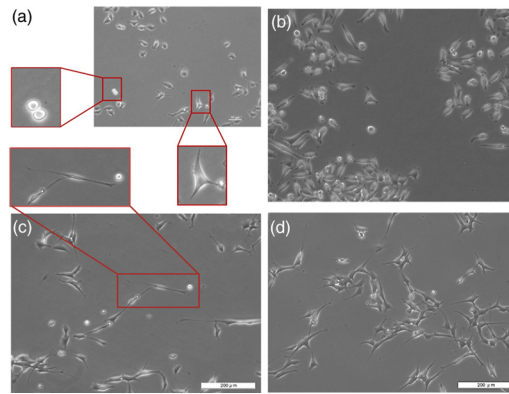


Fig. 2 (a) An example of a cell culture image before treatment. SH-SY5Y cells were treated with a differentiation promoting factor (retinoic acid); however, some cells continued to proliferate (left zoom: a dividing cell). Several of the neural-like cells in this image have started to sprout neurites (bottom zoom: a cell with three neurites). (b) An example of a cell culture image immediately after treatment. (c) An example of a cell culture image 24 h after treatment. Some cells developed into elongated cells (upper zoom: a cell with high aspect ratio, length versus width). (d) An example of a cell culture image of control untreated cells 24 h after treatment.

In addition, comparison between irradiated and control cultures revealed a significantly higher percentage of elongated cells, 12 as well as 24 h after treatment for the phototreated dishes (Fig. 4).

To further investigate the effect of illumination on neuronal differentiation and repair, the division rate of the cells was measured. Illuminated cells demonstrated a lower division rate compared to control untreated cells, 24 h after treatment [Fig. 5(a)]. Next, we examined the expression levels of β 3-tubulin, a protein that plays an important role in the differentiation process and is used as common marker for differentiation. β -actin levels were examined as a control, since its expression is not affected by differentiation. Figure 5(b) presents the expression levels of β 3-tubulin and β -actin in treated and untreated control cells, 24 h after irradiation treatment. The results clearly demonstrate that LED treatment increased the expression levels of β 3-tubulin compared to control cells.

An *in vitro* study of phototreatment using green LED illuminations (520 nm) was performed on SH cells cultured with RA, which promotes their differentiation. Cells that were illuminated for 30 min presented an increase in dendrite sprouting as compared to the control held at the same conditions without

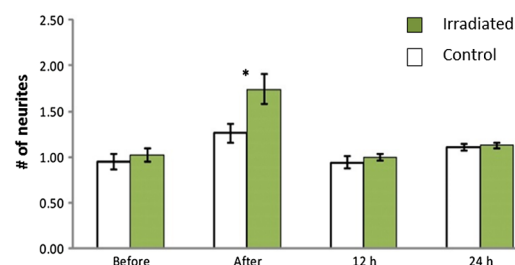


Fig. 3 The effect of low-level green LED illumination on neural sprouting. SH-SY5Y cells were illuminated by green LED at a power density of 100 mW/cm². The number of neurites per cell (green bars) was compared to untreated cells (white bars) counted at different time points. **p* < 0.05, Student's *t* test.

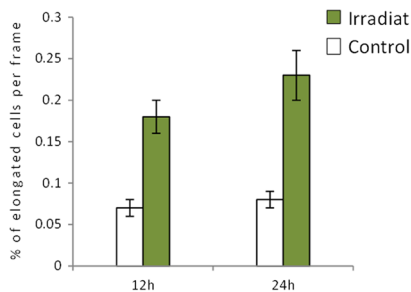


Fig. 4 The effect of low-level green LED illumination on the morphology of the cell body. The percentage of elongated cells per frame was measured 12 and 24 h after irradiation, (phototreated dishes are indicated by green bars and control dishes by white bars, *** $p < 0.001$, Students' t test).

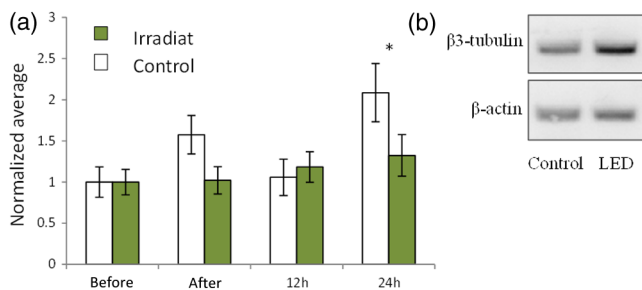


Fig. 5 (a) The effect of low-level green LED illumination on the division rate of the cells. The average number of cells per frame was counted before the light treatment, immediately after the light treatment, and at 12 and 24 h after light treatment. Phototreated dishes (green bars) show a consistently lower division rate than control dishes (white bars). * $p < 0.05$, Students' t test. (b) The effect of LED phototreatment on the cellular expression of the differentiation marker $\beta 3$ -tubulin.

phototreatment. However, this phenomenon disappeared 12 h after treatment. This indicates that for more effective treatment, a consecutive phototherapy protocol should be considered.

In addition, the percentage of elongated cells, was found to be much higher in irradiated dishes 12 and 24 h after treatment. The SH-SY5Y cells tend to elongate between divisions, enabling them to sense their surroundings and to move accordingly. This result may suggest that green LED illumination inhibits the cells' division rate and could also promote cell migration, which is important for nerve regeneration processes. These findings are in agreement with a previous work that demonstrated an increase in neurite sprouting and migration of cultured embryonic nerve cells, as well as cultured adult brain microexplants, within 24 h after laser phototherapy.⁸

In order to further study the effect of low-level green LED irradiation on cell division, the number of cells per frame was measured at several time points before and after illumination. The number of phototreated cells was significantly lower compared to control cells, indicating a lower division rate.

Moreover, the irradiation increased the expression levels of $\beta 3$ -tubulin, a well-known differentiation marker.

Together, the results indicate that phototherapy may be useful for promotion of neuronal repair, proposing a study on the use of a visible low-intensity LED that is economical, easy to use, and safe for the surrounding tissues as a tool for nerve regeneration.

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