

Photobiomodulation Effects of Single Near-Infrared (825 nm), Green (525 nm) and Combination of Wavelengths on Adipose-Derived Mesenchymal Stem Cell Physiology

Anine Crous

University of Johannesburg

Madeleen Jansen Rensburg

University of Johannesburg

Heidi Abrahamse (✉ habrahamse@uj.ac.za)

University of Johannesburg

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Abstract

Photobiomodulation (PBM) has been used as a bio stimulatory tool for adipose-derived mesenchymal stem cells (ADMSCs). The goal of this *in vitro* research was to examine the effects of combined and/or single applications of near infra-red (NIR) and green PBM using 5 J/cm² on ADMSCs. The results indicated that the viability of ADMSCs are not affected by single or combined wavelengths of 525 and 825 nm at 5 J/cm². However, PBM significantly stimulated cell metabolism seen by an increase in proliferation, it also upregulated intracellular ROS and MMP using 825 nm and 525 nm wavelengths. The combined wavelength irradiation mimicked results found for 825 nm during ATP measurement, cell concentration and migration rate, however significant MMP stimulation and increased ROS production was achieved. In conclusion, results indicate that the combination wavelengths of 525 and 825 nm can be used in the expansion and differentiation of ADMSCs for regenerative purposes.

Introduction

The use of adipose-derived mesenchymal stem cells (ADMSCs) have become widespread as they can be easily harvested with less-invasive means, are abundantly available and can, furthermore be differentiated into many phenotypes¹⁻³. Differentiation of ADMSCs can be triggered by the induction of biological growth factors or chemical inducers⁴⁻⁶. By this means, ADMSCs have a high differentiation potential *in vitro* into lineages that range from neuronal, osteogenic, chondrogenic, adipocyte, to myogenic⁴. For example, ADMSCs have been transdifferentiated into neuronal-like cells in media that contained inducers such as zinc chloride, fibroblast growth factor, nerve growth factor, forskolin, or 3-isobutyl-1-methylxanthine⁷⁻¹⁰. Other studies have also shown that ADMSCs can be transdifferentiated to osteoblasts by introducing inducers such as ascorbate-2-phosphate, dexamethasone, and β -glycerolphosphate⁶. Thus, ADMSCs can be applied to regenerative medicine in a vast number of ways, such as recovery from neurodegenerative diseases or osteoporosis^{11,12}.

Photobiomodulation (PBM) has become popular for its ability to either upregulate or downregulate biological processes at a molecular level depending on the laser parameters applied¹³. Parameters that are considered are wavelength, which can range from the visible to the near-infrared (NIR) spectrum of light at wavelengths between 400–1100 nm and fluence or energy applied¹⁴⁻¹⁷. Where the effects of various laser fluences are dose dependent. Fluences of 0.5–2 J/cm² has no significant effect on ADMSC proliferation¹⁸, 3–5 J/cm² has significant photobiomodulation¹⁹. Specifically, photobiomodulation triggers chromophores in the mitochondria, stimulating the electron transport chain to generate more adenosine triphosphate (ATP) and increase mitochondrial membrane potential (MMP)^{20,21}. Research showed that lower wavelengths, 451 and 540 nm, had no significant effects on cell proliferation, whereas higher wavelengths, 660 and 810 nm, upregulated proliferation of ADMSCs¹⁴. The use of green light between 450–580 nm has shown to stimulate differentiation of ADMSCs into osteoblasts^{22,23}, where green light has been seen to stimulate various transcription factors involved in cell differentiation²⁴.

Furthermore, the migration abilities of cells, important for homing, are also stimulated when exposed to NIR light ²⁵. However, the effect of combining upper and lower wavelengths remains to be explored.

Therefore, the current study explored the effect of green and NIR laser light at 525 and 825 nm respectively, as well as the combination of the two wavelengths using 5 J/cm². The effect on morphology, migration, proliferation, viability, cytotoxicity, intracellular ROS and MMP of the ADMSCs following photobiomodulation treatment was explored.

Results

Biochemical Analysis

Morphology of ADMSCs treated with PBM

Morphology results show a dense monolayer of ADMSCs that was relatively homogenous with a spindle-like shape and smooth cellular surface typically observed in healthy ADMSCs (Fig. 1). Experimental groups of ADMSCs that were exposed to laser irradiation at different wavelengths of 525 and 825 nm and fluence of 5 J/cm² had no visible morphological modifications in comparison to that of the control, they also showed a concentrated cell population. Of these experimental groups, the group treated with 825 nm NIR laser light was the densest 24 h, 48 h and 7 days post irradiation.

Cell viability and proliferation

To determine the effects of PBM on cell viability, we used the trypan blue dye exclusion assay. Viable cells inhibited penetration of the dye into the cells, indicative of their intact membrane, whereas cells with diminished membranes allowed for the dye to enter the cells. Results show (Fig. 2A) that there were no significant effects on cell viability when using NIR, green and NIR-green irradiation at 5 J/cm² on ADMSCs. All cells maintained a high cell viability. However, a negligible decrease in viability is seen using green irradiation after 24 and 48h.

Along with establishing cellular viability the effects of various PBM wavelengths on cell proliferation kinetics was measured by means of cell concentration. Viable cell counts of treatment groups were compared to their respective controls. Results show (Fig. 2B) that 24 h after PBM treatment using NIR and the combination had a significant increase in cell number. All treatment groups were significantly higher than their control after 48 h. Combination treatment indicated a statistical significance after 7 days, whereas other treatments had no significance compared to the control.

To characterize the effects of different wavelengths of PBM on the proliferation of ADMSCs relative to its metabolism, we used the ATP Luminescence Assay. This test uses luciferase to generate a luminescent signal that is equal to the amount of ATP in the sample. The amount of ATP present was proportional to the proliferation rate of the cells, where an increase in ATP suggests an increase in mitochondrial stimulation and cell proliferation and vice versa. Cell proliferation was determined over a period post PBM

treatment. Results show that NIR-PBM significantly increased the proliferation rate 24 and 48 h after irradiation (Fig. 2C). Green and combination wavelengths kept a slight upregulation in proliferation compared to the control cells after 24 h and a statistically significant increase is seen at 48 h and 7 days. It was however seen that the cells had reached a stationary phase after 7 days post NIR treatment, due to cells reaching confluency in the growth environment ²⁶.

Reactive oxygen species (ROS) detection

Although physiologically up-regulated ROS production is essential for maintenance of stem cell activities, abnormally high levels of ROS can harm MSCs ²⁷. Therefore, the expression of ROS in the ADMSCs was determined in order to establish what effects the PBM at various wavelengths have on intracellular ROS production. It is seen that (Fig. 3) control cells receiving no treatment, representing normal healthy ADMSCs have slight ROS activity. The increase in ROS activity over time is due to the increase in cell number. Treatment groups compared to the control group show an increase in ROS levels when using NIR-irradiation, green-irradiation, and their combination. After 24 h NIR-irradiation increased ROS levels the most. At 48 h it was green-irradiation and after 7 days the combination wavelengths increased ROS levels the most. It should be mentioned that the increase in intracellular ROS did not have a detrimental effect on the cells where previous results show increased proliferation activity, and healthy viable cells.

Mitochondrial membrane potential $\Delta\Psi_m$

Light is primarily absorbed in the mitochondria in mammalian cells specifically cytochrome c allowing for dissociation of nitric oxide from cytochrome c restoring electron transport and increasing mitochondrial membrane potential (MMP) ²⁸. In the following experiment, the effect of NIR 825 nm, Green 525 nm, and NIR-Green 825 + 525 nm laser light exposure on the MMP was measured by immunofluorescence microscopy 24 h, 48 h, and 7 days following irradiation (Fig. 4A). Results show that cells maintained their MMP over time after irradiation. With the cell mitochondria fluorescing a bright red. Analysis (Fig. 4B) revealed that NIR-Green PBM significantly ($P < 0.05$) stimulated the MMP at 24 h, 48 h, and 7 days post irradiation when compared to the unirradiated control group. Although NIR and green PBM did upregulate MMP in comparison to the untreated control group, it was not significant.

Cell migration: 'central scratch test' method

Cell migration was calculated using the 'central scratch method', where a centralized scratch was created to examine cell motility through closing of the scratch. Cell migration was observed morphologically over time 0, 24, 48 h and 7 days as the cells close the central scratch (Fig. 5).

The findings showed that all treatment groups had migrated significantly over time, compared to the untreated control cells, and that all treated samples travelled a similar distance (Fig. 6A). Irradiation using various wavelengths significantly promoted closure of the scratched area compared to cells not receiving irradiation treatment (Fig. 6B), where the combination treatment had the least amount of open area left.

Discussion

Various studies have explored the effects of PBM on ADMSCs. Studies included the use of single blue, green, red and NIR wavelengths. Results showed various outcomes depending on the laser wavelength and fluences used, along with power output^{14,25} and cell seeding density. The findings from this study using combination wavelengths revealed that ADMSCs exposed to PBM formed a denser monolayer following incubation when compared to the control group that was not exposed to PBM. Specifically, ADMSCs exposed to NIR 825 nm laser light displayed the densest monolayer formation 48 h and 7 days post irradiation. This is similar to Nurković et al that applied 808 nm laser light at 3 J/cm² and saw an increase in the number of cells when observing morphology²⁹. Although cells treated with combination 525 + 825 nm wavelengths showed a visible increase in the monolayer, it was not as dense as that of cells exposed to NIR 825 nm laser light.

Cell viability was not affected using PBM of 5 J/cm² and wavelengths in the green, NIR and their combination wavelengths. All cells maintained a high viability rate, indicating that these PBM parameters do not have a negative effect on the cells *in vitro*. Nurković et al also found that NIR treatment did not significantly affect MSC viability by itself²⁹. Similar findings were observed *in vivo* where no significant increase/ decrease was noted after PBM treatment using 890 nm accounting for the increase in proliferation measured thereafter³⁰. Similarly when using green irradiation, previous studies show that PBM at 470 nm³¹ and at 405 nm³² had no effect on cell viability. Therefore, the combination of 825 nm and 525 nm showed a persistent maintainable high viability insignificant of the untreated controls.

We found that NIR-PBM has a positive effect on ADMSC metabolism showing a significant increase in cell proliferation when using an energy density of 5 J/cm², corroborating results from previous studies using NIR-irradiation^{14,29}. However the use of green light (525 nm) had shown maintainable proliferation after 24 h and a significant increase after 48 h, when using 5 J/cm², this is similar to Anwer et al that saw an increase in proliferation following laser exposure of green PBM at 532 nm³³. The significant increase in cell proliferation is due to the stimulated metabolic activity seen in the increase in ATP proliferation. This is in contrast to Wang et al that saw a dose dependent decrease in proliferation¹⁴ and another study by Fekrazad et al that applied green light at 532 nm and saw an insignificant decrease in proliferation²², these results can be attributed to the difference in laser power density and initial cell seeding density. The combination wavelengths used in this study however, showed a time dependent increase in cell proliferation with a significantly higher rate after 48 h and 7 days compared to their respective controls.

Untreated ADMSCs maintained a small but steady upregulation in intracellular ROS over time, where ROS detection increases as cell numbers increase. However, treatment groups did show a slight increase compared to untreated controls in intracellular ROS at 24 h for NIR-PBM, at 48 h for green-PBM and at 7 days combination treatment. This indicates that PBM treatments maintained and upregulated ROS generation which can direct their stem cell fate and improve their therapeutic effect²⁷. This is seen during various differentiation directions leading to various degrees of ROS production. Where adipogenic³⁴, chondrogenic³⁵, osteogenic³⁶ and neural³⁷ differentiation had all shown physiologically increased ROS levels.

It was observed that the MMP of the cells were maintained throughout the incubation period. Further investigation, through fluorescent intensity measurement, combination wavelengths showed a significant increase in MMP 24 h, 48h, and 7 days post irradiation. NIR and green laser irradiation also showed an increase in fluorescent intensity when compared to the control, albeit insignificant. Interestingly, Wang *et al* found that NIR (810 nm) stimulated MMP, but green laser light (540 nm) inhibited the MMP¹⁴. The findings corresponded, not only to the NIR results recorded by Wang *et al* but also to that of Huang *et al*. in which an increase in the stimulation of the MMP was recorded at low fluences (0.03-3 J/cm²) when mouse models were treated with 810 nm NIR laser light³⁸. In this study, the combination of both NIR using 825 nm and green laser light of 525 nm significantly increased the MMP. Which leads to the bio stimulatory induction of ADMSC differentiation³⁹.

Cell homing is a significant feature of the ADMSCs because they are expected to relocate to the wounded site for treatment⁴⁰. ADMSCs treated using various wavelength of 525, 825 and their combination at a fluence of 5 J/cm² showed significant stimulation of cell migration, indicative of promoting cell homing. Research has shown that low-power lasers (red lasers at 660 nm) have been successful at facilitating the migration of ADMSCs to the injury site and facilitate the repair process²⁵. Specifically, El Gammal *et al*. found that the homing capabilities of bone marrow MSCs cultivated *in vitro* were significantly increased when exposed to NIR laser light at 804 nm⁴¹. A study by Rohringer *et al*. showed that green laser light at 516 nm and red laser light at 635 nm significantly increased the migration rate of human umbilical vein endothelial cells⁴². Making the use of both NIR and green irradiation a viable tool during stem cell homing for regenerative purposes.

In conclusion the effects of using various single and combination PBM wavelengths for possible use in regenerative medicine when using ADMSCs as a cell source for *in vitro* proliferation and differentiation was evaluated. As discussed PBM using NIR irradiation showed significant bio stimulatory effects on the cells aiding in cell proliferation, viability and migration which can be applied prior to differentiation of the cells to achieve an abundant stem cell source. Furthermore, green light showed significant bio stimulation promoting cell proliferation, although not as significant as NIR-PBM. Notably the combination uses of NIR- and green light significantly promotes the induction of ADMSC differentiation through bio stimulation of intracellular ROS production and MMP. Concluding that using combination wavelengths of NIR and green PBM can aid in the proliferation, differentiation and possible maintenance of the cells differentiated state prior to terminal differentiation and confirmation of cell function. Furthermore, NIR light could be used to increase the cell yield of the terminally differentiated cells, where future recommendations should include the evaluation of the combination treatment *in vivo* in order to establish the effects on cell transplantation for regenerative purposes and cell homing.

Methods

Cell culture

Adipose derived mesenchymal stem cells immortalized with hTERT ASC52telo (ATCC® SCRC-4000™) were cultured in Dulbecco's Modified Eagle Media (DMEM) (Sigma-Aldrich, D5796) supplemented with 10% foetal bovine serum (FBS Superior) (Biochrom, S0615) and 1% antibiotics: 0.5% Penicillin-Streptomycin (Sigma-Aldrich, P4333) and 0.5% Amphotericin B solution (Sigma-Aldrich, A2942). All cultured cells were maintained and incubated at 37°C in 5% CO₂ and 85% humidity (Heracell™ 150i CO₂ Incubator, Thermo Scientific™, 51026280), where they were cultured in Corning® cell culture flasks (Sigma, CLS430639/ CLS430641/ CLS431080).

Photobiomodulation

Following the culture of immortalized ADMSCs, 1×10^5 cells were seeded onto 35 mm diameter culture dishes (Corning, 430165) in complete medium and allowed to attach for 24 h before irradiation. The cells were irradiated with a Near infra-red (NIR) 825 nm Diode Laser (National Laser Centre of South Africa, SN 070900108) with a 1000 mA LaserSource, (ArroyoInstruments, 4210) and/or green (G) 525 nm Diode Laser (National Laser Centre of South Africa, EN 60825-1:2007) with a 100–240 VAC, 47–63 Hz 5A LaserSource, (OptoElectronics Tech.CO.,LTD). The laser output power (mW) was determined with a FieldMate Laser Power Meter (Coherent, 1098297). A High-Sensitivity Thermopile Sensor PM3 (Coherent, 1098336) was used to calculate the laser exposure time based on the fluence. The magnitude of the laser spot covered the whole cell monolayer. All laser parameters are shown in Table 1.

Table 1
Laser parameters

	Near infra-red (NIR)	Green (G)
Wavelength (nm)	825	525
Type	Diode	Diode
Emmision	CW	CW
Power (mW)	100	574
Power density (mW/cm ²)	10.394	59.66
Fluence (J/cm ²)	5	5
Time of irradiation (s)	8 min 1 sec	1 min 23 sec
Spot size (cm ²)	9.62	9.62
Cell cultures were divided into 3 study categories: 1) cells treated with NIR irradiation at 825 nm and 5 J/cm ² , 2) cells treated with G irradiation at 525 nm and 5 J/cm ² and 3) combination of NIR and G irradiation at 5 J/cm ² of each wavelength for a total of 10 J/cm ² . Cells that didn't receive irradiation (0 J/cm ²) served as controls.		

The time exposure of each respective laser wavelength was calculated using the following formula:

$$mW / cm^2 = \frac{mW}{\pi(r^2)}$$

$$W / cm^2 = \frac{mW / cm^2}{1000}$$

$$Time \text{ (seconds)} = \frac{J/cm^2}{W/cm^2}$$

Biochemical Analysis

Morphology: Inverted light microscopy

Distinctions in morphology were recorded and analysed 24 h and 48 h after irradiation via inverted light microscopy (OLYMPUS, CKX41) and captured with a microscope-connected digital camera (OLYMPUS, SC30) that uses the getIT program.

Cell viability: Trypan Blue exclusion assay

A colour exclusion assay using Trypan Blue Stain (0.4%) (Invitrogen™, T10282) was performed to assess cell survival. An Automated Cell Counter (ThermoFischer, AMQAX1000) that graphically displays cells and measures the percentage of viable cells was used.

Proliferation kinetics – Cell Concentration

A colour exclusion assay using Trypan Blue Stain (0.4%) (Invitrogen™, T10282) was performed to determine the viable cell count. An Automated Countess Cell Counter (ThermoFischer, AMQAX1000) that graphically displays the number of viable cells was used. Proliferation kinetics of the treated cells was established by comparing the cell concentration post irradiation to that of the untreated control.

Proliferation: ATP luminescence assay

Cell proliferation was calculated utilizing the CellTiter-Glo® 2.0 (Promega, G9241) ATP luminescence test. The luminescent intensity was translated and measured using the VICTOR3 Multilabel Plate Counter (PerkinElmer, HH3522019094) which illustrates the luminescent signal in relative light units (RLUs).

Reactive oxygen species (ROS) detection – Fluorescence microscopy

Fluorometric Intracellular ROS Kit (Sigma-Aldrich, MAK142) was used to determine the production of ROS. Cells were seeded at a density of 1×10^5 cells/ml, following PBM treatment, cells were moved to a 96-well plate. After this, cells were treated with 1 µl of 500 x ROS reagent and incubated for 30 min at 37

°C and 5 % CO₂. The fluorescent intensity was determined with a BD Accuri™ C6 Flow Cytometer using a Cy5 filter (BD 468 Biosciences, BD ACCURI C6 PLUS).

Mitochondrial membrane potential $\Delta\Psi_m$

Mito Red (Sigma-Aldrich, 53271) dye is rhodamine-based and localizes and interacts with mitochondria revealing the membrane potential of the cell. Cells were seeded at a density of 5×10^4 cells/ml. Cells were incubated with 20 nM Mito Red buffer for 30 min at 37 °C. Fluorescent microscopy (Zeiss, Axio Observer Z1) with rhodamine filter was used to determine dye emission.

Cell migration: ‘central scratch test’ method

Cell migration was analysed using the ‘central scratch’ method. Cells were cultured in 35 mm petri dishes and incubated overnight at 37°C and 5% CO₂. A central scratch was made prior to irradiation using a sterile P-200 pipette tip. The cell motility was observed, using set positions and focal planes, by an Inverted Microscope (Wirsam, Olympus CKX41) and captured using a digital camera (SC30 Olympus Camera). Images were taken at 0 h, 24 h, 48 h and 7 days post irradiation.

Statistical Analysis

Biochemical assays were conducted in triplicate $n = 3$. Spectrophotometry studies were performed using a blank sample taken from the relevant data gathered. Statistical analyses were carried out on data sets, student's t-test was used to compare two groups and one-way ANOVA to compare all groups together with SigmaPlot program version 12. Data obtained from migration morphology is quantitatively analysed using ImageJ, a public Java domain image processing system (National Institute of Health Bethesda, MD USA). All data is expressed as a mean \pm SE. The statistical significances were expressed by tables and graphs $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) and the standard error was defined by dispersion bars.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding authors upon request.

Declarations

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AUTHOR CONTRIBUTIONS

Conceptualization, AC; methodology, AC; validation, H.A.; formal analysis, AC and MJVR; investigation, AC and MJVR; resources, AC, MJVR and HA; writing—original draft preparation, AC and MJVR; writing—review

and editing, AC, MJVR and HA; visualization, AC and MJVR; supervision, HA; project administration, AC, MJVR and HA; funding acquisition, AC, MJVR and HA. All authors have read and agreed to the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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Figures

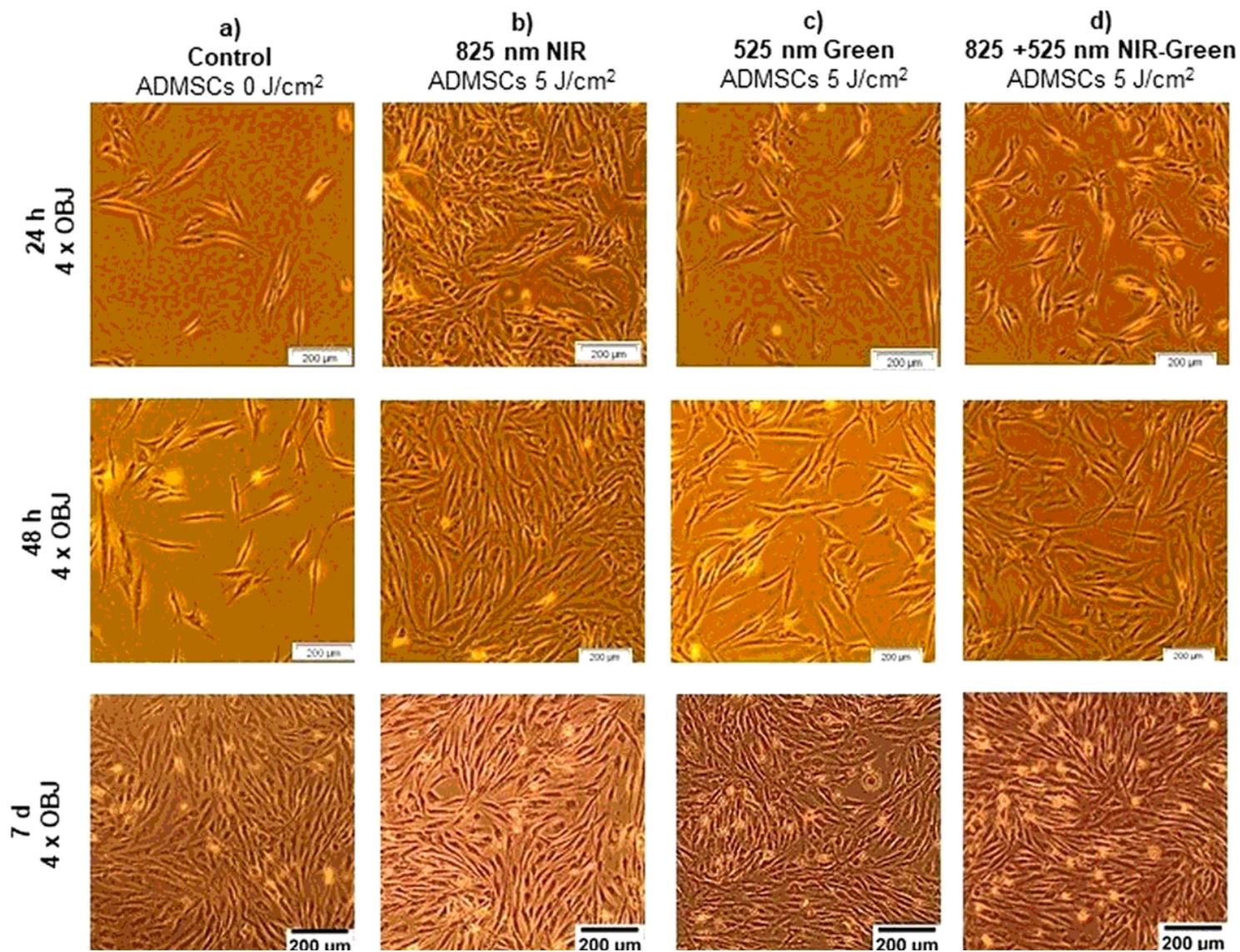


Figure 1

Morphology of ADMSCs 24 hours, 48 hours, and 7 days following laser irradiation. a) Untreated control cells; b) Experimental ADMSCs exposed to 825 nm NIR irradiation; c) Experimental ADMSCs exposed to 525 nm Green irradiation d) Experimental ADMSCs exposed to a combination of 525 nm Green and 825 nm NIR irradiation.

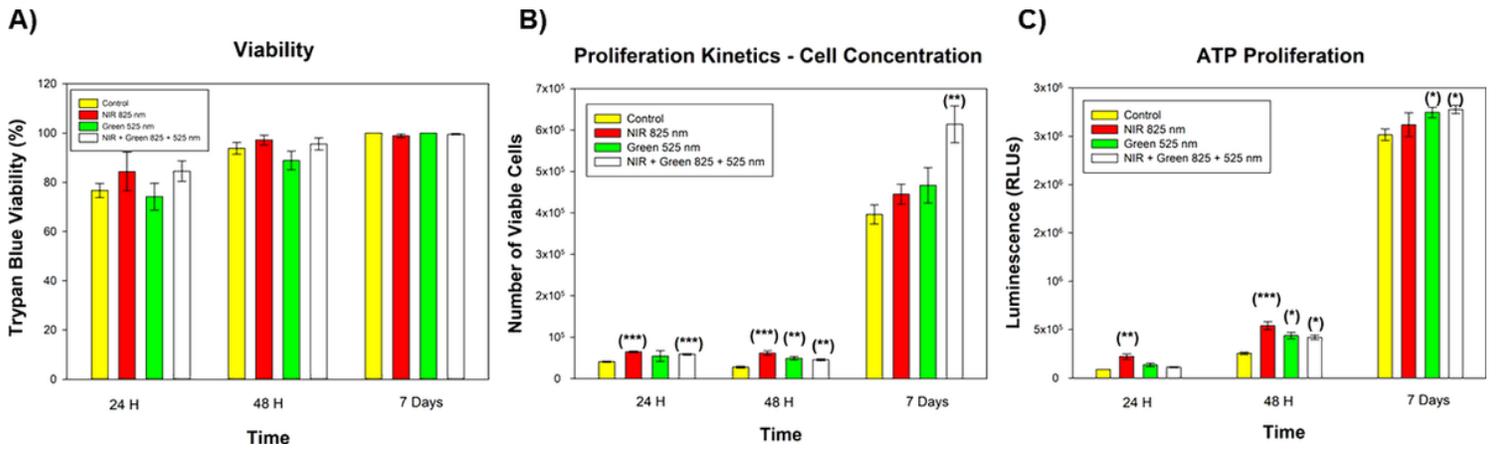


Figure 2

ADMSC viability and proliferation post PBM treatment. A) ADMSCs percentage viability 24 h, 48 h, and 7 days following laser irradiation using 825 nm, 525 nm and 825 + 525 nm. No statistically significant effects were seen on cell viability when comparing experiments to the respective controls. All cells maintained a high cell viability over time. Negligible decrease in viability is noted when using green irradiation after 24 and 48 h. B) Proliferation kinetics of ADMSCs post PBM treatment. Cell numbers were increased significantly after 24 h using 825 nm and the combination wavelengths (825 nm + 525 nm). An overall statistical significance was observed for all treatment groups compared to the controls after 48 h. The combination treatment had a significant increase in cell number 7 days post PBM treatment. C) The effect of various wavelengths (825 nm, 525 nm and 825 + 525 nm) on ADMSC ATP proliferation 24 h, 48 h and 7 days post PBM treatment. NIR-PBM significantly increased ADMSC metabolic activity 24 and 48 h post irradiation compared to control cells receiving no PBM. Green and combination wavelengths had no significant increase in ATP proliferation 24 h post irradiation. However, green and combination wavelengths showed significant metabolic stimulation 48 h and 7 days after PBM treatment.

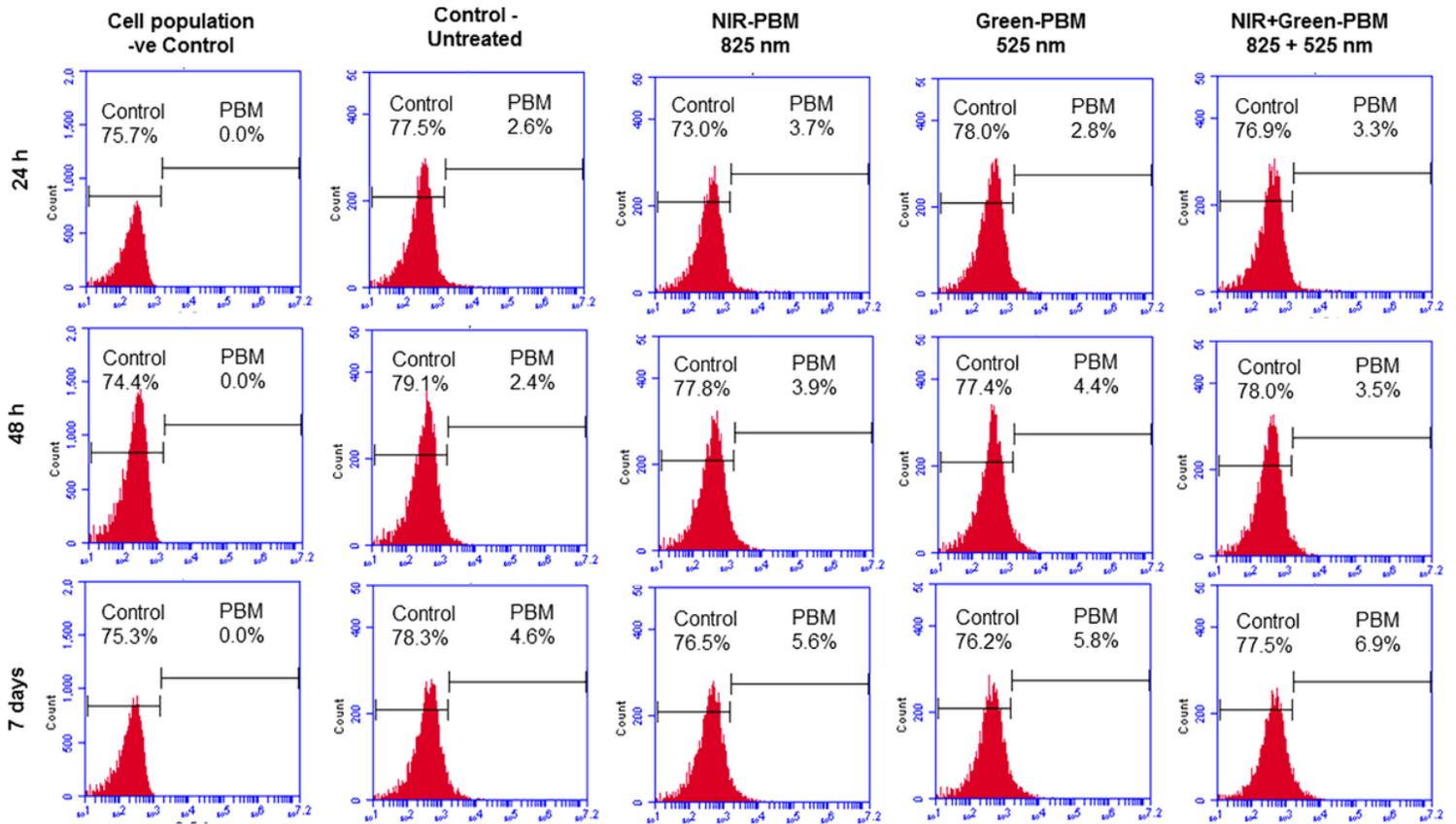


Figure 3

Intracellular ROS production of ADMSCs. The effect of various wavelengths (825 nm, 525 nm and 825 + 525 nm) on intracellular ADMSC ROS production at 24 h, 48 h and 7 days post PBM treatment. 24 h post NIR (825 nm) laser exposure, ROS levels had increased the most. At 48 h post green (525 nm) laser exposure, ROS levels had increased the most. Finally, at 7 days, ROS levels had the largest increase following exposure to combination (825 nm + 525 nm) laser wavelengths.

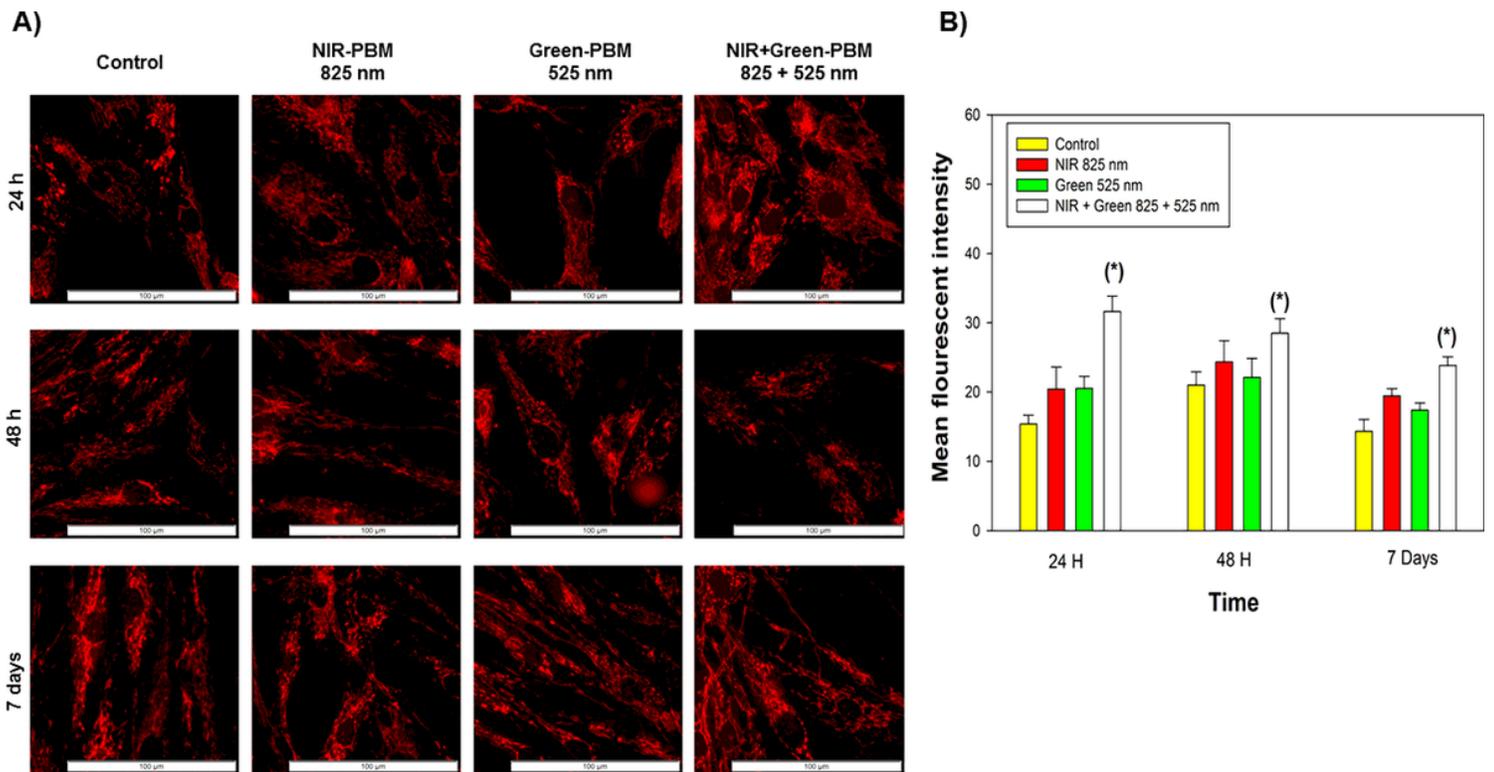


Figure 4

MMP of ADMSCs post laser irradiation. A) morphology MMP of ADMSCs 24, 48h and 7days post treatment with NIR 825 nm, Green 525 nm, and a combination of NIR and Green 825 + 525 nm irradiation. B) Mean fluorescent intensity of the MMP post laser treatment, where single wavelengths show a slight increase in MMP over time and combination wavelengths showed significant ($p < 0.05$) stimulation compared to their respective controls.

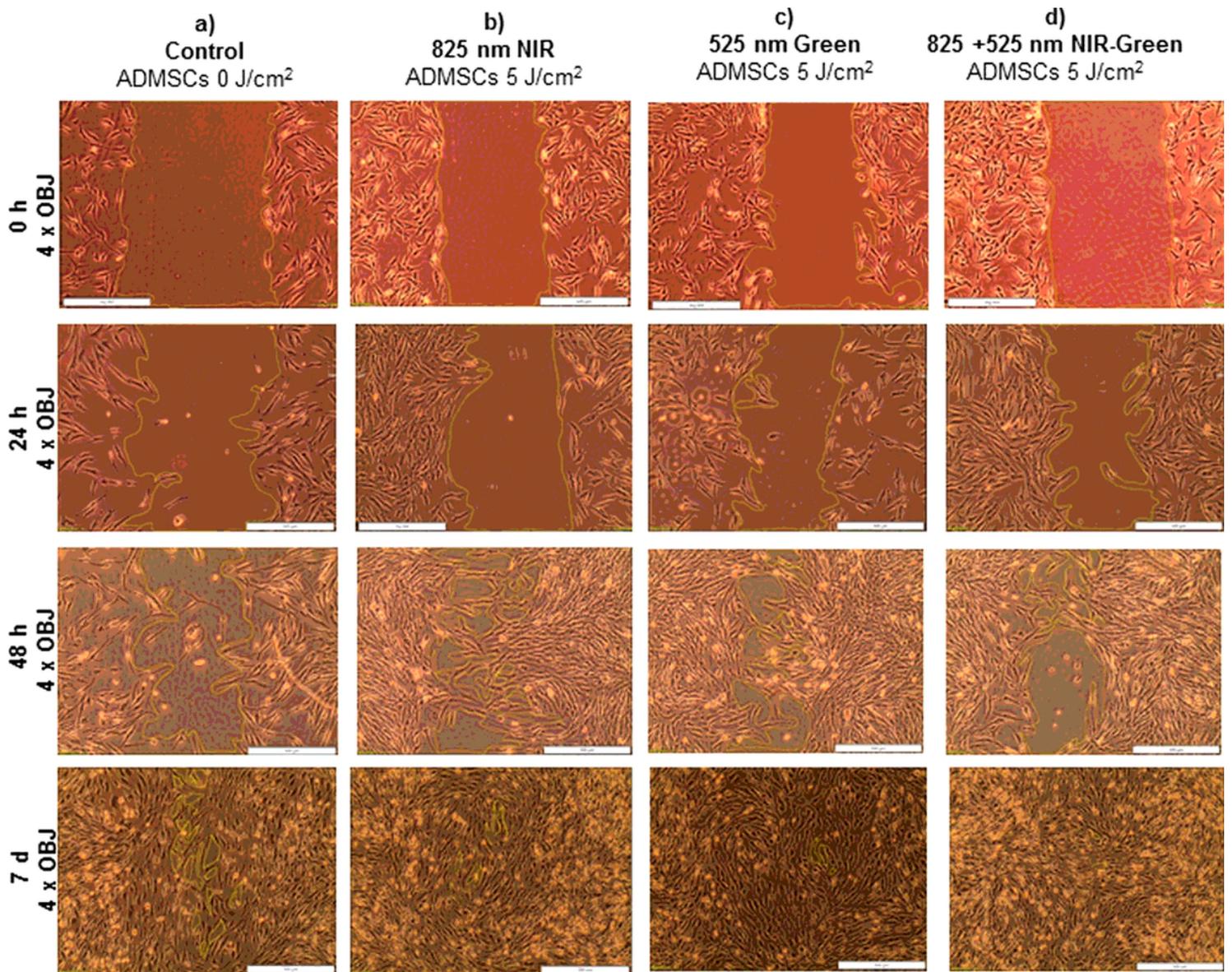


Figure 5

Morphology of ADMSC migration. Cells were irradiated with various wavelengths (825 nm, 525 nm and 825 + 525 nm) using 5 J/cm² and images taken at 0, 24, 48h and 7 days. Migration images show an increase in migration rate for treated cells compared to the untreated control.

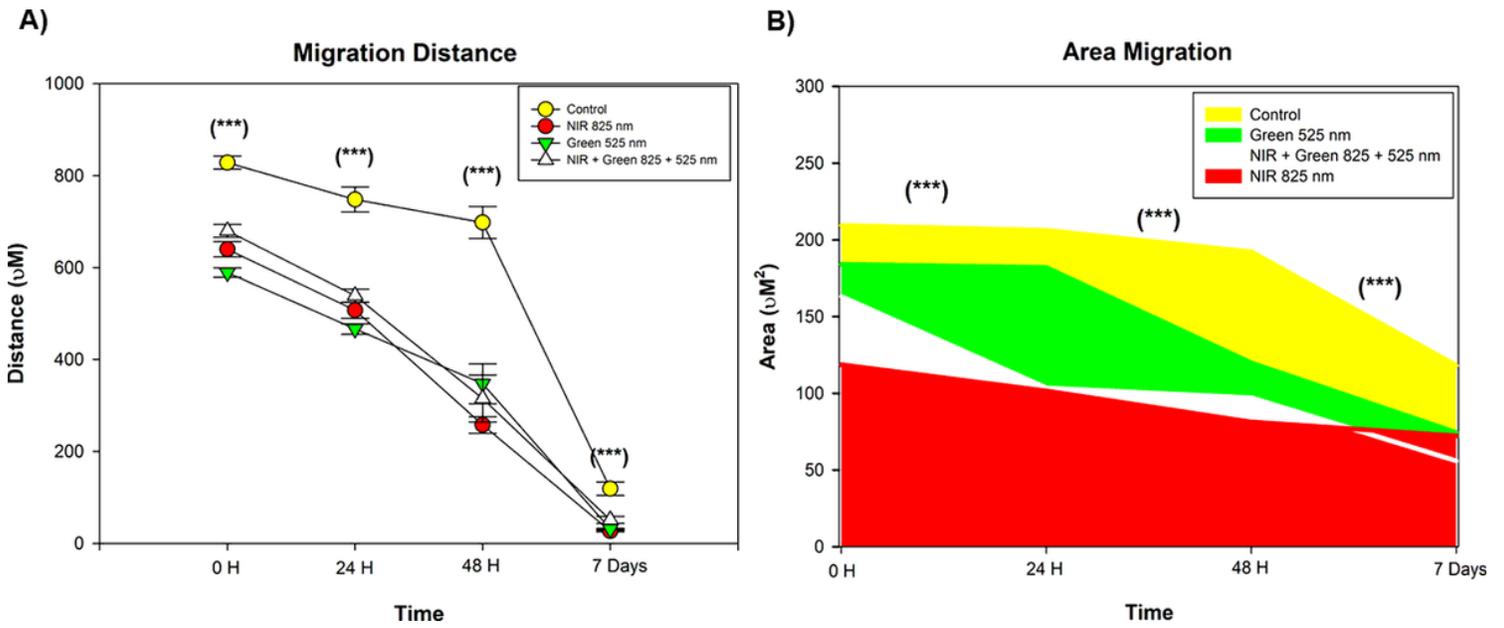


Figure 6

Migration analysis of ADMSCs 24 h, 48 h and 7 days post irradiation. A) All treatment groups showed a significant ($p < 0.001$) increase in the distance migrated compared to control cells. B) Area migration of the treated cells showed significant ($P < 0.001$) closure, compared to untreated controls, of the wounded area where migrating cells moved towards the central scratch.