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Abstract. Low-level light therapy has been shown to improve *in vitro* wound healing. However, well-defined parameters of different light sources for this therapy are lacking. The goal of this study was (1) to determine if the wavelengths tested are effective for *in vitro* wound healing and (2) to compare a laser and a light-emitting diode (LED) source at similar wavelengths. We show four wavelengths, delivered by either a laser or LED array, improved *in vitro* wound healing in A549, U2OS, and PtK2 cells. Improved wound healing occurred through increased cell migration demonstrated through scratch wound and transwell assays. Cell proliferation was tested by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-car-boxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay and was found generally not to be involved in the wound healing process. The laser and LED sources were found to be comparable when equal doses of light were applied. The biological response measured was similar in most cases. We conclude that the laser at 652 (5.57 mW/cm^2, 10.02 J/cm^2) and 806 nm (1.30 mW/cm^2, 2.334 J/cm^2) (full bandwidth 5 nm), and LED at 637 (5.57 mW/cm^2, 10.02 J/cm^2) and 901 nm (1.30 mW/cm^2, 2.334 J/cm^2) (full bandwidth 17 and 69 nm respectively) induce comparable levels of cell migration and wound closure. [DOI: 10.1117/1.JBO.19.3.038001]

Keywords: low-level light therapy; phototherapy; cell migration; wound healing; laser; light-emitting diode.

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1 Introduction

There are numerous reports of cell, animal, and clinical studies which claim to demonstrate the beneficial effects of low-level light therapy (LLLT). These include enhanced neovascularization,[1] promotion of angiogenesis,[2] increased collagen synthesis,[3] and promotion of healing in acute and chronic wounds.[4] All of these effects are thought to proceed via absorption of light by cytochrome C oxidase.[5] However, one of the challenges of LLLT that must be overcome is that the varied array of optical parameters used in the many different studies makes comparisons between studies as well as replication of studies, difficult, if not impossible.

In vitro assays are ideal for addressing this challenge as many conditions may be evaluated relatively quickly. Findings from *in vitro* assays include increased cellular proliferation,[6] cell migration,[7] and production of adhesion molecules.[8] Previous work has evaluated the effectiveness of laser and LED sources in the context of healing and cell migration.[9,10] However, each of these studies evaluated a set of optical parameters from only one light source, either a laser or an LED. No investigation has compared the two most common LLLT light sources in parallel studies.

We assess diode and a laser source at comparable wavelengths and light dosimetry for the induction of wound healing and cell migration in three different *in vitro* cell models: A549 (adenocarcinoma human alveolar epithelial cells), PtK2 (rat kangaroo renal epithelial cells), and U2OS (human osteosarcoma cells). The results demonstrate similar efficacy from both light sources. Researchers should be able to use either source interchangeably for LLLT as long as the desired wavelength is being applied regardless of coherence of the source being used. This may also prove to be useful for clinical applications especially since LED sources are generally more portable and user friendly than laser systems. Although other cell types such as dermal fibroblasts were not tested; here, it is likely that these optical parameters will also be effective for different cell types.

2 Material and Methods

2.1 *Cell Culture*

Cell lines were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, Maryland): A549 adenocarcinoma human alveolar epithelial cells, PtK2 rat kangaroo renal epithelial cells, and U2OS human osteosarcoma cells. Cells were grown in T-75 flasks (Corning, Fisher, Pittsburgh, Pennsylvania) in a 37°C, 5% CO_2/95% air incubator using Dulbecco's Modified Eagle's Medium (DMEM) for A549 and U2OS cells and Modified Eagle's Medium (MEM) for PtK2 cells. Both media types were supplemented with 10% fetal bovine serum (Mediatach, Manassas, Virginia). Cells were trypsinized with TrypLE (Invitrogen Life Technologies, Carsbad, California), seeded into 6-well or 96-well plates (Corning, Fisher, Pittsburgh, Pennsylvania) and grown to confluency.

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Table 1 Dimensions and dosimetry of light delivery.

Source	Wavelength (nm)	Full bandwidth (nm)	Spot diameter (cm)	Duration (s)	Power Density (mW/cm^2)	Energy Density (J/cm^2)
LED	637	17	NA	1800	5.57	10.02
LED	901	69	NA	1800	1.30	2.334
Laser	652	5	12.5	1800	5.57	10.02
Laser	806	5	12.5	1800	1.30	2.334

2.2 *Photoirradiation and Devices*

The two-light sources used were (1) a red (652 nm) and near-infrared (806 nm) emitting laser (Biolitec AG, Jena, Germany), and (2) an LED array with wavelengths at 637 and 901 nm (Biophotas Inc., Tustin, California). The LLLT phototherapy was delivered using the parameters shown in Table 1 and spectra given in Fig. 1(a)–1(d). The emission spectra from each light source were measured and the maximum intensity value was normalized to one. The full bandwidth was determined by measuring the bandwidth at half of the maximum intensity. Each of the four wavelengths was administered to a confluent 6-well dish. The light from the laser was coupled to an optical fiber terminating in a front distributing lens with a full angle of divergence of 34.7 deg and a beam diameter of 0.6 mm at 0 mm from the lens (FD, Medlight, Ecublens, Switzerland). The samples were exposed to a circular beam of light with the dimensions and dosimetry listed in Table 1. The LED array was placed 1.3 cm (measured from diode to cells) from the bottom of the sample dish. Each dish (well) was suspended above the LED array by a transparent stage. Laser and LED powers were measured using a FieldMate laser power meter (Coherent, Santa Clara, California). Output from either the optical fiber or LED was measured 1.3 cm from the detector, which has an area of 1 cm^2. Dosimetry values were calculated based on a 30-min light exposure. Samples were kept either in the dark or under low-level light for all other processing steps.

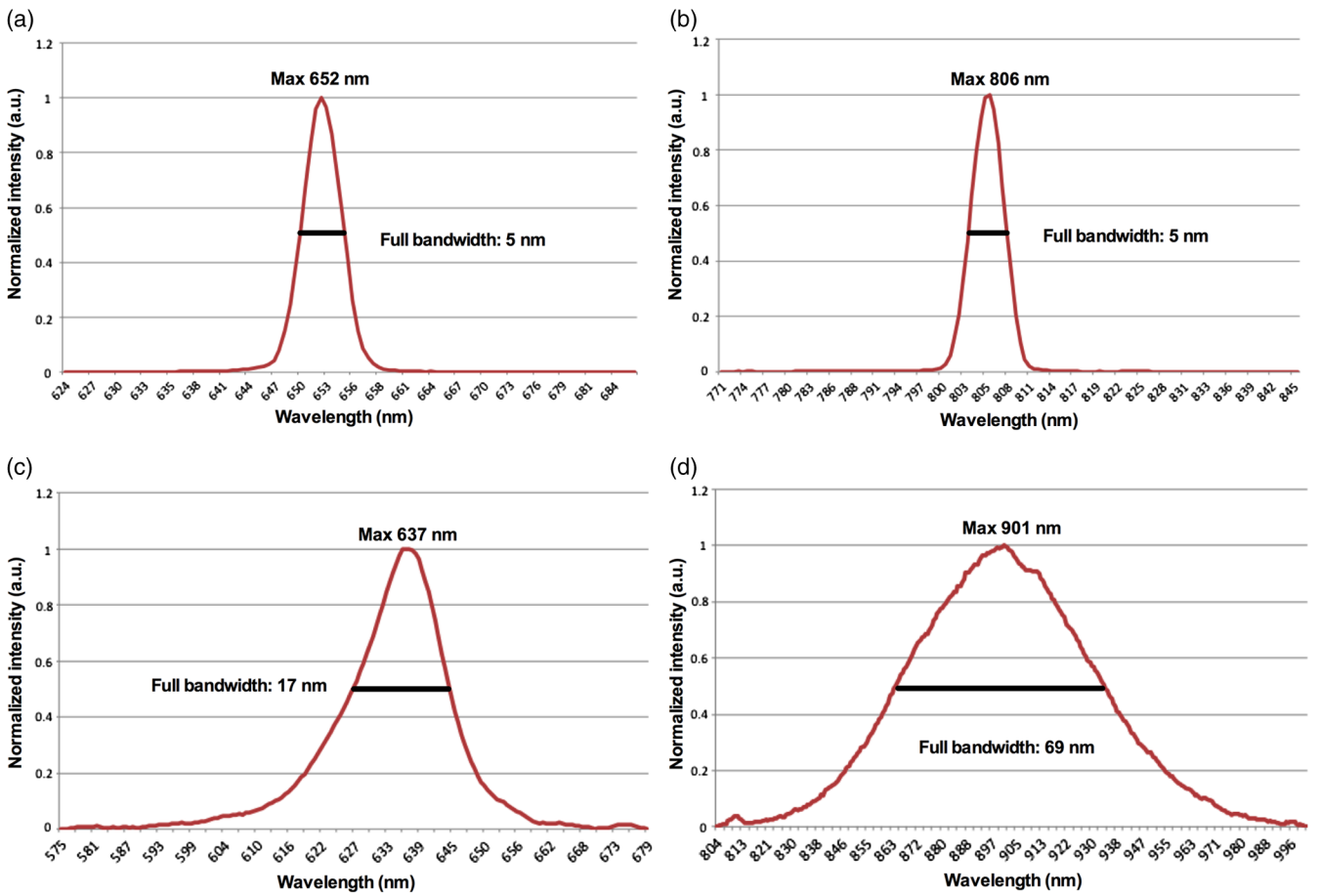

Fig. 1 Emission spectra of all four light sources. The emission spectra from either the laser (a and b) or LED (c and d) was measured and plotted. The maximum intensity value was normalized to one for all curves plotted and the full bandwidth was determined as the bandwidth at half maximum intensity (black line). The full bandwidth and maximum emission wavelength is indicated for all curves. Arbitrary units (a.u.).

2.3 *Heat Measurement*

A RAYNGER MX4 (Raytek, Santa Cruz, California) infrared-detecting thermometer was used to measure the temperature of the cell culture sample before and after LLLT. The laser temperature gun measures the selected area within the well with a relationship of 60:1 between the distance and spot size at the focal point. The aiming laser beam of the gun was aimed at multiple positions within each sample and for multiple culture dishes. Final temperature measurements were normalized to the initial (before any light treatment was administered) temperature.

2.4 *Scratch Wound Closure Assay*

The scratch wound closure assay method has been previously described.[11] Briefly 600 to 700-μm wide single-line scratches were mechanically generated in a cell monolayer by a 200-μl plastic tip. The area of the open wound was recorded for 24 h and quantified using a custom MATLAB® script. Cells from both edges of the wound migrated into the open wound area until closure was complete. The wound closure rate was determined by plotting changes in the open wound area as a function of time.

2.5 *Microscopy*

Phase contrast images were captured through a 10× magnification Ph1 NA 0.25 objective (Zeiss, Jena, Germany). Fluorescent images were captured through a 63 × Ph3 NA 1.4 objective (Zeiss, Jena, Germany). An inverted microscope (Axiovert 135, Zeiss, Jena, Germany) with a charge-coupled device camera (ORCA-R^2 Hamamatsu, Bridgewater, New Jersey) was used and images were acquired using custom Robolase II software developed previously.[12] The microscope stage was modified to accommodate a multiwell format for high-throughput analyses.

2.6 *Transwell Migration Assay*

A standard transwell migration assay method was used as previously described.[11] Briefly, cells were serum-starved overnight prior to being seeded (5×10^4 cells in 100 μl of DMEM or MEM/0.1% FBS) into the top chamber of a 24-well transwell plate with an 8.0-μm pore polycarbonate membrane (Corning, Fisher). MEM or DMEM/10% FBS was placed in the bottom chamber. Cells were allowed to adhere to the membrane for 24 h and then were exposed to light for 30 min. After an additional 24 h, the cells were fixed in 70% cold ethanol. A cotton swab was used to remove cells remaining on top of the membrane. Cell migration through the membrane was measured using the cell nuclei stain bisbenzimide H 33342 trihydrochloride (Hoechst, Invitrogen Life Technologies, Grand Island, New York). Cell number was quantified using the ImageJ (Wayne Rasband, NIH, Bethesda, Maryland) cell counter plug-in.

2.7 *MTS Cell Proliferation*

PtK2 and U2OS cells were seeded at 2×10^4 per well, and A549 cells were seeded at 1×10^4 per well in a 96-well plate format. Cells were incubated overnight to permit cell attachment. The cells were then exposed to light at all four wavelengths for 30 min (Table 1). At 24 h the level of cell proliferation was determined using a MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, Wisconsin) according to the manufacturer's instructions. The MTS dye was added directly to cell culture media and incubated with the cells for 2 h at 37°C. Dye absorbance at 490 nm was measured and plotted for both untreated (no-light) and light-treated cells.

2.8 *Statistical Analysis*

Data are presented as mean ± standard error of the mean (SEM). Student's *t*-tests were used for experiments that contained only two conditions, and one-way analysis of variance followed by Bonferroni's post-test was used for experiments containing three or more conditions. A p value <0.05 was considered to be statistically significant.

3 Results

3.1 *LLLT Enhances Wound Closure*

LLLT with either the laser or LED light source significantly enhanced *in vitro* wound closure in all three cell types at all four wavelengths tested. The initial wound size was evaluated and then compared with the wound 24-h postlight exposure [Figs. 2(a) and 2(b) and Figs. 3(a) and 3(b)]. All treatment conditions demonstrated statistically significant increases in wound closure rates when compared with the untreated controls.

In some cases (U2OS at 637 nm, U2OS at 901 nm, and PtK2 at 901 nm) the LED treatment produced a statistically greater closure rate than the laser. However, it should be noted that there is a small amount of heat given off by the LED device (3°C for 637 and 2°C for 901 nm), whereas there is no measurable temperature change during treatment with the laser source. Heat alone from the LED circuitry was tested for all assays run in this study, and it was found to have no effect in most cases. The U2OS cells showed no change in wound closure after heat treatment alone. This suggests that the differences observed between LED and laser treatments for U2OS cells are attributed to wavelength differences. In contrast PtK2 cells showed a significant increase in wound healing rate after heat treatment alone ($p < 0.05$ compared with the untreated control), which suggests that for this nontransformed marsupial cell type, at least some of the difference observed between the laser and LED, is likely due to the heat given off by the LED array. However, the marsupial cells did respond with increased wound healing when treated with the laser, which did not generate any heat.

3.2 *LLLT Improves Cell Migration*

Using the transwell assay LLLT was shown to significantly stimulate *in vitro* cell migration in all three cell types A549, PtK2, and U2OS [Figs. 4(a) and 4(b) and Figs. 5(a) and 5(b)]. Cells were serum starved prior to seeding onto a transwell insert and treated with laser 652 and 806 nm, and LED 637 and 901 nm. It should be noted that serum starvation was only used for the transwell assay, as it was the only assay carried out in this work requiring this pretreatment step. A549 and PtK2 cells demonstrated similar efficacy at all four wavelengths tested. U2OS cells, however, showed a statistically better response for the two LED wavelengths (637 and 901 nm) compared with the two laser wavelengths (652 and 806 nm). In U2OS cells the LED

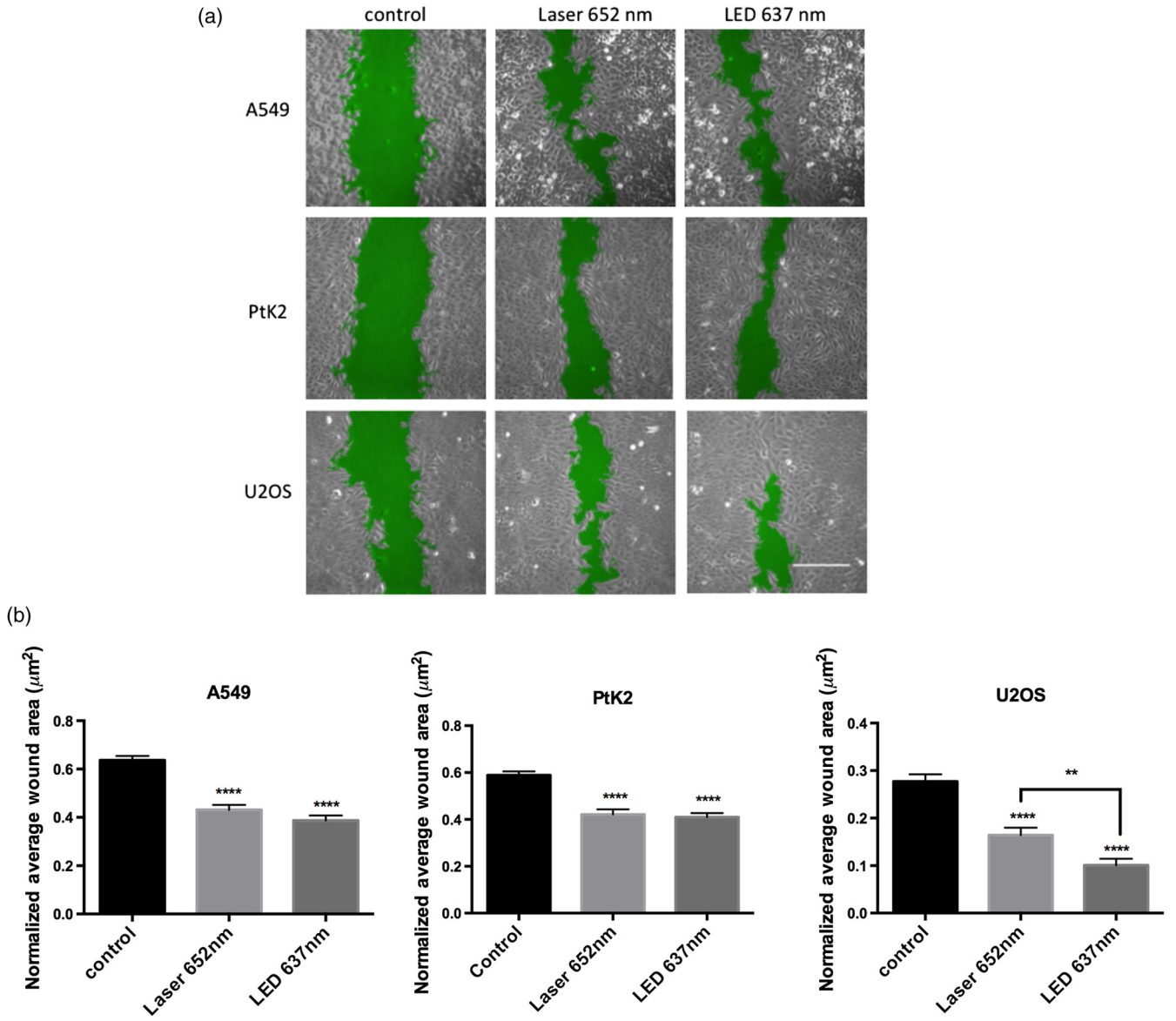

Fig. 2 Low-level light therapy (LLLT) increases *in vitro* wound healing in A549, PtK2 and U2OS using 637 and 652 nm. (a and b) Mechanical scratch wounds were generated in a monolayer of the respective cell type, and treated with LLLT 637 or 652 nm. Twenty-four hours later, the area of the wound was evaluated by a computer-based system as described in Sec. 2 and compared with the untreated control. The normalized average wound area was determined by dividing the final wound area by the initial wound area and averaging replicates. The green area marks the wound, bar, 100 μm. Data represent the mean $\pm$ standard error of the mean (SEM) of at least three separate experiments with $N = 12$; ****$p < 0.0001$, **$p < 0.01$ compared with control or for the indicated comparison.

and laser sources exhibited no difference in cell migration after heat treatment this was also true for A549 and PtK2 cells.

3.3 *LLLT and Cell Proliferation*

For three wavelengths (652, 637, and 901 nm), there was no measureable difference in proliferation in A549, PtK2, and U2OS cells compared to unexposed control cells. However, at 901 nm the A549 and U2OS cells exhibited no increase in cell proliferation compared with the controls, but the PtK2 cells did [Figs. 6(a) and 6(b)]. No difference was detected from heat treatment alone in all three cell types.

4 Discussion

Our results demonstrate that LLLT, whether from a laser or LED, accelerates wound healing in three *in vitro* cellular models: A549 adenocarcinomic human basal epithelial cells, PtK2 marsupial epithelial cells, and U2OS human osteosarcoma cells. All three cell types responded to a single 30-min treatment of LLLT. In particular, U2OS cells responded well to LED 637 nm and LED 901 nm possibly due to a difference in wavelength compared with the laser source or due to the cell type difference as both A549 and PtK2 are epithelial cells while U2OS are osteosarcoma. It should also be noted that A549 and U2OS are both human cancer cell lines, whereas PtK2 cells are a

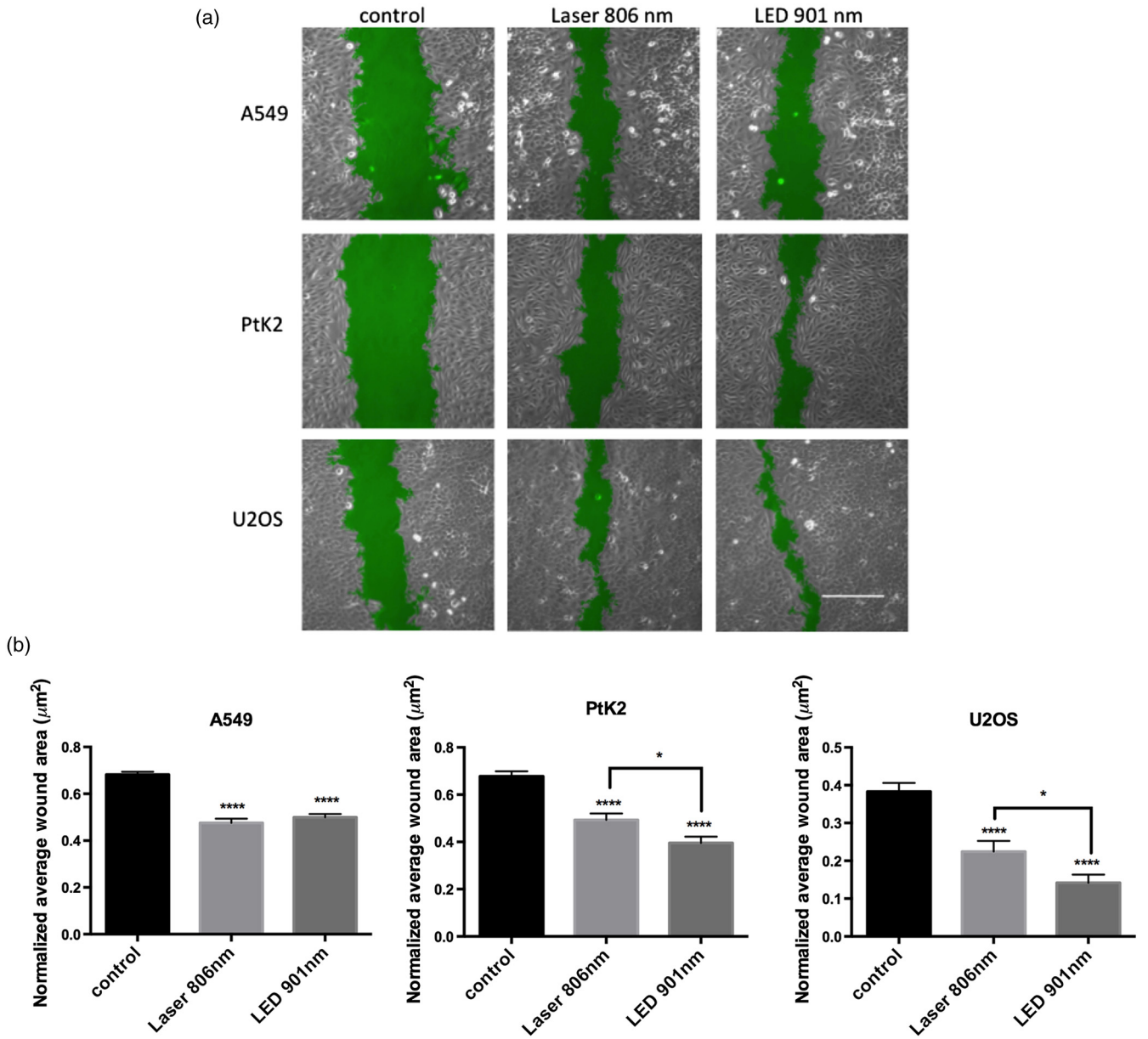

Fig. 3 LLLT increases *in vitro* wound healing in A549, PtK2, and U2OS using wavelengths 806 and 901 nm. (a and b) Cells were treated and evaluated as in (Fig. 2), but treated with LLLT 806 or 901 nm and wound healing was measured after 24 h. The green area marks the wound, bar, 100 μm. Data represent the mean $\pm$ SEM of at least three separate experiments with $N = 12$; $^{****}p < 0.0001$, $^{*}p < 0.05$ compared with control or for the indicated comparison.

transformed normal cell type from a marsupial. This phylogenetic and malignancy difference between the PtK2 and human cancer cells likely explains the differences in the cell responses to the heat generated by the LED. No form of stress, such as serum starvation was needed to induce a statistically significant increase in wound closure rate in any of the cell models used in the scratch wound assay prior to LLLT. We felt that it was more representative to treat unstressed cells being that a response was still observed in the scratch wound assay under our conditions.

The intermediate forms of cytochrome c oxidase redox cycle have been suggested as the photoacceptors of light delivery. The maximal absorption values are shown to be 820 nm for oxidized Cu_A, 760 nm for reduced Cu_B, 680 nm for oxidized Cu_B, and 620 nm for reduced Cu_A (Ref. 5). Although a biological response action spectra have been investigated for DNA synthesis rate it is still unclear which maximal absorbance peak(s) may correspond to different biological responses, such as the enhanced cell migration found in LLLT. Established action spectra fall within the 620 to 680 nm and 760 to 895 nm ranges. Thus, some wavelengths used in this study may be closer to the optimum action spectrum for wound healing than others. These may also vary between cell types. It is also important to note that the electronic circuitry of the LED array produces a ~2°C to 3°C (3°C for LED 637 and 2°C LED 901 nm) rise in temperature, while the laser source does not. U2OS and A549 cells did not demonstrate any measurable difference from heat alone treatments; however, the PtK2 cells did show a slight response to heat.

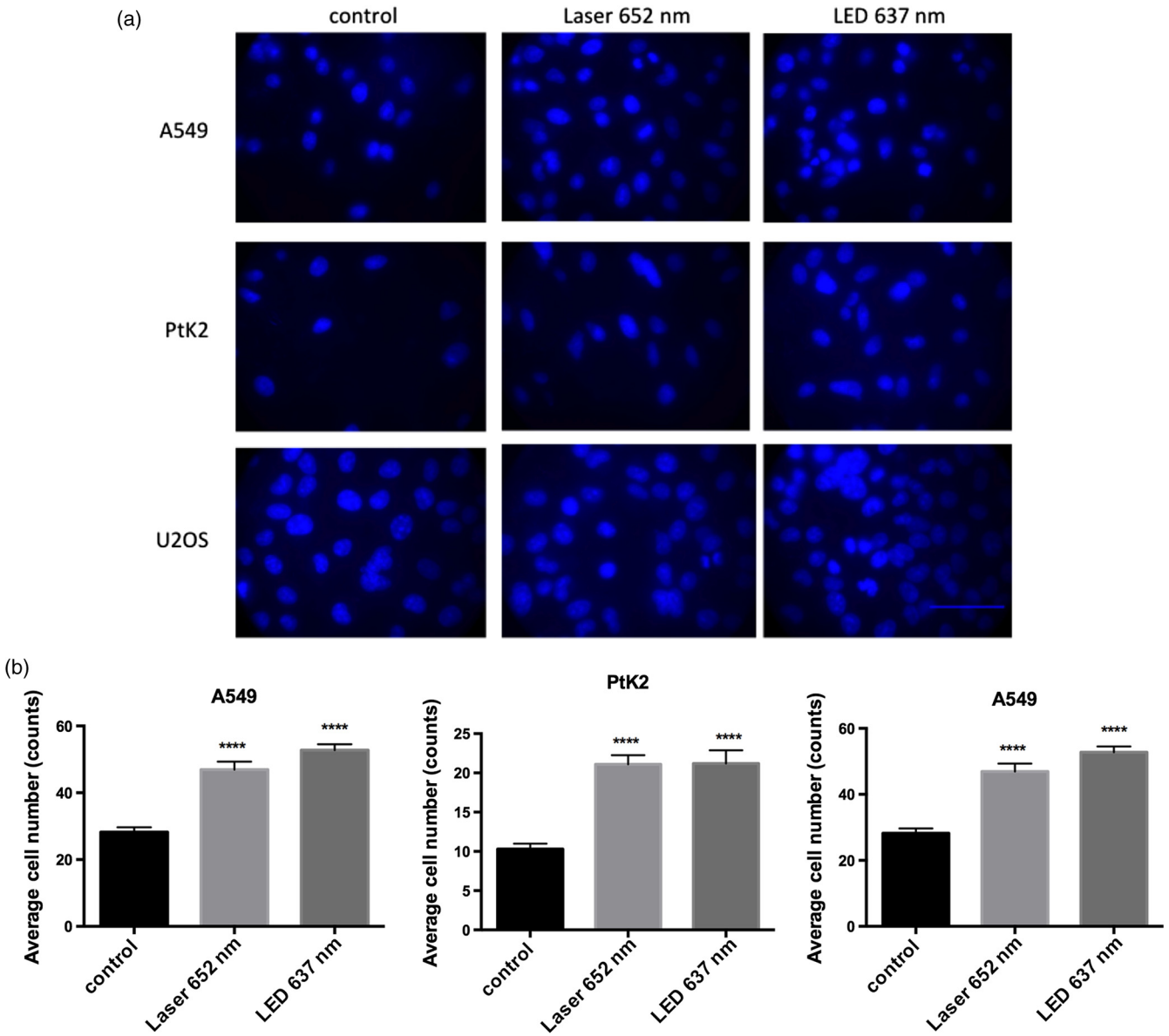

Fig. 4 LLLT 637 and 652 nm increase *in vitro* cell migration. (a and b) A549, PtK2, or U2OS cells were seeded onto a polycarbonate membrane and allowed to adhere to the transwell insert followed by treatment with either LLLT 637 or 652 nm. Transwell migration was visualized using Hoechst 33342 (blue) and quantified using the cell counter ImageJ plugin at 24-h posttreatment as described in Sec. 2. Bar 20 μm. Data represent the mean $\pm$ SEM of at least three separate experiments with $N = 3$ fields of view; ****$p < 0.0001$ compared with control or for the indicated comparison.

The LLLT increased cell migration in all three cell types with strong statistical significance (from $p < 0.05$ to $P < 0.0001$ respectively). The LLLT did not increase cell proliferation in U2OS and A549 but did in the marsupial PTK2 cells. However, considering the significant LLLT-enhanced cell migration in PtK2 cells (Figs. 4 and 5), it would seem likely that wound closure in this cell type as well as the other two cell types is predominantly via cell migration. However, it is possible that marsupial cells may respond differently than human (primate) cells. PtK2 cells are the only marsupial cell type used, as the other two cell types are human derived. In addition, PtK2 cells represent a normal-immortalized cell type, whereas A549 and U2OS cells are cancer cells. Normal-differentiated cells are known to primarily obtain mitochondrial metabolic energy through oxidative phosphorylation while cancer cells instead rely on aerobic glycolysis, a process known as the Warburg effect.[13] This is another likely reason for the difference observed in response of the PtK2 cells in regards to the scratch wound assay [Figs. 3(a) and 3(b)] and cell proliferation [Fig. 6(b)]. This point is increasingly important since the observed wound healing process is most likely Cox dependent, which is located within the electron transport chain.

Although under our conditions we did not observe an increase of cell proliferation with the exception of PtK2 cells in [Fig. 6(b)] others have reported an increase of cell proliferation following LLLT. Proliferation effects from LLLT have been reported using numerous methods including mitotic index,[14] colony forming ability,[15] and [^{3}H] thymidine incorporation.[16] The LLLT-induced cell increases in proliferation have been reported in fibroblasts,[17] keratinocytes,[18] HeLa cells,[19,20]

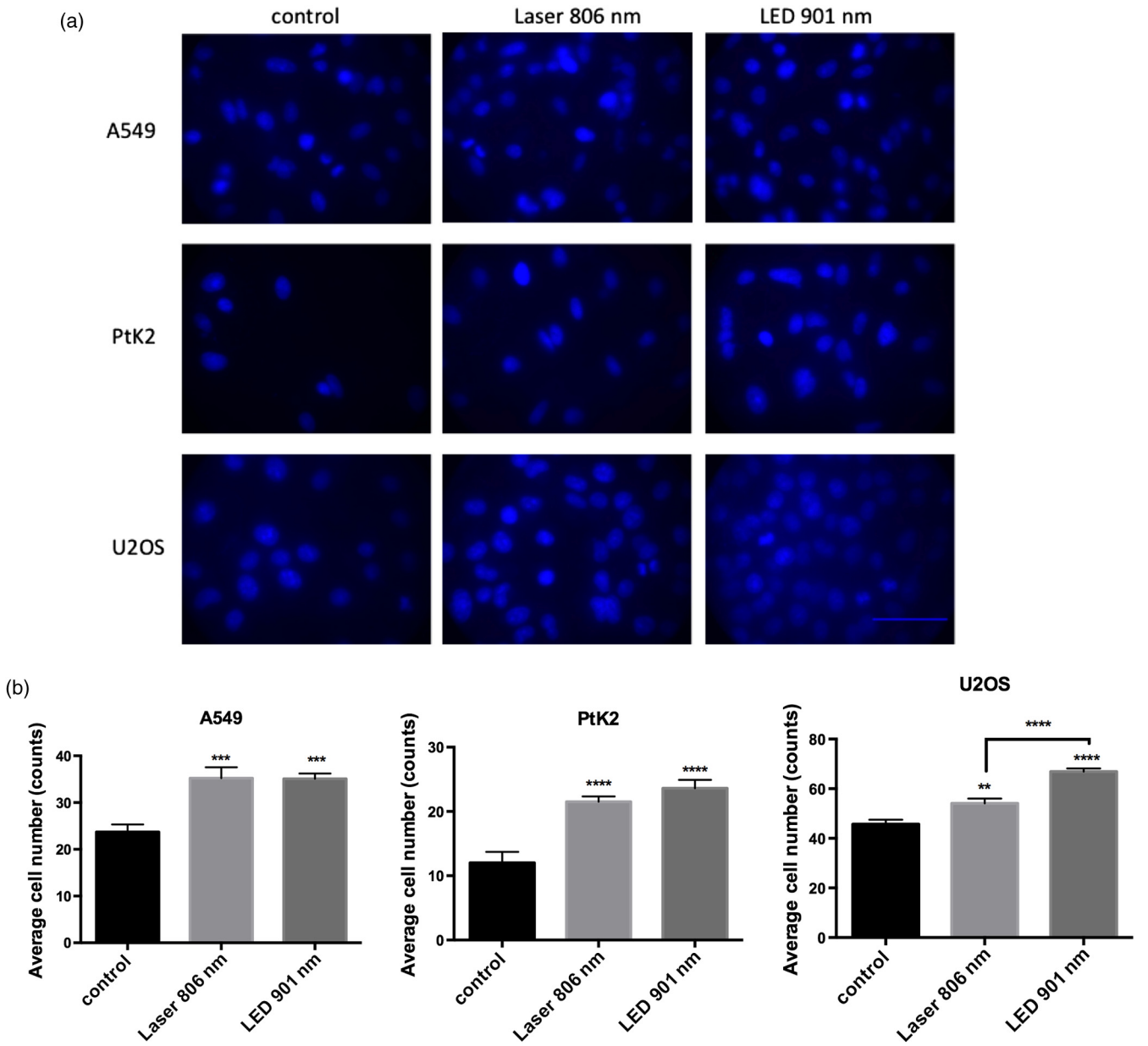

Fig. 5 LLLT 806 and 901 nm increase *in vitro* cell migration. (a and b) A549, PtK2, or U2OS cells were treated and quantified as in (Fig. 4), but using LLLT 806 or 901 nm. Bar 20 μm. Data represent the mean $\pm$ SEM of at least three separate experiments with $N = 3$ fields of view; $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$ compared with control or for the indicated comparison.

and many others. Although other reports demonstrate inhibition of cell proliferation in fibroblasts[21] or in some cases show no change in HeLa cells.[22] The large variability between reported results is likely due to the wide range of parameters possible for the treatment of cell culture and numerous assay methods. Also, the timing and treatment reagent seem to play a large role as well. For example, in the study presented here a single dose of light was applied to cells and effects were measured after 24 h, while studies that demonstrate proliferation generally treat multiple times and measure proliferation at much later time points post treatment.

PtK2 cells were used in this study as they have exhibited increased sensitivity to pharmacological treatment in our previous studies.[11] This cell type was primarily chosen because it was thought that these cells should be responsive to this therapy as well. Although the coherence of both sources differs, each source delivered an equal dose of light to the treatment area with comparable biologic responses. We conclude that both sources are equally effective at comparable light doses.

This study was conducted *in vitro*, so although it has potential applications for primary cultures, *in vivo* and clinical applications the work presented here is only suggestive of possible outcomes in other systems. Further investigation of the mechanism of action must be conducted to validate the proposed mechanism of action. It is also likely that other biochemical effects and down stream signaling is occurring in response to LLLT. The results presented here compare the functional response of wound healing only in the cell types tested. There are other factors that may be explored, such as ATP

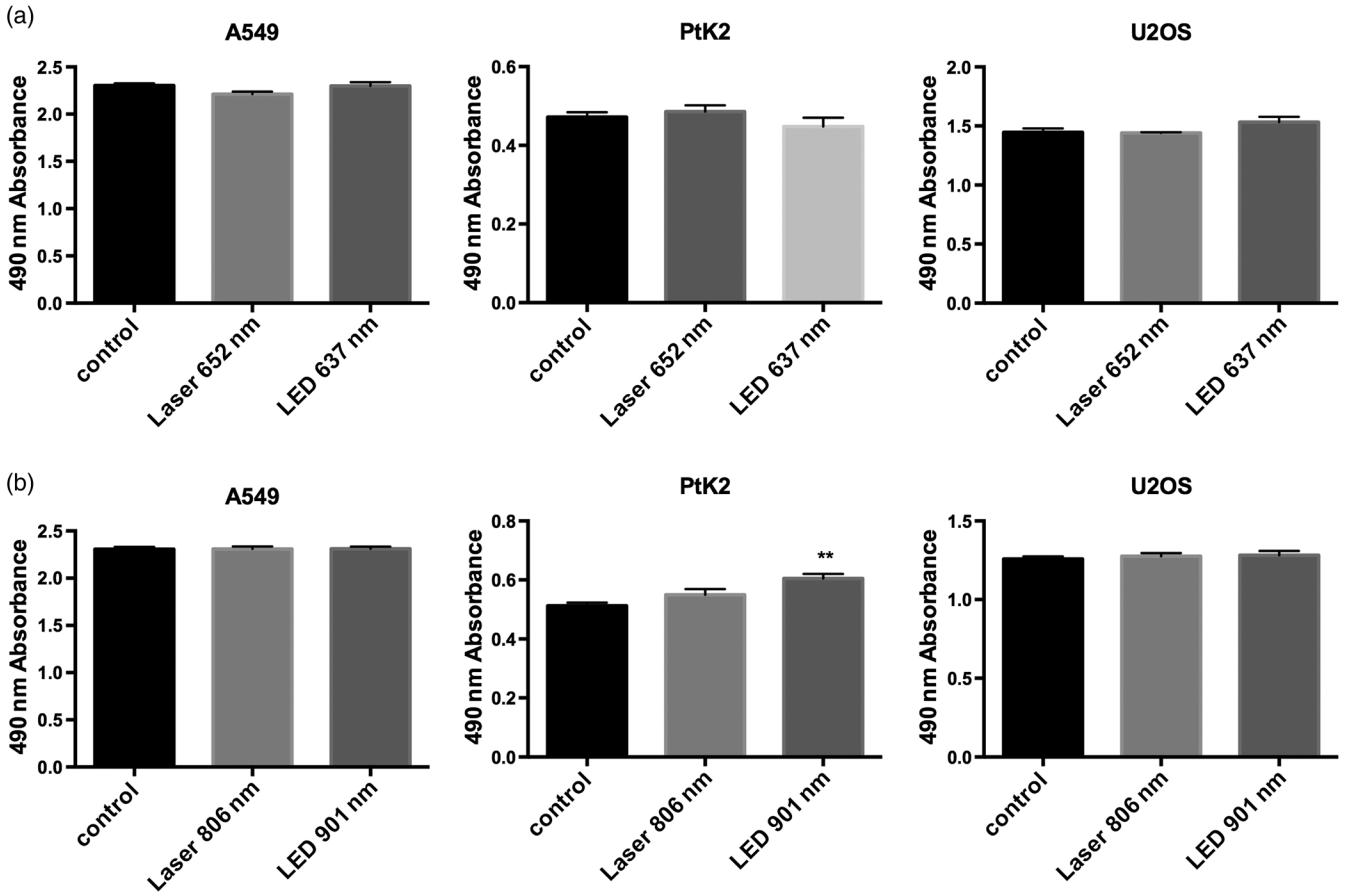

Fig. 6 LLLT does not increase *in vitro* cellular proliferation for most conditions. (a and b) A549, PtK2, or U2OS cells were treated with LLLT as indicated, and cell proliferation was measured 24-h posttreatment as described in Sec. 2. Data represent the mean ± SEM of at least three separate experiments with $n = 8$ $^{**}p < 0.01$ compared with control.

production, in order to provide a more comprehensive comparison of both light sources.

In summary, we have performed a side-by-side study comparing the LLLT effectiveness of laser and LED light sources for wound healing in three-different mammalian cell systems. Our results show that LLLT induces wound healing primarily through enhanced cell migration as opposed to cell proliferation. Although many studies have demonstrated that laser and LED light sources improve wound healing, we are unaware of any that compare these two sources side-by-side as reported here. A better understanding of the cellular and photobiological mechanisms of LLLT as well as standardization of treatment parameters will allow for the development of better therapies and wider acceptance of LLLT.

Acknowledgments

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