

Effect of low-level laser treatment of tissue-engineered skin substitutes: contraction of collagen lattices

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Abstract. Fibroblast-populated collagen lattices (FPCL) are widely used in tissue-engineered artificial skin substitutes, but their main drawback is that interaction of fibroblasts and matrix causes contraction of the lattice, reducing it to about 20% of its original area. The effect of low-level laser treatment (LLLT) on the behavior of 3T3 fibroblasts seeded in collagen lattices containing 20% chondroitin-6-sulphate was investigated to determine whether LLLT could control the contraction of FPCL. A He-Ne laser was used at 632.8 nm to deliver a 5-mW continuous wave with fluences from 1 to 4 J/cm². Laser treatment at 3 J/cm² increased contraction of collagen lattices in the absence of cells but decreased contraction of cell seeded lattices over a 7-day period. The effect was energy dependent and was not observed at 1, 2, or 4 J/cm². There was no alteration in fibroblast viability, morphology, or mitochondrial membrane potential after any laser treatments, but the distribution of actin fibers within the cells and collagen fibers in the matrices was disturbed at 3 J/cm². These effects contribute to the decrease in contraction observed. LLLT may offer a means to control contraction of FPCL used as artificial skin substitutes. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3127201]

Keywords: Low-level laser treatment (LLLT); fibroblast-populated collagen lattices (FPCL); contraction of cell-seeded collagen lattices; artificial skin substitutes; fibroblast morphology; He-Ne laser.

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1 Introduction

Tissue-engineered constructs are designed, and used, to replace diseased or damaged tissues, particularly the load-bearing soft connective tissues, such as cartilage,¹ tendon,² ligament,³ blood vessels,⁴ heart valves,⁵ and skin,^{6–8} including tissue-engineered skin products approved by the US Food and Drug Administration. Tissue-engineered structures replicate some aspects of tissue function, but the central aim is to allow the bioresorption of the construct and its replacement by natural body tissue.

Tissue-engineered skin substitutes generally consist of cells and extracellular matrix materials either alone or in combination. Epidermal keratinocytes facilitate the development of an epidermal layer on the surface of the skin substitute, and fibroblasts facilitate the development of a dermal layer. The extracellular matrix provides a three-dimensional (3-D) framework for the fibroblasts and provides biological support both for the fibroblasts and the overlying keratinocytes. Following the pioneering work of Burke et al.,⁹ collagen has been widely used as a matrix material in the form of sponges, gels, or lattices.¹⁰ Naturally occurring glycosaminoglycans (GAG), such as chondroitin-6-sulphate (Ch6SO₄), and cross-linking

agents may be added to matrix materials to improve their mechanical and biological properties.^{11–13}

Fibroblast-populated collagen lattices (FPCL) have been extensively studied and used in tissue-engineered skin replacements, and fibroblasts cultured in a lattice mimic the *in vivo* situation in the dermis, displaying similar cell shape, mitotic activity, and collagen synthesis compared to human dermis.¹⁴ The main drawback of FPCL is that the interaction between fibroblasts and the matrix produces contraction of the lattice, which can lead to a reduction up to 20% of the initial area.¹⁵ The contraction of the skin substitute reduces the area available for skin replacement and risks exposing the wound to tissue inflammation, maceration, or desiccation and bacterial infection.¹⁶

FPCLs have been used as models of healing wounds, in which contraction may also occur. Mester et al.¹⁷ reported that 1 to 4 J/cm² low-level laser irradiation induced healing of chronic nonhealing soft tissue ulcers, and this formed the genesis of clinical low-level laser treatment (LLLT). Other studies¹⁸ produced equivocal results because of the variability of clinical wounds and patient status, and other poorly controlled variables, such as patient mobility. *In vitro* studies, however, show that LLLT can modulate a variety of biological processes within cells.¹⁹ We investigated the use of LLLT *in vitro* to determine whether it represented a means of modi-

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fyng the contraction of FPCLs. In this context, we used FPCL following the compositions recommended by Yannas and coworkers,²⁰ which led to the collagen-chondroitin sulphate dermal regeneration template known as Integra. Contraction of the lattices was measured at a density of fibroblasts previously observed to accomplish a reduction in area of greater than 50% in 7 days in a model of wound healing.¹²

2 Materials and Methods

2.1 Preparation of Collagen Lattices

The test specimens were collagen lattices containing 0.3% (w/v) collagen supplemented with 20% w/v GAG. Lattices were formed by mixing 4.29 mg/ml type I collagen solution acid extracted from rat tail tendon,²¹ a mixture of 10×Dulbecco's Modified Eagle's Medium (DMEM) and 0.4 M NaOH (2:1), and the GAG, CH₆SO₄, prepared at 3 mg/ml in 1×serum-free DMEM, at a ratio of 7:1:2. The pH was adjusted to 8 to 8.5 by dropwise addition of 1 M NaOH.

Five ml aliquots of the collagen lattice solution were pipetted into a polycarbonate (Stockline Plastics Ltd., Glasgow, UK) cell chamber measuring 24×60×11 mm with an outer diameter of 100 mm. In order to have a hydrophobic cell chamber to inhibit cell attachment, the cell chamber was siliconized for 24 h in a fume cupboard using Sigmacote. Before cell seeding, the collagen lattices were washed twice by incubating them in complete DMEM containing 10% v/v fetal calf serum, 50 units/ml penicillin/50 μg/ml streptomycin, and nonessential amino acids. 3T3 mouse fibroblasts were seeded onto the set collagen lattice at a density of 10⁴ cells/cm² in 7 ml of complete DMEM and incubated for 4 h at 37 °C (Heraeus Electronics, Hanau, Germany) in an atmosphere of 5% CO₂ in air to allow cell attachment. After this time period, the FPCLs were detached from the walls of the cell chamber and floated freely in the culture medium. Laser treatment was carried out after this 4-h attachment period. Thereafter, cells were cultured for up to 7 days with a daily change of 2 ml of the 7 ml culture medium. Collagen lattices were also cultured in the absence of cells and laser treated by following the same protocol as described earlier, but omitting the cells.

2.2 Laser Irradiation

Lattices were irradiated using a 5-mW continuous wave, linearly polarized He-Ne laser (3225H-PC, Hughes Aircraft Co.) operating at 632.8 nm with a beam profile at TEM₀₀ mode delivering a laser beam with a spot size of 1.5 mm. The output power was measured using Moletron MAX 5200 and Metrologic power meters calibrated at 632.8 nm. The body of the laser was attached to a scanner, which was controlled by two linear actuator stepper motors. A host personal computer with a Pentium II processor running on LabVIEW 6.0 (Laboratory Virtual Instrument Engineering Workbench, National Instruments Corp.) and a development environment based on the graphical programming language G were used to control the scanning speed of both motors, V (cm/s), which determined the laser fluence or energy density, ρ (J/cm²), the relation being:

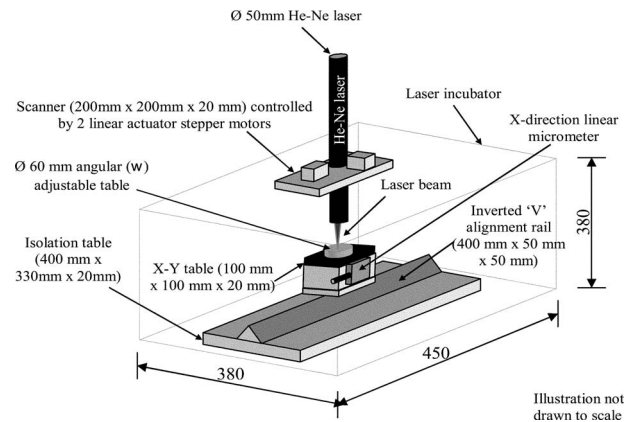


Fig. 1 The laser scanning system.

$$V = (S^* Pw) / (\rho^* A),$$

where S (cm) is the total distance travelled by the laser beam, Pw (W) is the output power, and A (cm²) is the area of treatment.

The laser, delivering an output power of 3.5 mW, was positioned 20 mm above the surface of the specimen. A holder specially fabricated to accommodate the cell chamber was attached to the top of the angular table together with a linkage connecting the linear micrometer with the x-y table, and an alignment rail enabled precise positioning of the fibroblast-populated collagen lattices (FPCL) for LLLT treatment (Fig. 1). The entire computer-assisted experimental setup was housed in a controlled environment of 37 °C.

2.3 Analysis of Cell Viability, Morphology, Mitochondrial Membrane Depolarization, and Collagen Fiber Content of the Matrix

To examine the viability of the fibroblasts in the FPCL, ethidium bromide (EB) and 5-carboxyfluorescein diacetate (CFDA) staining was used.²² CFDA stains the viable 3T3 fibroblasts green, and EB stains the nuclei of cells with damaged membranes red. The lattices were washed twice with phosphate buffered saline (PBS), and then 2 ml of 0.1% (w/v) EB in PBS was added. The lattices were kept in the dark at room temperature for 6 min before washing them again three times with PBS. Three ml of 25 μM CFDA in PBS was then added, and the lattices were kept in the dark at 4 °C. After 15 min, the lattices were again washed three times with PBS and viewed under the confocal laser scanning microscope (Leica CLSM). For the detection of cytoskeletal damage, fluorescein isothiocyanate (FITC)-labeled phalloidin was used to stain the actin, as described by Wulf et al.²³ The lattices were fixed by immersing them in PBS containing 4% formaldehyde for 20 min at room temperature. Fixed samples were then washed three times with PBS and incubated with 1:500 dilution of FITC-phalloidin for 1 h in the dark at room temperature, washed again three times with PBS, and examined by CLSM. To visualize the collagen fibers in the collagen-GAG lattices, both the LLLT-treated and control lattices were immersed for 5 min at room temperature in 0.1% (w/v) acriflavine, a mixture of 3,6-diamino-10-methylacridinium (acriflavine) and 3,6-diaminoacridine (proflavine). Samples were

washed three times with PBS to remove excess stain before viewing on the CLSM. Examination by CLSM for CFDA/EB, FITC-phalloidin, and acriflavine stained samples used 488-nm excitation with a 510-nm dichroic beamsplitter in place. Data from the two channels were collected, using a barrier filter of 590 nm in channel 1 and a bandpass filter of 530 nm in channel 2. The objective lens was a $25\times$ water immersion lens (NA 0.75).

To determine the mitochondrial membrane potential, accumulation of the fluorescent potentiometric dye tetramethylrhodamine ethyl ester (TMRE) was measured in the control and LLLT-treated 3T3 cells. For this method, the cells were cultured on polystyrene 60-mm Petri dishes and not in collagen lattices. Cells were seeded at 10^4 cells/cm² in 7 ml complete DMEM and treated with the laser after allowing 4 h for attachment as with the FPCL samples. As a positive control, cultured cells were also incubated with 100 μ M carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) for 1 h at 37 °C to disrupt the mitochondrial membrane potential. Cultures of control, LLLT-treated, and FCCP-treated cells were incubated with 100 nM TMRE in complete DMEM for 20 min, after which they were washed three times with PBS and imaged by z -sectioning. Each section was 2 μ m, and a total of 18 sections were imaged. The images were collected using Lasersharp 2000 software (Bio-Rad Laboratories) running OS/2. Images were recorded using an MRC 1024 CLSM (Bio-Rad) coupled to a Nikon E600FN upright microscope using a $60\times$ FLUOR water dipping lens (NA=10). TMRE was excited at 568 nm using a Krypton/Argon laser. A 580-nm LP filter (Chrom, Inc.) was used to collect emission of light greater than 580 nm. Time-dependent TMRE fluorescence changes were measured in frame scan mode. Projections of the image sections were created using a "maximum" algorithm in the Bio-Rad Lasersharp 2000 software. Subsequently, the intensity of the cells was quantified in Scion Image.

2.4 Measurement of Contraction

The area of the lattices in all the experiments was measured daily using a light box over which the cell chambers were placed on a standard millimeter grid. The area of the specimen was then calculated using digital planimetry and imaging software (Scion Image, www.scioncorp.com).

Using Scion Image, the file containing the digitized image of the collagen lattices was opened, the perimeter of the cell chamber outlined, and the original area calculated. The areas of the contracted lattices were measured in a similar way by first plotting the outline of the contracted lattices using a free-hand selection tool in Scion Image and then instructing the program to measure the outlined area. The contracted area of the lattices was expressed as a percentage of the original area.

3 Results

3.1 Lattice Contraction

Both the unseeded collagen lattices and the fibroblast populated lattices contracted, but the magnitude and time scale of the contraction were markedly different.

Seeded lattices showed progressive contraction over the 7 days for which it was monitored (Figs. 2 and 3). The rate of contraction was greatest initially, during the first day of moni-

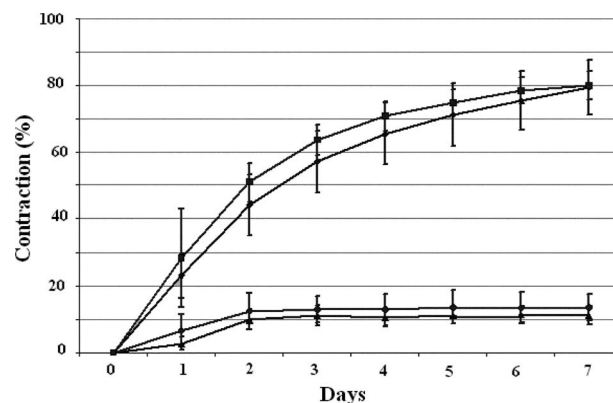


Fig. 2 Contraction of control and LLLT-treated unseeded collagen lattices and FPCL at 2 J/cm². Results are expressed as percentages of the original area of the collagen lattices and are the mean values \pm standard deviation (SD) of five repeated experiments. Diamonds indicate control (FPCL); triangles indicate control (collagen lattices); squares indicate LLLT at 2 J/cm² (FPCL); and circles indicate LLLT at 2 J/cm² (collagen lattices).

toring, and then it decreased nonlinearly. The maximum contraction occurred on day 7, the last day of monitoring, and this value was used to characterize the contraction of the FPCL. Collagen lattices in the absence of cells reached a maximum contraction on the second day and remained constant thereafter (Figs. 2 and 3).

The values of the parameters used to characterize the contraction of the lattices in control and LLLT-treated samples are shown in Table 1. Comparison of the values for control and laser-treated specimens using two-tailed Student's t -test showed that irradiation at fluences of 1, 2, and 4 J/cm² had no statistically significant effect. The effect of 2 J/cm² is shown on Fig. 3. Irradiation at 3 J/cm² produced significantly greater contraction of the unseeded lattices and reduced contraction of FPCL compared with values from the control un-

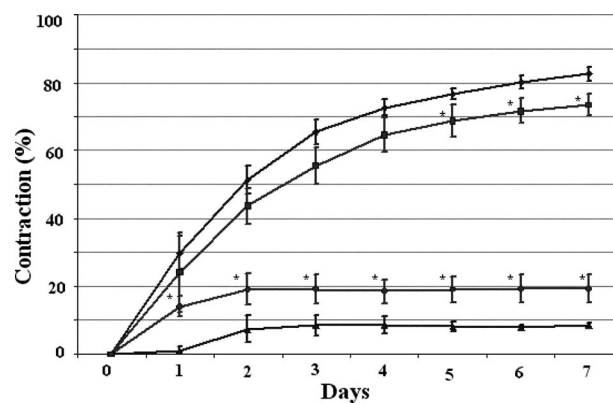


Fig. 3 Contraction of control and LLLT-treated unseeded collagen lattices and FPCL at 3 J/cm². Results are expressed as percentages of the original area of the collagen lattices and are the mean \pm SD, $n=5$. * $p < 0.05$, comparing values from the LLLT-treated lattices with and without cells with those from untreated seeded and unseeded collagen lattices, respectively, by two-tailed Student's t -test. Diamonds indicate control (FPCL); triangles indicate control (collagen lattices); squares indicate LLLT at 3 J/cm² (FPCL); and circles indicate LLLT at 3 J/cm² (collagen lattices).

Table 1 Contraction of FPCL and unseeded collagen lattices. Results are expressed as a percentage of the original area of the collagen lattice and represent the values after 7 days in culture. Values are means of five experiments, with the standard deviation in parentheses. * Denotes that the differences between laser-treated and control values are statistically significant, by two-tailed Student's *t*-test.

Fluence, J/cm ²	Unseeded, mean (%) contraction		FPCL, mean (%) contraction	
	Laser treated	Control	Laser treated	Control
1	6.5 (2.2)	6.6 (3.6)	82.6 (5.0)	76.3 (3.1)
2	13.2 (4.4)	11.0 (2.6)	79.3 (8.2)	76.9 (4.3)
3	19.0 (4.0)*	8.3 (1.7)	73.5 (3.1)*	82.7 (2.1)
4	12.0 (6.8)	9.2 (4.8)	80.0 (2.1)	81.9 (5.5)

seeded collagen lattices and FPCL, respectively ($p < 0.05$). The increase in contraction of the unseeded lattices was significant for all days after day 1, but the decrease in contraction of FPCL was significant for only the last 3 days of the monitoring period (Fig. 3).

Using CLSM, no significant visual difference was observed in the morphology and viability of the cells using CFDA and EB between the control and LLLT-treated FPCL [Figs. 4(a) and 4(b)]. Elongated fibroblastic morphology was maintained for up to 7 days after treatment, with typical alignment of cells in one direction. There was no discernable alteration in the number of nonviable cells after LLLT treatment.

3.2 Actin Filaments

Figure 5 shows the staining with FITC-labeled phalloidin after treatment with 2 and 3 J/cm². Intracellular actin filaments are less pronounced in cells treated with 3 J/cm² than in ei-

ther the controls or the cells treated with 2 J/cm². There are few prominent fibers crossing the interior of the cells, and actin staining appears to be mainly around the cell perimeter. Figure 6 shows that LLLT treatment has no significant effect on the accumulation of TMRE within cells, indicating that it does not alter the mitochondrial membrane potential. In contrast, FCCP treatment markedly decreased TMRE accumulation.

3.3 Collagen Fibers

The effect of LLLT treatment on the collagen fibers was investigated in cell-free matrices in order to distinguish the effect of laser irradiation from cell-induced changes. CLSM images of acriflavine stained collagen fibers at a depth of 20 μm into the collagen-GAG matrices are shown in fig. 7. The images show that there are fewer collagen fibers in the matrices treated with 3 J/cm² than in either controls or in samples treated with 2 J/cm². This was observed in three separate experiments, and Fig. 8 illustrates the mean fluorescent intensities measured in all experiments. After treatment with 3 J/cm², there was a significant decrease in fluorescent intensities measured at a depth of 20 μm in the matrices.

4 Discussion

The greatest rate of contraction of the FPCL was measured within the first day following cell attachment, and this is thought to be due to the forces generated during cell elongation.²⁴ Freyman and coworkers also reported that the contractile force developed by fibroblasts bending or buckling collagen fibrils in the matrix during elongation reached a maximum in the first few hours of culture.^{24,25}

Laser irradiation modifies the extent of the contraction of unseeded and suspension fibroblast seeded collagen lattices, but only at an energy density of 3 J/cm². The occurrence of significant photobiostimulation at 3 J/cm², which was not observed at other fluencies, could be explained by Karu et al.,^{26,27} who reported that the fluence versus response curve

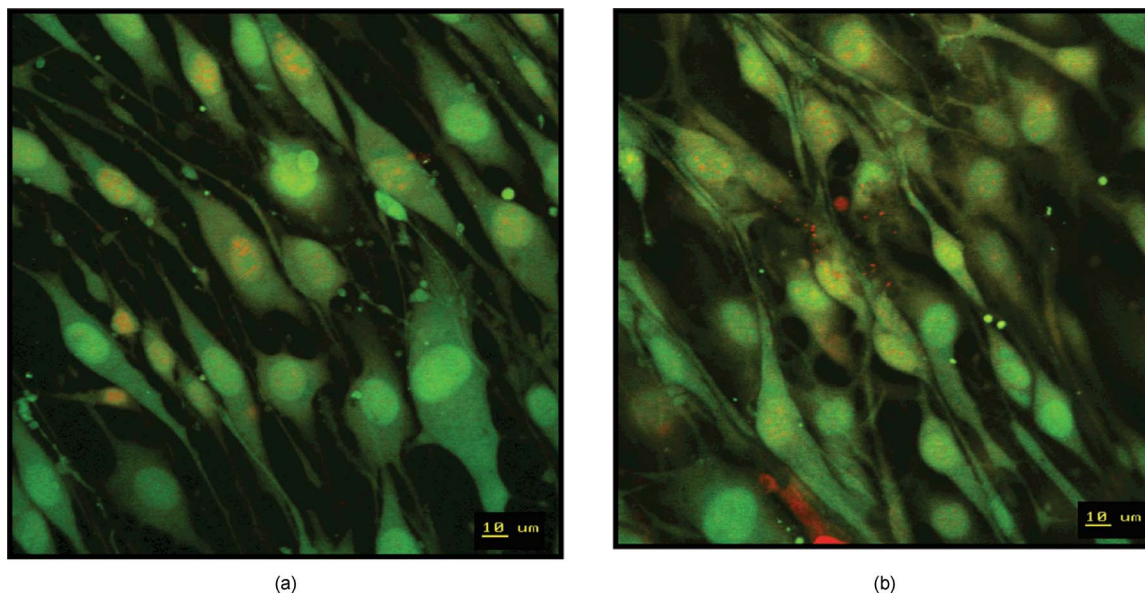


Fig. 4 CLSM images of the control FPCL (a) and the LLLT-treated FPCL (b) at 3 J/cm², on day 7.

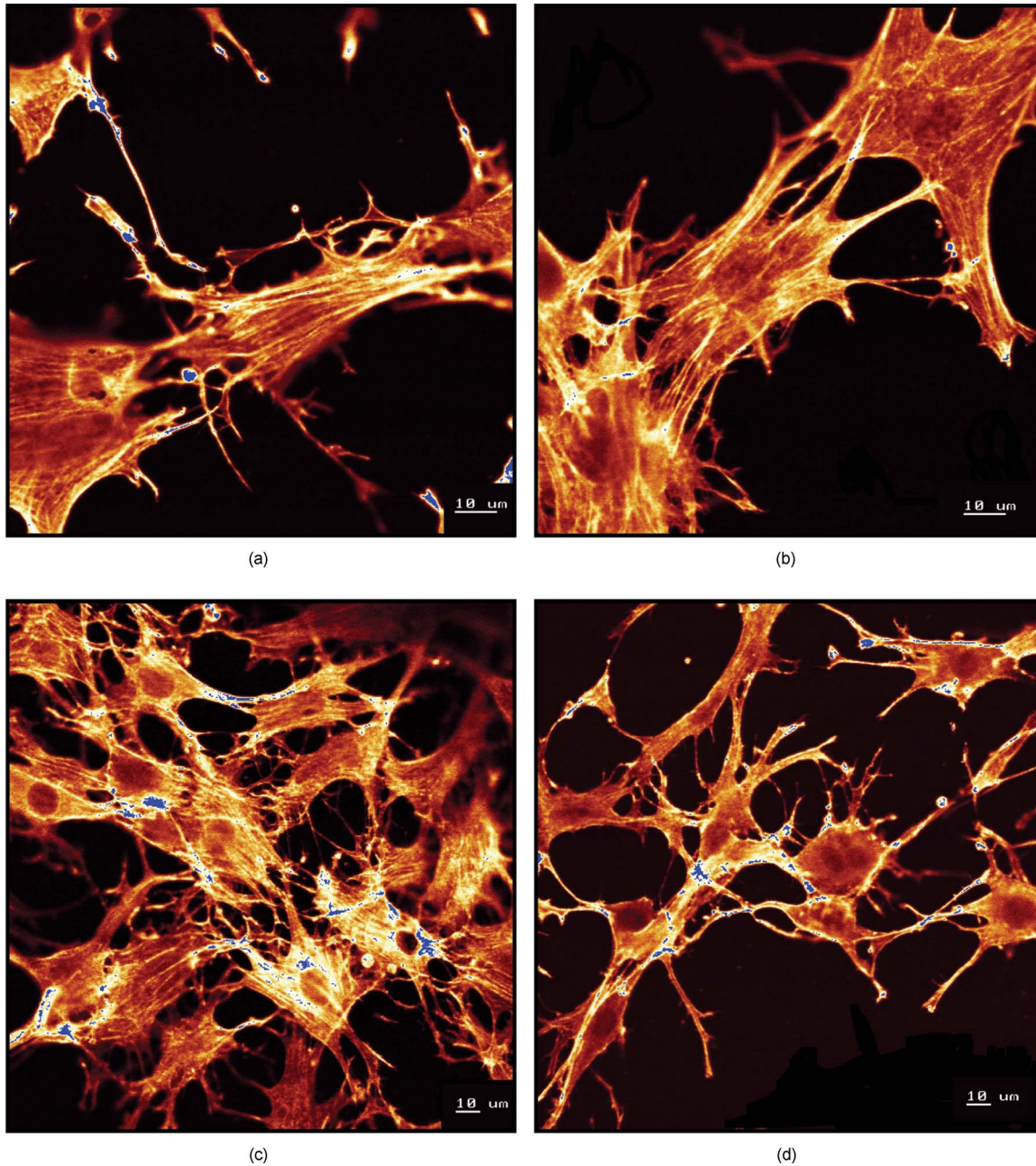


Fig. 5 CLSM images of actin stained with FITC-phalloidin in control and LLLT-treated FPCL. (a) and (c) are control untreated FPCL; (b) and (d) were treated with 2 and 3 J/cm², respectively.

was bell-shaped with a threshold, maximum, and phase of decline. Recent studies of the enhancement of porcine skin graft adherence using a light-activated process,²⁸ the effect of pulsed radiation on HeLa cell attachment to extracellular matrix,²⁹ and the effect of a He-Ne laser on the cloning efficiency of Chinese hamster ovary cells (CHO) and human skin fibroblasts (HSF)³⁰ further endorse a nonlinear fluence-response relationship.

The reduction in contraction in FPCL produced by laser irradiation at 3 J/cm² is statistically significant but is too small to be of great practical significance. Laser irradiation does, however, represent a simple applicable method of modifying contraction; it may be possible to further reduce con-

traction of FPCL by optimizing the procedure. Unfortunately, the mechanisms of the laser effect and contraction are not well understood. Nevertheless, both mechanisms must involve the fibroblast cell, the collagen substrate, or both.

Investigations of the influence of low-level irradiation of 3T3 mouse fibroblasts cultured on collagen-GAG substrates previously showed that postattachment cells showed no difference in proliferation rates, evaluated by 5-carboxyfluorescein diacetate, nor in cell viability, assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay.⁸ CLSM studies using CFDA and EB showed that LLLT treatment did not alter the fibroblasts, viability, morphology, or ability to align in culture.

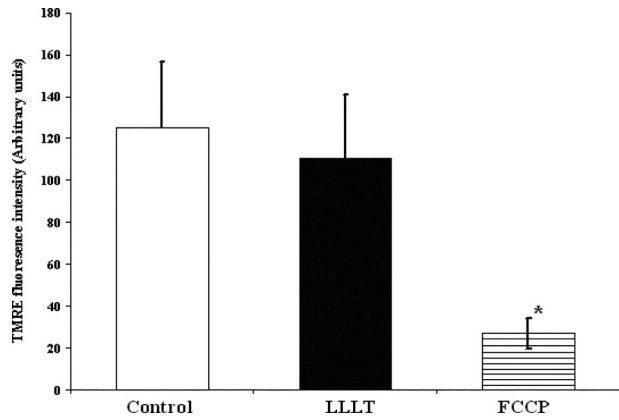


Fig. 6 TMRE fluorescence intensity of control and LLLT-treated (3 J/cm^2) 3T3 fibroblasts cultured on Petri dishes. Results are means \pm SD, $n=3$ experiments. * $p < 0.05$, comparing FCCP-treated cells with controls, by two-tailed Student's t -test.

There was some indication of an alteration in the distribution of actin with the cells after treatment with 3 J/cm^2 , and this may be related to their ability to contract in FPCL. The contractile activity of chondrocytes seeded in collagen-GAG matrices has been shown previously to parallel expression of smooth muscle actin,^{31,32} and treatment that depleted actin fibers reduced the ability of the cells to contract. Accumulation of actin around the perimeter of a cell has been associated with early apoptosis.³³ However, there was no evidence of an alteration in mitochondrial membrane potential after treatment with 3 J/cm^2 . Loss of the mitochondrial membrane potential is associated with the early stages of apoptosis and coincides with the opening of the mitochondrial permeability transition pores, allowing passage of ions and small molecules, resulting ultimately in release of cytochrome c into the cytosol.³⁴ There is no evidence from the data shown that the redistribution of

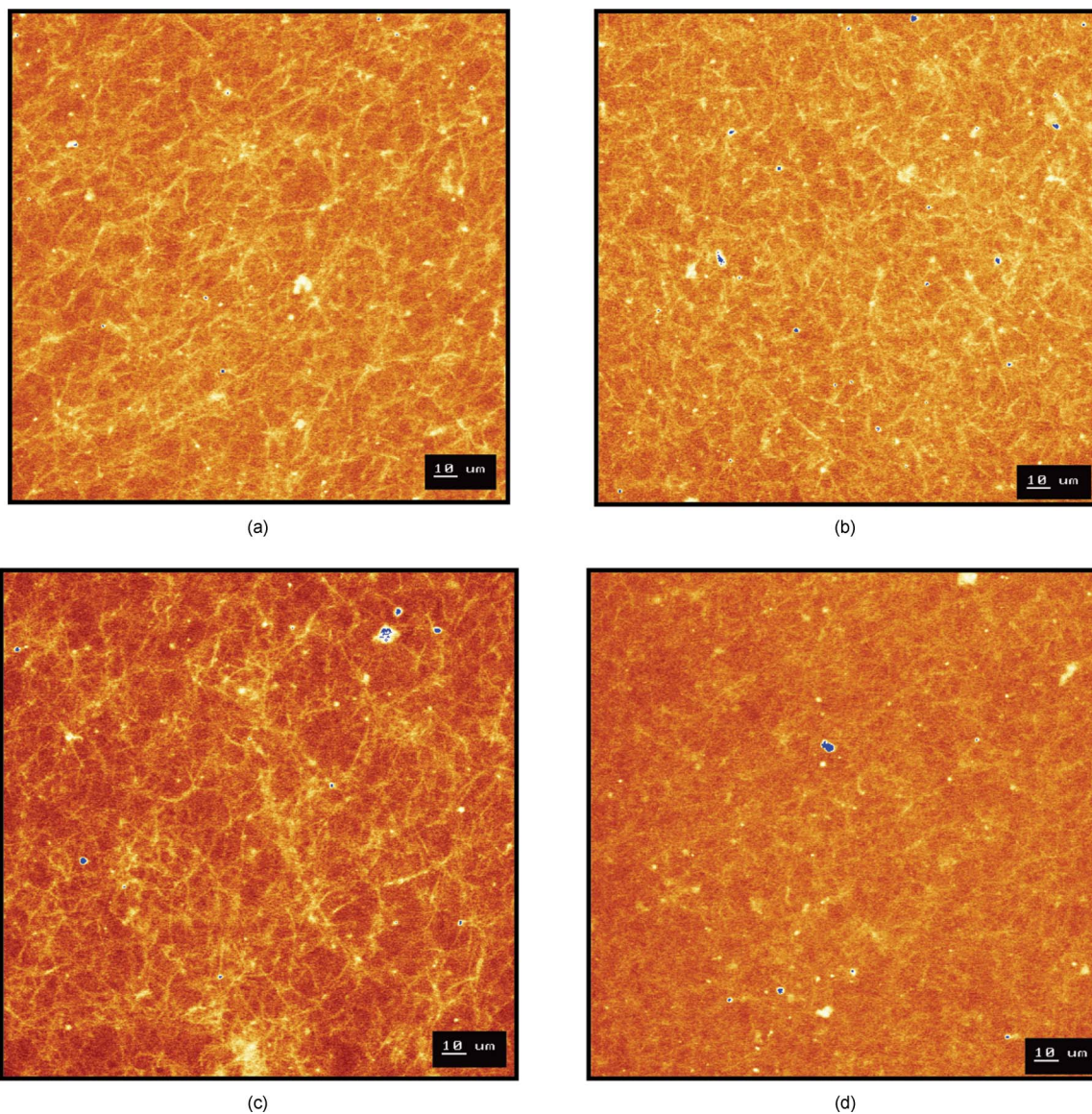


Fig. 7 Distribution of collagen fibers at a depth of $20 \mu\text{m}$ in control [(a) and (c)] and LLLT-treated [(b) is 2 J/cm^2 and (d) is 3 J/cm^2] collagen-GAG lattices.

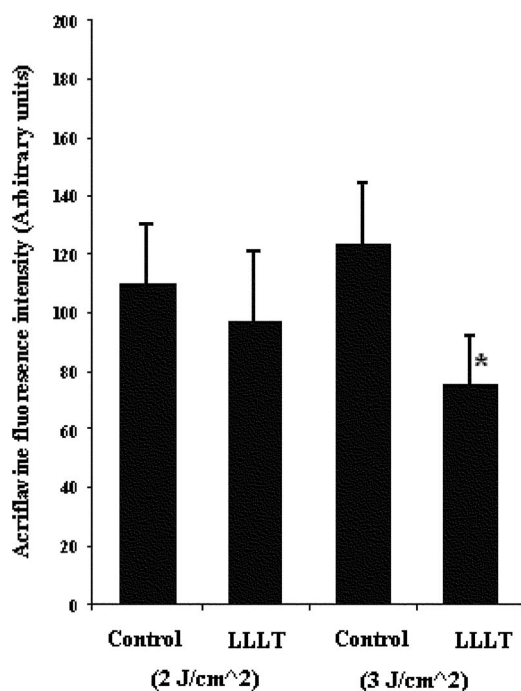


Fig. 8 Acriflavine fluorescent intensity of control and LLLT-treated collagen—GAG lattices at a depth of 20 μm using laser fluences of 2 and 3 J/cm^2 . Results are mean \pm SD, $n=3$. * $p<0.05$, comparing differences between control and LLLT-treated lattices using two-tailed Student's t -test.

actin inside cells treated with 3 J/cm^2 is accompanied by apoptotic events.

Acriflavine staining showed a decrease in the number of collagen fibers in the cell-free collagen-GAG matrices that had been treated with 3 J/cm^2 . When fibroblasts contact with fibrils of collagen in the matrix, they attach to them, and compact them by drawing the fibrils toward themselves, thereby causing contraction of the matrix. With fewer fibrils to attach to, the cell-mediated contraction of the irradiated matrices is decreased. There may, however, be an unwanted side effect. The stiffness and strength of collagen lattices are correlated with fibril density,³⁵ and fewer fibers may compromise the mechanical properties of FPCL.

The contraction of the unseeded collagen substrate was significantly increased by laser irradiation at 3 J/cm^2 . The mechanism responsible for this increase in the absence of cells is unclear. There may be physical cross-linking or laser-induced photobiostimulatory effects on the collagen fibers causing an increase in the volume of water expressed from the lattice, resulting in increased contraction.

It appears that the altered contraction of the FPCL caused by LLLT may be due to effects of the laser on both the collagen fibers of the matrix and the actin fibers inside the cell. Further study of the mechanical effects of laser irradiation of cell-free matrix may provide an explanation of the effect of irradiation and a route to the reduction of the contraction of FPCL.

5 Conclusions

Laser irradiation at an energy density of 3 J/cm^2 altered the contraction of FPCL over a subsequent period of 7 days. The

contraction of FPCL was significantly less than control unirradiated specimens on the last three days of monitoring. Energy density was a significant factor—1, 2, and 4 J/cm^2 produced no alteration in the magnitude of contraction. The effect of irradiation was accompanied by altered distribution of actin fibers intracellularly and collagen fibers in the matrix. This resulted in altered interaction between the two proteins, which contributed to the decrease in contraction.

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