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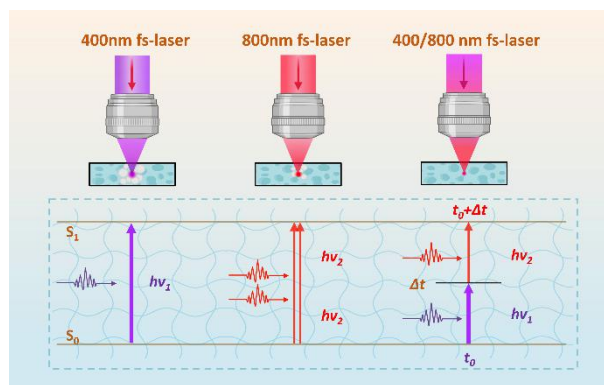
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A dual-wavelength dual-pulse femtosecond laser strategy decouples the trade-off between high stiffness and low thermal damage, enabling 20-layer bubble-free hydrogel scaffolds with cross-scale architecture and enhanced cellular responses.

Fabrication of cross-scale biomimetic 3D hydrogel scaffolds with thermo-mechanical optimized properties via femtosecond laser dual-wavelength dual-pulse regulation

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ABSTRACT: The mechanical properties and structural integrity of tissue engineering scaffolds demand high precision to replicate native extracellular matrix (ECM) cross-scale architecture. Femtosecond laser two-photon polymerization enables precise control of hydrogel scaffolds. Still, single-wavelength processing cannot simultaneously achieve high stiffness, low thermal damage, or cross-scale structures. This study proposes a dual-wavelength dual-pulse spatiotemporal synergy strategy. The results indicate that dual-wavelength processing reduces the polymerization threshold to 0.2 mW, which is 1/30 of 800 nm processing. More importantly, this strategy breaks through the single-wavelength fabrication limit, which only allows 3 layers at 400 nm or 10 layers at 800 nm, and achieves a homogeneous 20-layer structure. The scaffold exhibits an average compressive modulus of 148 kPa, higher than 27.7 kPa from single 800 nm and 88 kPa from single 400 nm. Concurrently, the scaffold surface exhibits uniform approximately 40 nm nanoscale topography, forming cross-scale architectures spanning nanometers to millimeters. Cellular experiments show that these high-modulus scaffolds with nanotopography induce the strongest actin fluorescence in L929 cells, demonstrating that the dual-wavelength scaffolds provide a more favourable microenvironment for cell adhesion and cytoskeletal organisation. This study achieved the synergistic fabrication of mechanically reinforced cross-scale hydrogel cell scaffolds with minimal thermal damage, offering promising applications in tissue engineering.

KEYWORDS: femtosecond laser dual-wavelength dual-pulse regulation; 3D hydrogel cellular scaffold; hierarchical nanostructure; thermal-mechanical synergistic optimization; tissue engineering

1. Introduction

The rapid development of tissue engineering and regenerative medicine has created an increasing demand for 3D biomimetic scaffolds that can accurately replicate the complexity of the native ECM [1-4]. An ideal scaffold requires not only possessing micro- and nanoscale structures [5, 6] and excellent biocompatibility [7-9] but also suitable mechanical properties, which serve as key physical signals that directly regulate cellular behavior [10-12]. Research has shown that matrix stiffness, as a core parameter of the mechanical microenvironment, influences cytoskeleton assembly and gene expression through the integrin-focal adhesion signaling pathway. Among these, actin is a core component of the cytoskeleton, whose polymerization

level directly reflects cellular perception and response to substrate mechanical signals [13-16]. On the other hand, native ECM is not a simple uniform structure but a multi-level cross-scale structure ranging from nanoscale collagen fibers and microscale pores to millimeter-scale tissue dimensions; such topographical features also play important regulatory roles in cell adhesion, spreading, and cytoskeletal organization. Therefore, developing advanced manufacturing technologies that can simultaneously achieve high-resolution 3D structuring, precise regulation of mechanical properties, and cross-scale structural biomimetics has become a core challenge in this field.

Femtosecond laser direct writing technology, with its nonlinear processing characteristics based on multiphoton absorption, provides a powerful tool for mask-free, true 3D micro- and nanofabrication within photosensitive hydrogel materials [17-21]. However, the current mainstream paradigm of this technology relies on single-wavelength excitation, which faces a series of inherent physical limitations when fabricating defect-free, complex multilayer scaffold structures [22-24]. Specifically, when using short-wavelength processing dominated by single-photon absorption, although its high linear absorption efficiency can lower the polymerization threshold, the energy deposition occurs too rapidly in the focal region and exhibits significant attenuation along the optical path. This makes it highly prone to localized heat accumulation and excessive polymerization, resulting in shallow processing depths. Furthermore, when attempting to print multi-layer structures, the repeatedly irradiated, already-crosslinked areas cannot dissipate heat, ultimately inducing hydrogel decomposition and the formation of microbubbles, which severely compromise structural integrity. Conversely, when employing long-wavelength processing based on two-photon absorption, while the nonlinear absorption can more tightly confine energy deposition to the focal center, effectively reducing the thermally affected zone, its lower two-photon absorption cross-section requires higher input power to achieve effective polymerization. Additionally, the volume and strength of a single crosslinking event are limited, making it difficult to construct deep-layer structures with sufficient mechanical strength [25-27]. Furthermore, single-wavelength processing modes cannot in situ form the nanoscale surface topography characteristic of the native ECM on the scaffold surface, making it difficult to achieve cross-scale structures spanning from nanometers to millimeters. Therefore, how to synergistically achieve efficient excitation, low heat accumulation, and cross-scale structural biomimetics has become the key scientific issue to overcome the application bottleneck of single-wavelength femtosecond laser technology in fabricating high-performance multi-layer cell scaffolds.

In recent years, researchers have made significant progress in extending the capabilities of femtosecond laser processing through temporal and spatial pulse shaping techniques. In terms of temporal shaping, dividing single-pulse energy into multiple sub-pulse sequences, enables effective control over energy deposition efficiency and thermal accumulation effects and has been applied to improve processing quality and efficiency [28, 29]. In terms of spatial shaping, Bessel beams can extend the focal depth and significantly enhance the achievable aspect ratio of processing [30]. In addition, machine learning-based process optimization methods have been used to establish rapid mapping relationships between laser parameters and processing quality, substantially shortening the process development cycle [31-33].

2. Experimental

2.1 Experimental Materials

Method for preparing the hydrogel precursor solution used in this study: Add 1 g of N-isopropylacrylamide (NIPAM, 97%) to 4 mL of poly (ethylene glycol) acrylate (PEGDA, Mn 700) and stir thoroughly until completely dissolved. Then, take 1 mL of the resulting mixture, add 2.5 mg of methylene blue (MB, 82%) and 50 μ L of ethylene glycol (EG, 99%), and stir under a magnetic stirrer for 12 hours to ensure uniform mixing of all components.

Meanwhile, the synergistic interplay between surface plasmon polaritons and material anisotropy has been revealed as a means to achieve directional modulation of nanostructures [34]. Although the above studies have greatly expanded the repertoire of femtosecond laser processing techniques, effective solutions are still lacking for simultaneously achieving high mechanical properties and cross-scale biomimetic structures with minimal thermal damage during the fabrication of hydrogel cell scaffolds.

Inspired by the aforementioned temporal shaping strategies, this study introduces a dual-wavelength dual-pulse processing strategy into the hydrogel system. By precisely controlling the time delay between two femtosecond laser pulses of different wavelengths, offers the potential for the spatiotemporal decoupling of energy deposition efficiency and the thermal relaxation process. Theoretically, when a short-wavelength pulse first excites the photoinitiator to an intermediate state, followed by a long-wavelength pulse after a specific time delay to further advance it to the reactive state, it is expected to increase the radical yield while reducing the instantaneous energy density, thereby achieving more efficient and gentle energy deposition [35-38]. However, the successful implementation of this strategy relies heavily on the optimal selection of the delay time parameter. Currently, there remains a lack of systematic mechanistic explanations and experimental validation regarding how the delay time affects the chemical homogeneity, macroscopic mechanical properties, and cellular response of the photopolymerization network.

Thus, this study systematically elucidated the mechanism governing the delay time in dual-wavelength, dual-pulse femtosecond laser processing and applied it to the fabrication of high-performance hydrogel cell scaffolds. The study found that a 50 ps delay time represents the optimal parameter window. Under these conditions, the dual pulses produce a significant synergistic effect, enabling the 3D printing of a 20-layer bubble-free structure with a total energy of only 0.6 mW, significantly surpassing the processing limits of single-wavelength femtosecond lasers. At the same time, due to the nonlinear response of the material induced by the spatiotemporal coupling of the dual pulses, a uniform nanoscale topological structure with a characteristic size of approximately 40 nm formed in situ on the scaffold surface, successfully creating a cross-scale structure ranging from the nanometer to the millimeter scale. The scaffolds prepared under these conditions exhibited optimal Young's modulus. Cell experiments revealed that L929 cells cultured on dual-wavelength scaffolds exhibited the most developed actin cytoskeleton and the strongest fluorescence intensity, confirming that this dual-wavelength cross-scale structure provides a more suitable microenvironment for cell adhesion and spreading. This work provides a theoretical basis and technical support for the precise fabrication of high-performance cellular scaffolds.

After preparation, the precursor solution was stored under yellow light to avoid unnecessary light exposure. PEGDA and MB were purchased from Sigma-Aldrich; NIPAM was purchased from TCI Co., Ltd.; and EG was supplied by Alfa Aesar.

The L929 cells used in the cell experiments were purchased from Cellverse Co., Ltd.; the cell culture medium was L929-specific medium; and the CCK-8 assay kit was purchased from SonGon. Major equipment included a CO₂-controlled incubator, a biosafety cabinet, and a refrigerated high-speed centrifuge.

2.2 Femtosecond Laser Processing System

The femtosecond laser used in the experiment was a Ti:sapphire femtosecond laser (Spectra-Physics, USA), consisting of an oscillator and an amplifier. The laser beam was a Gaussian beam with a central wavelength of 800 nm, a pulse width of 130 fs, a repetition rate adjustable in the range of 1–1000 Hz, and a maximum output power of 3 W. In the processing optical path, an objective lens (Olympus LMPLFLN 20X) with a numerical aperture of 0.4 was used for focusing, and the processing process was monitored in real time via a CCD camera. The displacement control system employed a six-degree-of-freedom parallel Stewart platform (Physik Instrumente, Germany) capable of achieving nanometer-level precision positioning in the six dimensions of X/Y/Z translation and $\theta_x/\theta_y/\theta_z$ rotation.

2.3 Single-Wavelength Femtosecond Laser Processing

In the single-wavelength processing experiments, a continuous-wave femtosecond laser was used for the additive manufacturing of PEGDA/NIPAM composite hydrogels. The scanning speed was fixed at 500 $\mu\text{m/s}$, and the layer thickness was set to 20 μm . The 400 nm processing was achieved by converting the 800 nm fundamental light to 400 nm using a BBO frequency-doubling crystal (1 mm thick, Fujian Hanguang Optoelectronics Co., Ltd.), while single 800 nm processing utilized the fundamental light directly. The polymerization thresholds were determined for both wavelengths. The polymerization thresholds were determined for both wavelengths. The polymerization threshold was defined as the minimum average laser power required to initiate observable polymerization at a fixed scanning speed of 500 $\mu\text{m/s}$. After optimizing the parameters near their respective thresholds, multi-layer stacking experiments were conducted. The structural integrity and bubble formation at different layer counts were observed using an optical microscope (Olympus BX53).

2.4 Dual-Wavelength Dual-Pulse Processing

The dual-wavelength dual-pulse optical path uses a beam splitter to divide the emitted laser beam into two beams. One beam is processed by a BBO and a short-pass filter (Thorlabs, FESH0600) to frequency-double the femtosecond laser with a central wavelength of 800 nm and filter out other wavelengths, resulting in a femtosecond laser with a central wavelength of 400 nm. The relative delay time between the two pulses is adjusted using a one-dimensional precision delay control platform (Zolix, PSA100-11-X). The successful tuning of the dual pulses is indicated by the simultaneous detection of both 400 nm and 800 nm wavelength signals using a spectrometer, with the corresponding spectrum shown in **Fig. S1**, the spectrum confirms the coexistence of both wavelength components after the dual-pulse alignment. During the fabrication process, the energy ratio between the two laser beams and the delay time were adjusted to systematically investigate their effects on scaffold formation quality and to determine the optimal processing parameter combination. The polymerization threshold under dual-wavelength conditions was determined using the same criterion as described in Section 2.3.

2.5 Evaluation of the Cytocompatibility of Extracts

2.5.1 Preparation of Extracts

Hydrogel scaffolds fabricated by dual-wavelength dual-pulse laser processing were immersed in basal DMEM medium and incubated in a shaking incubator at 37 °C for 48 h. Subsequently, the extracts were collected by centrifugation, and

the supernatants were filtered through a 0.22 μm filter. The filtered extracts were then supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum, and 89% post-extraction DMEM to prepare the extracts for experimental use.

2.5.2 Morphological Observation of Cells Exposed to Extracts

L929 cells were seeded at a density of 5,000 cells per well in a 96-well plate and cultured overnight. The medium was then replaced with the extract-containing medium described above. After 3 days of incubation, the medium was aspirated, and the cells were washed with PBS. The cells were stained with DAPI and phalloidin to visualize the nuclei and actin cytoskeleton, respectively, and observed under a fluorescence microscope. Cells cultured in standard complete medium served as the control group.

2.5.3 Cell Proliferation Assay

L929 cells were seeded at a density of 5,000 cells per well in a 96-well plate and cultured overnight. The medium was then replaced with the extract-containing medium described above. After 1, 3, and 5 days of culture, CCK-8 reagent was added to each well, and the plates were incubated at 37°C for 1.5 hours. Absorbance was measured at 450 nm. Cells cultured in normal medium served as the control group, and blank medium served as the blank group. Cell proliferation curves over the 5 days were subsequently plotted.

2.6 Cell Seeding on Hydrogel Scaffolds and Cytoskeleton Staining

To evaluate the suitability of hydrogel cell scaffolds fabricated by different processing methods as cell culture substrates, L929 fibroblasts were seeded onto the surfaces of three groups of scaffolds. Those processed with single 800 nm, single 400 nm, and dual-wavelength dual-pulse methods, respectively. The scaffolds were first fabricated on glass substrates via femtosecond laser direct writing and were directly used for cell culture after UV sterilization.

L929 cells in the logarithmic growth phase were seeded at a density of 6×10^3 cells per well onto glass dishes containing the scaffolds and cultured in a 37°C, 5% CO_2 incubator. On day 3 of culture, the culture medium was aspirated, and the cells were gently rinsed 2–3 times with PBS. DAPI and phalloidin staining solutions were added for incubation to label the cell nuclei and cytoskeleton. After incubation, excess staining solution was washed away with PBS, and the morphology and spreading status of the cells surrounding the scaffolds were observed and photographed under a fluorescence microscope.

3. Results and discussion

3.1 Single-Wavelength Femtosecond Laser Fabrication of 3D Cellular Scaffolds

To determine the photopolymerization wavelength suitable for this hydrogel system, ultraviolet-visible absorption spectroscopy was first performed on the material. As shown in **Fig. 1a**, the hydrogel exhibits a distinct absorption peak near 400 nm, attributed to the linear absorption of the photoinitiator in the short-wavelength region. Simultaneously, it also demonstrates strong absorption capability around 800 nm, originating from the two-photon absorption characteristics of the photoinitiator at this wavelength. These results indicate that both 400 nm and 800 nm

can serve as processing wavelengths for this hydrogel system.

At a scanning speed of 500 $\mu\text{m/s}$, the polymerization thresholds for single-wavelength processing at 400 nm and 800 nm were determined (**Fig. 1b**). The results show that the polymerization threshold for 400 nm processing is 0.6 mW. This is because 400 nm photons possess higher energy, and the hydrogel's photoinitiator exhibits high efficiency in linear single-photon absorption at this wavelength; therefore, only a low laser power is required to generate sufficient free radicals to initiate polymerization. In contrast, the polymerization threshold for 800 nm processing is as high as 6 mW, a tenfold difference. This is because 800 nm photons have only half the energy of 400 nm photons, and the linear absorption of the hydrogel material at this wavelength is extremely weak, necessitating reliance on the nonlinear process of two-photon absorption. To trigger two-photon absorption, the laser must be highly focused, and a high peak power is required to increase the probability of two photons being absorbed simultaneously. Consequently, its average power threshold is much higher than that of the linear process at 400 nm.

After optimizing the processing parameters near their respective thresholds, we examined the 3D shaping capabilities of 400 nm and 800 nm single-wavelength processing when applied to multilayer stacks. The experiment employed a layer-by-layer scanning approach to construct a 3D structure, with each layer approximately 20 μm thick. The structural integrity at different layer counts was observed using an optical microscope. It can be observed that for single-wavelength 800 nm processing, the structure remained largely intact when stacked to the 10th layer, though slight morphological distortion began to appear in local regions. By the 15th layer, line edges became blurred, interlayer interfaces were indistinct, and numerous bubbles appeared, indicating a significant decline in shape fidelity. These phenomena suggest that while 800 nm processing can achieve 3D construction to a certain number of layers, the issue of insufficient polymerization efficiency gradually becomes apparent as the processing depth increases, limiting its ability to fabricate high-aspect-ratio structures (**Fig. 1c**). Single-wavelength processing at 400 nm yielded good formation quality in the first three layers, with clear lines and a dense structure. However, when stacked to the fifth layer, microbubbles began to appear in the cross-linked regions, mainly distributed at the intersections of the lines. As the number of layers increased to the 10th layer, the number of bubbles increased significantly, with bubble clusters appearing in some grid areas. By the 12th to 15th layers, bubbles had spread throughout the entire structure, severely compromising the 3D integrity of the scaffold (**Fig. 1d**). An attempt was made to reduce the processing power to mitigate the bubble problem, but it was found that polymerization could not proceed stably at powers below the threshold, while maintaining power near the threshold still resulted in the bubble phenomenon. This indicates that bubble formation is not simply caused by excessively high power but rather is an inherent defect of the 400 nm processing mode. This phenomenon stems from the volumetric heating effect caused by the linear absorption characteristics of 400 nm processing. As the 400 nm laser penetrates the material, all initiators, monomers, and even water molecules along its path continuously absorb photons. A significant portion of the light energy is converted into heat, leading to a sharp rise in local temperature. During the layered

printing process, heat continuously accumulates within the material, causing water to boil and form vapor bubbles or triggering the thermal decomposition of components to produce gas. Once bubbles form, they scatter and block subsequent laser beams, damaging the already formed structure. Furthermore, as the beam penetrates deeper, the path length increases, resulting in greater total heat absorption and more uncontrollable thermal damage, thereby strictly limiting the number of printable layers.

Based on the above experimental results, the main limitations of single-wavelength femtosecond laser processing for hydrogel scaffolds can be summarized. Although 400 nm processing exhibits high linear absorption efficiency, its pronounced volumetric heating effect leads to frequent bubble formation during multi-layer fabrication, severely compromising structural integrity. While 800 nm processing, with its lower thermal effect, enables 3D construction to a certain number of layers, its insufficient two-photon absorption efficiency results in relatively low shape fidelity of the scaffolds. Furthermore, as depth increases, optical aberrations and material diffusion effects cause a significant decline in this fidelity. These results indicate that, under the single-wavelength processing mode, it is difficult to simultaneously achieve high fidelity and low thermal damage, and there is an urgent need to develop new processing strategies to resolve this inherent contradiction.

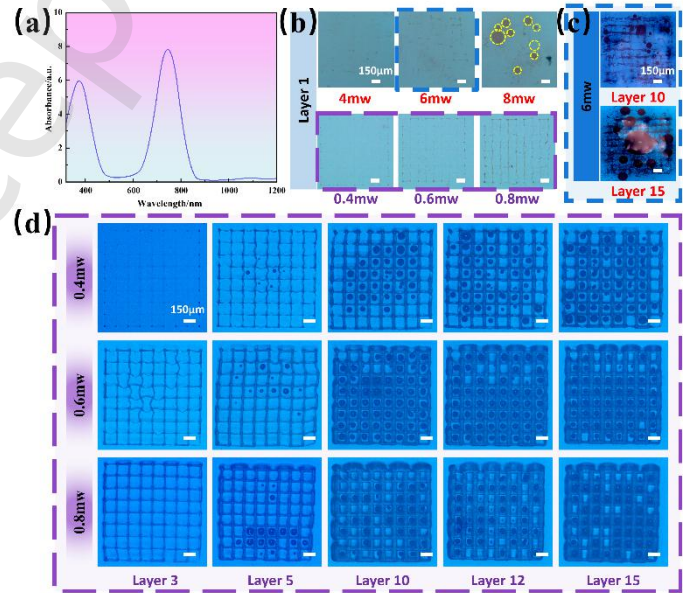


Figure 1 Performance characterization of hydrogel cellular scaffolds fabricated by single-wavelength femtosecond laser processing. (a) UV-Vis absorption spectrum of the hydrogel material (b) Comparison of polymerization thresholds for single-wavelength processing at 400 nm and 800 nm (c) Microscopic morphology of structures fabricated by single-wavelength 800 nm processing at different layers (d) Microscopic morphology of structures fabricated by single-wavelength 400 nm processing at different layers

3.2 Dual-Wavelength Dual-Pulse Synergistic Processing of 3D Cellular Scaffolds and Parameter Optimization

Based on the inherent contradictions identified in single-wavelength processing, this study employs a dual-wavelength dual-pulse temporal-spatial synergy strategy, aiming to achieve the fabrication of 3D multi-layered cellular scaffolds with high fidelity and low thermal damage under lower energy input conditions. The dual-pulse optical path design is shown in **Fig. 2a**, where the relative delay between the two pulses

is adjusted via a precise time-delay control platform. Based on the experimental results from single-wavelength processing, a temporal design is adopted where the 400 nm pulse precedes to efficiently excite the photoinitiator, followed by the 800 nm pulse to complete the crosslinking polymerization to achieve a highly efficient synergistic effect under conditions of low thermal accumulation.

3.2.1 The Effect of Energy Ratio on Processing Results

First, we investigated the effect of the energy ratio between pulses of two different wavelengths on the processing results. The number of processing layers was fixed at 10, and the total energy at 12 mW to ensure stable polymerization under different energy ratios, thereby clearly contrasting differences in fabrication quality. Based on this, the effects on cell scaffold fabrication quality were tested under two energy ratio conditions, 800 nm dominant (**Fig. 2b**) and 400 nm dominant (**Fig. 2c**). To ensure the generalizability of the conclusions across different delay times, each energy ratio group was tested at ten delay time points spanning the femtosecond to picosecond scale, including 0 fs, 100 fs, 500 fs, 1 ps, 5 ps, 10 ps, 50 ps, 100 ps, 200 ps, and 400 ps.

Experimental results indicate that the energy ratio has a dominant influence on the morphological integrity of the 3D structure, and this influence demonstrates good consistency across different delay times. When the processing was dominated by 800 nm wavelength laser energy, the fabricated cellular scaffold structures exhibited significant thermally induced bubbles at all tested delay time points, resulting in low shape fidelity, which is similar to the performance of single 800 nm processing at greater depths. This is because this ratio overly relies on the two-photon absorption process of 800 nm, whose excitation efficiency is much lower than the linear excitation of 400 nm. The energy that is not utilized effectively is released as heat dissipation, aggravating thermal accumulation in the focal region and leading to the common appearance of bubbles across different delay times. In contrast, under the condition dominated by 400 nm wavelength laser energy, the cellular scaffold structures showed no significant bubbles at any delay time, and the overall morphology was markedly superior to that of the 800 nm-dominant group. This advantage stems from the optimized energy allocation within the dual-pulse synergy mechanism. When the 400 nm energy proportion is higher, the 400 nm-dominant energy configuration prioritizes the limited energy for excitation, ensuring a sufficient concentration of excited-state photoinitiators, thereby enhancing the crosslinking efficiency of the subsequent 800 nm pulse.

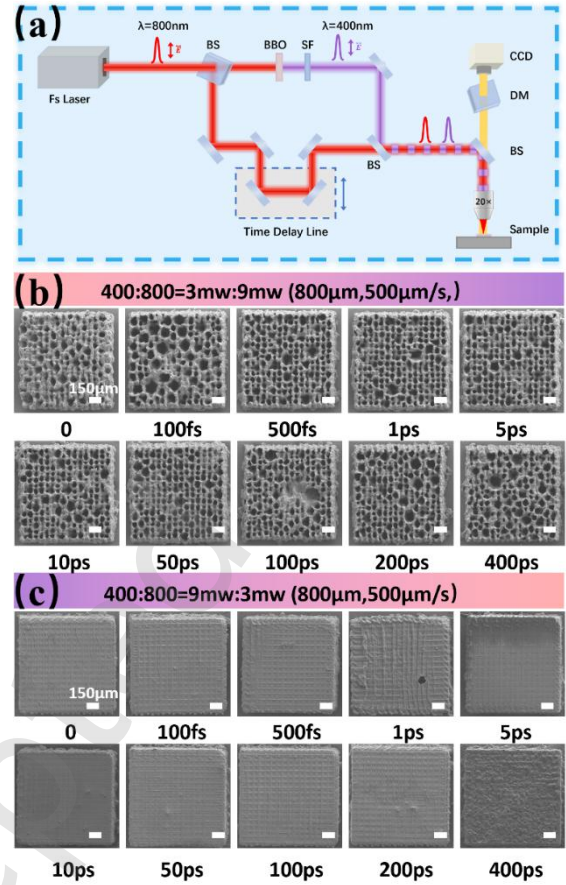


Figure 2 Effect of energy ratio on the quality of dual-wavelength synergistic processing. (a) Schematic diagram of the dual-wavelength dual-pulse processing optical path (b) Morphology of cell scaffolds at different delay times under 800 nm energy-dominated conditions (c) Morphology of cell scaffolds at different delay times under 400 nm energy-dominated conditions

3.2.2 Optimization of Delay Time and Analysis of Synergistic Mechanisms

Based on the results of the energy ratio optimization, a 400 nm-dominant energy ratio was adopted to further investigate the effect of delay time on processing quality. With the total energy fixed at 2 mW and the number of processing layers set to 10, the formation quality of the cell scaffolds was tested at ten delay time points ranging from 0 fs to 400 ps. As shown in **Fig. 3a**, the number of bubbles exhibits a significant non-monotonic dependence on the delay time, first decreasing and then increasing. In the short-delay range (0 fs to 10 ps), a large number of bubbles appeared within the scaffold structure, with bubbles nearly covering the entire grid under the 100 fs and 500 fs conditions. As the delay time increased to the range of 10 ps to 100 ps, the number of bubbles gradually decreased, reaching the minimum around 50 ps and 100 ps, where the scaffold morphology was most complete and clear. When the delay time was extended to 200 ps and 400 ps, the bubble count increased dramatically again, and the structural integrity deteriorated significantly.

To explain the above phenomenon at the molecular level, Fourier transform infrared spectroscopy (FTIR) analysis was performed on scaffolds prepared at ten different delay times (**Fig. 3b**). After normalization, the relative intensities of the characteristic peaks associated with the polymerization conversion rate were both at relatively low levels under the 50 ps and 100 ps delay conditions, indicating that both delay windows

can achieve a high degree of crosslinking and chemical uniformity. This is consistent with the results from morphological observations, which showed good formation quality near 50 ps and 100 ps.

Based on the above results, both the 50 ps and 100 ps delay windows demonstrated good fabrication quality during 10-layer processing. To further distinguish the relative strengths of their synergistic effects, the number of processing layers was increased to 15 while reducing the total energy, to amplify the differences in thermal accumulation effects through greater processing depth, thereby enabling a more sensitive evaluation of the 3D fabrication capabilities of the different delay windows (**Fig. 3c**). The experimental results show that when the total energy was 1 mW, the scaffold fabricated with a 50 ps delay largely maintained a good morphology with clear interlayer bonding, whereas the scaffold processed with a 100 ps delay exhibited severe thermal effects, with the top region showing obvious carbonization and blackening, resulting in compromised structural integrity. When the total energy was further reduced to 0.8 mW, thermal damage to the top of the scaffold remained evident under the 100 ps delay, while the 50 ps delay continued to maintain stable fabrication quality. At an even lower energy of 0.6 mW, the 50 ps delay still preserved good structural integrity, while the 100 ps delay continued to show evident thermal damage. This is consistent with the trends observed at 1.0 mW and 0.8 mW, confirming that the superior performance of the 50 ps delay is sustained across a broad range of energy levels. Based on the above results, 50 ps is determined as the optimal delay time window.

At a 50 ps delay, the processing power was further optimized. As shown in **Fig. 3d**, the material polymerization threshold under this parameter can be as low as 0.2 mW, which is 30 times lower than the 6 mW required for single 800 nm processing. This fully demonstrates the significant enhancement in energy utilization efficiency achieved by the dual-pulse synergistic effect. When the power is increased to 0.6 mW, the optimal cellular scaffold morphology is achieved, characterized by clear lines and a dense, uniform structure, indicating that the density of excited states and the cross-linking efficiency reach an ideal balance under these power conditions.

To further verify the kinetic origin of the 50 ps optimal delay observed above, we performed femtosecond transient absorption spectroscopy on the hydrogel precursor (**Fig. S2**). The results revealed that the most negative signals consistently appeared in the 650–700 nm range across all delay times, matching the characteristic absorption wavelength of the photoinitiator methylene blue in the hydrogel [39]. This confirms that the observed signals indeed originate from the excited-state dynamics of the photoinitiator. Tri-exponential fitting ($R^2 = 0.9917$) resolved three time constants: $\tau_1 = 12.98$ ps, $\tau_2 = 50.33$ ps, and $\tau_3 = 418.39$ ps. Notably, $\tau_2 = 50.33$ ps agrees with the 50 ps optimal delay from our fabrication experiments. We assign this component to the conformational relaxation of the excited-state intermediate, indicating that the photoinitiator reaches its most stable excited-state configuration at this time. This provides kinetic evidence that 50 ps corresponds to a favorable window for the subsequent 800 nm energy transfer step in the dual-pulse synergistic mechanism. Based on the above experimental results, subsequent studies employed a dual-wavelength synergistic processing approach dominated by 400 nm laser energy and further systematically optimized the energy and delay time

parameters to achieve optimal cell scaffold formation quality and mechanical properties.

After determining the optimal power and energy ratio, the boundary conditions of this ratio were further validated. The scaffold morphologies obtained under different energy ratios are presented in **Fig. S3**. The study found that, while maintaining a constant total power of 0.6 mW to fabricate 15-layer cellular scaffolds, increasing the 400 nm to 800 nm energy ratio from 3:1 to 5:1 resulted in bending of the scaffold lines and a significant decrease in shape fidelity. This demonstrates that the dual-wavelength synergistic process has an optimal energy ratio range. A higher proportion of 400 nm energy is not necessarily better, and energy allocation that is either too high or too low is detrimental to the stable construction of multi-layer structures. Based on the optimized parameters described above (total power of 0.6 mW, 50 ps delay, and 400 nm to 800 nm energy ratio of 3:1), multi-layer stacking experiments were conducted. The results, shown in **Fig. 3e**, demonstrate that the scaffold could be stably stacked up to 20 layers, reaching a total height of approximately 400 μm , which maintained good shape fidelity with clear lines and tight interlayer bonding, exhibiting no significant thermal effects or bubble formation. High-resolution SEM observation of the scaffold surface prepared under optimal parameters is shown in **Fig. 3f**. The results reveal a dense and uniformly distributed nanoscale topological structure on the scaffold surface, with a characteristic size of approximately 40 nm. We think that this nanostructure arises from an ordered competition between the material's nonlinear response and surface instability induced by the spatiotemporally coupled dual pulses, thereby inducing a regular nanoscale topography on the polymer surface.

To eliminate random variations in local observations made with a SEM and to clarify the origin of this nanostructure, we further performed atomic force microscopy (AFM) to analyze the cross-sectional profiles of the scaffold surfaces under different processing conditions at the submicron scale (**Fig. S4**). The results show that under the single 400-nm processing condition, the cross-sectional profile exhibits sharp, isolated spikes, which are attributed to random defects caused by bubble rupture triggered by volume heating effects dominated by linear absorption; under the single 800-nm processing condition, the profile exhibits a nearly horizontal, flat baseline, resulting from surface colloidal relaxation caused by low crosslinking density. In contrast, the cross-sectional profile curves of the scaffold surface at three different locations under dual-wavelength, dual-pulse irradiation almost completely overlap, exhibiting highly regular, equidistant sinusoidal undulations with a peak-to-peak spacing of tens of nanometers, which is essentially consistent with the aforementioned experimental results. This ordered structure appears only under dual-pulse conditions and is absent in both single-wavelength processing methods, indicating that its emergence depends on the introduction of dual pulses. This confirms that the nanostructure originates from the ordered competition between the nonlinear response driven by dual pulses and surface instability.

This result breaks through the limitations of 3D fabrication using single-wavelength processing. Compared to single-wavelength 400 nm processing, which can stack only 3 layers before bubbles appear, and single-wavelength 800 nm processing, which suffers from severe morphological degradation

after stacking 10 layers, the dual-wavelength synergistic processing could be stably stacked up to 20 layers, double the stable stacking limit of 800 nm processing and more than six times that of 400 nm processing, with structural integrity superior to that achieved by single-wavelength processing. Meanwhile, the approximately 40 nm nanoscale topological structure formed in situ on the scaffold surface, together with the microscale linewidth and millimeter dimensions, collectively constructs a cross-scale structure, in which the nanoscale topological structure provides cells with additional local adhesion sites and contact guidance signals. This breakthrough demonstrates that by optimizing the energy deposition path through spatiotemporal coordination of dual pulses, it is possible to fabricate complex cross-scale 3D cell scaffolds with high aspect ratios and minimal thermal damage at extremely low power inputs, providing an ideal microenvironment for subsequent cell culture.

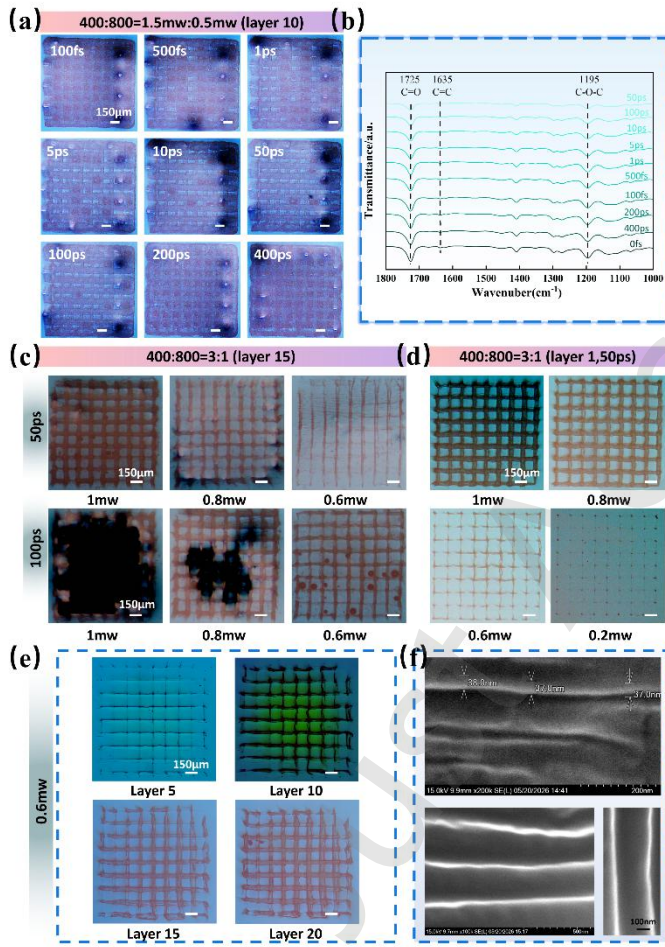


Figure 3 Optimization of dual-wavelength dual-pulse delay times and characterization of processing performance (a) Cellular scaffold morphology at different delay times (b) Normalized FTIR spectra of the scaffold at different delay times (c) Morphology comparison of 15-layer cell scaffolds prepared at 50 ps and 100 ps delay times (d) Optimization of processing power and determination of polymerization threshold at a 50 ps delay (e) Height measurement results of cell scaffolds under optimal parameters (f) Nanotopological structures (total power of 0.6 mW, 50 ps delay, 400 nm to 800 nm energy ratio of 3:1)

3.3 Mechanism Explanation

To reveal the internal mechanisms of dual-wavelength dual-pulse synergistic processing, we used MATLAB simulations to analyze the focal intensity distribution across different processing modes. The 3D intensity distribution in **Fig. 4a** and

the contour distribution in **Fig. 4b** illustrate the characteristics of energy distribution in space from different perspectives. The single 400 nm laser exhibits a sharp, steep 3D peak shape, with contours that are extremely dense in the central region and converge inward, indicating that the energy is highly confined within an extremely small volume at the very center of the focal region. This extremely concentrated energy distribution enables polymerization to be driven at a low power of 0.6 mW, but it also results in excessively high local energy density and rapid heat accumulation, which is consistent with the observation of bubble formation during processing at the third layer in the experiment. In contrast, the 3D distribution of the 800 nm laser is excessively broad, flat, and gentle, with energy dispersing and expanding in space. Its contour lines are sparse and spread outward, reflecting a severe deficiency in energy density. This characteristic results in its high polymerization threshold of 6 mW. Simultaneously, the excessive energy dispersion causes blurred polymerization reaction boundaries and makes it difficult to precisely control the crosslinked region, corresponding to the unclear structural edges and unstable morphology observed in experiments. The distribution of the dual-wavelength laser exhibits a uniquely optimized state, where the interference effect modulates its 3D morphology, ensuring it is neither too sharp nor too flat. The contour lines display moderate spacing and radius, indicating a more rational spatial redistribution of energy. This maintains a sufficient gradient in the central region to drive polymerization while avoiding extreme energy concentration. This optimized distribution pattern allows the dual-wavelength system, at a total power of 0.6 mW, to precisely control the energy within a balanced range that achieves clear and robust polymerization while effectively suppressing thermal damage. This balanced energy deposition, arising from the spatiotemporal coupling of the two pulses, induces a controlled material nonlinear response and surface instability, thereby leading to the ordered nanoscale topography with a characteristic size of approximately 40 nm on the scaffold surface.

The difference in the spatial energy distribution characteristics mentioned above originates from the distinct photophysical mechanisms of the three processing modes. As shown in **Fig. 4c**, single 400 nm processing is dominated by single-photon absorption. The photoinitiator absorbs energy along the entire beam path, resulting in high linear absorption efficiency but severe heat accumulation, which corresponds to the sharply concentrated energy distribution in the simulation and the bubble phenomenon in experiments. Single 800 nm processing relies on two-photon absorption, where energy deposition is strictly confined to the focal center. It has low thermal effects but insufficient absorption efficiency, corresponding to the gentle, dispersed energy distribution in the simulation and the high threshold and morphological degradation in experiments. The dual-wavelength dual-pulse strategy employs a temporal sequence where the 400 nm pulse excites first, followed by the 800 nm pulse for crosslinking. The leading pulse efficiently elevates the photoinitiator to an excited state using single-photon absorption, while the trailing pulse completes the crosslinking progression via two-photon absorption. The synergistic action of these two mechanisms achieves an optimized allocation of energy in both time and space. Consequently, stable 20-layer processing with low thermal damage and high fidelity was obtained experimentally. FTIR spectroscopy analysis of the cured

structures prepared by the three processing modes further confirmed this. FTIR spectra of the cured structures prepared under the three processing modes are presented in **Fig. S5**. The results indicate that the dual-wavelength sample spectrum exhibited the smoothest baseline, indicating a more complete crosslinking reaction and higher chemical homogeneity of the polymer network. This verifies, at the molecular level, that the dual-pulse synergistic mechanism, through optimized energy allocation, enables a more thorough and uniform crosslinking reaction.

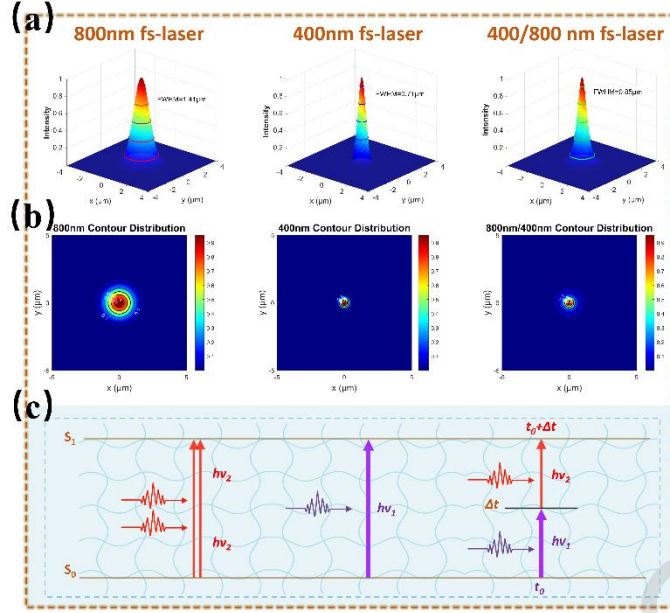


Figure 4 Mechanism of dual-wavelength dual-pulse synergistic processing (a) Simulation of 3D intensity distributions for different processing modes (b) Simulation of contour distributions for different processing modes (c) Schematic illustration of the mechanism of different processing modes

3.4 Performance Characterization

3.4.1 Mechanical Characterization and Surface Morphology of Cell Scaffolds

Based on the parameter optimization results described above, 10-layer 3D cellular scaffolds were fabricated using the optimal parameters for the three processing modes, and their Young's modulus was tested. Single 400 nm processing used a power of 0.6 mW, single 800 nm processing used a power of 6 mW, and for dual-wavelength dual-pulse processing, the optimal parameter combination of 0.45 mW for the 400 nm laser wavelength, 0.15 mW for the 800 nm laser wavelength, and a 50 ps delay time was adopted. To ensure comparability, all scaffolds were fabricated with 10 layers, resulting in a total height of approximately 200 μm (**Fig. 5a**).

The mechanical testing results are shown in **Fig. 5b**. The scaffolds prepared using the three processing modes exhibit significant differences in Young's modulus. The Young's modulus of the scaffold prepared by single 800 nm processing was predominantly concentrated around 27.7 kPa; this relatively low value was primarily attributed to the low crosslinking density resulting from insufficient two-photon absorption efficiency. The average modulus of the scaffold prepared by single 400 nm processing reached 88.8 kPa, demonstrating the crosslinking advantage conferred by high linear absorption efficiency. However, due to bubble defects during multi-layer fabrication, the uniformity and repeatability of its mechanical properties were

affected. The average compressive modulus of scaffolds fabricated by the dual-wavelength dual-pulse processing reached 148 kPa, which is 1.6 times that of the single 400 nm processing and 5.4 times that of the single 800 nm processing. AFM analysis further revealed disparities in the microstructure homogeneity of the scaffolds (**Fig. 5c**), with specific parameters detailed in Table S1. Compared to single-wavelength processing, scaffolds fabricated via dual-wavelength dual-pulse laser processing exhibited marginally superior overall surface flatness relative to the single 400 nm group and significantly outperformed the single 800 nm group. This demonstrates that dual-pulse processing yields a flatter surface and a more uniform microstructure.

The above mechanical and morphological characterizations collectively demonstrate that this technology significantly enhances mechanical properties while maintaining structural integrity. The hardness of the fabricated scaffolds is close to the lower limit of natural dermis and cartilage tissues [40, 41], providing a more suitable mechanical microenvironment for cell adhesion and spreading. This further confirms the superiority of this technology in the preparation of tissue engineering scaffolds.

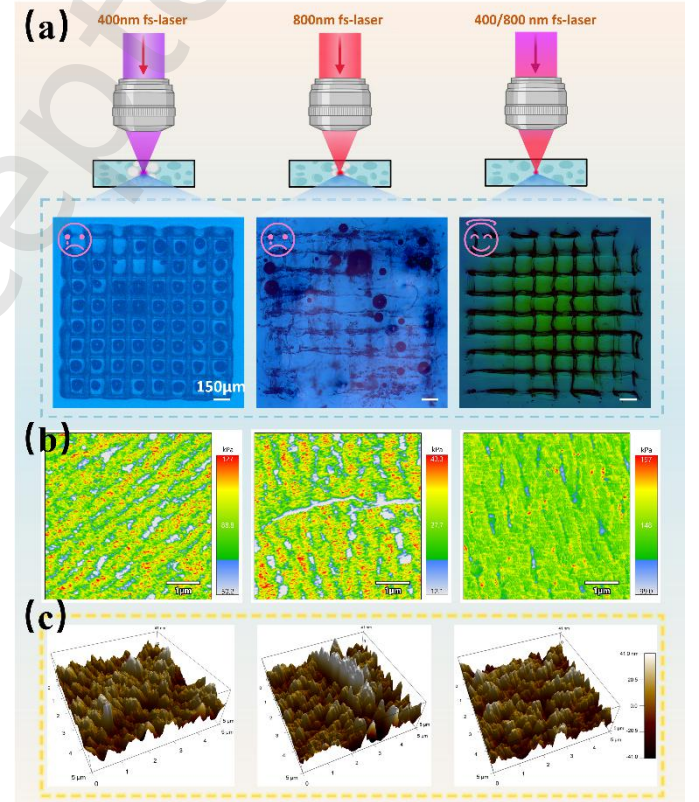


Figure 5 Results of mechanical and Surface Morphology testing (a) Morphological characterization of a 10-layer cell scaffold under different processing modes (b) Young's modulus test results (c) AFM images of the surface microtopography of the scaffold under different processing conditions

3.4.2 Biological Performance Characterization

We first evaluated the basic biosafety of the scaffolds. L929 cells were cultured in the scaffold extracts, followed by morphological observation and proliferation assays. As shown in **Fig. 6a**, the cells exhibited typical polygonal spreading with well-defined actin cytoskeletons. The fluorescence intensity showed no significant difference compared to the control group, indicating that the scaffold extracts had no adverse effects on cell morphology.

We further evaluated cell proliferation using the CCK-8 assay. As shown in **Fig. 6b**, cell viability remained above 75% at all 3 time points (1, 3, and 5 days). It exhibited a gradual upward trend over time, demonstrating good cellular metabolic activity and sustained proliferation capacity. These results confirm that the dual-wavelength dual-pulse scaffold is non-cytotoxic and possesses good biosafety.

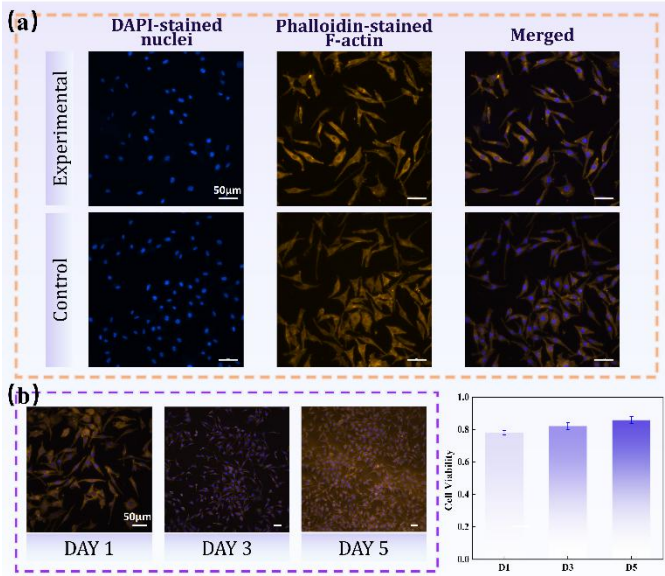


Figure 6 (a) Fluorescence staining images of the cells (b) Cell viability after culturing in extract-conditioned medium for 1, 3, and 5 days

To evaluate the suitability of hydrogel scaffolds fabricated by different processing methods as cell culture substrates, we seeded L929 fibroblasts onto the surfaces of three groups of scaffolds processed by single 800 nm, single 400 nm, and dual-wavelength dual-pulse methods. Cytoskeleton staining and morphological observation were performed on the third day of culture (**Fig. 7a**).

Observation on the third day of culture revealed significant differences in the morphological stability of scaffolds fabricated by different processing methods when immersed in the culture medium (**Fig. 7b**). Scaffolds produced by single 800 nm processing, due to insufficient crosslinking density and low modulus, could not maintain their structural integrity during immersion, exhibiting significant curling deformation, with edges curled and lifted, and some areas had already delaminated from the substrate. Although the 400 nm processed scaffold exhibited a higher modulus, structural discontinuities caused by air bubbles during the multi-layer processing led to stress concentrations at these defects, triggering localized deformation. Consequently, it also exhibited noticeable curling, albeit to a slightly lesser extent than the 800 nm group. In contrast, scaffolds fabricated by dual-wavelength dual-pulse processing, owing to their high modulus and highly homogeneous, bubble-free structure, largely retained their original printed morphology in the liquid environment, exhibiting structural integrity and clear boundaries without significant visible curling or delamination. Fluorescent cell staining observations conducted on this basis (**Fig. 7c**) revealed that the L929 cells on the dual-wavelength scaffold exhibited the strongest actin cytoskeleton fluorescence signal. In contrast, the fluorescence signals from the cell scaffolds processed using single 800 nm and single 400 nm wavelengths were significantly weaker due to the inhibitory effects of their

insufficient mechanical properties or structural defects on cytoskeletal development. This result demonstrates that the dual-wavelength scaffolds provide a more favorable microenvironment for cell adhesion and cytoskeletal organization compared to single-wavelength counterparts.

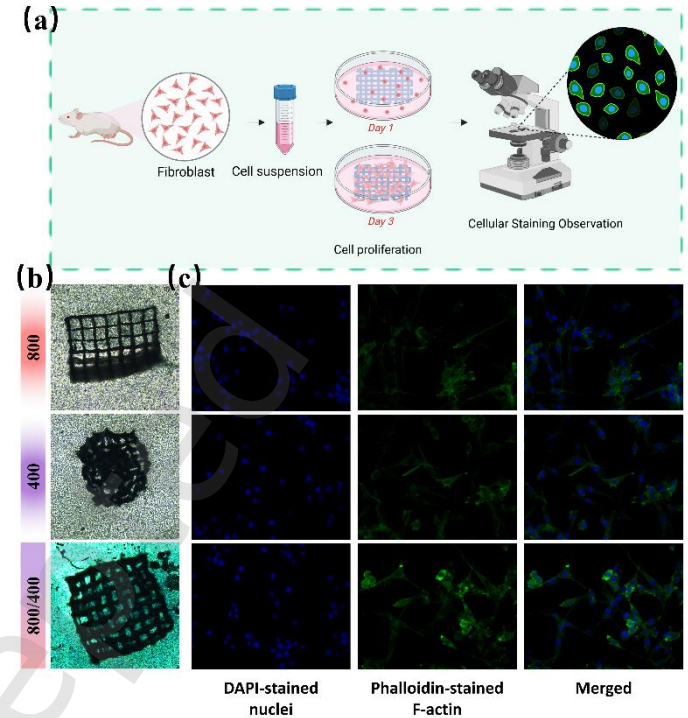


Figure 7 Results of L929 cell culture on different scaffolds (a) Schematic diagram of the experimental procedure (b) Morphological characterization of cells on different scaffolds on day 3 of culture (c) Fluorescence staining results of cells on day 3 of culture

4. Conclusions

This study addresses the inherent trade-off between high hardness and low thermal damage during the processing of hydrogel cell scaffolds using single-wavelength femtosecond lasers and proposes a dual-wavelength dual-pulse spatiotemporal synergy strategy based on thermomechanical coupling control. Through a temporal sequence design in which the 400 nm pulse initiates excitation and the 800 nm pulse follow to complete cross-linking, this strategy achieves spatiotemporal decoupling between energy deposition efficiency and thermal relaxation processes. Consequently, this enables the dual-pulse synergistic effect to drive efficient polymerization with a total power of only 0.6 mW, reducing the polymerization threshold by 30 times compared to single 800 nm processing, while simultaneously eliminating the thermally accumulated bubbles that are difficult to suppress in single 400 nm processing, thereby increasing the number of defect-free stacking layers from 3 to 20. Building on this, the dual-wavelength scaffold achieved an average compressive modulus of 148 kPa, which is 1.6 times and 5.4 times that of the single 400 nm and single 800 nm scaffolds, respectively. Meanwhile, a uniform nanoscale topological structure of approximately 40 nm was formed in situ on the scaffold surface, creating a cross-scale structure together with the micrometer-scale line width and millimeter-scale dimensions. Cell experiments further demonstrated that L929 cells cultured on the dual-wavelength scaffolds exhibited stronger actin

fluorescence signals compared to the single-wavelength groups, indicating more favorable support for cytoskeletal development.

However, the current experimental design does not allow for the individual contributions of modulus, surface roughness, crosslinking degree, and structural stability to cellular responses to be studied separately, as the dual-pulse processing simultaneously modulates multiple scaffold properties. Therefore, future work will systematically design experiments with independently varied surface topography and substrate stiffness to deconvolute the individual contributions of mechanical and topographical cues to cellular responses, which will provide deeper mechanistic insight into the observed cell-scaffold interactions. Building on this mechanistic understanding, further investigations will involve introducing functional cell types such as chondrocytes and osteoblasts to systematically evaluate the scaffold's regulatory effects on cell differentiation and functional expression. Its in vivo tissue regeneration capability will also be verified through animal implantation experiments. Concurrently, we will further expand the range of dual-wavelength dual-pulse parameter control to enable precise, controllable fabrication of scaffolds with stiffness within the physiological ranges of different tissues, thereby promoting the practical application of this technology in the field of tissue engineering.

Data availability

The data are available upon reasonable request.

Acknowledgements

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Declaration of competing interest

The authors declare no competing interests.

Author contribution statement

Yan Tan: Conceptualization, Methodology, Investigation, Writing - Original draft preparation. Ze Pei: Investigation, Writing - Original draft preparation. Yaning Wei: Formal analysis, Investigation. Chong Zhang: Formal analysis. Shuwei Zhang: Software. Ruonan Li: Writing - Review & editing. Baoshan Guo: Resources, Supervision, Project administration, Funding acquisition.

Use of AI statement

None

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Electronic Supplementary Material

Fabrication of cross-scale biomimetic 3D hydrogel scaffolds with thermo-mechanical optimized properties via femtosecond laser dual-wavelength dual-pulse regulation

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Supplementary figures

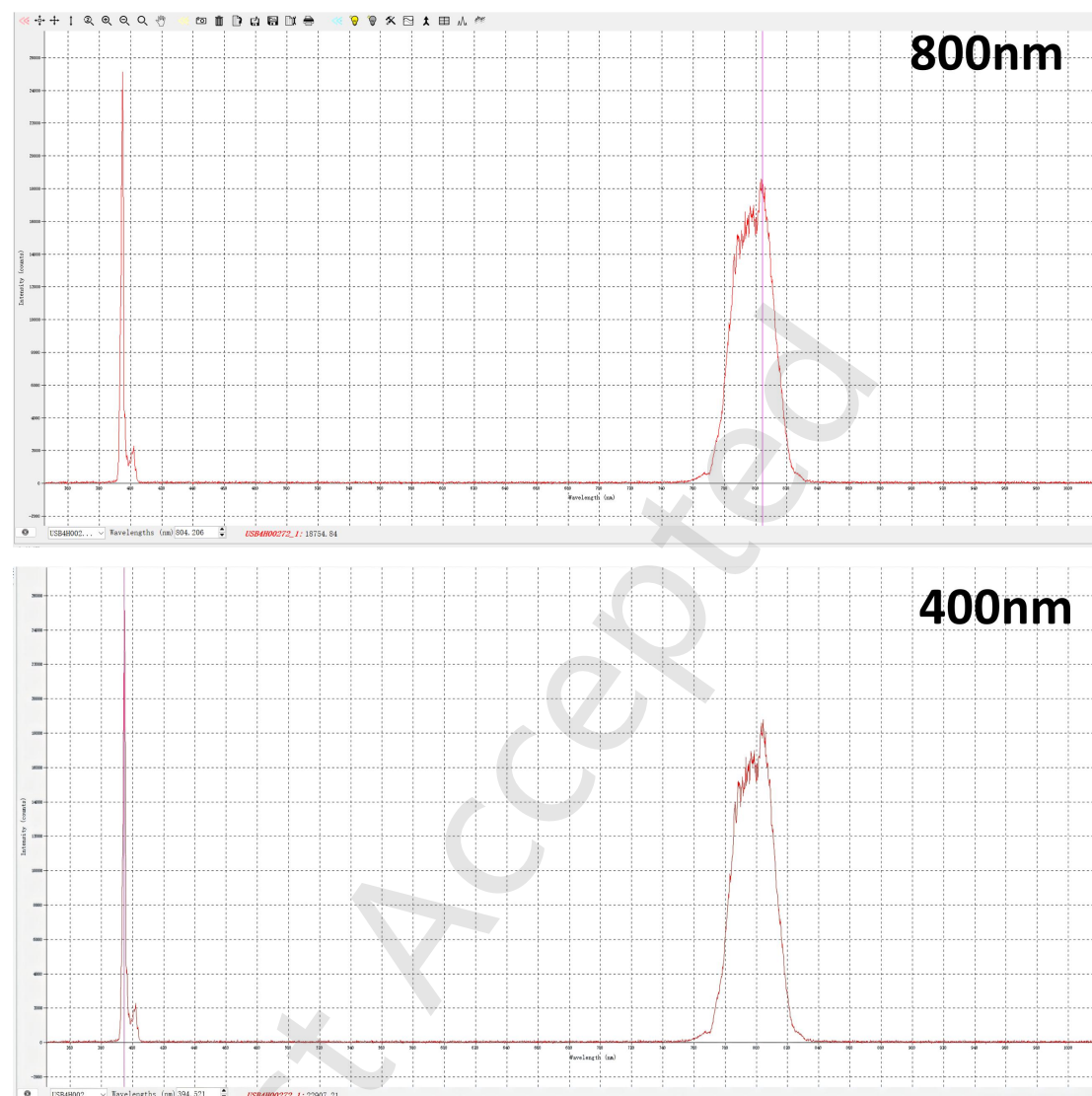


Figure S1 Spectral verification of two-pulse alignment

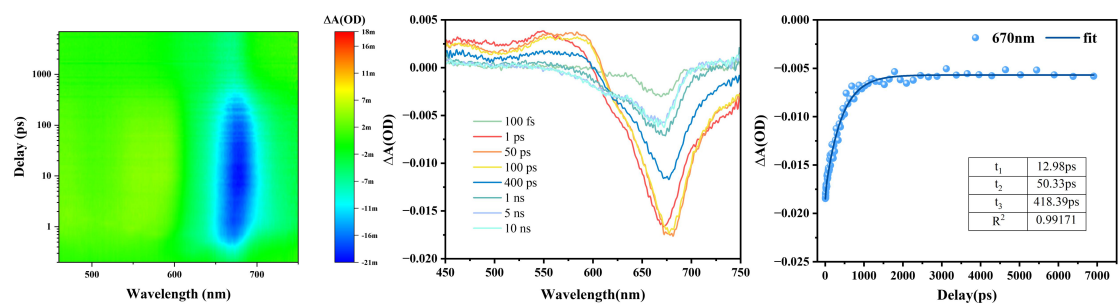
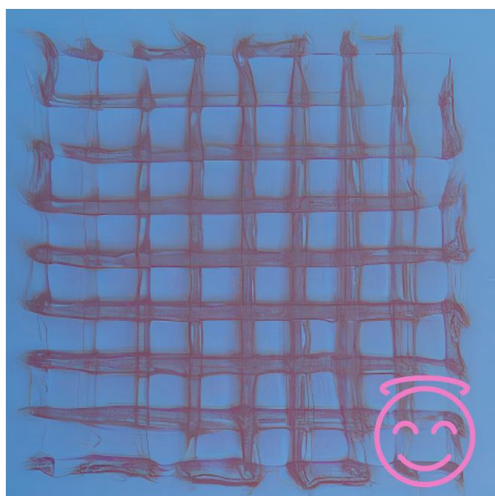
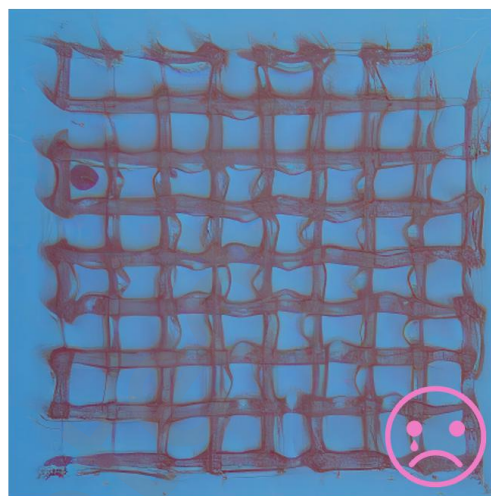


Figure S2 Femtosecond transient absorption characterization of the hydrogel precursor solution

Layer 15: 0.6mw, 500 μ m/s, 50ps



400:800=0.45:0.15



400:800=0.5:0.1

Figure S3 Morphology comparison of 15-layer scaffolds under different energy ratios

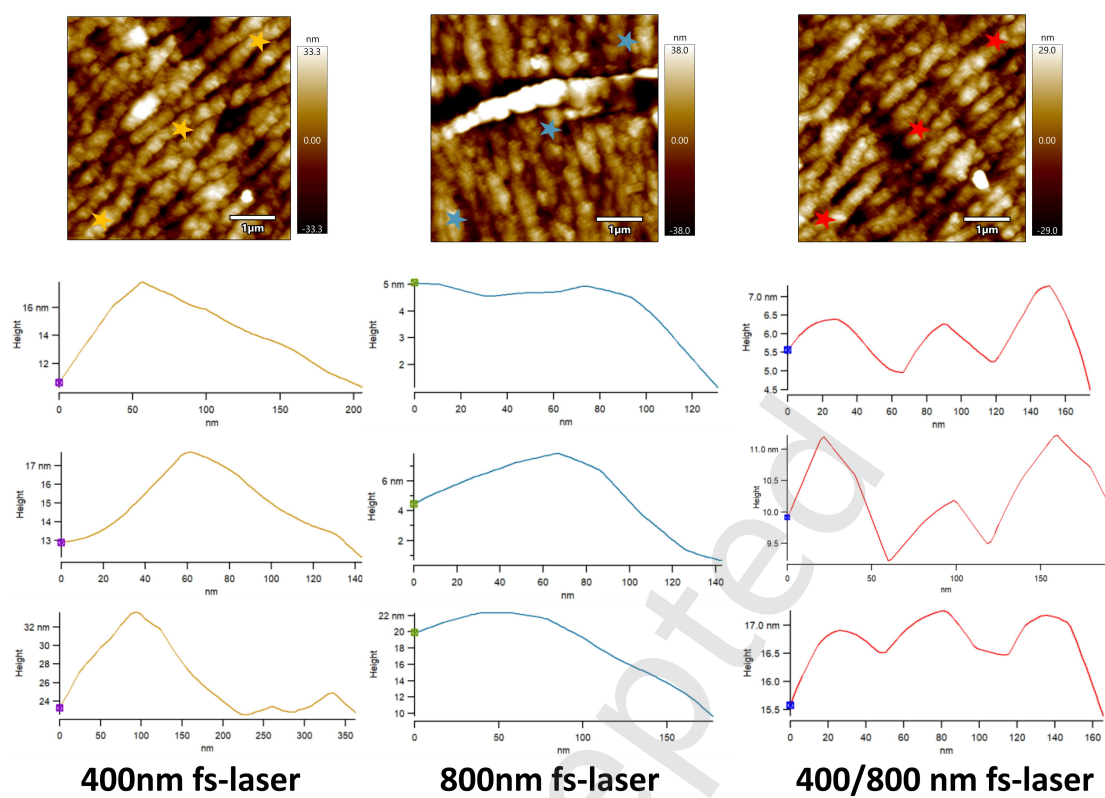


Figure S4 AFM cross sectional profiles of scaffolds under different processing conditions

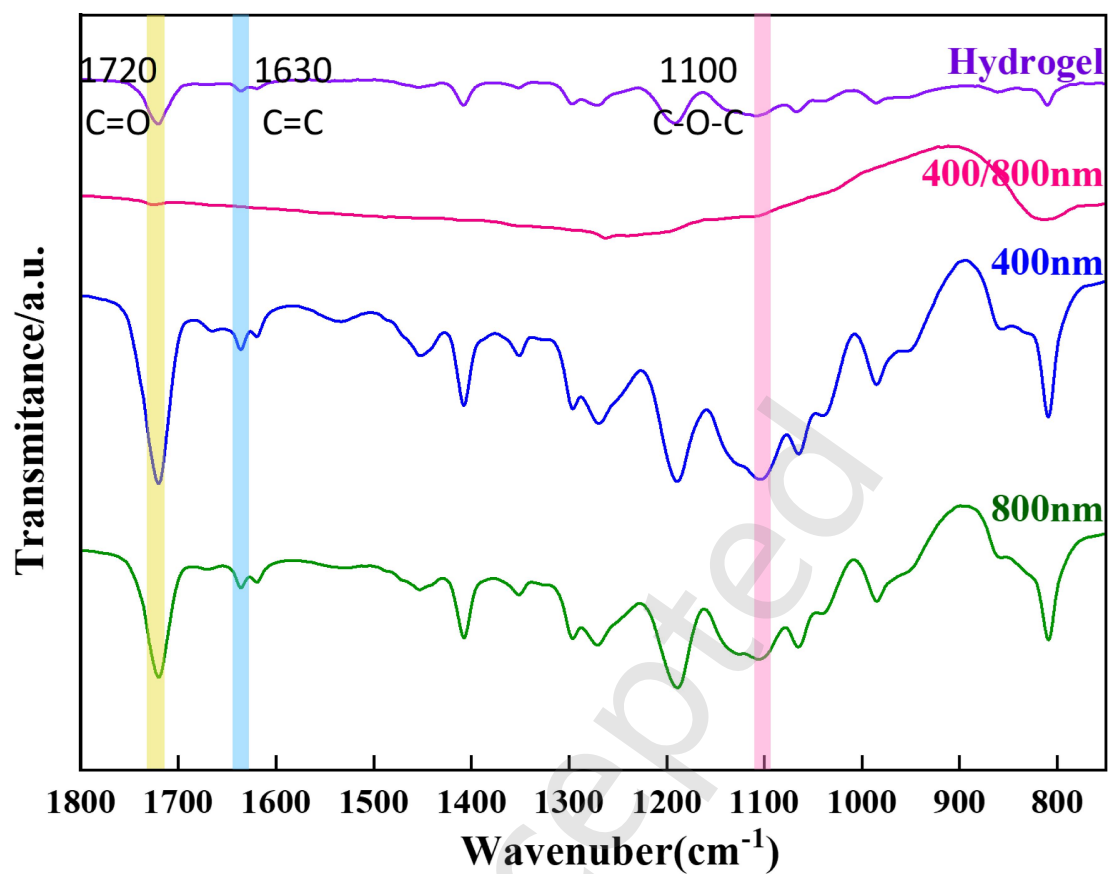


Figure S5 FTIR spectra of scaffolds fabricated by three processing modes

Supplementary tables

Table S1 Surface roughness parameters of hydrogel scaffolds fabricated by different laser processing modes

	Rq (nm)	Ra (nm)	Max (nm)	Min (nm)
Dual-wavelength	13.62	10.09	42.11	-54.96
800 nm	19.05	13.82	73.06	-90.35
400 nm	13.85	11.09	50.35	-58.57