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Multiscale 3D printing in tissue engineering: From micro/nanoscale precision to functional organoid fabrication

Running title: Multiscale 3D printing in tissue engineering

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Abstract

Three-dimensional biofabrication has become a central strategy in tissue engineering and regenerative medicine, driven by the need to recreate the spatial, mechanical, and biochemical complexity of native cellular microenvironments. Light-based 3D printing technologies are particularly powerful in this context, since they enable contactless fabrication with programmable resolution and volumetric control across multiple length scales. In this review, the evolution of optical biofabrication strategies, focusing on the complementary and interdependent roles of volumetric bioprinting (VBP) and two-photon polymerization (TPP) is discussed. VBP addresses the challenge of organ-scale construction, enabling rapid fabrication of centimeter-scale, perfusable tissue architectures, while TPP provides subcellular precision for engineering the microscale niches that govern cell behavior. The review critically analyzes these technologies in tandem, outlining a multiscale integration framework that bridges the gap between microscale control and organ-scale function. VBP is first discussed as an emerging paradigm for organ-scale fabrication. By delivering spatially modulated light doses throughout an entire photosensitive volume, VBP enables layer-free formation of centimeter-scale, cell-laden constructs within seconds. This approach minimizes mechanical stress and preserves high cell viability. It also enables the fabrication of complex internal architectures, including perfusable vascular networks. Key advances in computed axial lithography, holographic patterning, and bioink engineering are reviewed, with emphasis on optical transparency, light scattering control, and rheological stability in cell-dense systems. In the second part of the work, two-photon polymerization is analyzed as a complementary high-resolution strategy for micro-nanoscale biofabrication. Owing to nonlinear optical confinement, TPP provides submicrometer spatial resolution and precise three-dimensional control over scaffold architecture, mechanics, and biochemical patterning. These capabilities enable deterministic regulation of cell–material interactions and have been applied to stem cell niche engineering, neural and vascular interface design, tumor microenvironment modeling, and biohybrid microsystems. Finally, by integrating the information gathered on VBP and TPP, we outline a multiscale integration framework is presented in which the two volumetric and two-photon approaches are combined to address distinct but interdependent biological requirements for functional organoids, ranging from organ-scale architecture and perfusion to microscale niche definition and molecular-level signaling. Remaining challenges in material heterogeneity, optical–biological coupling, and long-term tissue maturation are discussed, highlighting future directions toward predictive design and translational biofabrication of functional living systems.

Keywords: 3D printing; Two-photon polymerization; Biofabrication; Volumetric bioprinting; Organogenesis; Functional organoids

1 Introduction

The ultimate goal of tissue engineering and regenerative medicine is to reconstruct living tissues that faithfully reproduce the structure and function of native organs^{[1]-[3]}. This goal is rooted in the fundamental principle that cell fate and behavior are strongly regulated by the three-dimensional microenvironment, which consists of a dynamic and hierarchical combination of biochemical and biophysical cues provided by the extracellular matrix^{[4]-[6]}. Methods like solvent casting and gas foaming, which represent traditional scaffold fabrication, provide limited control over the microenvironment. Consequently, they often produce constructs with poor reproducibility, limited functionality, and restricted translational potential^{[7][8]}.

The development of 3D printing technologies, particularly light-based additive manufacturing^[9], has significantly advanced the field by enabling precise control over scaffold geometry, mechanical properties, and biological function. Nevertheless, a central challenge remains unsolved: the need to balance high spatial resolution for reproducing fine cellular and extracellular features, high fabrication speed to preserve cell viability and enable large constructs^[10], and sufficiently large build volumes for clinically relevant tissues. Achieving all three simultaneously continues to be a major technical barrier in biofabrication^{[11]-[13]}.

This review analyzes the evolution of light-based 3D printing technologies, outlining a clear progression from micro- and nanoscale precision fabrication to emerging approaches for organ-scale volumetric biofabrication. The discussion begins with two-photon polymerization, a technology based on nonlinear two-photon absorption that enables true three-dimensional writing with feature sizes below 100 nm^[14]. Owing to its exceptional resolution and broad compatibility with materials ranging from synthetic photoresists to natural biopolymers, two-photon polymerization has become a key tool for engineering microenvironments at the subcellular scale.

This technique enables the fabrication of biomimetic scaffolds that actively influence cell behavior, including well-defined porous networks for studying nuclear mechanics and stem cell differentiation, submicron surface features for contact guidance, and spatially confined microstructures for modeling cancer invasion and neural growth. These capabilities have supported applications ranging from stem cell niche engineering and organoid guidance to the development of biohybrid microsystems and integrated sensing platforms within three-dimensional scaffolds^{[15]-[18]}.

Despite its exceptional fabrication precision, the serial point-by-point nature of two-photon polymerization limits fabrication speed, restricting its use to relatively small-scale constructs. This limitation has encouraged the adoption of projection-based methods like digital light processing and continuous liquid interface production, which polymerize entire layers simultaneously, thereby substantially increasing the printing speed^{[19][20]}. The working principles and trade-offs of these layer-based approaches are discussed, including surface stepping effects, the coupling between resolution and build area, and mechanical disturbances associated with repeated layer formation, which together limit their suitability for fabricating large, complex, cell-laden tissues.

To overcome the constraints of layer-by-layer fabrication, recent efforts have focused

on volumetric bioprinting. In particular, computed axial lithography has emerged as a promising layer-free approach^[21]. By projecting a series of dynamically calculated light patterns from multiple angles into a rotating volume of photosensitive bioink, this method enables the rapid formation of complete three-dimensional structures within seconds^[22]. The absence of mechanical layering reduces shear stress, allows the fabrication of complex internal channels without support materials, and maintains high cell viability even at elevated cell densities. Emerging holographic bioprinting strategies are also reviewed that use optical or acoustic wavefront control to organize cells and materials prior to polymerization, enabling spatial heterogeneity and offering new possibilities for dynamic and responsive tissue constructs.

The effectiveness of these advanced fabrication strategies relies heavily on continued progress in bioink development^[23]. Rather than serving solely as cell carriers, next-generation bioinks must integrate optical, rheological, and biological functions. Some researchers discuss approaches to optimizing photoinitiator systems to balance polymerization efficiency^[24] and cytocompatibility, reducing light scattering through refractive index matching in cell-dense formulations, and controlling cell sedimentation during volumetric printing through rheological design^[25].

As fabrication processes and material systems become more complex^[26], intelligent^[27] and adaptive manufacturing strategies are increasingly important^[28]. Recent advances in real-time monitoring techniques are reviewed, as well as the use of adaptive optics to compensate for optical distortions during printing. In addition, we highlight the growing role of deep learning in addressing challenges related to light propagation, parameter optimization, and process robustness, enabling computationally guided fabrication that accounts for interactions between light, materials, and living cells.

In summary, this review highlights how the convergence of multiscale light-based manufacturing technologies, advanced bioink design, and data-driven process control is shaping a viable pathway toward the fabrication of functional living tissues. Figure 1 summarizes this concept as a hierarchical strategy in which different fabrication modalities contribute with distinct but complementary levels of control across multiple length scales. We define this approach as a multiscale integration framework (Figure 1). Macro-scale volumetric bioprinting (Figure 1A) establishes the overall tissue geometry (anatomy) and perfusable channel network, while micro-scale two-photon polymerization (Figure 1B) refines this framework allowing fine control of specific microscale features such as vascular niches and spatially confined microstructures (e.g., porous networks). These structural elements can then be further augmented at the nano/molecular level (Figure 1C) through the incorporation into the scaffold of bioactive signals, which may specifically direct cell-scaffold interaction; these are molecular cues including adhesive ligands, growth factors, morphogens, and other biochemical regulators that may reconstitute a functional microenvironment which governs cell-material interactions and helps maintain or directs biological key requirements for functional tissue (as for example, cell fate and identity). Thus, panels A and B represent the fabrication of the physical and spatial framework, whereas panel C reflects the biological instruction layer that converts a printed construct into a functional microenvironment. From a prospective standpoint, this review indicates that

integrating these complementary structural and molecular levels of control (that combine organ-level geometry, microscale structural precision, and molecular guidance) will be critical for generating living tissue constructs.

The review concludes by discussing key remaining challenges, particularly in the establishment of vascular networks and the long-term functional maturation of organ-scale constructs. Addressing these challenges will require a shift from design of uniform structures toward spatially programmed heterogeneity, together with the integration of bioprinting with bioreactor-based conditioning systems. Looking ahead, predictive digital models that couple optical, mechanical, and biological factors may further accelerate the translation of light-based biofabrication toward patient-specific regenerative therapies.

To systematically address this challenge, this review first examines volumetric bioprinting as the technology that pushes the boundaries of scale and speed, enabling the rapid construction of organ-sized, perfusable tissue scaffolds (Chapter 2). We then turn to two-photon polymerization, which pushes the boundaries of resolution and precision, allowing us to engineer the microscale and subcellular features that determine cell fate^[29] and tissue function (Chapter 3). Finally, in Chapter 4, we propose a multiscale integration framework that combines these complementary technologies to achieve hierarchical control over tissue architecture, from organ-scale geometry to molecular-level signaling. This structure—from macro to micro—reflects our view that functional tissue engineering requires both top-down architectural control and bottom-up microenvironmental precision.

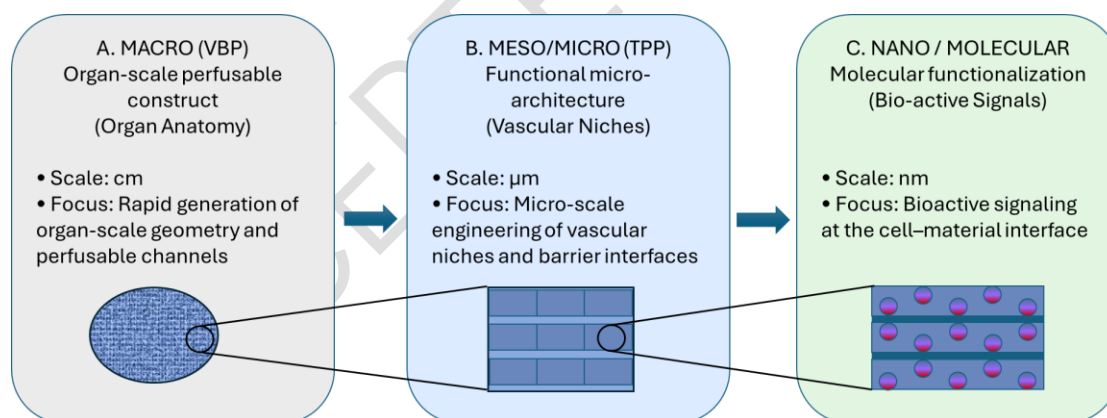


Fig. 1 Hierarchical Multiscale Integration Framework for Generating Functional Living Tissues Using Biofabrication Strategies: The figure illustrates a prospective multiscale framework in which distinct but complementary light-based bio fabrication approaches operate across different length scales to progressively build spatial, structural, and molecular complexity. (A) Macroscale volumetric bioprinting enables the rapid fabrication of organ-scale tissue geometries and perfusable channel networks. (B) Microscale two-photon polymerization refines this framework through the precise engineering of microscale features, including vascular niches and spatially confined microstructures such as porous networks or biological barrier-like structures. (C) At the nano/molecular level, scaffold functionalization introduces bioactive cues that establish a functional microenvironment capable of modulating cell–

material interactions and fulfilling the biological requirements necessary for tissue functionality, such as cell fate and identity. Together, these complementary levels of control enable the integration of physical architecture and biological instruction, offering a viable route toward the fabrication of functional living tissues.

2 Volumetric Bioprinting for Organ-Scale Fabrication

2.1 Evolution of Photocuring Technologies from Layer-by-Layer to Volumetric Fabrication

The pursuit of organ-scale biomanufacturing is fundamentally constrained by competing biological requirements (see more details in, Chapter 4). These requirements span multiple aspects of biological performance, including cell viability and phenotype stability (conditions which typically demand high fabrication speeds) together with the need for a physiologically relevant microenvironment, best supported by fine control of spatial technique resolution. In addition, the environmental, chemical and mechanical culture conditions must be appropriately managed to enable sustained, measurable, and reproducible functional outputs over time. The evolution of light-based bioprinting therefore represents a sequence of paradigm shifts aimed at addressing all or most of these requirements, progressing from point-scanning approaches to layer-projection methods, and ultimately toward true volumetric, layer-less fabrication. At the microscale, two-photon polymerization (TPP) exploits nonlinear optical absorption to achieve submicrometer resolution (typically 100–500 nm), enabling precise replication of cellular microstructures that guide cell growth and differentiation^[30]. Ovsianikov et al. demonstrated the encapsulation of cells within highly hydrated, photosensitive gelatin matrices using water-soluble photoinitiators^[31], highlighting TPP's utility for three-dimensional cell culture. However, as a point-scanning direct-write technique, TPP suffers from intrinsically low throughput, with fabrication time scaling cubically with volume. Typical scanning speeds of 30–550 mm/s result in printing cycles lasting hours, exposing cells to prolonged non-physiological conditions and severely limiting viability.

Projection-based digital light processing (DLP) bioprinting addresses this limitation by curing entire layers simultaneously, thereby substantially increasing throughput. Studies have demonstrated its capability to fabricate nanocomposite hydrogels^[32] and pre-vascularized, high-cell-density tissues^[33]. DLP remains constrained by its layer-by-layer nature: mechanical peeling and recoating introduce shear stresses^[34], while extended print times promote cell sedimentation and spatial inhomogeneity. These effects have been shown to compromise membrane integrity and

functional uniformity, particularly for sensitive cell types such as iPSCs and organoids^{[35][36]}, motivating the transition toward truly volumetric bioprinting paradigms.

2.1.1 Projection-Based Technologies (DLP-SLA): The Speed-Precision Trade-off in Layered Manufacturing

Projection-based DLP-SLA uses a digital micromirror device (DMD) as a dynamic mask to project an entire two-dimensional light pattern onto a photosensitive resin, enabling each layer to be polymerized in parallel rather than scanned point by point. This optical architecture is the main reason why DLP-SLA achieves a much higher resolution-to-time ratio than conventional serial writing approaches and remains attractive for rapid fabrication of hydrogel scaffolds, organ-on-chip components, and pre-vascularized tissue constructs. In typical systems, the light source is modulated by the DMD and focused through projection optics onto the resin vat, after which the build platform advances by one layer thickness and the exposure cycle is repeated. As illustrated in Figure. 2, this workflow preserves the simplicity of slice-based manufacturing while substantially increasing throughput relative to laser-scanning SLA.

This area-projection mechanism offers clear manufacturing advantages, but it also establishes the central physical trade-off of DLP-SLA. Because the number of DMD pixels is fixed, improvements in lateral resolution usually require a smaller projection area, whereas larger build areas inevitably increase effective pixel size and reduce feature fidelity^[37]. DLP systems therefore occupy an intermediate regime: they are faster than point-scanning methods and can routinely achieve lateral resolutions on the order of tens of micrometers^[38], yet they still remain challenging to simultaneously maximize resolution, build volume, and geometric complexity. This trade-off is especially relevant in tissue engineering, where print size and throughput must be balanced against the need to preserve local microenvironmental fidelity^[39].

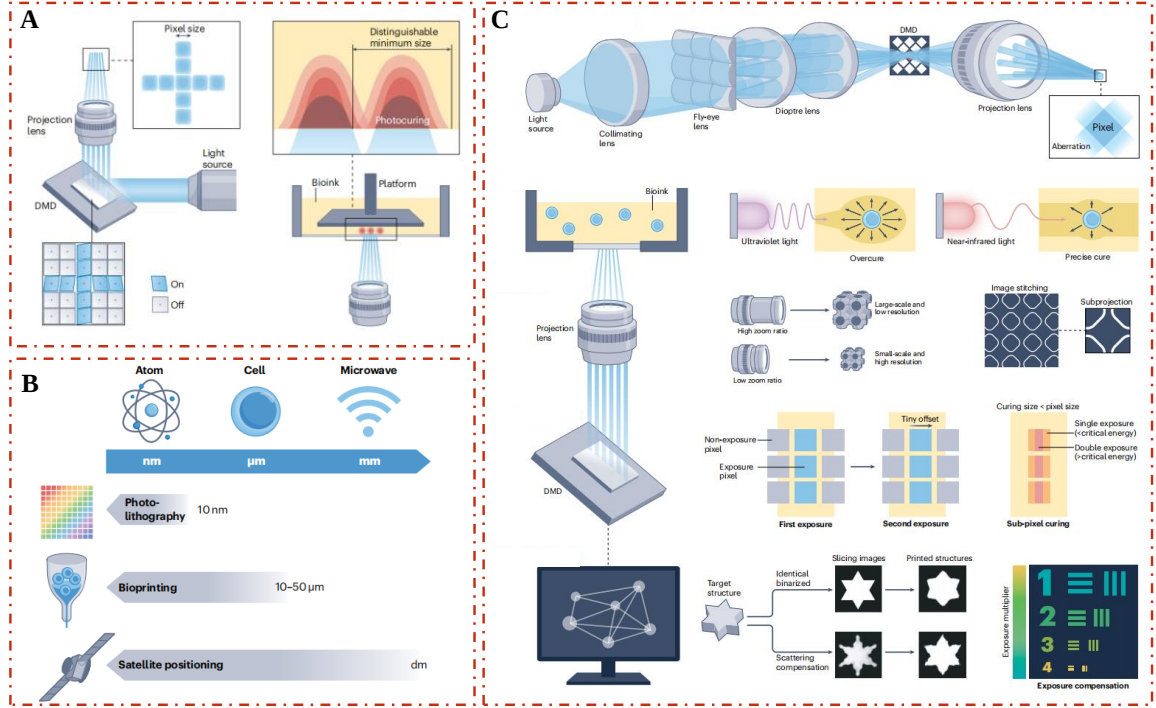


Fig.2. DLP-SLA technology principles and workflow. (a) Optical configuration of a projection-based DLP-SLA system. (b) Layer-by-layer fabrication sequence, including patterned light projection, resin curing, and synchronized platform motion. Adapted with permission from ref.[36] Copyright © 2025 Nature CC-BY 4.0.

From a biological perspective, the relevance of DLP-SLA is not defined by speed alone, but by how exposure conditions are translated into cell-instructive material properties. Exposure time, irradiance, photoinitiator concentration, and resin composition collectively regulate crosslink density, pore structure, elastic modulus, and curing depth. These parameters then influence cell spreading, cytoskeletal organization, nuclear deformation, and lineage commitment, meaning that optical parameters are indirectly coupled to mechanobiological outcome^{[40][41]}. In this sense, the central question is not simply the achievable printing speed, but whether the process can generate a sufficiently controlled three-dimensional microenvironment without compromising cytocompatibility.

The main biological penalties arise from the layered nature of the process. Repeated peeling and recoating can introduce cumulative mechanical stress, while prolonged fabrication time increases the probability of cell sedimentation and spatial heterogeneity, particularly in high-cell-density bioinks or delicate cell systems such as iPSC-derived tissues and organoids. In parallel, the staircase effect produces discontinuous surfaces and local topographical irregularities that can influence adhesion, migration, nutrient transport, and contact-guidance behavior^[42]. Thus, in

layered light-based bioprinting, geometric artifacts are not merely manufacturing imperfections; they can propagate into altered cell-matrix interactions and ultimately influence functional tissue performance.

CLIP represents an important refinement of this platform rather than a conceptual departure from it. By introducing an oxygen-permeable dead zone at the vat interface, CLIP suppresses adhesion between the cured layer and the substrate, allowing quasi-continuous part motion and greatly reducing peel-induced shear stress^[43]. This improvement can substantially increase fabrication speed and improve compatibility with fragile cell populations. Nevertheless, CLIP remains a layered projection process and therefore does not remove the underlying resolution-build area trade-off, the tendency toward relatively homogeneous material output, or the residual topographical limitations associated with sequential slicing. For this reason, the progression from DLP-SLA to CLIP is best interpreted as an optimization of layered photocuring, whereas the search for a more complete solution to the speed-stress-viability dilemma ultimately motivated the development of true volumetric bioprinting.

2.1.2 True Volumetric Bioprinting (VBP)

Volumetric Bioprinting (VBP) represents a significant advancement in additive manufacturing, with its core concept being the transition from the traditional "layer-by-layer" manufacturing paradigm to "layer-less" manufacturing. In traditional additive manufacturing, three-dimensional objects are realized through continuous stacking of two-dimensional layers, a process typically accompanied by inherent issues such as slow printing speeds, staircase effects, the need for support structures, and anisotropic mechanical properties^[44]. Mathur et al., in their review, noted that these limitations of traditional layer-by-layer printing severely constrain its industrial application in cell printing^[45]. "Layer-less" manufacturing means that objects are no longer stacks of discrete layers, but rather are cured in a single step within seconds to tens of seconds inside a three-dimensional volume of photosensitive material through precise three-dimensional spatial control of light energy, as shown in Figure. 3. Research by Whyte. D et al.^[46] demonstrated that this method can fabricate objects from any direction within the medium volume and can print complex overhanging structures without the need for supports, pioneering a new frontier in "layer-less" 3D printing.

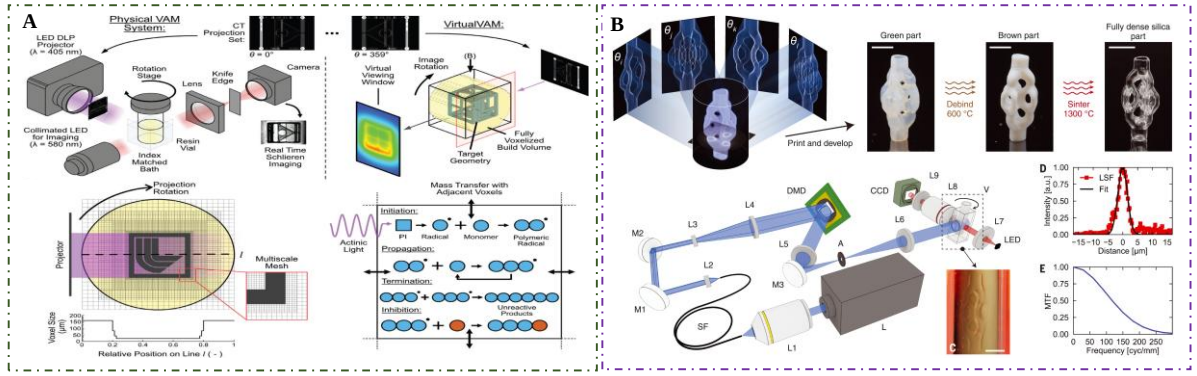


Fig.3 True Volume 3D Printing Principle. a) VirtualVAM simulates reaction and diffusion in all voxels within VAM. Adapted with permission from ref. ^[47] Copyright © 2025 Nature CC-BY4.0; b) Printing transparent fused silica glass with micro-CAL. Adapted with permission from ref. ^[46] Copyright © 2024 Elsevier CC-BY4.0.

When the resin container rotates, the projector continuously projects optimally computed light patterns from different angles. Within the resin volume, only those voxels that need to be cured receive sufficient accumulated light dose from all angles. By precisely controlling the light dose received by each voxel, photopolymerization reactions can be triggered at predetermined positions, thereby achieving synchronous curing of the entire three-dimensional object within the photosensitive resin liquid volume^[48]. The pioneering research by Kelly et al.^[49] demonstrated that this paradigm shift "from layers to volumes" enables printing speed to be reduced from hours to seconds or even tens of seconds, achieving a substantial improvement in printing efficiency. Figure. 3 illustrates the fundamental principles of true volumetric 3D printing. Figure. 3(a) shows the VirtualVAM simulation system, which can model photopolymerization reactions and mass diffusion behavior in all voxels during the volumetric additive manufacturing process, providing theoretical guidance for optimizing printing parameters. Figure. 3(b) demonstrates an example of printing transparent fused silica glass using micrometer-scale Computed Axial Lithography (CAL) (micro-CAL) technology, proving the feasibility of this technology in manufacturing high-precision optical components.

CAL, first proposed by Kelly *et al.* in 2019, represents one of the earliest and most influential implementations of volumetric additive manufacturing (VAM)^[49]. By adopting a tomographic reconstruction framework, CAL enables the synchronized projection of angular light patterns to achieve the simultaneous curing of an entire three-dimensional object within a volume of photosensitive resin in a matter of seconds^[50]. This computationally driven strategy established the technical foundation for subsequent developments in volumetric bioprinting.

Building upon the tomographic principle introduced by CAL, Tomographic Volumetric Bioprinting further demonstrated the fabrication of macroscopic tissue constructs through the controlled delivery of three-dimensional light dose distributions across the full print volume, effectively circumventing the constraints associated with sequential layer deposition. Further advancing this approach, Xolography, introduced by Regehrly *et al.*, achieved linear volumetric confinement via the intersection of structured light fields^[51] enabling non-contact volumetric printing with enhanced spatial selectivity. This strategy not only avoids shear-induced cellular damage arising from mechanical peeling processes but also facilitates more homogeneous mass transport conditions during fabrication.

The biological feasibility of volumetric bioprinting was subsequently demonstrated by Bernal *et al.*, who reported the fabrication of a human auricle model within 22.7 seconds^[47]. Moreover, functional organoids and complex vascularized constructs were produced at high cell densities ($>10^7$ cells/mL) while maintaining cell viabilities exceeding 85–90%. More recent studies have further shown that optimized bioresin formulations, 5% GelMA combined with 0.05% LAP, enable the fabrication of complex perfusable constructs within 30 seconds with cell viabilities above 90%^[52].

One of the key advantages of true VBP, particularly CAL technology, is its ability to print complex overhanging geometries without the need for support structures (support-free). In traditional layer-by-layer printing technologies, to prevent the collapse of overhanging portions during printing, additional support structures must be designed and printed, which not only increases material consumption and printing time but also requires complex post-processing steps to remove supports, with potential damage to the printed parts during removal. CAL technology, by directly curing within the liquid resin volume, eliminates the need for support structures, enabling easy fabrication of the following types of complex structures :

a) Complex internal channels: fluid channels mimicking human vascular networks, lymphatic systems, or microfluidic chips, which are difficult to achieve with traditional methods or require extremely complex support designs;

b) Hollow or porous structures: high-porosity scaffolds for tissue engineering, or organ models containing internal cavities, which better promote cell growth and nutrient exchange;

c) Delicate overhanging geometries: heart valves, complex biosensors, micro-mechanical components, etc., which may have fragile overhanging portions or complex curved surface structures that are difficult to process with traditional methods.

VAM technology has demonstrated broad application potential across robotics, healthcare, consumer electronics, and pharmaceuticals. In particular, within healthcare and biomedical engineering, VAM enables the fabrication of precision optical components, on-demand customized eyeglasses and contact lenses, experimental photonic devices, and laser implants, as well as the functional upgrading of existing devices through overprinting strategies that integrate additional functional layers or embedded electronic circuits. Beyond optical and photonic applications, VAM has been increasingly explored for customized surgical instruments, patient-specific implants and prostheses, and bioprinting of tissues and organs aimed at regenerative medicine and transplantation, thereby offering notable flexibility for precision medicine and personalized healthcare.

The volumetric fabrication paradigm provides several important advantages for biofabrication, including ultrafast curing that reduces fabrication time from hours to seconds, support-free manufacturing of complex internal and overhanging geometries within a liquid medium, preservation of cell viability even at high cell densities, and isotropic mechanical properties arising from single-step curing. Nevertheless, despite these compelling strengths, the translation of VAM from proof-of-concept demonstrations to robust clinical-scale applications faces critical challenges, particularly associated with bioink formulation. Current CAL and most VBP approaches predominantly rely on single-component, optically transparent bioinks, leading to constructs with homogeneous and isotropic material and cellular properties. This intrinsic limitation hampers the faithful replication of the hierarchical, multi-material, and spatially heterogeneous architectures inherent to native tissues, including graded tendon–bone interfaces and complex vascular networks, an issue that Section 4.2 will analyze in depth.

2.1.3 Holographic Bioprinting: Towards Spatially Heterogeneous Volumetric Construction

To address the homogeneity limitation of current VBP, holographic bioprinting technology incorporating wavefront engineering has emerged, with its core principle being the utilization of light or acoustic wave interference to achieve precise energy distribution in three-dimensional space. These methods aim to inject controlled heterogeneity into the volumetric printing process by using light or acoustic interference patterns to pre-organize materials or cells before curing.

Although tomographic VBP has successfully broken through the speed limits of macroscopic tissue construction, current implementation schemes primarily rely on single-component hydrogel precursors, resulting in fabricated constructs that typically

exhibit isotropic homogeneous characteristics (Homogeneity). Research by Gehlen et al. noted that this homogeneity limitation represents one of the main obstacles to clinical translation of current volumetric bioprinting technologies^[53].

Acoustic holography technology has achieved breakthrough advances in recent years. Melde et al. first proposed the concept of acoustic holograms in 2016^[54], establishing the theoretical foundation for this field. The emerging acoustic holography-assisted optical 3D printing technology reported by Zhang et al. developed an acoustically transparent and programmable metamaterial system that utilizes interference potential wells (acoustic radiation force fields) formed by ultrasonic waves in fluid fields to manipulate cells or micro/nanoparticles into arbitrary preset patterns in three-dimensional space before second-scale photocuring occurs^[55]. Figure. 4(c-d) shows the acoustic holography-assisted printing technology: utilizing Physics-Informed Neural Network (PINN) algorithms to optimize acoustic field distribution, programmable three-dimensional acoustic potential wells are generated through ultrasonic transducer arrays, achieving non-contact precise manipulation and patterned arrangement of cells or microparticles. This hybrid "acoustic field assembly-optical field curing" strategy endows volumetric printing with considerable control over internal microstructural heterogeneity and cell positioning, providing a potential strategy for constructing functionalized tissues with spatial gradients. The holographic direct sound printing technology reported by Derayatifar et al. further expanded the application boundaries of acoustic holography in additive manufacturing^[56]. This hybrid strategy of "acoustic field assembly-optical field curing" endows volumetric printing technology with considerable control capability over internal microstructural heterogeneity, providing new avenues for constructing functionalized tissues with spatial gradient properties.

The precise definition of internal microstructural orientation and compositional gradients by holographic technology provides a physical foundation for transcending "static manufacturing," driving biofabrication toward the idea of 4D bioprinting—introducing "time" as the fourth dimension above three-dimensional spatial structures, utilizing the responsiveness of smart hydrogels to external stimuli to achieve dynamic reconstruction and functional evolution of tissue scaffolds. By pre-programming local swelling rates or stiffness differences (Local Anisotropy) within materials through holographic or high-precision lithography techniques, printed constructs can undergo self-folding, shape memory, or directional deformation *in vitro* or *in vivo*. The Xolography technology^[51] published by Regehr et al. represents an important breakthrough in this direction, achieving linear volumetric 3D printing. This evolution from "static biomimicry" to "dynamic morphogenesis" enables the manufacture of

deployable scaffolds with minimally invasive delivery capabilities or artificial tissues that simulate physiological mechanical behaviors like heartbeat, representing the highest-dimensional pursuit of biofabrication technology [57].

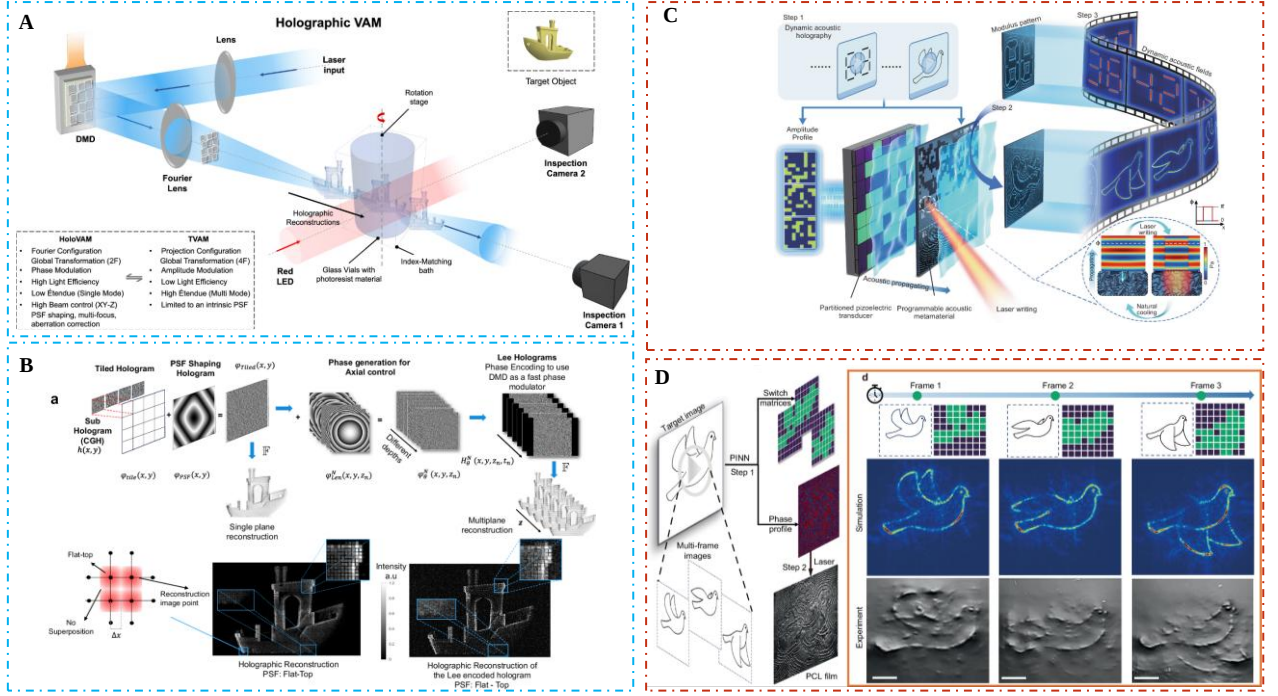


Fig. 4 Holographic 3D printing principle. a) Optical holographic 3D printing principle; b) Optical holographic 3D printing steps. Adapted with permission from ref. [58] Copyright © 2025 Nature CC-BY4.0. c) Acoustic holographic control principle. d) PINN acoustic holographic algorithm and effects. Adapted with permission from ref. [55] Copyright © 2025 Nature CC-BY4.0.

Holographic 3D printing technology utilizes the interference principle of light to fabricate microstructures, while the integration of Computer-Generated Holography (CGH) further enhances its precision and flexibility. The core concept of CGH is to use computer algorithms to generate holograms, which are then presented through a Spatial Light Modulator (SLM). The SLM can modulate the phase and/or amplitude of incident light waves in real-time, thereby generating complex light fields in three-dimensional space, forming multiple focused beams known as three-dimensional Holographic Optical Traps^[58], as shown in Figure.4(a-b). The optical holographic 3D printing principle and steps are: phase modulation of incident laser through a Spatial Light Modulator (SLM) generates computer-designed holographic patterns, forming multi-focal light fields in three-dimensional space for precise curing. Álvarez-Castaño et al. achieved Holographic Tomographic VAM for the first time^[59], combining holographic beam shaping technology with tomographic reconstruction to significantly

enhance the precision and efficiency of volumetric printing. These optical traps can capture and manipulate micrometer-scale particles, including living cells, microbeads, or nanoparticles. During the photocuring process, optical traps can serve as miniature "tools" to precisely position cells in photosensitive materials to predetermined three-dimensional coordinates and induce photopolymerization reactions at these positions, thereby achieving *in-situ* cell curing and simultaneous construction of three-dimensional structures^[60]. This technology enables the manipulation of individual cells with extremely high precision, constructing functional tissues with specific cell arrangements and microstructures. For example, CGH can be used to create complex microfluidic channel networks, highly sensitive biosensors, or three-dimensional cell arrays for high-throughput drug screening, demonstrating broad application prospects in cell biology research, tissue engineering, and micro/nanofabrication^[61].

In summary, the evolution from DLP to CLIP to CAL and holographic methods represents a clear trajectory from optimizing layered manufacturing to transcending it altogether. Table 1 highlights the key comparative advantages and persistent limitations of these core technologies in the context of organ-scale biofabrication:

Table 1. Comparative Analysis of Photocuring Technologies for Organ-Scale Bioprinting

Technology	Key Principle	Max Advantage	Limitation	Cell Viability Challenge
DLP-SLA	Layer-by-layer area projection	High speed vs. point-scanning; Good XY resolution	Staircase effect; Resolution-Build Volume trade-off	Shear stress from peeling; long process time
CLIP	Continuous layer growth via dead zone	Extreme speed; Reduced shear stress	Still layered; Homogeneous material output	Improved, but sedimentation during long builds
CAL/VBP	Volumetric curing via tomographic projection	Second-scale speed; No supports; High cell viability	Homogeneous, isotropic output; Scattering in dense inks	Optimized; challenge shifts to biopink engineering
Holographic VBP	Volumetric curing + wavefront patterning	Spatial heterogeneity & cell patterning	Computational complexity; Multi-material integration	Acoustic/optical forces must be cell-compatible

This analysis underscores that while true volumetric printing (CAL) solves the speed-stress-viability trilemma of layered methods, it creates a new set of challenges: mastering the opto-rheo-biological properties of bioinks to enable high-fidelity, heterogeneous, organ-scale construction.

2.2 Bioink Engineering for Volumetric Curing with Unified Optical and Biological Properties

The success of volumetric bioprinting hinges on the bioink's ability to behave as an effective "photonic medium" for accurate light field reconstruction while also serving as a physiological "cellular microenvironment." This requires an integrated engineering approach moving beyond mere biocompatibility towards simultaneous optimization of optical, rheological, and biological performance. Although true volumetric printing technologies represented by CAL have established absolute advantages in fabrication speed over traditional layer-by-layer techniques, advancing them from "proof-of-concept" to "clinical-scale functional tissue construction" poses notable physical and biological challenges for bioink systems engineering. As Bernal et al. noted in their review^[62], successful implementation of volumetric printing depends on precise reconstruction of light fields in three-dimensional space, which is directly constrained by the optical properties of materials (absorption and scattering) as well as the physical behavior of cells in suspension media (sedimentation and distribution)^{[62][63]}. Therefore, the development of next-generation bioinks must transition from mere "biocompatibility" toward integrated "Opto-Rheo-Biological" performance regulation.

2.2.1 Biocompatibility and Initiation Efficiency of Photoinitiator Systems

Photoinitiators are essential components that initiate photopolymerization reactions in light-based bioprinting, with their biocompatibility and initiation efficiency having decisive impacts on cell viability and print quality^[64]. Elkhoury et al. in their comprehensive review systematically elucidated the classification and application principles of photoinitiators^[65]. Based on their initiation mechanisms, photoinitiators are typically classified into two major categories: Type I and Type II.

a) Type I photoinitiators: These initiators undergo direct homolytic cleavage upon light exposure to generate active free radicals. Representative compounds include phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (BAPO) and 2,4,6-trimethylbenzoyl diphenylphosphine oxide (TPO) derivatives. Among these, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), owing to its excellent water solubility (up to 2.5% w/v) and relatively low cytotoxicity, has become the most widely used Type I photoinitiator in the bioprinting field. However, research has shown that when LAP concentration exceeds 0.1% (w/v) in combination with light exposure, significant cytotoxicity occurs, primarily due to the accumulation of free radicals generated by photoexcitation.

b) Type II photoinitiators: These initiators generate active free radicals through hydrogen abstraction reactions in synergy with co-initiators. Eosin Y is a typical visible light-responsive Type II initiator (excitation wavelength 500-550 nm), commonly used in combination with triethanolamine (TEOA) and N-vinylpyrrolidone (NVP) to form a ternary initiation system, achieving crosslinking within minutes while maintaining cell viability exceeding 85%. Lian et al.^[66] demonstrated that ruthenium-based initiator systems (tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate/sodium persulfate) oxidize tyrosine side chains to form dityrosine covalent bonds, making them particularly suitable for rapid volumetric bioprinting of decellularized extracellular matrix (dECM) bioinks, enabling construction of complex cardiac and meniscus tissue-like structures within 30-60 seconds with cell viability exceeding 85%.

The selection of photoinitiators requires comprehensive consideration of multiple factors including molar absorptivity, excitation wavelength, quantum yield, cytotoxicity, and water solubility. Topa-Skwarczyńska et al. noted that high-performance^[64] and low-toxicity novel photoinitiator systems are key to enhancing printing resolution and cell viability. Wolfel et al.^[67] developed Bioxolography technology based on diphenyliodonium chloride and N-vinylpyrrolidone. This technology utilizes dual-color photopolymerization principles, successfully achieving fabrication of living materials exceeding 1 cm³, with positive resolution of approximately 20 µm, negative resolution of approximately 125 µm, and overall printing time of only minutes, providing a new technical pathway for high-throughput volumetric bioprinting. Determining the optimal photoinitiator concentration in bioinks is not a simple single-variable optimization, but rather a involving multiple interrelated parameters including elastic modulus, pore structure, cell viability, and ROS levels. To systematically elucidate the coupling relationships among these parameters, Table 2 summarizes the comprehensive performance metrics of different photoinitiators within their respective optimal concentration windows.

Table 2: Multi-parameter comparison of photoinitiator systems in GelMA-based bioinks

Parameter	LAP (0.01%)	LAP (0.1%)	I2959 (0.5%)	Eosin Y(0.1 nM)
Excitation wavelength (nm)	365/405	365/405	365 (UV)	500-550 (Visible)
Curing time @ 10 mW/cm ²	4-5 min	2-3 min	3-4 min	4-5 min
Elastic modulus (kPa)	2-3	5-8	3-5	3-4
Pore size (SEM, μ m)	5-15	2-8	4-10	3-8
Crosslink density	Low	High	Medium	Medium
MSC viability at Day 1 (%)	>95%	~60%	~80%	>90%
MSC viability at Day 7 (%)	>90%	~50%	~70%	>85%
Relative ROS intensity	1.0 (baseline)	4-5 $\times$	2-3 $\times$	1.2-1.5 $\times$
Cell spreading area	Large	Restricted	Medium	Large
Focal adhesion number	High	Low	Medium	High
Co-initiator required	No	No	No	TEOA + NVP
Preferred application	Long-term culture	Rapid prototyping	General purpose	Cell-sensitive

Table 2 reveals the performance trade-offs among different photoinitiator systems. Low-concentration LAP (0.01%) maintains >90% MSC viability with minimal ROS levels, but yields hydrogels with only 2-3 kPa elastic modulus, larger pore sizes (5-15 μ m), and relatively low crosslinking density. High-concentration LAP (0.1%) increases modulus to 5-8 kPa with reduced pore size, but causes viability to drop to ~50% with 4-5 $\times$ higher ROS intensity. I2959 at medium concentration (0.5%) shows balanced performance with ~70% viability and 3-5 kPa modulus, though its UV-only activation poses potential DNA damage risks. Eosin Y at optimal concentration (0.1 nM) achieves >85% cell viability with ~4 kPa modulus and low

ROS levels, demonstrating superior overall performance for cell-sensitive applications .

The minimum photoinitiator loading strategy has been demonstrated as an effective approach to address the above trade-off dilemma while achieving multiple objectives. The core concept of this strategy is to strictly control ROS generation by maintaining the lowest possible photoinitiator concentration, while ensuring adequate crosslinking and ideal mechanical properties through precise control of exposure time and light intensity. Specifically, for the LAP system, using an optimal exposure time of 2.5-3 minutes at 10 mW/cm² under 365 nm illumination can achieve crosslinking effects comparable to high-concentration formulations at low initiator concentrations. Experimental data from Dogan et al. demonstrate that this strategy can improve MSC survival rates from 60% to over 85% during a 7-day culture period while maintaining good structural integrity and appropriate elastic modulus of the hydrogel^[68]. This strategy is particularly suitable for tissue engineering applications requiring long-term cell survival, which can be applied in vascularized tissue construction and functional organoid cultivation that require weeks of culture .

2.2.2 Optical Transparency and Refractive Index Matching in Complex Media

Light scattering is the primary obstacle limiting penetration depth and high-fidelity printing in light-based bioprinting (especially volumetric bioprinting)^[69]. S. You et al.^[70] systematically analyzed light field control in scattering materials. When building high cell density or thick tissues, cells themselves act as scattering centers, causing light path deflection and optical energy attenuation, thereby reducing printing resolution and penetration depth. This effect is particularly pronounced in bioinks with high cell loading (e.g., greater than 10⁷ cells/mL), manifesting primarily as Mie scattering, which disrupts precise focusing of the light field and subsequently affects the precision and uniformity of constructs.

To overcome the limitations of light scattering, researchers have proposed the Refractive Index Matching strategy. This strategy aims to minimize light scattering in the medium by adjusting the optical properties of the bioink to match the refractive index of encapsulated cells. S. You et al. demonstrated that the cytoplasmic refractive index typically ranges from 1.36 to 1.39^[70]. By supplementing common bioinks like gelatin methacryloyl (GelMA) with iodixanol or OptiPrep®, the bioink's refractive index can be effectively matched to this range, reducing the scattering coefficient approximately tenfold from 11.76 mm⁻¹ to 1.377 mm⁻¹. Bernal et al. confirmed that this method not only significantly improves printing resolution but also enables achievement of approximately 50 µm feature sizes at high cell densities (100 million

cells/mL), laying the foundation for constructing larger and more complex tissue engineering scaffolds^[71].

Table 3. Optical parameters of cell-laden GelMA bioinks with varying iodixanol concentrations (40 M cells/mL, 405 nm)

IDX Conc. (w/v)	Refractive Index (n)	μ_s (mm^{-1})	μ_s' (mm^{-1})	Anisotrop y (g)	Scatterin g Reductio n	Light Penetratio n Depth
0%	1.333	11.76	0.164	0.986	Baseline	<1 mm
10%	1.355	8.5	0.089	0.978	~28%	~1.5 mm
20%	1.370	4.8	0.046	0.968	~59%	~2.5 mm
30%	1.385	1.377	0.014	0.952	~88% (10×)	>5 mm
35%	1.395	1.1	0.009	0.945	~91%	>6 mm

Table 3 reveals several key trends. First, as iodixanol concentration increases from 0% to 30%, the scattering coefficient μ_s drops substantially from 11.76 mm^{-1} to 1.377 mm^{-1} , achieving approximately 10-fold scattering suppression. This improvement is sufficient to restore clarity to print features that would otherwise be largely blurred by scattering. Second, the anisotropy factor g remains consistently above 0.95, indicating that residual scattering is predominantly forward scattering—where scattered photons continue roughly along their original direction—which is beneficial for maintaining print precision. Monte Carlo simulations demonstrate this effect: at 0% iodixanol, photons diverge extensively after penetrating 1 mm, with spot diameter expanding several-fold; at 30% iodixanol, the photon beam maintains high collimation with minimal spot expansion.

Furthermore, the introduction of photoabsorbers and photoinhibitors provides effective means for precise control of light penetration depth and improved Z-axis resolution. He et al. demonstrated that adding biologically inert dyes as photoabsorbers enables precise regulation of light penetration depth in bioinks^[72], which is particularly important for achieving complex multilayer structures and high-precision microscale features. The simultaneous photoabsorption and free-radical reaction photoinhibition method developed in this study effectively suppresses light scattering effects, enabling

high-fidelity printing of fine structures in diluted hydrogels. This strategy helps achieve finer printing results while maintaining cell viability.

2.2.3 Rheology and Sedimentation Control in Static Volumetric Printing

A unique challenge for VBP like CAL is that the bioink must remain optically clear and physically stable in a static vat for the duration of the printing cycle (seconds to minutes), without the replenishment or agitation common in layer-based systems. Cell sedimentation and maintenance of cell viability during printing represent another core challenge in bioink engineering^[73]. Jain et al. systematically analyzed the impact of cell density on the printability of GelMA bioinks^[74]. During prolonged large-scale printing, cells suspended in bioinks tend to undergo non-uniform sedimentation due to gravity, resulting in uneven cell distribution within printed tissues and subsequently affecting tissue functional homogeneity^[75]. Cell sedimentation can also cause nozzle clogging, affecting the reliability and quality of printing.

To effectively address cell sedimentation, rheological modification has been widely applied in bioink design. Introducing yield-stress fluids as suspension matrices is one effective strategy^[76]. These materials exhibit solid-like behavior under low shear stress, effectively supporting cell suspension and preventing sedimentation; however, when shear stress is applied, their viscosity decreases significantly, enabling smooth flow and printing. Research has demonstrated that Laponite nanoclay (1-2% w/v) and xanthan gum (0.3-0.5% w/v) can both provide optimal yield stress ranges of 1-10 Pa, achieving excellent sedimentation control for over 24 hours while maintaining cell viability exceeding 85%. Liu et al. demonstrated that optimizing bioink rheological properties can significantly reduce cell sedimentation rates and improve cell survival during printing^[77]. The review by An et al. noted that beyond yield-stress fluids^[78], thickening agents or nanoparticles can be added to increase the low-shear viscosity of bioinks, enabling prolonged cell suspension.

VBP technology demonstrates unique advantages in maintaining cell viability. Duquesne et al. demonstrated VBP applications in bone-like tissue construction^[79]. Compared to traditional layer-by-layer printing technologies, VBP can complete curing of entire structures within extremely short "second-scale" timeframes. This rapid manufacturing window minimizes cell exposure time to hypoxic and non-physiological environments, thereby helping to preserve higher cellular metabolic activity and survival rates. Qiu et al. utilized VBP to print functional ultra-soft hydrogel structures within 7-15 seconds, with the low polyvinyl alcohol content (1.5%) printing matrix providing favorable stress-relaxation microenvironment for rapid cell spreading, stem cell differentiation, and matrix mineralization^[80]. This is crucial for constructing tissue engineering scaffolds with complex three-dimensional microenvironments and

maintaining long-term function of encapsulated cells. Based on the detailed discussion of various rheological modifiers above, **Table 4** systematically compares the key performance parameters of commonly used modifiers in cell sedimentation control, providing quantitative guidance for researchers to select appropriate modifiers based on specific application requirements. For VBP, the optimal rheological modifier must provide sufficient yield stress to prevent sedimentation over the print time, yet not significantly increase light scattering^[81].

Table 4. Comparison of five rheological conditions for cell sedimentation control.

Modifier	Concentration Range	Yield Stress (σ_y)	Viscosity@ 1s ⁻¹	Shear-thinning g (n)	Cell Compatibility	Sedimentation Control	Primary Application
Laponite RD	1-2% w/v	1-5 Pa	100-500 Pa·s	0.3-0.5	High (>85% viability)	Excellent (24h+)	GelMA reinforcement
Xanthan gum	0.3-0.5% w/v	1-10 Pa	50-300 Pa·s	0.2-0.4	High (>85% viability)	Excellent (24h+)	Low-cost universal
Carbopol 940	0.5-2% w/v	1-3 Pa	200-800 Pa·s	0.4-0.6	Medium-High	Good	Embedded extrusion bath
Gellan gum	0.3-0.8% w/v	0.5-5 Pa	20-100 Pa·s	0.3-0.5	High	Good	Support bath material
No additive	-	0 Pa	~1 mPa·s	1.0 (Newtonian)	N/A	Poor (minutes)	Short-term printing only

Table 4 compares five rheological conditions for cell sedimentation control. Both Laponite RD (1-2% w/v) and xanthan gum (0.3-0.5% w/v) provide excellent 24-hour sedimentation control with >85% cell viability, operating within the optimal 1-10 Pa yield stress range. Xanthan gum offers a cost-effective universal solution, while Laponite is preferred for GelMA reinforcement. Carbopol serves primarily as an embedded extrusion bath material. The optimal design criteria specify yield stress of 1-10 Pa, extrusion pressure <20 psi, and maximum shear rate <100 s⁻¹ to protect cell membrane integrity.

VBP technology, through its advantages of rapid, layer-less printing, is revolutionizing the biofabrication field^[82]. Overcoming light scattering, cell

sedimentation, and optimizing photoinitiator systems are key to achieving organ-scale, high cell density functional tissue construction. Refractive index matching strategies can effectively reduce light scattering and improve printing resolution. Rheological modification can address cell sedimentation issues, while VBP's rapid printing characteristics also help maintain cell viability. Biocompatible and efficient photoinitiator systems are fundamental to achieving high-precision printing and good cellular function. Akbari and Khademhosseini^[83] noted future research would further explore novel bioink components, advanced optical regulation technologies, and more efficient low-toxicity photoinitiators.

2.3 Intelligent and Adaptive 3D Bioprinting Manufacturing Systems Driven by Computation

The complexities introduced by VBP—managing light scattering in heterogeneous bioinks, ensuring cell placement accuracy, and correcting for optical distortions—necessitate a new level of process intelligence. The next paradigm is the integration of real-time sensing, adaptive control^[84], and AI-driven computation directly into the fabrication loop. With the co-evolution of processing techniques and materials, light-based bioprinting technology has shown significant potential in constructing complex biological structures, leveraging its advantages of high resolution and rapid fabrication. Wonjin Kim^[85] reported the breakthrough progress of DLP bioprinting in organ fabrication resolution, they demonstrate a new potential 3D liver model that can replicate native liver tissues. While Zhang et al.^[86] further elaborated on the synergistic applications of light-based 3D bioprinting and photoactive biomaterials in tissue engineering. With continuous advances in processing and materials, multi-material DLP systems have successfully replicated the mechanical gradients of native tissues. Yang et al.^[87] developed a multi-material DLP bioprinting platform based on PEGDA-AAm bioink, successfully fabricating hydrogel constructs with perfusable networks and heterogeneous mechanical properties, achieving precise replication of mechanical moduli for various biological tissues including bone, liver lobules, and vascular networks. In osteochondral interface regeneration research, researchers utilized bioinks with stiffness gradients to integrally print biphasic scaffolds^[88]. Zhou et al. demonstrated that such scaffolds not only exhibit excellent interfacial bonding strength but also possess the capability to induce cell differentiation, providing potential strategies for integrated osteochondral repair^[89].

Regarding high cell density functional tissue construction^[90], the light scattering effect in high cell density bioinks represents a significant challenge for traditional photocuring technology, leading to reduced printing precision and shape fidelity. Xiang et al.^[91] demonstrated that iohexol can serve as an effective refractive index tuning

agent for high cell density bioprinting. Guan et al.^[92] further confirmed that cell-induced light scattering significantly affects DLP bioprinting precision, while Li et al.^[93] reported an efficient light-based bioprinting strategy using rutin nanoparticle photoinhibitors. To overcome this challenge, the innovative "light-through-suspended-support-bath" strategy has emerged. This method involves printing high-concentration tissue-specific bioinks into transparent yield-stress support baths, utilizing in-situ photocuring technology to fabricate bone-like tissue precursors with high shape fidelity and cell-cell contact. Lee et al. , in their review, noted that this approach effectively addresses the limitations of traditional photocuring at high cell densities^[94]. Bernal et al. further demonstrated that optically tuned hydrogel systems can successfully construct liver-like metabolic biofactories, establishing the foundation for *in vitro* construction of functional solid organs^[71].

2.3.1 Optical Feedback and Closed-Loop Control Systems

To ensure high precision and high-fidelity during 3D bioprinting processes, real-time process monitoring and adaptive control are crucial. Yang et al.^[95] systematically elucidated the application of Optical Coherence Tomography (OCT) for *in-situ* process monitoring and automated multi-parameter evaluation in extrusion-based bioprinting. Regarding real-time process monitoring, integrating OCT and high-speed cameras represents key technologies for process monitoring. Tashman et al. demonstrated the application of OCT for *in-situ* volumetric imaging and analysis of FRESH 3D bioprinted constructs^[96]. As a non-contact, high-resolution imaging technique, OCT can monitor real-time changes in bioink refractive index, layer thickness, porosity, and internal structural integrity during gelation^[97]. High-speed cameras can capture the dynamic behavior of bioink deposition and curing during printing, enabling timely detection of material deposition errors and structural defects. Yang et al. further developed the 3D extrusion-based bioprinter-associated OCT (3D P-OCT) system^[95], achieving real-time defect detection, wide-field image mosaicking through the simplified iterative closest point algorithm, and reducing deviations between printed parts and design models to acceptable ranges through feedback control.

Regarding adaptive optics correction, cells and biomolecules in bioinks during deep tissue construction cause light scattering, leading to degraded spot quality and affecting printing resolution and precision. He et al. developed a simultaneous photo absorption and free-radical reaction photoinhibition method that effectively enhances the fidelity of light-based bioprinting^[72]. Adaptive optics technology, through combiners and deformable mirrors, can correct wavefront aberrations caused by turbid bioinks in real-time. Levato and Lim discussed various application modes of light in biofabrication^[98]. Wavefront sensors can precisely measure deformations in incident

light wavefronts, while deformable mirrors dynamically adjust their surface shapes according to measurement results, thereby compensating for optical path differences. Guo et al.^[99] demonstrated that deep learning methods can significantly improve the phase retrieval accuracy of Shack-Hartmann wavefront sensors—a technique that can be transferred to the bioprinting field to maintain high-quality spots in the deep layers of bioinks. This technology can effectively enhance the precision of light-based bioprinting in complex, multilayer structures. Florczak et al. demonstrated that the combination of multi-material integration with advanced optical correction techniques is particularly suitable for complex scaffold fabrication scenarios requiring fine control of cell arrangement and microstructure formation^[100].

2.3.2 Deep Learning-Assisted Light Field Reconstruction

Deep learning has become indispensable for overcoming the central optical challenge in bioprinting: light scattering. This is especially critical for high-density volumetric printing and holographic patterning^[101]. Rather than relying solely on physical refractive index matching, deep learning models offer a computational correction strategy. They can learn the complex nonlinear relationship between the intended light pattern and the distorted pattern after passing through a scattering bioink. Deep learning, particularly Convolutional Neural Networks (CNN), has demonstrated powerful application potential in the bioprinting field, especially in light field reconstruction, printing parameter optimization, and defect compensation. The study by Meyer et al. demonstrated how ML could handle the complexity of biomaterial design^[102], efficiently managing datasets of 50–100 polymers. AI's role extends beyond pre-print compensation to in-situ adaptation. Ng et al. provided in-depth analysis of the application prospects of deep learning in the fabrication and maturation of 3D bioprinted tissues and organs^[103], proposing practical guidelines for integrating deep learning into image processing and segmentation, printing parameter optimization and *in-situ* correction, and tissue maturation process optimization.

Traditional hologram computation methods are typically computationally intensive and time-consuming, while CNNs can learn the complex nonlinear relationships between light fields and printing results, thereby accelerating the hologram computation process and achieving faster printing speeds. Deep learning models can optimize projection light dose distributions, dynamically adjusting illumination intensity and exposure time according to bioink characteristics and target structure requirements. Guan et al. developed a deep learning-based method using a learning-based data augmentation strategy that requires only a small number of training samples to generate deformed masks for compensating scattering effects in cell-laden bioinks^[92]. This study further utilized a 3D printer simulator as a data augmentation

tool, reducing required training samples by 10-fold and significantly improving intra-layer printing resolution in DLP bioprinting. This approach exemplifies "computation as fabrication," where the printed object is not defined by the original design file, but by a computationally optimized pattern that accounts for the biological medium's interference, ensuring the final cured structure matches the intended design. Li et al. confirmed that such optimization helps improve printing efficiency while avoiding over-curing or under-curing, ensuring structural integrity and cell viability^[93].

The presence of cells and biomaterials causes optical inhomogeneity in bioinks, leading to light scattering effects (primarily manifested as Mie scattering caused by refractive index mismatch between cytoplasm and external hydrogel environment, as well as Rayleigh scattering caused by nuclei and organelles), which subsequently affects printing resolution and structural fidelity. Deep learning models can predict artifacts caused by light scattering based on bioink composition, cell density, and optical path parameters, and generate corresponding compensation strategies. Specifically, by training CNNs through genetic algorithms and neural networks to identify and correct scattering artifacts, projection light patterns can be optimized to effectively counteract the effects of light scattering on printing results, thereby achieving high-precision bioprinting in deep and high cell density environments.

In summary, intelligent and adaptive 3D bioprinting manufacturing systems, through the integration of optical feedback, closed-loop control, and deep learning-assisted light field reconstruction, are progressively overcoming the limitations of existing bioprinting technologies, particularly in complex heterogeneous tissue construction, high cell density functional tissue fabrication, and deep structure printing. Lee et al. ^[94]systematically summarized the clinical application progress and prospects of 3D bioprinting for engineered tissue constructs and patient-specific models. Maharjan et al. ^[94]emphasized that the combination of advanced 3D imaging and organoid bioprinting technologies has opened new avenues for biomedical research and therapeutic applications. Bhardwaj et al. ^[105] noted that bioprinted *in vitro* tissue models are emerging as a new platform for developing therapeutic interventions and disease modeling. These cutting-edge technologies provide powerful tools for the *in vitro* construction of functional tissues and organs in the regenerative medicine field, and are expected to drive further development of personalized medicine and drug screening models.

2.4 Synthesis and Future Outlook of Organ-Scale Volumetric Bioprinting

VBP has effectively resolved long-standing limitations in fabrication speed and cell viability, but its rapid development has shifted the primary bottleneck from hardware performance to material intelligence and computational complexity. Future progress hinges on moving from homogeneous constructs toward programmable heterogeneity, requiring multi-material volumetric strategies enabled by sequential printing, spatially selective curing chemistries, or the integration of complementary techniques including acoustic holography and multi-wavelength volumetric exposure.

At the organ scale, the dominant challenges center on vascularization and functional maturation. The fabrication of perfusable, hierarchical vascular networks remains the field's central objective, and volumetric bioprinting is uniquely suited to generate the required complex and support-free architectures. Realizing this potential, however, depends on advanced bioink systems that support simultaneous printing of sacrificial vascular templates and surrounding tissue, or "4D volumetric bioprinting" approaches in which printed channels dynamically mature into functional endothelium. Crucially, structural fidelity alone is insufficient; clinically relevant constructs require integration with biomanufacturing systems that enable long-term maturation under controlled mechanical, perfusive, and biochemical conditions.

As volumetric bioprinting systems grow in complexity, empirical trial-and-error becomes impractical, making standardization and *in silico* validation essential for translation. High-fidelity digital twins incorporating multiphysics models of light transport, fluid flow, cell mechanics, and reaction kinetics will be critical for predictive optimization and reproducibility^[106]. Collectively, volumetric bioprinting marks a paradigm shift from layer-by-layer fabrication to true volumetric synthesis, with the convergence of wavefront engineering, intelligent bioinks, and AI-driven manufacturing defining a credible pathway toward on-demand, patient-specific fabrication of functional living organs. While VBP provides the macroscopic architecture and perfusable networks essential for organ-scale viability, the functional units of tissues—the cellular microenvironments—require a level of precision beyond what volumetric methods alone can achieve. This gap is filled by two-photon polymerization, which we explore in the next chapter.

3 Two-Photon Polymerization For Tissue Engineering: Subcellular-Scale Precision Manufacturing

Having established how volumetric bioprinting enables rapid, organ-scale fabrication, the discussion now turns to microscale. Two-photon polymerization (TPP) offers the

subcellular resolution necessary to engineer the niches that direct cell behavior, complementing the macroscopic control provided by VBP. The ultimate objective of tissue engineering and regenerative medicine is the reliable reconstruction of functional, living tissues that recapitulate native physiological performance. This objective is guided by a well-established paradigm: cellular behavior, phenotype, and fate are exquisitely sensitive to the three-dimensional physical and biochemical characteristics of their surrounding microenvironment^[107]. An optimal cellular niche must therefore extend beyond the mere provision of soluble biochemical factors and instead reproduce the intricate, hierarchical architecture of the native extracellular matrix (ECM), which supplies essential topographical, mechanical, and spatial cues across multiple length scales.

Conventional scaffold fabrication strategies—including solvent casting/particle leaching, gas foaming, and freeze-drying—have historically provided foundational platforms for 3D cell culture and early tissue engineering efforts. However, these approaches are inherently limited by their stochastic nature, offering only coarse control over critical architectural parameters including pore size distribution, geometry, interconnectivity, and global microscale spatial organization^[108]. This lack of deterministic architectural control inevitably results in heterogeneous cell seeding, poorly defined cell–cell and cell–matrix interaction profiles, and the formation of non-uniform gradients of nutrients, oxygen, and metabolic waste. As a consequence, tissues engineered using such scaffolds often exhibit compromised functionality, poor reproducibility, and limited translational relevance.

Subsequent advances in microfabrication—including soft lithography, micro-electrodeposition, and micro-molding—introduced a higher degree of spatial precision but largely remained confined to a two-dimensional (2D) or quasi-2.5D fabrication paradigm, namely approaches based on planar micropatterning with limited out-of-plane relief or stacked patterned layers, rather than direct free-form fabrication of arbitrarily interconnected 3D structures. These methods typically rely on the fabrication of planar micro-patterned layers that are later stacked or assembled, a strategy that

frequently suffers from imperfect vertical integration and discontinuous interfaces. As a result, achieving seamless 3D interconnectivity and complex internal geometries remains challenging. Thus, a critical technological gap persisted: the absence of a fabrication modality capable of direct, arbitrary 3D structuring with resolution commensurate with cellular and subcellular dimensions.

The emergence of TPP, also known as direct laser writing or multiphoton lithography, has fundamentally altered this landscape, effectively bridging the divide between nanofabrication and cell biology. As emphasized by Shangting You et al.^[109], TPP is a laser-based additive manufacturing technique that enables maskless, free-form fabrication of complex 3D micro- and nanostructures, with feature sizes routinely below one micrometer and extending into the sub-100 nm regime. This extraordinary capability originates from the nonlinear optical phenomenon of two-photon absorption (TPA). By exploiting this effect, TPP enables true volumetric, “voxel-by-voxel” writing within a photosensitive material, with polymerization confined exclusively to the focal volume of a tightly focused femtosecond laser beam^[110].

This intrinsic 3D confinement liberates fabrication from the layer-by-layer constraints that characterize most additive manufacturing techniques, allowing the realization of complex, unsupported, and high-fidelity 3D geometries that were previously inaccessible or prohibitively difficult to produce. Moreover, TPP’s compatibility with an ever-expanding palette of materials—ranging from mechanically robust synthetic photoresists to biocompatible hydrogels and even native biological macromolecules—has unequivocally positioned TPP as the preeminent platform for subcellular-scale precision manufacturing. These attributes have catalyzed TPP’s rapid adoption for the construction of biomimetic cellular niches, the engineering of advanced organotypic and disease models, and the development of sophisticated biohybrid microsystems, thereby accelerating both fundamental biological discovery and translational innovation (Shown in Figure 5) .

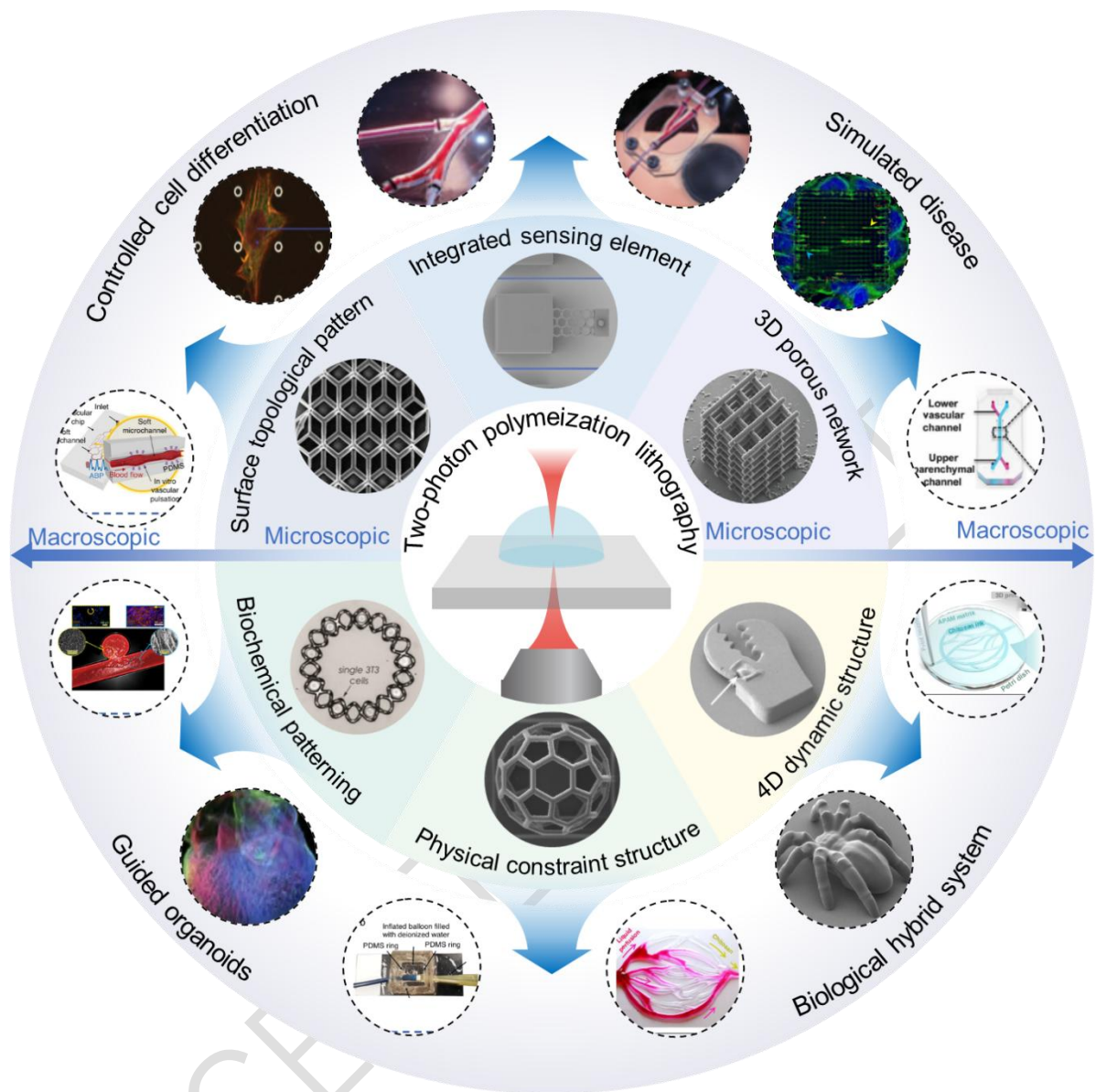


Fig. 5 This schematic illustrates the translational pipeline enabled by two-photon polymerization. TPP provides three-dimensional deterministic manufacturing with subcellular resolution (Layer 1: TPP platform). This capability is deployed to fabricate a suite of micro/nano-scale cell culture scaffolds (Layer 2: Scaffold Library), including 3D porous networks for cell distribution, surface topographies for contact guidance, confinement structures mimicking physiological spaces, and biochemical patterns for spatial signaling Adapted with permission from ref.^[111] Copyright © 2017 WILEY. After culturing various cells on the printed tissue scaffolds, functional differentiation occurs^[112], leading to the formation of more complex tissue structures such as organoids. Building on this foundation, the approach advances to the direct printing of organoid

structures, as illustrated in the third circle of the figure. These engineered microenvironments support the culture of various cell types, which can subsequently be integrated via bioassembly or volumetric bioprinting to build complex, heterogeneous tissue constructs (Layer 3: Cell Culture Models and Tissue Integration). Representative cell culture models (Layer 3) include: neural networks on topographically patterned scaffolds; Endothelialized vascular structures^[113]; Hepatic organoids maintaining metabolic function^[113]; and tumor spheroids in confined microenvironments.

3.1 Fundamental Mechanisms of Two-Photon Polymerization

The exceptional spatial resolution and genuine three-dimensional design freedom characteristic of TPP arise directly from its underlying physical mechanism, which stands in marked contrast to conventional single-photon photopolymerization processes^[114]. In a typical TPP system, a mode-locked femtosecond-pulsed laser—most commonly operating in the near-infrared (NIR) spectral range (approximately 780–850 nm)—is tightly focused through a high-numerical-aperture objective into a volume of photosensitive resin containing TPA-capable photoinitiator molecules^[115]. Two-photon absorption is a nonlinear optical process in which a photoinitiator molecule simultaneously absorbs two lower-energy photons within an extremely short temporal window. The combined energy of these photons is equivalent to that of a single higher-energy photon, typically in the ultraviolet range, and only when this combined energy exceeds the polymerization threshold are reactive species generated to initiate monomer or macromer cross-linking. Crucially, the probability of TPA scales with the square of the incident light intensity. As a result, polymerization occurs with appreciable probability only at the very center of the laser focus, where photon density is maximal, and rapidly diminishes with distance from this focal point.

This nonlinear intensity dependence confines polymerization to a sub-femtoliter voxel that is substantially smaller than the wavelength of the writing light itself. Regions of the resin that are traversed by the laser beam but lie outside the focal voxel experience

insufficient intensity to trigger TPA and therefore remain unpolymerized. This precise volumetric confinement is the cornerstone of TPP, enabling true 3D writing without physical masks, layer-by-layer exposure, or intermediate recoating steps. Structures can be fabricated anywhere within the bulk of a resin simply by scanning the laser focus in three dimensions^[116].

The strategic use of NIR light is particularly advantageous for biological applications. Most biological constituents—including cells, proteins, nucleic acids, and common hydrogel precursors—exhibit minimal linear absorption in this spectral window, resulting in reduced scattering and, critically, minimized photothermal damage. This optical transparency enables TPP to perform fabrication within pre-existing biological environments, such as inside cell-laden hydrogels or sealed microfluidic devices containing living cells. This unique “writing-inside-matter” capability underpins important applications, including *in situ* scaffold fabrication around cell aggregates and the integration of functional microstructures directly within established 3D tissue models.

3.2 Photosensitive Materials for Two-Photon Biofabrication

The versatility and scientific impact of TPP are greatly amplified by its compatibility with a broad and continuously expanding materials portfolio. Material selection not only dictates achievable resolution and mechanical performance but also governs biological functionality, cytocompatibility, and long-term integration.

3.2.1 High-Resolution Photoresists and Organic–Inorganic Hybrids.

For applications requiring maximal structural fidelity, dimensional stability, and surface smoothness, synthetic photoresists remain the materials of choice. Widely used systems like SU-8, the IP-series (e.g., IP-S, IP-L), and organic–inorganic hybrid materials likeOrmocer® enable feature sizes at or below 100 nm^[117]. Ormocer®, a silicate-based polymer network modified with polymerizable organic groups, combines exceptional mechanical robustness, chemical stability, and optical transparency. These attributes make it particularly suitable for long-term cell culture, master structures for

replication, and mechanically durable implantable devices^[118]. Such materials have been extensively employed to fabricate highly ordered 3D lattice architectures—including woodpile, cubic, and hexagonal geometries—to systematically interrogate the role of pore size and geometry in regulating cellular processes. Exemplary studies have demonstrated their utility in directing hMSC osteogenic differentiation^[119], modeling cancer cell invasion through confined microchannels^[120], and guiding neural network formation^[121]. Z. Yao et al^{Error! Reference source not found.} demonstrated that scaffold type dictates overall cell shape including elongated and triangular, while pore size critically influences nuclear morphology and differentiation potential. Larger pores promoted more rounded nuclei and enhanced osteogenic differentiation. The work provides novel insights into designing 3D microenvironments for controlling stem cell fate in tissue engineering. Alexandra Bran et al^[122] employed TPP to fabricate woodpile-like SU-8 scaffolds with submicrometer-scale pores, mimicking the confined spaces of tumor microenvironments. The study systematically investigated how a collagen coating and pore size (0.70–1.66 μm) influence the adhesion, invasion, and focal adhesion dynamics of A375 melanoma cells. The work establishes a novel, architecturally defined 3D platform for quantitatively analyzing cancer cell invasion mechanisms, with implications for developing personalized therapeutic strategies. Rengaraj A. et al. engineered a microscale niche for pancreatic tumor cells by combining 3D-printed microscale scaffolds with a bioactive layer-by-layer polyelectrolyte film coating containing tunable matrix stiffness and bound signaling proteins to simulate early-stage metastatic pancreatic cancer tissue^[124]. Angelo Accardo et al. fabricated high-precision 3D polymeric freestanding scaffolds by multiphoton direct laser writing and combined them with advanced 3D fluorescence imaging to study the growth, 3D colonization, and neuritic extension of neuroblastoma cells in biomimetic microenvironments^[125]. Beatriz N. L. Costa et al. reported^[126] the interaction of bone marrow mesenchymal stem cells (BM-MSCs) with 3D microscaffolds fabricated by TPP. Woodpile structures made from polymers IP-S, SZ2080 and hydrogel PEGDA were tested. Larger gap sizes 100 μm promoted higher cell proliferation and migration compared to smaller gaps 25 μm . Functionalization

with fibronectin improved cell adhesion. The scaffolds show promise for creating 3D models of bone marrow disorders and for co-culture studies in tissue engineering.

3.2.2 Biofunctional Hydrogels.

To better approximate the soft, hydrated, and bioactive milieu of native tissues, photocrosslinkable hydrogels are increasingly employed^[127]. GelMA derived from denatured collagen and retaining intrinsic RGD motifs, is among the most prominent examples. Its mechanical stiffness and degradation behavior can be precisely tuned by controlling methacrylation degree and polymer concentration^[128]. Complementary platforms include poly(ethylene glycol) diacrylate (PEGDA), which provides a bio-inert and highly customizable matrix, and methacrylated hyaluronic acid (MeHA), a principal ECM glycosaminoglycan. Through modulation of laser dose, scanning strategy, and macromer concentration, the elastic modulus of these hydrogels can be adjusted over several orders of magnitude—from approximately 100 Pa to beyond 100 kPa—allowing faithful matching of tissue-specific stiffness profiles. Researchers^[129] introduced a step-growth photopolymerization resin without additives, enabling tunable soft-to-stiff 3D microstructures via two-photon laser printing by adjusting exposure dose, spanning over two orders of magnitude in Young's modulus for multi-material applications.

Recent advances have pushed GelMA-based TPP into the sub-200 nm resolution regime, enabling the replication of fine biological architectures for capillary-scale networks. He et al.^[130] reported the fabrication of GelMA hydrogel scaffolds with feature sizes down to about 200 nm, successfully constructing complex structures that mimic mouse vasculature. Neuronal cells were subsequently seeded and cultured on these scaffolds, demonstrating their feasibility for *in vitro* tissue engineering applications. Further improvements in resolution and mechanical performance were achieved by Fu et al.^[131], who increased the degree of methacrylation and introduced a secondary crosslinker (PEGDA). This strategy enabled the fabrication of 3D micro-/nano-hydrogel structures with feature sizes below 120 nm while simultaneously enhancing mechanical strength. The authors systematically investigated the effects of

laser power, scanning speed, and layer spacing on structural fidelity. In vitro studies using MC3T3-E1 cells confirmed the cytocompatibility of the printed constructs, indicating their potential for tissue engineering applications. Beyond scaffold fabrication, significant challenges remain in integrating hydrogels into 3D-printed microfluidic cell-culture platforms, particularly with respect to material selection, precise hydrogel positioning, dynamic cell stimulation, and real-time monitoring. Recent reviews have summarized emerging solutions to these challenges and outlined future directions toward more functional and integrated hydrogel-based microphysiological systems^{Error! Reference source not found.}.

3.2.3 Native Biopolymers and Composite Smart Materials.

A particularly compelling frontier is the direct TPP of unmodified, native biopolymers, thereby eliminating the need for synthetic cross-linkers. Seminal work by Gebinoga et al. demonstrated that intense femtosecond laser irradiation can directly cross-link proteins between collagen I and fibrinogen, as well as complex biological fluids including serum^[113]. This approach preserves native bioactivity, ligand presentation, and enzymatic degradability. In parallel, TPP readily accommodates composite and stimuli-responsive systems. The resulting structures are water-insoluble, bioactive, and can immobilize cells. The technique enables "cell gluing" and the creation of biomimetic microenvironments, showing potential for advanced 3D tissue models, organ reconstruction, and applications in regenerative medicine and stem cell research. Functional nanoparticles, for antimicrobial activity, magnetic actuation, or therapeutic enhancement, can be incorporated, while polymers poly(N-isopropylacrylamide (pNIPAM) enable 4D structures that undergo reversible, stimulus-induced shape changes. TPP facilitates the creation of advanced composites and "smart" materials. Santos et al.^[132] develops novel acrylic resin nanocomposites incorporating Brazilian red propolis nanoparticles (RPNP) for wound dressing. The materials exhibit potent antibacterial activity against *S. aureus* and tunable drug release profiles. Microgroove scaffolds fabricated via two-photon polymerization support potential applications in

advanced wound healing. Stimuli-responsive polymers pNIPAM can be printed via TPP to create 4D dynamic structures that change shape (e.g., swell, bend, curl) in response to environmental triggers like temperature, enabling dynamic cell culture environments or soft micro-robotics, as explored by Yarali et al.^[134]. By tuning printing parameters and material composition, reversible shape morphing is achieved and modeled via finite element analysis, demonstrating potential for soft robotics and microfluidics.

3.3 Three-Dimensional Scaffold Architectures via Two-Photon Polymerization in Cellular Microenvironment

The true power of TPP resides in its ability to function as a “three-dimensional pixel editor” for cellular microenvironments, enabling the direct translation of digital design blueprints into physical scaffolds with feature dimensions that are comparable to, or even smaller than, those of cellular and subcellular components. This notable level of control allows researchers to systematically interrogate how discrete architectural parameters act not merely as passive supports, but as active physical directives that instruct cellular behavior, organization, and fate (Shown in Figure 6).

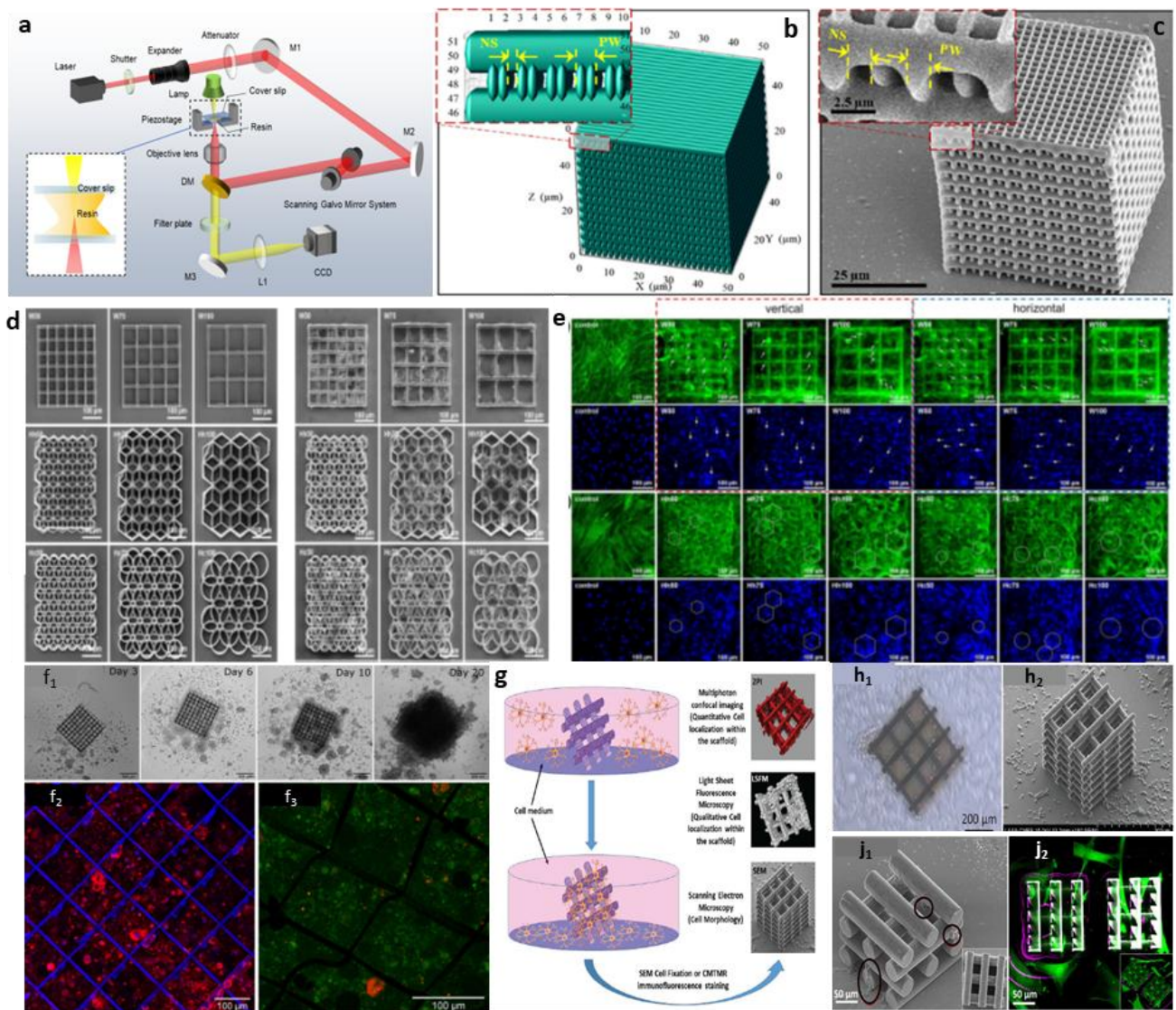


Fig. 6 (a) Schematic optical path of the two-photon micro-nanofabrication setup. Adapted with permission from ref. ^[121] Copyright © 2024 Elsevier. (b) Design of biomimetic cell culture scaffolds; (c) SEM image. Adapted with permission from ref. ^[122] Copyright © 2025 American Chemical Society. (d) SEM images of the 3D scaffolds and hMSCs cultured for 6 days on scaffolds. Structures from top to bottom: woodpile, honeycomb (hexagonal), and honeycomb (circular) scaffolds. (e) Fluorescent images of hMSCs cultured for 6 days on woodpile scaffolds. Nuclei are stained blue with DAPI, actin filaments green with phalloidin. Adapted with permission from ref. ^[124] by open access CC-BY4.0. (f) Culture of PANC1 cells on a bioactive scaffold loaded with 5 μg/mL fibronectin. (f₁) Optical microscopy images showing cell growth after direct culture for 3, 4, 10, and 20 days. (f₂) Confocal microscopy images after 11 days in culture with nucleus stained blue (DAPI) and cytoskeleton stained red (rhodamine). (f₃)

Live/Dead assay after 20 days in culture: live cells labeled green, dead cells labeled red. Adapted with permission from ref.^[124] Copyright © 2022 American Chemical Society. (g) Experimental approach for cell colonization of the 3D scaffold and multi-technique imaging based on SEM, LSM, and TPP. (h) Optical microscopy and SEM images of the scaffold. (j) Images of cell scaffolds with different types of woodpile stacking structures. Adapted with permission from ref.^[111] Copyright © 2017 WILEY CC-BY4.0.

3.3.1 3D Porous Networks and Mechanical Gradients.

By fabricating lattice architectures with precisely defined pore sizes—ranging from a few micrometers to several hundred micrometers—TPP enables deterministic control over cellular confinement, spatial organization, and the diffusion of soluble factors. Seminal studies by Zheyu Yao et al.^{Error! Reference source not found.} and Costa et al.^[126] demonstrated that pore size within woodpile scaffolds directly modulates nuclear morphology in human mesenchymal stem cells (hMSCs). Specifically, larger pores promote a more rounded nuclear geometry, which correlates strongly with enhanced osteogenic differentiation, thereby establishing a direct mechanistic link between spatial architecture, nuclear mechanics, and lineage-specific gene expression. Beyond uniform architectures, TPP uniquely enables the fabrication of continuous mechanical gradients within a single construct by spatially modulating laser exposure dose. Such gradients provide an exquisitely controlled platform for investigating durotaxis—directed cell migration along stiffness gradients—within a fully three-dimensional context. This study introduces a step-growth photopolymerization resin without additives, enabling tunable soft-to-stiff 3D microstructures via two-photon laser printing by adjusting exposure dose, spanning over two orders of magnitude in Young's modulus for multi-material applications.

3.3.2 Surface Topographies and Contact Guidance.

The capacity to pattern submicron-scale surface features—including ridges, grooves, pits, and pillars—onto both planar and curved 3D substrates allows for precise

regulation of contact guidance phenomena. Cells actively sense these topographical cues, reorganizing their cytoskeleton, reorienting nuclear alignment, and adopting anisotropic morphologies that mirror the underlying pattern. Jiaxi Song et al.^[135] demonstrates controlled cell alignment using two-photon direct laser writing (DLW) to pattern 2D and 3D hydrogel microstructures. By alternating laser scan speeds, alternating stiff and soft regions are created in cell-adhesive hydrogels, guiding hMSC cytoskeleton alignment. A width of 5 μm for these patterns induced the highest alignment. Combined with cell-repellent hydrogels, the method enables selective cell adhesion and precise organization on flat and curved 3D surfaces, offering a versatile platform for tissue engineering applications requiring directed cell growth. Jeon et al.^[136] uses TPP to fabricate high-aspect-ratio polymer ridge patterns for investigating contact guidance of fibroblast cells. By adjusting laser focus and power, parallel and orthogonal ridge patterns of varying heights (0.5–8 μm) were created on a single substrate. Cell alignment and elongation increased with ridge height, revealing a threshold near 1 μm for effective contact guidance. The method enables rapid production of complex topographies on large areas, offering a versatile tool for designing biomaterial surfaces to control cell behavior in tissue engineering and single-cell studies. Importantly, such topographical cues can be seamlessly integrated into the internal surfaces of microchannels or onto freestanding microfibers, enabling guided axonal extension and directed cell migration within true 3D environments.

3.3.3 Spatial Confinement for Disease Modeling.

Many essential physiological and pathological processes—including immune cell transmigration, cancer invasion, and axonal pathfinding—occur within highly confined microenvironments *in vivo*. TPP enables the fabrication of microchannels, pores, and confinement cages with dimensions as small as 0.7 μm , faithfully recapitulating these restrictive physical spaces. Bran et al employed two-photon polymerization to fabricate woodpile-like SU-8 scaffolds with submicrometer-scale pores^[122], mimicking the confined spaces of tumor microenvironments. The study systematically investigated how a collagen coating and pore size (0.70–1.66 μm) influence the adhesion, invasion,

and focal adhesion dynamics of A375 melanoma cells. The work establishes a novel, architecturally defined 3D platform for quantitatively analyzing cancer cell invasion mechanisms, with implications for developing personalized therapeutic strategies. These findings underscore the importance of physical boundary conditions in regulating disease progression and highlight TPP-based platforms as more physiologically realistic alternatives for metastasis research and anti-invasive drug screening.

Beyond topographical control, TPP affords unparalleled spatial precision in the placement of biochemical signals. Through strategies like multi-material printing, sequential resin infiltration, and localized photopatterning of bioactive ligands, distinct biochemical cues can be positioned within a single 3D construct with micrometer-scale resolution^[137]. For example, Benjamin Richter et al. ^[137] employed a multi-resin TPP strategy to fabricate micro-scaffolds selectively functionalized with fibronectin and vitronectin in discrete regions. This spatial segregation enabled preferential adhesion and positioning of different cell populations within a shared 3D architecture, representing a critical step toward engineering complex tissue interfaces and multicellular assemblies with controlled cellular hierarchies.

3.4 Important Applications in Cell-Instructive Scaffolds and Biological Interfaces

The convergence of nanoscale architectural precision, extensive material versatility, and increasing biocompatibility has propelled TPP to the forefront of advanced in vitro modeling, enabling the construction of physiologically relevant systems that transcend the limitations of conventional two-dimensional culture (Shown in Figure 7).

3.4.1 Stem Cell Niche Engineering and Organoid Guidance.

Native stem cell niches are defined by tightly regulated 3D architectures that impose specific physical constraints on resident cells. TPP-fabricated scaffolds, exemplified by the “Nichoid”—a complex cubic lattice with subcellular-scale features—provide an artificial 3D niche that more effectively preserves stem cell pluripotency and directs lineage commitment than planar substrates. In parallel, TPP-printed microwells and encapsulating microstructures enable precise control over organoid size, geometry, and

spatial organization. By reducing morphological heterogeneity, these platforms significantly enhance reproducibility and experimental consistency in organoid-based studies^[138]. They develop a submillimeter 3D device via TPP, combining elastic PDMS and gelatin-based hydrogel to mechanically stimulate encapsulated multicellular systems^[138]. Organotypic co-cultures and Medaka retinal organoids are directly printed within the hydrogel and subjected to controlled cyclic stretch. Results show rapid morphological changes and actin remodeling in cells under low-frequency stimulation, demonstrating the potential of this high-resolution platform to mimic *in vivo* mechanical stresses and study mechanobiology in complex 3D microenvironments.

3.4.2 Neural Engineering and Interface Design:

The regeneration and interfacing of neural tissue demand stringent control over neuronal adhesion, polarization, and neurite extension. TPP-fabricated tubular microtowers, fractal architectures, and three-dimensional microcages provide physical guidance cues that promote sustained neuronal adhesion and directed neurite outgrowth, supporting the formation of complex neural networks over extended culture periods. Research by Turunen et al.^[139], Eren et al.^[140], and Accardo et al.^[125] has shown the superiority of 3D TPP scaffolds for neural culture. Turunen et al. develop a 3D neuronal culture platform using TPP direct laser writing to fabricate tubular microtowers from Ormocomp^[139]. The microtowers, featuring intraluminal guidance cues and wall openings, support long-term culture, adhesion, and oriented growth of human pluripotent stem cell-derived neurons. The platform promotes neurite alignment and 3D network formation, offering a promising scaffold for neuroscience and tissue engineering applications. Hohmann et al. explain how 3D microstructures created by direct laser writing and coated with TiO₂ influence osteoblast-like cells^[141]. Results show that specific topographies, particularly with 25 μ m post spacing and horizontal rods, enhance cell proliferation by up to 170% and affect cell morphology, while maintaining osteogenic function. These findings suggest that controlled 3D surface designs could improve implant osseointegration. Accardo et al. compare neural cell (N2A) growth on 3D scaffolds versus 2D micro-ridges and flat surfaces^{Error! Reference source not found.}. The 3D scaffold shows higher cell occupancy and promotes better cell

interconnection and neurite extension, highlighting the advantage of 3D microenvironments for neural cell culture and tissue engineering. This is invaluable for studying neural development, modeling neurodegenerative diseases, and designing next-generation substrates for neural implants that better integrate with host tissue, as demonstrated by Paradowska S et al. using Dental LT Clear resin^[115]. The scaffold supports cell viability, differentiation into cortical neurons and glia, and functional maturation over 120 days. Calcium imaging and network analysis reveal spontaneous neuronal activity and scaffold-promoted local connectivity, offering a reliable model for studying neural development and disease *in vitro*.

3.4.3 Engineered Microenvironments for Functional Cell Culture Models

Cell behavior is governed by a complex microenvironment defined by mechanical properties, oxygen availability, and spatiotemporally heterogeneous biochemical cues. Two-photon polymerization offers an unparalleled level of control over microscale architecture, enabling the independent and precise modulation of these parameters within three-dimensional culture systems. By integrating patient-derived cells with TPP-fabricated scaffolds, it becomes possible to construct highly customized *in vitro* models that better capture native cell–matrix interactions and improve the predictive power of functional assays.

An illustrative example is provided by Koroleva et al.^[141], who combined TPP-printed Ormocomp microscaffolds with bioactive polyelectrolyte multilayer coatings to engineer a physiologically relevant microenvironment for pancreatic cell culture. This modular coating strategy enabled independent tuning of scaffold stiffness and controlled presentation of matrix-associated proteins, including fibronectin and BMP2/4. Experiments using both patient-derived and immortalized cell lines revealed distinct, cell-type-specific responses to biochemical and mechanical cues, underscoring the utility of this platform for high-content screening and functional phenotype analysis.

Beyond solid microenvironments, TPP has been widely applied to the fabrication of structured interfaces that regulate cell transport, polarization, and barrier function. The ability to produce lumenized tubular architectures and porous membranes with micrometer-scale precision provides instructive templates for the formation of organized cell layers. Such approaches have been successfully implemented in biomimetic barrier models that exhibit well-defined tight junctions and quantifiable trans-endothelial electrical resistance (TEER), offering robust platforms for permeability assessment, pharmacological evaluation, and toxicity screening. Marino et al.^[143] demonstrated a 1:1 scale barrier model fabricated by TPP, consisting of 10 μm porous microtubes co-cultured with endothelial and glioblastoma cells, which collectively formed functional permeability barriers and electrically resistive interfaces suitable for in vitro transport studies.

In parallel, TPP has enabled the integration of dynamic mechanical cues into three-dimensional culture systems through the fabrication of elastic, micron-scale components. One representative mechanobiology platform incorporates a TPP-fabricated elastic PDMS membrane coupled to a gelatin-based hydrogel chamber containing retinal organoids. Controlled cyclic pneumatic actuation of the membrane applies reproducible mechanical strain to the organoids, allowing real-time investigation of mechanotransduction processes within a complex 3D tissue context.

TPP further enables the fabrication of intricate, cell-scale wireframe scaffolds that reproduce key geometrical features of the extracellular matrix with submicrometer precision. When combined with optical tweezers or high-resolution micropatterning strategies, these TPP-fabricated architectures allow the deterministic placement of individual living cells at predefined three-dimensional locations. In this way, such platforms can be used to recapitulate spatially organized multicellular microenvironments and to probe cell–cell and cell–scaffold interactions under geometry-defined constraints. Gullo et al. demonstrated this capability by fabricating three-dimensional wireframe microscaffolds designed to mimic ECM-like architectures^[144], followed by the precise positioning of multiple cell types using optical

tweezers. Optical tweezers play a critical role in this context by enabling non-contact, force-controlled manipulation of single cells with micrometer accuracy, thereby decoupling cellular organization from stochastic seeding processes and allowing direct interrogation of how spatial arrangement governs collective cell behavior. Using this approach, the authors investigated cell migration dynamics within confined architectures, identifying a preferred protrusive extension length of approximately 28.6 μm and demonstrating that scaffold geometry can strongly constrain cell spreading, migration pathways, and growth directionality. Only after establishing this controlled multicellular organization did the study reveal important biological constraints: co-culturing multiple cell types at extremely close distances within rigidly confined wireframe geometries led to rapid loss of cell viability, underscoring the intense competition for space and resources, as well as stress arising from highly confined cell–cell communication. Together, these results provide a foundational experimental framework for the rational design of advanced multicellular biohybrid systems. This work provides a foundational approach for designing advanced multicellular biohybrid systems, applicable to microscale robots and biomedical implants.

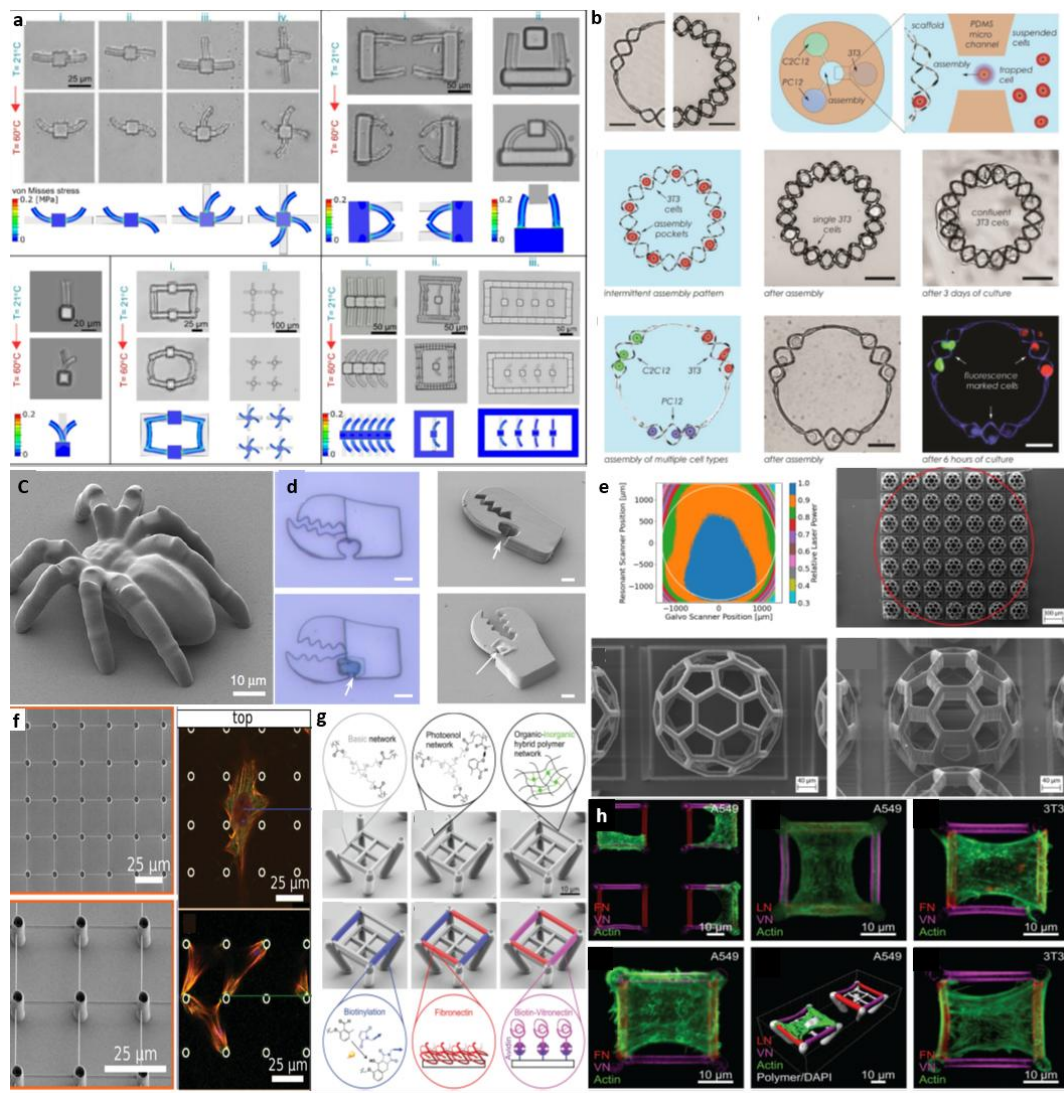


Fig. 7 (a) Optical images of shape-morphing behavior in proof-of-concept applications of 2PP 4D-printed microstructures with their corresponding motion prediction Adapted with permission from ref.^[134] Copyright © 2025 Nature CC-BY4.0.; (b) A scaffold structure specifically designed for 3T3 cell culture, where cells are confined within ring-shaped culture chambers. Fluorescence images showing the growth of cells labeled with different fluorescent proteins, along with an optical image of positioned 3T3 cells and an optical image after three days of culture. Adapted with permission from ref.^[144] Copyright © 2017 Wiley-VCH GmbH; (c) SEM images of the micro-spider; (d) SEM images of the crab claw-muscle system and of the claw after BSA integration (right) Adapted with permission from ref.^[145] Copyright © 2020 Nature CC-BY4.0; (e) Hexagonal cage-structured cell culture scaffolds fabricated via parallel printing into a 7×7 matrix array Adapted with permission from ref ^[146] Copyright © 2024

Elsevier CC-BY4.0; (f) Scanning electron micrographs of square grids with 25 μm spaced posts, investigating the influence of different geometries and grid spacing on cell culture. Adapted with permission from ref.^[140] Copyright © 2014 Wiley-VCH GmbH. (g) Various bioactive ECM proteins conjugated onto a scaffold framework printed withOrmocomp, with distinct colors representing the distribution of different proteins across the scaffold. (h) Examples of 3D microstructures functionalized with distinct ECM proteins after culturing epithelial (A549) or fibroblast (3T3) cell lines. Images are maximum intensity projections of LSM image stacks. Cells were labeled for actin (green). Adapted with permission from ref. ^[137] Copyright © 2016 Wiley-VCH GmbH.

3.5 Biohybrid Microsystems and Functional Living Constructs

A particularly compelling demonstration of TPP's multifunctional potential is the integration of sensing capabilities directly into 3D scaffolds. In a landmark study, van der Sanden et al. ^[147] developed a smart, three-dimensional cell culture matrix using two-photon polymerization. The matrix was fabricated from GelMA and type I collagen, with ruthenium complexes incorporated as both the photo-initiator for TPP and a covalent, bio-optical oxygen sensor. This design enabled the *in-situ*, real-time monitoring of local oxygen tension (pO_2) within the 3D microenvironment. The fabricated matrixes featured a controlled cubic alveoli structure ($50 \times 50 \mu\text{m}$) and demonstrated excellent stability in culture. Using U87MG glioma cells, the system successfully detected dynamic, localized decreases in pO_2 during critical cellular activities, including division, migration, and tumor mass formation, as demonstrated by a 2 to 3-fold increase in ruthenium fluorescence. This work establishes a novel platform for creating advanced 3D co-culture systems with integrated biosensors, promising for detailed studies of cell metabolism and for high-throughput drug screening applications. Such "lab-on-a-scaffold" systems represent a significant conceptual advance toward intelligent, self-reporting in vitro models.

A major limitation of TPP has been its inherently serial writing process, which constrains throughput and scalability. The field has responded to this challenge through

a range of engineering innovations. Spatial light modulators (SLMs) have been employed to generate multiple laser foci simultaneously, enabling holographic or parallel TPP. Zandrini^[148] introduces a spatial light modulator-enabled multi-foci two-photon polymerization system to fabricate 3D “Nichoid” scaffolds for stem cell expansion. The method achieves a fivefold increase in production speed while maintaining structural integrity. Cell viability tests confirm that scaffolds produced by multi-foci and single-focus methods exhibit similar biocompatibility, supporting their use in large-scale regenerative medicine applications. Binder et al. reported over a 50-fold increase in voxel writing rate, enabling rapid fabrication of large arrays of tissue engineering micro-scaffolds without compromising resolution^[146]. Collectively, these advances are progressively shifting TPP from a purely exploratory tool toward a viable platform for specific high-throughput applications, including drug screening and scaffold library production^[149]. However, material development remains constrained, particularly by the limited availability of photoresists that possess high refractive indices and tailored functionalities, such as conductivity. In terms of processing, resolution is fundamentally bounded by the optical diffraction limit; although super-resolution approaches exist, they involve intricate system modifications. Throughput is inherently low due to the serial point-scanning nature of TPP, rendering large-scale or volumetric fabrication time-inefficient. Surface roughness and shape fidelity are critical for optical applications but difficult to consistently achieve at the nanoscale. However, the community has recently proposed using the two-photon grayscale lithography method to improve surface roughness^[150]. Two-photon grayscale lithography (2GL) is an advanced additive manufacturing technique that enhances standard TPP. Modulating the exposure dose while scanning the laser focus across the scanning plane changes the voxel size within the photoresin, resulting in finely controllable size changes of the polymerized voxels. This is the result of synchronizing laser power modulation and high-speed galvo scanning with accurate lateral stage movement. To accomplish this, a grayscale image is converted into a spatial variation of the exposure levels, resulting in different voxel heights being printed in one plane. The key innovation of 2GL is the dynamic, real-time adjustment of laser power during scanning (grayscale exposure).

This allows for continuous modulation of the polymerization degree, enabling the creation of optically smooth surfaces and fine topographical gradients without requiring extremely small layer heights.

Wagner et al.^[151] presents a scalable fabrication chain integrating 2GL, electroplating, and injection molding to transition complex aspherical micro-optics from prototyping to mass production. Using 2GL, a high-resolution diffractive micro-lens array master is 3D-printed, then replicated via nickel electroplating to create a durable mold. Injection molding with cyclic olefin copolymer yields polymer replicas that retain sub-micron accuracy and low surface roughness (~10 nm). Optical testing confirms that replicated lenses match the master's focusing and imaging performance, demonstrating a viable route for high-volume manufacturing of advanced micro-optical systems. Additionally, achieving full photonic bandgaps in the visible spectrum is challenging due to resolution and refractive-index constraints. Lastly, accurate characterization of transparent 3D micro/nanostructures remains non-trivial, as nondestructive metrology tools with sufficient resolution are still underdeveloped.

The future development of two-photon polymerization in biofabrication is expected to follow several converging directions. Continued progress in photoresin design will likely yield advanced bio-inks with enhanced bioactivity, tunable degradation kinetics, and adaptive or self-healing properties, enabling closer replication of native extracellular matrices^[152]. At the same time, the integration of TPP with complementary biofabrication modalities is anticipated to facilitate hierarchical constructs that couple macroscale architecture with microscale functionality. Key enabling techniques include extrusion-based bioprinting, which permits large-scale material deposition, and microfluidic systems, which allow for controlled perfusion and gradient generation. In parallel, the optical transparency of biological tissues in the near-infrared window presents opportunities for *in situ* and potentially minimally invasive *in vivo* fabrication, supporting localized tissue repair or the formation of microstructured medical devices directly within living systems. Finally, the incorporation of artificial intelligence and generative design approaches is expected to shift scaffold development from empirical

trial-and-error toward predictive, outcome-oriented design, enabling the computational optimization of microarchitectures tailored to specific biological functions.

3.6 Conclusion

Three-dimensional TPP has emerged as a powerful and increasingly indispensable platform for precision manufacturing at the subcellular scale. By combining high spatial resolution, genuine three-dimensional design freedom, and a growing range of compatible materials, TPP provides a versatile toolkit for engineering cellular microenvironments. This approach enables systematic investigation of the physical principles that influence cell behavior and supports the rational design of engineered niches capable of directing cell fate, recapitulating key aspects of disease, and interfacing with biological systems at physiologically relevant length scales. To date, TPP has contributed to advances in predictive human *in vitro* models, high-content screening platforms, and the development of biohybrid micro structure devices. As the technology continues to evolve, with ongoing improvements in fabrication speed, the development of responsive and bioactive materials, and closer integration with complementary biofabrication strategies, its role is expected to expand further, supporting advances in regenerative medicine, personalized therapeutics, and fundamental studies of cellular and subcellular biology.

4 Functional in vitro models by 3D Bioprinting for Tissue Engineering and Biohybrid Microsystems

While two-photon polymerization offers high spatial resolution and architectural precision at the micro- and nanoscale, functional tissue and biohybrid systems ultimately require coordination across multiple length scales. Bridging microscale niche engineering with meso- to macroscale structural organization is therefore essential for translating optical biofabrication strategies toward physiologically relevant and application-oriented constructs. As anticipated in Figure 1, this multiscale integration operates within a hierarchical framework spanning three levels: (i) the macro scale, where organ-scale volumetric bioprinting defines the overall structural organization and perfusable architectures; (ii) the meso- to microscale, where two-photon polymerization provides spatially resolved microarchitectural guidance and mechanical boundaries; and (iii) the nanoscale, where scaffold functionalization with bioactive cues modulates cell–material interactions, thereby providing localized biochemical signaling. Together, these scales define a hierarchical multiscale integrated framework that may lead to the fabrication of organs and tissues with physiologically relevant functions.

In this chapter, we first review the biological conditions (requirements) necessary to ensure organ and tissue functionality (Section 4.1) and then analyze the main bioprinting strategies within this hierarchical multiscale integrated framework that may partially or fully fulfill these requirements in the realization of functional organs and tissues (Section 4.2). Specifically, Section 4.2 explains how various biofabrication strategies address these requirements, moving from supportive strategies that primarily maintain cell viability and cell system reproducibility, to enabling strategies that promote self-organization through instructive cues, and finally to emerging strategies that encode system-level architecture, mechanics, and function through multiscale design. Focused examples are presented showing approaches that combine high-resolution light-based printing with volumetric, modular, or hybrid manufacturing schemes to enable system-level functionality, ranging from perfusable tissue models to

biohybrid microsystems. The chapter concludes with a brief synthesis of the key concepts and future prospects for functional bioprinted systems.

4.1 Scientific Evidence Supporting Emerging Biological Conditions for Functional 3D Bioprinting

Building on the detailed analysis of the technical features and recent technological advances of VBP and TPP (as shown in Sections 2 and 3), it emerges that these classes of techniques, through distinct mechanisms, can partially or fully support, and in some cases even transform, the properties of 3D *in vitro* biological systems. However, evaluating these technologies solely from a technical perspective is insufficient to determine their relevance for functional tissue modeling. Instead, here we examine the biofabrication strategies through a biological lens, considering the fundamental cellular requirements that underpin robust and demonstrable organ- or tissue-like functions. Accordingly, in this section we report and derive a set of core biological requirements from seminal literature in organoid and tissue engineering research, which will serve as the analytical framework for evaluating representative studies of bioprinting strategies in the following sections (4.2). In their seminal review, Brassard and Lutolf emphasized that the functional relevance of organoids and related 3D models is not guaranteed by 3D architecture alone^[153]; it critically depends on whether the system achieves and sustains appropriate cell types and states, including lineage hierarchy, maturation, and phenotype maintenance over time in a controlled and reproducible manner. In this view, a functional 3D *in vitro* system requires the ability to maintain physiologically meaningful differentiation states over time of proper lineage-relevant cell populations, avoiding undesired drift toward non-physiological phenotypes during prolonged culture. Based on this information, we summarized this concept as (1) first biological requirement “Lineage-relevant cell composition and stable differentiation states”. Beyond cellular composition and state, Brassard and Lutolf also highlighted that native tissue function relies on precise 3D organization, including compartmentalization, polarity, lumen formation, and zonation, which are features necessary to enable coordinated multicellular behavior. While self-organization can indeed generate

rudimentary architectures driven by stochastic outcomes, the ability to impose a spatial patterning emerges as a key biological requirement to increase functional relevance and to reduce structural variability across constructs (in this review this concept is exemplified by requirement 2: “Reproducible tissue-like 3D architecture and spatial patterning”). In addition, *in vivo* tissue function arises from dynamic interactions among multiple cell types in the context of continuous biochemical gradients, mechanical inputs, and extracellular matrix (ECM) signal, therefore, functional 3D models require microenvironments that actively guide and sustain physiologically relevant intercellular communication. Gjorevski et al., in a landmark paper^[154], showed, for the first time, that organoid patterning can be made predictable and reproducible by designing the microenvironment as an instructive system coupled to programmable tissue geometry. Rather than leaving cells to self-organize in a largely stochastic way, they engineer the initial tissue shape and implement selected ECM cues that locally define where morphogenesis is permitted. This approach generates stable spatial differences in cell packing and mechanical signaling (including YAP activity), which coordinate cell–cell communication pathways such as Notch/DLL1, promote the localized emergence of niche cell types, and ultimately drive the formation of organized crypt–villus-like domains. Overall, this concept can be translated into a third core biological requirement: an instructive microenvironment that enables stable, physiologically relevant cell–cell crosstalk (Requirement 3: Instructive microenvironment enabling stable cell–cell crosstalk). As a final point, Brassard and Lutolf also emphasized that organ- or tissue-like claims must be supported by sustained, measurable, and reproducible functional outputs over time^[153]. Many current models display early functional signatures (e.g., marker expression or partial physiological activity) but fail to maintain these during prolonged culture, while variability in size, architecture, and maturation complicates quantitative benchmarking. Without stable and reproducible functional outputs, even highly organized 3D systems remain limited in translational and predictive value. Accordingly, we summarize this aspect as the fourth core biological requirement: “Sustained, measurable, and reproducible functional outputs over time”

Based on this scientific evidence, in the following section we analyzed seminal studies that generate different types of 3D *in vitro* systems (e.g., organoids^[155] and engineered tissues^[156]) critically examining the role of 3D bioprinting technologies in reaching the system functionality. In our critical analyses, we assigned distinct roles depending on whether the 3D bioprinting technologies (i) support or enable the biological requirements and associated functions (supportive or enabling role), or (ii) actively transform system functionality by introducing structural or functional capabilities that would not be achievable otherwise (defined here as a transformative role, shown in Figure 8). Additionally, we include selected examples of seminal biologically driven approaches that do not rely on bioprinting techniques but provide benchmarks against which future improvements enabled by 3D bioprinting can be quantitatively assessed [157].

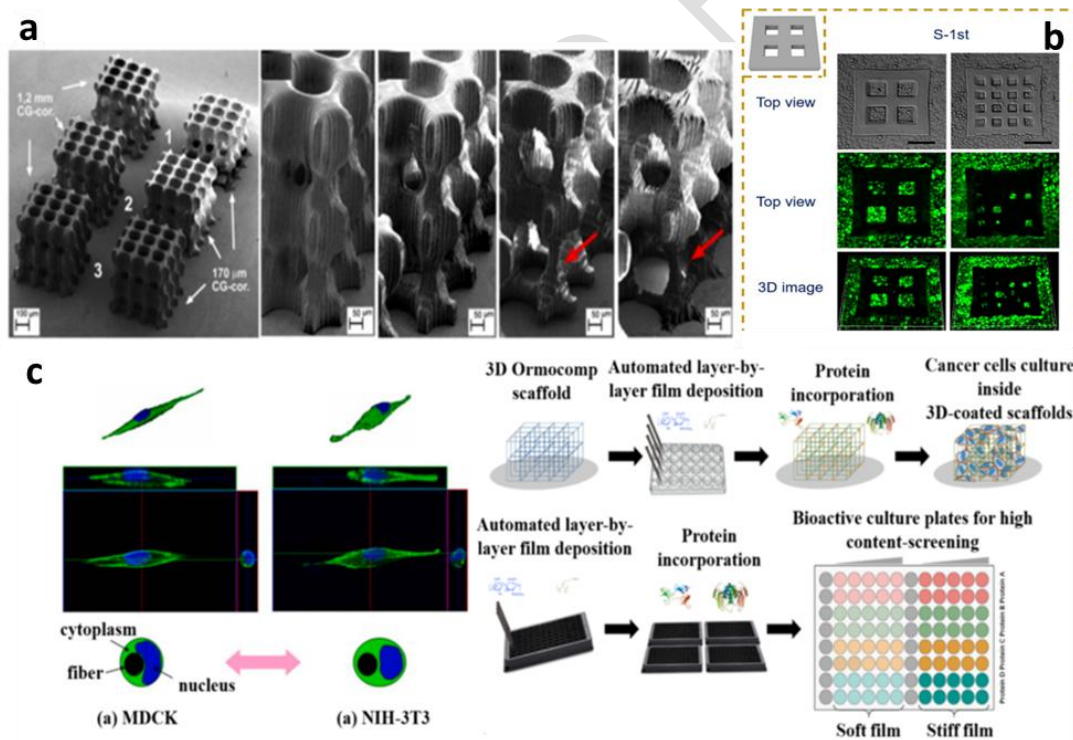


Fig. 8 (a,c) Two-photon polymerization of Ormocer® materials for biological tissue culture. The printed bio-scaffolds enable the growth of cancer cells within a 3D encapsulated structure. Adapted with permission from ref.^[158] Copyright © 2010 Japan Laser Processing Society and ref.^[159] Copyright © 2009 Springer Nature. (b) 3D hydrogel scaffolds encapsulating normal mouse fibroblast cells (L929) were fabricated via two-photon

polymerization using the hydrophilic photoinitiators Y4 and GelGM. The culture results demonstrate favorable biocompatibility. Adapted with permission from ref.^[158]
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4.2 From supportive biofabrication to transformative control of tissue function

4.2.1 Supportive strategies

The techniques and related approaches that mainly help maintain cell identity and lineage fidelity and contribute, to various extent, to sustaining functionality over time (for example with stable and comparable readouts across replicates) can be referred to as supportive of the biological functionalities. In this framework, Bernal et al. applied a VBP for the rapid creation of centimeter-scale liver scaffolds while preserving cellular identity during the printing phase^[71–24], providing also the necessary structural and mechanical stability of the organ (Figure 9); in another study, extrusion has been successfully employed by Lawlor et al. to incorporate kidney organoids while preserving their biological functions and differentiation stability^[155]. In the two examples, these techniques allow a precise high volume printing ensuring lineage fidelity before progressing to more complex integration. Other studies using microfluidic-based encapsulation (Bouwmeester et al. and Huang X. et al.^[155]) and hydrogel-mediated 3D cultures (Zhang et al.^[156]) successfully ensured lineage fidelity. It is important to note that in these examples, the biological functionalities are largely governed by the intrinsic biological programs. For example, spheroid deposition based on microarray approaches, while enhancing batch-to-batch cellular uniformity and experimental reproducibility, does not impose a coordinated tissue architecture. Another study applying TPP-based bioinks enabled high-resolution encapsulation of viable cells and tunable mechanics for material characterization; however, the system was not designed to actively guide cell differentiation or functional specialization. In another study, Taale et al. developed confinement-based spheroid assays enabling real-time monitoring of self-assembly processes and collective 3D cell migration^{Error! Reference source not found.}. The approach relies on the in situ fabrication of dome-shaped micro-scale confinements around pre-formed spheroids using two-photon lithography.

Here, bioprinting is used to optimize the physicochemical properties of the culture environment or to improve experimental observability, rather than actively guiding morphogenesis. In another application, Vitkūnaitė et al. showed that two-photon polymerization (TPP)–assisted scaffolds improved 3D tumor spheroid formation allowing precise control of architecture, pore size, and viability, providing a platform for angiogenesis studies and 3D drug screening^[158]. Furthermore, photocrosslinkable, kidney-derived bioinks have been employed to support cell viability, spheroid formation, and multilayer printing, while recapitulating the native microenvironment and enabling structurally stable constructs for renal tissue modeling^[162]. Overall, the discussed techniques in association to the commented methodological strategies enhanced the robustness and reliability of the readout but are implemented as essential parts of experimental infrastructure rather than drivers of tissue transformation.

4.2.2 Enabling strategies: reinforcing self-organization through instructive cues

We analyzed a second group of studies describing various techniques and related methodological approaches that can lead to a reinforced, biologically driven self-organization through instructive biochemical, mechanical (through geometry), or dynamic cues. These examples were referred to as enabling techniques.

Within this category, Brassard et al. published a seminal work where developed an integrating systems called Bioprinting-Assisted Tissue Emergence (BATE) that allows the scaling of functional units into mature tissues with sustained functional outputs^[163]. In particular, they produced macro-scale intestinal tubes, demonstrating that engineered spatial constraints can guide organoid self-organization into physiologically relevant architectures. With a comparable objective, Skylar-Scott et al. ^[163] applied high-density organoid-based biofabrication (SWIFT) and obtained perfusable vascularized tissues, demonstrating the rapid assembly of patient-specific organoids into large-scale functional organs. Overall, these seminal studies demonstrate the connection of functional units within complex biological systems, ranging from intestinal tissues to assembled organoids. In another key study, Fumandó Navarro et al. ^[165] applied VBP for developing neurovascular unit (NVU) models, thus bridging functional units in

mature systems and ensuring stable readouts over time. In a complementary but distinct approach, Antich et al.^[166] utilized extrusion-based bioprinting to integrate chondrocytes and MSCs, focusing on the mechanical and biological synergy of multi-lineage systems.

Representative enabling examples also include kidney organoid-on-chip platforms^[167], where extrusion-based fabrication is combined with controlled fluidic shear stress. In these systems, dynamic flow acts as a biophysical cue that promotes endothelial expansion, podocyte maturation, and epithelial–endothelial crosstalk, thereby strengthening microenvironmental signaling and functional maturation (that addresses the requirement 3) without explicitly encoding tissue architecture (requirement 2). Similarly, droplet-based strategies, which enable the timed delivery of endothelial cells to developing brain organoids^[165], support the formation of neurovascular units and reduce diffusion-limited necrosis, stabilizing emerging barrier functions through controlled cell–cell interactions rather than predefined geometry .

Importantly, biologically driven approaches that do not rely on biofabrication techniques can also be referred to as enabling strategies addressing many of the needed core biological requirements^[168]. In these cases, functional organization arises from carefully tuning biochemical or cellular inputs rather than engineered spatial constraints. Co-assembly of organoids within tissue-specific decellularized extracellular matrix (dECM) hydrogels exemplifies this principle, as the biochemical complexity of the native matrix promotes endogenous vascularization and lineage-specific maturation, effectively addressing the requirement 3, while leaving global architecture dependent on self-organization. Another landmark example is provided by the seminal work of Reza HA et al. ^[169]. In this work, chemically primed hepatic progenitors self-organize into liver organoids displaying metabolic zonation, a key feature of native liver physiology, without relying on bioprinting guidance. Lineage fidelity is strongly maintained through controlled progenitor specification, while spatially limited metabolic functions develop through biochemical preconditioning and naturally forming gradients rather than geometric constraints. This approach effectively

addresses some of the core biological requirements by supporting functionally relevant hepatocyte–hepatocyte interactions within a zoned microenvironment (requirements 1 and 3), but partially meets the spatial patterning as this results from self-organized biochemical signals (requirement 2). Sustained metabolic activity after transplantation is obtained, though the lack of explicit architectural control restricts reproducibility and scalability (requirement 4 partially addressed).

Another important example appears in the applicative field of environmental toxicology, where evaluating human-relevant effects of persistent and bioaccumulative pollutants strongly relies on *in vitro* models capable of supporting repeated and long-term exposure protocols^[170]. For types of highly persistent contaminants (e.g. per- and polyfluoroalkyl substances, PFAS), biological relevance is fundamentally connected to exposure duration and total dose. Caporale et al. developed human cortical brain organoids derived from pluripotent stem cells (hPSCs) that through not bioprinted, three-dimensionally self-assembly in a Matrigel support under dynamic suspension culture^[171], producing neuroectodermal tissues that reflect early human cortical development. These organoids were used to examine the effects of a mixture of PFAS at concentrations directly sourced from a mother–child cohort, thereby establishing a strong quantitative link between epidemiological exposure data and mechanistic *in vitro* outcomes.

Romaldini et al. developed a multilineage liver organoid by self-assembling Upcyte® hepatocytes^[172], liver sinusoidal endothelial cells, and bone marrow-derived mesenchymal stromal cells on Matrigel matrix. The 3D model demonstrated structural stability and long-term functionality of metabolic biomarkers, enabling repeated exposure studies and modeling of chronic nanomaterial toxicity while maintaining albumin secretion and CYP3A4 activity for up to 29 days (Figure 9).

The non-bioprinting reported examples, while are valuable biological benchmarks, demonstrate the cannot reach completely the biological functionality. Incorporating emerging biofabrication strategies provides a clear opportunity to improve structural control, temporal stability, and functional robustness, ultimately producing more predictive and translatable data especially fields of complex exposure scenarios as in

the case of environmental toxicology. By stabilizing tissue architecture, facilitating the integration of vascular and immune-relevant compartments, and extending functional lifespan under chronic dosing conditions, these approaches could significantly improve the relevance of advanced in vitro models for toxicology applications of emerging environmental contaminants where cumulative exposure and long-term effects are crucial for human risk assessment.

Together, these studies show that enabling strategies, whether biofabrication-assisted or purely biologically driven, work by enhancing intrinsic self-organizing processes rather than by programming tissue architecture. While they markedly increase physiological relevance compared to supportive methods, especially by strengthening cell-cell interactions and microenvironmental signaling, they only partly control the spatial architecture and long-term functional integration.

4.2.3 Emerging strategies: encoding architecture, mechanics, and system-level function

A third category of studies that we analyzed includes important biofabrication strategies, which fundamentally differ from supportive and enabling approaches by explicitly encoding tissue architecture, mechanical constraints, or boundary conditions using design parameters instead of relying solely on intrinsic self-organization. These emerging strategies utilize multiscale design principles, linking macro-structural control, micro-architectural guidance, and molecular-level functionalization to achieve system-level tissue functionality, thus exemplifying what we term a “hierarchical multiscale integrated framework” (Figure 1). These strategies have the potential to affect a range of core biological processes.

A defining characteristic of transformative strategies is that spatial organization is no longer an emergent property but a programmable input. This conceptual shift becomes evident with the use of TPP when used to explore its unique ability to achieve sub-micrometer precision necessary to actively guide morphogenesis. For example Urciolo

et al, demonstrated that dynamic in situ fabrication of hydrogel-in-hydrogel structures around organoids can provide instructive mechanical and geometrical cues to guide morphogenesis^[173], cell polarity, and axon directionality in human liver, intestinal, cancer, and spinal cord organoids (Figure 10). Notably, this work illustrates how architecture and mechanical boundary conditions can be deliberately encoded into the culture environment via dynamic fabrication, transforming spatial organization into a design parameter that directly shapes system-level functional outcomes. Gjorevski et al, through the combination of photopatterning and self-assembly demonstrated that engineered tissue geometry can deterministically pattern intestinal organoids^[154], controlling crypt formation, epithelial organization, and multicellular composition. Additionally, the work by Colombo et al. illustrates how TPP can impose controlled mechanical stimulation in mechanobiology models, transforming the system's mechanics into a design parameter^{Error! Reference source not found.}. Such capabilities of geometric and topographical control enable the simultaneous stabilization of lineage fidelity and the guidance of neurite alignment or network formation in spinal and neural constructs, as shown in the works of Crowe et al. ^[174], that developed TPP-fabricated scaffolds for human iPSC-derived cortical neuronal networks to provide topographical neurite guidance and enable reproducible network formation, while maintaining compatibility with optical interrogation. Furthermore, Harris et al.^[175] created Hilbert microcapillary scaffolds via TPL to guide human neural stem cell differentiation and alignment, resulting in interconnected neural networks with precise control over spatial organization and cell fate.

In contrast, the self-organized organoid systems, such as those first reported by Lancaster and Knoblich ^[176], in which complex neural tissue architectures emerge without any externally imposed geometric or mechanical constraints, remain inherently stochastic in spatial organization. On the other hand, this deterministic control is exemplified by the organotypic spinal cord (oSpC) models developed by Urciuolo et al.^[173], where axons follow printed pathways (Figure 10).

In our article analysis, the emerging approach is also achieved through engineering

boundary conditions. The convergent VBP + TPP approach is shown effective to enable the creation of hierarchical vascular networks, from macro-vessels (mm) to micro-capillaries (μm), defining the resolution limits needed to integrate vascular systems into macro-scaffolds, as demonstrated by He et al.^[130] and by the perfusable neurovascular unit models of Navarro et al.^[165]. These studies, also supported by evidence from Han et al.^[177], demonstrate how combining different techniques can create complex vascularized tissue models. Likewise, microfabricated villus-crypt geometries enable deterministic symmetry breaking in intestinal organoids, shifting tissue architecture from an emergent property to a design parameter.

From an operational standpoint, the convergence of VBP and TPP should be understood as a staged manufacturing workflow rather than a single fabrication event. A practical sequence begins with VBP to generate the centimeter-scale bulk geometry, perfusable primary channels, and global transport pathways within seconds, thereby establishing the anatomical envelope and mass-transfer capacity of the construct. TPP can then be deployed selectively within predefined regions, such as vascular interfaces, stem-cell niches, epithelial barriers, or neural guidance zones, where subcellular control over pore geometry, stiffness gradients, and ligand presentation is required. The resulting hybrid construct is subsequently matured under perfusion, mechanical conditioning, or organ-on-chip culture so that macroscale transport function and microscale cell-instructive control are integrated into a single biological system. In this framework, VBP primarily solves the problems of scale, speed, and support-free internal architecture, whereas TPP resolves the problems of local boundary conditions, cell guidance, and deterministic microenvironmental patterning.

Beyond micro-scale architectural programming, volumetric and layerless bioprinting methods offer another compelling emerging technique by tackling tissue organization and function at the macro scale. In these systems, transformation results from a shift in physical regime: centimeter-scale constructs with controlled internal topology surpass diffusion-limited constraints typical of conventional organoids, allowing for sustained metabolic and detoxification functions over time. At the same time, the hybrid VBP-

TPP workflow is constrained by several unresolved limitations, including phototoxicity and ROS accumulation from photocuring chemistries, local heat or dose accumulation during prolonged TPP writing, limited throughput at the TPP stage, registration accuracy between macro- and microscale fabrication steps, and the need for material systems that remain compatible across both platforms. These engineering constraints are accompanied by translational barriers related to reproducibility, process standardization, sterility control, and eventual regulatory qualification of complex photo-crosslinked living constructs.

We also highlight that an additional element is provided by dynamic and perfused culture platforms, especially when combined with biofabricated architectures. By applying controlled flow, shear stress, or time-specific dosing, these systems turn static cultures into dynamic environments where functional outputs are maintained under physiologically relevant conditions. Sequential drug exposure platforms^[182] demonstrate how temporal control enhances functional stability and coordination over time, while also preserving organized tissue structure and instructive microenvironmental signals (most of the biological requirements addressed).

Collectively, these strategies share a common principle: they replace spontaneously forming tissue organization with engineered architectural and physical constraints, allowing reproducible and causally interpretable structure–function relationships across biological scales. As summarized in Table 5, biofabrication becomes significant when it broadens the biological design space of 3D in vitro models, turning tissue architecture, mechanics, and dynamics into controllable variables rather than downstream effects of self-organization. This direct programming of multiple biological needs directly addresses the limitations seen in supportive and enabling strategies.

Table 5 Summary of multiscale bioprinting strategies and their contributions to organoid functionality and biological requirements. The table compares various bioprinting technologies, ranging from organ-scale volumetric bioprinting (VBP) and extrusion-based printing to microscale TPP. It highlights how each technique, used individually or in combination with other approaches (for example microfluidics), enhances the biological functionality of 3D organoids by linking a structural design across different length scales to achieve tissue complexity within a hierarchical multiscale integrated framework spanning from macro- to meso-/microscales.

Technique	Impact	References
Volumetric Bioprinting (VBP)	Scaling and macro-architectural control; sustained tissue-level function to prevent core necrosis in centimeter-scale biofactories	Núñez Bernal et al. [71]
Two-Photon Polymerization (TPP/2PP)	Programmable control of micro-architecture, mechanics, and topography to guide axonal alignment and cell niches	Gjorevski et al. [154], Urciuolo et al. [173], Colombo et al. Error! Reference source not found. , Crowe et al. [174], Harris et al. [175]
Convergence (VBP + TPP)	Creation of hierarchical vascular networks (from macro-vessels to capillaries) for multi-scale integration.	He et al.[130], Navarro et al. [165]
Microfluidic + Dynamic Culture	Overcoming diffusion-limited metabolic decay; turn static constructs into physiologically responsive environments.	Schuster et al. [170]
Photopatterning + Microfabrication	Inducing morphogenesis and deterministic symmetry breaking via geometric constraints.	Gjorevski et al. [154]

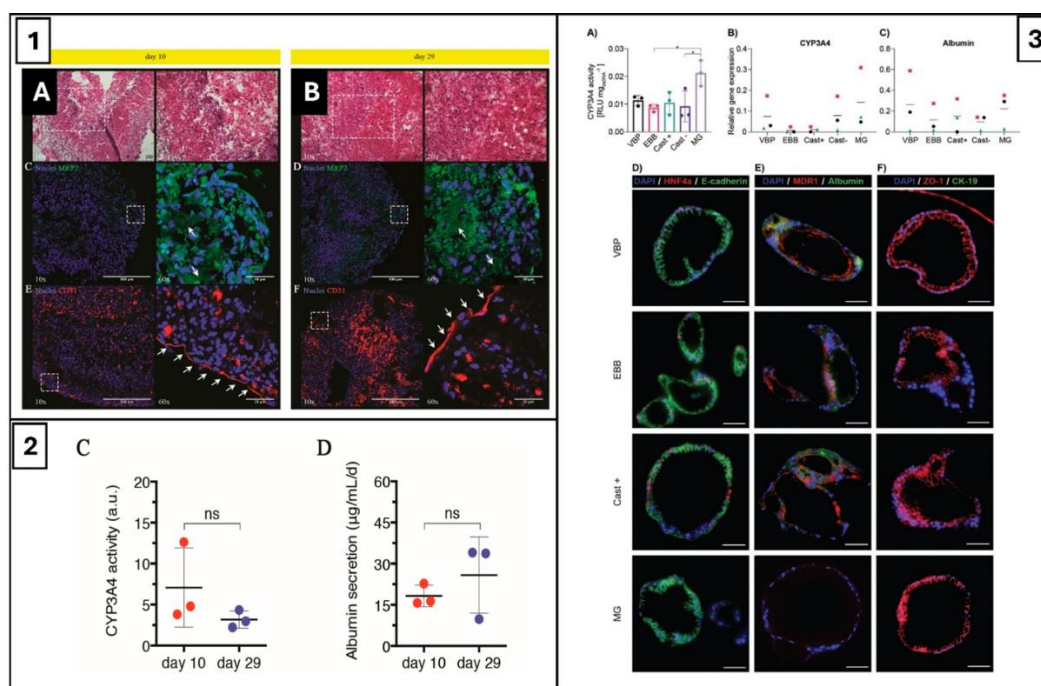


Fig. 9 (Liver Models): (Panel 1, Figures A and B) Non bioprinted self-assembled liver organoids generated by co-culturing upcyte® human hepatocytes (UHHs), liver sinusoidal endothelial cells (LSECs), and human bone-marrow-derived mesenchymal stromal cells (hbmMSCs) in a fixed ratio of 1:1:0.2 on Matrigel. The mixed cell population spontaneously reorganizes into compact three-dimensional aggregates within 7–10 days of culture, reaching approximately a projected surface area of about 10–13 mm². The resulting millimeter-scale liver organoids exhibit spatial organization of hepatic and endothelial cells forming primitive sinusoid- and biliary-like structures and maintain hepatic functionality, including albumin production and CYP3A4 expression for about a month (Panel 2, Figures C and D), enabling long-term culture and repeated exposure to nanomaterials for hepatotoxicity studies. Growth is limited by diffusion constraints, which restrict size and internal organization. Figure reproduced from Romaldini et al.^[172] © 2025 MDPI, distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0). (Panel 3, Figures D, E, F) Centimeter-scale liver bio factories. Reproduced from ^[71]. Generated by embedding human liver epithelial organoids (derived from primary intrahepatic bile duct stem/progenitor cells) within a photoresponsive gelMA-based bioresin by layerless volumetric bioprinting. The layerless, high-speed fabrication enables complex internal geometries, improved nutrient perfusion, and metabolic exchange, maintaining lineage fidelity and extending functional performance (CYP450 activity, ammonia detoxification) (Panel 3, figures A, B, C).

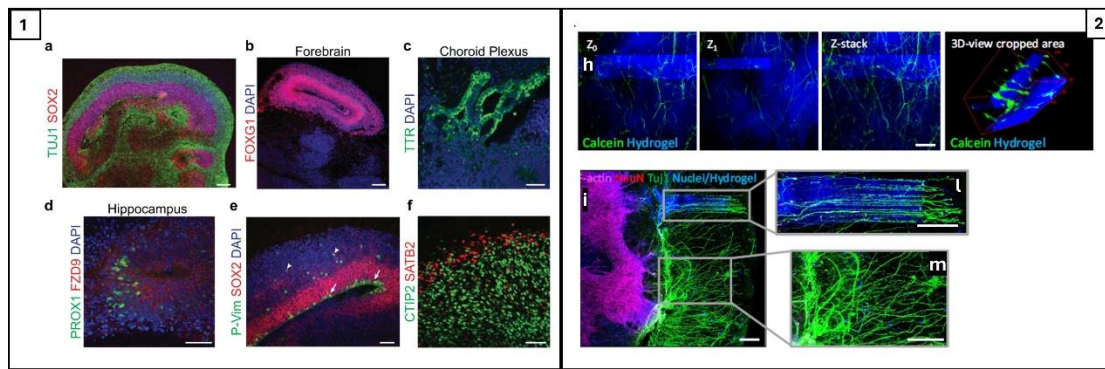


Fig. 10 (Neural Models): (Panel 1, a-f): Cerebral organoids (Lancaster and Knoblich ^[176] Figure reproduced from Lancaster & Knoblich, Nature Protocols 2014, 9, 2329–2340, © 2014 Nature.) generated from human pluripotent stem cells recapitulating key features of early human corticogenesis through spontaneous neuroepithelial self-organization. (a) Immunostaining reveals several brain regions and neuronal cell identities such as SOX2-positive apical neural progenitors lining ventricle-like luminal regions and TUJ1-positive neurons, distributed more basally, consistent with the polarized architecture of developing cortical tissue; Staining for (b) organoids extended forebrain regions, (c) choroid plexus, (d) hippocampal regions, (e) mitotic radial glia domains; (f) staining for cortical layer identities of long term cultured organoids (75 days). Formal permission obtained from Springer Nature (CCC licensed). (Panel 2, h-l): Organotypic spinal cord (oSpC) constructs Figure adapted from Urciuolo et al.^[173], Nature Communications 2023, 14, 3128, © 2023 Nature, distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0.) generated via hydrogel-in-hydrogel live bioprinting. In this ex vivo system, pre-formed organotypic spinal cord cultures preserve the native tissue architecture, while anisotropic bioprinted hydrogel cues align axonal outgrowth into defined trajectories. Instead of explicitly replicating organoid self-organization, this methodology emphasizes the potential of bioprinting-based guide to spatially arrange pre-formed neural tissues and organoid-derived structures; h) Confocal fluorescence images (acquired at ΔZ planes via Z-stack imaging analysis) showing multiple hydrogel layers (blue) fabricated at different Z planes, embedding alive (calcein-positive, green) oSpC previously cultured in 3D matrigel droplet for 7 days before bioprinting. Scale bars, 100 μm ; i) Fluorescence images of a spinal cord culture show aligned axons in the hydrogel (l) in contrast to randomly oriented axons when the hydrogel is absent (m). Scale bar, 200 μm .

4.3 Conclusion

This chapter evidences a clear progression in the role of biofabrication within functional 3D *in vitro* models. Supportive strategies mainly improve reproducibility, throughput, and material reliability, reinforcing the biological requirements which call for cell identity and lineage fidelity together with more stable and comparable functional readouts .

Enabling strategies advance this paradigm by reinforcing biologically driven self-

organization through dynamic, biochemical, or tissue-specific cues. These methods significantly enhance cell–cell interactions and instructive microenvironments and help stabilize tissue architecture although any external design.

Finally, these strategies establish a more deterministic relationship between design and biological outcome, enabling greater control over tissue organization and function across scales. This shift strengthens the reproducibility, physiological relevance, and translational potential of advanced 3D in vitro models.

5 Conclusion and Future Perspectives

Light-based three-dimensional biofabrication has become a key technological approach in tissue engineering and regenerative medicine, significantly influencing how biological structure, function, and scale can be integrated within engineered living systems. As reviewed in this work, the field has followed a clear evolutionary trajectory, progressing from subcellular-scale two-photon polymerization to ultrafast volumetric bioprinting approaches capable of organ-scale construction. Each of these technologies addresses distinct yet complementary biological and manufacturing requirements, collectively forming a multiscale fabrication framework that spans nanometer-resolution control of cell–matrix interactions to centimeter-scale fabrication of complex, cell-laden tissues. (Technology roadmap Shown in Figure 11)

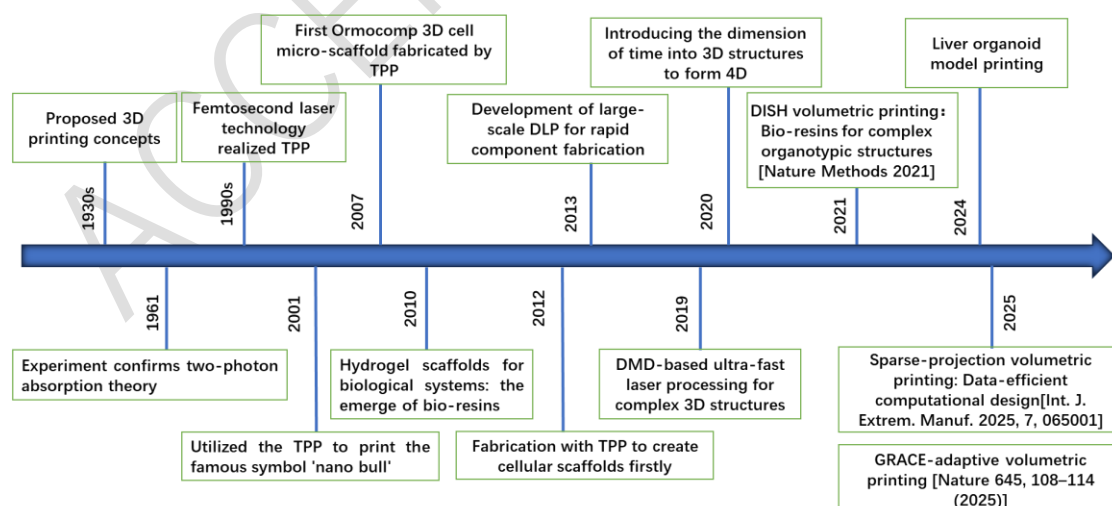


Fig. 11 Technology roadmap: A comprehensive milestone diagram depicting the developmental trajectory of these novel 3D printing technologies^{[21][178][179]} Adapted with permission by open access CC-BY4.0.

At the microscale, two-photon polymerization has emerged as an unparalleled platform for precision engineering of cellular microenvironments. By exploiting nonlinear optical confinement, this technique enables deterministic regulation of scaffold architecture, mechanical properties, surface topography, and biochemical patterning at length scales comparable to subcellular features. These capabilities have been instrumental in advancing the understanding of how physical cues regulate stem cell fate, nuclear mechanics, contact guidance, and invasive behavior. TPP has enabled the construction of advanced disease models, neural and vascular interfaces, and biohybrid microsystems with integrated sensing functions. Recent progress in parallelized writing strategies, grayscale lithography, and smart material development is progressively mitigating intrinsic throughput limitations, thereby extending the relevance of two-photon polymerization from exploratory microfabrication toward scalable platforms for high-content screening and mechanobiological studies.

At larger length scales, projection-based and VBP technologies have addressed the long-standing challenge of balancing resolution, fabrication speed, and construct size. Digital light processing and continuous liquid interface production have significantly increased manufacturing throughput, yet they remain constrained by the inherent limitations of layer-by-layer fabrication, including staircase effects and cumulative mechanical stress. In contrast, true volumetric bioprinting approaches, exemplified by computed axial lithography and emerging Xolographic and holographic strategies, represent a fundamental shift toward layer-less fabrication. By curing entire three-dimensional constructs within seconds, these methods minimize shear-induced cellular damage, preserve high cell viability even at elevated cell densities, and enable the formation of complex, support-free internal architectures. As a result, volumetric bioprinting provides a credible technological foundation for organ-scale tissue engineering and rapid manufacturing of living systems.

Despite these advances, several critical challenges must be overcome before light-based biofabrication can be broadly translated into clinical and industrial applications^[180]. Chief among these is the development of next-generation bioinks that simultaneously satisfy optical fidelity, rheological stability, and biological functionality^[178]. Volumetric printing in particular imposes stringent constraints related to light scattering, optical absorption, cell sedimentation, photoinitiator cytotoxicity, and ROS accumulation, whereas TPP remains vulnerable to local heat build-up, dose accumulation, and intrinsically limited throughput during prolonged serial writing. These constraints necessitate an integrated design philosophy in which optical, mechanical, and biological parameters are optimized in concert rather than in isolation. In parallel, there is a growing need to move beyond homogeneous constructs toward spatially programmed heterogeneity that more faithfully reproduces the graded,

anisotropic, and multicellular organization of native tissues. Emerging holographic and acoustic-field-assisted strategies provide promising routes for three-dimensional pre-patterning of cells and materials prior to curing, enabling controlled heterogeneity, interface engineering, and enhanced functional complexity.

Looking ahead, the future of light-based biofabrication is likely to be shaped by hybrid and multiscale manufacturing ecosystems rather than by a single dominant technology. A realistic operational route for VBP-TPP integration is a sequential workflow in which VBP first establishes the organ-scale bulk geometry and perfusable transport network, after which TPP is applied only to biologically strategic regions requiring micron- or submicron-scale control over architecture, mechanics, and biochemical presentation. Subsequent maturation under perfusion, dynamic loading, or organ-on-chip control then converts this geometrically integrated construct into a functionally coupled living system. At the same time, the convergence of biofabrication with computational modeling, machine learning^[182], and digital twin frameworks is expected to shift scaffold design from empirical trial-and-error toward predictive and outcome-oriented strategies. In such approaches, optical parameters, material composition, biological responses, and even fabrication order can be co-optimized *in silico* prior to manufacturing. For translation, however, this multistage workflow will also require robust inter-platform registration, reproducible maturation protocols, standardized quality-control metrics, and regulatory pathways capable of evaluating complex living constructs produced through sequential photo-crosslinking processes.

An additional emerging direction lies in the development of dynamic and adaptive biofabrication strategies. The incorporation of stimuli-responsive materials, together with volumetric and holographic patterning techniques, opens pathways toward four-dimensional bioprinting, in which engineered constructs evolve over time in response to mechanical, biochemical, or physiological cues. These systems offer the potential for minimally invasive deployment, self-organization, and long-term functional maturation, thereby bridging the gap between static biomimicry and developmental bioengineering. In conclusion, light-based three-dimensional biofabrication has evolved from a precision microfabrication tool into a comprehensive platform for constructing functional living tissues across multiple length scales. Continued advances in materials science, optical engineering, computational design, and biological integration will be essential to translate these technologies from sophisticated *in vitro* models toward clinically relevant regenerative therapies and patient-specific tissue reconstruction. The convergence of multiscale fabrication, intelligent design, and dynamic biological

control positions light-based biofabrication as a foundational technology in the next generation of tissue engineering and regenerative medicine.

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Conflict of interest

The authors declare they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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