

REVIEW ARTICLE

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

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Abstract

Three-dimensional (3D) biofabrication is increasingly used in tissue engineering and regenerative medicine to recreate the spatial, mechanical, and biochemical complexity of native cellular microenvironments. Among light-based 3D printing technologies, volumetric bioprinting (VBP) and two-photon polymerization (TPP) offer complementary capabilities across distinct length scales. This review examines their evolution, key advances, and potential integration within a multiscale biofabrication framework. VBP enables rapid, layer-free fabrication of centimeter-scale, cell-laden constructs by delivering spatially modulated light doses throughout a photosensitive volume. This approach reduces mechanical stress, supports high cell viability, and permits complex internal architectures, including perfusable vascular networks. Advances in computed axial lithography, holographic patterning, and bioink engineering are reviewed, with emphasis on optical transparency, control of light scattering, and rheological stability in cell-dense systems. TPP, by contrast, uses nonlinear optical confinement to achieve submicrometer resolution and precise 3D control over scaffold architecture, mechanics, and biochemical patterning. These capabilities support deterministic regulation of cell–material interactions and applications in stem cell niche engineering, neural and vascular interface design, tumor microenvironment modeling, and biohybrid microsystems. Integrating VBP and TPP may bridge organ-scale architecture and perfusion with microscale niche definition and molecular-level signaling, thereby supporting the development of functional organoids and living tissues and better addressing distinct but interdependent biological requirements across organ, tissue, cellular, and molecular scales. Remaining challenges include material heterogeneity, optical–biological coupling, scalability, and long-term tissue maturation. Future progress will require

coordinated advances in materials, optical design, computational modeling, and predictive design strategies to enable the 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 (3D) microenvironment, which comprises a dynamic, hierarchical combination of biochemical and biophysical cues provided by the extracellular matrix (ECM).⁴⁻⁶ Methods such as solvent casting and gas foaming, which are traditional scaffold fabrication techniques, 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 first examines volumetric bioprinting (VBP), which addresses organ-scale fabrication, before turning to two-photon polymerization (TPP), a technology based on nonlinear two-photon absorption (TPA) that enables true 3D 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, TPP 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 3D scaffolds.¹⁵⁻¹⁸

Despite its exceptional fabrication precision, the serial point-by-point nature of TPP limits fabrication speed, restricting its use to relatively small-scale constructs. This limitation has encouraged the adoption of projection-based methods such as digital light processing (DLP) and continuous liquid interface production (CLIP), which polymerize entire layers simultaneously, thereby substantially increasing 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 (VBP). 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 3D 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. Current bioink-engineering strategies focus on optimizing photoinitiator systems to balance polymerization efficiency²⁴ 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 increasingly complex,²⁶ intelligent and adaptive manufacturing strategies are becoming more important.^{27,28} Recent advances in real-time monitoring techniques are reviewed, along with 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 in 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 provide distinct but complementary levels of control across multiple length scales. We define this approach as a hierarchical multiscale integration framework. Macro-scale VBP ([Figure 1A](#)) establishes the overall tissue geometry (anatomy) and the perfusable channel network, while microscale TPP ([Figure 1B](#)) refines this framework, allowing fine control over 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](#)) by incorporating bioactive signals into the scaffolds, including adhesive ligands, growth factors, morphogens, and other biochemical regulators that may direct cell-scaffold interactions and help reconstitute a functional microenvironment. From a prospective standpoint, this review indicates that integrating these complementary levels of control, which 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 establishing vascular networks and in 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 VBP as the technology that pushes the boundaries of scale and speed, enabling the rapid construction of organ-sized, perfusable tissue scaffolds (Section 2). We then turn to TPP, which pushes the boundaries of resolution and precision, allowing us to engineer microscale and subcellular features that determine cell fate²⁹ and tissue function (Section 3). Finally, in Section 4, we propose a hierarchical 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.

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 Section 4). These requirements span multiple aspects of biological performance, including cell viability and phenotype stability (which typically demand high fabrication speeds), as well as the need for a physiologically relevant microenvironment, best supported by fine control of spatial 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, 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.*³¹ demonstrated the encapsulation of cells within highly hydrated, photosensitive gelatin matrices using water-soluble photoinitiators, highlighting TPP's utility

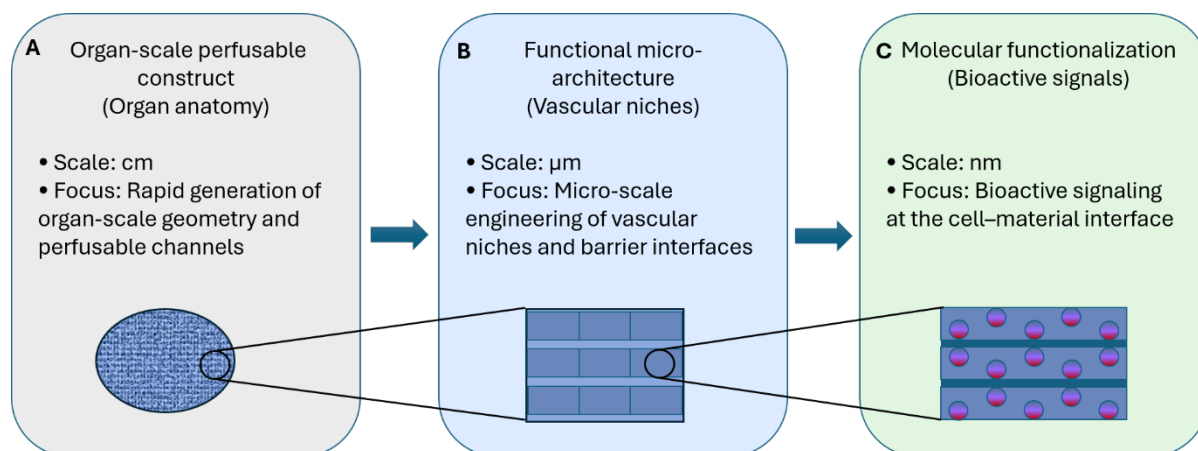


Figure 1. Hierarchical multiscale integration framework for generating functional living tissues using biofabrication strategies. The figure illustrates a prospective multiscale strategy in which distinct but complementary light-based biofabrication approaches operate across different length scales to progressively establish 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 provides precise control over 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 support the establishment of a functional microenvironment by modulating cell–material interactions and regulating key biological processes, 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.

for 3D cell culture. However, as a point-scanning direct-write technique, TPP has intrinsically low throughput, with fabrication time increasing approximately with the number of voxels written and, at a fixed resolution, with the processed volume. For sufficiently large or densely written constructs, typical scanning speeds of 30–550 mm/s can result in fabrication time of several hours, exposing cells to prolonged non-physiological conditions and severely limiting viability.

Projection-based DLP bioprinting addresses this limitation by curing entire layers simultaneously, thereby substantially increasing throughput. Studies have demonstrated its capability to fabricate nanocomposite hydrogels³² 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 induced pluripotent stem cells (iPSCs) and organoids,^{35,36} motivating the transition toward truly VBP paradigms.

2.1.1. Projection-based technologies (digital light processing stereolithography): The speed–precision trade-off in layered manufacturing

Projection-based digital light processing stereolithography (DLP-SLA) uses a digital micromirror device (DMD) as a

dynamic mask to project an entire two-dimensional (2D) 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 substantially higher throughput at a given spatial resolution 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 stereolithography (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 the 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 simultaneously maximizing resolution, build volume, and geometric complexity remains challenging. This trade-off is especially relevant in tissue engineering, where print

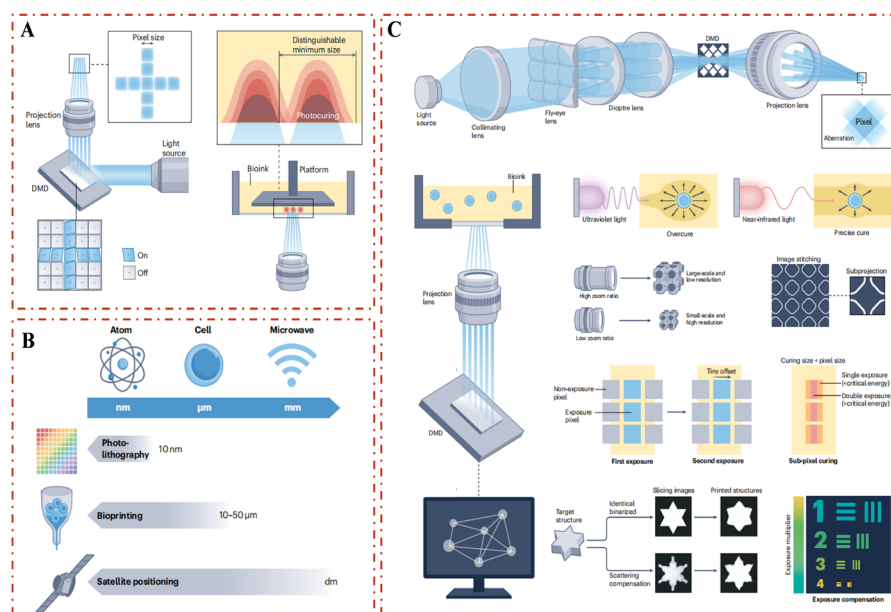


Figure 2. Digital light processing stereolithography (DLP-SLA) technology principles and workflow. (A) Optical configuration of a projection-based DLP-SLA system. (B) Layer-by-layer fabrication sequence, including patterned light projection, resin curing, and synchronized platform motion. (C) Optical and computational strategies for improving the resolution and printing fidelity of DLP-SLA, including wavelength selection, zoom-ratio adjustment, image stitching and subprojection, subpixel curing, scattering compensation, and exposure compensation. Adapted with permission from Ref.³⁶ Copyright © 2025, The Society of Manufacturing Engineers.

Abbreviation: DMD: Digital micromirror device.

size and throughput must be balanced with the need to preserve local microenvironmental fidelity.³⁹

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 outcomes.^{40,41} In this sense, the central question is not simply the achievable printing speed, but whether the process can generate a sufficiently controlled 3D 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.

Continuous liquid interface production 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 VBP.

2.1.2. True volumetric bioprinting

Volumetric bioprinting 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, 3D objects are fabricated by sequentially stacking 2D layers, a process that is typically accompanied by inherent issues such as slow printing speeds, staircase effects, the need for support structures, and anisotropic mechanical properties.⁴⁴ A review by Mathur *et al.*⁴⁵ noted that the limitations of

traditional layer-by-layer printing severely constrain its industrial applications in cell printing. In layer-less manufacturing, the object is formed volumetrically rather than by sequential stacking of discrete layers. Spatially controlled light doses trigger polymerization at predefined locations throughout a 3D photosensitive volume, allowing complete structures to be formed within seconds to tens of seconds (Figure 3). Whyte *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.

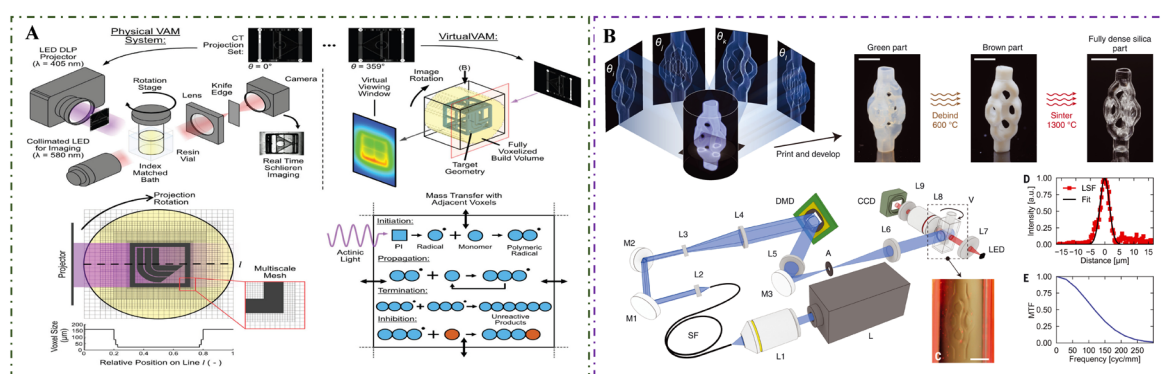


Figure 3. Principles underlying true volumetric bioprinting. (A) VirtualVAM simulation system for modeling polymerization reactions and mass diffusion in all voxels during VAM. Adapted with permission from Ref.⁴⁷ Copyright © 2025, Springer Nature Limited. (B) Printing of transparent fused silica glass using micro-CAL, illustrating the fabrication of high-precision optical components. Adapted from Ref.⁴⁶

Abbreviations: CCD: Charge-coupled device; CT: Computed tomography; DLP: Digital light processing; DMD: Digital micromirror device; LED: Light-emitting diode; LSF: Line spread function; micro-CAL: Microscale computed axial lithography; MTF: Modulation transfer function; PI: Photoinitiator; SF: Square-core fiber; VAM: Volumetric additive manufacturing; VirtualVAM: Virtual volumetric additive manufacturing.

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 3D object within the photosensitive resin liquid volume.⁴⁸ Computed axial lithography (CAL), first demonstrated by Kelly *et al.*⁴⁹ in 2019, is one of the earliest and most influential implementations of volumetric additive manufacturing (VAM). By adopting a tomographic reconstruction framework, CAL enables rapid volumetric fabrication within seconds rather than through conventional layer-by-layer processing.^{49,50} This computationally driven strategy established an important technical foundation for subsequent developments in VBP. Figure 3 illustrates representative principles and applications of this approach.

Building on the tomographic principle introduced

by CAL, Tomographic VBP further demonstrated the fabrication of macroscopic tissue constructs by controlling the delivery of 3D light dose distributions across the entire print volume, effectively circumventing the constraints of sequential layer deposition. Further advancing this approach, xolography, introduced by Regehy *et al.*,⁵¹ achieved linear volumetric confinement via the intersection of structured light fields, 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 VBP was subsequently reviewed by Bernal *et al.*,⁴⁷ who reported the fabrication of a human auricle model in 22.7 s. Moreover, functional organoids and complex vascularized constructs were produced at high cell densities ($>10^7$ cells/mL), with cell viability exceeding 85–90%. A recent study further showed that optimized bioresin formulations, 5% gelatin

methacryloyl (GelMA) combined with 0.05% lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), enable the fabrication of complex perfusable constructs within 30 s, with cell viability above 90%.⁵²

One of the key advantages of true VBP, particularly CAL technology, is its ability to print complex overhanging geometries without support structures. 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.

Volumetric additive manufacturing 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-fabricated 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 the 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, translating VAM from proof-of-concept demonstrations to robust clinical-scale applications faces critical challenges, particularly those associated with bioink formulation. Current CAL and most VBP approaches predominantly rely on single-component, optically transparent bioinks, resulting in constructs with homogeneous, 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: Toward 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 3D 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 overcome the speed limits of macroscopic tissue fabrication, current implementation schemes primarily rely on single-component hydrogel precursors, resulting in fabricated constructs that typically exhibit isotropic homogeneous characteristics (homogeneity). Gehlen *et al.*⁵³ noted that this homogeneity limitation represents one of the main obstacles to the clinical translation of current VBP technologies.

Acoustic holography technology has achieved breakthrough advances in recent years. Melde *et al.*⁵⁴ first proposed the concept of acoustic holograms in 2016, establishing the theoretical foundation for this field. The emerging acoustic holography-assisted optical 3D printing technology reported by Zhang *et al.*⁵⁵ 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 3D space before second-scale photocuring occurs. Figure 4A and 4B show the principles and implementation of acoustic holography-assisted

printing. Physics-informed neural network algorithms are used to optimize the acoustic-field distribution, after which ultrasonic transducer arrays generate programmable 3D acoustic potential wells, 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 spatially graded properties. The holographic direct-sound printing technology reported by Derayatifar *et al.*⁵⁶ further expanded the application boundaries of acoustic holography in additive manufacturing.

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 3D 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 developed by Regehy *et al.*⁵¹ 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 of artificial tissues that reproduce dynamic physiological behaviors, such as cyclic cardiac-like contraction, representing the highest-dimensional pursuit of biofabrication technology.⁵⁷

Holographic 3D printing technology utilizes the interference principle of light to fabricate microstructures, and integrating 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

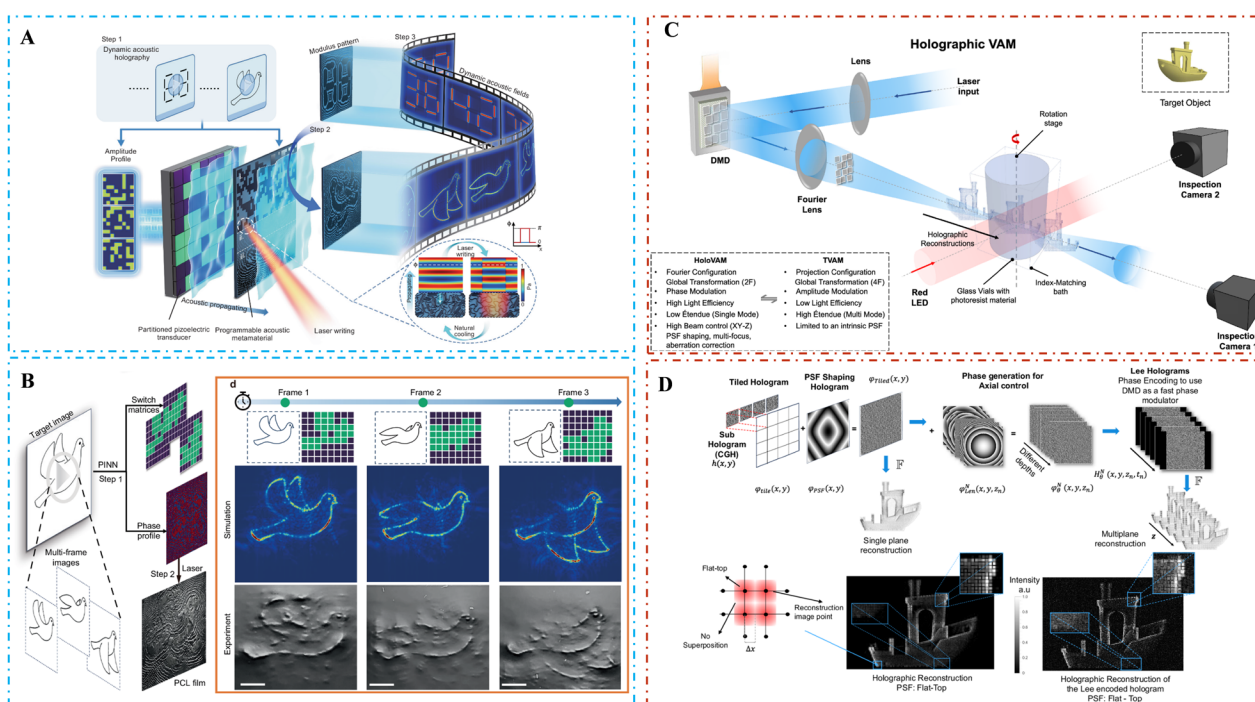


Figure 4. Holographic 3D printing principles. (A) Acoustic holographic control principle. (B) Physics-informed neural network-based acoustic holographic algorithm and resulting effects. (A,B) Adapted with permission from Ref.⁵⁵ Copyright © 2025, The Author(s). (C) Optical holographic 3D printing principle. (D) Optical holographic 3D printing workflow. (C,D) Adapted with permission from Ref.⁵⁸ Copyright © 2024, Society of Photo-Optical Instrumentation Engineers (SPIE).

Abbreviations: CGH: Computer-generated hologram; DMD: Digital micromirror device; HoloVAM: Holographic volumetric additive manufacturing; LED: Light-emitting diode; PINN: Physics-informed neural network; PCL: Polycaprolactone; PSF: Point-spread function; TVAM: Tomographic volumetric additive manufacturing; VAM: Volumetric additive manufacturing.

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 3D space and forming multiple focused beams known as 3D holographic optical traps,⁵⁸ as shown in Figure 4C and 4D. In optical holographic 3D printing, phase modulation of incident laser light by an SLM generates computer-designed holographic patterns, producing multifocal light fields for spatially controlled curing. Álvarez-Castaño *et al.*⁵⁹ achieved holographic tomographic VAM for the first time, 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 at predetermined 3D coordinates within a photosensitive material and induce localized photopolymerization of the surrounding matrix, thereby immobilizing or encapsulating the positioned cells while simultaneously constructing the 3D structure.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 3D 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 summarizes the key comparative advantages and persistent limitations of these core technologies in the context of organ-scale biofabrication.

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 VBP hinges on the bioink’s ability to serve as an effective “photonic medium” for accurate light-field reconstruction while also providing a physiological cellular microenvironment. Achieving this dual functionality requires an integrated engineering approach moving beyond mere biocompatibility towards simultaneous optimization of optical, rheological, and biological performance. Although true volumetric printing technologies, such as 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.*⁴⁷ noted in their review, successful implementation of volumetric printing depends on the precise reconstruction of light fields in 3D 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

Table 1. Comparative analysis of photocuring technologies for organ-scale bioprinting

Technology	Key principle	Major advantage	Limitation	Cell viability challenge
DLP-SLA	Layer-by-layer area projection	High speed vs. point-scanning; excellent 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	Fabrication in seconds; no support structures required; high cell viability	Homogeneous, isotropic output; scattering in dense inks	Optimized; challenge shifts to bioink engineering
Holographic VBP	Volumetric curing + wavefront patterning	Spatial heterogeneity & cell patterning	Computational complexity; multi-material integration	Acoustic/optical forces must be cell-compatible

Abbreviations: CAL: Computed axial lithography; CLIP: Continuous liquid interface production; DLP-SLA: Digital light processing stereolithography; VBP: Volumetric bioprinting.

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 a decisive impact on cell viability and print quality.⁶⁴ Elkhoury *et al.*⁶⁵ in their comprehensive review systematically elucidated the classification and application principles of photoinitiators. Based on their initiation mechanisms, photoinitiators are typically classified into two major categories: Type I and Type II.

Type I photoinitiators undergo direct homolytic cleavage upon light exposure to generate active free radicals. Representative compounds include phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide and 2,4,6-trimethylbenzoyl diphenylphosphine oxide derivatives. Among these, 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) and is combined with light exposure, significant cytotoxicity occurs, primarily due to the accumulation of free radicals generated by photoexcitation.

Type II photoinitiators generate active free radicals via 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 and *N*-vinylpyrrolidone 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 VBP of decellularized ECM bioinks, enabling construction of complex cardiac and meniscus tissue-like structures within 30–60 s 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.*⁶⁴ noted that high-performance and low-toxicity novel photoinitiator systems are key to enhancing printing resolution and cell viability. Wolfel *et al.*⁶⁷ developed bioxography technology based on diphenyliodonium

chloride and *N*-vinylpyrrolidone. This technology utilizes dual-color photopolymerization principles, successfully fabricating 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 VBP. Determining the optimal photoinitiator concentration in bioinks is not a simple single-variable optimization, but rather involves multiple interrelated parameters including elastic modulus, pore structure, cell viability, and reactive oxygen species (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 reveals the trade-offs in performance among different photoinitiator systems. Low-concentration LAP (0.01%) maintains >90% mesenchymal stem cell (MSC) viability with minimal ROS levels, but yields hydrogels with only 2–3 kPa elastic modulus, larger pore sizes (5–15 µm), 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× higher ROS intensity. Irgacure 2959 at a medium concentration (0.5%) shows balanced performance with ~70% viability and a 3–5 kPa modulus, though its ultraviolet-only activation poses a potential risk of DNA damage. Eosin Y at optimal concentration (0.1 nM) achieves >85% cell viability with a ~4 kPa modulus and low ROS levels, demonstrating superior overall performance for cell-sensitive applications.

A strategy based on using the lowest effective photoinitiator concentration has been demonstrated to be an effective approach for addressing 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, an optimal exposure time of 2.5–3 min at 10 mW/cm² under 365 nm illumination can achieve crosslinking effects comparable to those of high-concentration formulations at low initiator concentrations. Experimental data from Dogan *et al.*⁶⁸ 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 an appropriate elastic modulus of the hydrogel. This strategy is particularly suitable for tissue engineering applications requiring long-term cell survival, such as vascularized tissue construction and functional

Table 2. Multiparameter comparison of photoinitiator systems in GelMA-based bioinks

Parameter	LAP (0.01%)	LAP (0.1%)	I2959 (0.5%)	Eosin Y (0.1 nM)
Irradiation wavelength (nm)	365/405	365/405	365 (UV)	500–550 (visible)
Curing time at 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 measured by SEM (μm)	5–15	2–8	4–10	3–8
Crosslinking density	Low	High	Medium	Medium
MSC viability on day 1 (%)	>95%	~60%	~80%	>90%
MSC viability on day 7 (%)	>90%	~50%	~70%	>85%
Relative ROS intensity	1.0 (baseline)	4–5×	2–3×	1.2–1.5×
Cell spreading area	Large	Small	Medium	Large
Number of focal adhesions	High	Low	Medium	High
Co-initiator required	No	No	No	TEOA + NVP
Preferred application	Long-term culture	Rapid prototyping	General-purpose applications	Cell-sensitive applications

Abbreviations: GelMA: Gelatin methacryloyl; I2959: Irgacure 2959; LAP: Lithium phenyl-2,4,6-trimethylbenzoylphosphine; MSC: Mesenchymal stem cell; NVP: *N*-vinylpyrrolidone; ROS: Reactive oxygen species; SEM: Scanning electron microscopy; TEOA: Triethanolamine; UV: Ultraviolet.

organoid cultivation, which 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 VBP).⁶⁹ 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, deflecting light paths and attenuating optical energy, thereby reducing printing resolution and penetration depth. This effect is particularly pronounced in bioinks with high cell loading (e.g., >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 bioink's optical properties to match the refractive index of encapsulated cells. You *et al.*⁷⁰ demonstrated that the cytoplasmic refractive index typically ranges from 1.36 to 1.39. By supplementing common bioinks such as 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.*⁷¹ confirmed that this method not only

significantly improves printing resolution but also enables feature sizes of approximately 50 μm at high cell densities (100 million cells/mL), laying the foundation for constructing larger and more complex tissue engineering scaffolds.

Table 3 reveals several key trends. First, as iodixanol concentration increases from 0% to 30%, the scattering coefficient μ_s decreases substantially from 11.76 mm⁻¹ to 1.377 mm⁻¹, resulting in approximately a 10-fold reduction in scattering. 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.*⁷² demonstrated that adding biologically inert dyes as photoabsorbers enables precise control of light penetration depth in bioinks, which is particularly

Table 3. Optical parameters of cell-laden gelatin methacryloyl bioinks with varying iodixanol concentrations (4×10^7 cells/mL, 405 nm)

Iodixanol concentration (w/v)	Refractive index (n)	μ_s (mm ⁻¹)	μ_s' (mm ⁻¹)	Anisotropy (g)	Scattering reduction	Light penetration 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

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 such as 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.*⁷⁴ systematically analyzed the impact of cell density on the printability of GelMA bioinks. During prolonged large-scale printing, cells suspended in bioinks tend to undergo non-uniform sedimentation under gravity, resulting in uneven cell distribution within printed tissues and subsequently affecting tissue functional homogeneity.⁷⁵

To effectively address cell sedimentation, rheological modification has been widely applied in bioink design. Introducing yield-stress fluids as suspension matrices is an effective strategy.⁷⁶ Such 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.*⁷⁷ demonstrated that optimizing bioink rheological properties can significantly reduce cell sedimentation rates and improve cell survival

during printing. The review by An *et al.*⁷⁸ noted that beyond yield-stress fluids, thickening agents or nanoparticles can be added to increase the low-shear viscosity of bioinks, enabling prolonged cell suspension.

Volumetric bioprinting technology demonstrates unique advantages in maintaining cell viability. Duquesne *et al.*⁷⁹ demonstrated VBP applications in bone-like tissue construction. 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.*⁸⁰ utilized VBP to print functional ultra-soft hydrogel structures within 7–15 s, with the low polyvinyl alcohol content (1.5%) in the printing matrix providing a favorable stress-relaxation microenvironment for rapid cell spreading, stem cell differentiation, and matrix mineralization. Such a stress-relaxation microenvironment is crucial for constructing tissue-engineering scaffolds with complex 3D microenvironments and for maintaining the 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 during printing, yet not significantly increase light scattering.⁸¹

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-h 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

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

Modifier	Concentration range	Yield stress (τ_y)	Viscosity at 1 s^{-1}	Flow behavior index (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 (>24 h)	Gelatin methacryloyl reinforcement
Xanthan gum	0.3–0.5% w/v	1–10 Pa	50–300 Pa·s	0.2–0.4	High (>85% viability)	Excellent (>24 h)	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

Laponite is preferred for GelMA reinforcement. Carbopol serves primarily as an embedded extrusion bath material. The optimal design criteria specify a yield stress of 1–10 Pa, an extrusion pressure < 20 psi, and a maximum shear rate < 100 s^{-1} to protect cell membrane integrity.

Volumetric bioprinting technology, thanks to its rapid, layer-less printing, is revolutionizing the field of biofabrication.⁸² Overcoming light scattering and 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 print 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 Khademhosseini⁸³ noted that 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 artificial intelligence (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 for constructing complex biological structures, leveraging its high resolution and rapid fabrication. Kim and Kim⁸⁵ reported breakthrough progress in DLP bioprinting for organ fabrication; they demonstrated a new potential 3D liver model that can replicate native liver tissue. 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 a poly(ethylene glycol) diacrylate (PEGDA)–acrylamide 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. For osteochondral interface regeneration, stiffness-gradient bioinks have been used to fabricate biphasic scaffolds in a single printing step.⁸⁸ Zhou *et al.*⁸⁹ demonstrated that such scaffolds not only exhibit excellent interfacial bonding strength but also possess the ability to induce cell differentiation, providing potential strategies for integrated osteochondral repair.

High-cell-density functional tissue fabrication poses

an additional challenge because cell-induced light scattering can reduce printing precision and shape fidelity.⁹⁰ 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.*,⁹⁴ in their review, noted that this approach effectively addresses the limitations of traditional photocuring at high cell densities. Bernal *et al.*⁷¹ 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.

2.3.1. Optical feedback and closed-loop control systems

To ensure high precision and fidelity during 3D bioprinting, 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. Real-time process monitoring can combine OCT with high-speed imaging to detect deposition errors and structural defects during printing. Tashman *et al.*⁹⁶ demonstrated the application of OCT for *in situ* volumetric imaging and analysis of FRESH 3D-bioprinted constructs. 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.*⁹⁵ further developed an OCT-integrated extrusion-bioprinting system, 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 reduced printing resolution and precision. He *et al.*⁷² developed a simultaneous photoabsorption and free-radical

reaction photoinhibition method that effectively enhances the fidelity of light-based bioprinting. Adaptive optics technology, using combiners and deformable mirrors, can correct wavefront aberrations caused by turbid bioinks in real time. Levato and Lim⁹⁸ discussed various applications of light in biofabrication. Wavefront sensors can precisely measure wavefront aberrations, while deformable mirrors dynamically adjust their surface shapes based on these measurements, 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.*¹⁰⁰ demonstrated that the combination of multi-material integration with advanced optical correction techniques is particularly suitable for complex scaffold fabrication scenarios that require fine control of cell arrangement and microstructure formation.

2.3.2. Deep learning-assisted light field reconstruction

Deep learning has become indispensable for overcoming the central optical challenge in bioprinting: light scattering. The need to mitigate light scattering 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, including convolutional neural networks (CNNs), has demonstrated strong potential for bioprinting applications, particularly in light-field reconstruction, printing parameter optimization, and defect compensation. The study by Meyer *et al.*¹⁰² demonstrated that machine learning could handle the complexity of biomaterial design, efficiently managing datasets of 50–100 polymers. AI's role extends beyond pre-print compensation to *in situ* adaptation. Ng *et al.*¹⁰³ provided an in-depth analysis of the application prospects of deep learning in the fabrication and maturation of 3D-bioprinted tissues and organs, 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, whereas CNNs can learn complex nonlinear relationships between light fields and printing results, thereby

accelerating hologram computation and enabling faster printing speeds. Deep learning models can optimize projection light dose distributions by dynamically adjusting illumination intensity and exposure time based on bioink characteristics and target structure requirements. Guan *et al.*⁹² 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. The 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.*⁹³ confirmed that such optimization improves printing efficiency while avoiding over-curing or under-curing, ensuring structural integrity and cell viability.

The presence of cells and biomaterials causes optical inhomogeneity in bioinks, leading to light scattering (primarily Mie scattering from refractive-index mismatches between the cytoplasm and the external hydrogel environment, as well as Rayleigh scattering from 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 with 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, 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.*¹⁰⁴ 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

Volumetric bioprinting 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 VBP is uniquely suited to generate the complex, support-free architectures required. 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 VBP 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, VBP 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 TPP, which we explore in Section 3.

3. Two-photon polymerization for tissue engineering: Subcellular-scale precision manufacturing

Having established how VBP enables rapid, organ-scale fabrication, the discussion now turns to the microscale. TPP provides subcellular resolution 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 3D physical and biochemical characteristics of the 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 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 overall 3D 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 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 fabricating planar micro-patterned layers that are later stacked or assembled, a strategy that often 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 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 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 (Figure 5).

3.1. Fundamental mechanisms of two-photon polymerization

The exceptional spatial resolution and genuine 3D design freedom characteristic of TPP arise directly from its underlying physical mechanism, which stands in marked contrast to conventional single-photon

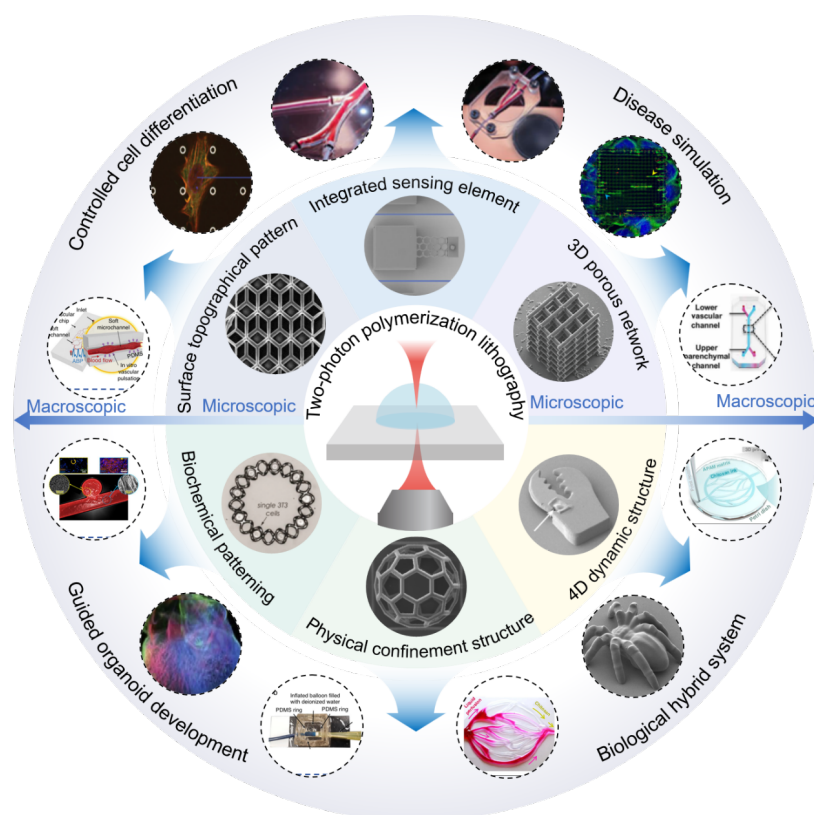


Figure 5. Schematic illustrating the translational pipeline enabled by two-photon polymerization (TPP). TPP provides 3D deterministic manufacturing with subcellular resolution (Layer 1: TPP platform). This capability is used to fabricate a suite of micro- and nanoscale cell culture scaffolds (Layer 2: 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 from Ref.¹¹¹ 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 VBP 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;¹¹² and tumor spheroids in confined microenvironments.

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 TPA 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 reactive species are generated only when this combined energy exceeds the polymerization threshold. 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 whose characteristic dimensions can be substantially smaller than the excitation wavelength. 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 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 fabricate within pre-existing biological environments, such as within 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 enhanced by its compatibility with a broad, 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 such as SU-8 and the IP-series (e.g., IP-S, IP-L), as well as organic–inorganic hybrid materials such asOrmocer® 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 human mesenchymal stem cell (hMSC) osteogenic differentiation,¹¹⁹ modeling cancer cell invasion through confined microchannels,¹²⁰ and guiding neural network formation.¹²¹ Yao *et al.*¹²² 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 the design of 3D microenvironments for controlling stem cell fate in tissue engineering. Bran

*et al.*¹²³ 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 *et al.*¹²⁴ engineered a microscale niche for pancreatic tumor cells by combining 3D-printed microscale scaffolds with a bioactive layer-by-layer polyelectrolyte film coating that provides tunable matrix stiffness and bound signaling proteins to simulate early-stage metastatic pancreatic cancer tissue. Accardo *et al.*¹²⁵ 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. Costa *et al.*¹²⁶ reported the interaction of bone marrow 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 than smaller gap sizes (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 arginine–glycine–aspartic acid motifs, is among the most prominent examples. Its mechanical stiffness and degradation behavior can be precisely tuned by controlling the methacrylation degree and polymer concentration.¹²⁸ Complementary platforms include PEGDA, which provides a bioinert and highly customizable matrix, and methacrylated hyaluronic acid, a principal ECM glycosaminoglycan. By modulating 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. Edmondson *et al.*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 and He¹³⁰ 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 and Yu,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 in material selection, precise hydrogel positioning, dynamic cell stimulation, and real-time monitoring. A foundational review summarized emerging solutions to these challenges and outlined future directions toward more functional and integrated hydrogel-based microphysiological systems.¹³²

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.*¹¹³ demonstrated that intense femtosecond laser irradiation can directly cross-link proteins such as collagen I and fibrinogen, as well as complex biological fluids including serum. 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 such as 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.*¹³³ developed novel acrylic resin nanocomposites incorporating Brazilian red propolis nanoparticles for wound dressing. The materials exhibit potent antibacterial activity against *Staphylococcus aureus* and tunable drug release profiles. Microgroove scaffolds fabricated via TPP support potential applications in advanced wound healing. Stimuli-responsive polymers, such as pNIPAM, can be printed via TPP to create 4D dynamic structures that change shape (e.g., swell, bend, curl) in response to environmental triggers, such as 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 lies in its ability to serve as a “three-dimensional pixel editor” for cellular microenvironments, enabling the direct translation of digital design blueprints into physical scaffolds with feature dimensions 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 (Figure 6).

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 Yao *et al.*¹²² and Costa *et al.*¹²⁶ demonstrated that pore size within woodpile scaffolds directly modulates nuclear morphology in 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 3D context. A study introduced a step-growth photopolymerization resin without additives, enabling tunable soft-to-stiff 3D

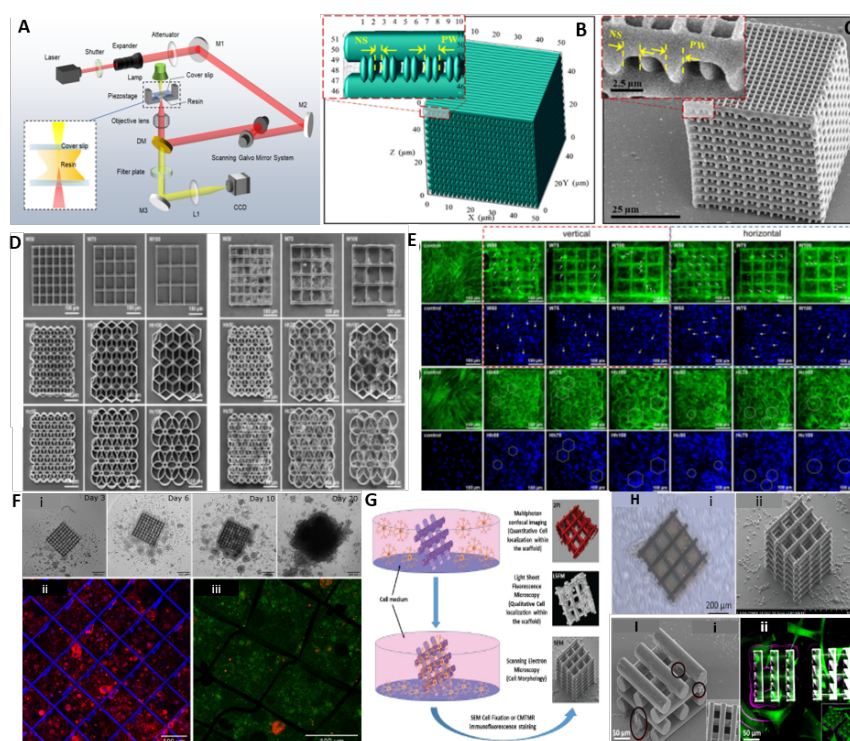


Figure 6. Two-photon fabrication biological scaffold structure. (A) Schematic optical path of the two-photon micro-nanofabrication setup. Adapted with permission from Ref.¹²¹ Copyright © 2024, Elsevier. (B) Design of biomimetic cell culture scaffolds; (C) Scanning electron microscopy (SEM) image. Scale bars: 25 μm ; inset: 2.5 μm . (B,C) Adapted with permission from Ref.¹²³ Copyright © 2025, American Chemical Society. (D) SEM images of the 3D scaffolds and human mesenchymal stem cells (hMSCs) cultured for six days on scaffolds. Structures from top to bottom: woodpile, honeycomb (hexagonal), and honeycomb (circular) scaffolds. Scale bars: 100 μm . (E) Fluorescent images of hMSCs cultured for six days on woodpile scaffolds. Nuclei are stained blue with DAPI and actin filaments green with phalloidin. (D,E) Adapted with permission from Ref.¹²² Copyright © 2025, The Author(s). (F) Culture of PANC1 cells on a bioactive scaffold loaded with 5 $\mu\text{g}/\text{mL}$ fibronectin. Scale bars: 100 μm . (i) Optical microscopy images showing cell growth after direct culture for 3, 4, 10, and 20 days. (ii) Confocal microscopy images after 11 days in culture with nucleus stained blue (DAPI) and cytoskeleton stained red (rhodamine). (iii) Live/Dead assay after 20 days in culture: live cells labeled green, dead cells labeled red. Scale bars: 100 μm . 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. Scale bars: 200 μm . (G,H) Adapted with permission from Ref.¹²⁵ Copyright © 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (I) Images of cell scaffolds with different types of woodpile stacking structures. Scale bars: 50 μm . Adapted from Ref.¹²⁶

microstructures via two-photon laser printing by adjusting exposure dose, spanning over two orders of magnitude in Young's modulus for multi-material applications.¹²⁶

3.3.2. Surface topographies and contact guidance

The ability to pattern submicron-scale surface features—including ridges, grooves, pits, and pillars—on both planar and curved 3D substrates enables precise control 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. Song *et al.*¹³⁵ demonstrated controlled cell alignment using two-photon direct laser writing to pattern 2D and 3D hydrogel microstructures. Alternating the laser-scanning speed created alternating stiff and soft regions within the cell-

adhesive hydrogels, thereby 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, providing a versatile platform for tissue-engineering applications that require directed cell growth. Jeon *et al.*¹³⁶ used 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.*¹²³ 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 the development of 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 such as 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.¹³⁶ For example, Richter *et al.*¹³⁷ employed a multi-resin TPP strategy to fabricate microscale 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 2D culture (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 Colombo *et al.*¹³⁸ developed a submillimeter 3D device via TPP, combining elastic polydimethylsiloxane and gelatin-based hydrogel to mechanically stimulate encapsulated multicellular systems. 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 3D 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. Studies by Turunen *et al.*,¹³⁹ Eren *et al.*,¹⁴⁰ and Accardo *et al.*¹²⁵ have shown the superiority of 3D TPP scaffolds for neural culture. Turunen *et al.*¹³⁹ developed a 3D neuronal culture platform using TPP direct laser writing to fabricate tubular microtowers fromOrmocomp. 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.*¹⁴¹ explain how 3D microstructures created by direct laser writing and coated with TiO_2 influence osteoblast-like cells. Results show that specific topographies, particularly with 25 μm 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.*¹²⁵ compare N2A neural cell growth on 3D scaffolds versus 2D micro-ridges and flat surfaces. 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. These advantages are valuable for studying neural development, modeling neurodegenerative diseases, and designing next-generation neural implant substrates that better integrate with host tissue. Paradowska-Stolarz *et al.*¹¹⁵ further demonstrated the potential of such substrates using Dental LT Clear resin. The scaffold supports cell viability, cortical neuron and glial differentiation, and functional maturation over 120 days. Calcium imaging and network analysis reveal spontaneous neuronal activity and scaffold-promoted local connectivity, providing a reliable *in vitro* model for studying neural development and disease.

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. TPP offers an unparalleled level of control over microscale architecture, enabling the independent and precise modulation of these parameters within 3D 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.*,¹⁴² who combined TPP-printed Ormocomp microscallops 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 the controlled presentation of matrix-associated proteins, including fibronectin and bone morphogenetic protein 2/4. 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 used to fabricate 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, 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 3D culture systems by fabricating elastic, micron-scale components. One representative mechanobiology platform incorporates a TPP-fabricated elastic polydimethylsiloxane membrane coupled to a gelatin-based hydrogel chamber containing retinal organoids.¹³⁸ 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.

Two-photon polymerization further enables the fabrication of intricate, cell-scale wireframe scaffolds that reproduce key geometrical features of the ECM 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 3D 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.*¹⁴⁴ demonstrated this capability by fabricating 3D wireframe microscallops designed to mimic ECM-like architectures, 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, Gullo *et al.*¹⁴⁴ 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 findings provide an experimental framework for designing multicellular biohybrid systems, which is applicable to microscale robots and biomedical implants.

3.5. Biohybrid microsystems and functional living constructs

A particularly compelling demonstration of TPP's

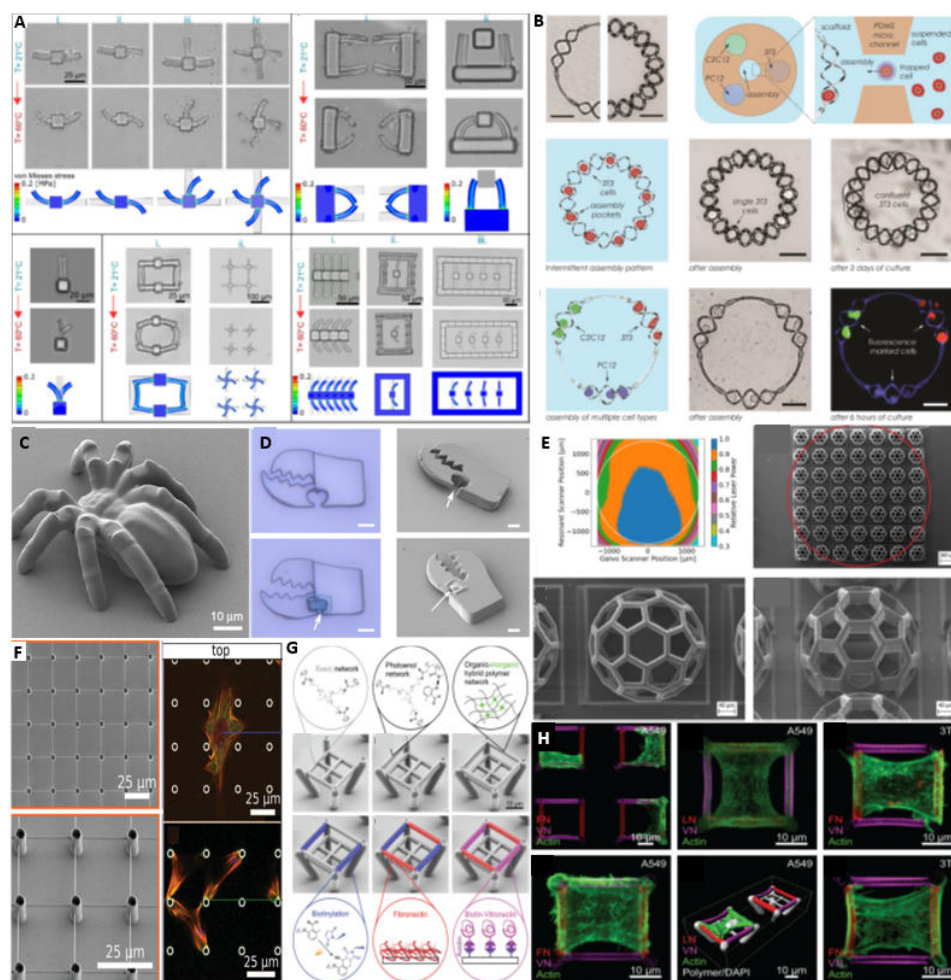


Figure 7. The engineered microenvironment of functional cell culture model. (A) Optical images of shape-morphing behavior in proof-of-concept applications of two-photon polymerization 4D-printed microstructures with their corresponding motion prediction. Scale bars: 25/50/100 μm . Adapted with permission from Ref.¹³⁴ Copyright © 2025, The Author(s). (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. Scale bars: 30 μm . Adapted with permission from Ref.¹⁴⁴ Copyright © 2017, Wiley-VCH GmbH & Co. KGaA, Weinheim. (C) Scanning electron microscopy (SEM) images of the micro-spider. Scale bars: 10 μm . (D) SEM images of the crab claw-muscle system and of the claw after bovine serum albumin integration. Scale bars: 10 μm . (C,D) Adapted from Ref.¹⁴⁵ (E) Hexagonal cage-structured cell culture scaffolds fabricated via parallel printing into a 7×7 matrix array. Scale bars: 100/40 μm . Adapted from Ref.¹⁴⁶ (F) SEM images of square grids with 25 μm -spaced posts, investigating the influence of different geometries and grid spacing on cell culture. Scale bars: 25 μm . Adapted with permission from Ref.¹⁴¹ Copyright © 2014, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (G) Various bioactive extracellular matrix (ECM) proteins conjugated onto a scaffold framework printed with Ormocomp, with distinct colors representing the distribution of different proteins across the scaffold. Scale bars: 10 μm . (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). Scale bars: 10 μm . (G,H) Adapted with permission from Ref.¹³⁷ Copyright © 2016, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

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, 3D cell culture matrix using TPP. The matrix was fabricated from GelMA and type I collagen, with ruthenium complexes incorporated as both the photoinitiator for TPP and a covalent, optical oxygen sensor. This design enabled the *in situ*, real-time monitoring of local oxygen tension (pO_2) within the 3D microenvironment. The fabricated matrices featured a controlled cubic alveolar structure ($50 \times 50 \mu m$) 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. The work establishes a novel platform for creating advanced 3D co-culture systems with integrated biosensors, promising for detailed studies of cell metabolism and high-throughput drug screening. 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. SLMs have been employed to generate multiple laser foci simultaneously, enabling holographic or parallel TPP. Zandrini *et al.*¹⁴⁸ introduced an SLM-enabled multi-foci TPP system to fabricate 3D “Nichoid” scaffolds for stem cell expansion. The method achieved a fivefold increase in production speed while maintaining structural integrity. Cell viability tests confirmed that scaffolds produced by multi-foci and single-focus methods exhibited similar biocompatibility, supporting their use in large-scale regenerative medicine applications. Binder *et al.*¹⁴⁶ reported an over 50-fold increase in voxel writing rate, enabling rapid fabrication of large arrays of tissue engineering microscale scaffolds without compromising resolution. 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 with 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 TPP’s serial point-scanning nature, 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 (2GL) method to improve surface roughness.¹⁵⁰ 2GL is an advanced additive manufacturing technique that enhances standard TPP. Modulating the exposure dose during laser scanning, focusing across the scanning plane changes the voxel size within the photoresin, enabling finely controlled changes in the size of polymerized voxels. This precise voxel-size control is achieved by 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 distribution of exposure levels, resulting in different voxel heights being printed in a single plane. The key innovation of 2GL is the dynamic, real-time adjustment of laser power during scanning (grayscale exposure). This dynamic laser-power modulation 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 presented 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 TPP in biofabrication is expected to follow several converging directions. Continued progress in photoresin design will likely yield advanced bioinks with enhanced bioactivity, tunable degradation kinetics, and adaptive or self-healing properties, enabling closer replication of native extracellular matrices.¹⁵² At the same time, integrating TPP with complementary biofabrication modalities is anticipated to facilitate the formation of hierarchical constructs that couple macroscale architecture with microscale functionality. Key enabling techniques include extrusion-based

bioprinting, which enables 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 NIR window offers opportunities for *in situ*, 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 AI 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. Summary

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 3D 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 TPP 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 shown in **Figure 1**, this multiscale integration operates within a hierarchical framework spanning three levels: (i) the macro scale, where organ-scale VBP defines the overall structural

organization and perfusable architectures; (ii) the meso- to microscale, where TPP 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 integration framework that may lead to the fabrication of organs and tissues with physiologically relevant functions.

In this section, 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 reproducibility of the cell-based systems, to enabling strategies that promote self-organization through instructive cues, and finally to transformative strategies that encode system-level architecture, mechanics, and function through multiscale design. Focused examples demonstrate how high-resolution light-based printing, combined with volumetric, modular, or hybrid manufacturing schemes, enables system-level functionality ranging from perfusable tissue models to biohybrid microsystems. The section 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), the preceding analysis shows that these technologies, 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, we examine biofabrication strategies through a biological lens, considering the fundamental cellular requirements that underpin robust, demonstrable organ- or tissue-like functions. Accordingly, in this section we 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 bioprinting studies in Section 4.2. In their seminal review, Brassard and Lutolf¹⁵³ emphasized that the functional relevance of organoids and related

3D models is not guaranteed by 3D architecture alone. Rather, it critically depends on whether the system can establish and sustain appropriate cell types and states, including lineage hierarchy, maturation, and phenotype maintenance, in a controlled and reproducible manner. From this perspective, a functional 3D *in vitro* system must maintain physiologically meaningful differentiation states in appropriate lineage-relevant cell populations over time while avoiding undesired drift toward non-physiological phenotypes during prolonged culture. We therefore define this concept as Requirement 1: “Lineage-relevant cell composition and stable differentiation states.”

Beyond cellular composition and state, Brassard and Lutolf¹⁵³ also highlighted that native tissue function relies on precise 3D organization, including compartmentalization, polarity, lumen formation, and zonation, all of which are necessary for coordinated multicellular behavior. Although self-organization can generate rudimentary architectures, its stochastic nature may result in substantial structural variability. The ability to impose spatial patterning therefore represents a key biological requirement for increasing functional relevance and improving reproducibility across constructs. We define this concept as Requirement 2: “Reproducible tissue-like 3D architecture and spatial patterning.”

In addition, *in vivo* tissue function arises from dynamic interactions among multiple cell types within microenvironments characterized by continuous biochemical gradients, mechanical inputs, and ECM signals. Functional 3D models therefore require microenvironments that actively guide and sustain physiologically relevant intercellular communication. Gjorevski *et al.*,¹⁵⁴ in a landmark paper, demonstrated, for the first time, that organoid patterning could be made more predictable and reproducible by designing the microenvironment as an instructive system coupled with programmable tissue geometry. Rather than allowing cells to self-organize in a largely stochastic manner, they engineered the initial tissue geometry and introduced selected ECM cues to locally define where morphogenesis could occur. This approach generated stable spatial differences in cell packing and mechanical signaling, including YAP activity, which coordinated cell–cell communication pathways such as Notch/DLL1 signaling, promoted the localized emergence of niche cell types, and ultimately drove the formation of organized crypt–villus-like domains. We therefore define this concept as Requirement 3: “Instructive microenvironment enabling stable cell–cell crosstalk.”

Finally, Brassard and Lutolf¹⁵³ emphasized that claims of organ- or tissue-like functionality must be supported by sustained, measurable, and reproducible functional outputs over time. Many current models display early functional signatures (e.g., marker expression or partial physiological activity) but fail to maintain these features during prolonged culture. Moreover, variability in construct size, architecture, and maturation complicates quantitative benchmarking. Without stable and reproducible functional outputs, even highly organized 3D systems remain limited in their translational and predictive value. Accordingly, we define this aspect as Requirement 4: “Sustained, measurable, and reproducible functional outputs over time.”

Based on this scientific evidence, in the following section, we analyze seminal studies that generate different types of 3D *in vitro* systems (e.g., organoids¹⁵⁵ and engineered tissues¹⁵⁶) and critically examine the role of 3D bioprinting technologies in achieving system-level functionality. In our critical analyses, we assign distinct roles to these technologies depending on whether they (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 otherwise not be achievable (transformative role; Figure 8). Additionally, we include selected examples of seminal biologically driven approaches that do not rely on bioprinting but provide benchmarks against which future improvements enabled by 3D bioprinting can be quantitatively assessed.¹⁵⁷

4.2. From supportive biofabrication to transformative control of tissue function

4.2.1. Supportive strategies

The techniques and related approaches that primarily help maintain cell identity and lineage fidelity and, to varying degrees, sustain functionality over time (for example, with stable and comparable readouts across replicates) can be considered supportive of biological functionality. In this framework, Bernal *et al.*⁷¹ used VBP for the rapid creation of centimeter-scale liver scaffolds while preserving cellular identity during the printing phase, also providing the necessary structural and mechanical stability of the organ (Figure 9); in another study, extrusion has been successfully employed by Lawlor *et al.*¹⁵⁵ to incorporate kidney organoids while preserving their biological functions and differentiation stability. In the two examples, these techniques allow precise, high-volume printing, ensuring lineage fidelity before progressing to more complex integration. Other studies using microfluidic-

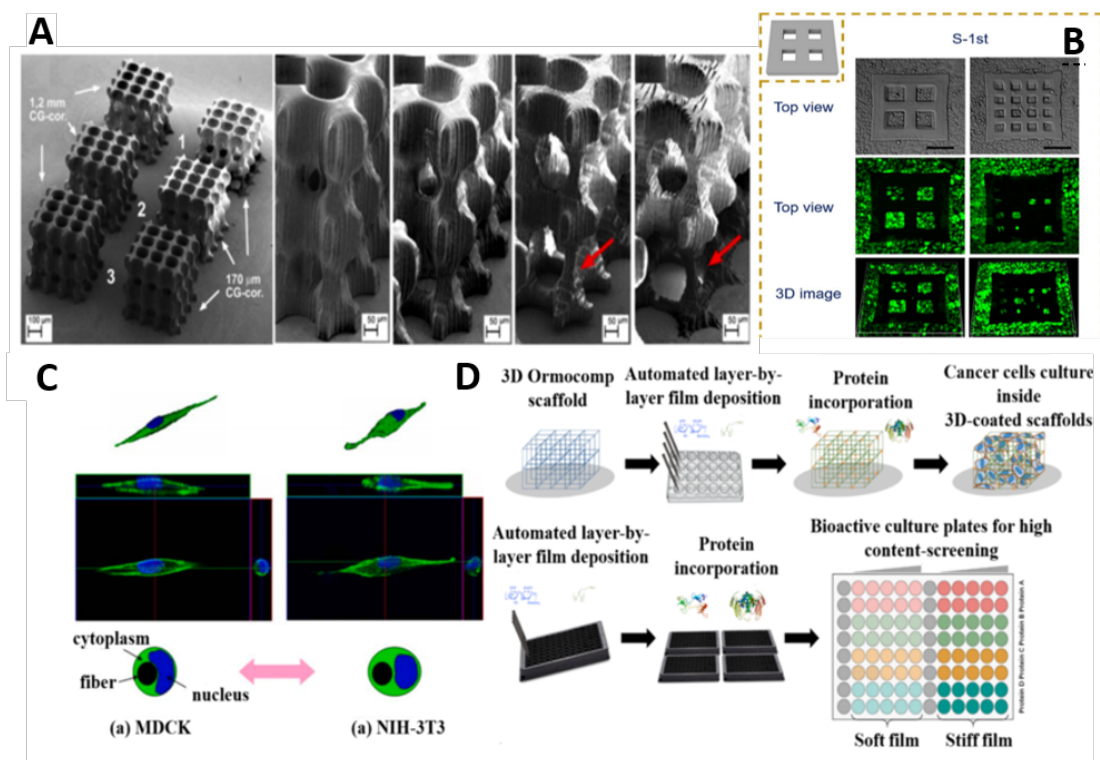


Figure 8. Cell scaffold-assisted cell differentiation. (A) Two-photon polymerization of Ormocore® materials for biological tissue culture, showing printed bioscaffolds supporting cancer cell growth within 3D structures. Scale bars: 100/50 μm. Adapted with permission from Ref.¹⁵⁸ Copyright © 2010, Japan Laser Processing Society. (B) 3D hydrogel scaffolds encapsulating normal mouse fibroblasts (L929), fabricated by two-photon polymerization using the hydrophilic photoinitiators Y4 and glycidyl methacrylate modified gelatin. The culture results demonstrate favorable biocompatibility. Scale bars: 100 μm. Adapted with permission from Ref.¹⁶⁰ Copyright © 2021, Elsevier. (C) Cell morphology of MDCK and NIH-3T3. Adapted with permission from Ref.¹⁵⁹ Copyright © 2009, The Author(s). (D) Schematic representation of the overall process of microtumor development on 3D bioactive scaffolds. Adapted with permission from Ref.¹²⁴ Copyright © 2022, American Chemical Society.

based encapsulation (Bouwmeester *et al.*¹¹² and Huang *et al.*¹⁶⁰) and hydrogel-mediated 3D cultures (Zhang *et al.*¹⁵⁶) successfully ensured lineage fidelity.

It is important to note that in these examples, biological functions are largely governed by 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. A study using TPP-based bioinks enabled high-resolution encapsulation of viable cells and tunable mechanics for material characterization;¹³⁰ however, the system was not designed to actively guide cell differentiation or functional specialization. In another study, Taale *et al.*¹¹¹ developed confinement-based spheroid assays that enable real-time monitoring of self-assembly processes and collective 3D cell migration. The approach relies on the *in situ* fabrication of dome-shaped microscale confinements around preformed spheroids using two-photon lithography. Here, bioprinting is used to optimize the physicochemical properties of

the culture environment or to enhance experimental observability, rather than to actively guide morphogenesis. In another application, Vitkūnaitė *et al.*¹⁶¹ showed that 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. 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 Together, these methodological strategies improve the robustness and reliability of experimental readouts but function primarily as components of the experimental infrastructure rather than as direct drivers of tissue morphogenesis.

4.2.2. Enabling strategies: Reinforcing self-organization through instructive cues

A second group comprises enabling strategies that reinforce

biologically driven self-organization through biochemical, geometric, mechanical, or dynamic cues. These examples were referred to as enabling techniques.

Within this category, Brassard *et al.*¹⁶³ published a seminal work in which they developed an integrated system called bioprinting-assisted tissue emergence (BATE) that allows the scaling of functional units into mature tissues with sustained functional outputs. 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.*¹⁶⁴ 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, Navarro *et al.*¹⁶⁵ applied VBP to develop neurovascular unit 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 (which addresses 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 considered enabling strategies that address many of the 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 ECM hydrogels exemplifies this principle, as the biochemical complexity of the native matrix promotes endogenous vascularization and lineage-specific maturation, effectively

addressing Requirement 3 while leaving the global architecture dependent on self-organization. Another landmark example is provided by the seminal work of Reza *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 patterned 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 requirement, as this results from self-organized biochemical signals (Requirement 2). Sustained metabolic activity is maintained after transplantation, although the lack of explicit architectural control restricts reproducibility and scalability (Requirement 4 partially addressed).

Another important example appears in the field of environmental toxicology, where evaluating human-relevant effects of persistent and bioaccumulative pollutants strongly relies on *in vitro* models capable of supporting repeated, long-term exposure protocols.¹⁷⁰ For highly persistent contaminants (e.g., per- and polyfluoroalkyl substances [PFAS]), biological relevance is fundamentally linked to exposure duration and total dose. Caporale *et al.*¹⁷¹ developed human cortical brain organoids derived from pluripotent stem cells that were not bioprinted, but formed 3D structures through self-assembly in a Matrigel support under dynamic suspension culture, producing neuroectodermal tissues that reflect early human cortical development. These organoids were used to examine the effects of a PFAS mixture at concentrations directly derived from a mother–child cohort, thereby establishing a strong quantitative link between epidemiological exposure data and mechanistic *in vitro* outcomes.

Romaldini *et al.*¹⁷² developed a multilineage liver organoid by self-assembling Upcyte® hepatocytes, 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).

Although these nonbioprinted systems provide valuable biological benchmarks, they may offer limited control over 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 in fields with complex exposure scenarios, such as 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 the toxicological assessments of emerging environmental contaminants, for which cumulative exposure and long-term effects are particularly relevant to human risk assessment.

Together, these studies show that enabling strategies, whether biofabrication-assisted or purely biologically driven, enhance intrinsic self-organizing processes rather than 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. Transformative strategies: Encoding architecture, mechanics, and system-level function

A third category of studies we analyzed includes important biofabrication strategies that fundamentally differ from supportive and enabling approaches by explicitly encoding tissue architecture, mechanical constraints, or boundary conditions using design parameters rather than relying solely on intrinsic self-organization. These transformative strategies utilize multiscale design principles, linking macrostructural control, microarchitectural 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 when TPP is used to explore its unique ability to achieve sub-micrometer precision necessary to actively guide morphogenesis. For example, Urciolo *et al.*¹⁷³ demonstrated that dynamic *in situ* fabrication of hydrogel-in-hydrogel structures around organoids can provide instructive mechanical and geometrical cues to guide morphogenesis, 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.*,¹⁵⁴ through the combination of photopatterning and self-assembly, demonstrated that engineered tissue geometry can deterministically pattern intestinal organoids, controlling crypt formation, epithelial organization, and multicellular composition. Additionally, the work by Colombo *et al.*¹³⁸ illustrates how TPP can impose controlled mechanical stimulation in mechanobiology models, transforming the system's mechanics into a design parameter. Such geometric and topographical control capabilities enable simultaneous stabilization of lineage fidelity and guidance of neurite alignment or network formation in spinal and neural constructs, as shown in the work of Crowe *et al.*,¹⁷⁴ who 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 and Zou¹⁷⁵ created Hilbert microcapillary scaffolds via two-photon lithography 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. This deterministic control is exemplified by the organotypic spinal cord cultures developed by Urciolo *et al.*,¹⁷³ where axons follow printed pathways (Figure 10).

In our article analysis, the emerging approach is also achieved through the application of engineering boundary conditions. The convergent VBP + TPP approach is shown to be effective in enabling the creation of hierarchical vascular networks, from large vessels (mm) to microcapillaries (μm), defining the resolution limits needed to integrate vascular systems into macroscale scaffolds, as demonstrated by He and He¹³⁰ 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.¹⁷⁷

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, thereby integrating macroscale transport function and microscale cell-instructive control into a single biological system. In this framework, VBP primarily addresses the problems of scale, speed, and a support-free internal architecture, whereas TPP addresses the problems of local boundary conditions, cell guidance, and deterministic microenvironmental patterning.

Beyond microscale architectural programming, volumetric and layer-less 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 overcome 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 photocrosslinked living constructs.

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

Collectively, these strategies share a common principle: they replace spontaneously forming tissue organization with engineered architectural and physical constraints, thereby enabling reproducible and causally interpretable structure–function relationships across biological scales. As summarized in Table 5, these approaches span organ-scale VBP, microscale TPP, and their integration with complementary strategies such as microfluidics, dynamic culture, and photopatterning. Individually or in combination, these approaches expand the biological design space of 3D *in vitro* models by linking structural control across multiple length scales within a hierarchical multiscale framework. Biofabrication therefore becomes particularly significant when it transforms tissue architecture, mechanics, and dynamics into controllable variables rather than treating them as downstream effects of self-organization. This direct programming of multiple biological needs addresses the limitations of supportive and enabling strategies.

Table 5. Summary of multiscale bioprinting strategies and their contributions to organoid functionality and biological requirements

Technique	Impact	References
Volumetric bioprinting (VBP)	Scaling and macro-architectural control; sustained tissue-level function to prevent core necrosis in centimeter-scale biofactories.	Bernal <i>et al.</i> ⁷¹
Two-photon polymerization (TPP)	Programmable control of micro-architecture, mechanics, and topography to guide axonal alignment and cell niches.	Gjorevski <i>et al.</i> , ¹⁵⁴ Urciuolo <i>et al.</i> , ¹⁷³ Colombo <i>et al.</i> , ¹³⁸ Crowe <i>et al.</i> , ¹⁷⁴ Harris <i>et al.</i> ¹⁷⁵
Convergence (VBP + TPP)	Creation of hierarchical vascular networks (from large vessels to capillaries) for multi-scale integration.	He <i>et al.</i> , ¹³⁰ Navarro <i>et al.</i> ¹⁶⁵
Microfluidic + dynamic culture	Overcoming diffusion-limited metabolic decay; enabling the transformation of static constructs into physiologically responsive environments.	Schuster <i>et al.</i> ¹⁷⁰
Photopatterning + microfabrication	Inducing morphogenesis and deterministic symmetry breaking via geometric constraints.	Gjorevski <i>et al.</i> ¹⁵⁴

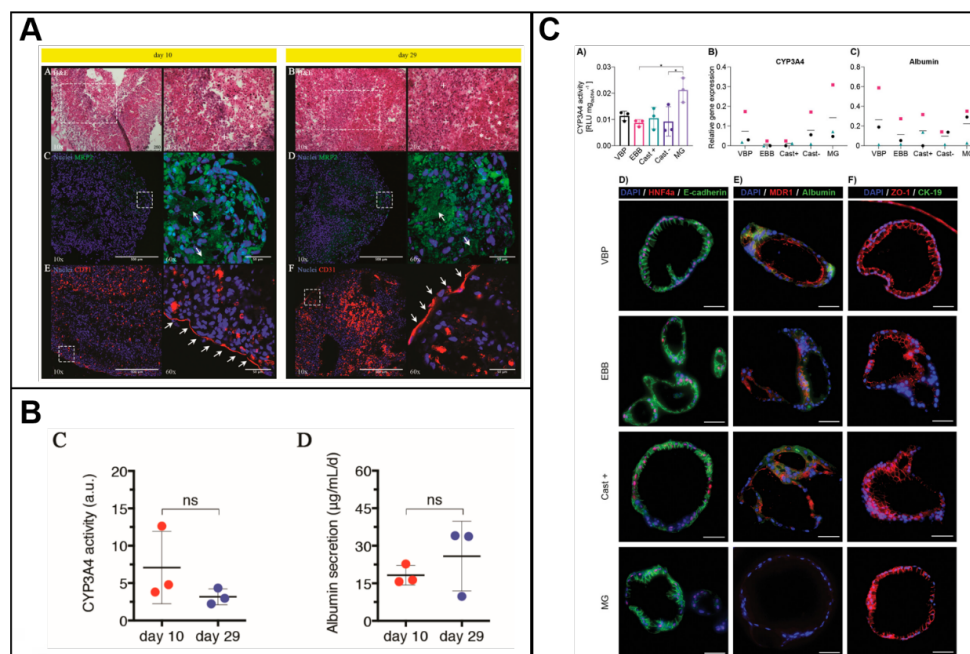


Figure 9. Liver organoid models. (A) Nonbioprinted, self-assembled multilineage liver organoids generated by co-culturing Upcyte® human hepatocytes, liver sinusoidal endothelial cells, and human bone marrow-derived mesenchymal stromal cells at a fixed ratio of 1:1:0.2 in Matrigel-coated culture wells. The organoids reached morphological stability after 7–10 days of culture, with a surface area of approximately 10–13 mm², and displayed spatial organization of hepatic and endothelial cell populations into primitive sinusoid- and biliary-like structures. Scale bars: 250/500/50 μm. (B) CYP3A4 activity and albumin secretion at days 10 and 29, demonstrating maintenance of key hepatic functions during long-term culture. Growth is limited by diffusion constraints, which restrict size and internal organization. (A,B) Adapted from Ref.¹⁷² (C) Human liver epithelial organoids derived from primary intrahepatic bile duct stem/progenitor cells and incorporated into a photoresponsive GelMA-based bioresin for layer-less volumetric bioprinting. The resulting constructs maintained liver-specific phenotype and functionality. Layer-less, high-speed fabrication enabled the generation of complex internal geometries that facilitated nutrient perfusion and metabolic exchange while maintaining lineage fidelity and prolonged functional performance, including CYP450 activity and ammonia detoxification. Adapted from Ref.⁷¹

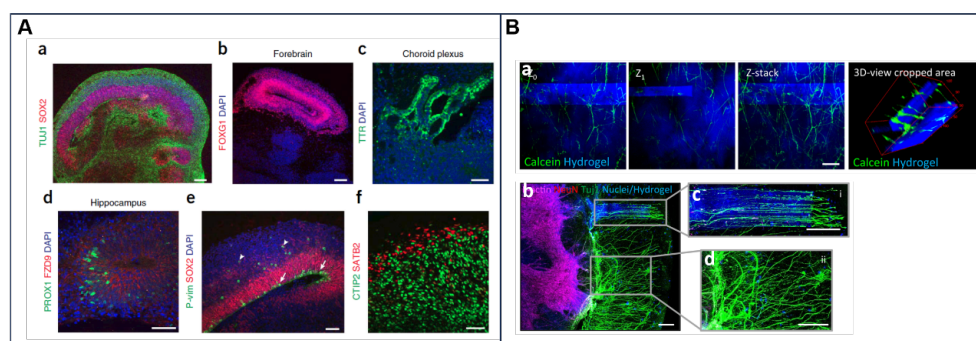


Figure 10. Neural organoid models. (A) Cerebral organoids generated from human pluripotent stem cells recapitulate key features of early human corticogenesis through spontaneous neuroepithelial self-organization. (a) Immunostaining showing SOX2-positive apical neural progenitors lining ventricle-like luminal regions and TUJ1-positive neurons distributed more basally, consistent with the polarized architecture of developing cortical tissue. Immunostaining further demonstrates (b) forebrain regions, (c) choroid plexus, (d) hippocampal regions, (e) mitotic radial glia domains, and (f) cortical layer identities in long-term cultured organoids (75 days). Scale bars: (a,b) 100 μm/(c–f) 50 μm. Adapted with permission from Ref.¹⁷⁶ Copyright © 2014, Springer Nature Limited. (B) Organotypic spinal cord (oSpC) constructs generated via hydrogel-in-hydrogel live bioprinting. In this *ex vivo* system, preformed organotypic spinal cord cultures preserve native tissue architecture, while anisotropic bioprinted hydrogel cues guide axonal outgrowth along defined trajectories. Rather than relying on organoid self-organization, this approach demonstrates the potential of bioprinting-based guidance to spatially organize preformed neural tissues. (a) Confocal fluorescence images acquired at different Z-planes and reconstructed as Z-stack, showing multiple hydrogel layers (blue) fabricated at different Z-positions and embedding live, calcein-positive (green) oSpC constructs that had been cultured in 3D Matrigel droplets for seven days before bioprinting. Scale bars: 100 μm. (b) Fluorescence image of an oSpC culture showing the regions analyzed for axonal organization. Higher-magnification images show (c) aligned axons within the hydrogel and (d) randomly oriented axons in the absence of the hydrogel. Scale bar: 200 μm. Adapted from Ref.¹⁷³

4.3. Summary

This section demonstrates a clear progression in the role of biofabrication within functional 3D *in vitro* models. Supportive strategies primarily improve reproducibility, throughput, and material reliability, reinforcing the biological requirements for cell identity and lineage fidelity, as well as for 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, regardless of the external design.

Finally, transformative 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 3D 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 TPP to ultrafast VBP 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. The technology roadmap is shown in Figure 11.

At the microscale, TPP 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 TPP 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. DLP and CLIP 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 VBP approaches, exemplified by computed axial lithography and emerging xolographic and holographic

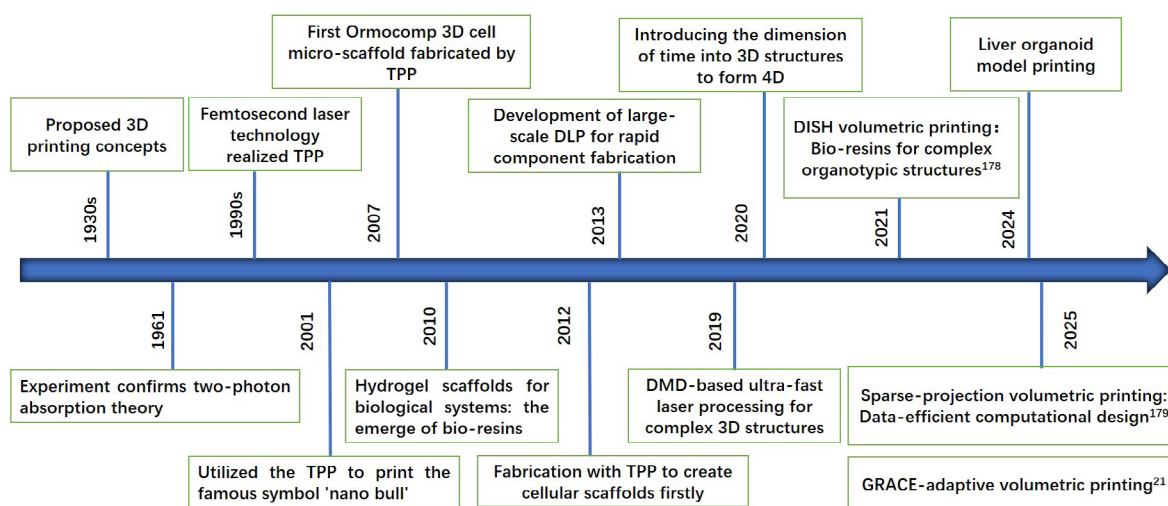


Figure 11. Technology roadmap: A comprehensive milestone diagram depicting the developmental trajectory of these novel 3D printing technologies

strategies, represent a fundamental shift toward layer-less fabrication. By curing entire 3D 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, VBP 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 A major challenge is the development of next-generation bioinks that simultaneously satisfy optical fidelity, rheological stability, and biological functionality.¹⁸¹ Volumetric printing in particular imposes stringent constraints on light scattering, optical absorption, cell sedimentation, photoinitiator cytotoxicity, and ROS accumulation, whereas TPP remains vulnerable to local heat buildup, 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 3D 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, multiscale manufacturing ecosystems rather than 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 learning¹⁸² and digital twin frameworks is expected to shift scaffold design from empirical trial-and-error toward predictive, 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 the way for four-dimensional bioprinting, in which engineered constructs evolve 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 3D 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.

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