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Organoid bioprinting to pattern the matrix microenvironment

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The development of organoid cultures has propelled the fields of cell biology, tissue engineering, and regenerative medicine forward. These cultures better mimic *in vivo* tissue structure and function compared to 2D cell culture; however, organoids are limited in size and do not inherently allow precise control over tissue architecture and cell heterogeneity. Hand-wrought organoid biofabrication approaches enable the production of larger and more complex tissues, but they still lack reproducible control of spatiotemporal tissue patterns. In contrast, bioprinting is a collection of machine-wrought technologies that are emerging as powerful tools in tissue engineering and disease modeling, but have not yet been widely applied to organoids. When combined with advances in biomaterials science, bioprinting offers the possibility to control spatiotemporal cellular and microenvironmental features. The interactions between biomaterial inks, support baths, and embedded cells provide the opportunity to guide the maturation and functionality of engineered tissues. This review describes how recent advances in organoid technology, bioprinting, and biomaterials science can be integrated to achieve spatiotemporal patterning of four aspects of the microenvironment: matrix structure and mechanics, matrix ligands and morphogens, co-culture of multiple cell types, and incorporation of vasculature. These insights underscore the potential for organoid bioprinting to advance the fabrication of *in vitro* tissue mimics for applications in drug screening, disease modeling, and regenerative medicine.

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Introduction

Organoids are three-dimensional (3D) *in vitro* cultures derived from stem cells (pluripotent, embryonic, or adult), or patient-derived cells (progenitor or differentiated, healthy or diseased) that self-organize into tissue-like structures [1]. Compared to two-dimensional (2D) cultures, organoids better mimic the biological, structural, and functional complexity of human tissues [2]. Hence, they are a promising tool to advance personalized medicine, drug screening, and regenerative medicine [1].

Despite their advantages, organoids cannot grow beyond the millimeter length-scale, which hinders the acquisition of macroscopic features present in life-size organs. Moreover, organoids are typically grown within homogeneous microenvironments that do not recapitulate the spatiotemporal features present *in vivo* [3]. Furthermore, organoids frequently suffer from large batch-to-batch variability. Organoid bioprinting has recently emerged as a strategy to overcome both of these limitations, guiding tissue morphogenesis of complex structures across more physiologically relevant size scales [4]. Printing organoids offers tremendous potential for tissue engineering, since it allows for spatial and temporal organization of microenvironmental cues, including biochemical and physical signals from the extracellular matrix (ECM) [5], morphogens [6], inclusion of multiple cell types [7], and patterning of vasculature [8], all of which assist in the formation of larger tissue constructs.

In organoid bioprinting, there are two generally recognized categories: continuous bioprinting, which includes extrusion and volumetric bioprinting, and pick-and-place bioprinting, which includes aspiration and magnetic bioprinting [3,9]. Extrusion-based bioprinting is currently the most common approach for tissue engineering applications, in which cells (or organoids) are loaded into a syringe and extruded in a layer-by-layer pattern to build a construct [10].

Extrusion-based bioprinting can be direct or embedded. As its name indicates, direct bioprinting allows for direct deposition of a printed construct in air, usually over a glass slide or a mold of defined geometry. Through direct bioprinting, filament and cell alignment can be achieved in many constructs, such as printed heart tissues [11]. On the other hand, embedded bioprinting

makes use of a support bath to enable printing of three-dimensional structures with free-form printing [10]. Embedded bioprinting methods are often designed to be a form of additive manufacturing, but this processing can also be subtractive, where material is removed to create, for example, hollow networks [12,13].

Volumetric bioprinting involves the use of light to cure centimeter-scale constructs in seconds [14]. Aspiration bioprinting enables manipulation of organoids or cell spheroids by vacuum pressure to precisely position them into a construct [15]. Depending on the cell type and size of the organoids, the application of vacuum may cause plastic deformation and degradation of the organoids [9]. To avoid this, organoids temporarily coated with magnetic nanoparticles can be positioned using a magnetized 3D printer into larger tissue constructs [9].

For most of these organoid bioprinting techniques, the process typically includes both a printable ink and a support bath, into which the ink is printed. Both inks and support baths are commonly yield-stress materials (i.e., they fluidize upon the application of force) that can recover their shape after printing. Engineering inks and baths that present these mechanical properties have been one of the main limitations of bioprinting. Moreover, when the ink and/or support bath contains a living cellular component, scalability constitutes another key limitation. Producing sufficient volumes of living materials becomes even more challenging when cells are used at a high density, especially if they are derived from stem cells or patient samples. Organoids (or individual cells capable of forming organoids) can be included either in the ink material and/or the support bath. When living cells are included within the ink, this is termed a bioink [16,17]. Ideal bioinks can shield cells from the potential detrimental effects of the bioprinting process (e.g., exposure to shear stress, ultraviolet light, or chemical crosslinking reagents) [17]. Furthermore, after printing, bioinks must undergo a cell-compatible solidification process to maintain their prespecified geometry [17]. In some cases, the printed inks are sacrificial in nature, meaning they are removed after printing. Alternatively, support bath materials can be used to provide temporary structural support to the printed construct. Yield-stress fluids are extensively used as support baths because their rapid solid−liquid transition induced by mechanical movement of the print head enables the free-form deposition of a variety of inks, including bioinks and sacrificial inks [18]. In organoid bioprinting, the structural, chemical, and biophysical environment provided by both the ink and support bath components can be considered as an engineered extracellular matrix (eECM), opening up new avenues for microenvironment patterning.

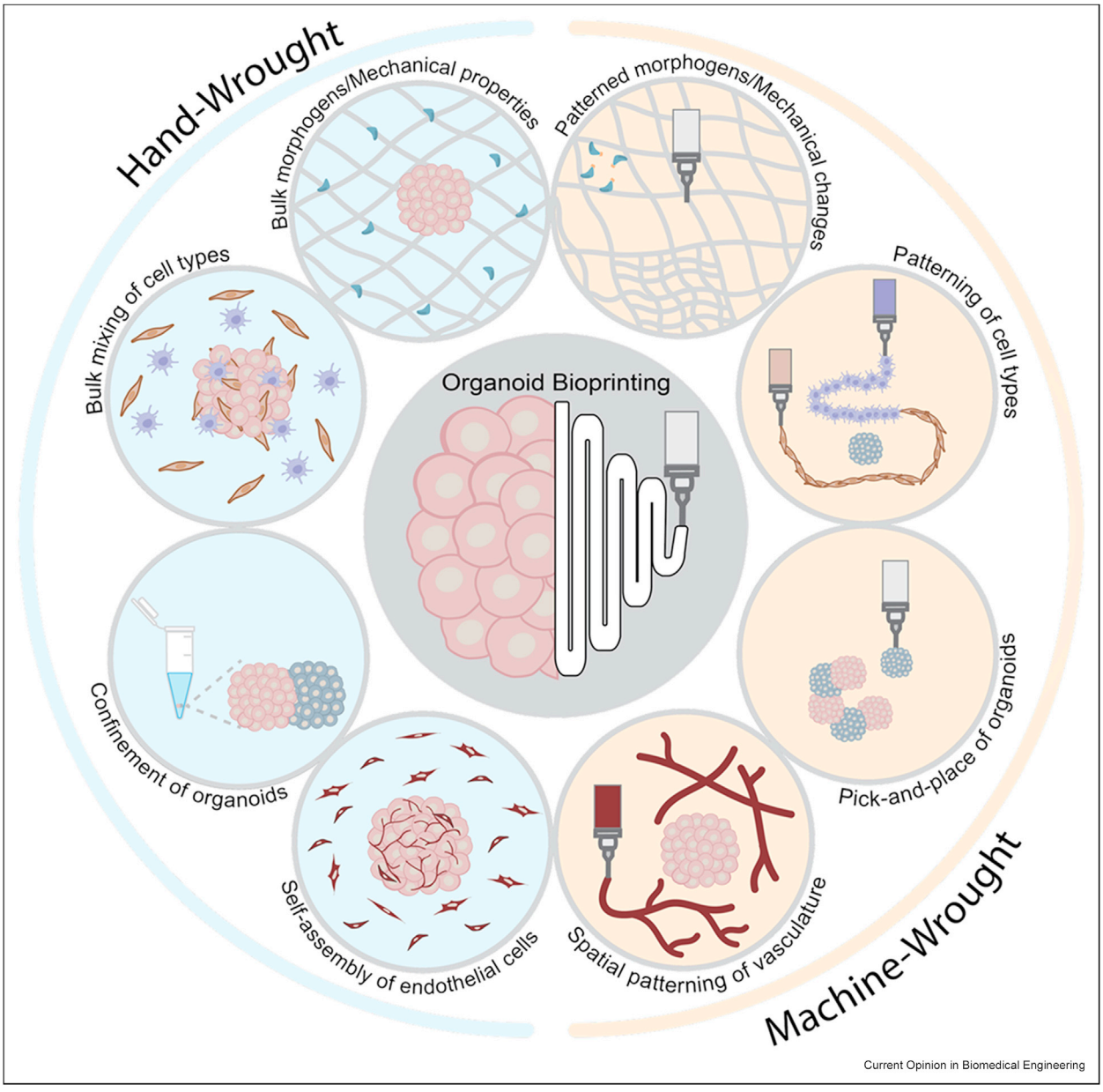
While several clever strategies have been reported to fabricate organoids into tissue-like structures with microenvironmental control, these have typically employed hand-wrought methods that lack microscale resolution (Figure 1, left). In contrast, biomaterials and 3D bioprinting offer opportunities for more accurate microenvironment recapitulation, although this family of precise machine-wrought technologies is still in its early stages of development and frequently does not include organoids. This review aims to explore recent advancements in bioprinting and designer biomaterials for applications in organoid bioprinting, focusing on strategies for engineering microenvironmental cues to enhance tissue functionality. Specifically, we will discuss recent advances in eECM structure and mechanical properties, the incorporation of cytokines and ligands into the microenvironment, as well as the combination of different cell types and vascular channels into the printed structure (Figure 1, right). This work serves as a forward-looking perspective meant to inspire the field and encourage the further development of novel organoid bioprinting strategies.

eECM structure and mechanics

The endogenous ECM is a complex fibrous protein and proteoglycan network, whose specific composition and post-translational modifications vary among tissues (e.g. ECM mineralization in teeth and bones [19]). The ECM presents a highly organized nano- and microstructure that provides mechanical and biochemical support to cells within tissues, helping to guide cell spreading, differentiation, migration, and proliferation. Due to its essential role in cell and tissue functionality, the majority of hand-wrought biofabrication approaches involve the use of an eECM, commonly employing harvested biopolymers, such as collagen, hyaluronic acid, alginate, and Matrigel [20].

Compared to most hand-wrought biofabrication techniques, where the properties of eECMs are modulated in bulk with limited spatiotemporal control, organoid bioprinting opens future opportunities to pattern the structural and mechanical features of the eECM to guide tissue morphogenesis. The library of biomaterials that have been utilized as ink components in 3D bioprinting can generally be divided into naturally derived polymers (e.g., gelatin, alginate, fibrin) [21,22] and synthetically derived polymers (e.g., polyethylene glycol (PEG), Pluronic) [21,22], each of which can be chemically modified to control the eECM structural and mechanical properties. For instance, a tetra-culture model of glioblastoma was printed with a mixture of gelatin methacryloyl (GelMA) and glycidyl methacrylate-HA (GMHA), which provided a microenvironment with mechanical stiffness relevant to the disease model [23]. Bioprinting has also been used to recapitulate the mechanical properties of healthy tissues. In a recent example, a neuron-laden bioink was printed into a soft astrocyte-laden GelMA support bath

Figure 1



Comparison of hand-wrought and machine-wrought approaches to patterning organoid microenvironments, demonstrating the potential of organoid bioprinting. Microenvironment properties that impact organoid growth include morphogen presentation, mechanical properties, inclusion of different cell types, inclusion of multiple organoid types, and vascularization of cultures. While many hand-wrought protocols (left) are being developed to control these microenvironment properties, they typically lack the spatial resolution that can be achieved with machine-wrought bioprinting approaches (right).

matrix, mimicking the soft nature of healthy brain tissue [24].

While these bioprinting examples showcase the potential of eECM engineering to mimic the mechanical microenvironment, further improvements in ink resolution, shape fidelity, and dynamic control are still required. Achieving high-resolution during printing is crucial to fabricating complex, large-scale, heterocellular constructs. Two-photon polymerization is the bioprinting approach that offers the highest resolution, with features smaller than 100 nm; however, its characteristic dimension is limiting for tissue engineering [25]. Decreasing the printed layer thickness, pixel size, or single line size are different approaches that can help increase print resolution, with the final resolution

commonly depending on the ink viscoelastic properties, the selected printing parameters, and the kinetics of the crosslinking or curing process [25]. An ongoing goal within the bioprinting field is to finely tune the spatiotemporal features of eECMs to closely replicate the properties of the physiological ECM. Below, we highlight recent approaches to control eECM nano- and microstructure and mechanics.

Controlling the structure of printed cell-laden inks enables the regulation of cell function. Introducing pores of defined sizes into the eECM provides physical spaces that assist tissue functionality through mechanotransduction [26]. In one demonstration, nanoparticle colloidal hydrogels were designed to form pores that promoted cell spreading and migration through a flexible GelMA hydrogel network better than in macroporous or homogeneous GelMA hydrogels. When chondrocytes were grown in these scaffolds with larger pores, they were observed to increase their proliferation and production of cartilage matrix [26] (Figure 2a). GelMA has also been fabricated in the form of microgels photocrosslinked through volumetric bioprinting to create microporous gels [27]. When microgels are used as a support bath for extrusion bioprinting, the choice of printing speed can be tuned to introduce micropores into the printed ink, due to mixing between the support bath and ink. In a demonstration with collagen inks, porosity was regulated through the ratio between the ink's shear viscosity and the microgel support bath's zero-shear viscosity [28].

Besides porosity, topographical guidance can direct cells to enhance anisotropic tissue formation. Many tissues in the human body present structural and cellular anisotropy that ultimately improves tissue functionality, including skeletal muscle, skin, and bone [29,30]. For example, in skeletal muscle, the alignment of muscle fibers has downstream effects on the global synchrony of muscle contraction [31]. Moreover, loss of anisotropy is a frequent feature of disease; for example, changes in tissue anisotropy have been reported in cancerous lesions of the uterus, bladder, kidney, liver, and colon [29]. Cell alignment within engineered tissues has been recently achieved through a variety of innovative bioprinting techniques. One strategy is Filamented Light (FLight) biofabrication, which creates hydrogels composed of unidirectional microfilament networks, aiding the maturation of engineered heart tissue [32]. In a different approach, applying mechanical load during the printing process can align hydrogel fibers and the cells printed with them, as demonstrated with norbornene-modified hyaluronic acid and GelMA inks [33]. In another study, combining embedded printing of collagen and myoblasts within a PEG solution with uniaxial mechanical loading generated fully aligned fibers that supported muscle regeneration [34]. Finally, volumetric bioprinting offers the ability to define microscale features within centimeter-scale constructs, as demonstrated with hepatic organoids printed into GelMA. This method produced liver mimics featuring cyst-like structures with inner hollow lumens surrounded by a cell monolayer—characteristics typically absent in constructs derived from monodispersed cells [14].

Just like porosity and topography, the mechanical properties of eECMs are vital to creating structures that are functional and biologically relevant (Figure 2). Because matrix-induced mechano-signaling can direct cell and organoid behavior [35,36], many strategies are being developed to control the spatiotemporal patterning of eECM mechanics. In one non-printed example, patterning of PEG hydrogel mechanics enabled the predictable construction of intestinal organoids with controlled shapes and sizes (Figure 2b) [30]. Specifically, a photo-degradation reaction was used to locally soften the matrix and hence bias the location of crypt formation [30]. In complementary work with alginate-based eECMs, the stress-relaxation rate of the matrix was shown to impact crypt formation in intestinal organoids [37]. In another non-printed example with alginate-based eECMs, a stress-relaxing matrix induced loss of symmetry for breast tissue spheroids and formation of invasive finger-like protrusions [38] (Figure 2c). Recent bioprinting efforts are opening the door towards spatiotemporal control of eECM mechanics. For example, spatial mechanical gradients have been formed by printing two inks, fully carboxylated agarose and native agarose, into one construct with human embryonic kidney cells (HEK-293) [39].

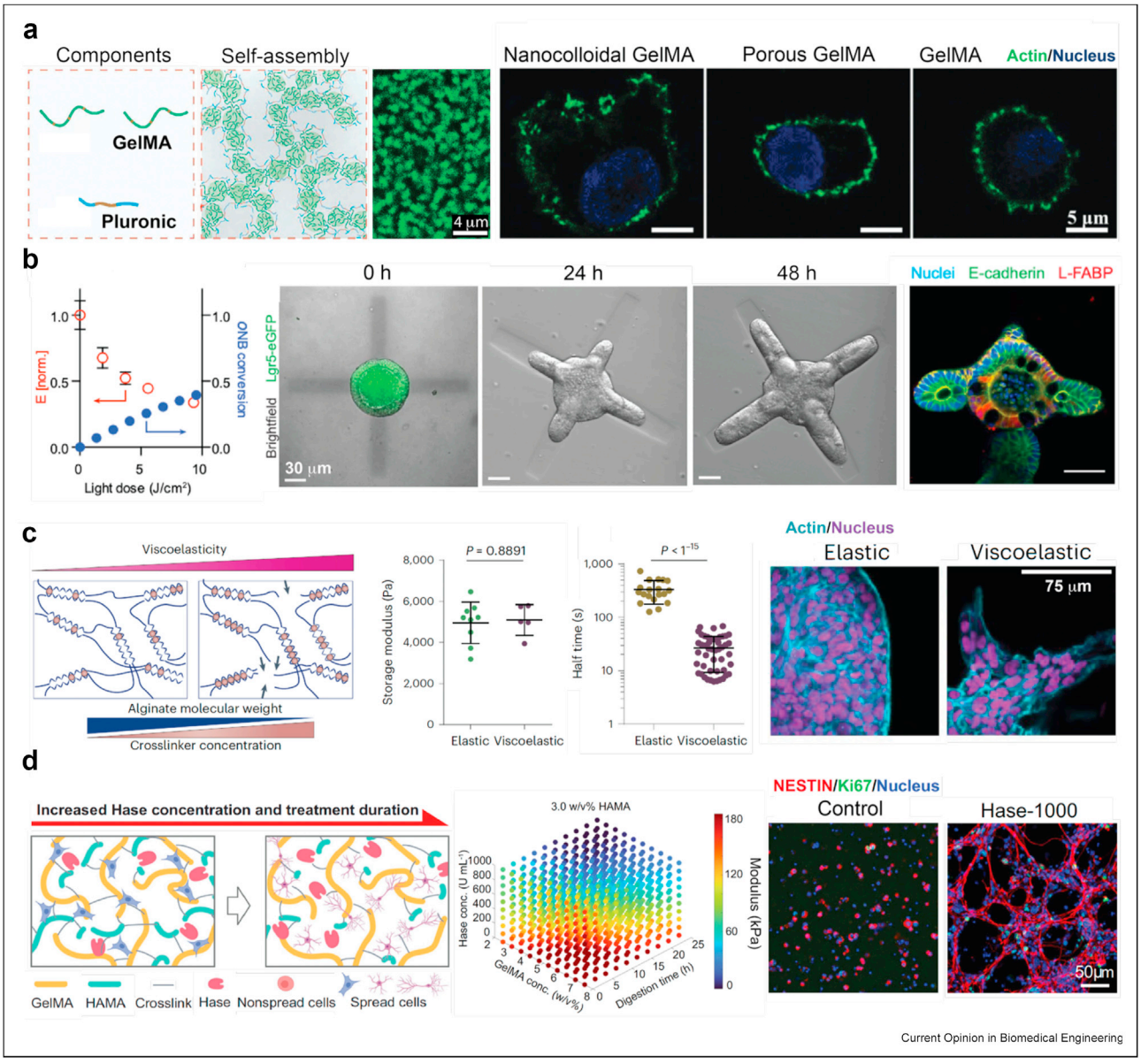
In another study, after printing of GelMA/hyaluronic acid-methacrylate (HAMA) matrices, they were subsequently exposed to enzymatic digestion to achieve dynamic softening to match tissue stiffness, changing the spreading behavior of encapsulated neural progenitor cells [40] (Figure 2d).

To date, most examples of dynamically changing the mechanical cues of an eECM have involved softening of the matrix [40]. In contrast, in numerous physiological tissues, stiffening of the matrix is commonly experienced during aging [41] and disease progression, such as cancer [42]. Thus, future efforts to design inks that reversibly stiffen on demand would have tremendous value for the bioprinting of human organoid disease models.

Matrix ligands and morphogens

In addition to different tunable structures and mechanics, matrix inks also have tailorable biochemical properties with the potential to influence cell phenotype when applied to organoid culture. Biochemical signaling in organoid culture is often applied through bulk morphogen exposure with molecules that drive the

Figure 2



The structural and mechanical properties of the matrix modulate cell behavior in machine- and hand-wrought constructs. (a) Matrix porosity. (From left to right) Schematic of the formation of a nanocolloidal gelatin methacryloyl (GelMA) and Pluronic hydrogel. Fluorescence 3D reconstruction of the hydrogel structure, with fluorescently labeled GelMA (in green). Chondrocytes at day 5 post-printing within 3D-printed tissue constructs fabricated with GelMA formulated to be nanocolloidal, porous, or homogeneous. Reproduced with permission. Copyright 2024, Wiley-VCH GmbH. **(b) Matrix mechanics patterning.** (From left to right) Mechanical characterization of RGD− and laminin-1−containing PEG−based hydrogels with atomic force microscopy reveals that there is a reduction in the Young's modulus **(e)** after light exposure, corresponding to conversion of photocleavable ortho-nitrobenzyl (oNB) moieties within the photosensitive PEG. Composite image showing the intestinal stem cell marker expression (Lgr5-GFP expression in green) in a symmetric colony and photopatterned matrix immediately after spatially restricted light exposure (0 h). In brightfield, spatially defined crypts form within photopatterned gels 24 h and 48 h after light-induced patterning. Enterocytes (L-FABP in red) are found in the central regions of the organoids. Scale bars 30 μm. Reproduced with permission. Copyright 2022, The American Association for the Advancement of Science. No claim to original U.S. Government Works. **(c) Matrix viscoelasticity.** (From left to right) schematic showing that alginate molecular weight and extent of crosslinking allow for modulation of alginate viscoelasticity. Quantification of the storage modulus and stress relaxation half time for two different alginate formulations. Spreading of a breast epithelial cell line (MCF10A) is more prominent in viscoelastic matrices; F-actin cytoskeleton (phalloidin, cyan), nuclei (Hoechst, magenta). Reproduced with permission. Copyright 2022, Springer Nature Limited. **(d) Bioprinted matrix stiffness.** (From left to right) enzymatic digestion of a bioprinted GelMA/hyaluronic acid-methacrylate (HAMA)-matrix with hyaluronidase (Hase) results in HA degradation, reducing matrix stiffness, and enhancing cell spreading. Parameter map of matrix moduli depending on GelMA concentration, Hase concentration, and digestion time. Fluorescence micrographs of neural progenitor cells cultured in GelMA/HAMA matrix without or with Hase digestion (nestin neural stemness marker in red, Ki67 proliferation marker in green, nuclei in blue). Reproduced with permission. Copyright 2022, Springer Nature Limited.

development of the organoids of interest. Since true ECM biochemical signaling in the body is finely tuned to occur with exact spatial and temporal specificity, spatiotemporally controlling the exposure of cells to biochemical signaling cues is often more physiologically relevant. Spatiotemporal patterning of morphogens has not been thoroughly explored in either organoid culture or the bioprinting field. However, several recent examples in designer biomaterials suggest that future demonstrations will become common for both fields. This section will specifically highlight works that control the patterning of morphogens in biomaterials, with an outlook towards applying these methods for organoid bioprinting work on the horizon.

Mixing morphogens into culture medium does not always induce the desired signaling pathway, since activation of some receptors requires physical forces that are only possible upon interaction with an immobilized ligand. Immobilizing proteins within hydrogel scaffolds can address this limitation, allowing for controlled organoid morphogenesis. For example, immobilizing full-length proteins like Jagged1 onto chemically modified eECMs can induce Notch cell signaling in organoids [43] and has been a popular way to include biochemical signaling in matrices. However, permanent immobilization of factors through bulk mixing does not allow for time-dependent control or spatial patterning control over their presentation to organoids, hindering the reproduction of complex, native tissue dynamics.

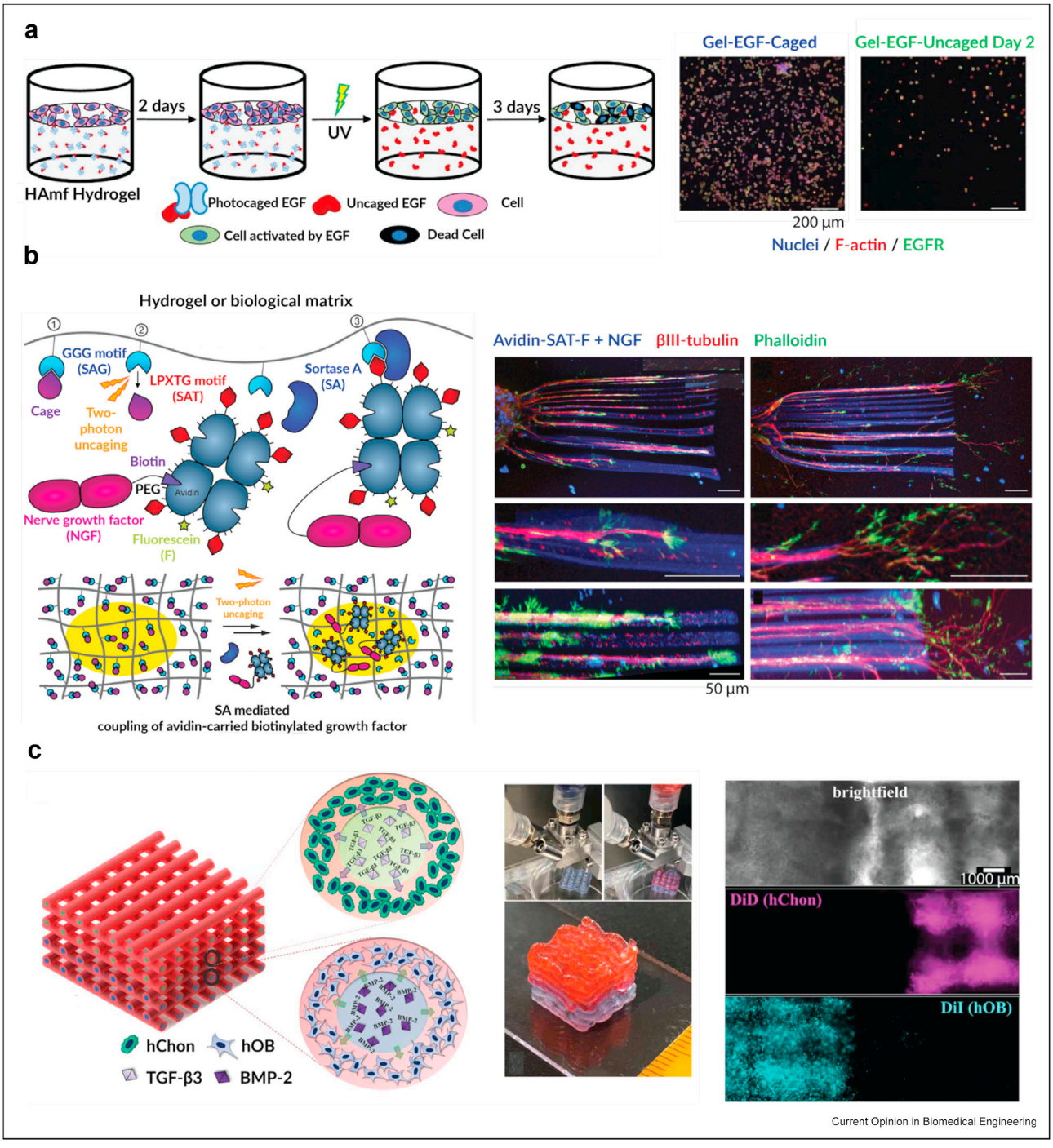
Since organoids have the potential to mimic the biological complexity of human organs, spatiotemporal patterns of gene expression are highly desired. For example, the intestinal stem cell niche is maintained with differential expression of WNT, BMP, and other cues [44]. One non-printed example of an effort to generate patterned organoids with time-controlled activation includes the usage of optogenetics to locally induce Sonic Hedgehog (*SHH*) signaling. This work aimed to study the contribution of *SHH* to gene regulation in neurodevelopment, and the tools they employed successfully resulted in patterned activation of the pathway [45]. Similarly, light patterning can also be employed to induce spatiotemporal patterning of morphogens within an eECM. For example, light-based volumetric printing was used to spatially pattern vascular endothelial growth factor (VEGF) using thiol-ene click chemistry within the hydrogel, allowing for region-specific adhesion of endothelial cells (ECs) [46]. This work highlights the precise, spatially controlled biochemical editing afforded by light-based printing techniques. In addition to spatial patterning, temporal control has also been explored in designer eECM materials. In one work, photocaged, immobilized proteins were uncaged on demand to control cell fates in breast cancer and lung cancer models [47], exhibiting the potential for temporal control of morphogens important in inducing cell fate (Figure 3a). Combining the goal of spatial *and* temporal selectivity, one example showed the utilization of two-photon patterning for selective, on-demand anchoring of nerve growth factor (NGF) in a hydrogel to guide axon extensions [6] (Figure 3b).

Although spatiotemporal control of matrix-bound factors in organoid culture has not been explored extensively, the bioprinting field has introduced several versatile methods with the potential to be applied to organoid culture. In particular, bioprinted tissue-engineered constructs for transplantation often have engineered parameters for controlled growth factor or drug release due to the short lifetime of these bioactive molecules and the need for persistent exposure to elicit desired effects. Depending on the type of tissue and biological process, the length-scale of morphogen and ligand patterning could be subcellular or span more than a few hundred micrometers in length. Similarly, the time scale of applied cues can range from relatively short to more durable in time [48].

Methods to slow down and spatially control the release of bioactive factors have been developed to improve the recruitment, support, and differentiation of cells in transplantable constructs. The release of growth factors, ions, and drugs have all been explored in different biological applications. Examples of morphogen release for vascular applications include layer-by-layer assembly of growth factors onto bioprinted blood vessels, allowing for increased myofibroblast recruitment to the constructs post-transplantation, improving functionality [49]. An osteochondral application example is the utilization of core–shell bioprinting methods to spatially segregate sustained delivery of growth factors to adjacent bone and cartilage zones in osteochondral constructs [5] (Figure 3c). Bioprinting has also been employed for spatiotemporal control of factors in *coupled* angiogenesis and osteogenesis [50].

Bioprinting also enables the copatterning of construct topology together with spatial release of bioactive factors. For example, nerve conduits that slowly released factors isolated on microgrooved channels allowed for enhanced neurogenic differentiation [51]. In addition to controlling the organization of growth factors, controlled release of other biomolecules has also been explored in biomaterials design and bioprinted constructs. For example, bioprinting of a vascular construct that achieved a slow, sustained release of heparin resulted in improved support of ECs and an antithrombotic environment compared to high doses of heparin [52]. Finally, spatiotemporal patterning of bioactive factors is not limited to organic molecules. For example, the bioprinting of a vertical gradient of calcium phosphate has been used to control the extent of osteogenic differentiation of mesenchymal stem/stromal cells (MSCs)

Figure 3



Spatial and temporal patterning of matrix ligands and morphogens. (a) (left) Temporally controlled activity of epidermal growth factor (EGF) can be achieved by transient photocaging within a methylfuran-modified hyaluronic acid (HAmf) hydrogel and photo-uncaging with ultraviolet (UV) light. (right) MDA-MB-468 breast cancer cells seeded on top of HAmf hydrogels undergo cell death in the presence of photo-uncaged EGF (nuclei in blue, F-actin cytoskeleton in red, epidermal growth factor receptor (EGFR) in green). Reproduced with permission. Copyright 2021, American Chemical Society. **(b)** (left) A hydrogel displaying photocaged Sortase A glycine donor peptides (SAG) can be spatially patterned with two-photon microscopy. Sortase A (SA) mediates ligation of SAG to SAT-labeled avidin, followed by docking of biotinylated nerve growth factor (NGF). (right) Neurite guidance from chick dorsal root ganglia (DRG) into transglutaminase crosslinked hyaluronan matrix with spatially patterned NGF (NGF in blue, βIII-tubulin in red, phalloidin staining of F-actin cytoskeleton in green). Reproduced with permission. Copyright 2024, Advanced Materials. **(c)** (left) Coaxial extrusion enables core–shell bioprinting of human mesenchymal stromal cells within two distinct bioinks containing either bone morphogenetic protein-2 (BMP-2) or transforming growth factor-β3 (TGF-β3) within the core. Over time, protein release results in differentiation into either chondrogenic (hChon) or osteogenic (hOB) cells. Photographs show bioprinting of the biphasic construct. Microscopy characterization shows the interface between the two distinct cell layers at day 21. Reproduced with permission. Copyright 2022, IOP Publishing.

[53]. In addition to affecting cell fate, the inclusion of calcium phosphate can also alter the stiffness of the matrix [54].

To date, many of the methods used to pattern ligands and morphogens have relied on ultraviolet/visible light or diffusion of enzymes. In engineered tissues with high cellularity, both of these strategies will be challenged by limited penetration depth. Looking ahead, new strategies to modulate biochemical features of the matrix across physiologically relevant length scales are needed. For example, ultrasound-induced chemistry [55] and infrared light-induced photochemistry [56], both of which can penetrate several millimeters into tissue, are promising strategies. Applying these biomaterials technologies to organoid cultures has the potential to improve reproducibility by mimicking the spatial and temporal dynamics that occur in native tissue during development.

Patterning of multiple cell types

In addition to the spatial organization of morphogens, bioprinting also allows for the opportunity to spatially organize different cell types. Several cell types, including fibroblasts, immune cells, and ECs, have been cultured alongside organoids in order to uncover important cell—matrix and cell—cell communications. A common approach in organoid co-culture is to simply mix the cell types together and rely on cellular self-assembly. In a recent example that used this strategy, the establishment of endometrial organoids co-cultured with stromal cells allowed for specific cell—cell crosstalk analyses in the context of applied hormone changes [7]. While mixing of cell types is straightforward, the complex spatial positioning of cells cannot be easily controlled. For example, spontaneous cell segregation frequently relies on differential cell adhesion to form patterns with minimized interfacial energy [57]. In contrast, bioprinting offers a method to spatially organize different cell types within one model. This section will highlight recent organoid bioprinting examples as well as bioprinting methods that could be applied in the future to increase cell type complexity in organoid co-culture models.

Spatial arrangement of co-cultures may spontaneously arise in a “bottom-up” fashion or be experimentally controlled through “top-down” methods. Current “bottom-up” approaches to control spatial arrangements of cells are reliant on controlling discrete cell properties, and the underlying mechanisms are still being discovered [58]. When these methods are applied at the scale of real tissues, the work may become unwieldy. Thus, the application of “top-down” methods through bioprinting may be more efficient in larger constructs. As a demonstration of self-assembly, co-aggregation of MSCs and ECs to form bone marrow organoids resulted in spontaneous organization of ECs into an interconnected vessel-like network surrounded by MSCs [59]. Within the field of organoid biology, co-culture of two or more organoids, spheroids, or dispersed cell types, combined, has been popularized, and recent developments include spatially arranging organoids by hand to better recapitulate interactions between different tissue regions. For example, to study the cell dynamics at the foregut-midgut boundary, anterior and posterior gut spheroids were separately derived and then fused to study morphogenesis [60].

Bioprinting represents a promising approach to spatially pattern distinct cell types (including organoids) into multicellular co-culture models. While examples of bioprinting organoids in co-culture are limited, bioprinting of single cell suspensions has been used to spatially organize different cell types to study cell—cell dynamics, which could be readily extended to organoid applications. For example, embedded bioprinting was used to print a murine melanoma cell ink within a microporogen-structured collagen matrix embedded with antigen-specific cytotoxic T-cells, where T-cells migrated to the cancer sites and caused cell death [61]. A stromal co-culture example included the 3D bioprinting of islands of pancreatic cancer cells surrounded by rings of cancer-associated fibroblasts, which mimics the desmoplastic stroma that surrounds these tumors. The spatially patterned co-culture induced both cell types to make dynamic alterations to their differentiation and secretory profiles compared to mono-cultures [62]. 3D bioprinting also enables studies of cell migration across specific geometries, exemplified by an assay that patterned human umbilical vein endothelial cells (HUVECs) and MSCs at different distances and found that angiogenesis is deeply influenced by crosstalk between these two cell types [63]. These examples highlight the potential for bioprinting as a tool for probing cell—cell interactions in a spatially relevant manner.

While exerting spatial control on organoid co-cultures allows for the study of mechanistic cell behavior, combining bioprinting with organoid co-culture can also enable the creation of more physiologically relevant tissue engineered constructs, with applications towards pharmaceutical testing or personalized medicine. In one case, combining pancreatic cell progenitors, ECs, and MSCs in different formations within a microgel support bath yielded vascularized exocrine and endocrine pancreatic lineages, which enabled complex recapitulation of pancreatic tissue for disease modeling [64]. Another example used bioprinting to create a cardiac model from a triculture of induced pluripotent stem cell (iPSC)-derived cardiomyocytes, MSCs, and ECs on printed constructs with adjustable curvature, which demonstrated successful myocardial maturation [65]. Both studies foreshadow future studies with multicell-

type bioprinted constructs for personalized medicine drug screening.

Another promising application for multi-cell-type bioprinted tissue constructs is regenerative medicine, which is currently at the preclinical stage of development. In one bioprinting example, human epidermal keratinocytes were seeded onto encapsulated human dermal fibroblasts to form a full-thickness skin model that demonstrated successful wound closure in a rat model [66]. As a demonstration of an even larger engineered tissue, a bioprinted autologous auricular cartilage construct was fabricated with a prevascularized dermo-epidermal skin substitute. When transplanted onto immunocompromised rats, the fabricated tissue successfully connected with the recipient vascular system [67].

The combination of organoid culture and bioprinting will enable the fabrication of more spatially relevant models for capturing multitissue interactions. To date, few bioprinting examples have included the spatial organization of multiple organoid types. One example is a pick-and-place bioprinting platform to spatially control the construction of neural assembloids from different types of neural organoids using magnetic forces [9]. In another notable example, bladder assembloids were created through extrusion bioprinting of bladder tumor organoids mixed with cancer-associated fibroblasts and ECs [68]. These two demonstrations of bioprinting organoid co-cultures highlight the immense potential for growth in the field of multi-cell-type organoid bioprinting.

Advancements in techniques to pattern organoids at high density are currently limited by the lack of scalability in organoid production. The cost and time needed to culture enough cells at such high density prove to be a barrier in creating physiologically relevant tissue prints. This is especially challenging when printing large volumes of organoids derived from patient samples. Moving forward, the field will require new strategies to lower the cost [69] and increase the throughput of organoid biomanufacturing to realize the promise of organoid bioprinting. Such strategies may include the use of automated cell culture robots [70], engineered cell culture supplements, and artificial intelligence to identify optimized cell culture conditions.

Vasculature and perfusion

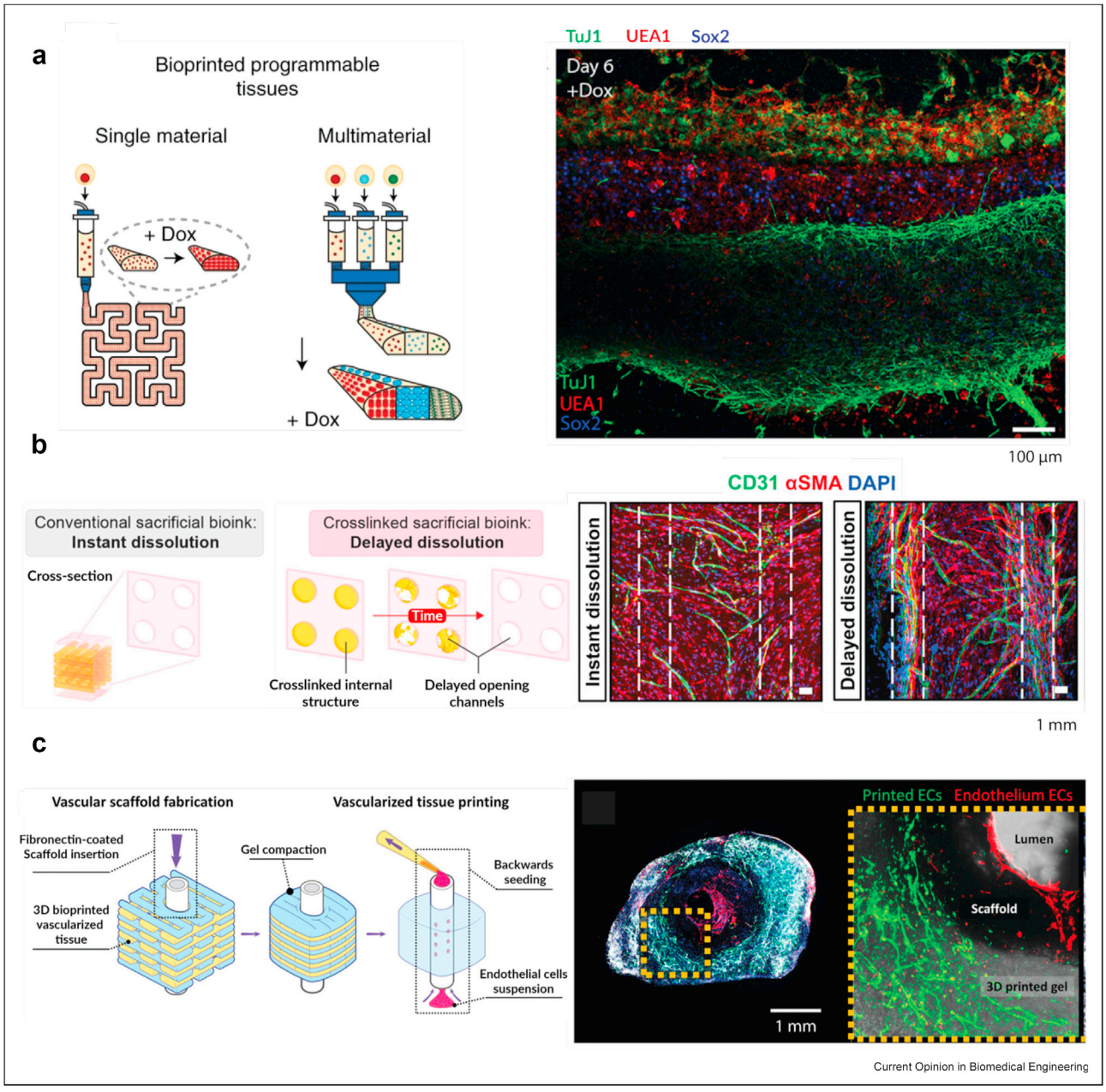
As organoid cultures begin to include more cell types and larger constructs, it becomes increasingly important to perfuse them to ensure cell survival. Methods to vascularize organoid cultures have included adding ECs through bulk mixing [71] or genetic programming [72]. These approaches rely on the spontaneous organization of ECs to form capillary-sized structures. In contrast, native vasculature is a hierarchical structure that spans multiple length scales. Thus, bioprinting can enable the patterning of larger-sized vessels to facilitate the anastomosis of organoid models with external vessels, either *in vitro* or *in vivo*. In this section, we highlight examples of bioprinted vasculature spanning several orders of magnitude in size that may be utilized in organoid models in the future.

A recent innovation in the genetic programming approach is the development of a one-pot protocol to orthogonally induce cell differentiation and spatially pattern multicellular organoids through transcription factor overexpression [73]. Spatial organization of cell types within cortical organoids was achieved through stepwise aggregation of inducible neuronal and endothelial human iPSCs, which yielded core–shell cortical organoids. While core–shell organoids have radially symmetric spatial organization, bioprinting has the potential to yield more complex spatially organized vasculature. These same preprogrammed cells were also used as high-cell-density bioinks to fabricate a layered architecture of multiple cell types post-differentiation (Figure 4a). This demonstration certainly calls attention to the spatial patterning benefits that bioprinting methods could offer to genetically programmed organoid cultures. Spatial patterning can also be used to guide the spontaneous self-assembly of ECs into vascular-like networks. In one demonstration, bioprinting of ECs was used to fabricate vascular and avascular regions within a single bioprinted construct [8].

A complementary strategy to relying on the self-organizing capacity of ECs, which results in small capillary-like channels, is the bioprinting of perfusable, hollow structures using sacrificial bioinks, which results in larger channels. For example, a sacrificial Pluronic ink can be printed into a microgel suspension and then removed to reveal hollow structures that can be seeded with HUVECs to create vascular linings [74]. Further innovation for printing hollow structures includes programming delayed dissolution of the sacrificial ink to better recapitulate the dynamic nature of tissues [75] (Figure 4b). Hollow structures can also be built by depositing supporting templating bioink next to matrix bioinks layer-by-layer [76], allowing for the future endothelialization of more complex hollow structures.

Bioprinting methods to vascularize engineered tissue are particularly critical for applications in implantable tissues. Implantable constructs must be well vascularized in order to maintain viability and proper functionality after implantation. This is especially true for the fabrication of thick tissues in which diffusion alone is insufficient to maintain appropriate oxygen and nutrient levels, concerns that are also common within the organoid biology community. Thus, we envision that bioprinting advances to achieve vascularized, implantable tissues could be readily applied to the

Figure 4



Multimaterial bioprinting enables the formation of complex tissue mimics. (a) (left) Doxycycline (Dox)-induced overexpression of transcription factors in engineered human-induced pluripotent stem cells (hiPSCs) allows for spatial patterning of different cell types by bioprinting. (right) Multimaterial bioprinting exhibits clear separation of different cell layers after 6 days of culture (Tuj1 neuronal marker in green, UEA1 lectin marker in red, Sox2 neural stemness marker in blue). Reproduced with permission. Copyright 2023, Springer Nature. **(b)** (left) Delayed dissolution of an extruded sacrificial bioink allows for spatiotemporal introduction of microchannels within hydrogel constructs fabricated by volumetric bioprinting. (right) While mesenchymal stromal cells (labeled in red with aSMA marker) and human umbilical vein endothelial cells (labeled in green with CD31 marker) are randomly distributed in instant dissolution constructs after 10 days of culture, endothelial cells align along the microchannels in constructs with delayed sacrificial templates. Reproduced with permission. Copyright Advanced Functional Materials, 2023. **(c)** (left) Fabrication of implantable, perfusable, vascularized tissues includes inserting a printed synthetic polymer scaffold (shown in white) into a bioprinted vascularized tissue made of recombinant human collagen methacrylate. After gel compaction due to cellular forces, the scaffold is seeded with endothelial cells (ECs). (right) Fluorescent microscopy of the vascularized scaffold after culture shows network formation of the printed ECs (green) and lumen formation of the seeded ECs (red). Reproduced with permission. Copyright Advanced Materials, 2021.

vascularization of organoid models in the future. Often, these advanced bioprinting strategies rely on the creative use of multiple, specialized biomaterial inks. For example, a vascularized construct with bioinstructive microchannels was fabricated by coating sacrificial alginate cores with multiple layers of chitosan and cell-adhesive biomaterials. Exposure to a calcium chelating solution then caused the alginate cores to dissolve away, revealing perfusable channels with cell-adhesive surfaces for ECs [77].

Finally, an emerging research area in bioprinted vasculature is the combination of "top-down" and "bottom-up" approaches to achieve networks with both large and small vessels, respectively. For example, the bioprinting of a basement membrane-like biomaterial promoted the adhesion and self-organization of ECs into capillary beds between larger, bioprinted vessels [78]. Such complex networks that combine micro- and mesoscale vessels can also be designed to promote anastomosis with the host vasculature upon implantation. In an exemplary demonstration, bioprinted self-assembled microvascular networks were connected to a larger vascular scaffold, enabling the microvessels to receive nutrients from the larger vessel (Figure 4c) [79]. Upon *in vitro* maturation, the construct formed a fully vascularized tissue flap that was successfully anastomosed with a rat femoral artery, showcasing the ability of bioprinted vasculature to perfuse large tissue constructs. The application of these newly emerging biomaterials and bioprinting methods to organoid cultures will allow for the construction of more complex, physiologically relevant organoid models with multiscale vascular networks.

In addition to current efforts to merge the different, hierarchical length scales required for true vasculature within organoids, another area of focus for future work is the ability to support active perfusion. Currently, engineered vasculature is either perfused through passive diffusion or is driven by an external pump. In the future, vascularized organoid-based tissues could be designed to include contractile cell types that actively pump, allowing for the creation of closed-loop circulatory systems [80].

Conclusion

The advent of organoids has advanced the complexity of tissue mimics, offering a more accurate representation of human tissue than 2D cultures [2]. Organoids can be interrogated for mechanistic studies in tissue development and disease modelling, as well as applications in drug discovery, personalized medicine, and regenerative medicine [1]. Despite their advantages, organoids are limited by their small size, their patient-to-patient variability, and the lack of methods to control their spatiotemporal features [3]. To overcome these limitations, emerging technologies like 3D bioprinting are rapidly gaining traction. By using organoids as building blocks, 3D bioprinting allows the creation of more intricate, physiologically relevant structures.

The implementation of organoid bioprinting is still in its infancy, although recent examples already suggest the tremendous potential that this strategy offers for controlling the organoid microenvironment— a complex, dynamic 3D space. Exerting control over microenvironmental features will facilitate the fabrication of constructs more representative of *in vivo* conditions. Here, we highlighted recent efforts to pattern complexity into the cellular microenvironment, selecting case studies from organoid bioprinting where they exist. We also described exciting advances in organoid culture (without printing) and cellular printing (without organoids), when those studies could be readily applied to organoid bioprinting in the future. As cutting-edge biomaterials strategies become integrated with advanced bioink design, we envision that organoid bioprinting will be able to achieve on demand, spatiotemporal patterning of the matrix microstructure [27], topographical features [32], and mechanical properties [39,40] at cellular resolution. Similarly, advances in light-based biomaterials techniques and controlled release of soluble factors will allow for spatiotemporal patterning of matrix ligands and morphogens [46,47] for organoid bioprinting. Another emerging strategy to enhance engineered tissue complexity is the patterned co-culture of multiple cell types, although demonstrations with organoid bioprinting remain largely on the horizon. Work in this space will likely be inspired by current examples of coprinting different single-cell types [61—63]. Finally, as the complexity and size of these engineered tissues grow, efforts to pattern the vasculature and introduce perfusion will become increasingly important [77—79]. Early applications of organoid bioprinting will include the fabrication of human models of development and disease, especially for those tissues where animal models have significant limitations, such as neurodevelopment. For example, the improved reproducibility of organoid bioprinting will enable high-throughput testing for the identification of novel drug targets and assessment of potential drug efficacy and toxicity. Longer term, organoid bioprinting will be applied in the fabrication of transplantable tissues for regenerative medicine, including self-contractile constructs [79,80] and eventually whole organs, where hand-wrought methods would be too laborious and cumbersome. Overall, the development and use of machine-wrought bioprinting technologies is poised to transform our ability to control the organoid microenvironment, enabling the production of physiologically relevant tissue models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could

have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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