Patterning hydrogels in three dimensions towards controlling cellular interactions

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The 3-dimensional (3D) interaction between cells and extracellular matrix (ECM) constitutes a dynamic regulatory system for directing tissue formation and homeostasis, as well as regeneration in response to injury. As such, significant progress has been made in the field of tissue engineering to develop 3D hydrogels capable of promoting cell viability and many of these important ECM interactions. However, the spatial patterning of hydrogels in 3D, motivated by the microscale heterogeneity of native tissue architectures, has only recently been a focus. Here, we review efforts to date to engineer structurally and/or biochemically patterned 3D hydrogels to control cellular behavior for regenerative medicine applications. Such techniques can be separated into two categories: stereolithographic "bottom-up" methods that pattern structures during layer-by-layer fabrication and post-gelation techniques involving modification of a uniform, pre-formed hydrogel. Many techniques in each group are further compatible with cell encapsulation, providing a valuable set of models for studying cell-cell signaling or for the engineering of new tissues.

Introduction

Tissues are heterogeneous structures on a wide range of length scales, with the average being on the order of 100 μm.[1] As such, biomaterials for regenerative medicine applications must be capable of facilitating the reconstruction of this heterogeneity upon implantation into a defect site, including towards integration with the native tissue. In many instances, this heterogeneity will develop through tissue morphogenesis, provided the right cell types and cues are available. However, it is also thought that the replication of these structures through engineered materials (*e.g.*, hydrogels) may aid in reconstruction and accelerate healing. Hydrogels are water-swollen, crosslinked polymers that are attractive as scaffolding materials for regenerative medicine applications due to their ability to mimic many physical properties of tissues.[2] Another particular advantage of hydrogels are their typically facile and gentle processing conditions, enabling cell encapsulation directly in the gel. The ability to seed or encapsulate cells within 3-dimensional (3D) constructs is a paradigm of growing interest,[3,4] as these materials better replicate *in vivo* microenvironments when compared to cell seeding atop 2-dimensional (2D) substrates.[5,6]

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While significant progress has been made in engineering 3D hydrogels that promote cell-matrix interactions *via* incorporation of biochemical cues,[7,8] a shortcoming of these largely uniform and static materials is their inability to recapitulate the spatial heterogeneity of matrix found in tissues.[9] Due to their thickness and extensive swelling, spatial patterning of hydrogels in 3D is not trivial and these challenges and difficulties may have limited technology development and implementation. However, the past decade has witnessed the introduction of a number of such patterning techniques that are now well established and continually being improved. Here, we review efforts to date toward the patterning of hydrogels in 3D, primarily toward controlling cell interactions for regenerative medicine applications.

For the purpose of this review, 3D patterning is defined as the incorporation of some controlled heterogeneity, either biochemical (*e.g.*, the spatially patterned incorporation of cell adhesive ligands without alteration of the uniform gel structure) or structural (*e.g.*, patterned gel structure using stereolithography (SLA)), within the interior of a hydrogel. Cells that interact with these features may either be encapsulated directly into the hydrogel or seeded atop the constructs and migrate into the gel using patterned guidance cues (*e.g.*, photoablated microchannels). Thus, techniques limited to the creation of patterns only on surfaces, for example using dynamic wrinkling techniques, fall outside this scope.[10–12] Techniques using soft lithography, although capable of creating heterogeneous 3D hydrogels, also fall outside this scope because they are limited to basic 2D patterns with depth and relatively large features sizes.[13] Additionally, because we focus here on cell-material interactions, some acellular techniques[14,15] involving 3D patterned gel formation have also been omitted.

The quality of patterning throughout the depth of the hydrogel systems reviewed here is typically assessed by direct visualization using techniques such as laser scanning confocal microscopy (LSCM), as well as by the response of encapsulated or seeded cells to the patterned features. Metrics of cell behavior used to evaluate the response to patterning range from cell morphology and guided migration to directed stem cell differentiation. Generally, it is clear that technology has developed rapidly in recent years and will continue to improve to provide better control of cellular behavior towards advanced tissue engineering (TE) constructs.

Bottom up *versus* post-gelation patterning techniques

3D patterning techniques developed to date can be separated into two categories: those that incorporate patterning during or as a result of fabrication (*i.e.*, using layer-by-layer stereolithography, SLA) or those that involve processing following the initial gelation of a pre-formed, uniform hydrogel (*e.g.*, the use of two-photon microscopy to pattern bioactive moieties into a formed hydrogel). Importantly, and as will be described below, many of these systems feature sufficiently rapid and gentle processing conditions to afford cell encapsulation directly within the hydrogel. Alternatively, cells are seeded atop hydrogels to assess 3D migration into the scaffold in a patterned manner.

Bottom-up patterned hydrogel fabrication

The majority of reports to date describing the synthesis of 3D patterned hydrogels employ layer-by-layer fabrication using SLA, a rapid prototyping technique. This approach uses light irradiation of individual, patterned printed layers of hydrogel precursor solution, with washing of uncrosslinked solution between layers. The use of SLA to create patterned constructs is beneficial to study cell interactions compared to conventional lithography on a silicon chip, which suffers from many processing steps and low cytocompatibility. In particular, the limitations associated with conventional lithography make cell encapsulation difficult.[16]

Within SLA, the majority of studies can be further separated into two sub-categories: those that cure a liquid prepolymer solution (in some cases, containing cells) with a laser source immediately upon deposition with a printer, and those that flood irradiate a film of prepolymer solution through a photomask to acquire the desired patterned layer (under the premise that only unmasked regions of the solution will undergo light exposure and crosslinking). For both cases, after the initial patterned layer is fabricated, uncrosslinked polymer is washed away, and the process is repeated for subsequent layers. In many cases, the processing conditions allow for the deposition of cells directly within each layer to give a cell-encapsulated hydrogel. For other studies, cells are seeded atop the constructs after fabrication, and cell migration occurs into the gels in a 3D, patterned fashion. Because thin layers are fabricated individually in a stereophotolithographic approach, numerous concerns that exist for post-gelation patterning, such as light diffraction through a thick sample or uniformity of patterning through the hydrogel depth, are minimized with this approach.[17]

Microfluidic systems are also commonly used for patterning cells in 3D. These approaches may involve patterned photopolymerization of portions of a cell-encapsulated hydrogel within a microfluidic device.[18] Medium is then perfused through adjacent empty channels, and variations in the channel width and perfusion rate allows the study of morphogen transport on cell viability or cell-cell communication. While we focus here on studies examining cell-cell communication for possible therapeutic manipulation, similar systems have been used for other applications including modeling of *in vitro* pharmacokinetics[19] and for immunoassay development.[20]

Maskless stereophotolithography. Maskless SLA is the layer-by-layer fabrication of hydrogels using polymerization of a crosslinkable polymer solution immediately after printing onto a substrate. Typically, the desired shape to be printed is drawn with computer aided design (CAD) software, followed by automated printing. Maskless SLA, which is sometimes referred to as layered manufacturing (LM) or 3D plotting, was one of the first techniques used to fabricate 3D scaffolds for TE applications.[21,22] From the perspective of studying cellular interactions in hydrogels, this approach is unique in that different cell populations can be introduced in either the same or a different polymer, potentially providing a facile method for studying cell-cell signaling in 3D. Also, since the hydrogel solutions are polymerized immediately upon deposition, these systems offer low minimum feature sizes and excellent pattern fidelity even

after swelling of the hydrogel. The major drawback of this technique is the time required to fabricate a large number of samples, since the hydrogels are processed individually.

Dhariwala *et al.*[23] employed maskless SLA in one of the first reports demonstrating its compatibility with cell encapsulation. A commercially available SLA system was used to photoencapsulate Chinese hamster ovary (CHO) cells within donut-shaped PEGDA hydrogels with high viability. With this approach, the macroscopic construct shape was patterned with good resolution (~150 μm feature size) and cells within the hydrogel were presented with a relatively uniform 3D microenvironment. However, the modified commercial SLA system used required admittedly tedious manual addition of precursor solution for each layer. Despite this limitation, this important report illustrated the feasibility of cell encapsulation within 3D hydrogels using SLA.

Mapili *et al.*[24] developed a technique in which a laser was used to irradiate a micromanipulator stage coated with a photopolymerizable prepolymer solution. By manually translating the stage, a pre-designed pattern could be created in a single layer of crosslinked hydrogel. The uncrosslinked solution was then washed and the process repeated with different macromer solutions to provide the final 3D construct (Fig. 1). OP-9 mouse stromal cells seeded onto the scaffolds attached in a patterned fashion only when the PEG macromers were pre-conjugated with RGD.

A similar approach was used by Arcaute *et al.*[25] to construct cell adhesive PEG hydrogels. In this study, a He-Cd laser was used to cure a patterned subvolume of a reactive PEG solution in a flat top container. The simultaneous curing of four patterns took less than 10 s, affording high viability (~90%) of human dermal fibroblasts encapsulated within the constructs. Additionally, the authors demonstrated good control of crosslinking depth (*i.e.*, hydrogel thickness) of each layer by varying such parameters as the polymer concentration, photoinitiator type and concentration, and laser intensity and time of exposure.

Another adaptation of this approach was developed by Barry *et al.*,[26] who used a deposition microneedle to write up to six layers of hydrogel-based ink with simultaneous photopolymerization to create a patterned 3D scaffold. 3T3 fibroblasts seeded atop the patterned scaffolds aligned themselves with the striped or cuboidal patterns, compared to a random orientation on a control substrate.

Chan *et al.*[27] employed a vertically translating platform to fabricate multi-layered patterned hydrogels using either a top-down (in which the platform was immersed in the bulk prepolymer solution, which was irradiated by a laser to create a new layer) or bottom-up (in which prepolymer solutions are pipetted one layer at a time onto the growing gel and simultaneously cured) technique. Using the bottom-up approach, which enables multiple polymers and/or cell types to be deposited, the authors were able to photoencapsulate multiple cell types in adjacent layers, as well as demonstrate good cell spreading when 5 mM RGDS peptides were incorporated into the gel.

A recent report by Hsieh *et al.*[28] further developed the use of two photon laser scanning photolithography (TPLSP) for the fabrication of patterned constructs. A photocurable resin transparent to UV illumination and compatible with cells was printed layer-wise with CAD and immediately polymerized by the focused laser to produce the constructs. The authors demonstrated that seeding of primary hepatocytes on the 3D scaffolds led to higher albumin and urea levels relative to 2D controls.

A similar approach was employed by Chang *et al.*[29] to fabricate alginate "microorgans" with a proprietary bioprinting process. A syringe-based direct cell writing process was used to fabricate the microorgan in the presence of HepG2 liver cells in a layerwise fashion. A soft lithographic technique was then used to construct a microfluidic housing device for the microorgan. The authors demonstrated good cell viability and proliferation, as well as superior cell-specific function (urea production per hepatocyte) within the 3D construct relative to 2D controls. The system features four printing nozzles with independent process parameters, affording the deposition of different polymers, cells, and biological factors with great spatial versatility and complexity. Because the microfluidic device allows for the

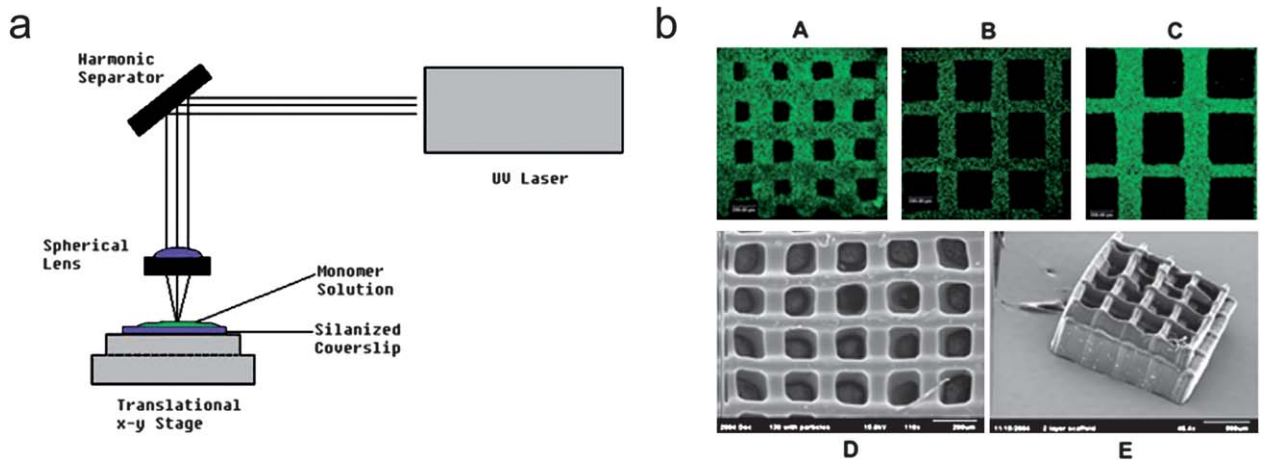

Fig. 1 (a) Experimental setup for layerwise stereolithographic fabrication of patterned scaffolds and (b) confocal microscopy images (A-C) and SEM micrographs (D-E) showing PEG scaffolds with precise internal geometries fabricated using a stereolithographic approach. 1% (w/w) FITC-labelled latex particles were included in the precursor solution. Scale bars = 200 μm. Reproduced from Mapili *et al.*, ref. 24, with permission.

localization of perfusion streams to separate regions of the gel, this system may thus be powerful for screening drug toxicity and efficacy in 3D.

Mask-based stereolithography. Mask-based SLA uses the inverse procedure of the maskless techniques described above. A prepolymer solution containing photoinitiator is exposed to light through a photomask, resulting in gelation only in unmasked regions (*i.e.*, regions under clear portions of the mask). The uncrosslinked solution is then rinsed away and the steps are repeated (with either the same or different polymer and mask) until the final multi-layered, patterned structure is achieved. Masked-based approaches do not require printers, thereby eliminating concerns of clogging and other issues that limit the cytocompatibility of some maskless techniques. However, relative to maskless SLA, masked-based approaches suffer from a lack of automation (*i.e.*, masks are applied manually to the precursor), the resolution that can be obtained with masks, and the need to pre-fabricate a large number of photomasks.

In 2002, Liu *et al.*[30] used this general paradigm in one of the first examples of patterned microstructure fabrication with locally encapsulated cells. A prepolymer solution containing cells was injected into a mold, followed by UV exposure through a photomask to polymerize unmasked portions of the solution onto a glass wafer. Subsequent layers were then added by washing the unbound solution, injecting a different solution, and repeating the process. The report demonstrated that populations of cells could be encapsulated in different layers, with the patterned shapes of each layer retaining high resolution (within 10% of the feature size).

In another report from Bryant *et al.*,[31] photopolymerization of porous poly(hydroxyethyl methacrylate) (poly(HEMA)) hydrogels was performed through photomasks permitting light transmittance at different intensities in clear *versus* opaque mask regions. In gel regions exposed to high intensity light (transparent regions), the macromers remained soluble due to poor polymerization kinetics, while masked regions exposed to lower intensity light crosslinked into a gel. The gels were then washed extensively, removing the poly(HEMA) in unmasked regions, resulting in a gel with well defined cylindrical macro-channels. The authors then demonstrated the circumferential alignment of seeded C2C12 myoblasts along the macro-channel walls.

In another example, Papavasiliou *et al.*[32] functionalized a glass substrate with eosin. A photopolymerizable PEG precursor solution was placed on the substrate and cured through a photomask, and excess solution was then rinsed. Repeating these steps gave a patterned construct in which the individual layer thicknesses were controlled *via* the polymerization conditions. Although this work was acellular, the authors noted that cells could easily be incorporated into the system by pre-conjugating with cell adhesive ligands.

A similar technique was demonstrated by Tsang *et al.*,[33] who additively photopatterned successive layers of PEG gels (in this example, in the presence of primary hepatocytes) using the same general paradigm of polymerization through a photomask, rinsing, and repeating (Fig. 2). Importantly, hepatocyte encapsulation in a 3D layered hexagonal hydrogel eliminated the decrease in cell viability observed in the center of uniform hydrogel disks when both constructs were perfused with media in a bioreactor. The authors attributed this difference to superior nutrient transport in the hexagonal hydrogels, mediated by the perfused channels infiltrating the construct. Beyond viability, albumin and urea production by hepatocytes was also higher in the patterned *versus* non-pattened hydrogels. Thus, this report illustrated that the spatially designed incorporation of channels into 3D hydrogels may be critical to support the viability and function of fragile, adhesion-dependent primary cells.

While the above studies used SLA to produce patterned constructs comprised of multiple layers, the approach can also be used to generate single layer constructs with thicknesses that better mimic tissue length scales. In one example, Underhill *et al.*[34] synthesized a replicate array of cylindrical 500 μm thick

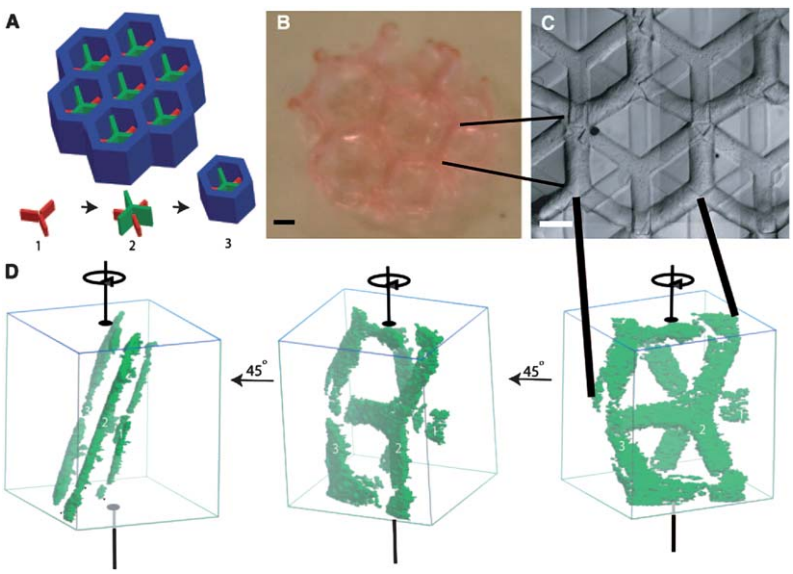

Fig. 2 Additive photopatterning of hydrogels with 3D tissue microarchitectures. (A) Design of the photopattern for each layer: red (1), green (2), blue (3). (B) Photograph (Scale bar = 1 mm) and (C) phase contrast micrograph (Scale bar = 500 μm) of the resultant 3D microscale hepatic tissue. (D) 3D reconstruction of the tissue with fluorescently labelled cells. Reproduced from Tsang *et al.*, ref. 33, with permission.

PEG hydrogels containing encapsulated bipotential mouse embryonic liver (BMEL) cells or primary hepatocytes atop a methacrylated slide. The void regions between cylinders was then filled in with a fibroblast-containing precursor solution and polymerized to give a single layer patterned construct. A recently reported[35] dielectrophoretic patterning technique was then used in the system to generate hydrogels with hepatocyte and fibroblast localization defined at the cellular scale.

Microfluidic approaches. From the perspective of scaffold fabrication, the most basic microfluidic approaches employ photopolymerization (nominally in the presence of cells) of a prepolymer solution through a patterned photomask onto a photoreactive glass substrate (*e.g.*, a methacrylated coverslip). Unreacted precursor solution corresponding to regions under the light-impermeable regions of the mask are then washed away, and media is perfused through the empty channels. Using this approach, Nichol *et al.*[36] created patterned stamps of gelatin methacrylate hydrogels with encapsulated 3T3 fibroblasts or HUVECs with channel widths as low as 100 μm. Encapsulation of these cells led to binding, proliferation, and elongation, including formation of an aligned endothelium by HUVECs.

More elaborate techniques employing microfluidic devices as housings for 3D patterned hydrogels have also been developed. Trkov *et al.*[37] developed a novel co-culture system in which different cell types encapsulated in fibrin hydrogels were localized within microfluidic channels separated by desired spacing distances (in this report, spacing ranged from 500 to 2000 μm apart). This model, which was used by the authors to demonstrate the distance-dependent migration of MSCs toward human umbilical vein endothelial cells (HUVECs), offers a valuable new method to study cell-cell communication in the presence of shear forces in 3D.

Cuchiara *et al.*[38] created a microfluidic device for 3D cell culture in which the patterning element was not the material itself, but the exponential loss of diffusivity with changes in distance of the cells from the media perfusion channel. The report demonstrated increased viability in the microfluidic system relative to 2D controls, as well as strict control of solute effective diffusivity based on the solute molecular weight and polymer concentration. This system may thus be useful for studying the importance of different morphogens and their transport conditions for optimal cell viability and phenotype within hydrogels.

Lee *et al.*[39] developed a membrane-mounted microfluidic, multilayer hydrogel fabrication platform to study co-culture and cell-cell communication between cell types. While traditional SLA suffers from a trade off between vertical resolution and field of view, the authors combine a low numerical aperture optical system for photopolymerization with a soft membrane for height control to impart a maximum area of lithography while maintaining resolution. The ability of the system to house multiple cell types in adjacent regions with high viability and spatial resolution was confirmed by co-culturing HeLa cells (labelled with red or blue dyes) in patterned hydrogels.

In one example from Chueh *et al.*,[40] a microfluidic device was loaded with an alginate precursor solution. Patterned, reversible gelation of the alginate was then achieved by light exposure through a photomask, which released caged calcium crosslinker only in exposed regions of the device. The procedure was repeated with MC3T3 fibroblasts present in the alginate solution. Following washing of uncrosslinked alginate, an endothelial cell (EC) suspension was flowed into the fluidic device in contact with the fibroblast containing patterned gels, illustrating the potential value of this technique for studying cell-cell communication. However, the authors cited the prohibitive cost of the calcium caging agent (DM-nitrophen™) as a limitation of the study.

In contrast to those described, techniques involving microfluidics have also been developed that do not require UV light or photoinitiators. One such approach is to use microfluidics to clear a patterned sacrificial polymer from the interior of the chemically crosslinked hydrogel to create microchannels in 3D. This technique is advantageous compared to SLA in that the absence of void space in each layer during fabrication provides optimum structural stability for subsequent layers. Also, the use of chemical crosslinkers diminishes concerns of non-uniform crosslinking (due to such factors as radical scavenging by ambient oxygen) in photopolymerization. In one example, Lee *et al.*[41] used a custom bioprinter to first print a uniform collagen layer, which was crosslinked into a gel using $NaHCO_3$. Successive collagen layers were then printed with patterned empty channels in which sacrificial gelatin was used to fill in the void space. After completion of all layers, the gelatin was selectively liquefied. The report demonstrated that fibroblasts could be printed directly with the collagen precursor solution, encapsulated upon crosslinking, and then retained high viability when the constructs were perfused with media.

Wong *et al.*[42] employed an approach that does not require light exposure to create microenvironments in which multiple cell types could easily be patterned with simultaneous application of gradients of soluble factors across the gel. In this approach, laminar flow is used to partition microfluidic channels with microslabs of hydrogels. This geometry was achieved by delivering a stream of hydrogel precursor flanked by two streams of buffer in a microfluidic device. A rapid temperature increase from 4 °C to 37 °C caused thermal gelation of the hydrogel, resulting in spatially precise slabs of gel adjacent to empty channels. Because laminar flow limits transport between streams due to diffusion, the method allowed the production of soluble factor gradients in a facile manner. Also, since the source of soluble factors can be either the encapsulated cells or external injection, this system represents a potentially useful model for the study of cellular communication through soluble factor diffusion.

Moon *et al.*[43] developed a method of printing cell-encapsulated droplets within a layered collagen scaffold. After first printing a uniform collagen substrate on a glass slide, a mechanical valve was used to deposit cell laden collagen droplets within the layer, and the process was repeated to produce the multi-layered hydrogel. The report demonstrated the ability to print overlapping cell laden droplets with good viability and controlled spacing as a method to recapitulate the multiple cell layer composition of native tissues.

Other techniques. A study by Albrecht *et al.*[44] describes the novel use of dielectrophoretic (DEP) forces to localize live cells within 3D micropatterned PEG/agarose hydrogels. The ability to localize cells in structures with good control of the microscale

architecture was reported in earlier work.[35] In the present example, the authors formed cell-containing microgels which were mixed with a bulk phase agarose precursor solution. Application of positive DEP forces first localized microgels to regions of high electric field strength. The gels were then trapped in place by exposure of the bulk-phase polymer to light or a change in temperature. This technology was demonstrated to support higher viability of seeded liver progenitor cells relative to the earlier single phase systems, and may provide a useful tool for studying the influence of 3D construct architecture on cell behavior.

Post-gelation patterning techniques

The other class of photolithographic patterning techniques are those that incorporate patterning post-gelation, either immediately following network formation or after an incubation period with cells. This group of techniques employs irradiation with laser or lamp light to alter the chemistry or crosslinking in spatially defined subvolumes of the gel, with the goal of incorporating photoreactive moieties (*e.g.*, photoreactive cell adhesive oligopeptides) swelled into the hydrogel or directly altering the network structure. Since photopolymerization is used to additively incorporate features into the gel, photolithography necessitates that the preformed gel retains at least some reactive groups following the initial crosslinking. However, even when gelation techniques are used that theoretically consume 100% of reactive groups (*e.g.*, free radical polymerization), additive photopatterning is still possible since full conversion of reactive groups is typically not obtained.[45]

Again, these techniques can be further categorized into two groups: those that afford patterning fully in 3 dimensions using two-photon laser scanning photolithography (TPLSP), and those that flood-irradiate samples through photomasks using lamp based light sources. The advantages and disadvantages of each approach are similar to those of the respective bottom-up techniques. Maskless photolithography affords the creation of a user-defined pattern of arbitrary shape due to the precision of the laser focal point, but suffers from greater time and expense needed to process each sample. With masks, a shortcoming is that the desired patterning is essentially a 2D pattern incorporated through the depth of the hydrogel. Also, light diffraction issues with depth limits the maximum thickness (typically on the order of mm) that can be accommodated using this technique. However, relative to TPLSP, this approach enables multiple samples to be processed simultaneously (limited only by the light source beam width) and is relatively inexpensive.

Post-gelation patterning with TPLSP. Using single-photon laser scanning photolithography (SPLSP), light of the appropriate excitation wavelength passes through the entire sample before reaching the focal point, thus limiting the applied pattern to uniformity in the cross section through the depth of the sample. In contrast, TPLSP combines two lower energy photons to provide the energy needed for excitation only at the focal point. Thus, any photoreactive moiety can theoretically be incorporated into a photoreactive polymer with complete spatial precision. Typically, a photoinitiator and the desired molecule conjugated to a photoreactive group are swelled into the gel, which itself contains unconsumed photoreactive groups. The desired pattern is then designed using software to create a virtual mask, which is in turn converted into instructions for the automated patterned irradiation of the sample.

Hahn *et al.*[46] illustrated this approach using TPLSP of poly(ethylene glycol) (PEG) hydrogels. Preformed PEG hydrogels were first fabricated *via* light initiated photopolymerization of poly(ethylene glycol) diacrylate (PEGDA) prepolymers. A precursor solution of fluorescently labeled and acrylated peptides (ACRL-PEG-peptide) was then swelled into the hydrogel, and a virtual mask was used to implement 3D patterns with irradiation from a Ti:sapphire 720 nm laser. The authors demonstrated a well-defined 3D spiral staircase and parallelogram pattern incorporation.

A later study from the same group focused on guided cell migration with this approach. Lee *et al.*[47] incorporated fluorescent RGDS-conjugated PEG (FITC-RGDSK-PEG-arcylate) into a pre-formed, collagenase-sensitive PEG hydrogel only within the focal volume of the applied two-photon laser. The authors used the immobilized fluorescence to demonstrate precise control of the amount of immobilized RGDS based on the amount initially present in the prepolymer solution. When fibrin clusters containing human dermal fibroblasts (HDFs) were encapsulated into a network subsequently patterned with RGDS, outgrowth from the clusters was observed only into the RGDS-containing regions within the interior of the hydrogel (Fig. 3).

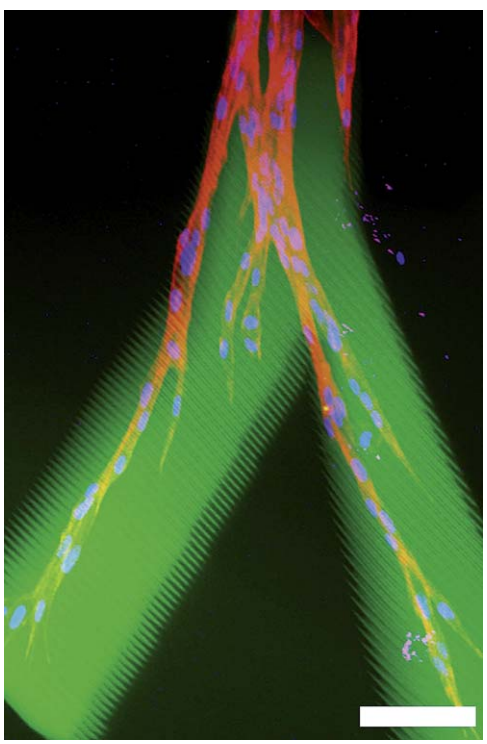

Fig. 3 Confocal microscopy images of fibroblasts (labelled with rhodamine phalloidin and DAPI for cellular actin and nuclei, respectively) migrating within RGDS-patterned (labelled with FITC) regions inside enzyme-sensitive PEG hydrogels. Scale bar = 100 μm. Reproduced from Lee *et al.*, ref. 47, with permission.

A similar approach was employed by Aizawa *et al.*[48] to guide primary endothelial cells (ECs) in a 3D patterned scaffold. In this study, coumarin chemistry was used in conjunction with a multi-photon patterning technique to incorporate a gradient of photoreactive vascular endothelial growth factor isoform (VEGF165) within cell-adhesive agarose-sulfide hydrogels. ECs encapsulated within these gels formed tubule-like structures as a result of gradient-induced migration. In comparison, little migration was observed within uniform hydrogels not containing VEGF165. While past studies have demonstrated encapsulated cell migration by providing a path of cell adhesivity within the gel, this work is the first to guide EC tubule formation based on growth factor concentration gradients within a 3D gel.

Techniques using laser photoablation. While the above described techniques incorporate bioactive moieties (*i.e.*, cell adhesive domains) into pre-formed, proteolytically sensitive hydrogels, another approach is to use TPLSP to cleave photodegradable elements within non-degradable hydrogels to direct cell behavior in 3D. In one example, Luo *et al.*[49] developed an agarose hydrogel functionalized with a sulfhydryl-containing group coupled to a 2-nitrobenzyl-protected cysteine. Focused laser irradiation was used to cleave the 2-nitrobenzyl groups in specific regions of the gel, exposing the free sulfhydryl groups for covalent reaction with a cell adhesive (GRGDS) oligopeptide segment. Neurite outgrowth from dorsal root ganglia (DRG) seeded atop the agarose gels followed the adhesive channels with high spatial precision. In a follow up study,[50] cell guidance was achieved in thick (1.5 mm) HA gels using a laser technique that incorporated concentration gradients within channels of varying diameters.

A similar approach using DRG encapsulation within 3D patterned PEGylated fibrinogen hydrogels was used by Sarig-Nadir *et al.*[51] In this study, guidance microchannel formation with micron scale control of channel diameter was achieved by focused laser photoablation of a pre-formed, DRG encapsulated gel. Spatially directed outgrowth was observed only into the ablated microchannels adjacent to the cell source, regardless of the pattern complexity (Fig. 4). The authors cite the transparency of the materials used in the study as an important advantage, since this property has been shown to significantly enhance optical control of neural activity.[52,53]

A recent report from Tibbitt *et al.*[54] used photodegradation by a two-photon laser to study the interaction between cells and soft PEG gels. In this report, light was focused on the interface between the photodegradable PEG macromers and encapsulated hMSCs, inducing subcellular detachment only at the focal point of the laser. The authors also demonstrated that the area of eroded features could be directly varied by varying the duration of pulsed light exposure. Because this approach affords user-controlled cell detachment of ECM at the anterior or posterior of polarized cells, it may be a useful tool to study cell motility and the dynamics of individual filopodia during cell migration. More applied directions for the technology include the directed migration of adhesion-dependent cells.

Mask-based photolithography of 3D hydrogels. The other general category of techniques to pattern pre-formed hydrogels in 3D is the exposure of the entire sample through a physical photomask to selectively irradiate unmasked regions of the gel. In this approach, a uniform hydrogel is first formed using macromers functionalized with reactive groups capable of undergoing photopolymerization (*e.g.*, acrylates). The initial gelation occurs through these same reactive groups (*e.g.*, acrylate consumption *via* Michael-type addition reactions) or through another set of groups using orthogonal chemistry (*e.g,* "click" Huisgen cycloaddition reactions). In the former case, the cross-linking is performed such that at least a portion of photoreactive groups remains available for photopatterning. Spatial patterning is then achieved by applying a photomask to the gel surface and irradiating with a lamp. In contrast to two-photon techniques, this approach enables rapid processing of multiple samples simultaneously (the number of samples that can be fabricated together is limited only by the area of the lamp exposure).

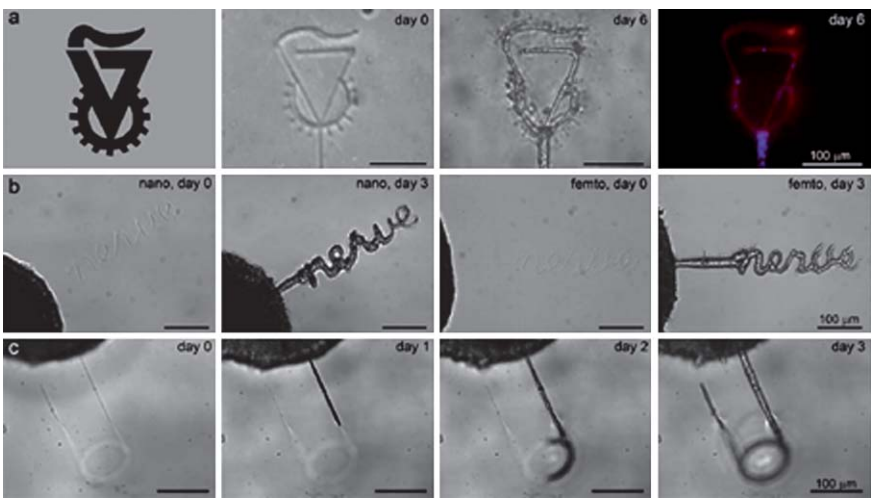

Fig. 4 Directed dorsal root ganglia (DRG)- cell outgrowth into microchannels of various patterns: (a) (left to right) Technion University symbol, DRG-cell micrographs at day 0 and 6, immunolabelling for β3-tubulin (neural marker, red) and DAPI (nuclei, blue). (b) Inscription of the word "nerve" using either a nanosecond or femtosecond laser system, illustrating DRG-cell outgrowth at day 0 and 6. (c) DRG-cell migration into a microchannel at day 0, 1, 2 and 3. All scale bars = 100 μm. Reproduced from Sarig-Nadir *et al.*, ref. 51, with permission.

However, because energy of the excitation wavelength is passed through the entire sample, some background level of conjugation outside the focal plane is typically observed.

This technique has been used to pattern cell adhesive domains into a pre-formed hydrogel. In one example, DeForest *et al.*[55] encapsulated fibroblasts within an acrylated PEG hydrogel using a Huisgen cycloaddition reaction. A thiol-functionalized RGD peptide was then swelled into the network. The gel was irradiated using 365 nm UV light through photomasks, covalently conjugating the cell adhesive ligand to the network only in exposed regions of the gel *via* a thiolene reaction. Because proteolytic degradability was incorporated into the network backbone, the photopatterning of RGD led to patterned cell spreading with good resolution. This approach features two chemistries – the "click" cycloaddition and thiolene reactions – that are completely orthogonal, affording a high degree of tunability. In particular, a wide range of biochemical cues can be incorporated during the secondary step in a patterned fashion, provided they contain compatible reactive groups.

Under a similar route, photopatterning has also been used to switch the hydrogel structure itself from a degradable to non-degradable state. Khetan *et al.*[56] demonstrated this using a sequential crosslinking technique. A uniform hydrogel "permissive" to remodeling was first formed in the presence of human mesenchymal stem cells (hMSCs) using michael type reactions between multi-acrylate HA macromers and cell adhesive and degradable oligopeptide crosslinkers. This primary crosslinking consumed only a portion of total acrylate groups. In the presence of a photoinitiator, the gel was then irradiated through photomasks, resulting in crosslinking of remaining acrylate groups and a switch to a gel structure "restrictive" to remodeling only in exposed regions of the gel. The authors used the technique to demonstrate spatially patterned remodeling from cells in suspension (hMSCs) (Fig. 5), and in a subsequent report,[57] from *ex vivo* tissues (chick aortic arches). Additionally, this approach was used to spatially control hMSC differentiation in 3D hydrogels.

Mask-based techniques have also been used with photoablation to guide cell behavior in 3D gels. In one example, Kloxin *et al.*[58] fabricated an initially uniform PEG hydrogel from photodegradable macromers in the presence of fibrosarcoma cells. Subsequent light exposure of the gel through photomasks with straight line patterns resulted in cell migration into the eroded channels corresponding to the unmasked regions. A follow-up study[59] used the attenuation of flood irradiation with depth from absorption by photolabile groups to generate a gradient of hydrogel erosion in the z-direction. When hMSCs were encapsulated within these hydrogels, the cell area was found to decrease (*i.e.*, decreased cell spreading) with depth (*i.e.*, translating downward into the hydrogel where progressively fewer photolabile groups were cleaved). This approach may be useful to study cell behavior within microenvironments that mimic *in vivo* gradients of tissue density in response to injury.[60,61]

Discussion and conclusions

Increased understanding of the influence of microscale heterogeneity on tissue repair and function *in vivo* has motivated the development of 3D hydrogel patterning techniques. Fabrication of these materials, particularly in a manner compatible with cell encapsulation, presents some unique challenges compared to the preparation of 2D substrates. As reviewed here, however, significant progress has been made in engineering constructs with designed heterogeneity towards guiding cellular responses, including cell migration and differentiation. A continued challenge associated with 3D studies in general is the evaluation of cell behavior within the interior of the hydrogels. In contrast to studies with uniform materials, quantitative assays are difficult or impossible to perform with patterned constructs because they typically require digestion of the gel. For this reason, the continued development and improvement of advanced optical imaging techniques capable of imaging within gels – most notably, LSCM – in a non-destructive manner is critical to reduce the costs associated with 3D constructs and move closer to clinical feasibility.

Although the cellular behavior used for validation of current 3D patterned hydrogels is typically limited to adhesion or migration, a future goal of this field is the use of patterning to direct advanced cellular phenotypes (*e.g.*, multi-lineage stem cell differentiation in single samples) based on patterned material cues alone. These capabilities have been demonstrated in relatively few 3D systems, and even in these cases, soluble differentiation factors common to both lineages of interest are required. Despite these shortcomings, the collective work reviewed here indicates great promise for the engineering of 3D hydrogels with

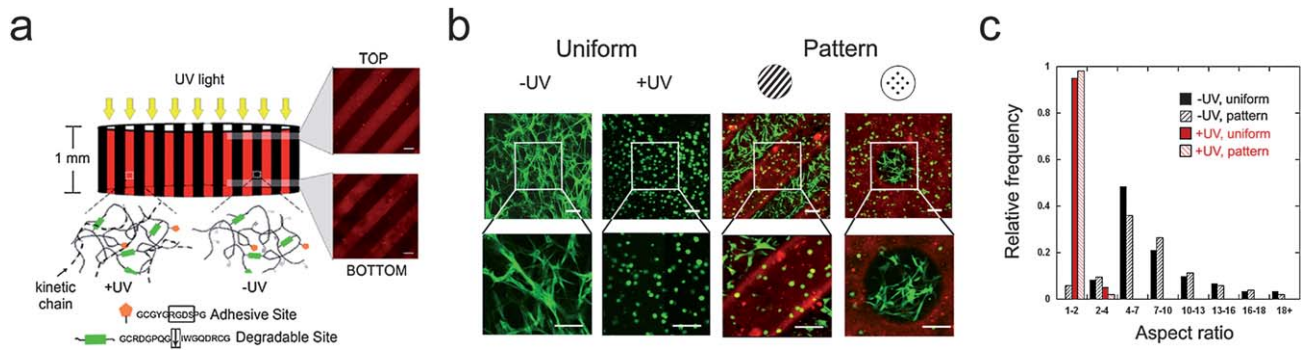

Fig. 5 Hydrogel remodelling by encapsulated hMSCs. (a) Schematic of patterning process (regions exposed to 2° crosslinking fluoresce red from methacrylated rhodamine incorporation) to alter crosslink type in gels formed from multi-functional macromers to spatially control cell-mediated remodelling of hydrogels. (b) Calcein-stained hMSCs in uniform or photopatterned hyaluronic acid hydrogels. Scale bars = 100 μm. (c) Histograms of the cellular aspect ratio for these same groups. Reproduced from Khetan *et al.*, ref. 56, with permission.

precise and controlled microscale heterogeneity to direct cell behavior towards regenerative applications.

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