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4D Biochemical Photocustomization of Hydrogel Scaffolds for Biomimetic Tissue Engineering

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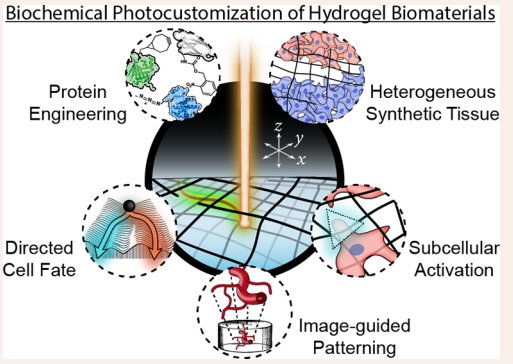
CONSPECTUS: Programmable engineered tissues and the materials that support them are instrumental to the development of next-generation therapeutics and gaining new understanding of human biology. Toward these ends, recent years have brought a growing emphasis on the creation of "4D" hydrogel culture platforms—those that can be customized in 3D space and on demand over time. Many of the most powerful 4D-tunable biomaterials are photochemically regulated, affording users unmatched spatiotemporal modulation through high-yielding, synthetically tractable, and cytocompatible reactions. Precise physicochemical manipulation of gel networks has given us the ability to drive critical changes in cell fate across a diverse range of distance and time scales, including proliferation, migration, and differentiation through user-directed intracellular and intercellular signaling. This Account provides a survey of the numerous creative approaches taken by our lab and others to recapitulate the dynamically heterogeneous biochemistry underpinning *in vivo* extracellular matrix (ECM)−cell interactions via light-based network (de)decoration with biomolecules (e.g., peptides, proteins) and *in situ* protein activation/generation. We believe the insights gained from these studies can motivate disruptive improvements to emerging technologies, including low-variability organoid generation and culture, high-throughput drug screening, and personalized medicine. As photolithography and chemical modification strategies continue to mature, access to and control over new and increasingly complex biological pathways are being unlocked.



The earliest hydrogel photopatterning efforts selectively encapsulated bioactive peptides and drugs into rudimentary gel volumes. Through continued exploration and refinement, next-generation materials now boast reversible, multiplexed, and/or Boolean logic-based biomolecule presentation, as well as functional activation at subcellular resolutions throughout 3D space. Lithographic hardware and software technologies, particularly those enabling image-guided patterning, allow researchers to precisely replicate complex biological structures within engineered tissue environments. The advent of bioorthogonal click chemistries has expanded 4D tissue engineering toolkits, permitting diverse constructs to be independently customized in the vicinity of any cell that is amenable to hydrogel-based culture. Additionally, the adoption of modern protein engineering techniques including genetic code expansion and chemoenzymatic alteration provides a roadmap toward site-specific modification of nearly any recombinant or isolated protein, affording installation of photoreactive and click handles without sacrificing their bioactivity. While the established bind, release, (de)activate paradigm in hydrogel photolithography continues to thrive alongside these modern engineering techniques, new studies are also demonstrating photocontrol of more complex or nonclassical operations, including engineered material-microorganism interfaces and functional protein photoassembly. Such creative approaches offer exciting new avenues for the field, including spatial control of on-demand biomolecule production from cellular depots and patterned bioactivity using a growing array of split protein pairs. Taken together, these technologies provide the foundation for truly biomimetic photopatterning of engineered tissues.

1. INTRODUCTION

Multicellular life emerges from the heterogeneous complexity displayed by living tissue through three spatial dimensions and time (i.e., 4D). Each individual cell receives and interprets a myriad of unique physicochemical cues, challenging engineers that seek to mimic living tissue across scales.[1] Although conventional biomaterials permit bulk physical tunability and the presentation of soluble factors *in vitro*, there is considerable and growing interest in developing robust methods to

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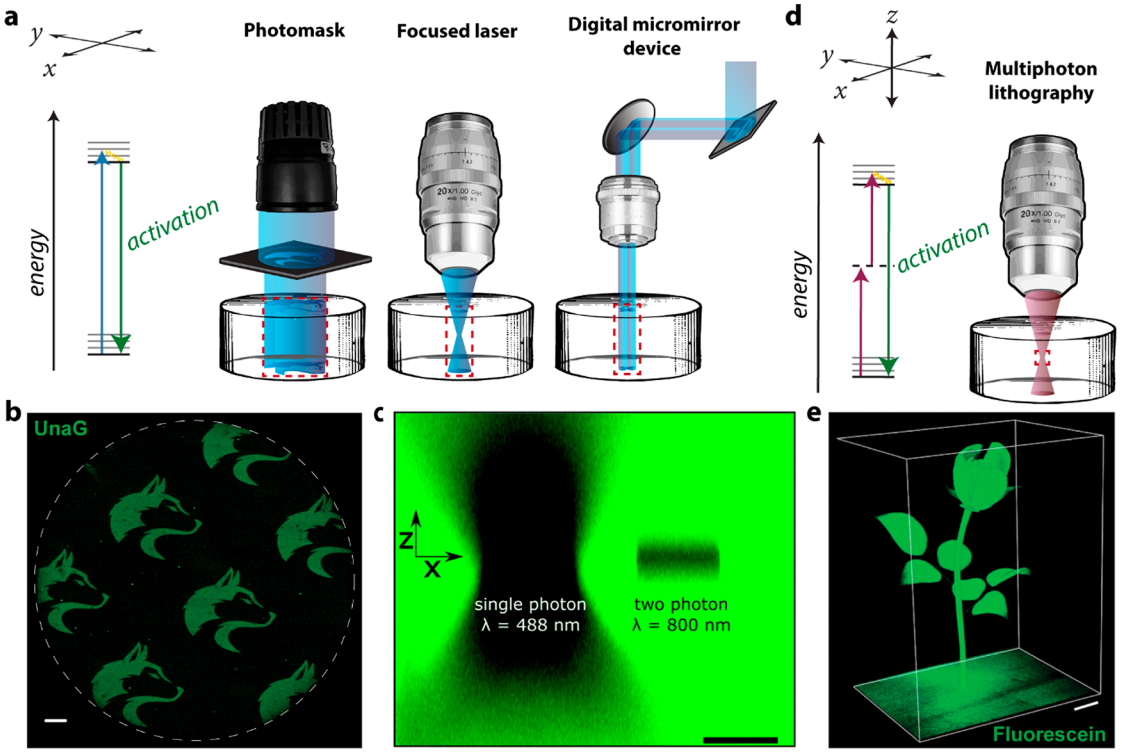

Figure 1. Lithographic strategies for hydrogel photopatterning. (a) Single-photon techniques can use photomasks, focused lasers, and digital micromirror devices for material patterning. Here, a single absorbed photon provides sufficient energy to initiate the photochemical reaction. Each method affords excellent resolution in two spatial dimensions (i.e., x and y) with mostly uniform activation parallel to incident light. (b) Typical pattern generated using a collimated 365 nm light source and stationary photomask. Image is a representative z-projection through the z-axis of a cylindrical hydrogel, depicting locally patterned functional assembly of a fluorescent protein (UnaG, green). Scale bar = 500 μm. Reproduced with permission from ref 61. Copyright 2023 The Author(s). (c) Comparison of single- and multiphoton activation volumes (depicted with loss of green fluorescence via gel photobleaching) using focused laser light. Scale bar = 25 μm. Reproduced with permission from ref 16. Copyright 2017 Royal Society of Chemistry. (d) Multiphoton lithography features full 3D photochemical control. Two high-wavelength photons absorbed in rapid succession provide sufficient energy to activate molecules confined to a small volume at the focal point. (e) Representative multiphoton pattern demonstrating complete 3D control of reaction photoactivation, here through controlled immobilization of fluorescein-modified peptide (green). Scale bar = 50 μm. Reproduced with permission from ref 40. Copyright 2020 Wiley-VCH.

customize network biochemistry, cell−cell, and cell−extracellular matrix (ECM) interactions in 4D. Photolithographic processing of hydrogels has further provided a means for spatiotemporally specifying complex cell fates, including migration and invasion, (trans)differentiation, and multicellular assembly at the nano- and microscales. With nearly three decades of history, these maturing techniques are now poised to revolutionize efforts in fundamental biological investigation, next-generation therapeutic development, and organoid-based disease modeling.[2,3] Light is a uniquely powerful stimulus for user-directed material modification because it affords near-instantaneous and spatially resolved modulation within user-specified subvolumes of cell-laden gels.[4] These parameters are of the highest importance to modern tissue culture: scaffold modification must occur much more quickly than the cellular processes it seeks to affect ($\ll 10^3$ s) and with subcellular resolutions ($\ll 10^1$ μm). By harnessing light, advanced photolithographic processing techniques, and a growing wealth of bioorthogonal and photolabile chemistries to biochemically (de)decorate polymeric scaffolds or generate active biomolecules, we seek to precisely probe and direct cell fates within engineered microenvironments whose 4D complexity approaches that of living tissue. This Account highlights past efforts and outlines future opportunities in engineering heterogeneous tissue via biochemical photocustomization of hydrogel biomaterials.

2. METHODS FOR PHOTOCHEMICAL HYDROGEL CUSTOMIZATION

Hydrogels are broadly defined as water-swollen polymer networks, formed generally through physical or chemical cross-linking of hydrophilic precursors. Such gels are typically soft materials with highly tunable stiffness and viscoelasticity and have become ubiquitous in medical sciences, biotechnology, and an array of other fields. Their porosity, biocompatibility, optical transparency, and mechanical similarity to living tissue has also led to a long history of use as scaffolds for 3D cell culture. Photochemical customization of this diverse material class provides a powerful route toward dynamically controlling 4D cell fate.

2.1. Bulk Photopolymerization of Functionalized Gel Scaffolds

The adaptation of macromolecular building blocks and photoinitiated radical polymerization to create chemically tractable hydrogel materials immensely benefited 3D tissue engineering efforts and teased the existence of a near-infinite combinatorial library of chemistries. The first hydrophilic polymers modified with acryl end-groups[5] effectively decoupled chemical properties of the gel backbone from its

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cross-linking groups and enabled hydrolysis-mediated drug delivery, patterned photofabrication,[6] and biomolecule presentation. Key platforms including poly(ethylene glycol) (PEG) di(meth)acrylate,[5] gelatin methacrylamide (GelMA),[7] methacrylated hyaluronic acid (MeHA),[8] and subsequent click-based systems[9] also consist of chemically modified hydrophilic macromolecules with cross-linkable handles to enable tunable fabrication of basic, homogeneous ECM. This chemical flexibility foreshadowed the ability to engineer materials capable of multiple orthogonal processes (e.g., patterned protein presentation alongside tunable bulk viscoelasticity) in the presence of living cells[10] and enabled covalent integration of biomolecular cues throughout the culture volume. Soon after the first examples of photoencapsulated cell culture,[11] acrylamide-modified cell-adhesion peptides were tethered into acrylate gels either as pendants or as bifunctionalized cross-linkers, simplifying cell encapsulation through a one-pot, one-chemistry process.[12] Additionally, enzymatically degradable peptide backbones remedied early limitations associated with 3D cell morphology and activity in photoinitiated gels, yielding synthetic ECM that were more biomimetic and mechanically dynamic.[13] With growing understanding of how cells behave in a uniformly tunable synthetic ECM, spatiotemporal control of network biochemistry would prove to be a logical next step.

2.2. Lithographic Customization of Hydrogels

Optical lithography as a tool for biomaterials fabrication precedes 3D cell encapsulation. Surfaces, commonly tissue culture-treated plastic, have long been functionalized with photosensitive molecules and patterned using collimated or coherent light sources (e.g., photomasks, lasers, digital micromirror devices). Applied to biochemical gel patterning, these techniques generate activated columnar volumes through the material with x-y tunability at the single-micron scale (Figure 1a−c).[14] Optical clarity and homogeneity of the material are highly valued, leading to the sharpest and most consistent patterns. For this reason, perfectly defined, transparent, and biochemically inert "blank slate" backbones like PEG continue to be favored testbeds, although photolithographically tunable natural and protein-based hydrogels have also been engineered.

The depth and integrity of patterning through the axis parallel to incident light—the z dimension—is dependent on light scattering and attenuation. As such, uninformed introduction of large-absorption coefficient additives at high concentrations or in thick layers can lead to non-negligible artifacting or heterogeneous activation. While uniform patterning through the column is often preferred, attenuation in strongly absorbing materials has been suggested as a means for generating biochemical gradients.[15] Living tissue experiences both continuous and discrete spatiotemporal fluctuations in biochemical signaling, so it is vital to generate such gradients in biomimetic models. Natural attenuation is perhaps the only means to generate variable z-activation using single-photon-based lithography. Although the photochemical response may be nonlinear, these dosage gradients are fundamentally limited by the Beer−Lambert law and not fully customizable.

Pattern formation in x-y space is far easier to manipulate. Opaque moving photomasks, predefined masks with spatially variable transparency, differential laser powers, and dynamically adjustable digital micromirrors are all capable of complex gradient formation by delivering spatially variable light dosage (Figure 1a,b).[4] Although patterned volumes typically need to be z-dimensionally thin to avoid attenuation, arbitrarily large $x-y$ patterns can be generated rapidly so long as the researcher selects a sufficiently sensitive chemistry and can deliver light over a large enough area. Simple hardware such as a collimated light source and hand-cut paper photomask can provide submillimeter spatial resolutions, while focused lasers are a thousandfold more precise but with processing times that scale with pattern size.

Total 3D control is attainable through focused laser light and higher order absorption phenomena. In accordance with the aforementioned processes, a focused laser concentrates photons around a small, z-specified focal plane within the material, but the objective-defined light cone still extends vertically through the sample.[16] By drastically increasing laser power and wavelength to encourage near-simultaneous absorption of two or more comparatively low-energy photons, activation is effectively limited to a small 3D volume centered around the focal plane (Figure 1c−e). Controlled laser scanning/rastering enables materials to be patterned with full spatial complexity following 3D input geometries, whereby submicron patterning resolution in x-y and single-micron in z is often achievable.[17] The prerequisites for this exquisite control have historically been prohibitively expensive equipment and comparatively slow fabrication times (<1 μL per hour), but emerging photochemical and optics-based hardware advances promise to make this technology faster and more versatile.[18]

Image-guided patterning, the superposition of digitally processed images onto a sample as virtual or real photomasks, is well poised to benefit from lower multiphoton fabrication costs. Beyond simple 2D/3D shapes (e.g., cylinders, cubic rectangles), image-guided approaches permit flexible material customization through a variety of inputs (e.g., tissue microscopy, data storage patterns, user-generated artwork) (Figure 2a). For example, the West lab demonstrated native microvasculature-informed 3D photoablation of hydrogels with high fidelity (Figure 2b).[19,20] The ability to faithfully reproduce these and other biological structures within engineered matrices will be invaluable to fields such as personalized medicine that emphasize miniscule variations between patient tissue microenvironments.

An additional layer of sophistication afforded by modern image-guided techniques is the ability to encode nonbinary images. Exploiting the dose-dependence of many photochemistries, sequential illumination of a material through a series of binary patterns can afford spatially varied functionalization according to the amount of light delivered to each region. The Zenobi-Wong group recently demonstrated this via two-photon-assisted immobilization of a fluorescent peptide-tagged avidin, whereby a 2D-defined biochemical pattern with adjacent regions spanning many discrete concentrations was created (Figure 2c).[40] Though fabrication times in this approach scale with pattern complexity (i.e., a pattern with 100 intensity levels will take at least 100× as long to fabricate as one with just a single pattern intensity), sequential illumination of gel regions uniquely affords high-resolution nonbinary customization of biomaterials.

3. 4D CUSTOMIZATION OF GEL BIOCHEMISTRY

Combining the lithographic techniques outlined above with photolabile and bioorthogonal chemistries affords 4D control of cell fate in engineered materials. Creative iterations of a simple patterning logic—bind, release, (de)activate, and

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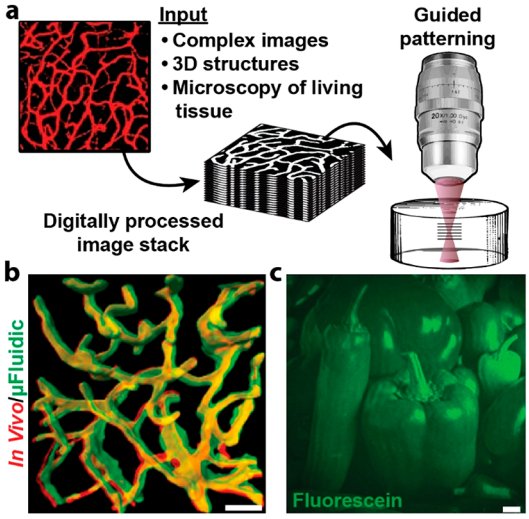

Figure 2. Image-guided biochemical patterning of hydrogels. (a) User-defined 2D and 3D images are digitally sliced and processed into stacks. A multiphoton microscope individually patterns each slice into discrete hydrogel volumes. Retinal vasculature adapted with permission from ref 19. Copyright 2012 Wiley-VCH. (b) Fluorescently labeled dextran (green) is perfused through 3D patterned microvasculature. The patterning input image is overlaid in red. Scale bar = 25 μm (estimated). Reproduced with permission from ref 20. Copyright 2016 Wiley-VCH. (c) Nonbinary image-guided biomolecule (here, fluorescein peptide-avidin) immobilization using a series of virtual photomasks each corresponding to discrete laser power settings. Scale bar = 50 μm. Reproduced with permission from ref 40. Copyright 2020 Wiley-VCH.

assemble—give rise to highly dynamic and diverse materials that can be tuned for many 4D culture applications. Importantly, signals and stimuli are delivered, removed, or (in)activated with negligible consequence to either the bulk or local mechanical properties, freeing bandwidth for further sophistication with orthogonally controlled matrix degradation or stiffening. Dynamic modulation of hydrogel mechanics is also of great consequence to 4D tissue engineers and has been covered in detail elsewhere.[21] Well-engineered photopatterning of biochemical cues also strives for orthogonality to other biological and environmental factors, such as off-target signaling pathways and cytotoxic reagents.

3.1. Photoimmobilization of Biochemical Signals within Gels

A decade after the first live-cell encapsulation in photoinitiated hydrogels, Luo and Shoichet decorated hydroxyls extant on ultralow melting point agarose with an *ortho*-nitrobenzyl (*o*NB)-photocaged cysteine probe to selectively bind soluble peptide pendants. Application of a focused laser and maleimide-tagged RGD cell adhesion peptides successfully guided process growth of dorsal root ganglia from the gel surface into defined volumes.[22] Soluble agarose conveniently forms physical (i.e., noncovalent) cross-links upon thermal initiation, requiring no optical input for initial material formation, but new strategies were needed to access the rapidly diversifying array of gel materials. Most early hydrogels capable of live-cell encapsulation required radical photoinitiation with near-ultraviolet (UV) or blue light, complicating efforts to ligate additional photoactive species in the precursor solution. Contemporary platforms addressing this hurdle included hyaluronan gels chemically cross-linked using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) for guided neurite outgrowth from surface-seeded dorsal root ganglia[23] and photopatterned labeling of unreacted "defect" handles within preformed acryl hydrogels.[24] The first approach was ultimately unsuitable for 3D encapsulation due to a toxic cross-linking chemistry, and the second, while generally applicable to synthetic cross-linkers lacking internal handles, presented some difficulties with excessive radical generation and poorly defined degree-of-functionalization. Nevertheless, these studies laid important groundwork for creative techniques combining complex gel mechanics with photopatterned peptides. For example, fibrous electrospun hyaluronan gels cross-linked and photopatterned with thiol-norbornene were selectively labeled to study cell adhesion in the presence of heterogeneous cues and a spatially aligned ECM.[25] Additionally, these early reports generated interesting and relevant 3D-patterned biomolecules using both single- and multiphoton lithography, including branched cell-adhesive channels to probe migration and invasion through synthetic ECM.[26]

The tension between photomediated gelation and patterning has since been alleviated through several routes. First, red-shifted photopolymerization via visible light-responsive radical initiators manifests little or no crosstalk when combined with traditional photocaging groups that respond to near-UV exposure.[27] Although there are few such literature reports, one exciting implication is that popular acrylated hydrogels (e.g., GelMA) could be preloaded with a known concentration of near-UV-photolabile pendant groups that are unaffected by visible light used for gelation, providing a straightforward method of dosage control. More generally, the continued pursuit of red-shifted and biocompatible chemistries breathes life into photoinitiated biomaterials that are immensely popular beyond the development of 4D patterning techniques. Though often framed for subdermal drug delivery due to its minimal absorption and scattering by living tissue, red and near-IR light could be leveraged for future *in vitro* culture of very large or optically dense tissues or for implantation of photocustomizable organoids.[28] Additionally, employing several wavelength-orthogonal species in a single material enables multiplexed and sequential patterning strategies.

The second generation of patterned biomolecule tethering arrived in the wake of bioorthogonal click chemistries, particularly strain-promoted azide−alkyne cycloaddition (SPAAC)[29] and oxime ligation. These spontaneous covalent interactions upended and expanded the gelation chemistry toolbox available to tissue engineers, effectively negating requirements for radical-mediated polymerization,[30,31] although such materials remain useful. The use of ill-defined defects in the gel matrix as pendant tethering sites was superseded by stoichiometrically modified and chemically defined macromers such as multiarm polymer and peptide cross-linkers (Figure 3a−c). The former can be prereacted with a small but known quantity of a photocaged pendant species prior to gelation, while multiple orthogonal handles are easily engineered into the latter via on-resin modification. Additionally, the use of spontaneous gelation greatly simplified strategies involving photoinitiated patterning, such as thiol−ene chemistries introduced by the Anseth lab (Figure 3b).[32] Multiplexed patterning has also been achieved through mutually orthogonal chemistries by further functionalizing click macromers with additional orthogonal handles (Figure 3d).[9,30,33,34] The photocaged oxime chemistry first pioneered

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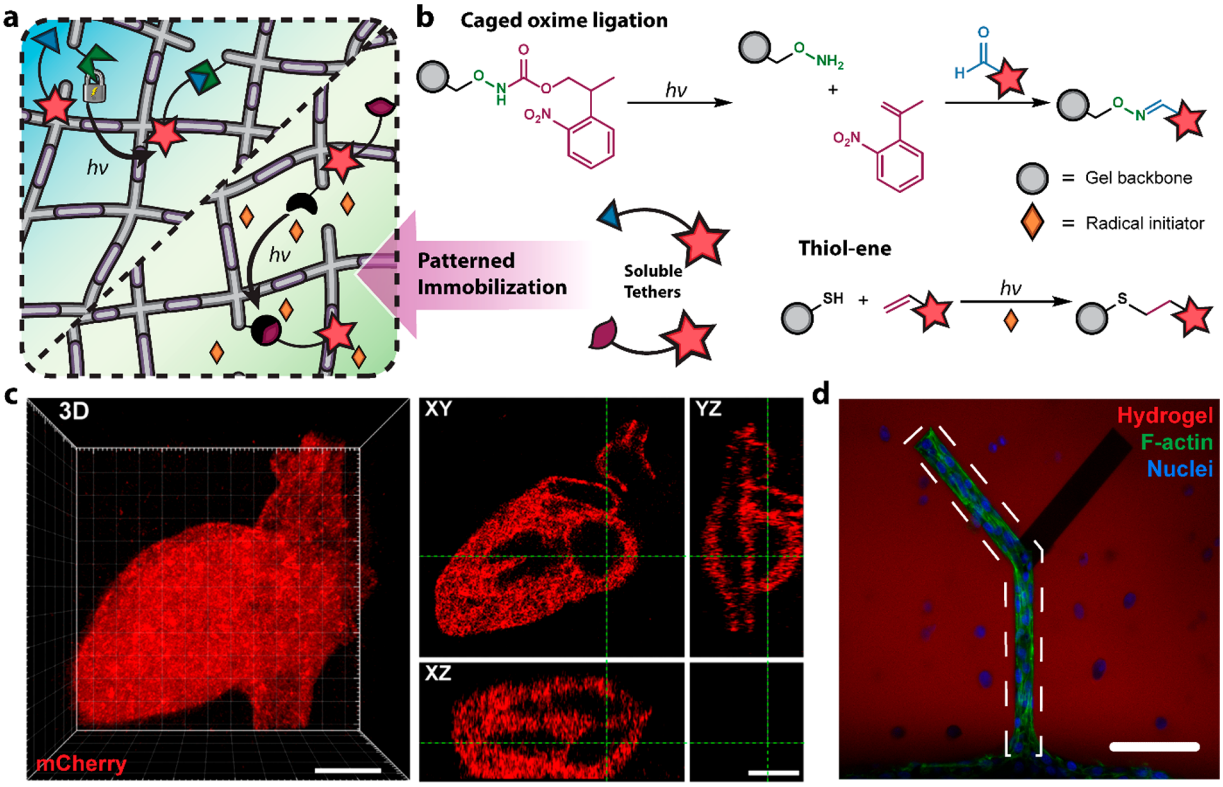

Figure 3. Photopatterned immobilization of bioactive cues within gels. (a) Two strategies for photoimmobilization. Soluble tethers are ligated to photoresponsive sites on the gel backbone either spontaneously (upper left) or through radical-mediated addition (lower right). (b) Representative chemistries for each class shown in Figure 3a. 2-(2-Nitrophenyl)prop-1-oxycarbonyl (NPPOC)-caged oxyamines selectively ligate with soluble aldehyde-functionalized cargo (red star) while alkenes and thiols couple in the presence of light and a radical initiator. (c) 3D and cross-sectional views of a protein-patterned anatomical heart (mCherry, red) via image-guided multiphoton lithography and caged oxime chemistry in collagen I hydrogels. Scale bars = 50 μm. Reproduced with permission from ref 17. Copyright 2021 National Academy of Sciences. (d) Precisely controlled 3T3 fibroblast (green = actin, blue = nuclei) outgrowth in an encapsulated coculture with hMSCs using combined gel (red) degradation and patterned thiol−ene immobilization of RGD adhesion peptide in a click hydrogel. Dashed lines show the region of the branched channel that was functionalized with RGD. Scale bar = 100 μm. Reproduced with permission from ref 9. Copyright 2011 The Author(s).

by DeForest and Tirrell and later advanced by our lab is a potent example of this.[17,35,36] Recent advances in synthetic chemistry are again expanding this space: the inverse electron-demand Diels−Alder (IEDDA) reaction is highly tunable, orthogonal to most other click chemistries, and shows particular promise for biomaterials development.[37]

Photomediated hydrogel decoration can also be performed with the aid of enzymes. The Lutolf and Segura laboratories proposed a photocaged activated transglutaminase factor XIII (FXIIIa) substrate to selectively capture Q-peptide labeled biomolecules.[38,39] Remarkably, this technique allowed patterning of site-specifically modified whole growth factors through recombinant expression of N-terminal Q-peptide fusions. Prior studies tended to rely on residue-specific modifications (e.g., activated esters to lysine and maleimides to cysteine) which are often stoichiometrically undefined at the protein level and more likely to hinder protein function due to the loss of critical side chains. Recombinant handles can generally be engineered to avoid such losses in bioactivity through the use of selective positioning with peptide linkers/spacers. Nevertheless, synthetic modification remains popular due to the wide array of bioorthogonal handles that are offered on commercially available proteins.

In a recent *tour de force*, an intricately engineered system using highly multiphoton-sensitive coumarin photocages and several stages of biochemical and enzymatic adaptation enabled the precise immobilization of biotinylated proteins.[40] While not directly genetically encoded (though potentially synthesized chemoenzymatically),[41] biotinylated morphogens and other proteins of interest are readily available for purchase. Additionally, any losses in bioactivity can be easily recuperated through the addition of more protein from the outset. The report demonstrated impressive control over axonal guidance through the hydrogel using tethered nerve growth factor.

3.2. Photoremoval of Biochemical Cues from within Gels

Photodegradable linkers are the natural complement to photoimmobilized tethers and are similarly employed in the construction of spatiotemporally varied anisotropic materials. Toward this end, such linkers were initially adapted from 2D photopatterning applications for on-demand drug delivery and cell-adhesion peptide release from hydrogels.[42] As early 3D cell encapsulations enabled by homo- and heterogeneous hydrogel modification gained traction, photodegradation displayed great promise as a means to selectively cleave other molecular cargoes for drug delivery[15] (Figure 4a), control hydrogel mechanical properties and for cell release.[42] Mechanical modulation via homobifunctional cross-linkers engineered with photolabile cores is a robust research area in its own right, but similarly constructed heterobifunctional linkers can join and release hydrogels and their biomolecular cargo. For example, negative patterning, or selective removal of tethered cues, allows the user to release signaling molecules from within

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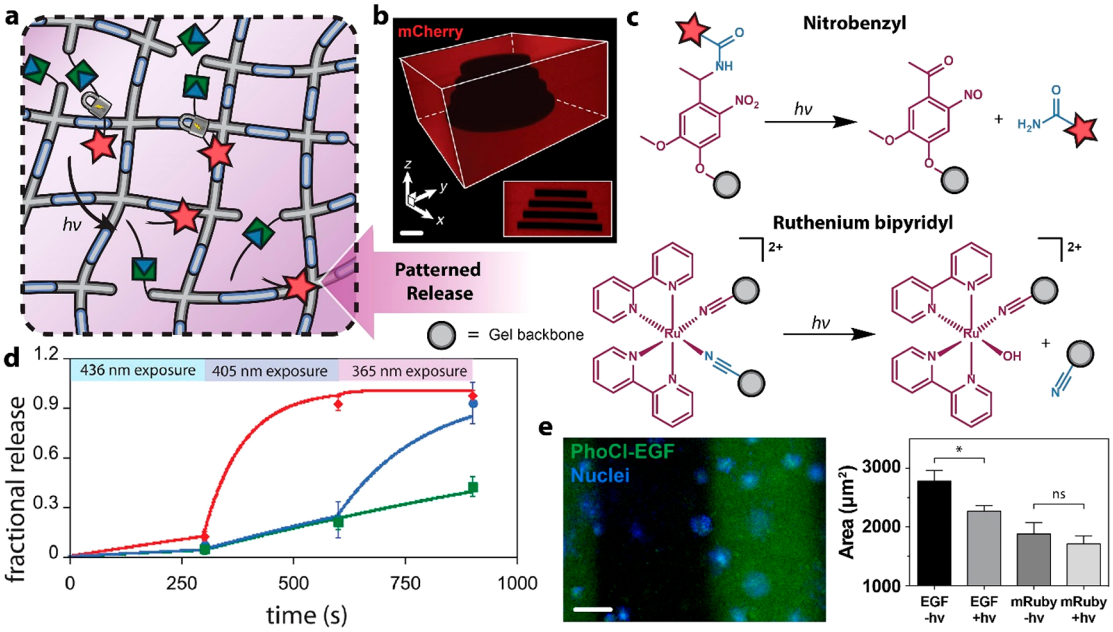

Figure 4. Patterned photorelease of bioactive cues from gels. (a) Selective release of tethered molecules from the gel backbone is achieved using bifunctionalized, photodegradable caging groups. (b) 3D and side-projected view of multiphoton patterned release of mCherry (red) from a hydrogel using an *o*NB-based linker. Scale bar = 100 μm. Reproduced with permission from ref 36. Copyright 2019 The Author(s). (c) Representative chemistries for two types of photodegradation. A classic *o*NB-based linker is shown (above), representing a popular option for photodegradation for both signal (red star) release and mechanical modulation. Other bifunctionalized photolabile groups, such as ruthenium bipyridyls (below) are emerging as specialized alternatives due to their red-shifted absorbance spectra or higher multiphoton sensitivity. (d) Wavelength-orthogonal release of multiple signals from a hydrogel using differently substituted nitrobenzyl linkers. Reproduced with permission from ref 45. Copyright 2012 American Chemical Society. (e) Photodegradable PhoCl-EGF tether (green) selectively released, leading to increased HeLa cell (blue) density and spheroid growth in regions with constitutively presented EGF. A control experiment using a PhoCl-mRuby control did not yield significant differences in cell fate (right). Scale bar = 200 μm. Adapted with permission from ref 50. Copyright 2019 American Chemical Society.

specific gel volumes, relying on liberated species to diffuse away from their tether sites (Figure 4b). The first and most prolific of these degradable linkers was derived from *o*NB,[15] but other photocleavable groups with red-shifted absorption or more favorable kinetics have been demonstrated. In fact, many synthetically tractable cages can be functionalized at multiple positions while maintaining photolability, giving rise to an array of degradable linker cores with unique characteristics ranging from the original nitroaryl derivatives to coumarins,[43] ruthenium bipyridyls,[44] and more (Figure 4b,c). The longevity of nitrobenzyl- and nitrophenethoxycarbonyl-derived photolabile groups despite their kinetic obsolescence is worth noting: to date, there simply is no other synthetically and biologically (via genetic code expansion) tractable option that retains such a diversity of cageable functional groups. This provides invaluable flexibility for linker design as well as a route to direct photoactivation, a process discussed in section 3.4.

Multiplexed and logic-based release is also attainable through the use of wavelength- or stimulus-orthogonal chemistries. Nitrobenzyl-derived linkers with varied substituent positions about the aromatic ring were shown to preferentially cleave at different wavelengths for drug delivery (Figure 4d),[45] and our lab recently sought to improve orthogonality and sensitivity using a pair of coordinated ruthenium linkers in tandem with the canonical *o*NB.[44] Given the wealth of available stimulus-responsive chemistries, we also demonstrated that Boolean logical response could be encoded into materials through varied cross-linker topology. By introducing orthogonal degradable moieties within linear/(bi)cyclic/tadpole peptide-based linkers, linker degradation was readily specified in response to precise YES/OR/AND-based logical combinations of light, enzymes, and/or redox inputs.[46−48] For example, a linear OR-gated cross-linker was constructed by arranging an *o*NB and matrix metalloproteinase-degradable peptide sequence in series while a cyclized AND linker had them arranged in parallel.

In addition to synthetic small molecule and peptide cores, it is also possible to assemble photodegradable linkers from more complex and cleavable biomolecules such as nucleotides[49] and recombinant proteins. Our lab recently engineered recombinant photocleavable linkers using synthetically modified PhoCl[50]—a circularly permuted photoconvertible fluorescent protein that cleaves upon blue light exposure. Tethered PhoCl-epidermal growth factor (EGF) chimera proteins were selectively released from hydrogels to spatially confine spheroid formation in HeLa cells (Figure 4e). A follow-up study from the West lab replaced the chemoenzymatically introduced azide cross-linking handle with an N-terminal SpyCatcher, offering a fully canonical protein-based approach through immobilization in a SpyTag-decorated gel.[51]

3.3. Reversible Photoimmobilization of Biochemical Cues within Gels

The stream of biochemical cues received by each cell *in vivo* must be overwhelmingly transient in order to encode dynamic and complex biological functions. Beyond the irreversible biomolecule immobilization/release strategies highlighted previously, more recent efforts have sought to establish methods that permit reversible hydrogel modification. Initial studies focused on exploiting reaction and/or wavelength-

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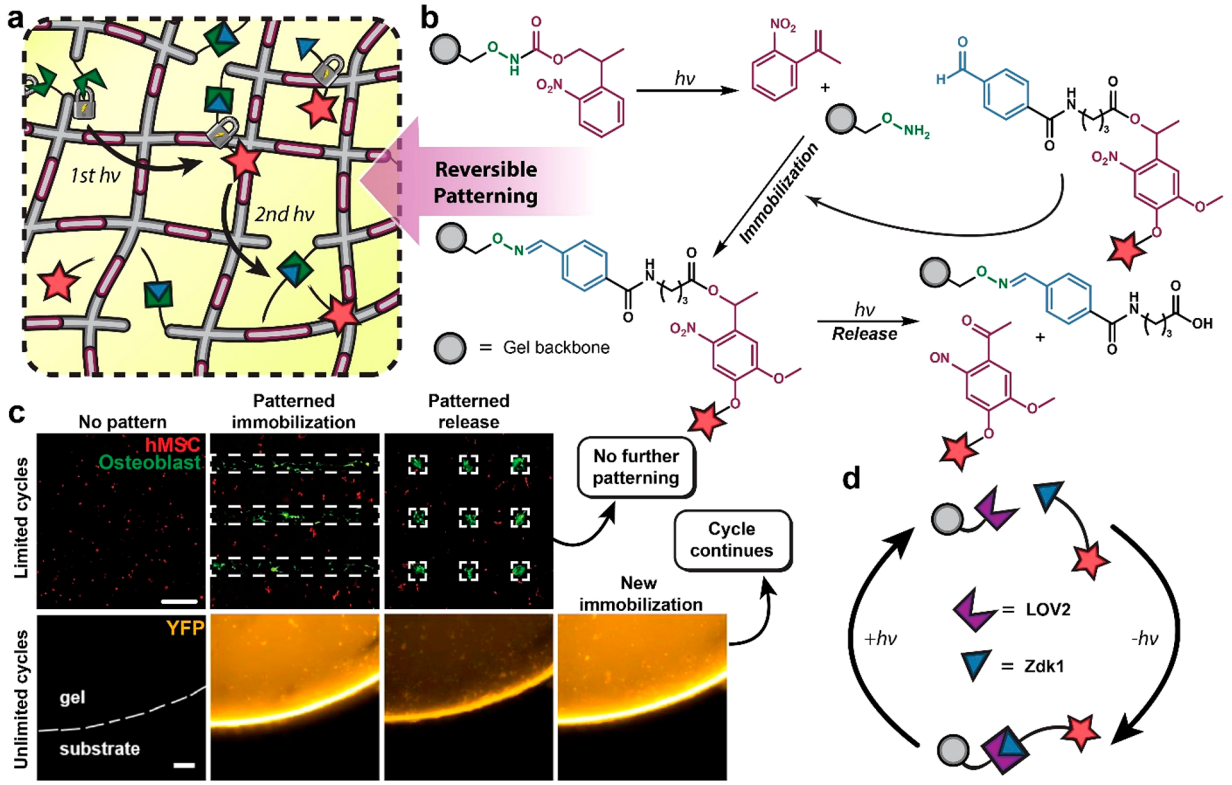

Figure 5. Cycle-limited and fully cyclic strategies for reversible hydrogel photopatterning. (a) Photochemical immobilization and release techniques are combined via patterned immobilization of biomolecules (red star) that include photodegradable linkers. (b) Representative cycle-limited but photoreversible chemistry using an NPPOC-caged oxime ligation and *o*NB-degradable linker. (c) Cycle-limited patterning strategies offer excellent resolution, bioorthogonality, and minimal background interaction but are indefinitely user-controllable. Fully cyclic techniques allow multiple rounds of photopatterning and release but currently face limitations such as background association (LOVTRAP). Scale bars = 100 μm. Adapted with permission from refs 35 and 54. Reference 35 Copyright 2015 The Author(s). Reference 54 Copyright 2022 American Chemical Society. (d) LOVTRAP chemistry applied to hydrogel photopatterning. Here, LOV2 and Zdk1 protein domains reversibly separate upon blue light exposure but spontaneously reassociate in the dark.

orthogonality to combine both photoimmobilization and subsequent photorelease into a single platform. For example, DeForest and Anseth previously demonstrated that a visible-light mediated thiol−ene reaction could be combined with a near-UV-mediated *o*NB cleavage to afford reversible tethering of synthetic peptides to PEG-based gels.[27] Our group has also achieved reversible photoimmobilization of fragile proteins including growth factors through *o*NB's combination with a photomediated oxime ligation in which a caged oxyamine present throughout the gel is photochemically unmasked to immobilize biomacromolecules modified either residue- or site-specifically with an *o*NB-photoreleasable aldehyde (Figure 5a,b).[35,36] Though such approaches have yielded spatiotemporal and reversible control over EGF and Notch (via Delta) signaling as well as differentiation of encapsulated human mesenchymal stem cells, cyclic functionalization is limited to one round. Nevertheless, functional assays including luminescent cell reporters and antibody staining indicate that proteins modified in this way maintain their bioactivity, and the system avoids radical chemistries entirely.

A small but important collection of approaches toward multicycle patterned biomolecule immobilization have also been established (Figure 5c). Inspired by the chemistries underlying reversible addition−fragmentation chain-transfer (RAFT) polymerization, the Anseth lab designed allyl sulfide linkers for thiol−ene photoligation such that a new allyl sulfide was generated upon ligation, permitting molecular photoexchange. This process was demonstrated using both thiolated small molecules and stochastically modified proteins.[52] Although these materials can theoretically be cycled more than prior approaches, they are practically limited by (1) nonspecific radical reactions and (2) statistical release of the reactive allyl group due to exchange between the incoming ligand and gel anchor, causing the number of gel-bound photoreactive allyl sulfides to decrease by half throughout each cycle.

Photopatterned noncovalent interactions provide the clearest route to date toward true cyclic material patterning. The LOVTRAP protein pair, consisting of the blue light-sensitive LOV2 domain from *Avena sativa* and engineered affibody Zdark1, was recently identified by the West lab as a powerful tool toward engineering photoreversible biomaterials.[53] LOV2 and Zdark1 strongly associate in the absence of light but separate reversibly upon illumination, bringing their respective cargoes in and out of proximity (Figure 5d). This approach is highly cytocompatible and fully cyclic but displays considerable and undesired background association in the illuminated state. Interestingly, these proteins have also been leveraged for fluorescent polymeric hydrogels (FPH) to store "grayscale" 2D patterns.[54] An alternative to LOVTRAP, photopatterned DNA hybridization is another highly tunable process that was used to encode shockingly complex and reversible functionality into hydrogel materials. Although only recently applied to 4D cell culture,[49] prior work has

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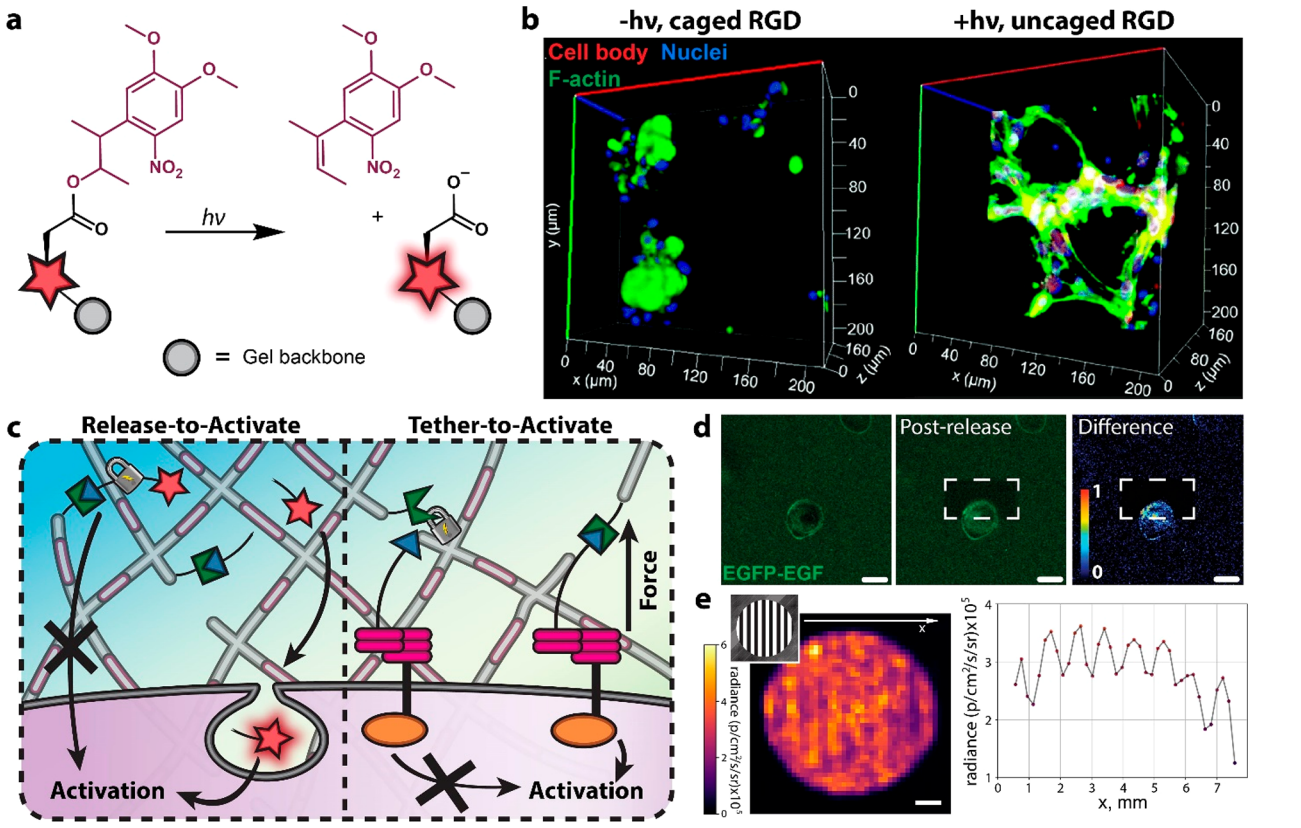

Figure 6. Context-dependent biochemical patterning via "release/tether-to-activate" methodologies. (a) Nitrophenethoxycarbonyl-derived photocage conditionally liberates an essential functional group (carboxylic acid) from a tethered biomolecule upon light exposure. (b) Photopatterned angiogenesis in encapsulated HUVEC cells using a photocaged cyclic-RGD peptide tether. Adapted with permission from ref 57. Copyright 2020 Royal Society of Chemistry. (c) Activation of two types of pendant molecules is photopatterned in different contexts. On the left, release of a tethered ligand allows it to interact with a nearby cell (membrane: gray, cytoplasm: purple) via endocytosis. On the right, photoimmobilization anchors a ligand to the gel matrix, enabling mechanotransduction. (d) Subcellularly resolved EGF (green) internalization by an A431 cell occurs upon multiphoton release from the hydrogel matrix. Scale bars = 10 μm. Reproduced with permission from ref 36. Copyright 2019 The Author(s). (e) Increased Notch signaling in the presence of tethered Dll1 is visualized using a U2OS dual luciferase reporter. Quantified average radiance along the labeled *x*-axis is shown on the right. Scale bar = 1 mm. Adapted with permission from ref 17. Copyright 2021 National Academy of Sciences.

successfully engineered gels with bidirectional wavelength multiplexing[55] and pattern edge detection features.[56] Photopatterned DNA hybridization in conjunction with toehold displacement may provide an alternative route toward cyclic modification of hydrogel biomaterials. Though such noncovalent modification strategies yield cyclic modification, careful consideration must be given to biomolecule/ECM binding affinities.

3.4. Photopatterned Biomolecule Activation/Generation

Many biomolecules are active at low concentrations in both soluble and tethered forms, potentially complicating the many hydrogel (de)decoration methods that rely on diffusive introduction/removal during 3D cell culture. Off-target signaling may even be different depending on the state of the signal. For example, some biomolecules have intra- and extracellular targets associated with separate pathways. Biological processes occur over a wide range of time scales, so it is often prudent to avoid prolonged exposure of encapsulated cells to active soluble factors as they pass in or out of the material. An emerging strategy to circumvent these complications seeks to tether inactive "photocaged" biomolecules throughout the gel volume prior to light exposure—either before or after gelation. Photocaged vascular endothelial growth factor (VEGF) peptidomimetic and cyclic RGD have been exploited in this way to selectively promote angiogenesis in cell populations upon light exposure (Figure 6a,b).[57,58] Moving beyond peptide activation, the Shoichet lab recently demonstrated effective temporary suppression of protein bioactivity through attachment of multiple streptavidin domains. A photocleavable NHS probe stochastically appends biotin moieties onto surface-accessible amines prior to incubation with streptavidin, and cleavage of the linker scarlessly reveals the cargo protein upon liberation of the streptavidin−biotin complex.[59] Though photocaging and subsequent patterning of any commercially available protein containing multiple lysine residues is theoretically tractable with no extra modifications, labeling must be performed using purified proteins and characterized for each batch. The Shoichet lab applied this strategy to Jag1-Notch signaling for temporal control over cholangiocyte differentiation and assembly into duct structures.[59]

In contrast to direct photoactivation, biomolecule signals that are active *only* in their soluble or tethered form can be activated through photopatterned changes in contextual presentation (Figure 6c). Our lab demonstrated that patterned EGF binds EGFR and promotes cell proliferation when immobilized extracellularly even though endocytosis and

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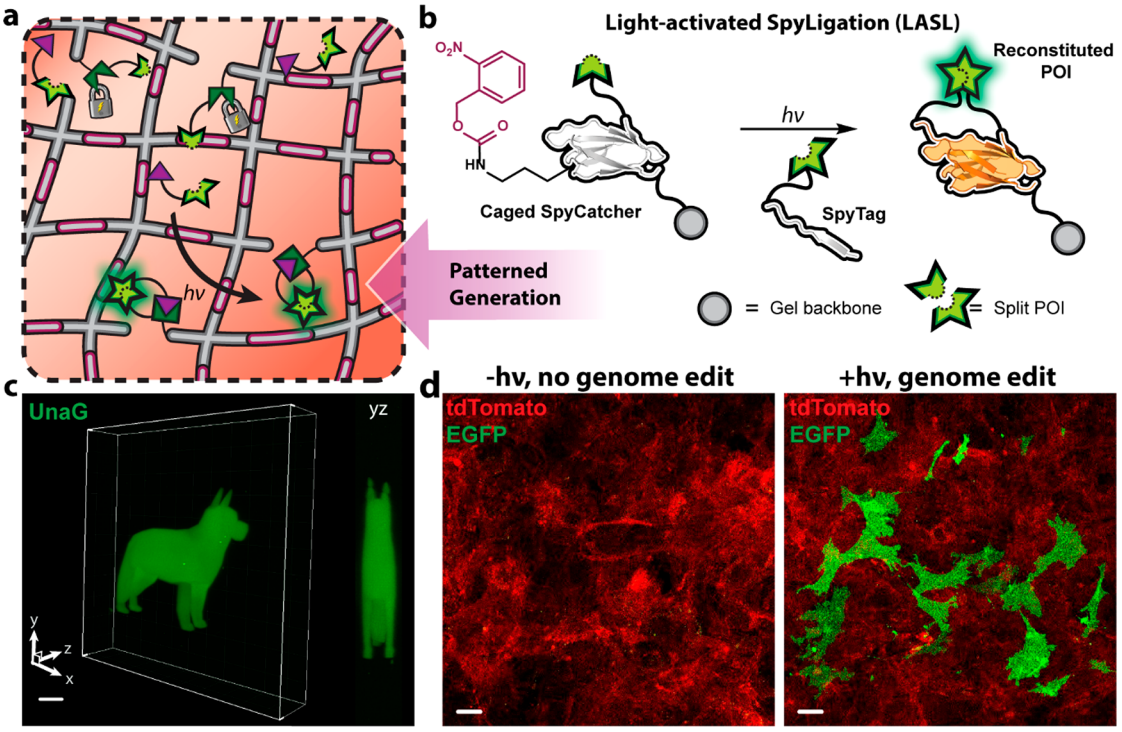

Figure 7. Patterned activation of biochemical signals through *in situ* split protein assembly via light-activated SpyLigation (LASL). (a) User-directed protein reconstitution is accomplished by coupling split protein of interest (POI) halves to a photocaged ligation system such as SpyTag-SpyCatcher. Induced proximity of the POI halves after photouncaging yields functional protein. (b) In LASL, a photocaged SpyCatcher is viable for both intra- and extracellular applications during 3D cell culture. (c) Multiphoton-patterned LASL reconstitution of split UnaG (green) in a synthetic hydrogel. Scale bar = 50 μm. Reproduced with permission from ref 61. Copyright 2023 The Author(s). (d) Patterned intracellular reconstitution of split Cre recombinase via LASL deactivated tdTomato (red) and activates EGFP (green) expression through irreversible genome editing in fibroblasts. Scale bar = 50 μm. Reproduced with permission from ref 61. Copyright 2023 The Author(s).

canonical signaling are inhibited. Upon photorelease, the newly soluble protein can be locally endocytosed, triggering canonical EGF-mediated signaling (Figure 6d).[36] This "release-to-activate" strategy could be leveraged with other cargoes, although it has yet to be demonstrated at large scale. Conversely, some extracellular ligands, notably those implicated in the Notch pathway, must be anchored to propagate intercellular signaling via mechanotransduction.[60] We demonstrated a "tether-to-activate" strategy using Delta-like protein 1, which performs one-sided Notch signaling when anchored to a gel matrix rather than another cell membrane (Figure 6e).[17] While less generalizable than other means of photopatterned signal activation, these "release/tether-to-activate" approaches directly draw from ongoing advances in photochemical immobilization/release from gels.

Another potentially powerful method enabling patterned protein activation relies on *in situ* functional assembly. We recently introduced the "light-activated SpyLigation" (LASL) as a genetically encoded photoclick reaction capable of site-specific protein−protein photoligation (Figure 7a).[61] Here, a photocaged lysine is incorporated during protein translation at SpyCatcher's catalytic reaction site via genetic code expansion. Upon light exposure, the uncaged protein undergoes isopeptide bond formation in perfect reaction specificity with its peptide partner SpyTag (Figure 7b). Genetically fusing the photocaged SpyCatcher and SpyTag to nonfunctional split fragment pairs, we demonstrated that LASL could stably reassemble bioactive proteins following short light exposures in solution, biomaterials, and living mammalian cells. Though this approach has enabled patterned genome editing and 4D biomaterial customization through specific and molecularly defined chemistries, functional assembly requires that a suitable split site has been identified for the to-be-activated protein of interest (Figure 7c,d).

Demonstrating another highly creative approach to patterned biomolecule generation, the del Campo lab engineered hybrid "living materials" composed of a synthetic matrix and coimmobilized cells designated for production of active signaling molecules.[62] Using engineered bacteria as biomolecule factories in coculture with living mammalian cells, protein production in the material was optogenetically regulated using light-responsive expression systems pDawn and pDusk. Hydrogel surface-seeded fibroblasts were seen to take up fluorescent proteins generated and secreted by the embedded bacteria, providing evidence of direct communication. It is likely that follow-up studies will demonstrate complete 4D control of bioproduction near encapsulated cells and leverage the ability to continuously and dynamically produce multiple biological cues including growth factors and antitumor agents, a feature that is not yet available in other materials. Additionally, living materials show great promise for the patterned application of multiple biochemical cues under differential promoters without the need to exogenously diffuse material into the gel or engineer complex linking molecules.

4. SUMMARY AND OUTLOOK

Light-mediated biochemical (de)decoration of and patterned protein activation/generation within hydrogel scaffolds are invaluable tools for engineers and researchers seeking to spatiotemporally guide cell fate *in vitro*. The field is ripe for

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translational innovation as many of the historical challenges associated with pattern orthogonality, scale, and biochemical modification have been addressed. Early pendant groups tended to be synthetically modified peptides or small molecule drugs, but these quickly broadened toward the use of residue-specifically modified full proteins. More recently, site-specific protein engineering methodologies and fully recombinant photolabile systems have greatly improved the biochemical activity, cytocompatibility, and sophistication of hydrogel pendants. Beyond engineered tethering and release strategies, reversible chemistries, *in situ* signal uncaging, and functional protein assembly have emerged as routes to more complex manipulation of the synthetic ECM.

Similarly, the macromolecular building blocks supporting these pendants have progressed. Many of the early challenges associated with performing photochemistry in photopolymerized gels are now moot due to spontaneous click chemistries and the availability of red-shifted initiators. Researchers have also adapted many patterning strategies to naturally derived or even recombinant protein-based hydrogels. Hardware advances have been modest, but recent improvements in multiphoton lithography, particularly for photofabrication, point to significantly improved resolution and volumetric patterning rate on the horizon. Together, the implications of these materials are far-reaching: teasing low-variance organoid panels for drug discovery, image-guided patterning of patient-derived tissue geometries for personalized medicine, and eventually customized *in vitro* growth of macroscopic tissues. Further advances will enable larger, more complex structures to be dictated with increasing precision. Beyond expanding the bounds of molecular and hardware technologies, what remains for the field is to begin implementing hydrogel optical lithography toward gaining new biological insights.

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Author Contributions

The manuscript was written through contributions of both authors. Each author has given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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