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Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments

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Click chemistry provides extremely selective and orthogonal reactions that proceed with high efficiency and under a variety of mild conditions, the most common example being the copper(I)-catalysed reaction of azides with alkynes[1,2]. While the versatility of click reactions has been broadly exploited[3–5], a major limitation is the intrinsic toxicity of the synthetic schemes and the inability to translate these approaches into biological applications. This manuscript introduces a robust synthetic strategy where macromolecular precursors react through a copper-free click chemistry[6], allowing for the direct encapsulation of cells within click hydrogels for the first time. Subsequently, an orthogonal thiol–ene photocoupling chemistry is introduced that enables patterning of biological functionalities within the gel in real time and with micrometre-scale resolution. This material system enables us to tailor independently the biophysical and biochemical properties of the cell culture microenvironments *in situ*. This synthetic approach uniquely allows for the direct fabrication of biologically functionalized gels with ideal structures that can be photopatterned, and all in the presence of cells.

An emerging paradigm in organic synthesis is a focus on highly selective and orthogonal reactions that proceed with high efficiency and under a variety of mild conditions. A growing number of these reactions are grouped under the term 'click chemistry', and have been used to produce a catalogue of functional synthetic molecules and subsequent materials[1,4]. Characteristics of modular click reactions include (1) high yields with fast kinetics, (2) regiospecificity and stereospecificity, (3) insensitivity to oxygen or water and (4) mild reaction conditions, solventless or in water.

While the versatility of click reactions has been broadly exploited in many fields including drug discovery[7,8], material science[9–11], and bioconjugation[3,12,13], a major limitation is the intrinsic toxicity of the synthetic schemes and the inability to translate these approaches to biological applications. Though the 1,3-dipolar Huisgen cycloaddition between azides and alkynes[2] is often seen as the quintessential click reaction, the crucial copper catalyst precludes its use with biological systems[14,15]. This drawback, however, was recently circumvented through the development of novel cyclooctyne moieties whose ring strain and electron-withdrawing fluorine substituents give rise to an activated alkyne. This molecule has been shown to react quickly with azides in the absence of a metal catalyst, enabling the use of traditional click chemistry in living systems[6,16]. Specifically, azide-labelled cell-surface glycans were reacted with fluorescently labelled cyclooctynes *in vivo* to enable the visualization of dynamic subcellular development within zebra-fish embryos[17]. Though this chemistry has been exploited in the labelling of biomolecules, it has not yet been used for biomaterial formation.

More recently, the radical-mediated addition of a thiol to an alkene known as the thiol–ene reaction has gained attention as an emerging click reaction[18]. In addition to being bio-orthogonal and biocompatible, the reaction is advantageous in that it is readily initiated with light, ultimately affording spatial and temporal control over where the reaction occurs[19]. This reaction has been used to create two-dimensional surface gradients of biomolecules[20] as well complex materials[21].

In alignment with the evolution of click chemistry, the combined utilization of multiple orthogonal reactions presents the opportunity to fabricate multifunctional and tunable materials without sacrificing synthetic simplicity or efficiency. While materials with highly defined structures have applications in microelectronics, membrane technology, and fuel cells, one increasingly important area of research is in developing biomaterial platforms that enable researchers to culture and study cells in three dimensions[22]. Though initial material development has proven successful in permitting cell growth, a growing topic of interest is the development of bioactive materials that promote and detect specific cell function through spatially presented biochemical and biomechanical cues[23]. Ultimately, a platform offering such versatility would be of particular note to those interested in well-defined niches for three-dimensional (3D) cell culture and understanding the role of biomechanical versus biochemical signals in cell function, as well as regenerating tissue structures[24]. Appropriately developed click chemistry can provide this versatility, enabling the fabrication of increasingly complex 3D culture constructs using just a few simple reactions.

Here, a hydrogel platform is introduced that uses two orthogonal click chemistries; one for hydrogel formation and another for biochemical patterning within the preformed material. The modular aspect of these reactions allows for independent control of the network structure and chemistry, and facile incorporation of biological epitopes. Network formation is accomplished using a recently developed Cu-free variant to the traditional click reaction, the Huisgen cycloaddition, between an azide ($-N_3$) and an alkyne ($-C{\equiv}C-$) to form a triazole[6]. This method uses a di-fluorinated cyclooctyne moiety (DIFO3), whose ring strain and electron-withdrawing fluorine substituents promote the [3 + 2] dipolar cycloaddition with azides without the use of a catalyst[25] (Fig. 1a). This reaction has been carried out under physiological conditions in the presence of living cells with no reported toxicity[17]. Beyond this bioconjugation approach for cell labelling, multifunctional macromolecular monomers were synthesized to create ideal

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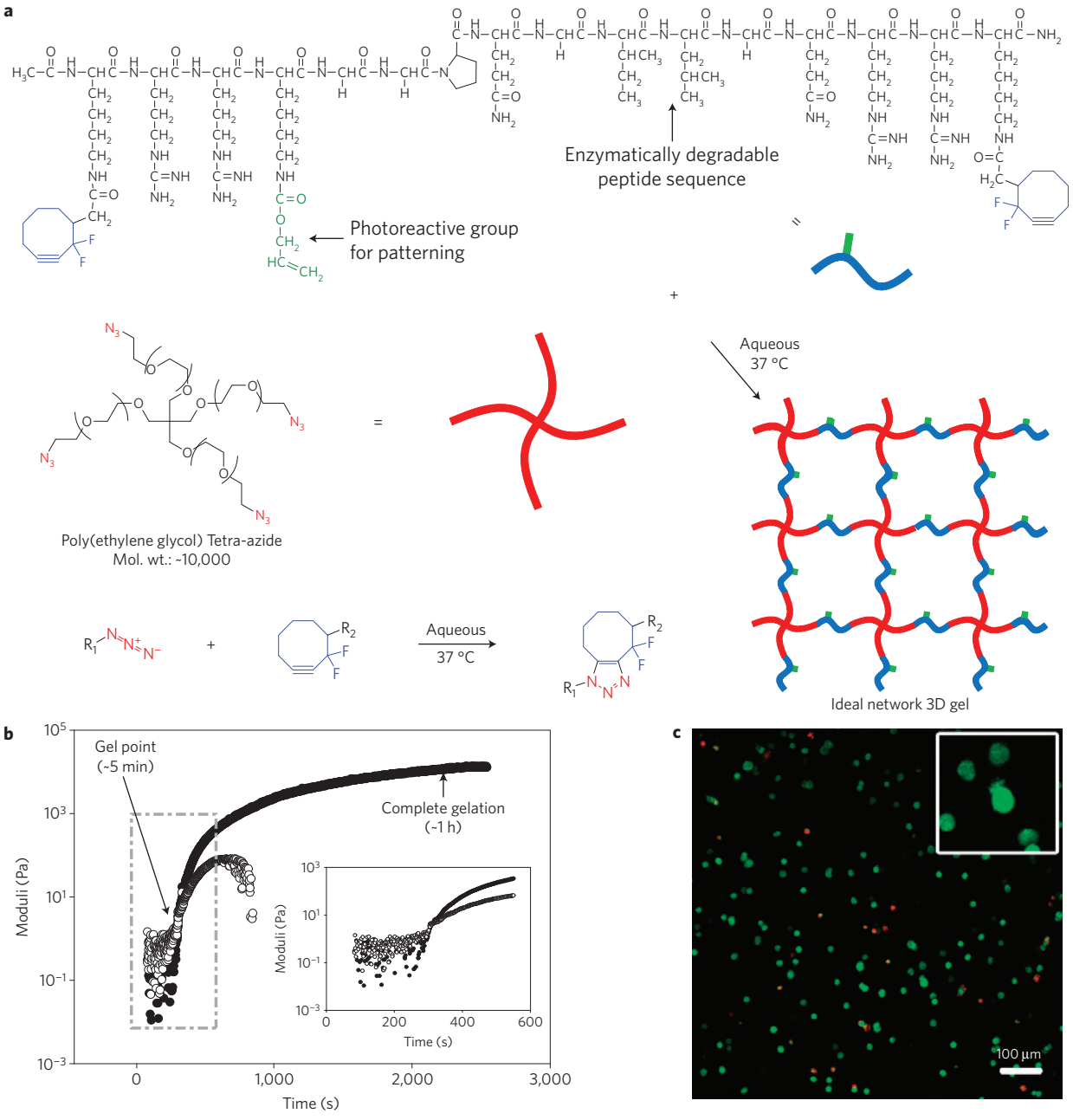

Figure 1 | Cytocompatible-click-hydrogel formation reaction and kinetics. a, Click-functionalized macromolecular precursors undergo the [3+2] Huisgen cycloaddition to form a 3D ideal network hydrogel through a step-growth polymerization mechanism. **b**, Rheology can be used to monitor dynamic network formation and indicates gelation within minutes and complete reaction occurring in less than 1 h at 37 °C for a 13.5 wt% monomer solution. G' is shown as closed circles, whereas G'' are open circles. **c**, A live/dead stain at 24 h of 3T3s encapsulated within this material indicates a predominantly viable population (live cells are shown in green, whereas dead cells are shown in red). The image represents a 200 μm confocal projection.

network structures with minimal defects and local heterogeneities. Specifically, multifunctional azides and activated alkynes were reacted in a one-to-one fashion to yield end-linked polymer gels, under reaction conditions that enable cell encapsulation and result in gels with initially uniform material properties.

A four-arm poly(ethylene glycol) (PEG) tetra-azide was reacted with bis(DIFO3) di-functionalized polypeptide in an aqueous environment at 37 °C (schematic shown in Fig. 1a). The choice of PEG enables us to tailor readily the biophysical properties of the gel, while eliminating non-specific interactions that often result when proteins adsorb to materials. Biological functionality can be readily introduced into the hydrogel backbone by the choice of the crosslinking peptide sequence. Here, a matrix metalloproteinase cleavable sequence (GPQG ↓ ILGQ) is selected, so that cells can actively remodel their surroundings through secreted enzymes[26]. Cells encapsulated in hydrogels containing an enzymatically degradable crosslinker sequence spread and migrate throughout the material, with dramatically increased viability compared with non-degradable alternatives[27,28].

Hydrogels were formed using a 13.5 wt% total macromer solution containing a 1:1 ratio of alkyne to azide functionalities. Ultimately, this gel composition affords a high water content, elasticity similar to many tissue matrices, and the ability to image cells in three dimensions. Dynamic time sweep rheological experiments were conducted to monitor network evolution during this step polymerization (Fig. 1b). The crossover point, an estimate

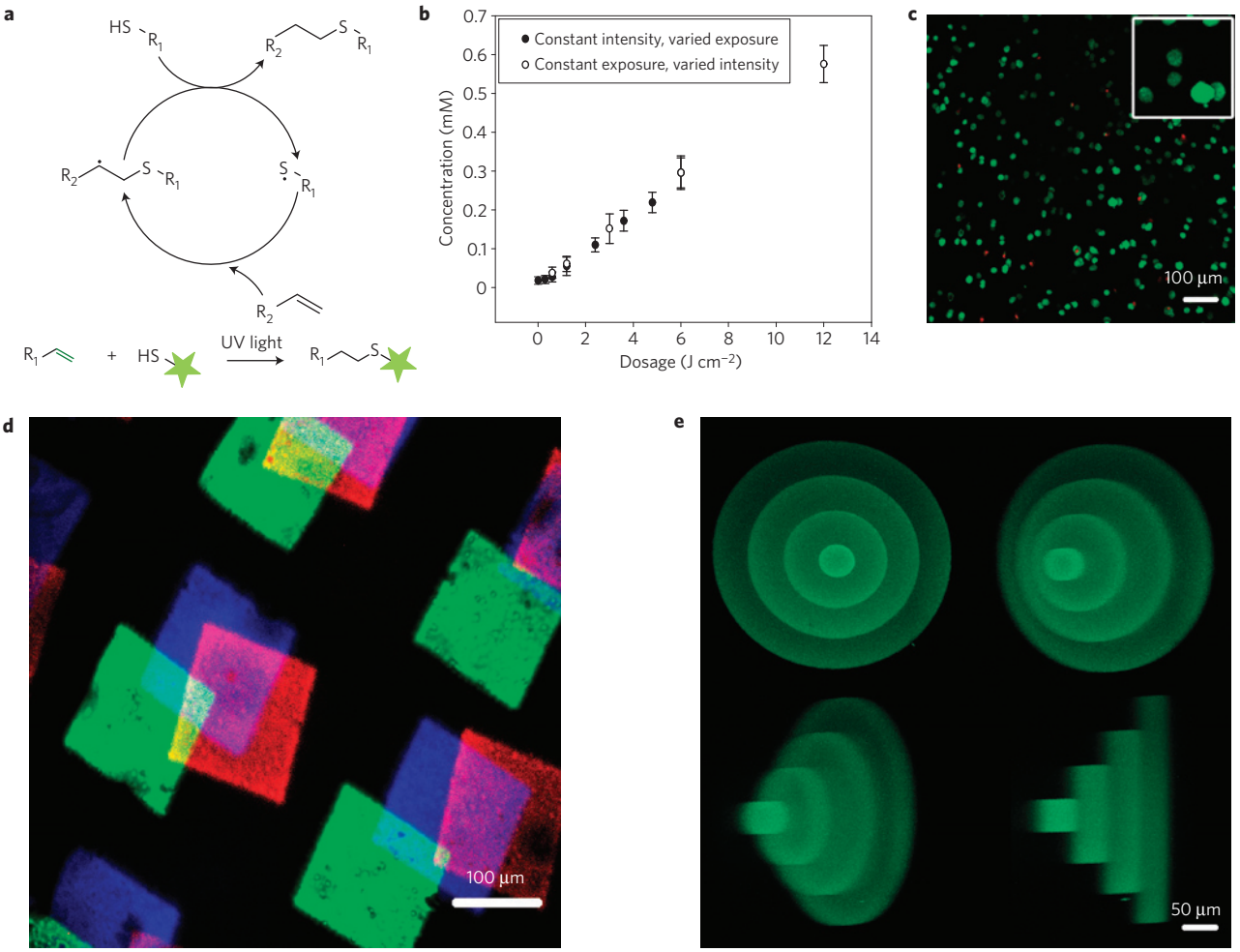

Figure 2 | Cytocompatible, biochemical patterning within preformed click hydrogels. a, The thiol–ene reaction mechanism provides a means to quantitatively couple sulphhydryls (–SH) with vinyl functionalities (–C=C) in the presence of light. **b**, On swelling into the material, relevant thiol-containing biomolecules are covalently affixed to the hydrogel network at varying concentrations by altering the dosage of exposed light (intensity and exposure time). **c**, A live/dead stain at 24 h after photolithographic patterning of 3T3s indicates a predominantly viable population (live cells are shown in green, whereas dead cells are shown in red) and that the patterning process is cytocompatible. **d**, The thiol–ene reaction is confined to user-defined regions in space using photomasks to introduce three different fluorescently labelled peptide sequences within the gel, a process that can be repeated at desired times and spatial locations to introduce additional biochemical cues. **e**, By controlling the focal point of the laser light in three dimensions using a confocal microscope, micrometre-scale spatial patterning resolution is achieved. Values in **b** are reported as mean ± s.d. ($n = 5$). The image in **c** represents a 200 μm confocal projection. The images in **d** and **e** represent confocal micrographs of fluorescently tagged peptides patterned within the networks.

of gelation at which the elastic modulus (G') is equal to the storage modulus (G''), occurs in less than 5 min (290 ± 30 s). Furthermore, the data indicate a final G' value of 12.0 ± 0.6 kPa at time $t \sim 1$ h, signifying a structurally robust network that maintains its 3D shape with loading. The step-growth mechanism was confirmed by the statistical gelation model for step-growth networks developed by Flory and Stockmayer (see Supplementary Fig. S1). In addition, dynamic magic-angle spinning ^{1}H NMR was carried out to further examine the reaction kinetics of network formation (see Supplementary Fig. S2). Under normal solution-phase NMR conditions, the NMR spectral lines would quickly become extremely broad, yielding useless spectra as the polymer network begins to form owing to dipolar relaxation in the motionally restricted (semisolid) phase being formed. With the sample oriented at the magic angle (about 54.736°), rotating at a frequency that exceeds the static dipolar linewidth, this dipolar line-broadening can be eliminated, yielding high-resolution ^{1}H NMR spectra throughout the polymerization reaction. Characteristic peaks associated with the alkyne DIFO3 functionality were found to completely disappear on reaction with azides within 1 h, with a second-order rate constant of 8.9×10^{-5} M^{-1} s^{-1}. Both the rheological and the magic-angle spinning NMR data suggest that the formed hydrogel is nearly ideal, agreeing with previous work on click-based networks[10]. Ultimately, the timescale and mechanism of this reaction are such that it permits cell encapsulation with high viability comparable to traditional hydrogel systems (>90% at 24 h post encapsulation, Fig. 1c and Supplementary Fig. S6).

Post network formation, a second click reaction enables facile modification of the cell's niche through the conjugation of biomolecules at specific locations with the gel. Specifically, by including a photoreactive allyl ester within the crosslinking peptide sequence through the commercially available Fmoc-Lys(alloc)-OH amino acid (Fig. 1a), relevant biochemical cues can be covalently incorporated within the hydrogel using the bio-orthogonal thiol–ene coupling reaction. Originally designed as an orthogonal protecting group for lysine[29], allyloxycarbonyl (alloc) contains a vinyl group capable of undergoing a thiol–ene photocoupling reaction with any thiol-containing compound, including cysteine[11,30]. The alloc protecting group is stable to Fmoc deprotection and peptide trifluoroacetic acid cleavage from resin, rendering it a suitable and

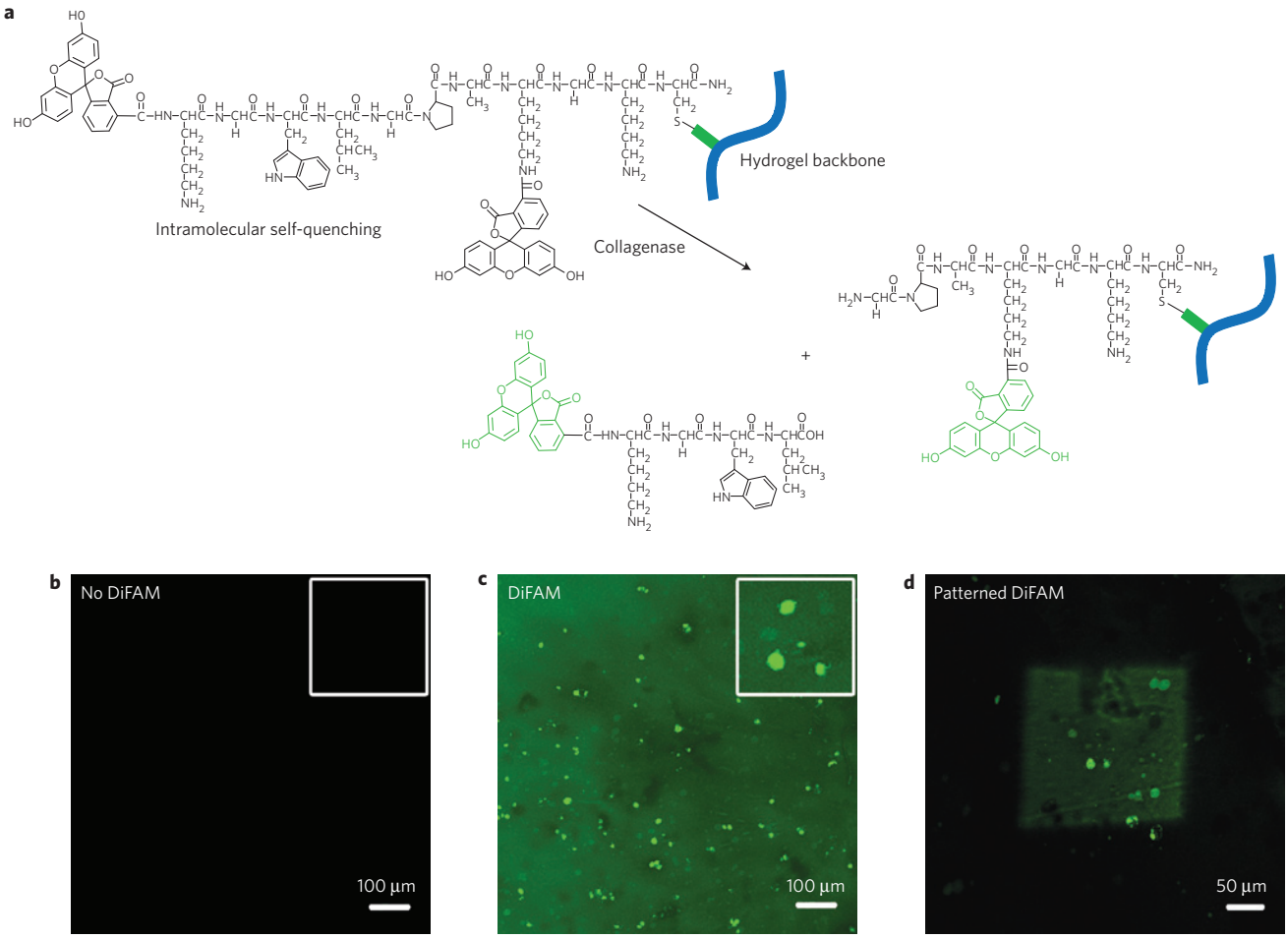

Figure 3 | Visualizing 3T3 collagenase activity through patterned detection peptide within 3D click hydrogels. a–d, 3T3s were encapsulated into hydrogel networks at 3×10^6 cells ml^{-1}. After 24 h, a di-fluorescein collagenase-sensitive peptide sequence (DiFAM), which shows intramolecular self-quenching until enzymatically cleaved (**a**), was swollen into networks at 0.5 mg ml^{-1} and exposed to 365 nm collimated light at 10 mW cm^{-2} for 10 min through a variety of photomasks: **b**, full mask; **c**, no mask; **d**, full mask with a 200-μm-square opening. Here, patterned regions gently fluoresce whereas areas of high collagenase activity (near the cell surface) fluoresce with greater intensity. Images represent 200 μm confocal projections at 3 days.

versatile choice as the photoreactive component of our hydrogel crosslinker. Additionally, the electron-rich alloc allyl ester is not susceptible to Michael-type addition, eliminating the possibility for non-specific chemical immobilization[31].

The thiol–ene reaction is a radical-mediated addition of a thiol to an alkene, involving the catalytic propagation of a thiyl radical through a vinyl functional group and the chain transfer from the resulting carbon radical to a thiol[32] (Fig. 2a). Thiols can be deprotonated to thiyl radicals using photolytically cleaved, hydrogen-abstracting initiator systems. Selectively exposing specific locations within the material to light affords spatial and temporal control of where this photocoupling reaction occurs in real time. The extent of patterning can ultimately be controlled by regulating the light intensity and exposure time (Fig. 2b) and uses cytocompatible wavelengths (365 nm) and intensities (~10 mW cm^{-2}). Light exposure can be controlled using conventional photolithographic, single-photon and multiphoton techniques, each affording a higher degree of reaction specificity than the last. This thiol–ene reaction is compatible with cells, as indicated by the high viability maintained throughout patterning (>90% at 24 h post encapsulation, Fig. 2c and Supplementary Fig. S6). Two-dimensional patterns were transferred throughout the z axis of a gel using stereolithography (Fig. 2d and Supplementary Fig. S8). We demonstrate that the reaction scheme is fully additive by incorporating three different peptides at varied positions within the gel (Fig. 2d and Supplementary Fig. S10). More complex 3D structures of arbitrary size and shape can be patterned within the gel by systematically scanning the focal point of a pulsed near-infrared laser where functionalization is desired. The latter technique affords micrometre-scale pattern resolution, as illustrated in Fig. 2e and Supplementary Fig. S9, and is carried out on timescales similar to that required for 3D confocal imaging.

The thiol–ene reaction enables any thiol-containing compound to be pendantly attached at user-defined locations within the hydrogel. While adding thiol functionality to small molecules is fairly straightforward, cysteine-containing peptides require no additional synthetic modifications to be patterned within a gel. To illustrate the versatility that is afforded by this type of patterning scheme, a detection molecule was covalently incorporated as a pendant functionality that increases its fluorescence when exposed to cellular protease activity within the network. Specifically, a di-fluorescein collagenase-sensitive peptide sequence (DiFAM) was selectively patterned into the gels. This peptide, FAM–KGWL↓GPAK(FAM)GKC–NH_2, shows intramolecular self-quenching until it is enzymatically cleaved (Fig. 3a). While the gel fluoresces slightly where the quenched molecule has been patterned, the probe is found to fluoresce with much higher intensity in regions of collagenase activity immediately surrounding the cells (Fig. 3b–d). This DiFAM probe serves as a proof of concept that these materials are able to report real-time information concerning local encapsulated

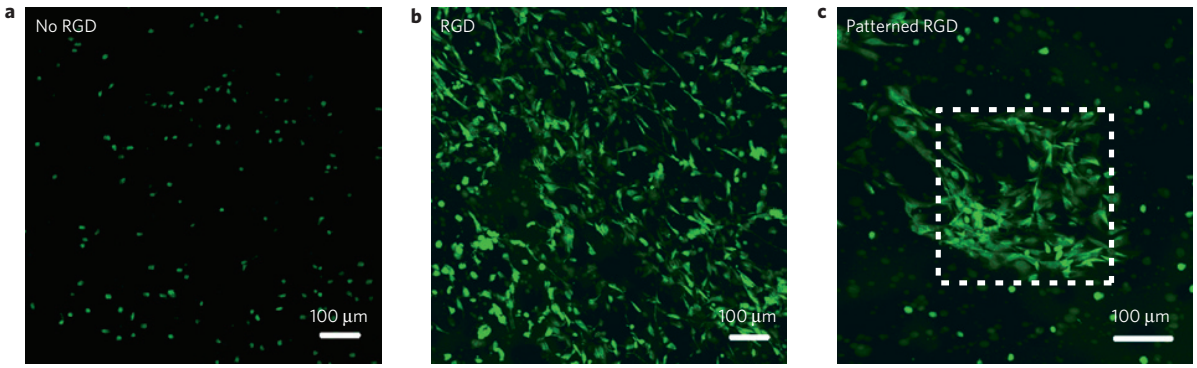

Figure 4 | Effect of patterned RGD on 3T3 population within 3D click hydrogels. a–c, 3T3s were encapsulated into hydrogel networks at 3×10^6 cells ml^{-1}. After 24 h, thiol-functionalized RGD, a fibronectin motif known to promote cell attachment, was swollen into networks at 3 mg ml^{-1} and exposed to 365 nm collimated light at 10 mW cm^{-2} for 10 min through a variety of photomasks: **a**, full mask; **b**, no mask; **c**, full mask with a 250-μm-square opening (illustrated by the dashed lines). 3T3s were stained at day 10 with CellTracker orange and imaged using confocal microscopy. Here, cells adopt a spread morphology only in user-defined regions of RGD. Images represent 200 μm confocal projections.

cell behaviour, and that these detection assays can be confined to user-defined regions within the gel.

Just as this system allows for the patterning of reporter probes, biochemical functionalities that direct cell behaviour can be incorporated within these materials in a location-specific manner. Incorporation of the RGD sequence, a fibronectin motif, has been extensively used to promote cell adhesion uniformly throughout biomaterials[33,34], as well as in patterned channels[35–37]. Here, a cysteine-containing, fluorescently labelled RGD sequence, AF_{488}–AhxRGDSC–NH_2, was selectively affixed within a cell-impregnated hydrogel. The fibronectin motif induces localized morphological and migratory changes within the patterned regions (Fig. 4). Where the RGD is present, cells are able to attach to and locally degrade the surrounding network, giving rise to a spread morphology. However, when this functionality is absent, cells maintain a rounded morphology. Figure 4c illustrates that these induced differences in cell behaviour can be selectively confined to patterned regions within a single gel.

As presented, this work uses two novel bio-orthogonal click-chemistry schemes to combine and exploit features of previously mutually exclusive technologies. Namely, the enzymatically degradable hydrogel platform provides an ideal network into which biomacromolecules can be photopatterned that detect, as well as promoting, specific cellular functions. The material chemistry affords a simplified synthetic microenvironment that captures critical aspects of extracellular matrices, allowing for the direct observation of cellular processes in three dimensions, including migration, proliferation and morphological changes. The ability to then spatially tune the material properties provides an additional tool to manipulate cell function. Since reactive monomer components can be easily exchanged, the material is readily tailorable with multiple functionalities for 3D cell studies.

Methods

Synthesis of click-functionalized macromolecular precursors. *Synthesis of PEG tetra-azide.* Four-arm PEG tetra-azide was synthesized following a published synthetic route[11]. In short, methanesulphonyl chloride (×5) was added to four-arm PEG (molecular mass $M_n \sim 10{,}000$ Da) (Jenkem) and subsequently reacted with sodium azide (×5). Additional detail is available in Supplementary Fig. S11.

Synthesis of bis(cyclooctyne)-functionalized peptide crosslinker. The enzymatically degradable, allyl ester-containing peptide Ac-KRRK(alloc)GGPQGILGQRRK–NH_2 was synthesized (ABI 433A peptide synthesizer) through standard Fmoc solid-phase methodology and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole (HBTU/HOBt) activation. Resin was treated with trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5) for 2 h and precipitated (×3) using ice-cold diethyl ether (see Supplementary Fig. S3). DIFO3 was coupled to the ε-amino groups of the terminal lysines through standard 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) coupling chemistry. Peptides were purified using semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) (Waters Delta Prep 4000) using a 70 min linear gradient (5–95%) of acetonitrile and 0.1% trifluoroacetic acid (Sigma). Peptide purity was confirmed by an analytical RP-HPLC and matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (Applied Biosystems DE Voyager) using α-cyano-4-hydroxycinnamic acid matrix (Sigma): calculated ($[M+H]^+$ 2329.7); observed ($[M+H]^+$ 2329.1) (see Supplementary Fig. S4).

Synthesis of self-quenched collagenase-sensitive detection peptide (DiFAM): H–KGWLGPAK(Dde)GKC–NH_2 (0.25 mmol) was synthesized through standard Fmoc solid-phase methodology and HBTU/HOBt activation. Carboxyfluorescein (1 mmol, Novabiochem) was coupled to the N-terminus through standard HATU coupling chemistry. The Dde protecting group was removed using 2% hydrazine in DMF, and a second carboxyfluorescein (1 mmol) was coupled to the ε-amino groups of the deprotected lysine using standard HATU coupling chemistry. Resin was treated with trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5) for 2 h and precipitated (×3) using ice-cold diethyl ether. Peptides were purified using semipreparative RP-HPLC (Waters Delta Prep 4000) using a 70 min linear gradient (5–95%) of acetonitrile and 0.1% trifluoroacetic acid (Sigma). DiFAM peptide purity was confirmed by an analytical RP-HPLC and matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (Applied Biosystems DE Voyager) using α-cyano-4-hydroxycinnamic acid matrix (Sigma): calculated ($[M+H]^+$ 1860.04); observed ($[M+H]^+$ 1861.94) (see Supplementary Fig. S5)

Synthesis of fluorescently labelled adhesive ligand. H–AhxRGDSC–NH_2 (0.25 mmol) was synthesized through standard Fmoc solid-phase methodology and HBTU/HOBt activation. Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (1 mg, Invitrogen), was dissolved in N-methylpyrrolidone with a catalytic amount of N,N-diisopropylethylamine and stirred with resin overnight at room temperature. Resin was treated with trifluoroacetic acid/triisopropylsilane/water/dithiothreitol (94.5:2.5:2.5:0.5) for 1 h and precipitated (×3) using ice-cold diethyl ether. This product, denoted AF_{488}–AhxRGDSC–NH_2, was used with no further purification.

Rheological experiments. Dynamic frequency-, time-, and strain-sweep rheology experiments were carried out on a TA Ares rheometer with parallel-plate geometry (20 mm diameter) at 25 °C. Initial gel network formation of a 13.5 wt% solution was monitored by observing G' and G'' at a constant frequency of 100 rad s^{-1} as a function of time. Gel properties were monitored through frequency-sweep measurements at fixed strain amplitude (10%) to measure the hydrogel storage, G', and loss, G'', moduli.

3T3 fibroblast cell culture. *General cell culture.* NIH 3T3s were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Invitrogen), 2% penicillin/streptomycin (Gibco), 0.4% fungizone (Gibco) and 0.2% gentamicin (Gibco) in 5% CO_2 at 37 °C.

Cell encapsulation. 3T3s were suspended at 3×10^6 cells ml^{-1} in a 13.5 total wt% monomer solution in media and allowed to react for 1 h to form a cell-laden hydrogel sheet.

Biochemical patterning. Hydrogels were swollen in phenol red-free media (pH = 7.4) containing 0.05 wt% Irgacure 2959 (Ciba) and 3 mg ml^{-1} patterning agent AF_{488}–AhxRGDSC–NH_2 for 1 h. Using conventional photolithographic techniques, gels were exposed to collimated ultraviolet light (365 nm wavelength at 10 mW cm^{-2}) through a patterned photomask for 10 min. Under these

photopatterning conditions, 1–2% of the alloc functional groups are consumed to yield ~0.1 mM of conjugated peptide, implying that multiple signals can be incorporated at biologically relevant concentrations. Alternatively, two-photon techniques were exploited for complex patterning by placing gels on a 710 LSM NLO confocal microscope stage (Carl Zeiss) and selectively exposing to pulsing focused 720 nm laser light through a ×20/0.8 Plan-Apochromat objective (Carl Zeiss), with x–y control afforded by region-of-interest scanning and z control by focal depth. Z planes were scanned at 1 μm increments with a laser power of 400 mW μm^{-2} and a scan speed of 2.4 μs μm^{-2}. For both the two-photon and photolithographic approaches, photocoupling of the patterning agent to the hydrogel network occurs only within areas exposed to light. After patterning is complete, the gel is washed for approximately two hours with fresh media to remove any unbound material, yielding the final patterned hydrogel (see Supplementary Figs S7 and S9). The process of swelling in the patterning agent, photopatterning and washing can be repeated for multiple cues within the same gel (see Supplementary Fig. S10). For gels that are of reasonable thickness for 3D cell culture (~200 μm to 1 mm), characteristic diffusion times of the unreacted peptides from the gel are of the order of a few minutes to a few hours.

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References

1. Kolb, H. C., Finn, M. G. & Sharpless, K. B. Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.* **40**, 2004–2021 (2001).
2. Huisgen, R. 1.3-Dipolare Cycloadditionen—Ruckschau und Ausblick. *Angew. Chem. Int. Ed.* **75**, 604–637 (1963).
3. Moses, J. E. & Moorhouse, A. D. The growing applications of click chemistry. *Chem. Soc. Rev.* **36**, 1249–1262 (2007).
4. Kolb, H. C. & Sharpless, K. B. The growing impact of click chemistry on drug discovery. *Drug Discov. Today* **8**, 1128–1137 (2003).
5. Hawker, C. J. & Wooley, K. L. The convergence of synthetic organic and polymer chemistries. *Science* **309**, 1200–1205 (2005).
6. Baskin, J. M. *et al.* Copper-free click chemistry for dynamic *in vivo* imaging. *Proc. Natl Acad. Sci. USA* **104**, 16793–16797 (2007).
7. Srinivasan, R., Li, J., Ng, S. L., Kalesh, K. A. & Yao, S. Q. Methods of using click chemistry in the discovery of enzyme inhibitors. *Nature Protocols* **2**, 2655–2664 (2007).
8. Evans, M. J., Saghatelian, A., Sorensen, E. J. & Cravatt, B. F. Target discovery in small-molecule cell-based screens by *in situ* proteome reactivity profiling. *Nature Biotech.* **23**, 1303–1307 (2005).
9. Franc, G. & Kakkar, A. Dendrimer design using Cu–I-catalyzed alkyne-azide 'click-chemistry'. *Chem. Commun.* 5267–5276 (2008).
10. Malkoch, M. *et al.* Synthesis of well-defined hydrogel networks using click chemistry. *Chem. Commun.* 2774–2776 (2006).
11. Polizzotti, B. D., Fairbanks, B. D. & Anseth, K. S. Three-dimensional biochemical patterning of click-based composite hydrogels via thiolene photopolymerization. *Biomacromolecules* **9**, 1084–1087 (2008).
12. Tornoe, C. W., Christensen, C. & Meldal, M. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **67**, 3057–3064 (2002).
13. Franke, R., Doll, C. & Eichler, J. Peptide ligation through click chemistry for the generation of assembled and scaffolded peptides. *Tetrahedron Lett.* **46**, 4479–4482 (2005).
14. Prescher, J. A. & Bertozzi, C. R. Chemistry in living systems. *Nature Chem. Biol.* **1**, 13–21 (2005).
15. Chen, J. K. Fish 'n clicks. *Nature Chem. Biol.* **4**, 391–392 (2008).
16. Agard, N. J., Prescher, J. A. & Bertozzi, C. R. A strain-promoted [3 + 2] azide–alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* **126**, 15046–15047 (2004).
17. Laughlin, S. T., Baskin, J. M., Amacher, S. L. & Bertozzi, C. R. *In vivo* imaging of membrane-associated glycans in developing zebrafish. *Science* **320**, 664–667 (2008).
18. Dondoni, A. The emergence of thiol–ene coupling as a click process for materials and bioorganic chemistry. *Angew. Chem. Int. Ed.* **47**, 8995–8997 (2008).
19. Hoyle, C. E., Lee, T. Y. & Roper, T. Thiol–enes: Chemistry of the past with promise for the future. *J. Polym. Sci. A* **42**, 5301–5338 (2004).
20. Khire, V. S., Benoit, D. S. W., Anseth, K. S. & Bowman, C. N. Ultrathin gradient films using thiol–ene polymerizations. *J. Polym. Sci. A* **44**, 7027–7039 (2006).
21. Killops, K. L., Campos, L. M. & Hawker, C. J. Robust, efficient, and orthogonal synthesis of dendrimers via thiol–ene 'click' chemistry. *J. Am. Chem. Soc.* **130**, 5062–5064 (2008).
22. Langer, R. & Vacanti, J. P. Tissue engineering. *Science* **260**, 920–926 (1993).
23. Cushing, M. C. & Anseth, K. S. Hydrogel cell cultures. *Science* **316**, 1133–1134 (2007).
24. Anseth, K. S. A biologist looks to 'click chemistry' for better three-dimensional tissue models. *Nature* **453**, 5 (2008).
25. Codelli, J. A., Baskin, J. M., Agard, N. J. & Bertozzi, C. R. Second-generation difluorinated cyclooctynes for copper-free click chemistry. *J. Am. Chem. Soc.* **130**, 11486–11493 (2008).
26. Nagase, H. & Fields, G. B. Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. *Biopolymers* **40**, 399–416 (1996).
27. Lutolf, M. P. *et al.* Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics. *Proc. Natl Acad. Sci. USA* **100**, 5413–5418 (2003).
28. Lutolf, M. P. & Hubbell, J. A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotech.* **23**, 47–55 (2005).
29. Kunz, H. & Unverzagt, C. The allyloxycarbonyl (aloc) moiety—conversion of an unsuitable into a valuable amino protecting group for peptide-synthesis. *Angew. Chem. Int. Ed.* **23**, 436–437 (1984).
30. Lee, T. Y., Smith, Z., Reddy, S. K., Cramer, N. B. & Bowman, C. N. Thiol–allyl ether–methacrylate ternary systems. Polymerization mechanism. *Macromolecules* **40**, 1466–1472 (2007).
31. Mather, B. D., Viswanathan, K., Miller, K. M. & Long, T. E. Michael addition reactions in macromolecular design for emerging technologies. *Prog. Polym. Sci.* **31**, 487–531 (2006).
32. Cramer, N. B., Reddy, S. K., O'Brien, A. K. & Bowman, C. N. Thiol–ene photopolymerization mechanism and rate limiting step changes for various vinyl functional group chemistries. *Macromolecules* **36**, 7964–7969 (2003).
33. Ruoslahti, E. & Pierschbacher, M. D. New perspectives in cell-adhesion—RGD and integrins. *Science* **238**, 491–497 (1987).
34. Burdick, J. A. & Anseth, K. S. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **23**, 4315–4323 (2002).
35. Hahn, M. S., Miller, J. S. & West, J. L. Three-dimensional biochemical and biomechanical patterning of hydrogels for guiding cell behaviour. *Adv. Mater.* **18**, 2679–2684 (2006).
36. Lee, S. H., Moon, J. J. & West, J. L. Three-dimensional micropatterning of bioactive hydrogels via two-photon laser scanning photolithography for guided 3D cell migration. *Biomaterials* **29**, 2962–2968 (2008).
37. Luo, Y. & Shoichet, M. S. A photolabile hydrogel for guided three-dimensional cell growth and migration. *Nature Mater.* **3**, 249–253 (2004).

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Author contributions

B.D.P., C.A.D. and K.S.A. developed the material concept, C.A.D., B.D.P. and K.S.A. designed the experiments, C.A.D. carried out the experiments, and C.A.D. and K.S.A. composed the manuscript.

Additional information

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