Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Spatially Resolved Coding of λ-Orthogonal Hydrogels by Laser Lithography

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Abstract: A λ-orthogonal reaction system is introduced where visible light induced radical thiol-ene and UV light induced NITEC (Nitrile-Imine mediated Tetrazole-Ene Coupling) ligations are consecutively employed to fabricate and functionalize PEG-based hydrogels. The fluorescent pyrazoline cycloadducts from the NITEC reaction are exploited to visualize the functionalized structures within the hydrogels as well as to anchor a RGD containing functional groups to promote spatially resolved cell attachment on the hydrogel surface.

Supporting Information

Materials

4-Dimethylaminopyridine (DMAP, 99%, Merck). *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC.HCl, TCI), 5-norbornene-2-methanol (>98%, mixture of isomers, TCI), 5-norbornene-2-carboxylic acid (98%, mixture of *endo* and *exo*, predominantly *endo*, Sigma), (-)-Riboflavin (98%, Sigma), tri(methoxypropylsilyl) methacrylate (98%, Sigma), DL-dithiothreitol (DTT, biotechnology grade, VWR), 4-arm poly(ethyleneglycol) 20,000 Da (JenKem USA), 4-formyl benzoic acid (96%, Acros), benzenesulfonyl hydrazide (98%, Sigma), pyridine (99%, Alfa Aesar), sodium nitrite (≥97%, Sigma), aniline (99%, Sigma), dichloromethane (DCM, 99.8%, extra dry, Acros), ethanol (99.8%, VWR), ethyl acetate (99.5%, VWR), pyridine (99%, Alfa Aesar), dimethyl sulfoxide (DMSO, Merck), hydrochloric acid (37%, Roth), toluene (99%, Alfa Aesar), NH2-Arg-Gly-Asp-Cys-OH (RGDC, Ontores Biotechnologies), Methanol (Ajax Finechem), Normal Human Dermal Fibroblasts (HDF: neonatal, Sigma Aldrich).

Characterisation and Instrumentation

Nuclear Magnetic Resonance (NMR) Spectroscopy

Compounds were analysed via ^{1}H NMR spectroscopy using a Bruker Avance III HD 400 (^{1}H, 400 MHz) spectrometer. Samples were dissolved in MeOD-d_4/D_2O. The δ-scale was referenced to residual solvent peaks (H_2O, δ = 4.79; $CHCl_3$, δ = 7.26; DMSO, δ = 2.55).

High Resolution Magic Angle Spinning NMR spectroscopy (HR MAS) was recorded on a Bruker 700 MHz spectrometer with TXI HRMAS probe at 3500 Hz with 64 scans and pre-saturation on the PEG resonance at 3.68 ppm. Sample preparation: The hydrogel precursor solution in D_2O was syringed into several 40µL HRMAS inserts and irradiated for specific time intervals under 455nm light at 27mW/cm^2.

Direct Laser Writing (DLW)

A home-built 3D DLW setup based on a Ti:Sapphire oscillator (Spectra-Physics Mai Tai HP) and an optical parametric oscillator (Spectra-Physics Inspire) delivering 150 fs pulses centered at 530 nm was employed. The pulse energy was controlled by means of an electro-optic modulator (EOM, Linos LM0202). The laser beam was focused through an oil-immersion lens with numerical aperture NA = 1.4 (Leica HCX PL APO 100x/1.4-0.7 OIL CS). All pulse energies were measured at the back focal plane of the objective lens through an aperture of 5.6 mm diameter. During the writing procedure, an

additional dedicated diode laser (532 nm wavelength) was used to find the z-position of the glass photoresist interface with high accuracy via a confocal detection scheme. The sample is held by a 3D piezo stage (Physik Instrumente (PI) GmbH und Co. KG) that allows for its relative translation with respect to the laser beam focus. An additional CCD camera is used for monitoring the writing process and sample alignment.

Writing conditions: All patterns were written with a scan velocity of 100 µm s^{-1} and average laser powers of typically 1 mW.

Fluorescence microscopy

Fluorescence micrographs of the micropatterns generated by DLW were recorded with a laser scanning microscope (Zeiss LSM 510 Meta) using a laser diode with an emission wavelength of 405 nm as excitation source and an oil immersion objective lens with a magnification of 63x and a numerical aperture of 1.4.

Cell adhesion observed under laser scanning confocal microscopy

The gels seeded with NHDF were stained with 10 µg/mL propidium iodide in PBS for 30 min. Then the gels were washed with PBS three times and mounted in PBS for observation with a Zeiss laser scanning confocal microscope (LSM 780). The observation was performed with a 10× objective lens. The excitation wavelengths for the gels and the cells were 405 nm and 514 nm, respectively. The emission bands for the gel and cells were 410-560 nm and 570-692 nm, respectively. The images were captured and processed with software Zen 2.3.

Rheometry

A Discovery HR-1 Hybrid Rheometer (TA Instruments) was used for rheological measurements. In-situ rheology was performed using parallel plate geometry with a quartz upper plate (50 mm) at 25 °C. All measurements were conducted with a 400 µm gap. To obtain the gel point, a time sweep was performed with constant stress at 50 Pa, conducted in the viscoelastic region. All gel point measurements were taken at frequency of 1Hz.

Experimental Procedures

Synthesis of 4-(2-phenyl-2H-tetrazol-5-yl) benzoic acid

Taken from a literature procedure[1]. 4-formylbenzoic acid (2.5g, 1.67mol) was dissolved in ethanol (150mL). Benzenesulfonyl hydrazide (2.87 g, 1.67 mol) was added to the pale-yellow solution and stirred for 30min. Water (130 mL) was added and a white precipitate formed. The precipitate was collected by filtration and dried under vacuum. A solution of sodium nitrite (1.15 g, 1.67 mol) in water (8 mL) was added to a cooled solution of aniline (1.55 g, 1.67 mol) in water/ethanol 26mL 1:1 and concentrated hydrochloric acid (5mL). The white precipitate was dissolved in pyridine (250 mL) and

the aniline solution slowly added to this solution at -10 ºC and stirred for 1 hr. Ethyl acetate (300 mL) was added to the resulting red solution and the top layer was collected. Concentrated hydrochloric acid (3N, up to 800 mL) was added to the ethyl acetate solution. A red precipitate formed at the phase interface and was collected by filtration and dried under vacuum. The desired product was obtained as a pale red solid (2.2 g, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 13.3 (s, 1H), 8.31 (m, 2H), 8.19 (m, 4H), 7.72 (m, 2H), 7.65 (m, 1H).

Synthesis of 4 arm Poly(ethyleneglycol)- 3NB-tetrazole macromer

4-(2-phenyl-2H-tetrazol-5-yl) benzoic acid (48 mg, 0.18 mmol) was dissolved in DCM (1mL). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (37.4 mg, 0.19 mmol) was added to the solution and stirred for 30min.A solution of 4-arm polyethyleneglycol 20 kDa (3 g, 0.15 mmol), dimethylaminopyridine (9 mg, 0.075 mmol) and triethylamine (19.6 mL, 0.18 mmol) in DCM (3mL) was slowly added to the tetrazole/EDC solution and the resulting solution was stirred overnight in darkness. 5-norbornene-2-carboxylic acid (124 mg, 0.9 mmol) and EDC.HCl (173 mg, 0.9 mmol) were added to the solution and stirred for 24hrs in darkness. The product was purified by dialysis (MWCO 6-8 kDa) against methanol, then water and freeze-dried. The product was obtained as a pale pink powder (2.7 g, 90%). Degree of functionalisation was found to be >95% by ^{1}H NMR spectroscopy and the ratio of norbornene to tetrazole end-groups was 3:0.7.

Synthesis of RGDC-maleimide

Arg-Gly-Asp-Cys peptide (RGDC) (30 mg, 0.067 mmol) was dissolved in DMSO (1 mL). 1,1'-((ethane-1,2-diylbis(oxy)) bis(ethane-2,1-diyl))bis(1H-pyrrole-2,5-dione) (20.6 mg, 0.067 mmol) and a catalytic amount of triethylamine were added to the solution. The solution was stirred for 24hrs. The solution was diluted with water (5 mL) and lyophilized to remove solvent and triethylamine. The reaction progression was monitored by ^{1}H NMR spectroscopy and stopped at 80% conversion to ensure the unreacted maleimide didn't undergo a Michael addition with the primary amines present on the peptide.

Cytotoxicity testing

Normal Human Dermal Fibroblast (NHDF) cell line was cultured in tissue culture flasks with RPMI 1640 medium (supplemented with 10 % foetal bovine serum, MEM, Sodium pyruvate and plasmocin) at 37 ℃ (5% CO2 atmosphere). After reaching 90% confluency, the cells were collected from the flasks using Trypsin/ EDTA. The cell suspension was then seeded into a 96-well cell culture plate at a cell density of 800,000 cells/mL with 100 µL/well^{-1}. After 24 h incubation, the old medium in the cell culture plate was discarded and replaced with 100 µL of RPMI1640 medium containing different concentrations of PEG-3NB-Tet and the cells were incubated for a further 48hrs. The culture medium

was discarded and 100 µL of 10% TCA was added and incubated for 30 min at 4 °C. The supernatant was discarded and the plates were washed 5 times with water and air-dried. 100 µL of SRB solution 0.4% (w/v) in 1% acetic acid was added to each well and the plates incubated for 15 min at room temperature. After staining, the unbound dye was removed by washing 5 times with 1% acetic acid and air-dried. Bound stains were dissolved with 200 µL 10 mM Tris Buffer and the absorbance was measured on a Bio-Rad BenchMark microplate reader (λ = 490 nm). The data was analyzed and plotted using GraphPad Prism 7.0.

Hydrogel fabrication

A solution of 10 wt% of PEG macromer, equimolar amount dithiothreitol (to NB units) and Riboflavin (0.3mM) was prepared and a droplet was placed on a methacrylate silanised glass slide. A thin square of PDMS was gently placed on top and the sample irradiated under blue light **(**OMNILUX PAR-20 240V E27 36 LED 5mm BLUE, δ = 455 nm, 27mW/cm^2**)** for 10min. The PDMS was removed and the gels immersed in water to remove riboflavin and to swell.

Swelling studies: For the swelling study, 300µL of the precursor solution was added into a circular mold (ID = 1.8cm).

Photopatterning (Lithography)

The hydrogel was immersed in a solution of 2-hydroxyethyl acrylate (5% vol in water) or RhBEA or RGDC-maleimide (1% vol) for 2 hrs. The hydrogel patted with a kimwipe and placed on a black surface. A photomask was placed on top of the hydrogel and was irradiated directly from above with an Arimed B6 lamp for 1-2 hrs. The gel was immersed in water (2L) for 48 h to remove unreacted acrylate with water changes twice daily. The photopatterns were imaged using optical and fluorescence microscopy methods.

Cell Adhesion

The photopatterned hydrogel (RGDC-maleimide) was sterilised in 70% ethanol solution for 30 min, then placed in sterile MilliQ or 30min to remove the ethanol, then placed in a well plate. Normal Human Dermal Fibroblasts (NHDF) (cultured in growth media, subcultured at 90% confluence and used between passages 7-9) were placed on the hydrogels (contained by a glass ring) at a density of 5x10^5 cell/mL in growth medium. After 3hrs, an additional 3mL of growth medium was added to the well and the incubated at 37^{o}C for 24 hrs. The glass ring was removed and the gels washed twice with PBS to remove non-adhered cells, then placed in 4% paraformaldehyde solution for 1hr. The gels were again washed with PBS and kept at 4^{o}C until imaged.

Growth Medium: RPMI 1640 supplemented with Fetal Bovine Serum (10%), Glutamax (1%), Sodium pyruvate (1%), MEM non-essential amino acids (1%) and plasmocin.

Substrate preparation (cleaning and silanisation)

All glass substrates were cleaned by activated during 10 min. in a plasma oven. Preactivated substrates were placed in vials containing a solution of 3-(trimethoxysilyl) propyl methacrylate dissolved in toluene (1 mM) for 60 min. at ambient temperature without stirring. The substrates were thoroughly washed in toluene, acetone and miliQ water to remove any physisorbed silane. The silanization process was performed to covalently bind the hydrogel to the substrate surface

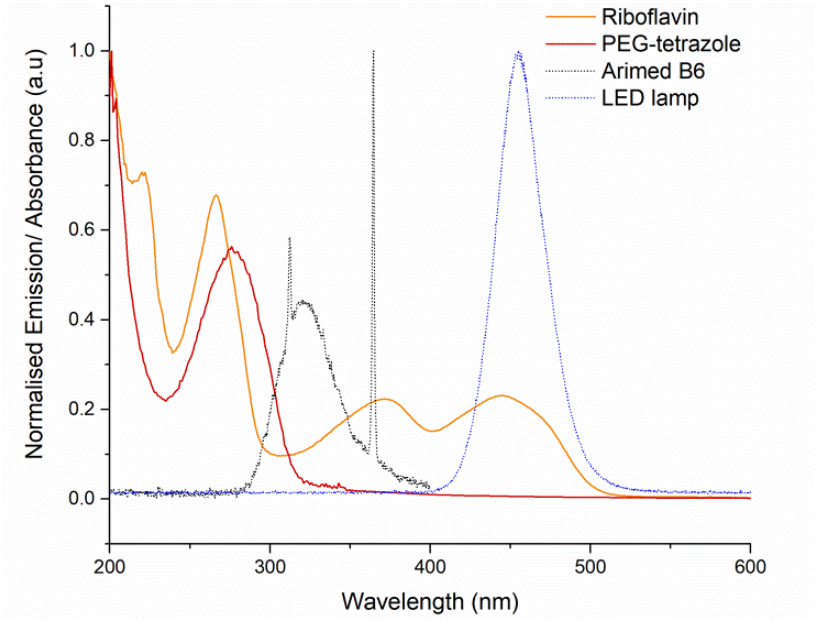

Figure S1. UV/Vis absorption spectrum of Riboflavin (0.18 mM in water) and Tetrazole capped poly (ethylene glycol) (0.2 mM in water) superimposed with the emission spectra of the Arimed B6 lamp and the Blue LED.

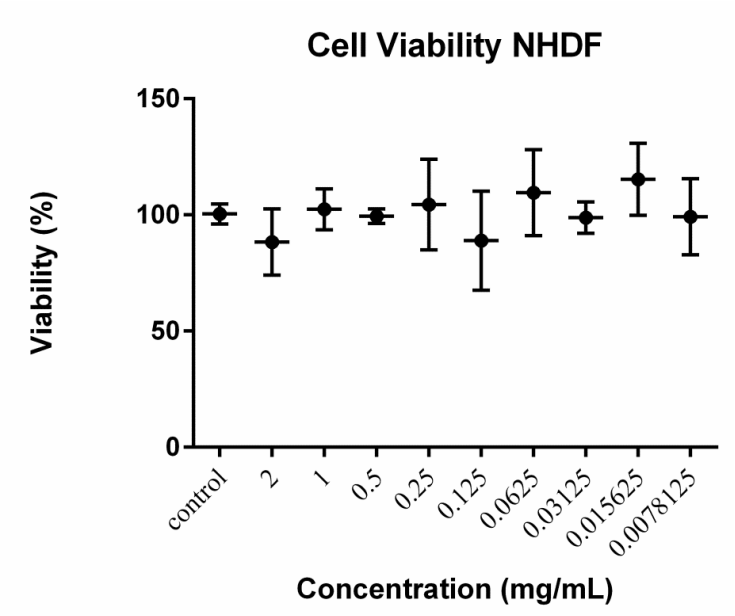

Figure S2. Cell viability of NHDF cells in the presence of PEG-3NB-Tet. Columns, mean (n=6), ± SEM.

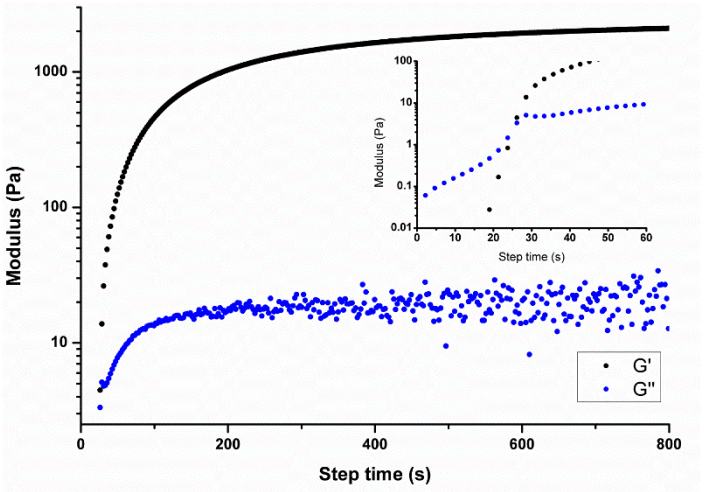

Figure S3. In-situ rheometry showing the crossover of the storage modulus (G') and the Loss modulus (G'') for the gelation of 10 wt% PEG-3NB-Tetrazole with DTT.

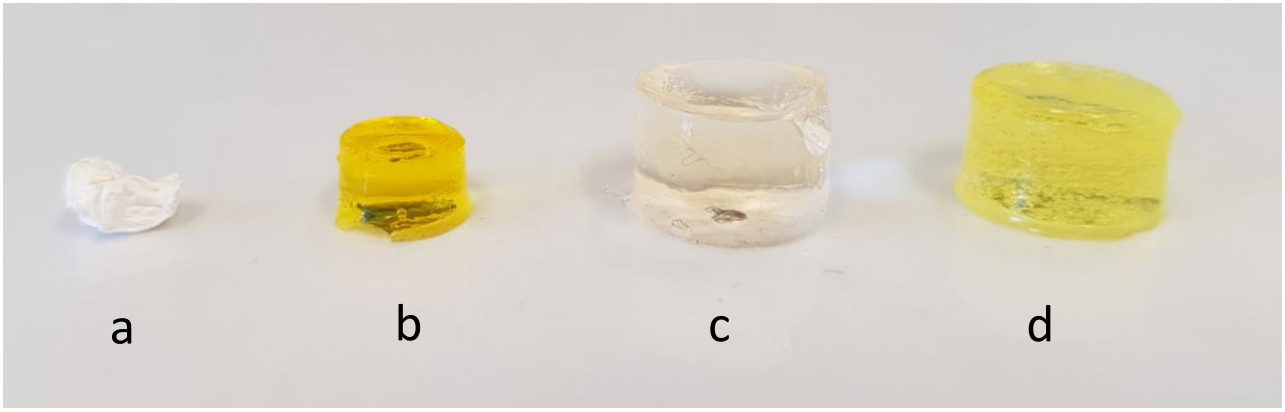

Figure S4. Hydrogel images. (a) Dried hydrogel (before NITEC reaction), (b) Hydrogel directly after gelation, (c) swollen hydrogel, riboflavin removed, before NITEC reaction, (d) swollen hydrogel after the NITEC reaction.

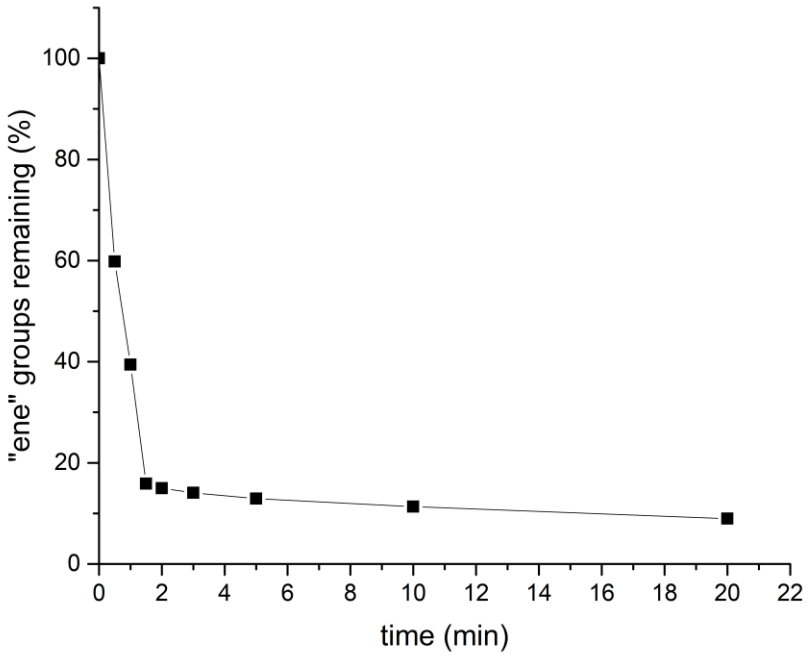

Figure S5. HRMAS data showing the amount of norbornene functional groups present at different time intervals. The remaining percentage was determined by comparison of the integrals of norbornene alkene resonances at 5.8-6.3 ppm and the ω- CH_2 resonances on the PEG chain at 4.2 ppm.

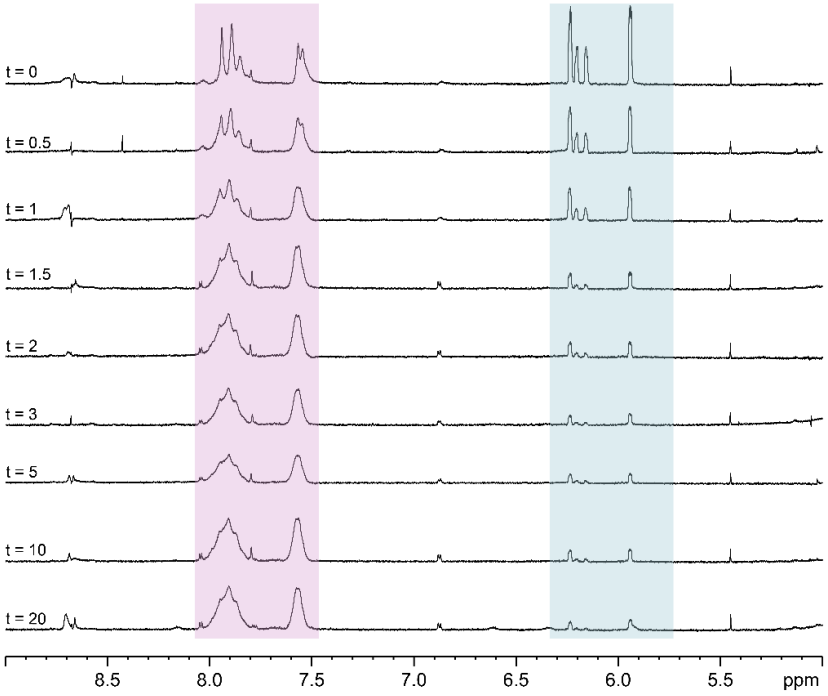

Figure S6. ^{1}H NMR spectra showing the disappearance of the norbornene alkene groups at 5.8-6.3ppm over time (min). The pink region displays the aromatic tetrazole resonances and the blue region displays the resonances associated with the alkene group of the norbornene.

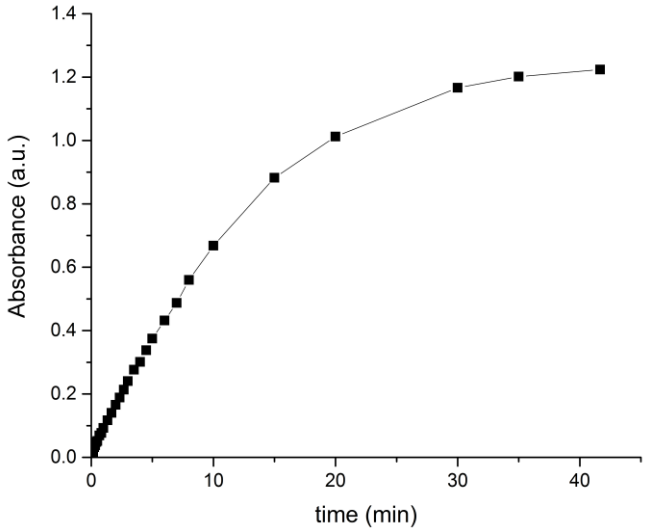

Figure S7. Kinetic plot displaying the absorbance of the pyrazoline at 445 nm vs reaction time.

The crosslinked gel made from 10wt% PEG macromer with equimolar amount of DTT (to norbornene units) and 0.03mM of riboflavin was dialysed against water then swollen with 2-hydroxy ethyl acrylate solution (0.1M in water) for 2 hours. After, the gel was placed into a quartz cuvette and irradiated with UV light (326 nm for specified time intervals). The production of the pyrazoline adduct results in an absorbance from 350-450 nm. The reaction was monitored by the absorbance at 455 nm.

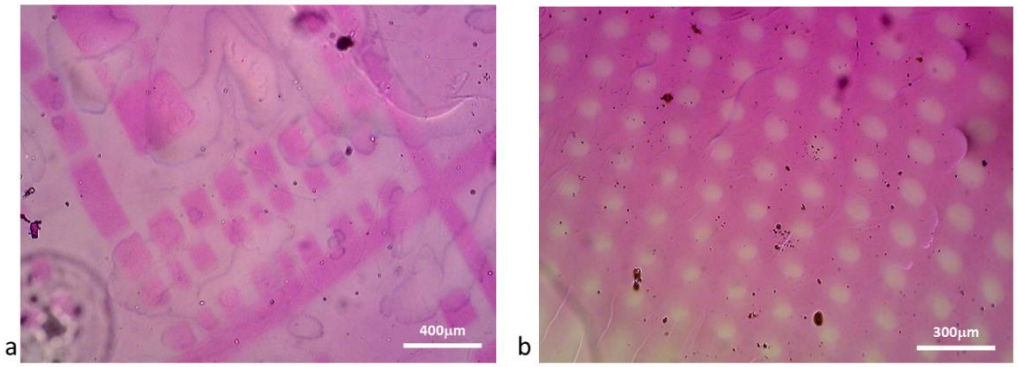

Figure S8. Photopatterned hydrogels using Rhodamine B ethyl acrylate. The pink areas indicate the irradiated sections.

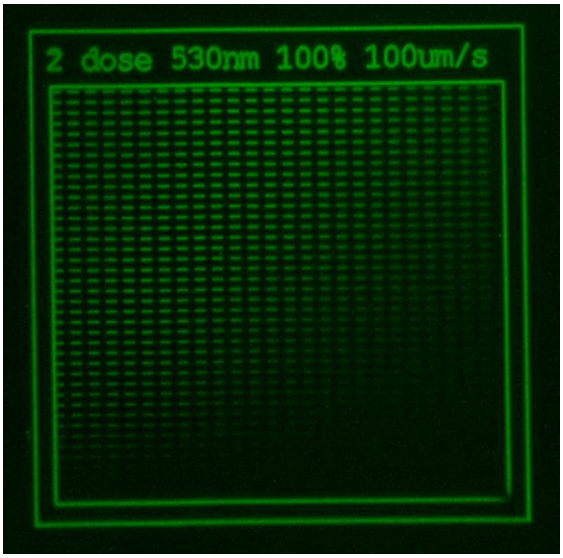

Figure S9. Example of dose test consisting on lines written at 530 nm at 100 μm s^{-1} with powers varying from 0 to 5 mW (down to top) and height from 0 to 2 μm (left to right)

[1] K. Hiltebrandt, T. Pauloehrl, J. P. Blinco, K. Linkert, H. G. Börner, C. Barner-Kowollik, *Angew. Chemie Int. Ed.* **2015**, *54*, 2838–2843.