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SUPPLEMENTARY INFORMATION

Enzyme-Assisted Photolithography for Spatial Functionalization of Hydrogel

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Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise specified, and were used as received. LIVE/DEAD® Cytotoxicity Kit, CellTracker™ Green CMFDA and CellTracker™ Orange CMTMR were purchased from Invitrogen. ImmunoPure® Streptavidin (Rhodamine tagged), Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) and EZ-Link Sulfo-NHS-LC-Biotin were purchased from Pierce. Peptide Ac-GCGYG**RGD**SPG-NH_2 was purchased from AnaSpec. Biotinylated mouse anti-human anti-CD19 and anti-CD5 were purchased from Invitrogen. The deionized water was prepared by a Millipore NanoPure purification system (resistivity higher than 18.2 $M\Omega \cdot cm^{-1}$).

Instruments. Absorbance for the Bradford assay was measured on a Thermo Scientific GENESYS 20. The UV treatment was carried out by a UV light source from Spot Light Curing, Omnicure Series 1000 (100 W, 365 nm). LC-MS was conducted with a Shimadzu 2010 EV Liquid Chromatography Mass Spectrometer using positive electrospray ionization and a Phenomenex Luna 5u 2.0 X100 mm C18 reverse-phase column. Samples were separated over a linear gradient of 5% to 95% CH_3CN (vol/vol) for 30 min and 95% CH_3CN (vol/vol) for an additional 30 min in H_2O supplemented with 0.1 % (vol/vol) trifluoroacetic acid at a flow rate of 0.1 mL/min at room temperature. Fluorescence images of cells were obtained with a Zeiss Axio Observer Z1 Inverted Microscope, equipped with a Zeiss Axiocam MRm CCD camera. Hydrogel thickness and surface profile were measured by Dektak 6 Surface Profile Measuring System. AFM images were recorded by VEECO Dimension 3100 by the tapping mode. The storage modulus and loss modulus of hydrogel were tested using and AR2000 rheometer (TA Instruments, New Castle, DE) under constant strain of 0.05 and frequency from 0.1 to 10 Hz. The photomask was designed by L-Edit and fabricated by Heidelberg DW66 laser writer with a two micron critical dimension.

Protein Expression and Purification. The wild-type CP3 expression plasmid pHC332 was a generous gift from Dr. A. Clay Clark (North Carolina State University). *Escherichia coli* BL21(DE3) *LysS* cells were transformed with plasmid pHC332.. Transformed cells were inoculated overnight at 37 ºC with shaking in Luria-Bertani medium containing 100 μg/mL ampicillin. Overnight cultures were diluted 1:200 and grown in Fernbach flasks containing 1 liter of LB medium with 100 μg/mL ampicillin at 37 ^{o}C with shaking at 270

rpm. When the cultures reached an absorbance *A*600 ~1.2, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce protein expression, and the cells were incubated at 16 ^{o}C overnight. The cells were harvested by centrifugation (2,000g, 4 ^{o}C, 15 min), resuspended in 30 mL Buffer A (50 mM Tris–HCl, pH 8.0, 2 mM DTT, 2 mM EDTA), and lysed by sonication. Cell debris and insoluble proteins were removed by centrifugation (20,000g, 4^{o}C, 30 min). After centrifugation, the cleared cell lysate was incubated with 0.5 mL of Ni-NTA resin (Qiagen) for 3 hours at 4 ºC. The protein was then purified using a step gradient of Buffer A with increasing concentrations of imidazole (10, 20, and 250 mM). CP3 was eluted with 5 mL Buffer A containing 250 mM imidazole. The protein concentration was qualitatively assessed by SDS-PAGE and quantitatively determined by the Bradford protein assay using bovine serum albumin (BSA) as the standard.

Synthesis of Photolabile Peptide Crosslinker. Photolabile modified aspartic acid (Fmoc-Asp(ODMNB)-OH) was synthesized by modifying a previously reported procedure[1]. Briefly, 2.06 g (5.0 mmol) of N-a-Fmoc-L-aspartic acid-a-t-butyl ester was dissolved in in 20 mL of dichloromethane (DCM). Then, 1.08 g of 4,5-dimethoxy-2-nitrobenzyl alcohol (DMNBA, 5.1 mmol) dissolved in 40 mL of DCM and 30 mg of 4-dimethylaminopyridine (DMAP, 0.21 mmol) were added. 1,3-dicyclohexylcarbodiimide (DCC, 1.03 g, 5.0 mmol) dissolved in 25 mL of DCM was then added dropwise over 2 minutes. The reaction mixture was stirred at room temperature for 3 h. After filtration, the crude product was washed three times by aqueous 2.5% $NaHCO_3$. The organic phase was separated and evaporated yielding a yellow oil, which was dissolved in 100 mL TFA:water (19:1) and stirred for 90 min to remove the α-carboxyl protecting group (tBu). The solvent was removed by vacuum and the crude product was dissolved in 50 mL DCM. Following an aqueous wash, the organic layer was isolated, evaporated and dried to give 2.22 g (~90% yield) of pure Fmoc-Asp(ODMNB)-OH, as confirmed by LC-MS. Peptide VDEV$\mathbf{D_m}$TK including photolabile aspartic acid $\mathbf{D_m}$ was assembled using standard solid-phase peptide synthesis procedures for Fmoc-chemistry on a custom-designed semiautomatic multireactor synthesizer. Wang Rink Amide MBHA resin was used as the solid support, and each coupling step was monitored using a ninhydrin test. Coupling of amino acids to the resin backbone was accomplished by a 0.9 equivalent of HBTU activation and 2 equivalents of DIEA. The Fmoc group was removed by 20% piperidine in DMF. Cleavage from the resin was achieved with a mixture of TFA/phenol/water/TIPS (88/5/5/2) for 2 h. The crude peptides were precipitated and washed with diethyl

ether before being lyophilized. The crude compound was further purified by HPLC equipped with a reverse-phase 10u 150 X 21.2 mm C18 column (Jupiter), using a linear gradient of 5% to 95% CH_3CN in H_2O (vol/vol) over 30 min and 95% CH_3CN (vol/vol) for an additional 20 min supplemented with 0.1% (vol/vol) trifluoroacetic acid at a flow rate of 5 mL/min. After lyophilization using a freeze-dryer (Labconco, Freezone 6 Plus), pale yellow powder **VDEVD$_m$**TK was obtained (LC-MS calc'd for $C_{48}H_{70}N_{10}O_{20}^+$ $[M+H]^+$ 999.5, found $[M+H]^+$ 999 and $[M+2H]^{2+}$ 500). The peptide crosslinker with bifunctional acrylic groups was obtained by reaction between the amine groups of valine and lysine residues in the peptide sequence and *N*-acryloxysuccinimide, as shown in Figure S1a. Specifically, 20 mg of peptide were dissolved in 3.0 mL of 50 mM aqueous sodium bicarbonate solution (pH = 8.0), and 10 mg *N*-acryloxysuccinimide dissolved in 50 μL dimethyl sulfoxide (DMSO) was slowly added to the solution with stirring. The reaction was kept at room temperature for 2 h. Subsequently, the resulting solution was dialyzed against deionized water for 24 h with periodic bath changes to remove unreacted compounds. The crude compound was further purified by HPLC. The final dialysis product was lyophilized overnight to obtain pale yellow solid (LC-MS calc'd for $C_{48}H_{70}N_{10}O_{20}^+$ $[M+H]^+$ 1107.5, found $[M+H]^+$ 1107 and $[M+2H]^{2+}$ 554). The evolutional changes of absorption integral area of 50 μg (1 mg/mL) **VDEVD$_m$**TK at 348 nm and the mass signal integral area weight of VDEVDTK upon irradiation with UV light (λ=365 nm, 100 W) was presented in Figure S2a. A detailed LC-MS analysis of the dual-cleavage process by UV exposure and caspase-3 digestion of V**DEVD$_m$**TK was shown in Figure S2b.

Preparation of PEG Hydrogel Film. In order to improve the adhesion between hydrogel and glass substrate during detachment, the glass cover slides (Fisher) were first modified by methacryloxypropyltrichlorosilane. The glass slides were first treated with piranha solution (H_2SO_4:H_2O_2 = 3:1; *Be careful!*) to produce hydroxyl termination and then soaking inside 0.2 wt % methacryloxypropyltrichlorosilane in toluene for 1 h. After the slides were removed from the solution, they were cleaned thoroughly with fresh toluene in an ultrasonic bath and dried with nitrogen gas. The PEG hydrogels were prepared by varying the amount of peptide crosslinker in the feed (defined by feed molar ratio percent to monomer) and remaining the total monomer amount of poly(ethylene glycol) methyl ether methacrylate (M_n:~475, Sigma) as 20% w/v. The aqueous mixture of peptide crosslinker and monomer was bubbled by dry nitrogen gas in a glass vial for 20 min to remove dissolved oxygen. ~ 10 μL mixture solution was gently dropped onto a piranha washed silicon wafer via a pipette. The polymerization was

initiated by adding 2 μL 10 wt % (based on total volume of the mixture solution) of ammonium persulfate (AP) in deoxygenated and 0.2 μL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). Immediately, the acrylated silane-treated glass (~ 1 cm^2) was gently covered onto the reaction solution. The polymerization was allowed to proceed for 15 min at room temperature in a nitrogen atmosphere. Finally, the slide was carefully detached from the silicon wafer and stored in the nitrogen atmosphere covered by aluminum foil to avoid light.

Characterization of PEG Hydrogel. Results of water content studies were listed in Table S1. PEG hydrogel samples prepared in the 96-well plate with different molar ratio of peptide crosslinker were freeze-dried overnight. The dried hydrogel samples were weighed upon removal from the freeze-dryer and thoroughly immersed in excess 1x PBS (pH= 7.4), or cell-culture medium (DMEM or RPMI-1640, with 10% (v/v) BGS) for 24 h at 37 ^oC. The water content was calculated based on the weight difference of the hydrogel samples before and after swelling, according to the equation[2]: water content= $(W_s - W_d)/W_s \times 100$, where W_s is the weight of the swollen gel and W_d is the weight of the dry gel. For Hydrogel rheology studies (Figure S3), the gels were prepared in the 12-well plate and swollen in the 6-well plate. Cut to a size of ~1.8 cm in diameter, the sample was loaded onto the lower plate of the rheometer (2.0 cm in diameter), the upper fixture was lowered, and a humidity chamber was placed around the sample to prevent dehydration during data collection. The data of storage modulus (G') and loss modulus (G") were collected in a constant strain mode (5%) over the frequency range from 0.1 to 10 Hz.

UV Exposure. For the hydrogel film treatment, noninverted mask was loaded to the Karl Suss MA6 photolithography machine, equipped with the UV light source (100 W, 365 nm). The hard contact model was applied to make gel film attached to the desired mask patterning. For 120 seconds treatment, shine UV for 40 seconds for three times with an interval of 20 seconds for each time. For the UV treatment of the *CL*-**VDEVD$_m$**TK dissolved in 1x PBS solution, the UV light source was fixed above the samples with a distance of 10 mm.

Enzymatic Development. After UV treatment and a thorough wash with 1x PBS, chips were completely immersed into PBS buffer with 0.5 mg/mL of caspase-3 for certain development time at room temperature

with gentle shaking. After each development, the chips were thoroughly rinsed with PBS and stored in 1x PBS for desired time. Typical topographies of patterns used in this study were summarized in Figure S4 and S5.

Bioconjugation to PEG Hydrogel Surfaces. To modify patterned PEG hydrogel surfaces with Ac-GCGYG**RGD**SPG-NH_2, developed hydrogel chips were placed into 1 mM solution of Sulfo-SMCC in 1x PBS (pH=7.4) at room temperature with gentle shaking. After 1h incubation, chips were thoroughly rinsed with 1x PBS and placed into 0.25 mM solution of Ac-GCGYG**RGD**SPG-NH_2 in 1x PBS (pH=7.4) at room temperature with gentle shaking. After 90 min incubation, chips were thoroughly rinsed with PBS and stored in 1x PBS for overnight before cellular experiments. To immobilize antibodies to the developed hydrogel, chips were first placed into 1 mM solution of Sulfo-NHS-LC-Biotin in 50 mM sodium bicarbonate solution (pH=8.5) at room temperature with gentle shaking. After 1 h incubation, chips were thoroughly rinsed with 1x PBS and placed into 20 μg/mL of ImmunoPure® Streptavidin (Rhodamine tagged) in 1x PBS solution (pH=7.4) at room temperature. After 30 min incubation, chips were thoroughly rinsed with PBS and gently dried by nitrogen. Then 50 μL of biotinylated mouse anti-human anti-CD19 or anti-CD5 monoclonal antibodies (200 μg/mL) was placed onto the chip surface (~50 μL/ cm^2). After 20 min incubation at room temperature, chips were rinsed with PBS for three times and stored in 1x PBS solution.

Cell Culture and Fluorescence Staining. HeLa cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 1.5 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were cultured at 37 ^oC, in 98% humidity and 5% CO_2. Cells were regularly subcultured using Trypsin-EDTA. Human acute lymphoblastic leukemia T-cells (MOLT 3) and human Burkitt's lymphoma B-cells (Raji) obtained from America Type Culture Collection (ATCC, Manassas, VA) were cultured in RPMI-1640 medium (MP Biomedicals) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 1.5 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin. The cell concentration was determined using a hemocytometer by Trypan blue exclusion. For fluorescence imaging, cells were stained with CellTracker™ Green CMFDA or CellTracker™ Orange CMTMR following the manufacturer instructions. Briefly, cells were first harvested by centrifugation at 300 g for 5min and then the supernatant

was carefully aspirated. The cells were resuspended in phenol red-free RPMI-1640 culture media with 10 μM CellTracker™ supplemented. Cells were kept cultured in a 5% CO2/95% air humidified incubator at 37 ^{o}C for 20 min. Then the aforementioned cell preparation procedure was followed for the cell patterning experiments.

Formation of Cell Arrays. For HeLa cell adhesion array, 5 mm x 10 mm Ac-GCGYG**RGD**SPG-NH_2 modified hydrogel chips were placed into a six-well plate, 2 mL of the prepared cell suspension (3 x 10^5/mL) was added to cover the whole substrate and allowed to seed for 1 h at 37^{o}. Chips were then transferred into another well with fresh medium. After 16 h incubation, chips with adherent cells were gently washed by PBS and stained by LIVE/DEAD® Cytotoxicity Kit. For individual HeLa cell culture in 100 μm-diameter well, 5 mm x 5 mm Ac-GCGYG**RGD**SPG-NH_2 modified hydrogel chips were placed into a six-well plate, 2 mL of the prepared cell suspension (5 x 10^4/mL) was added to cover the whole substrate and allowed to seed for 1 h at 37^{o}. Chips were then transferred into another well with fresh medium. Microscope images were taken after the incubation for 6 h, 24, 48 h and 72 h. Cells were stained by LIVE/DEAD® Cytotoxicity Kit at the last day. For B-cell patterning shown in Figure 3C, 5 mm x 10 mm andibodies immobilized hydrogel chips were placed into a six-well plate, 2 mL of the prepared cell suspension (5 x 10^5/mL, stained with LIVE/DEAD® Cytotoxicity Kit) was added to cover the whole substrate and allowed to seed for 30 min at room temperature. Upon the completion of patterning, the chips were turned over and gently dipped into fresh 1x PBS to remove the unbound cells. Cell occupancy in the microwells was calculated according to the equation[3]: Cell Occupancy (%) = (# of occupied wells /# of total wells) x 100. Typically, the array region comprised of 400 wells was randomly selected and manually counted under microscope with 100 x magnifications. For all experiments, the number of samples was $n \geq 4$. For B-cell sorting via micro-fluidics system, a 1.5-mm-wide, 70-μm-tall glass-based micro-fluidics system[4] was aligned over the patterned area of the PEG substrate for trapping B cells via flowing a mixture solution of CellTracker™ Green CMFDA stained B-cells and CellTracker™ Orange CMTMR stained T-cells (1 x 10^5/mL for each). The mixture was then transported through the microchannel with a flow rate of ~ 100 μL/min. Upon the completion of sorting, the chips were detached from the microchannel and turned over for a gentle wash in 1x PBS solution. B cell composition after sorting is based on counts of cells in 2000 wells within a randomly selected region.

Cytotoxicity Study Using MTS Assay. Cells were seeded into 96-well plates at a density of 5,000 cells per well and cultivated in 100 μL of DMEM with 10 % BGS. After incubation for certain time, the cells were washed with PBS solution and incubated with 100 μL fresh DMEM and 20 μL MTS solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay). The plates were incubated for an additional 3 h. The absorbance of the plates was read at 550 nm and a reference wavelength of 690 nm using a microplate reader (PowerWave X, Bio-tek Instruments).

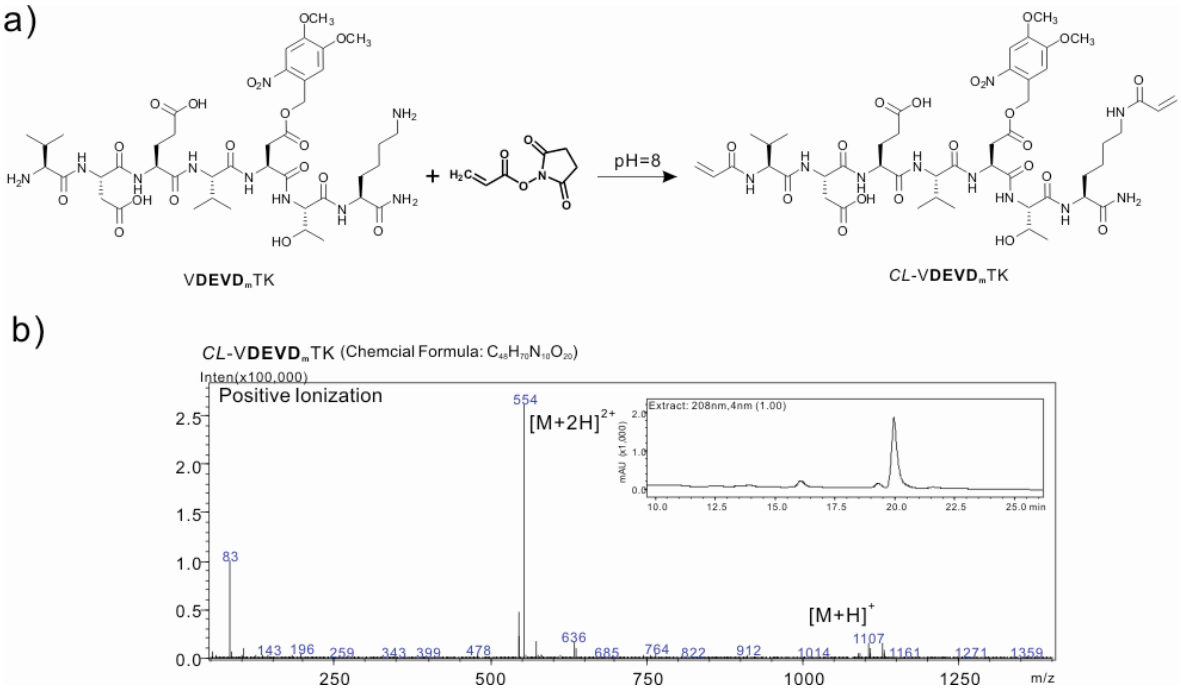

Figure S1. Schematic of synthesis of *CL*- V**DEVD$_m$**TK (a) and LC-MS analysis of *CL*- V**DEVD$_m$**TK (b).

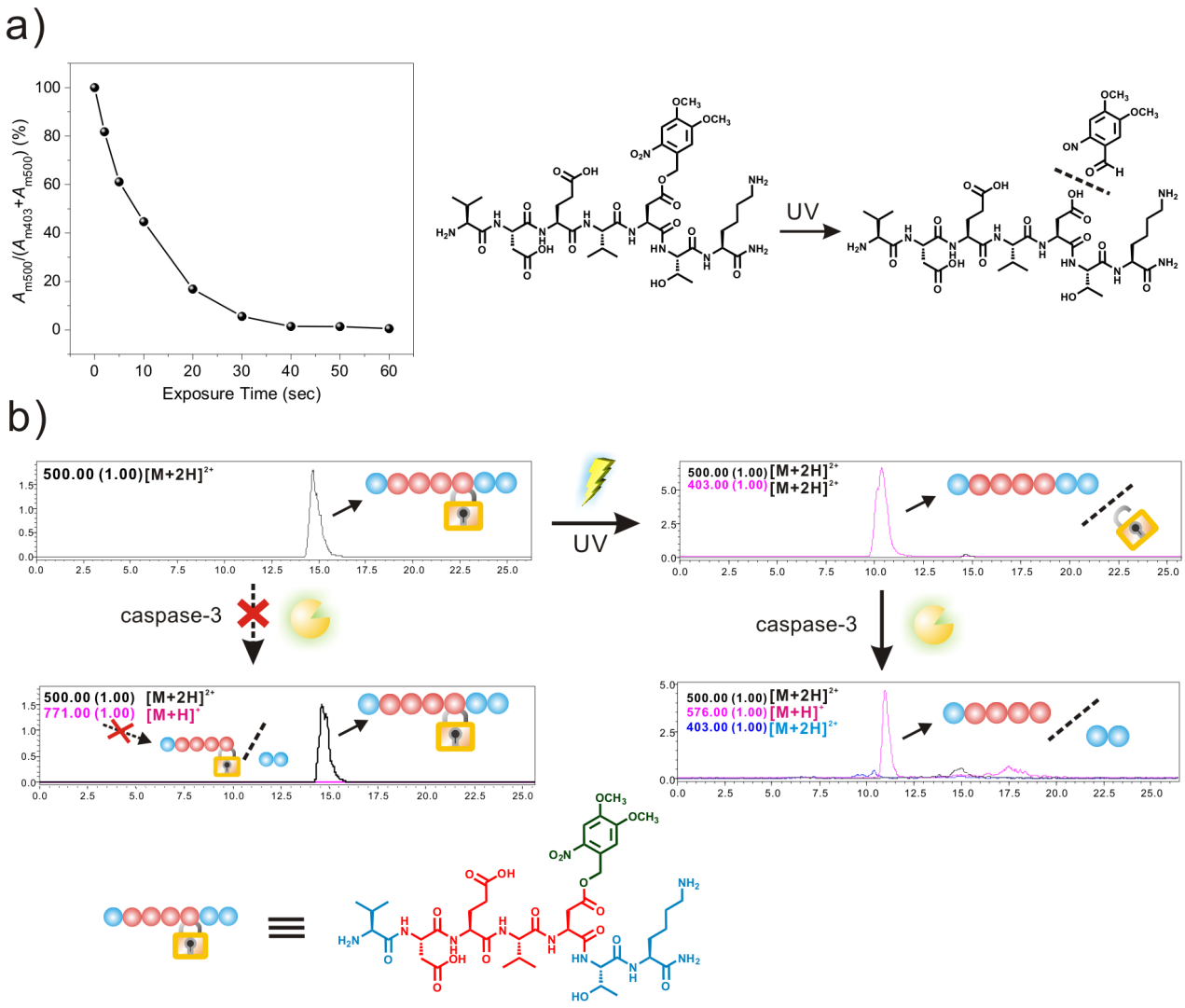

Figure S2. (a) The changes of mass signal integral area weight of 1 mg/mL V**DEVD$_m$**TK ([M+2H]$^{2+}$=500) upon irradiation with UV light (λ=365 nm, 100 W). (b) LC-MS analysis of the dual-cleavage process of V**DEVD$_m$**TK. 50 μg (1 mg/mL) V**DEVD$_m$**TK ([M+2H]$^{2+}$=500) in 1x PBS buffer was treated with UV for 40 s to produce V**DEVD**TK ([M+2H]$^{2+}$=403); V**DEVD**TK was further digested by 5 μg CP3 (1 mg/mL) for 60 min to produce V**DEVD** ([M+H]$^{+}$=576); while 50 μg (1 mg/mL) V**DEVD$_m$**TK in 1x PBS buffer was incubated with 5 μg CP3 (1 mg/mL) for 60 min, the assumed compound V**DEVD$_m$** ([M+H]$^{+}$=771) cannot be detected.

Table S1. Water Content of *CL*-V**DEVD**$_m$TK Crosslinked PEG hydrogels with Different Media

Hydrogel samples (crosslinker density)	**1x PBS**	**DMEM (10% BGS)**	**RPMI (10% BGS)**
0.48 mol %	96.3±0.25	96.0±0.21	95.6±0.52
1.08 mol %	95.8±0.12	95.4±0.11	95.4±0.24
1.80 mol %	94.8±0.64	95.0±0.09	94.8±0.15

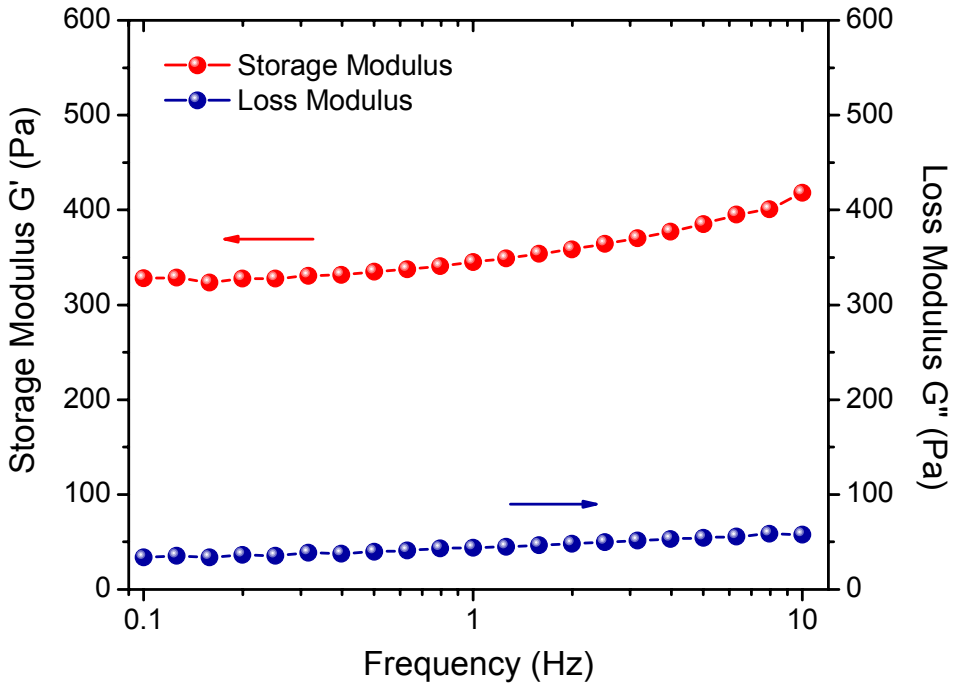

Figure S3. Storage (G') and loss modulus (G") of PEG hydrogel with a crosslinker density of 1.80 mol %, measured using the plate-to-plate rheometry at 37^{o}C.

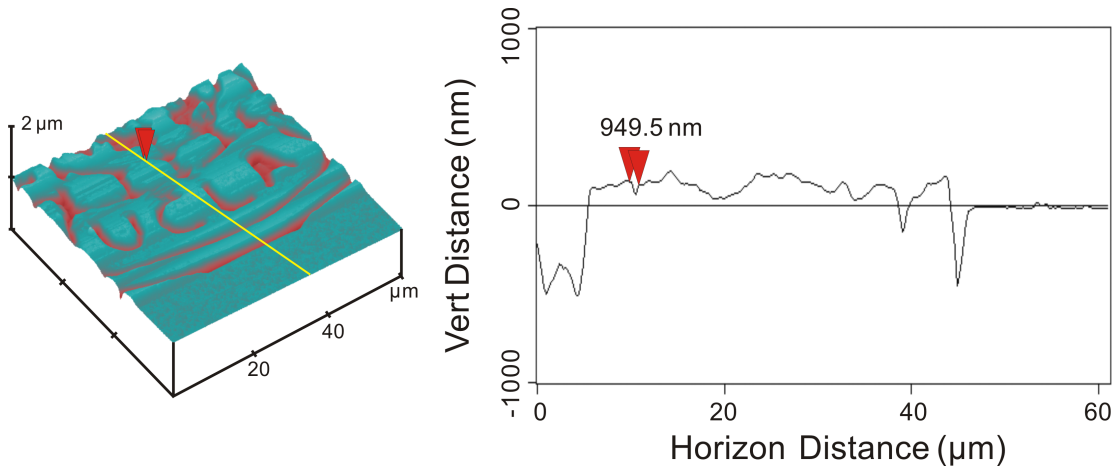

Figure S4 AFM section analysis of patterned PEG hydrogel surface with a sub-micron feature size. The crosslinker density used for this pattern was 2.64 mol %.

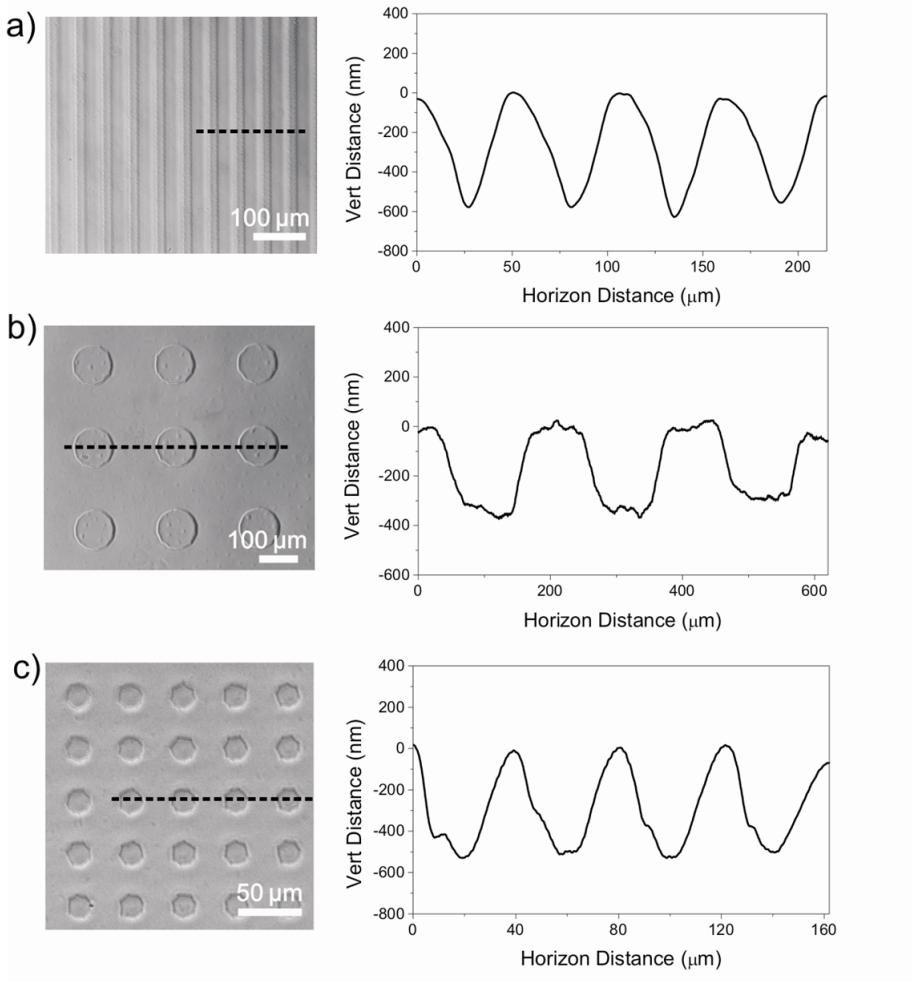

Figure S5. PEG hydrogel patterns by EAPL used in this study. a) 20 µm-width line array for HeLa cell adhesion, as shown in Figure 2b; b) 100 µm-diameter micro-well array for HeLa cell culture, as shown in Figure 3c; c) 20 µm-diameter micro-well array for B-cell patterning and sorting, as shown in Figure 4c,d. The crosslinker density used for these patterns was 1.80 mol %.

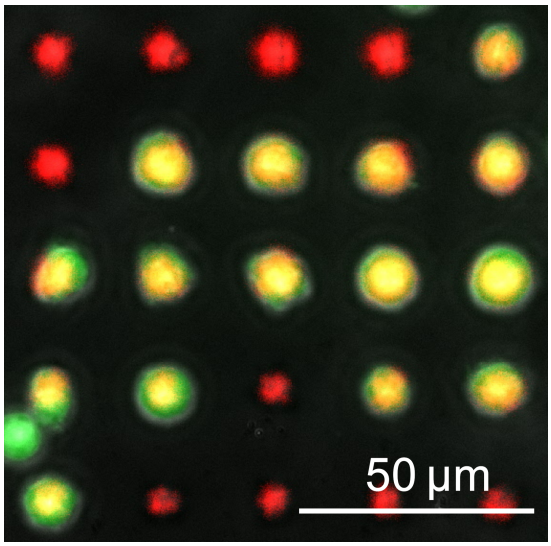

Figure S6. Merged fluorescence images of B-cells (green, stained by CellTracker® Green CMFDA) on anti-CD19 modified 10 µm-diameter micro-PEG-well using the same patterning conditions in the Figure 4c. Red patterning: rhodamine tagged streptavidin. Seeding cell density: 1×10^6 cells/mL.

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