# Supplementary Information for

## Bioactive Site-Specifically Modified Proteins for 4D Patterning of Gel Biomaterials

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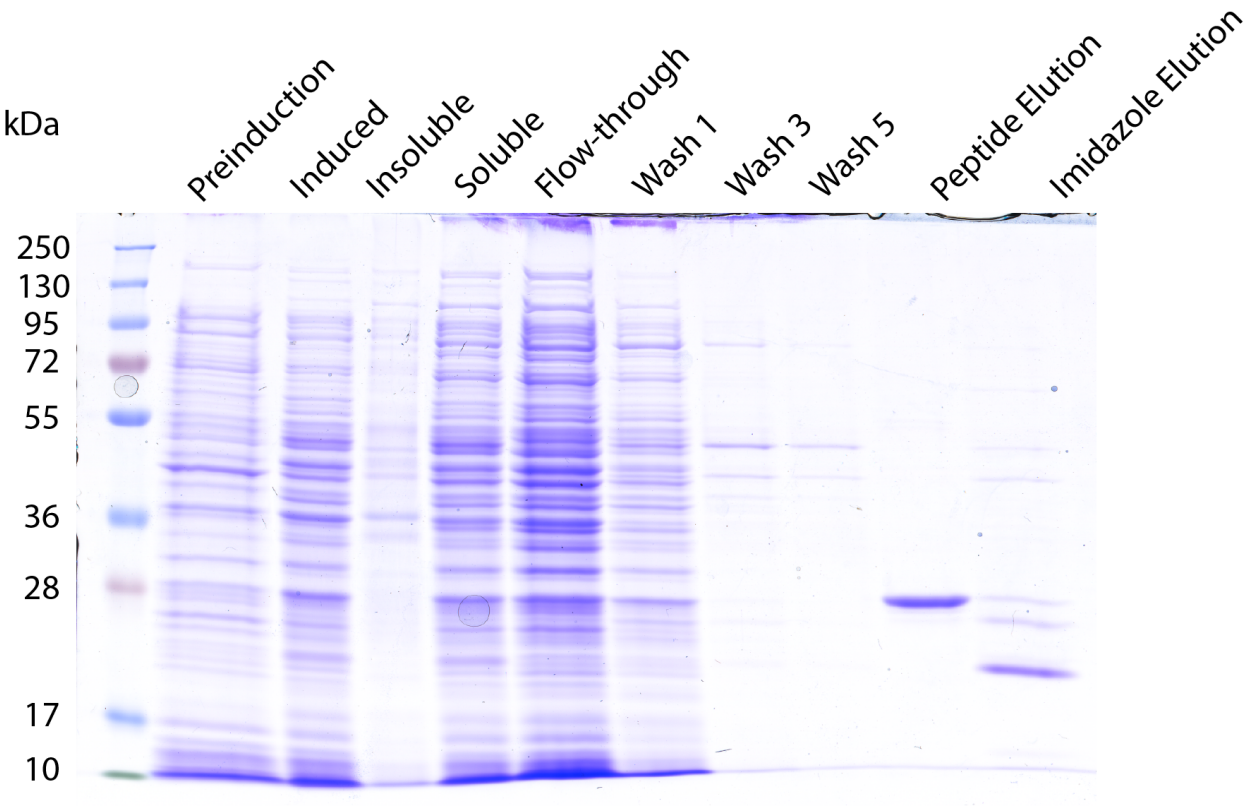
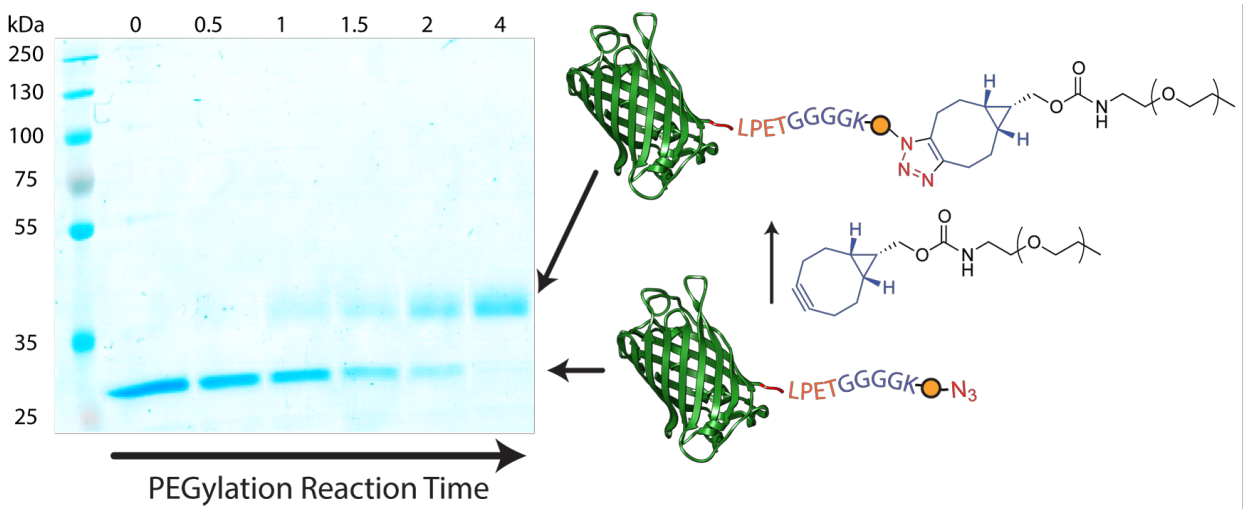
**Supplementary Figure 1:**



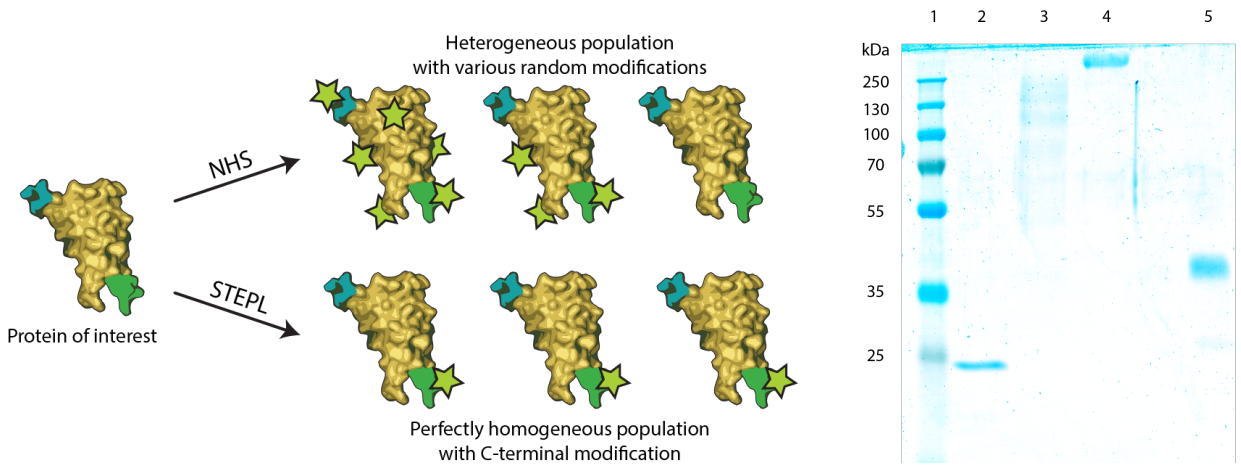

**STEPL purification of EGFP-$N_3$.** EGFP-$N_3$ is generated through STEPL involving EGFP and H-GGGGDDK($N_3$)-$NH_2$ peptide. The EGFP-SrtA-6xHis fusion appears as a ~50 kDa band post induction. After chromatographic immobilization on Ni-NTA, the resin is incubated with the polyglycine probe; the sortagged protein is collected in the eluent, while the SrtA-6xHis protein remains column-bound. Subsequent resin washes with imidazole displaces the SrtA-6xHis and regenerates the Ni-NTA. The peptide-eluted sortagged protein (EGFP-$N_3$) is an exceptionally pure (>95%) homogenous species appearing as a band ~29 kDa. The collected sortase enzyme appears as a band at ~17 kDa. Similar results were obtained for all protein-peptide conjugates purified throughout the reported studies.

**Supplementary Figure 2:**




**Assessing purity and reactivity of sortagged proteins by SDS-PAGE gel shift analysis.** To assess the ability for STEPL to generate homogenous protein samples with desired reactivity, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel shift assay[3] was performed C-terminal PEGylation by SPAAC. EGFP-*o*NB-$N_3$ (1 µg) was reacted with mPEG-BCN (100x, Supplementary Methods) in PBS (10 µL) for varying lengths of time (0-4 hr) at room temperature. The complete disappearance of the starting protein band after 4 hours of reaction, accompanied by the simultaneous appearance of a new band upshifted by the average molecular weight of a single PEG chain, is indicative of quantitative functionalization of purified proteins using STEPL. Similar results were obtained for $n = 3$ independent samples.

**Supplementary Figure 3:**

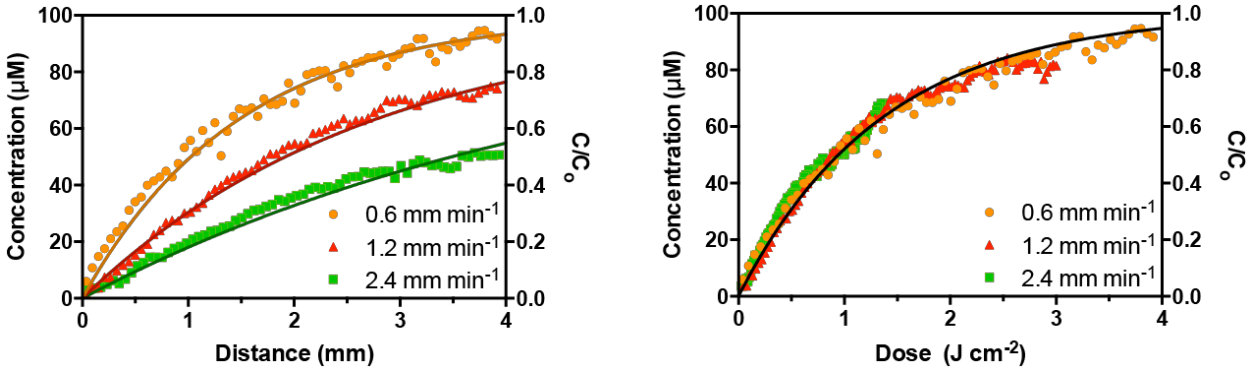



**Comparing purity and reactivity of sortagged proteins with those randomly modified by NHS chemistry.** To determine the extent of functionalization of EGFP randomly modified with azide moieties by NHS-chemistry, an SDS-PAGE gel shift assay was performed (Supplementary Figure 2**)**. Sortagged EGFP-$N_3$ as well as EGFP reacted with various amount of $N_3$-OSu (10, 100, 1000x, based on NHS:protein molar equivalents) were each treated with mPEG-BCN (100x) in PBS prior to analysis. PEGylation events resulting in protein band upshifting provide a lower bound for estimating the number of azides introduced.

Lane 1: Protein ladder of known molecular weights
Lane 2: EGFP + $N_3$-OSu (10x) + mPEG-BCN (100x)
Lane 3: EGFP + $N_3$-OSu (100x) + mPEG-BCN (100x)
Lane 4: EGFP + $N_3$-OSu (1000x) + mPEG-BCN (100x)
Lane 5: EGFP-$N_3$ + mPEG-BCN (100x)

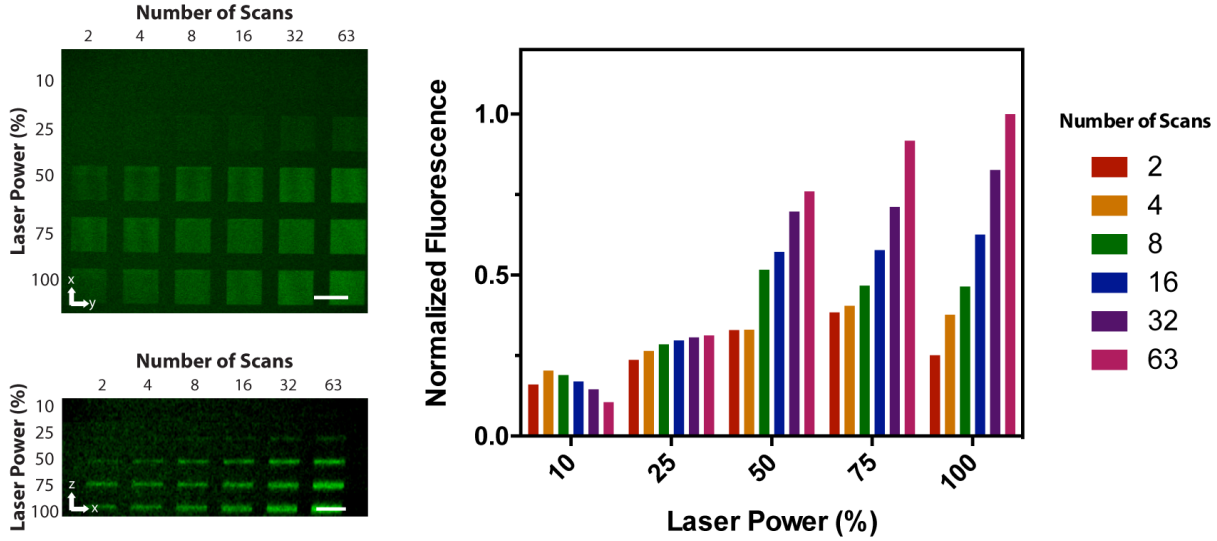
NHS treatment with $N_3$-OSu yielded a heterogeneous population with many species of varying azide content. 10-fold molar excess yielded no appreciable labeling; 100-fold excesses gave significant labeling but in an ill-defined manner; 1000-fold excesses resulted in uniform tagging (likely of every primary amine on the protein), whereby the extent of PEGylation prevented it from migrating far into the gel. By contrast, the sortagged variant showed only a single upshift indicating a perfectly homogenous population. Similar results were obtained for $n = 3$ independent samples.

**Supplementary Figure 4:**



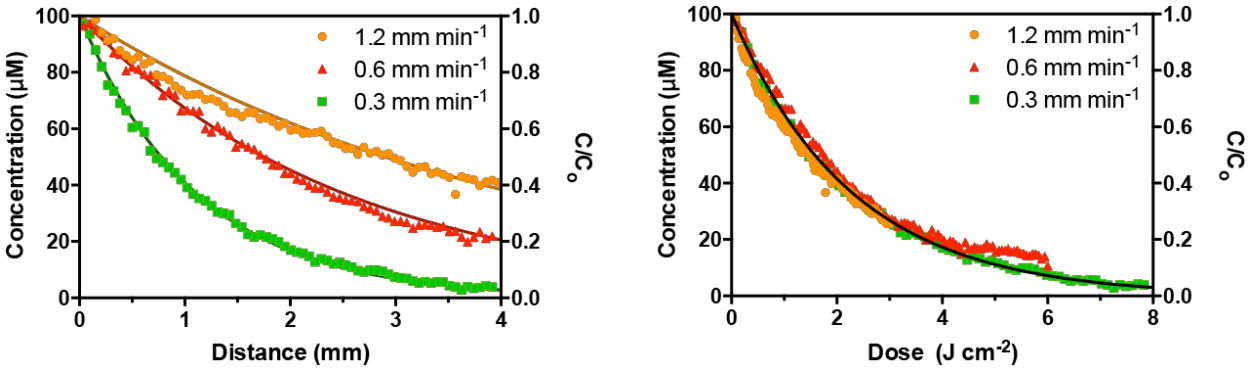

**Dose response for photo-mediated immobilization of proteins.** Dose represents the total amount of energy delivered to the system and was calculated as the product of exposure time and light intensity. Connected line represents predicted concentration based on NPPOC cleavage photokinetics[1]. Experiments were performed on a single gel for each gradient light condition.

**Supplementary Figure 5:**



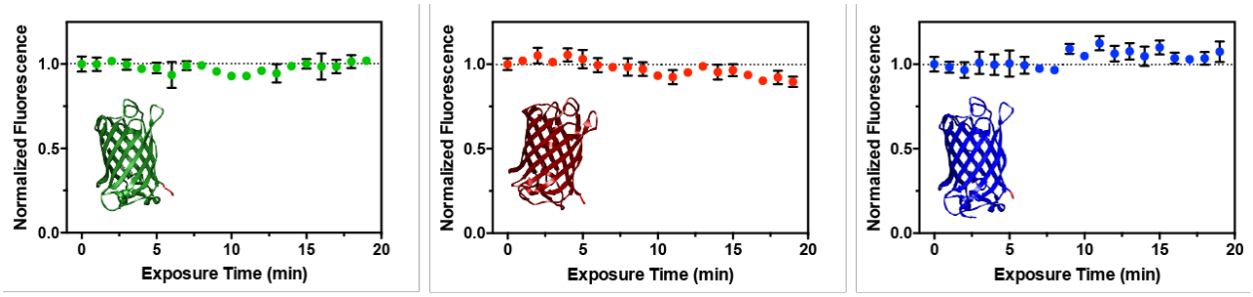

**Quantification of 3D protein patterning via photomediated oxime ligation.** Multiphoton laser-scanning lithographic techniques (λ = 740 nm) were exploited in the photomediated immobilization of EGFP-CHO within an $N_3$-TEG-ONH-NPPOC functionalized SPAAC-based gel over a variety of laser powers (10, 25, 50, 75, 100%) and scan repeats (2, 4, 8, 16, 32, 63). Here, 50 µm x 50 µm square region-of-interests (ROIs) were scanned at 2.5 µm z-increments for 10 µm before swelling in EGFP-CHO (100 µM) to create an array of protein-functionalized rectangular boxes, with each volume corresponding to a unique patterning condition. Samples were imaged using fluorescence microscopy (left), and quantification of immobilized protein concentration was performed for each light condition (right). Reported values correspond to the mean z-centered voxel fluorescence for each ROI across a single patterned sample. Optimal patterning conditions, maximizing total immobilized protein and z-resolution while minimizing patterning speed, were identified as 50% power with 16 scan repeats.

**Supplementary Figure 6:**



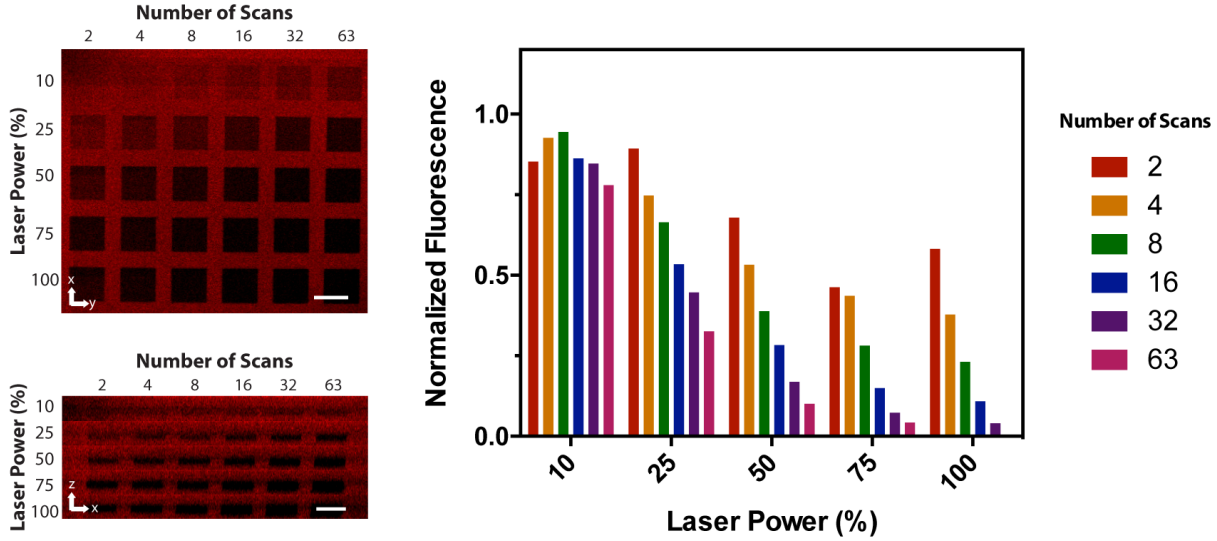

**Dose response for photorelease of immobilized proteins.** Dose represents the total amount of energy delivered to the system and was calculated as the product of exposure time and light intensity. Connected line represents predicted protein concentration based on the kinetics of *o*-nitrobenzyl ether (*o*NB) photocleavage[1]. Experiments were performed using a single gel for each gradient light condition.

**Supplementary Figure 7:**



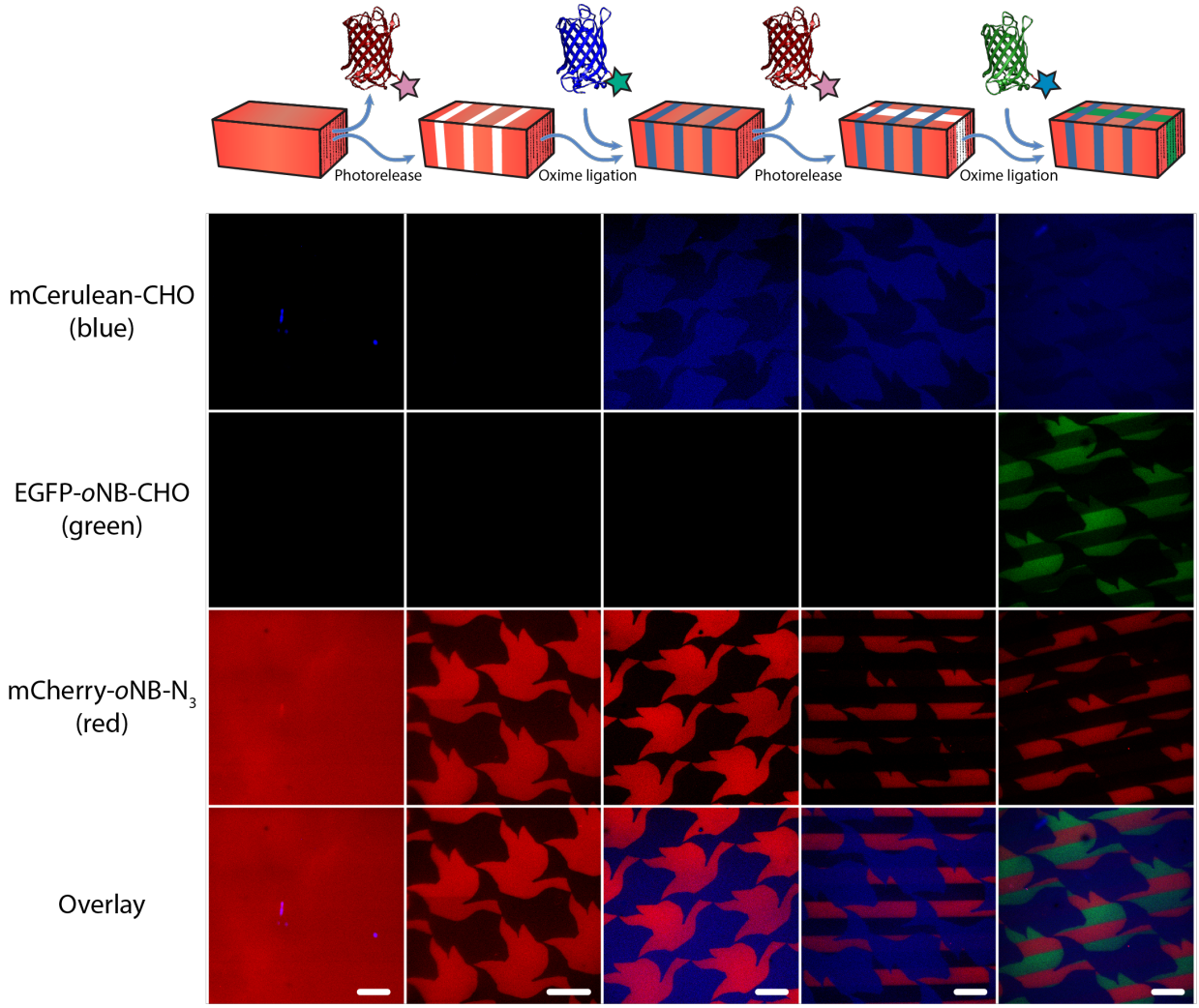

**Assessing photobleaching of fluorescent proteins in response to UV light**. To assess photobleaching kinetics of fluorescent proteins in response to patterning light conditions, samples of EGFP, mCherry, and mCerulean (each at 2 μM, in PBS) were exposed to far UV light (λ = 365 nm, 10 mW $cm^{-2}$) for varying amounts of time (0 – 20 min); fluorescence measurements were taken every minute and normalized to initial sample fluorescence. Analysis was performed in triplicate. Left plot corresponds to EGFP; middle to mCherry; right to mCerulean. Error bars correspond to the standard deviation about the mean for $n$ = 3 independent samples. EGFP and mCerulean remained fully stable throughout light exposure, while mCherry showed minor photobleaching (~10%).

**Supplementary Figure 8:**



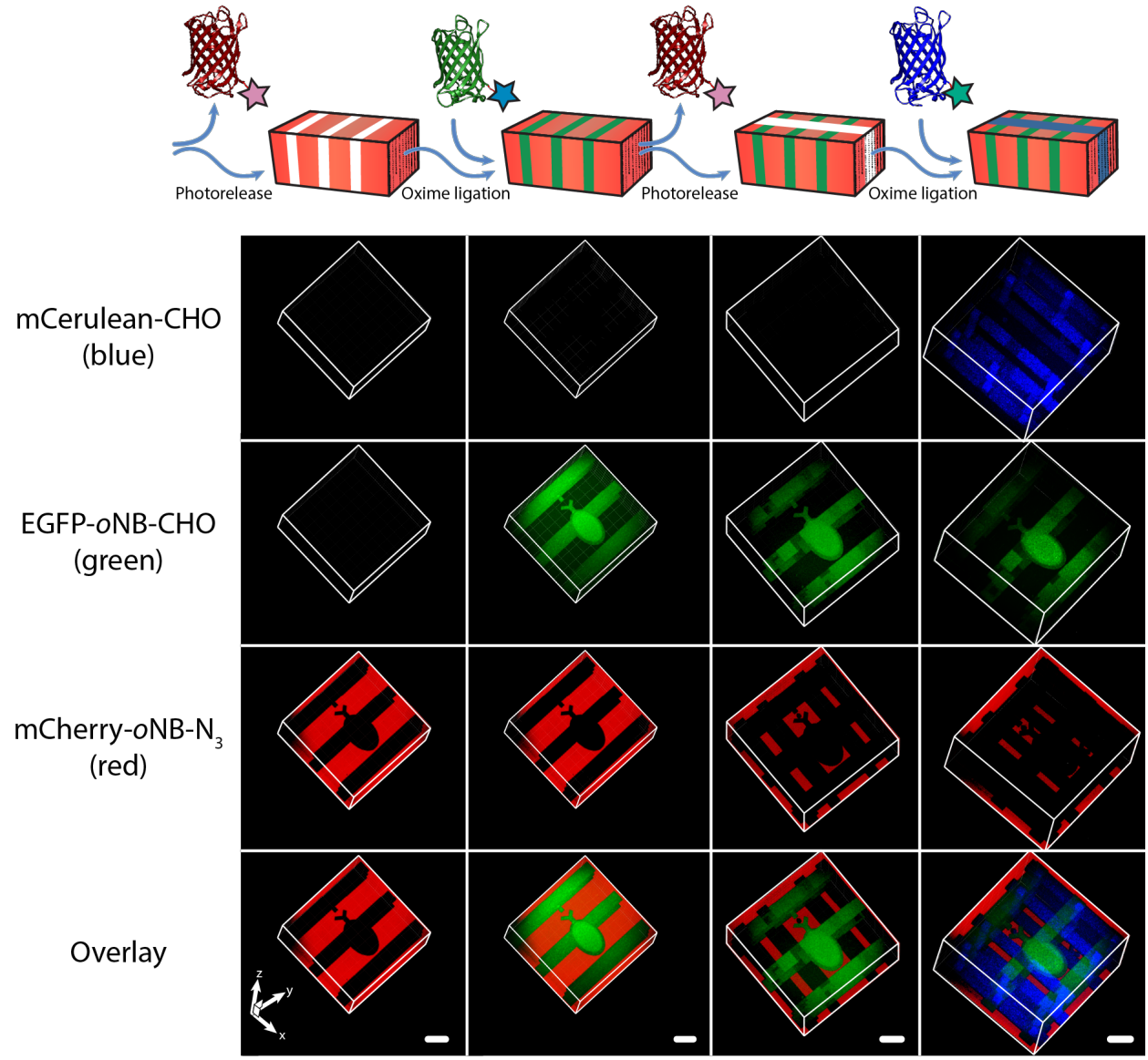

**Quantification of 3D protein photoremoval by multiphoton laser scanning lithography**. Multiphoton laser-scanning lithographic techniques (λ = 740 nm) were exploited to release immobilized mCherry-*o*NB-$N_3$ (initial concentration = 10 μM) from a SPAAC-based gel over a variety of laser powers (10, 25, 50, 75, 100%) and scan repeats (2, 4, 8, 16, 32, 63). Here, 50 μm x 50 μm square ROIs were scanned at 2.5 μm z-increments for 10 μm to create an array of protein-depleted rectangular boxes within gels, with each volume corresponding to a unique patterning condition. Samples were imaged using fluorescence confocal microscopy (left), and quantification of remaining immobilized protein concentration was performed for each light condition (right). Reported values correspond to the mean z-centered voxel fluorescence for each ROI across a single patterned sample. Optimal patterning conditions, maximizing total protein release and z-resolution while minimizing scan repeats, were identified as 50% power with 16 scans.

**Supplementary Figure 9:**



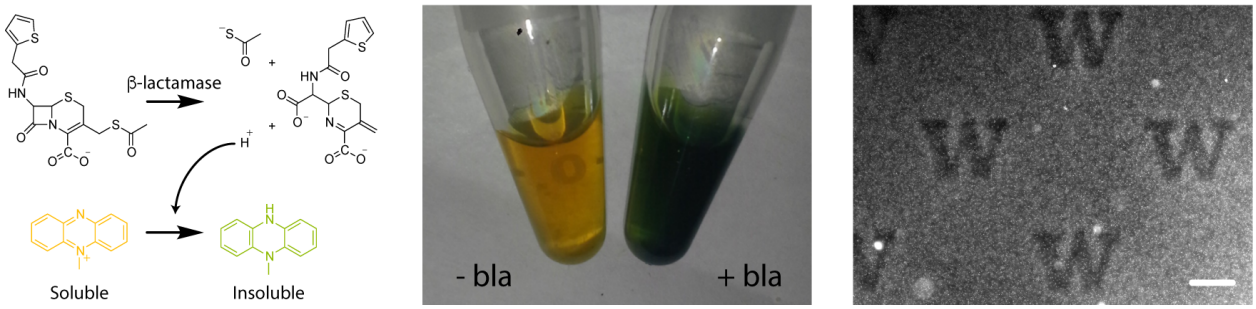

**Split-color images of multicolor patterning generated by mask-based lithography.** Images were taken throughout the photopatterning process as highlighted in Figure 4. For each stage of patterning, the red, green, and blue channels were separated to better show the localization of individual fluorescent proteins. Experiment was replicated ($n$ = 3) with similar results. Scale bars = 100 μm.

**Supplementary Figure 10:**



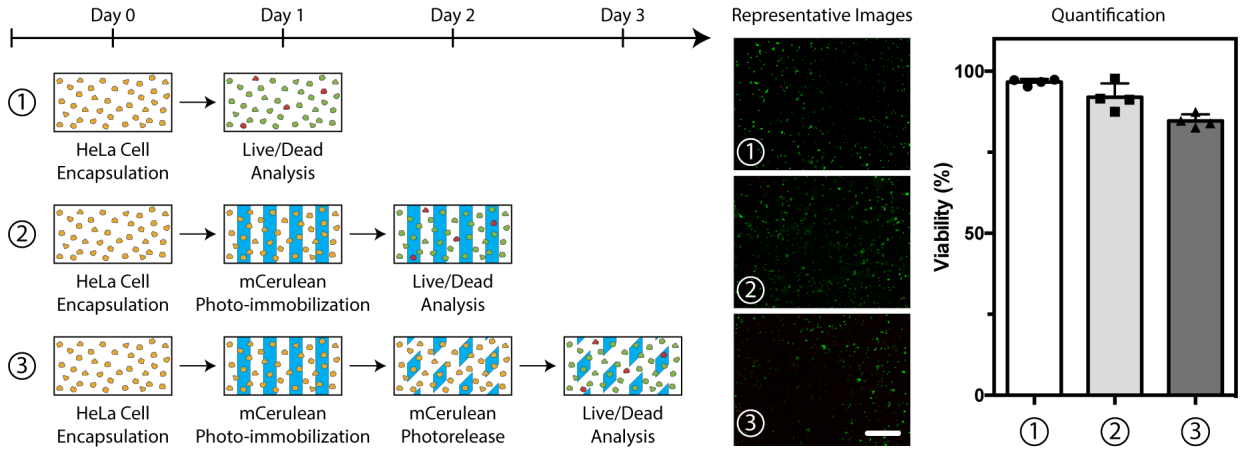

**Split-color images of multicolor patterning by multiphoton laser scanning lithography**. Images were taken throughout the photopatterning process as highlighted in Figure 4. For each stage of patterning, the red, green, and blue channels were separated to better show the localization of individual fluorescent proteins. Experiment was performed within a single gel. Scale bars = 100 μm.

**Supplementary Figure 11:**




**Gel-immobilized bla remains bioactive.** SPAAC-based gels uniformly functionalized with bla-*o*NB-$N_3$ (30 μM) were subjected to masked light (λ = 365 nm, 10 mW $cm^{-2}$, 10 min) through a chrome photomask containing transparent University of Washington Boundless 'W' logo features. Gels were equilibrated in PBS overnight prior to incubation with thioacetate cefalotin (5 mM, Supplementary Methods) and phenazine methosulfate (6.5 mM) in PBS (37 °C, 1 h). The gels were then washed with PBS (3x, 15 min) prior to visualization by phase contrast microscopy. Gels showed expected deposition of lightly colored precipitated product in regions where bla protein remained tethered. Experiment was replicated ($n$ = 6) with similar results. Scale bar = 250 μm.

**Supplementary Figure 12:**




**Cell viability throughout encapsulation and protein photopatterning.** To determine cell viability throughout encapsulation and protein photopatterning reactions, a series of three experiments were performed:

① HeLa cells were encapsulated (2 x $10^6$ cells $mL^{-1}$) in SPAAC-based gels (10 wt%, 5 µL) crosslinked with $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$ (Supplementary Methods, 8 mM) containing $N_3$-TEG-ONH-NPPOC (100 µM) attached to azide-functionalized glass slides (Supplementary Methods). Cell viability was assessed one day after encapsulation.

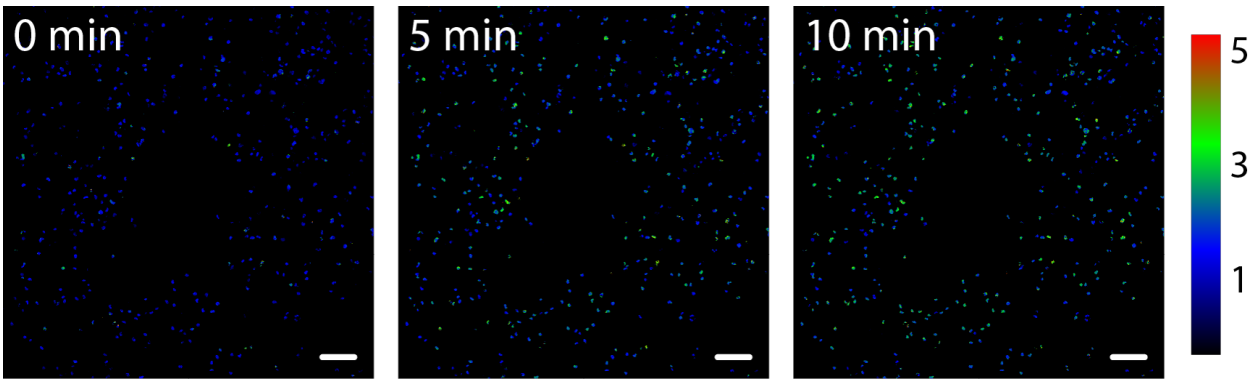
② HeLa cells were encapsulated in gels as in experiment ①. One day after encapsulation, gels were exposed to masked UV light (λ = 365 nm, 10 mW $cm^{-2}$, 10 min) through a slitted photomask containing 400 µm wide line features. Cell-laden gels were incubated in DMEM supplemented with FBS (10%), penicillin/streptomycin (1%), and mCerulean-CHO (0.3 µM). Following overnight conjugation, the gels were transferred to media lacking mCerulean-CHO. Cell viability was assessed one day after gel patterning was complete.

③ HeLa cells were encapsulated in gels as in experiment ①. One day after encapsulation, gels were exposed to masked UV light (λ = 365 nm, 10 mW $cm^{-2}$, 10 min) through a slitted photomask containing 400 µm wide line features. Cell-laden gels were incubated in DMEM supplemented with FBS (10%), penicillin/streptomycin (1%), and mCerulean-*o*NB-CHO (0.3 µM). Following overnight conjugation, the gels were transferred to media lacking mCerulean-*o*NB-CHO. The gels were again exposed to masked UV light (λ = 365 nm, 10 mW $cm^{-2}$, 10 min) through a slitted photomask containing 400 µm wide line features, this time rotated 45° from the initial protein pattern. Cells were maintained in mCerulean-free media for an additional day prior to assess viability.

Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Thermo Fisher) with Calcein AM (2 µM) and Ethidium homodimer-1 (2 µM) following manufacturer protocols. Standard fluorescent microscopy and image quantification were used to determine viability for each treatment. Overall cell viabilities were determined to be 96 ± 1% for experiment ①, 92 ± 4% for experiment ②, and 84 ± 2% for experiment ③ for four biological replicates ($n$ = 4). No significant difference using a two-tailed t-test assuming equal variance in viability was

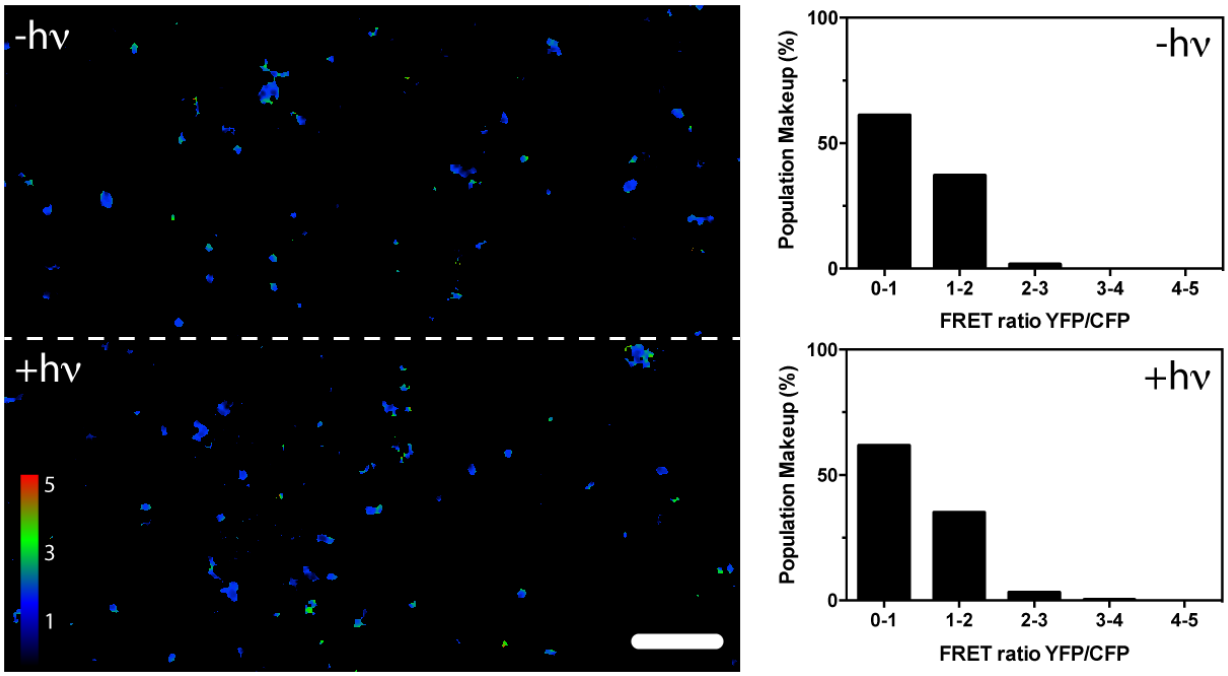
observed between UV-exposed and unexposed portions of the gel in all conditions. Bar plots correspond to the mean viability in each condition with error bars representing ±1 standard deviation. Scale bar = 250 μm.

**Supplementary Figure 13:**



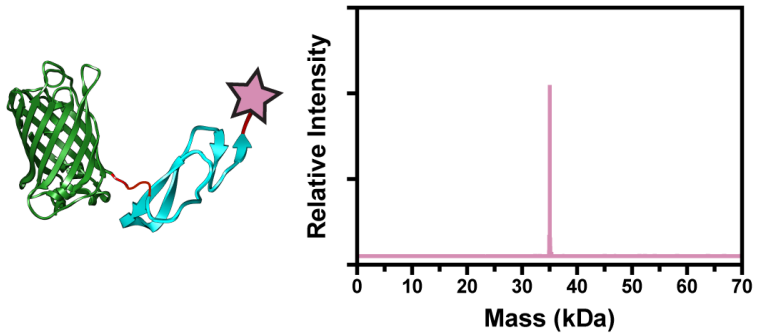

**EGF stimulation of MAPK-reporter cells.** HeLa cells with EKAREV linker treated with soluble EGF (100 ng in 600 µL total serum-free DMEM) and imaged using a Leica SP8X confocal microscope. EKAREV was excited using an argon laser (λ = 405 nm, 10% laser power); CFP excitation was monitored from $\lambda_{emission,CFP}$ = 450-484 nm, while YFP fluorescence was measured from $\lambda_{emission,YFP}$ = 520-524 nm. After image acquisition, background fluorescence was subtracted, and the YFP/CFP ratio for each pixel of the image was calculated using ImageJ. FRET response ratios were visualized as a blue-green-red color map. The left panel is prior to EGF stimulation; middle panel is 5 min post treatment; right panel is 10 min post treatment. Cells exhibit an increase in YFP/CFP ratio (represented as a color change from blue to green) upon EGF stimulation. Experiment was performed in triplicate ($n$ = 3) with similar results. Scale bar = 100 µm.

**Supplementary Figure 14:**



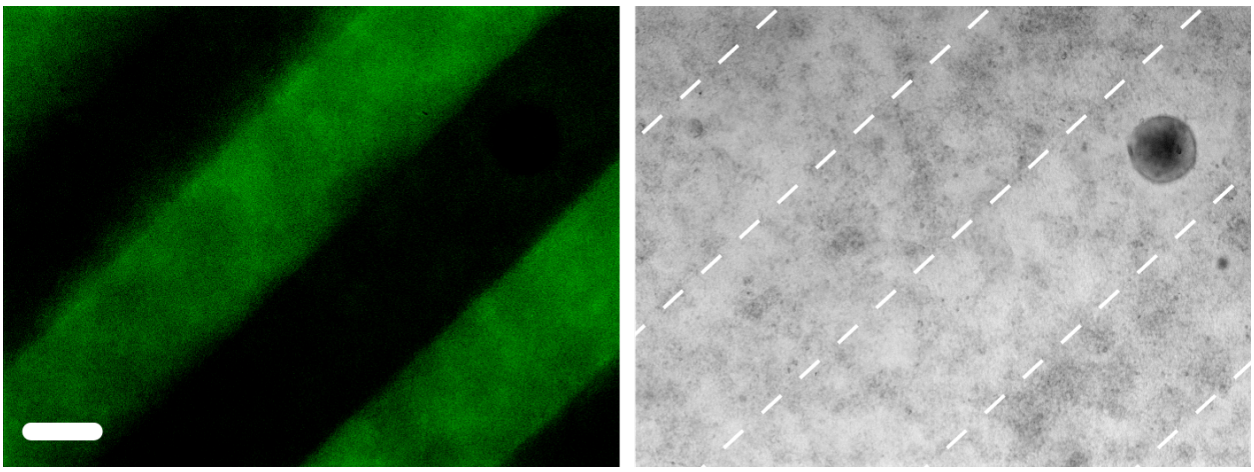

**Effect of light treatment on EKAREV biosensor function**. HeLa cells transfected with EKAREV FRET reporter for MAPK activation were encapsulated (2 x $10^6$ cells $mL^{-1}$) in hydrogels lacking immobilized EGF. Gels were exposed to patterned light (λ = 365 nm, 10 mW $cm^{-2}$, 10 min), switched to serum-free media overnight, and imaged to determine FRET values. Cells exhibited the same basal level of FRET activation independent of light exposure, indicating that the EKAREV FRET reporter is suitable for use in photopatterned hydrogels. Image represents color-coded FRET response normalized to basal MAPK activation, obtained from confocal z-slices. Experiment was performed in triplicate ($n$ = 3) with similar results. Scale bar = 100 μm.

**Supplementary Figure 15:**



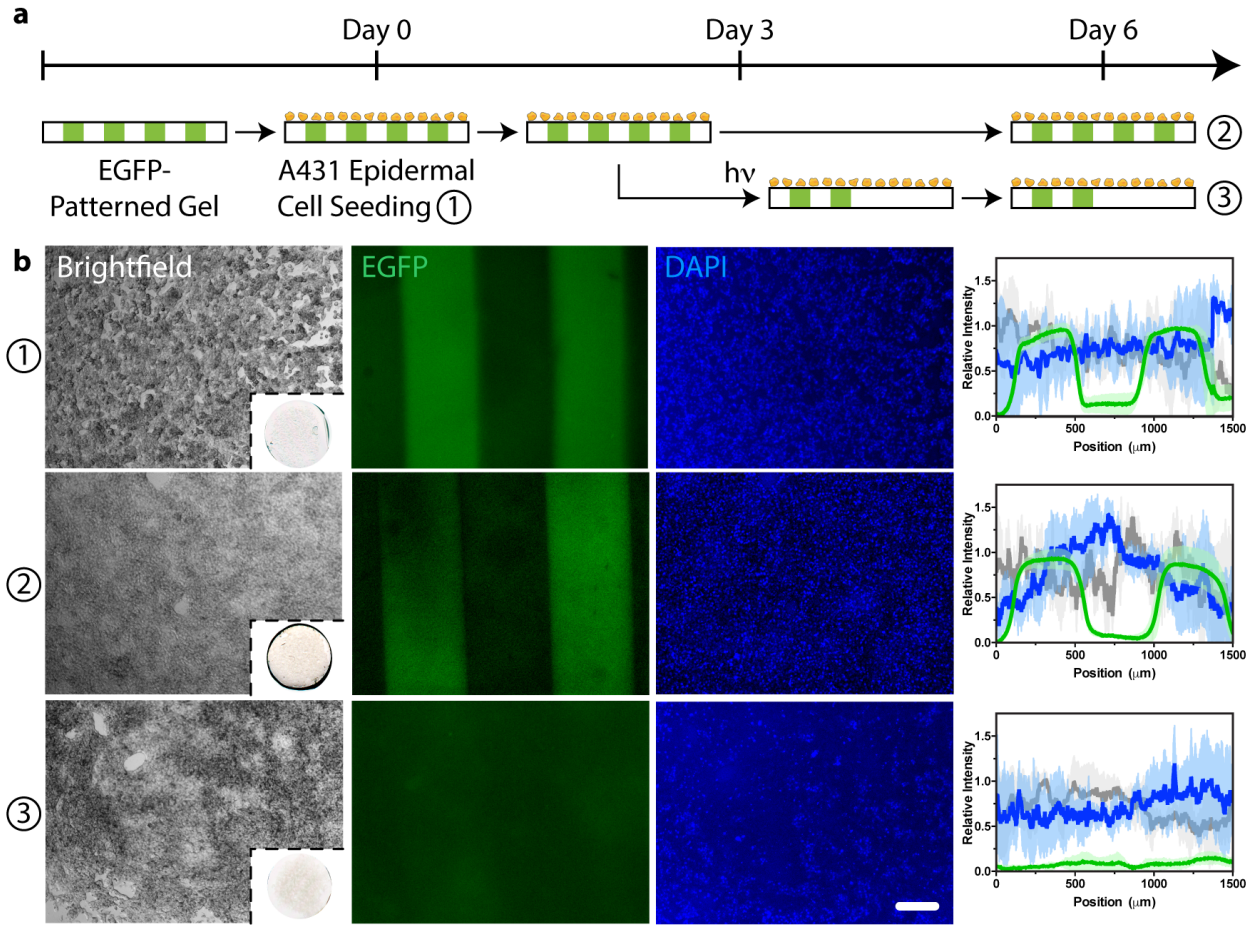

**Mass spectrum of purified EGFP-EGF-*o*NB-$N_3$.** EGFP-EGF-*o*NB-$N_3$ was synthesized and purified following methodologies outlined in Supplementary Methods using the STEPL plasmid for EGFP-EGF sortagged with H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ polyglycine probe (Supplementary Methods). Whole-protein mass spectrometry of a representative single purification indicated high sample purity and quantitative functionalization. Experiment was replicated ($n$ = 3) with similar results.

**Supplementary Figure 16:**



**Heterogenous cell density observed after three days of culture on patterned gels.** For the experiment described in Figure 6a, heterogenous cell distributions is observed after Day 3 in culture on patterned gels. Fluorescent images of EGFP-EGF (green, left) correspond to regions with higher cell densities observed by brightfield microscopy (right). Experiment was replicated ($n$ = 6) with similar results. Scale bar = 400 μm.

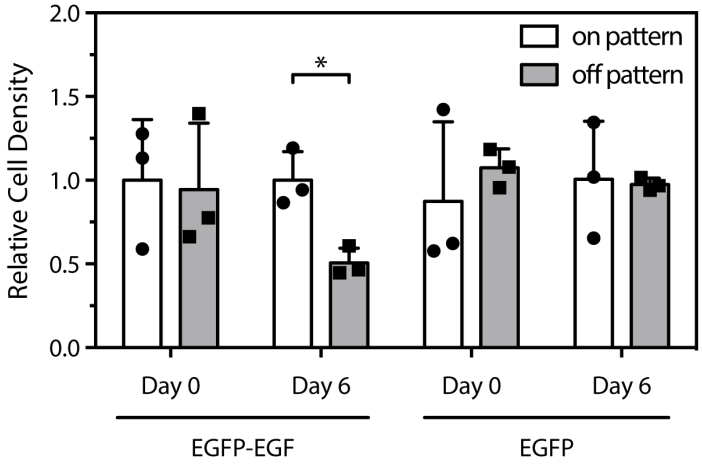
**Supplementary Figure 17:**




**2D cell response to dynamically patterned EGFP on gels.** EGFP-*o*NB-$N_3$ (25 μM) was conjugated (25 °C, 1 hr) to PEG-tetraBCN ($M_n$ ~ 20,000 Da, 4 mM). EGFP-modified hydrogels (5 μL) were formed (1 hr) between Rain-X®-treated glass slides with silicone rubber spacers (McMaster-Carr, 0.5 mm thick) with $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$ crosslinker (Supplementary Methods, 8 mM). After removal from the glass slide chamber, gels were exposed to masked collimated UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) through a slitted photomask containing 400 μm wide line features and soaked in PBS overnight. Gels were swollen (1 hr) in DMEM containing FBS (10%) prior to cell seeding. A431 cells (1 x $10^7$ cells $mL^{-1}$) were added to the top of gels as a droplet (10 μL) and allowed to attach (1 hr, 37 °C) prior to media addition. Gels were swollen in DMEM containing FBS (10%) and penicillin/streptomycin (1%) overnight prior to imaging (condition ①). Gel media was supplemented with bovine serum albumin (BSA, 0.1%) and cells maintained in culture (37 °C, 5% $CO_2$). Three days after cell seeding, a portion of the hydrogels were exposed to a second round of photopatterning; one half of each treated gel was exposed to UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) while the other half was left unexposed. In all cases, gels were maintained in DMEM containing FBS (10%), penicillin/streptomycin (1%), and BSA (0.1%) for an additional three days prior to analysis (conditions ② and ③).

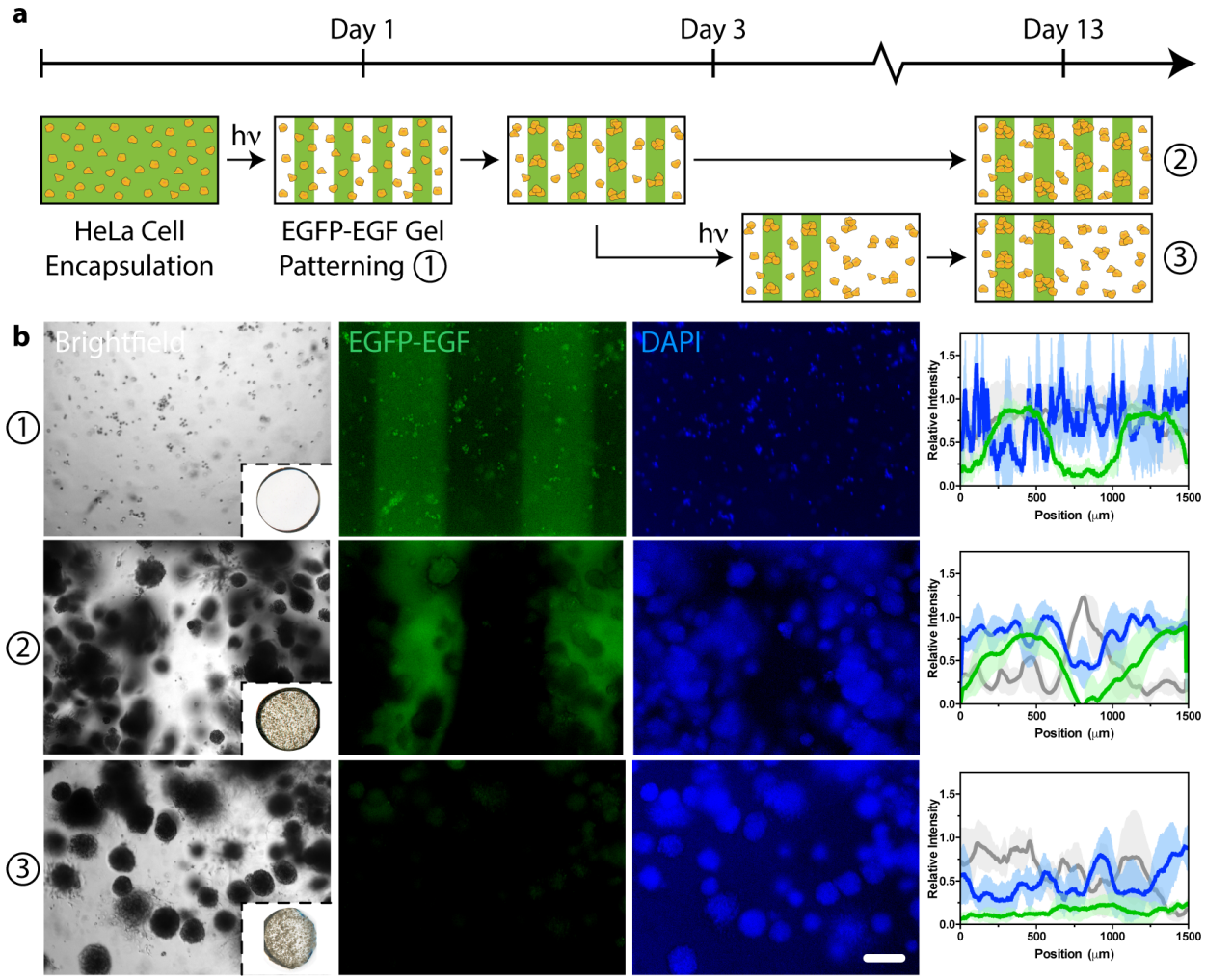
At the end of each treatment condition, cells were fixed in paraformaldehyde (1%, 30 min), stained with DAPI (0.15 μg/mL, 30 min), and visualized *via* fluorescence microscopy. Image analysis was performed for each condition using ImageJ, quantifying the normalized intensity profile for EGFP (green), nuclei (blue), and optical transmission (grey) across the gel perpendicular to photopatterned lines. Plots indicate average values (dark lines) and standard deviations (light error bars) from three biological replicates across the gel surface. Whole-gel images were performed on an Epson Perfection 4490 Photo document scanner. Unlike gels functionalized with EGFP-EGF, EGFP-only gels exhibit no variations in cell density matching the patterned region of tethered protein. Image insets correspond to gels roughly 0.5 cm in diameter. Scale bar = 200 μm.

**Supplementary Figure 18:**




**Variable 2D cell response throughout patterned gels.** Average 2D cell density was determined for A431 cells both on and off protein-patterned regions in conditions ① and ② of experiments comprising Figure 6a-b and Supplementary Figure 17. Cell density was calculated as the average total DAPI fluorescence in the center 200 μm portion of each region ($n$ = 3 biological replicates). To account for basal levels in cell proliferation throughout the experiment, data has been normalized such that the highest relative cell density for experiments on a given day for a given patterned protein has a value of 1. A statistically significant difference (unpaired two-tailed t-test, $*p = 1.1 \times 10^{-2}$) was observed for cell density on versus off the EGFP-EGF pattern on day 6, but not for EGFP protein. All Day 0 data are statistically indistinguishable, indicating that the initial protein pattern did not hamper uniform cell seeding. Bar plots correspond to the mean cell density in each condition with error bars representing ±1 standard deviation.

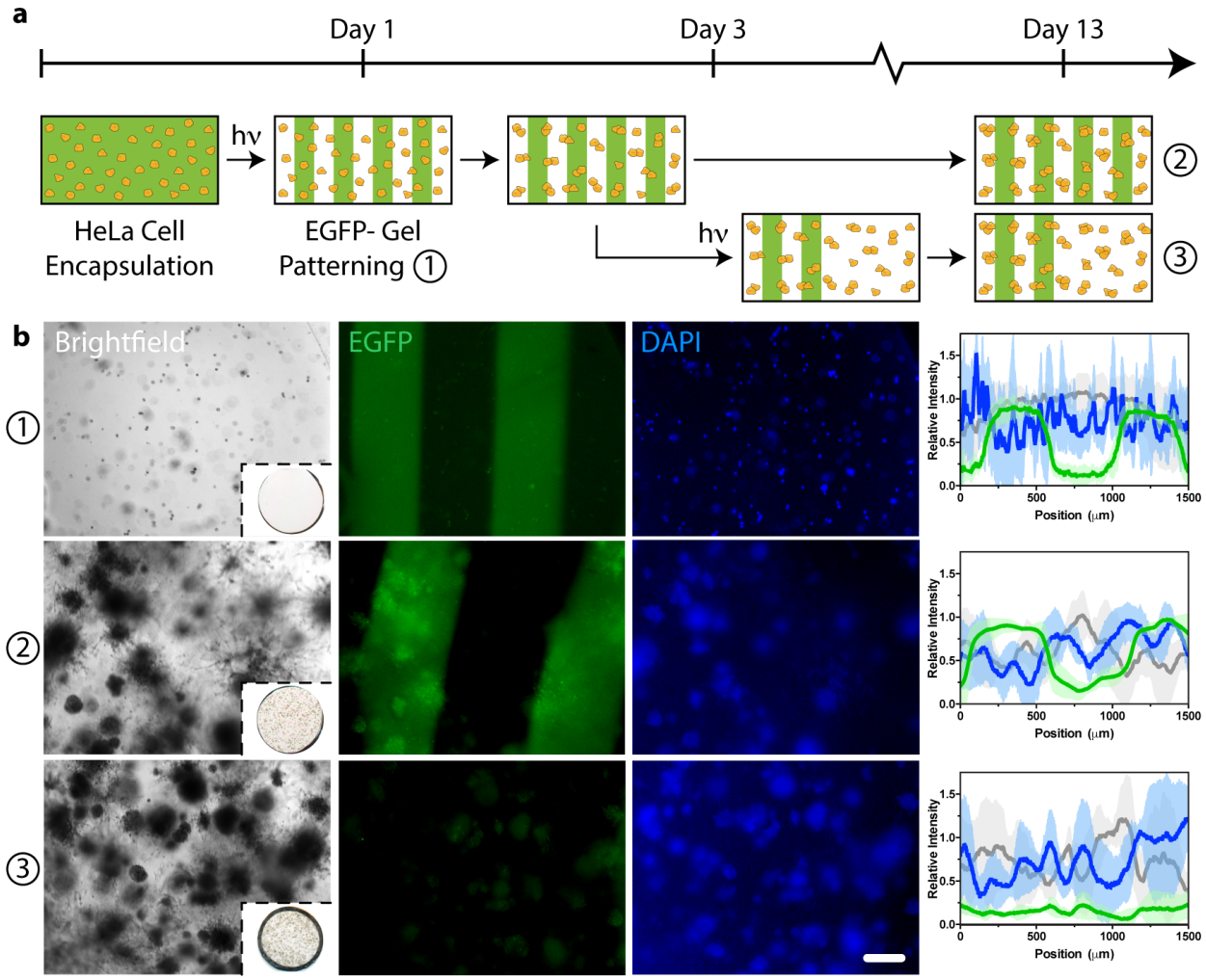
**Supplementary Figure 19:**




**3D cell response to dynamically patterned EGFP-EGF within gels.** EGFP-EGF-*o*NB-$N_3$ (10 μM) was conjugated (25 °C, 1 hr) to PEG-tetraBCN ($M_n$ ~ 20,000 Da, 4 mM). EGFP-modified hydrogels (5 μL) were formed (1 hr) between Rain-X®-treated glass slides with silicone rubber spacers (McMaster-Carr, 0.5 mm thick) with $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$ crosslinker (Supplementary Methods, 8 mM) and HeLa cell suspension (1 x $10^7$ cells $mL^{-1}$). After removal from glass slide chamber, gels were maintained in DMEM containing FBS (10%) and penicillin/streptomycin (1%) overnight prior to initial photopatterning. Gels were exposed to masked collimated UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) through a slitted photomask containing 400 μm wide line features. Gels were swollen in DMEM containing FBS (10%) and penicillin/streptomycin (1%) and maintained in culture (37 °C, 5% $CO_2$) overnight prior to imaging (condition ①). Three days after cell encapsulation, a portion of the hydrogels were exposed to a second round of photopatterning; one half of each treated gel was exposed to UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) while the other half was left unexposed. In all cases, gels were maintained in DMEM containing FBS (10%) and penicillin/streptomycin (1%) for an additional ten days prior to analysis (conditions ② and ③).

At the end of each treatment condition, cells were fixed in paraformaldehyde (1%, 30 min), stained with DAPI (0.15 μg/mL, 1 hr), and visualized *via* fluorescence microscopy. Image analysis was performed for each condition using ImageJ, quantifying the normalized intensity profile for EGFP-EGF (green), nuclei (blue), and optical transmission (grey) across the gel perpendicular to photopatterned lines. Plots indicate average values (dark lines) and standard deviations (light error bars) from three biological replicates across the gel volume. Cells exhibit staining in the green channel in condition ① due to EGFP-EGF binding with membrane receptors prior to being recycled. Whole-gel images were performed on an Epson Perfection 4490 Photo document scanner. Gel regions containing immobilized EGF are visually darker, attributed to enhanced spheroid growth. Image insets correspond to gels roughly 0.5 cm in diameter. Scale bar = 200 μm.

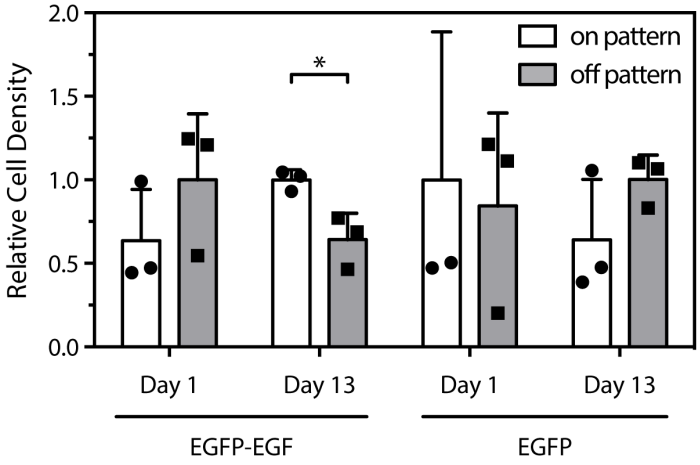
**Supplementary Figure 20:**




**3D cell response to dynamically patterned EGFP within gels.** EGFP-*o*NB-$N_3$ (10 μM) was conjugated (25 °C, 1 hr) to PEG-tetraBCN ($M_n$ ~ 20,000 Da, 4 mM). EGFP-modified hydrogels (5 μL) were formed (1 hr) between Rain-X®-treated glass slides with silicone rubber spacers (McMaster-Carr, 0.5 mm thick) with $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$ crosslinker (Supplementary Methods, 8 mM) and HeLa cell suspension (1 x $10^7$ cells $mL^{-1}$). After removal from glass slide chamber, gels were maintained in DMEM containing FBS (10%) and penicillin/streptomycin (1%) overnight prior to initial photopatterning. Gels were exposed to masked collimated UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) through a slitted photomask containing 400 μm wide line features. Gels were swollen in DMEM containing FBS (10%) and penicillin/streptomycin (1%) and maintained in culture (37 °C, 5% $CO_2$) overnight prior to imaging (condition ①). Three days after cell encapsulation, a portion of the hydrogels were exposed to a second round of photopatterning; one half of each treated gel was exposed to UV light (λ = 365 nm, 10 min, 10 mW $cm^{-2}$) while the other half was left unexposed. In all cases, gels were maintained in DMEM containing FBS (10%) and penicillin/streptomycin (1%) for an additional ten days prior to analysis (conditions ② and ③).

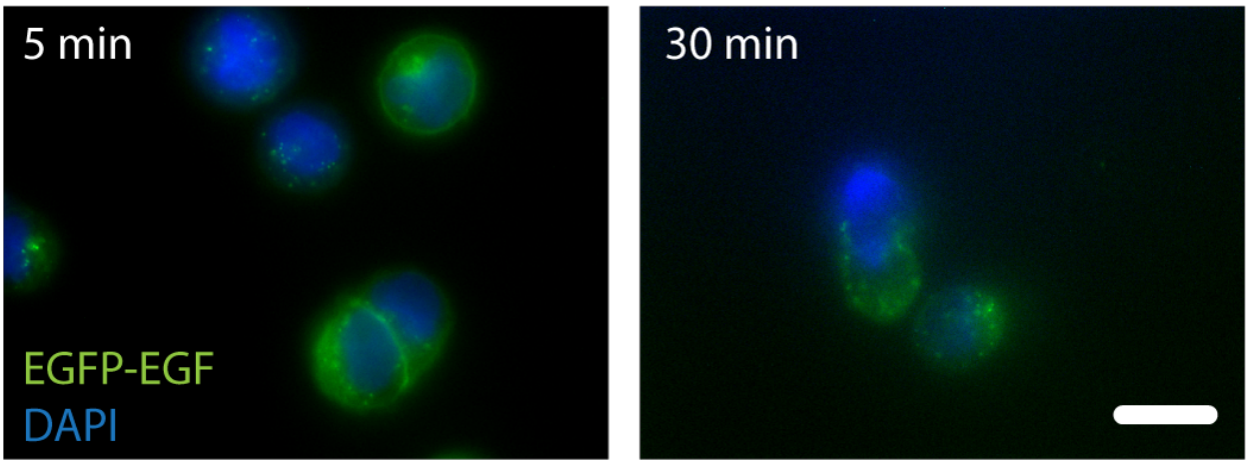
At the end of each treatment condition, cells were fixed in paraformaldehyde (1%, 30 min), stained with DAPI (0.15 μg/mL, 1 hr), and visualized *via* fluorescence microscopy. Image analysis was performed for each condition using ImageJ, quantifying the normalized intensity profile for EGFP (green), nuclei (blue), and optical transmission (grey) across the gel perpendicular to photopatterned lines. Plots indicate average values (dark lines) and standard deviations (light error bars) from three biological replicates across the gel volume. Whole-gel images were performed on an Epson Perfection 4490 Photo document scanner. EGFP-modified gels showed no increase in spheroid number or diameter between functionalized and unfunctionalized regions. Image insets correspond to gels roughly 0.5 cm in diameter. Scale bar = 200 μm.

**Supplementary Figure 21:**



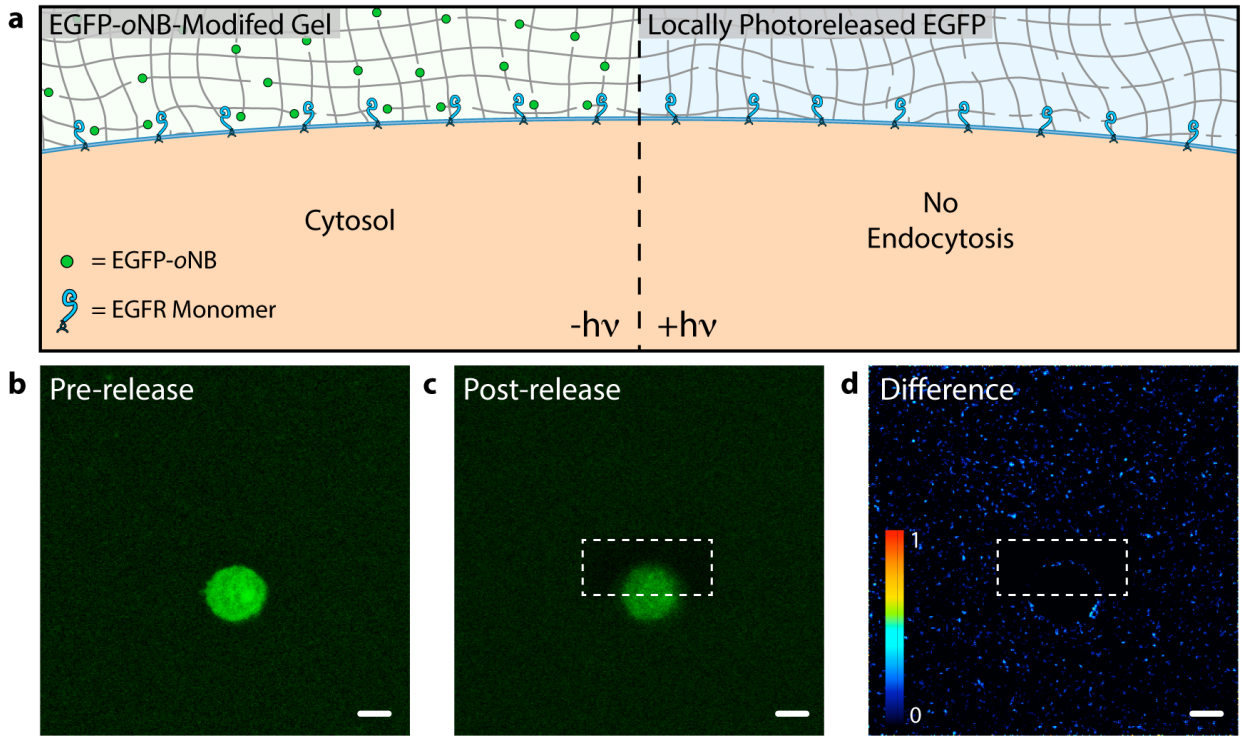

**Variable 3D cell response throughout patterned gels.** Average 3D cell density was determined for HeLa cells both on and off protein-patterned gel subvolumes in conditions ① and ② of experiments comprising Supplementary Figures 19 and 20. Cell density was calculated as the average total DAPI fluorescence in the center 200 μm portion of each region ($n$ = 3 biological replicates). To account for basal levels in cell proliferation throughout the experiment, data has been normalized such that the highest relative cell density for experiments on a given day for a given patterned protein has a value of 1. A statistically significant difference (unpaired two-tailed t-test, $*p = 2.9 \times 10^{-2}$) was observed for cell density within versus outside the EGFP-EGF pattern on day 13, but not for EGFP protein. All Day 1 data are statistically indistinguishable, indicating that HeLa cells were encapsulated uniformly in gels. Bar plots correspond to the mean cell density in each condition with error bars representing ±1 standard deviation.

**Supplementary Figure 22:**




**2D cell stimulation with soluble EGFP-EGF.** To test receptor binding and internalization of the EGFP-EGF-*o*NB-$N_3$ fusion protein, A431 cells were plated on tissue-culture polystyrene ($10^5$ cells $cm^{-2}$) and allowed to attach overnight in DMEM supplemented with FBS (10%) and penicillin/streptomycin (1%). The media was replaced with DMEM containing EGFP-EGF-*o*NB-$N_3$ (125 nM) and incubated for 30 minutes at room temperature before swapping into fresh media with no added growth factor and returned to 37 °C. At either 5 or 30 minutes later, cells were fixed in paraformaldehyde (1%, 30 min), stained with DAPI (0.15 µg/mL, 30 min), and visualized *via* fluorescence microscopy. Cells display strong membrane staining at early time points, followed by more diffuse staining as the EGFR receptor is internalized. Punctate staining in the green channel corresponds to concentrated EGFP-EGF within endosomal vesicles. Experiment was replicated ($n$ = 8) with similar results. Scale bar = 10 µm.

**Supplementary Figure 23:**




**Encapsulated cell response to subcellular photoreleased EGFP**. A431 cells ($1x10^7$ cells $mL^{-1}$) were encapsulated in SPAAC hydrogels (5 µL) containing EGFP-*o*NB-$N_3$ (125 nM) and $N_3$-GRGDS-$NH_2$ peptide (Supplementary Methods, 1 mM) and affixed to an azide-functionalized slide (Supplementary Methods). The cells were cultured for 48 hours in DMEM supplemented with FBS (10%) and penicillin/streptomycin (1%) before transferring to phenol red-free DMEM containing FBS (10%) and penicillin/streptomycin (1%). Protein photorelease was performed *via* multiphoton laser scanning lithography (Olympus FV1000 MPE BX61 Multi-photon Microscope, 20x objective, 5x zoom, λ = 740 nm, 25% laser power, 16 scan repeats, z-interval = 2.5 µm), confined to a 40 µm x 30 µm x 5 µm subvolume bisecting an individual cell. Images were taken (b) prior to release and (c) 30 minutes afterwards. (d) ImageJ was used to calculate the difference in pixel intensity accompanying photorelease. Gels containing EGFP-*o*NB-$N_3$ exhibit nonspecific staining of encapsulated cells. Photoreleased EGFP does not trigger endocytosis, as evidenced by the lack of punctate fluorescence within the cell following treatment. Experiment was performed within a single gel. Scale bar = 10 µm.

**Supplementary Table 1. Identification codes for all sortagged species**

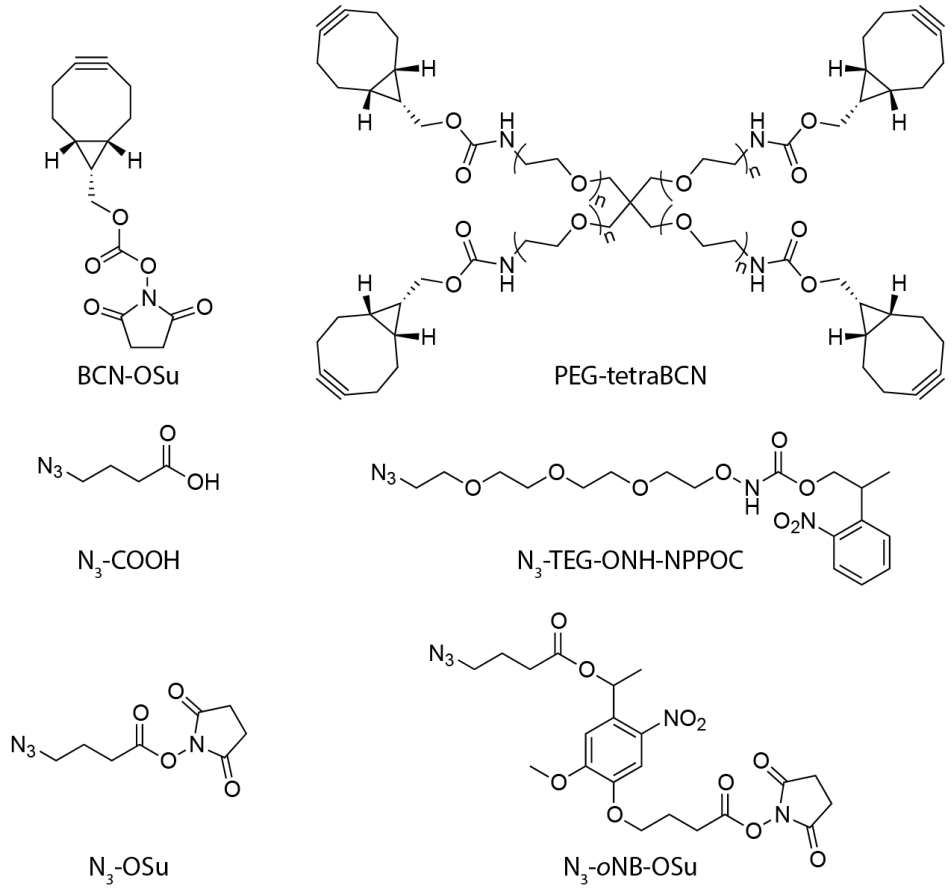
| Base Protein | Polyglycine Probe | Sortagged Protein Identity |
|---|---|---|
| *EGFP* | Triglycine | EGFP |
| | H-GGGGDDK($N_3$)-$NH_2$ | EGFP-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | EGFP-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | EGFP-*o*NB-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | EGFP-*o*NB-CHO |
| *mCherry* | Triglycine | mCherry |
| | H-GGGGDDK($N_3$)-$NH_2$ | mCherry-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | mCherry-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | mCherry-*o*NB-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | mCherry-*o*NB-CHO |
| *mCerulean* | Triglycine | mCerulean |
| | H-GGGGDDK($N_3$)-$NH_2$ | mCerulean-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | mCerulean-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | mCerulean-*o*NB-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | mCerulean-*o*NB-CHO |
| *EGF* | Triglycine | EGF |
| | H-GGGGDDK($N_3$)-$NH_2$ | EGF-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | EGF-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | EGF-*o*NB-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | EGF-*o*NB-CHO |
| *bla* | Triglycine | bla |
| | H-GGGGDDK($N_3$)-$NH_2$ | bla-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | bla-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | bla-*o*NB-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | bla-*o*NB-CHO |
| *MBP-FGF* | Triglycine | MBP-FGF |
| | H-GGGGDDK($N_3$)-$NH_2$ | MBP-FGF-$N_3$ |
| | H-GGGGDDK(CHO)-$NH_2$ | MBP-FGF-CHO |
| | H-GGGGDDK(*o*NB-$N_3$)-$NH_2$ | MBP-FGF-$N_3$ |
| | H-GGGG-*o*NB-DDK(CHO)-$NH_2$ | MBP-FGF-CHO |

**Supplementary Methods:**

**General synthetic information**

Chemical reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific and used as received unless otherwise noted. Peptide synthesis reagents were purchased from either ChemPep or Chem-Impex and used as received. Deionized water ($dH_2O$) was generated by a U.S. Filter Corporation Reverse Osmosis System with a Desal membrane. Synthetic chemical reactions were performed under a nitrogen atmosphere in oven-dried glassware and stirred with a Teflon-coated magnetic stir bar unless otherwise noted. Solvents were removed *in vacuo* with a Büchi Rotovapor R-3 equipped with a V-700 vacuum pump and V-855 vacuum controller and a Welch 1400 DuoSeal Belt-Drive high vacuum pump. $^1H$ nuclear magnetic resonance (NMR) data was collected at 298 K on Bruker instruments and chemical shifts are reported relative to tetramethylsilane (TMS, $\delta = 0$). Microwave-assisted peptide synthesis was performed on a CEM Liberty 1. Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed on a Dionex Ultimate 3000 equipped with a variable multiple wavelength detector, automated fraction collector, and Thermo 5 μm Synchronis silica 250 x 21.2 mm C18 column. Lyophilization was performed on a LABCONCO FreeZone 2.5 Plus freeze-dryer equipped with a LABCONCO rotary vane 117 vacuum pump. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was performed in reflectron positive ion mode or reflectron negative ion mode on a Bruker AutoFlex II using a matrix of α-cyano-4-hydroxycinnamic acid:2,5-dihydroxy benzoic acid (2:1). Whole-protein mass spectrometry was performed using a Waters Synapt – G2 QTOF. The light source for the photochemical cleavage was a Lumen Dynamics OmniCure S1500 Spot UV Curing system with an internal 365 nm filter and an external 360 nm cut-on longpass filter. Light intensity was measured using a Cole-Parmer Radiometer (Series 9811-50, $\lambda = 365$ nm). Biochemical gradients were created with the aid of a Harvard Apparatus PHD 2000 Syringe Pump. Fluorescence readings were acquired on a SpectraMax M5 spectrometer using Thermo Scientific Nunc black polypropylene 96-well plates. Confocal microscopy was performed at the University of Washington Keck Microscopy Center on a Leica SP8X confocal microscope. Multiphoton lithography was performed on an Olympus FV1000 MPE BX61 Multi-photon Microscope at the Garvey Imaging Center at the University of Washington. Polymerase chain reaction (PCR) was performed in a Bioer LifeECO thermal cycler. Protein expression was performed in a Thermo Scientific MaxQ 4000 shaker incubator. Cells were lysed using a Fisher Scientific Model 505 Sonic Dismembrator with a 1.27 cm diameter probe. Mammalian cell culture was performed in a NuAire LabGard ES NU-437 Class II Type A2 Biosafety Cabinet. Cells were maintained in a Sanyo inCu saFe® MCO-17AC incubator at 37 °C and 5% $CO_2$.

**Synthesis of previously reported compounds used in this work**




(1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (2,5-dioxopyrrolidin-1-yl) carbonate (BCN-OSu), poly(ethylene glycol) tetrabicyclononyne (PEG-tetraBCN, $M_n \sim 20{,}000$ Da), 4-azidobutanoic acid ($N_3$-COOH), 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate ($N_3$-OSu), 2-(2-nitrophenyl)propyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxycarbamate ($N_3$-TEG-ONH-NPPOC), and 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate ($N_3$-*o*NB-OSu) were synthesized as previously reported[1].

**Plasmid construction for protein expression and STEPL purification**

*Sources of DNA sequences*
The pSTEPL plasmid[2] containing EGFP and an ampicillin resistance gene was generously donated by the Tsourkas group (University of Pennsylvania). Plasmids containing gene sequences for mCherry, Beta-lactamase, and Maltose Binding Protein sequences were donated by the Baneyx group (University of Washington). A plasmid containing the mCerulean sequence was donated by Daniel Strongin (Fred Hutchinson Cancer Research Center). EGF-bio-His was a gift from Gavin Wright (Addgene plasmid # 53340). A codon-optimized gene sequence for FGF was purchased as a gBlock (Integrated DNA Technologies).

*STEPL Plasmid Construction*
For each protein to be expressed in the STEPL system, polymerase chain reaction (PCR) was used to amplify gene sequences of interest and introduce relevant restriction sites (5' NdeI and 3' XhoI) for subsequent cloning.

pSTEPL plasmid and PCR products were digested (4 hr, 37 °C) with NdeI and XhoI restriction enzymes (New England BioLabs) and purified by extraction following electrophoretic separation (0.8% agarose). The digested pSTEPL and protein insert were ligated (T4 DNA ligase, 16 hr, 16 °C), and transformed into chemically competent Top10 *E. coli* (Thermo Fisher) by heat shock, and plated onto agar plates (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, 15 g agar, 1 L $dH_2O$) containing ampicillin (100 μg $mL^{-1}$). Colonies were subsequently grown overnight in Miller's Lysogeny Broth (LB, 5 mL) containing ampicillin (100 μg $mL^{-1}$). Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and sequenced using a SimpleSeq DNA Sequencing Kit (Thermo Fisher). Plasmids corresponding to the STEPL construct of interest were purified and subsequently transformed into chemically competent BL21(DE3) *E. coli* (Promega) for expression.

Due to low solubility of the MBP-FGF-SrtA STEPL fusion protein, MBP-FGF containing a C-terminal sorting signal was also expressed separately from the sortase enzyme. The sequence for MBP was lifted out using PCR and adding a 5' NdeI site and a 3' LPETG motif followed by a HindIII site. The gBlock for FGF was ordered with a 5' HindIII site and a 3' XhoI site. Each insert was digested with their respective enzymes (New England BioLabs), and pET-21a(+) was digested with NdeI and XhoI. After gel extraction, the fragments were ligated using T4 DNA polymerase (16 °C, 16 h). The ligated product was transformed into chemically competent Top10 cells by heat shock. Purified plasmids from successful transformants were subsequently transformed into chemically competent BL21(DE3) *E. coli* for expression.

Below is a list of DNA open reading frame sequences (5' → 3') generated for and utilized in these studies. Nucleotides shown in black correspond to that for the specific protein of interest. Bases shown in orange correspond to STEPL portion common to all constructs, which features the C-terminal LPETG sortase recognition motif, a flexible $(GGS)_5$ linker, SrtA, and a 6xHis tag.

*EGFP-STEPL*:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG
GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGAC
CACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA
CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA
TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATG
GCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGC
CGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGT
CCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACT
CTCGGCATGGACGAGCTGTACAAGCTCGAGCTGCCGGAAACCGGTGGTGGTAGTGGTGGCTCTGGCGGTTCTGGTGG
CAGTGGCGGTAGCCAAGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATG
CTGATATTAAAGAACCAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAAGCTTTGCAGAAGAA
AATGAATCACTAGATGATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCGAACTATCAATTTACAAA
TCTTAAAGCAGCCAAAAAAGGTAGTATGGTGTACTTTAAAGTTGGTAATGAAACACGTAAGTATAAAATGACAAGTA
TAAGAGATGTTAAGCCAACAGATGTAGAAGTTCTAGATGAACAAAAAGGTAAAGATAAACAATTAACATTAATTACT
TGTGATGATTACAATGAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAACATCACCA
CCATCATCACTAA

*mCherry-STEPL*:

ATGGTTTCCAAGGGCGAAGAAGACAACATGGCGATCATCAAAGAATTTATGCGTTTTAAAGTTCACATGGAAGGTTC
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AAGTTACCAAAGGTGGTCCGCTGCCGTTCGCGTGGGACATCCTCAGCCCGCAGTTCATGTACGGTTCTAAAGCGTAC
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TGCGTGGCACCAACTTCCCGTCTGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCGTCTTCTGAACGT
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GGAGGTTAAAACCACCTACAAAGCGAAAAAGCCGGTTCAACTGCCGGGTGCGTACAACGTTAATATCAAGCTGGACA
TCACCTCTCACAACGAAGACTACACCATCGTTGAACAGTACGAACGTGCGGAAGGCCGTCACTCTACCGGTGGTATG
GACGAACTGTACAAGCTCGAGCTGCCGGAAACCGGTGGTGGTAGTGGTGGCTCTGGCGGTTCTGGTGGCAGTGGCGG
TAGCCAAGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATGCTGATATTA
AAGAACCAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAAGCTTTGCAGAAGAAAATGAATCA
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TTAAGCCAACAGATGTAGAAGTTCTAGATGAACAAAAAGGTAAAGATAAACAATTAACATTAATTACTTGTGATGAT
TACAATGAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAACATCACCACCATCATCA
CTAA

*mCerulean-STEPL*:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG
GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCGCCCGCTACCCCGAC
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GCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGC
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CCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACT
CTCGGCATGGACGAGCTGTACAAGCTCGAGCTGCCGGAAACCGGTGGTGGTAGTGGTGGCTCTGGCGGTTCTGGTGG
CAGTGGCGGTAGCCAAGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATG
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AATGAATCACTAGATGATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCGAACTATCAATTTACAAA
TCTTAAAGCAGCCAAAAAAGGTAGTATGGTGTACTTTAAAGTTGGTAATGAAACACGTAAGTATAAAATGACAAGTA
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TGTGATGATTACAATGAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAACATCACCA
CCATCATCACTAA

*EGF-STEPL*:

ATGAACAGCGACAGCGAGTGCCCACTGAGCCACGACGGCTACTGCCTGCACGACGGCGTGTGCATGTACATCGAGGC
CCTGGACAAGTACGCCTGCAACTGCGTCGTGGGCTACATCGGCGAGCGGTGCCAGTACCGGGACCTGAAGTGGTGGG
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AGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATGCTGATATTAAAGAAC
CAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAAGCTTTGCAGAAGAAAATGAATCACTAGAT
GATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCGAACTATCAATTTACAAATCTTAAAGCAGCCAA
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CAACAGATGTAGAAGTTCTAGATGAACAAAAAGGTAAAGATAAACAATTAACATTAATTACTTGTGATGATTACAAT
GAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAACATCACCACCATCATCACTAA

*bla-STEPL*:

ATGCACCCTGAAACGCTGGTGAAAGTAAAANATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGA
TCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGC
TATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGAC
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AACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGC
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GCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGT
TTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCC
TCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGG
TGCCTCACTGATTAAGCATTGGCTCGAGCTGCCGGAAACCGGTGGTGGTAGTGGTGGCTCTGGCGGTTCTGGTGGCA
GTGGCGGTAGCCAAGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATGCT
GATATTAAAGAACCAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAAGCTTTGCAGAAGAAAA
TGAATCACTAGATGATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCGAACTATCAATTTACAAATC
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AGAGATGTTAAGCCAACAGATGTAGAAGTTCTAGATGAACAAAAAGGTAAAGATAAACAATTAACATTAATTACTTG
TGATGATTACAATGAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAACATCACCACC
ATCATCACTAA

*MBP-FGF-STEPL*:

ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAA
GAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTG
CGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTG
GCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGCAA
GCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAA
CCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAGAA
CCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAA
AGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGA
ATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGG
GCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAA
ACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAA
ACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTAC
GAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACCATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACAT
CCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATG
AAGCCCTGAAAGACGCGCAGACTAATTCGAGCAAGCTTGCTGCTGGTTCTATCACCACCCTGCCGGCTCTGCCGGAA
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*MBP*-*FGF*-*LPETG*-*His6*:

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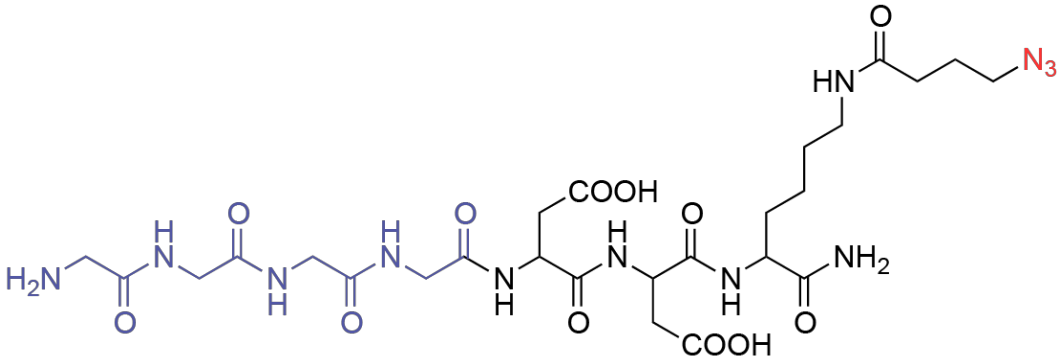
CACTGA

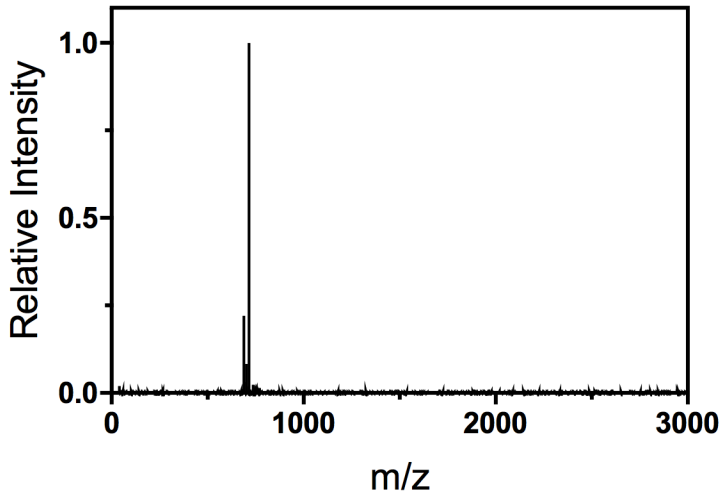
**Fmoc solid-phase peptide synthesis**

A CEM Liberty 1 was used to perform microwave-assisted Fmoc solid phase peptide synthesis (SPPS, 0.25 mmol scale). Fmoc deprotection was performed in 20% piperidine (v/v) in dimethylformamide (DMF) with 1-hydroxybenzotriazole (HOBt, 0.1 M, 90 °C, 90 sec). Amino acids were coupled to resin-bound peptides upon treatment (75 °C, 5 min) with Fmoc-protected amino acid (2 mmol, 4x), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 2 mmol, 4x), and *N*,*N*-diisopropylethylamine (DIEA, 2 mmol, 4x) in a mixture of DMF (9 mL) and *N*-Methyl-2-pyrrolidone (NMP, 2 mL). Room-temperature couplings were performed without microwave assistance (1 hr).

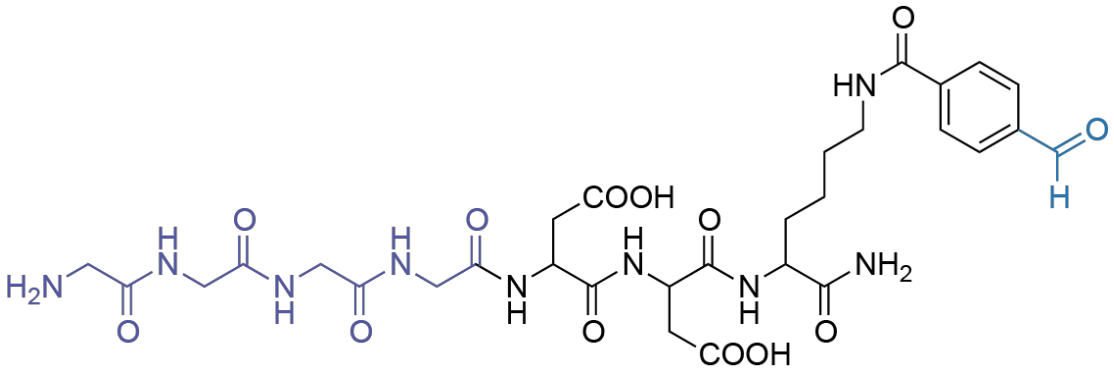
**Synthesis of H-GGGGDDK($N_3$)-$NH_2$**



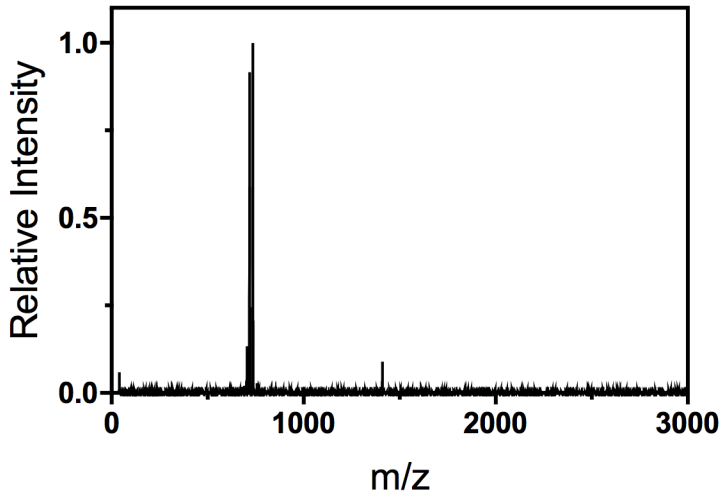

The resin-bound peptide Boc-GGGGDDK(Mtt)-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). The resin was washed with DMF (3x) and dichloromethane (DCM, 3x) prior to Mtt cleavage [2 min, 15 mL, 97:2:1 DCM:Triisopropylsilane (TIS):Trifluoroacetic Acid (TFA), 9x]. Resin was washed (DCM, 3x; DMF, 3x) prior to treatment (1 hr) with $N_3$-COOH (4x, 1 mmol, 129 mg) by HATU coupling (3.95x, 0.988 mmol, 188 mg) and N,N-Diisopropylethylamine (DIEA, 8x, 2 mmol, 174 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product (H-GGGGDDK($N_3$)-$NH_2$) as a white solid (62 mg, 0.087 mmol, 34% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{26}H_{43}N_{12}O_{12}^+$ $[M + {}^1H]^+$, 715.31; observed 715.40.



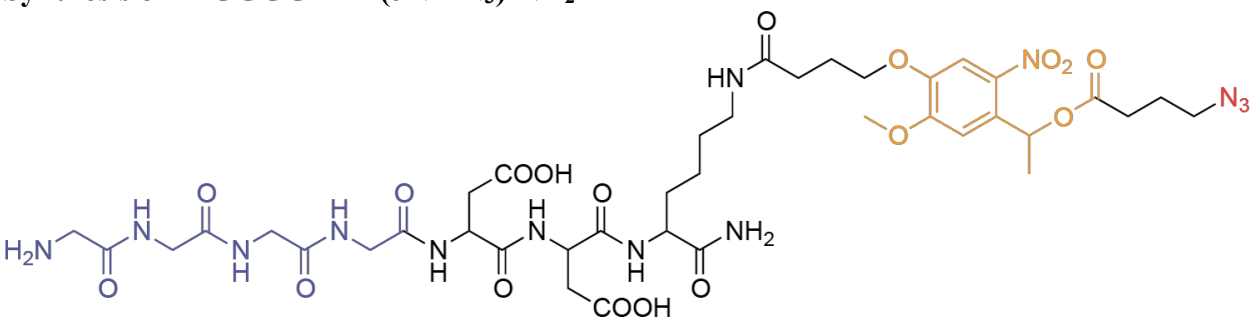
**Synthesis of H-GGGGDDK(CHO)-$NH_2$**



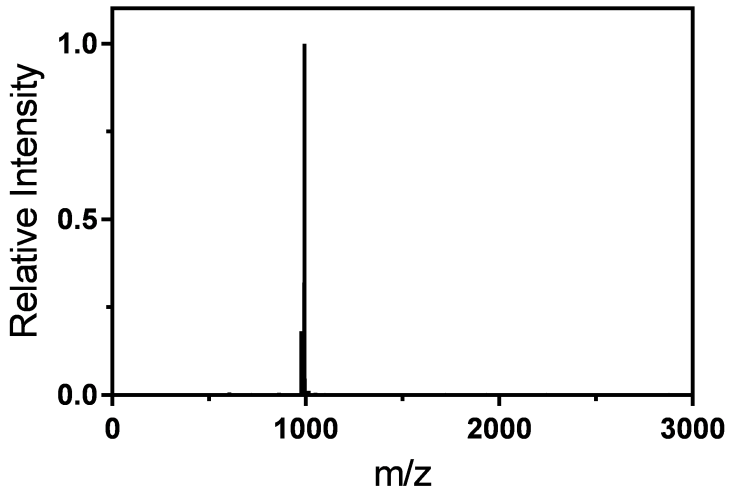
The resin-bound peptide Boc-GGGGDDK(Mtt)-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). The resin was washed with DMF (3x) and dichloromethane (DCM, 3x) prior to Mtt cleavage (2 min, 15 mL, 97:2:1 DCM:TIS:TFA, 9x). Resin was washed (DCM, 3x; DMF, 3x) prior to treatment (1 hr) with 4-formylbenzoic acid (4x, 1 mmol, 150 mg) by HATU coupling (3.95x, 0.988 mmol, 188 mg) and DIEA (8x, 2 mmol, 174 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product (H-GGGGDDK(CHO)-$NH_2$) as a white solid (39 mg, 0.053 mmol, 21% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{30}H_{42}N_9O_{13}^+$ $[M + {}^1H]^+$, 736.29; observed 736.39.



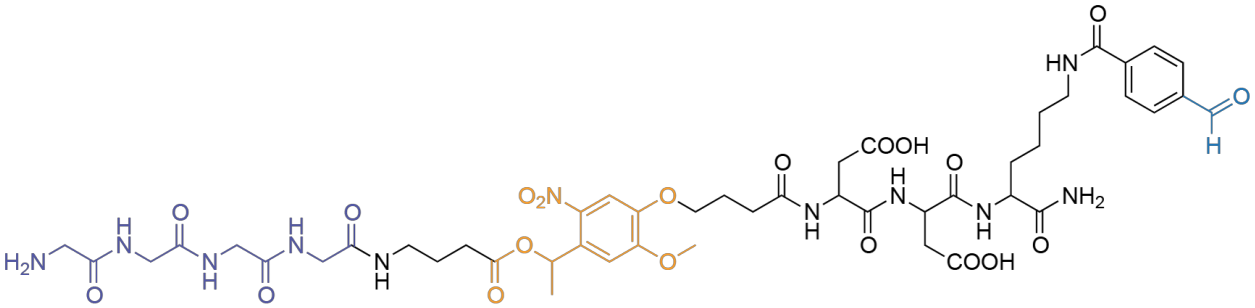
**Synthesis of H-GGGGDDK(*o*NB-$N_3$)-$NH_2$**



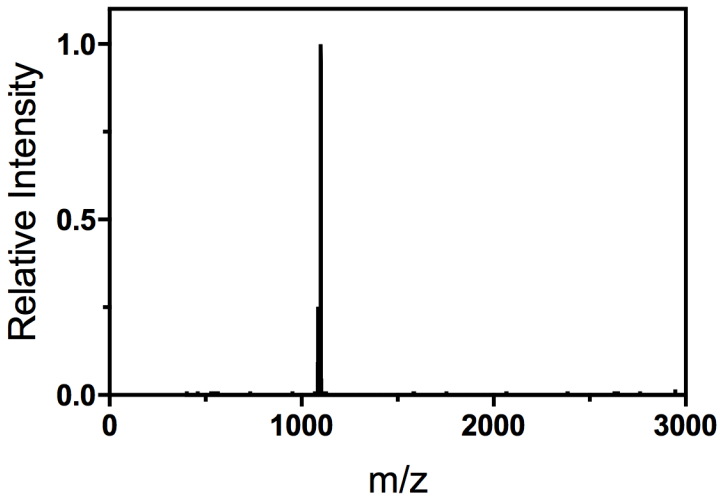
The resin-bound peptide Boc-GGGGDDK(Mtt)-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). The resin was washed with DMF (3x) and dichloromethane (DCM, 3x) prior to Mtt cleavage (2x, 9 min, 15 mL, 97:2:1 DCM:TIS:TFA). Resin was washed (DCM, 3x; DMF, 3x) prior to treatment (2 hr) with $N_3$-*o*NB-OSu (1.5x, 0.375 mmol, 90 mg) and DIEA (4x, 1 mmol, 87 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product (H-GGGGDDK(*o*NB-$N_3$)-$NH_2$) as a yellow solid (70 mg, 0.070 mmol, 27% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{39}H_{56}N_{13}O_{18}^-$ [M - $^1H$]$^-$, 994.39; observed 993.47.



**Synthesis of H-GGGG-*o*NB-DDK(CHO)-$NH_2$**



The resin-bound peptide H-DDK(Mtt)-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). Resin was washed (DMF, 3x) prior to treatment with $N_3$-*o*NB-OSu (1.5x, 0.375 mmol, 90 mg) and DIEA (4x, 1 mmol, 87 µL) in minimal DMF. A Staudinger reduction involving triphenylphosphine (5 wt% in 90:10 tetrahydrofuran:$H_2O$, 20 mL) was utilized to reduce the N-terminal azide to a primary amine. The *o*NB-containing peptide was elaborated further by Fmoc-based methodologies to introduce Boc-GGGG-OH onto the resin-bound species at room temperature. The resin was washed with DMF (3x) and dichloromethane (DCM, 3x) prior to Mtt cleavage (2 min, 15 mL, 97:2:1 DCM:TIS:TFA, 9x). Resin was washed (DCM, 3x; DMF, 3x) prior to treatment (1 hr) with 4-formylbenzoic acid (4x, 1 mmol, 150 mg) by HATU coupling (3.95x, 0.988 mmol, 188 mg) and DIEA (8x, 2 mmol, 174 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product (H-GGGG-*o*NB-DDK(CHO)-$NH_2$) as a yellow solid (39 mg, 0.053 mmol, 21% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{47}H_{62}N_{11}O_{20}^-$ [M - $^1H]^-$, 1100.42; observed 1099.95.



**Protein expression and purification by STEPL**

LB (500 mL) supplemented with ampicillin (100 μg $mL^{-1}$) was inoculated with an overnight cell culture (10 mL) and incubated (37 °C) with agitation (250 rev $min^{-1}$). After reaching an optical density at λ = 600 nm of 0.6, isopropyl β-D-1-thiogalactopyranoside was added (final concentration of 0.5 mM) and expression was continued overnight under reduced temperature (18 °C).

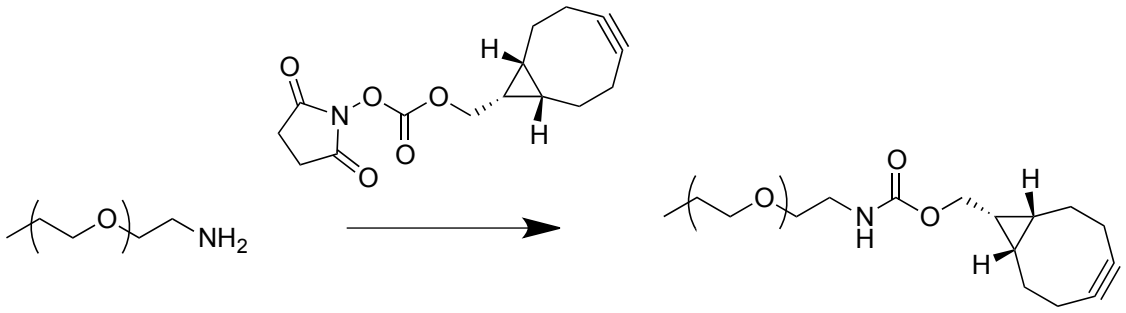
Cells were harvested *via* centrifugation (7,000 g, 10 min). The cell pellet was resuspended in lysis buffer (40 mL, 20 mM Tris, 50 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride) and sonicated on ice (6 cycles of 3 minutes at 30% amplitude 33% duty cycle and 3 min resting). Soluble and insoluble fractions were separated *via* centrifugation (5,000 g, 20 min).

Clarified lysate was applied to Ni-NTA resin (2.5 mL) and incubated under mild agitation (4 °C, 1 hr). The flow-through was discarded, and the resin was washed with wash buffer (20 mM Tris, 50 mM NaCl, 20 mM imidazole, 20 mL, 5x) and STEPL buffer (20 mM Tris, 50 mM NaCl, 20 mL, 1x). The polyglycine probe (20 molar excess) was added to resin in conjugation buffer (20 mM Tris, 50 mM NaCl, 100 μM $CaCl_2$, 2 mL) to promote intramolecular sortagging (37 °C, 4 hr).

The conjugated protein solution was collected, and the resin was washed with STEPL buffer (1 mL, 5x) to collect any remaining protein. The protein solution was dialyzed against STEPL buffer using ThermoFisher SnakeSkin Dialysis Tubing (molecular weight cut-off, MWCO ~ 10 kDa) to remove any unconjugated peptide and concentrated using an Amicon centrifugal spin column (MWCO ~ 10 kDa). Typical yields for purified proteins following STEPL were ~15 mg per liter of cell culture.

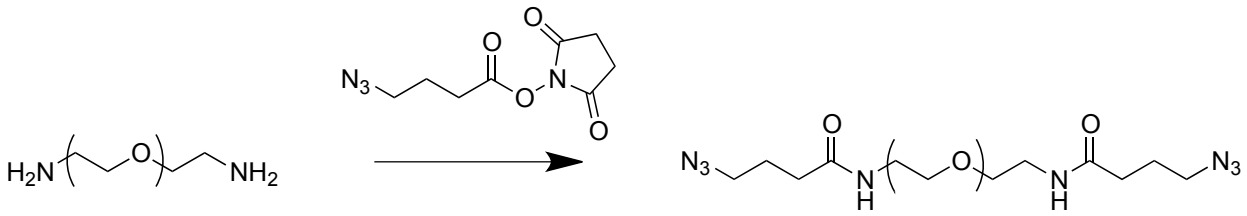
With 6 base proteins and 5 different polyglycine probes, a total of 30 sortagged proteins were generated. Identification codes denoting sortagged species identity are given in Supplementary Table 1.

**Synthesis of mPEG-BCN**



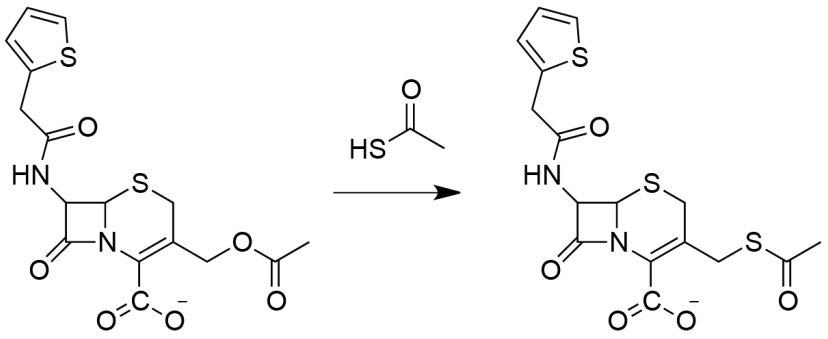
Methoxy poly(ethylene glycol) amine ($M_n$ ~ 10,000 Da, 200 mg, 0.02 mmol $NH_2$, 1x, Jenkem) and BCN-OSu (8.73 mg, 0.03 mmol, 1.5x) were dissolved in DMF (2 mL). DIEA (6.9 μL, 5.1 mg, 0.08 mmol, 4x) was added to the mixture, and the reaction was stirred overnight, dissolved in water, dialyzed (MWCO ~ 2 kDa, SpectraPor), and lyophilized to yield a white powder (200 mg, quantitative yield). $^1$H NMR (500 MHz, $CDCl_3$) δ 4.16 (d, 2H), 3.65 – 3.62 (m, 909H), 3.50 – 3.47 (m, 4H), 3.23 (s, 3H), 2.24 (m, 6H), 1.60 (m, 2H), 1.39 (m, 2H), 1.25 (m, 1H) 0.95 (m, 2H). Functionalization was found be >95% by $^1$H-NMR by comparing integral values for characteristic BCN peaks (δ 2.24, 1.60, 1.39, 0.95) with those from the methoxy peak (δ 3.23).

**Synthesis of PEG-diazide ($N_3$-PEG-$N_3$)**



Linear poly(ethylene glycol) diamine ($M_n$ ~ 3,500 Da, 1 g, 0.57 mmol $NH_2$, 1x, Jenkem) and $N_3$-OSu (194 mg, 0.86 mmol, 1.5x) were dissolved in dimethylformamide (5 mL). *N*,*N*-Diisopropylethylamine (398 µL, 294 mg, 2.28 mmol, 4x) was added to the mixture, and the reaction was stirred overnight, diluted in water (15 mL), dialyzed (MWCO ~ 2 kDa, SpectraPor), and lyophilized to yield a white powder (1.00 g, quantitative yield). $^1$H NMR (500 MHz, $CDCl_3$) δ 3.75 (m, 4H), 3.65-3.61 (m, 318H), 3.28 (m, 4H), 2.35 (m, 4H), 1.86 (m, 4H). Functionalization was found to be >95% by comparing integral values for hydrogens introduced upon azide coupling (δ 3.28, 2.35, 1.86) with those from the PEG backbone (δ 3.60-3.42).

**Synthesis of thioacetate cefalotin**



Thioacetate cefalotin was synthesized based on a known synthetic route[4]. Briefly, cefalotin (1x, 0.85 mmol, 357.5 mg), thioacetic acid (2.55 mmol, 194 mg), and sodium bicarbonate (3x, 2.55 mmol, 214 mg) were dissolved in water (10 mL) and stirred (30 hr) at elevated temperatures (50 °C). The reaction mixture was frozen, lyophilized, and redissolved in methanol (5 mL). The product was recrystallized in ice-cold diethyl ether (45 mL, 0 °C, 2x), isolated by vacuum filtration, washed with ethyl acetate, and dried over vacuum to yield the final product (thioacetate cefalotin) as a light brown solid (0.554 mmol, 224 mg, 64% yield). $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.64 (s, 1H), 6.99 (s, 2H), 5.55 (s, 1H), 5.03 (s, 1H), 4.02-3.99 (s, 2H), 3.90-3.86 (m, 3H), 3.84-3.81 (m, 1H), 2.38-2.34 (s, 3H).

**Plasmid construction for EGFP-EGF expression and STEPL purification**

A STEPL plasmid expression system was constructed for an EGFP-EGF fusion following the strategies outlined previously. PCR was used to amplify the gene sequences of EGFP and EGF and introduce relevant restriction sites (5' NdeI and 3' BamHI for EGFP and 5' BamHI and 3' XhoI for EGF) for cloning. The EGFP-EGF STEPL plasmid was purified and subsequently transformed into chemically competent BL21(DE3) *E. coli* (Promega) for expression.

Below is a list of DNA open reading frame sequences (5' → 3') generated for and utilized in these studies. Nucleotides shown in green correspond to EGFP, while those in purple correspond to EGF. Bases shown in orange correspond to STEPL portion, which features the C-terminal LPETG sortase recognition motif, a flexible $(GGS)_5$ linker, SrtA, and a 6xHis tag.

*EGFP*-*EGF*-*STEPL*:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG
GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGAC
CACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA
CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA
TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATG
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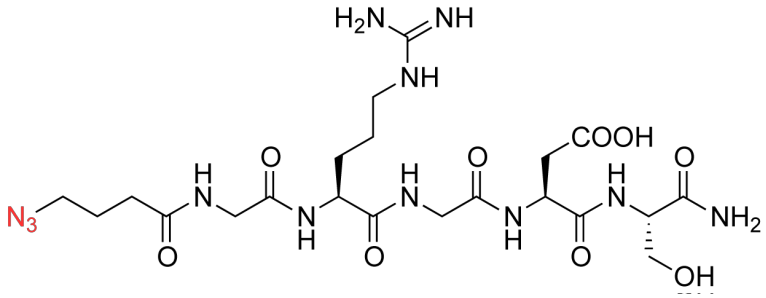
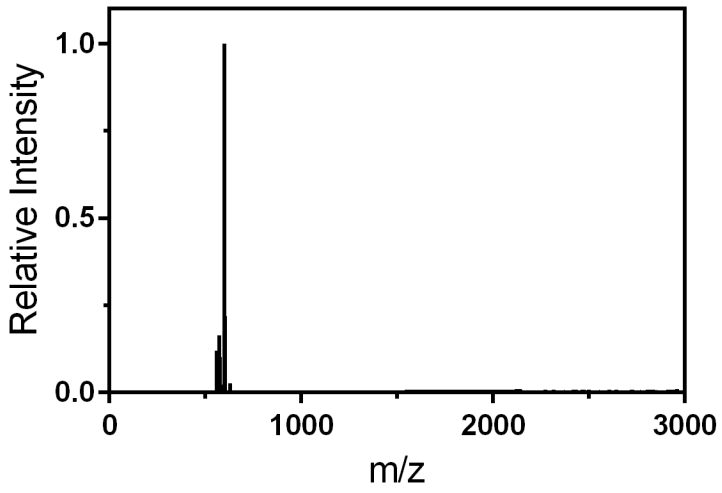
GGCGGTTCTGGTGGCAGTGGCGGTAGCCAAGCTAAACCTCAAATTCCGAAAGATAAATCAAAAGTGGCAGGCTATAT
TGAAATTCCAGATGCTGATATTAAAGAACCAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAA
GCTTTGCAGAAGAAAATGAATCACTAGATGATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCGAAC
TATCAATTTACAAATCTTAAAGCAGCCAAAAAAGGTAGTATGGTGTACTTTAAAGTTGGTAATGAAACACGTAAGTA
TAAAATGACAAGTATAAGAGATGTTAAGCCAACAGATGTAGAAGTTCTAGATGAACAAAAAGGTAAAGATAAACAAT
TAACATTAATTACTTGTGATGATTACAATGAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAA
GTCAAACATCACCACCATCATCACTAA

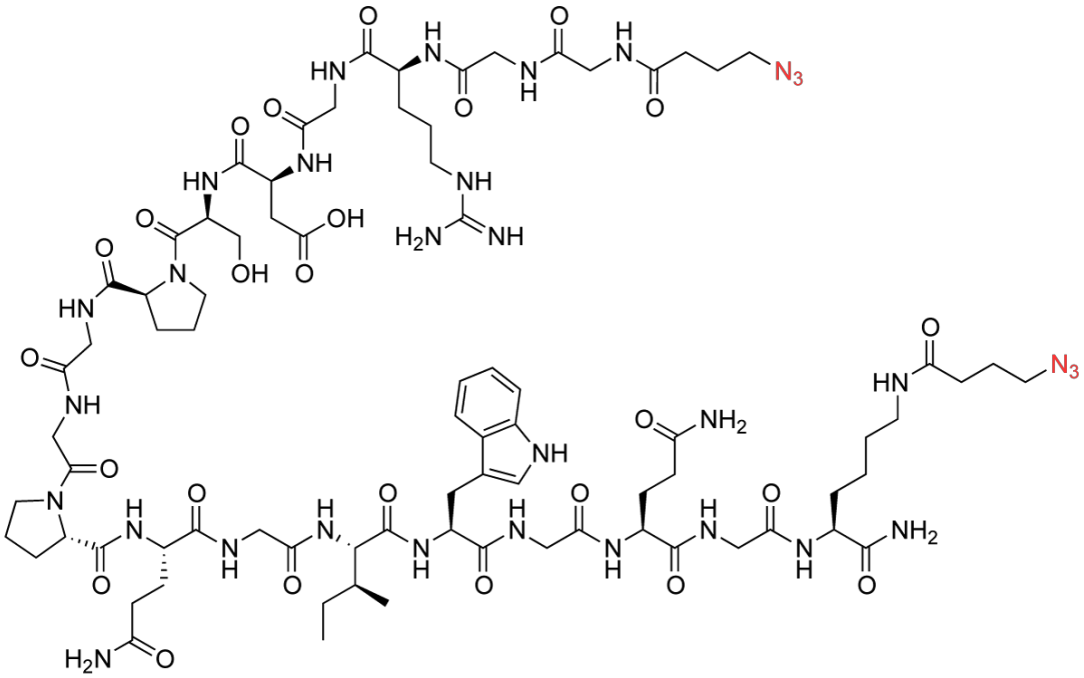
**Synthesis of $N_3$-GRGDS-$NH_2$**



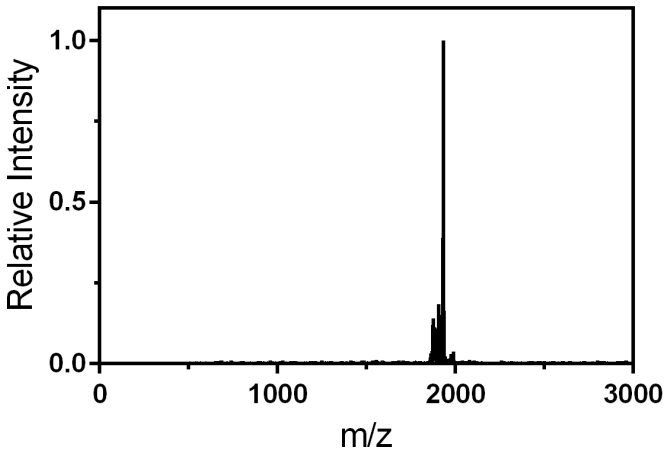
The resin-bound peptide H-GRGDS-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). Resin was washed (DMF, 3x) prior to treatment (1 hr) with $N_3$-COOH (4x, 1 mmol, 129 mg), HATU (3.95x, 0.988 mmol, 376 mg), and DIEA (8x, 2 mmol, 174 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product (denoted $N_3$-GRGDS-$NH_2$) as a white solid (72 mg, 0.12 mmol, 48% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{21}H_{37}N_{12}O_9^+$ $[M + {}^1H]^+$, 601.59; observed 600.86.



**Synthesis of $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$**

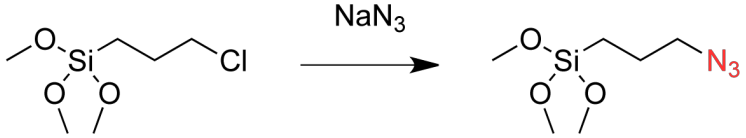


The resin-bound peptide H-GGRGDSPGGPQGIWGQGK(Dde)-$NH_2$ was synthesized by Fmoc SPPS (Supplementary Methods) on Rink amide resin (0.25 mmol scale). The resin was washed with DMF (3x) prior to Dde (1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl) deprotection (10 min, 30 mL, 2% hydrazine in DMF, 3x), yielding a peptide with free N-terminal amine and a free ε-amino group on the unprotected lysine. Resin was washed (DMF, 3x) prior to treatment (1 hr) with $N_3$-COOH (4x, 2 mmol, 258 mg), HATU (3.95x, 1.976 mmol, 752 mg), and DIEA (8x, 4 mmol, 348 μL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:$H_2O$, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified *via* RP-HPLC using a 55-minute gradient from 5-100% acetonitrile:$H_2O$; lyophilization yielded the final product [denoted $N_3$-GGRGDSPGGPQGIWGQGK($N_3$)-$NH_2$] as a light yellow solid (123 mg, 0.064 mmol, 25% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{80}H_{123}N_{32}O_{25}^+$ $[M + {}^1H]^+$, 1933.02; observed 1932.53.


Relative Intensity
1.0
0.5
0.0
0
1000
2000
3000
m/z

**Synthesis of azide-functionalized glass slides**

*Synthesis of (3-azidopropyl)trimethoxysilane*




(3-Chloropropyl)trimethoxysilane (11.9 mL, 12.9 g, 65.3 mmol), sodium azide (6.38 g, 98 mmol, 1.5x), and dry DMF (40 mL) were added to a flame-dried round-bottom flask (250 mL) and stirred overnight at 100 °C. The reaction was cooled to room temperature and diluted with diethyl ether:water (1:1, 150 mL). The organic layer was washed with water (3x) and brine before drying over $MgSO_4$. The mixture was filtered and concentrated under vacuum to yield product (3-azidopropyl)trimethoxysilane as a pale yellow oil (12.74 g, 95% yield). $^1H$ NMR (500 MHz, $CDCl_3$) δ 3.57 (s, 9H), 3.26 (t, J = 7.0 Hz, 2H), 1.71 (m, 2H), 0.70 (m, 2H). Characterization matched that previously reported[5].

*Surface cleaning and functionalization of glass slides*

Glass slides were cleaned (30 min) with piranha solution (35% $H_2O$, 15% $H_2O_2$, 50% $H_2SO_4$) before washing with water (3x) and acetone (3x). Dry slides were treated (90 min) with (3-azidopropyl)trimethoxysilane (70 mM) and n-butylamine (70 mM) in toluene (100 mL). Slides were rinsed with toluene (3x), wiped dry, and heated (80 °C) overnight. Azide-functionalized slides were stored at room temperature and used as needed.

**Supplementary Movie 1:**
Scanned XY planes of multiphoton "cell" pattern from Figure 4 **f-i** rendered using Imaris. A single gel was photopatterned to generate the given 3D image stack.

**Supplementary Movie 2:**
Scanned XZ planes of multiphoton "cell" pattern from Figure 4 **f-i** rendered using Imaris. A single gel was photopatterned to generate the given 3D image stack.

**Supplementary Movie 3:**
Time lapse FRET quantification of HeLa cells expressing EKAREV. Cells were treated with soluble EGF (100 ng in 600 μL total serum-free DMEM) at 3:00 minutes. Experiment was performed in triplicate ($n$ = 3) with similar results.

**Supplementary References**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