Supporting Information for the article:

Spatially Addressable Chemoselective C-terminal Ligation of an Intein Fusion Protein from a Complex Mixture to a Hydrazine-Terminated Surface

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Materials and Methods.

Materials and Reagents. $HSC_{11}(EG)_6OCH_2COOH$ (EG6-COOH) and $HSC_{11}(EG)_3OH$ (EG3-OH) were purchased from Prochimia (Poland). Glass cover slips, ethanol (200 proof), concentrated sulfuric acid, and hydrogen peroxide were purchased from VWR. Dimethyl formamide (DMF), sodium azide, Boc-protected 1-bromo-2-aminoethane, hydrochloric acid (HCl), Boc-(Trt)Cys-OH, trifluoroacetic acid, sodium chloride (NaCl), sodium hydroxide (NaOH), dioxane, tetrahydrofuran (THF), HEPES, PBS, copper sulfate hydrate, sodium ascorbate, propargylamine (alkyne-amine), 11-azido-3,6,9-trioxaundecan-1-amine (azide-amine), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(2-aminoethoxy) ethanol (AEE), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), dithiothreitol (DTT), 4-mercaptophenylacetic acid (MPAA) , and thioredoxin (Trx) used in control experiments were also obtained from Sigma. The HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) for surface plasmon resonance (SPR) was obtained from Biacore (GE). In order to use TCEP in protein samples, HBS-EP TCEP buffer was prepared by dissolving 50 mM TCEP in HBS-EP buffer and adjusted the pH to 7.4. The solution was then immediately stored at 4 degree and dark place. TCEP solution prepared and stored under such condition could be stable for a relatively long time, based on the statement from TCEP providers, Pierce and Sigma websites. Certain amount of HBS-EP TCEP buffer was added to samples to obtain desired concentration of TCEP in final protein buffer. MOPS-NaOH buffer was

prepared by dissolving 0.5 M 3-(N-morpholino)propanesulfonic acid (MOPS, Sigma) and 0.5 M NaCl in water, then adjusting the pH to 7 with concentrated NaOH. α-hydrazino-1-azido-2-aminoethane-ethanamide (referred to as hydrazine-azide) and cysteine-propargylamine-amide (referred to as cysteine-alkyne) were synthesized based on Raines' and Lin's reports, respectively,[1, 2] and described below. Thioredoxin (Trx)-intein-ELP, Trx-ELP, and Green Fluorescent Protein (GFP)-intein-ELP fusion proteins were expressed from pET32a vectors (Novagen) that harbored their genes in *E. coli* BLR cells and GFP-ELP fusion proteins were expressed from pET25b vector (Novagen) that harbored their genes in *E. coli* BLR cells. After expression, protein samples were purified by a non-chromatographic method –inverse transition cycling (ITC)– that we have developed for the purification of ELP fusion proteins.[3] An *E. coli* anti-Trx monoclonal antibody (mAb) was obtained from Assaydesign. A secondary antibody labeled with Alexa 647 fluorophore (Alexa 647-IgG) was purchased from Invitrogen. All proteins were aliquoted and stored at -20ºC. Reverse-osmosis purified water (Pureflow, Inc.) was used in all experiments.

Synthesis of hydrazine-azide. A detailed description of the procedure can be found in the literature.[1] First, Boc-protected 1-bromo-2-aminoethane was reacted with sodium azide in DMF for 12 h at 110°C to form Boc-protected 1-azido-2-aminoethane ($BocNHCH_2CH_2N_3$). Next, the Boc groups were removed by adding HCl (4N) in dioxane. The resulting free amine groups were then coupled with tri-Boc-protected α-hydrazino acetic acid [$(Boc)_2NN(Boc)CH_2COOH$] to produce Boc-protected hydrazine azide [$(Boc)_2NN(Boc)CH_2CONHCH_2CH_2N_3$], which was deprotected with HCl to yield the final α-hydrazino-1-azido-2-aminoethane-ethanamide ($H_2NNHCH_2CONHCH_2CH_2N_3$, referred to as hydrazine-azide, structure shown in Figure S1 and S3) used in the surface modification after purification with cation-exchange chromatography.

Synthesis of cysteine-alkyne. A detailed description of the procedure can be found in the literature.[2] First, Boc-(Trt)Cys-OH was reacted with propargylamine to form Boc-(Trt)Cys-alkyne. The resulting product was deprotected with trifluoroacetic acid (TFA) to remove Boc and Trt protecting groups. The final oil product, cysteine-propargylamine-amide (referred to as cysteine-alkyne, structure shown in Figure S2 and S3) was used for surface modification.

Surface modification steps to fabricate hydrazine surface. The glass cover slips were first cleaned with piranha solution, and then coated by a 45-nm thick gold layer with a 5-nm thick Cr adhesion layer between the glass and gold. ***Caution:*** *Piranha solution reacts violently with organic materials; it must be handled with extreme care, followed by copious rinsing with deionized water.* The gold-coated cover slips were stored in ethanol at -20 ºC until further use. For formation of alkanethiol self-assembled monolayers (SAMs), ethanol used to make the solutions was degassed for at least 30 min. The gold-

coated glass was cleaned by a UV-ozone cleaner (UVO cleaner, No. 42, Jellght Company Inc., CA, USA) for 2 min (the shortest distance from light source to glass surface was used during the cleaning process), then directly transferred into the thiol solution (2 mM total thiol concentration). The thiol solutions consisted of either pure EG6-COOH thiol in ethanol, or a mixture of EG6-COOH and EG3-OH thiols in ethanol. The solution was purged with N_2, sealed, and incubated overnight. The substrate was then rinsed with copious amounts of ethanol and sonicated in ethanol for 2 min. The SAM-modified gold substrate was then directly used in the next step or stored under N_2 at -20 ºC. For fabrication of the hydrazine-derivatized SAM, a pure EG6-COOH thiol SAM or a mixed EG6-COOH/EG3-OH thiol SAM was incubated in an EDC (0.1 M) / NHS (0.2 M) solution in water for 15 min to activate the -COOH groups. Next, the SAM was sequentially incubated in the following solutions at room temperature (RT) for 2 h: (1) 1 M alkyne-amine in THF; (2) 1 M AEE in HEPES buffer (50 mM, pH 8.0). Finally, the substrate was immersed in 100 mM hydrazine- azide solution in EDTA-free MOPS-NaOH buffer pH 7.4, with 0.5 mM copper sulfate and 7.5 mM sodium ascorbate added, and incubated overnight. Sodium ascorbate reduces Cu^{2+} to Cu^{1+}, and Cu^{1+} then catalyzes the click reaction. After the reaction, the substrate was soaked in HBS-EP buffer and shaken for 2 h to clean the surface thoroughly. The amount of hydrazine on the surface was controlled by the initial ratio of EG6-COOH to EG3-OH in the thiol solution during SAM formation. For example, 50% hydrazine-functionalized SAM refers to a mixed SAM in which the solution ratio of the EG6-COOH thiol to EG3-OH thiol was 1:1. There have been already a lot of papers addressing the relationship between the final SAM molar ratio on surface and the feeding thiol ratio in solution,[4] therefore, we will not explore this again in present work, considering it will be out of our key aim for this research.

Surface modification steps to fabricate cysteine surface. The modification steps to install the cysteine group on the SAMs were similar to those used for hydrazine derivatization. A gold-coated glass surface modified with a pure EG6-COOH thiol SAM or a mixed EG6-COOH/EG3-OH thiol SAM was first incubated in EDC (0.1 M) / NHS (0.2 M) in water for 15 min to activate the -COOH groups. Next, the substrate was sequentially incubated in the following solutions at room temperature for 2 h: (1) 1 M azide-amine in HEPES buffer (50mM, pH 7.4); (2) 1 M AEE in HEPES buffer (50 mM, pH 8.0). The substrate was then incubated in a 50 mM alkyne-cysteine solution in EDTA-free PBS (pH 7.4) with copper sulfate (0.5 mM) and sodium ascorbate (7.5 mM) overnight to complete the click reaction in high yield. The substrate was then soaked in HBS-EP containing 50 mM TCEP and shaken for 2 h to clean the surface and also to reduce any disulfide bonds. The surface concentration of cysteine on the surface was controlled by the initial ratio of EG6-COOH to EG3-OH in the thiol solution during SAM formation. For example, 50% cysteine refers to a mixed SAM in which the solution ratio of EG6-COOH to EG3-OH was 1:1.

Protein immobilization. The fusion proteins were expressed in *E. coli,* and the concentration was determined by UV-vis spectroscopy using the following calculated extinction coefficients[5] and molecular masses: Trx (13,940 $M^{-1}cm^{-1}$, 12 KDa), Trx-intein-ELP (33,360 $M^{-1}cm^{-1}$, 72 KDa), Trx-ELP (19,630 $M^{-1}cm^{-1}$, 60 KDa), GFP-intein-ELP (52,000 $M^{-1}cm^{-1}$, 88KDa), and GFP-ELP (22,000 $M^{-1}cm^{-1}$, 72KDa).

Protein immobilization for SPR experiments was accomplished according to the following procedures. For surface-triggered intein protein ligation on the hydrazine surface, purified Trx-intein-ELP fusion protein was reacted directly with the hydrazine SAM. For EPL on the cysteine surface, Trx was pre-cleaved from the Trx-intein-ELP fusion by adding either DTT (10 mM in HEPES pH 8.2 buffer, RT overnight) or MPAA (10 mM in HEPES pH 8.2 buffer, RT overnight) to the protein solution. The resulting Trx proteins modified with a thioester at the C-terminus were termed Trx-DTT and Trx-MPAA, respectively. Trx-DTT or Trx-MPAA was then separated from the mixture solution by ITC (details described below). The Trx-thioester sample was then used directly in the ligation experiment or stored at -20ºC for future use. All protein samples were characterized by SDS-PAGE with Coomassie Blue staining. Protein immobilization and antibody binding on the SAMs was measured by Surface Plasmon Resonance (SPR) spectroscopy. Protein solutions of various concentrations in HBS-EP buffer (pH 7.4, 0.5 mM TCEP) were injected into the flow cell at a flow rate of 1 μl/min, with a total injection volume of 100 μl. The surface was then rinsed with HBS-EP running buffer to remove non-specific adsorption. The SPR response unit (RU) increase indicated the extent of protein ligation. Antibody recognition was then evaluated by injecting anti-Trx mAb (1 μl/min, 100 μl) into the flow cell to bind to the Trx immobilized on the surface. For the EDC/NHS method, the EG6-COOH surface was activated with a freshly prepared EDC/NHS solution (5 μl/min, 75 μl) before Trx injection, and AEE was used to quench any unreacted sites (1 μl/min, 100 μl) after Trx injection.

Immobilization of proteins for the microcontact printing experiments was accomplished according to the following procedures. A gold-coated substrate was first cleaned by UV-ozone for 2 min, then patterned with EG6-COOH thiol by microcontact printing (μCP).[6] A drop of EG6-COOH (2mM in ethanol) was placed on a PDMS stamp surface for 5 min, then dried with N_2 gas for 3 min. The stamp was carefully put onto the gold surface and pressure was applied by hand for 25 s. Pressure was removed for 10 s, and then the stamp was carefully peeled away from the gold surface. The substrate was immediately incubated in an EG3-OH solution (2 mM in ethanol) overnight at RT to backfill the surface with an EG3-OH background, in order to render the background regions resistant to non-specific adsorption of proteins. The patterned, hydrazine-functionalized surface was obtained by following the remaining reaction sequence described above (hydrazine surface fabrication section). The substrate was then

incubated in the target protein solution of interest. For Trx immobilization experiments, the substrate was then incubated in anti-Trx mAb (0.01 mg/ml) for 2 h at RT, and then incubated in a solution of fluorescently-labeled secondary antibody (Alexa 647-IgG, 0.01 mg/ml) at 37ºC for 2 h to render the protein visible with fluorescence microscopy. Thorough rinsing with HBS-EP buffer (three cycles with each cycle consisting of at least 6 min continuous shaking) was performed between each step to remove any non-specifically adsorbed protein. For GFP immobilization, GFP was observed directly.

For protein printing experiments, hydrazine-functionalized substrates were obtained by the procedures described above (hydrazine surface fabrication section). Protein samples at different concentrations in printing buffer (PBS with 10% glycerol, 0.005% Triton X-100, and 50 mM TCEP added) were spotted using a contact mode microarrayer (AxSys MFC, Cartesian Tech) at room temperature and 80% humidity, and incubated in 80% humidity chamber for 40 hrs. The chip was then thoroughly rinsed with HBS-EP buffer (three cycles with each cycle consisting of at least 6 min continuous shaking). GFP spots were observed directly (with no fluorescent-molecule labeling) under the fluorescence microscope after rinsing, and Trx spots were visualized by further incubating in anti-Trx mAb (0.01 mg/ml) for 2 h at RT, and then a fluorescently-labeled antibody solution (Alexa 647-IgG) (0.005 mg/ml) at 37ºC for 2 h. Thorough rinsing with HBS-EP buffer (three cycles with each cycle consisting of at least 6 min continuous shaking) was performed between each step to remove any non-specifically adsorbed protein. For direct spotting from cell lysate solution, concentrated protein samples were diluted to the desired concentration with filtered cell lysate solution (BLR cells without pET plasmid that could lead to any expression of target proteins) with 10% glycerol, 0.005% Triton X-100, and 50 mM TCEP added.

Characterization. Protein concentrations were calculated from their UV-vis spectra (Nanodrop). SPR was performed on a Biacore X spectrometer (Biacore, GE). X-ray photoelectron spectra (XPS) were obtained on an AXIS Ultra (Kratos Analytical, NY) spectrometer with a monochromatic AlK_{α} X-ray source (1.4867 KeV) and a monochromator. Operational conditions for the X-ray source were 400μm nominal X-ray spot size operating at 15 kV, 8.9 mA for both survey and high-resolution spectra. Survey spectra, from 0 to 1200 eV binding energy (BE), were acquired at 100 eV pass energy with a step size of 1.0 eV, and a dwell time of 100 ms. High-resolution spectra were recorded at 20 eV pass energy with an energy step of 0.1 eV, a dwell time of 1.2 s, with a typical average of 50 scans. Fluorescence microscopy was performed with a Nikon Eclipse TE2000-U. The protein-immobilized surface was kept hydrated during fluorescence microscopy to prevent denaturation of the protein. For GFP observation, a B function filter set was used with the excitation wavelength at 488 nm and the emission wavelength at 509 nm. For Trx observation after labeling with Alexa 647-IgG, a Cy5 filter set was used with the excitation wavelength at 649 nm and the emission wavelength at 666 nm.

Fusion protein purification by ITC (Inverse Transition Cycling). The crude *E. coli* lysate containing Trx-intein-ELP, Trx-ELP, GFP-intein-ELP, or GFP-ELP protein was purified by the following cycles to get purified fusion protein:

1) Hot spin: 5M NaCl was added into the solution, and the resulting precipitate was collected by centrifuge spinning at room temperature for 10 min. The supernatant solution was then discarded and fresh HBS-EP buffer was added to resuspend the protein pellet.

2) Cold spin: After re-dissolving the protein into fresh buffer, the solution was centrifuged at 4°C for 10 min. Next, the transparent supernatant was transferred into a new tube.

3) Steps 1) and 2) were repeated at least seven times to guarantee high purity of the protein in buffer.

Trx-MPAA and Trx-DTT purification by ITC. The purified Trx-intein-ELP was diluted with HBS-EP (pH 7.4) containing 50 mM TCEP to 10 mg/ml, and then mixed with an equal volume of HEPES buffer (pH 8.2) containing 10 mM MPAA or DTT. MPAA or DTT was used to cleave the peptide bond between Trx and the intein to form Trx-MPAA or Trx-DTT, plus intein-ELP in the solution. The solution was incubated at RT overnight, and then at 37°C for 10 min. The increased temperature induced the phase transition of ELP and as a result, intein-ELP was precipitated. After centrifuge spinning at 37°C for 10 min, the intein-ELP pellet was discarded and the supernatant solution containing Trx-MPAA or Trx-DTT was used in subsequent protein ligation experiments or stored at -20°C.

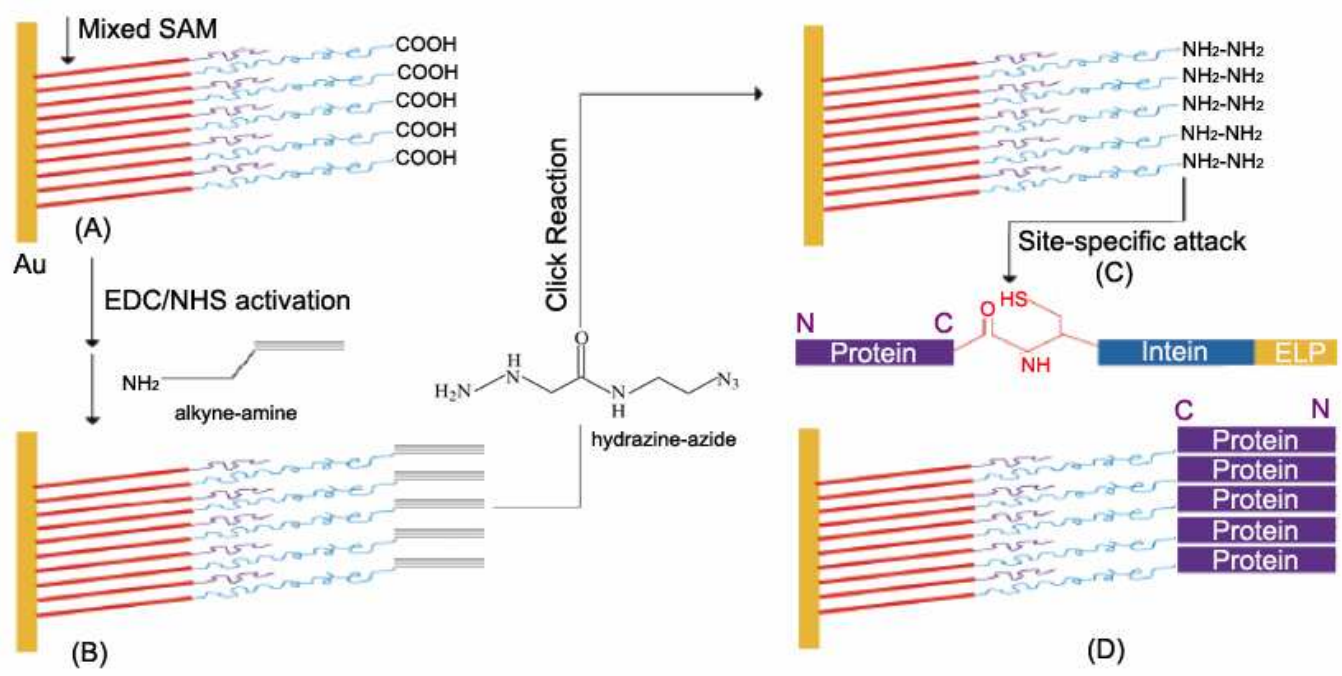

Figure S1. The detailed reaction mechanism for constructing a hydrazine surface, and the subsequent reaction with proteins: (A) a gold surface is first modified with a mixed SAM (EG6 and EG3); (B) after EDC/NHS activation of the carboxyl groups of EG6-COOH and coupling with an amine-terminated alkyne derivative, the surface is modified with an alkyne functional group; (C) the click reaction between the alkyne surface and the azide-terminated hydrazine results in a hydrazine-modified surface; and (D) the subsequent site-specific attack on the unnatural bond between the C-terminus of the protein and the N-terminus of the intein results in the site-specific attachment of the proteins to the surface with uniform orientation.

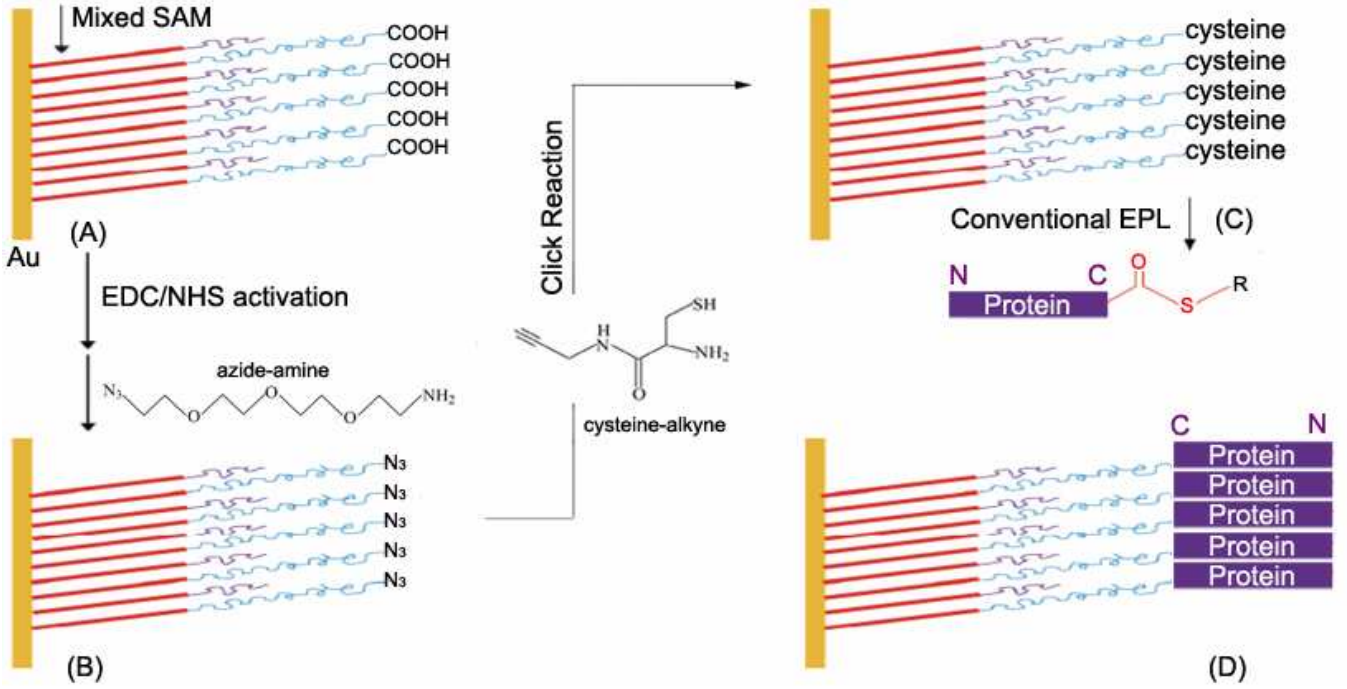

Figure S2. The detailed reaction mechanism for constructing a cysteine surface and the subsequent reaction with proteins: (A) a gold surface is first modified with a mixed SAM (EG6 and EG3); (B) after EDC/NHS activation of the carboxyl groups of EG6-COOH, and coupling with an amine-terminated azide derivative, the surface is modified with an azide functional group; (C) the click reaction between the azide surface and the alkyne-terminated cysteine results in a cysteine-modified surface, and (D) the sequential site-specific reaction with the thioester functional group on the C terminus of the target protein results in the site-specific attachment of the proteins to the surface with uniform orientation.

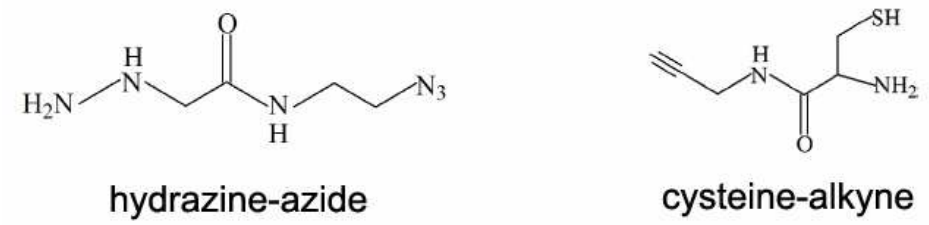

Figure S3. The chemical structures of hydrazine-azide and cysteine-alkyne used for surface modification.

Table S1. C, O, and N XPS experimental and stoichiometric (if 100% reaction yield were obtained) N/C signal ratios. The reaction yields for hydrazine and cysteine surface modification reactions were calculated by the ratio of the N/C (experimental) to N/C (stoichiometric).

Surface	C_{1s} (%)	O_{1s} (%)	N_{1s} (%)	N/C	N/C	Yield (%)
				experimental	stoichiometric	
EG6	73.6	25.6	0	-	-	-
Alkyne	73. 5	24.2	2.3	0.031	0.036	86
Hydrazine	67.8	22.0	10. 4	0.158	0.218	73
Azide	71.8	22.8	5.4	0.075	0.121	63
Cysteine	68.1	22.1	8.2	0.120	0.154	78

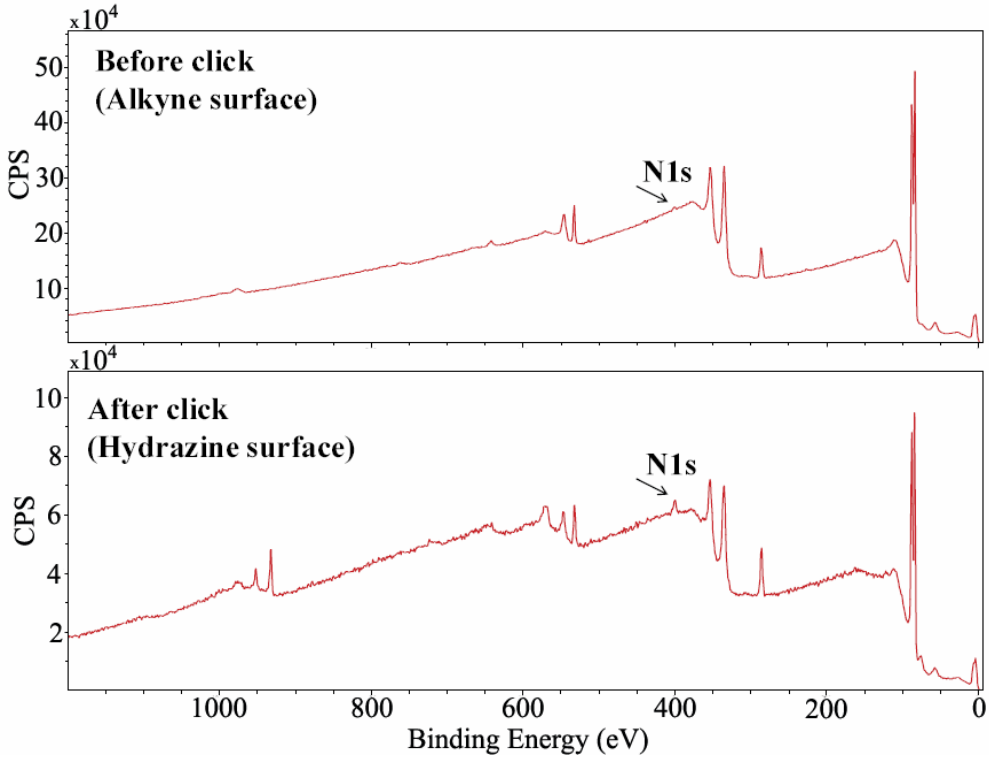

Figure S4. The XPS survey spectra of the surfaces before and after the click reaction to fabricate the hydrazine surface. In the spectrum of the surface before the click reaction (alkyne-grafted surface, top spectrum), only a small N_{1s} peak was observed, due to one carbonyl amide group between EG6-COOH and the alkyne groups in the SAM. In the spectrum of the surface after the click reaction (hydrazine-grafted surface, bottom spectrum), there was a much higher N_{1s} signal because the hydrazine-azide molecule containing five N atoms was introduced onto the surface.

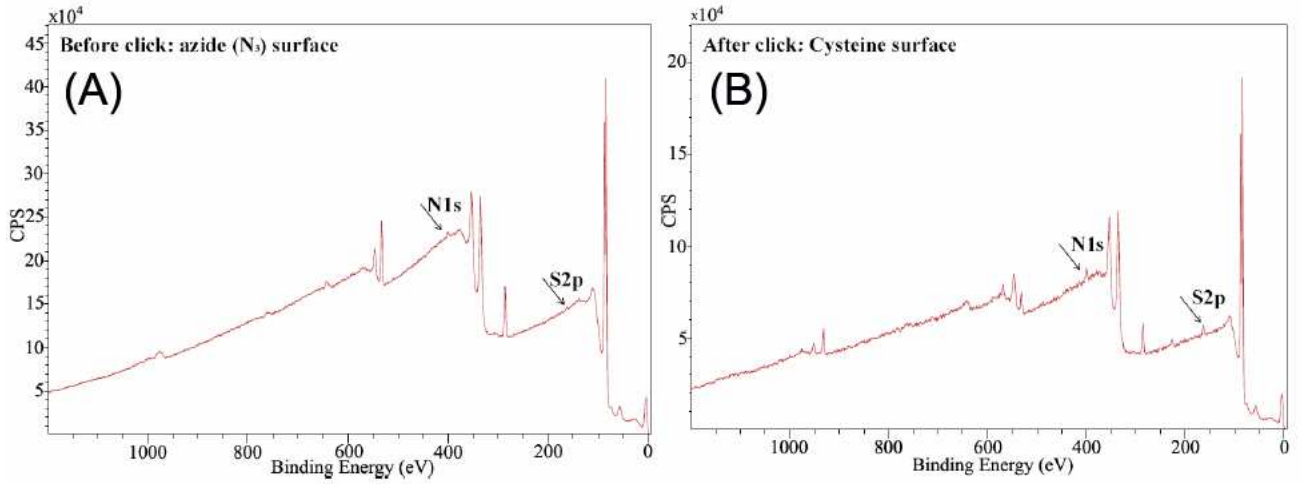

Figure S5. The XPS survey scan of (A) the azide surface (before click reaction) and (B) the cysteine surface (after click reaction with alkyne-cysteine). The N_{1s} and S_{2p} peaks, which were assigned to azide and Au-sulfur groups, can be seen in (A). The N_{1s} corresponds to the carbonyl amide and azide nitrogens. (B) is the XPS survey scan of the cysteine surface, which shows a larger N_{1s} signal due to the increased number of nitrogens on the surface. More importantly, the S_{2p} signal is also larger because cysteine sulfur groups were introduced onto the surface. Moreover, unlike the Au-sulfur bond, which was buried in the surface layer, the cysteine groups are at the solid-vacuum interface, resulting in a higher S_{2p} signal.

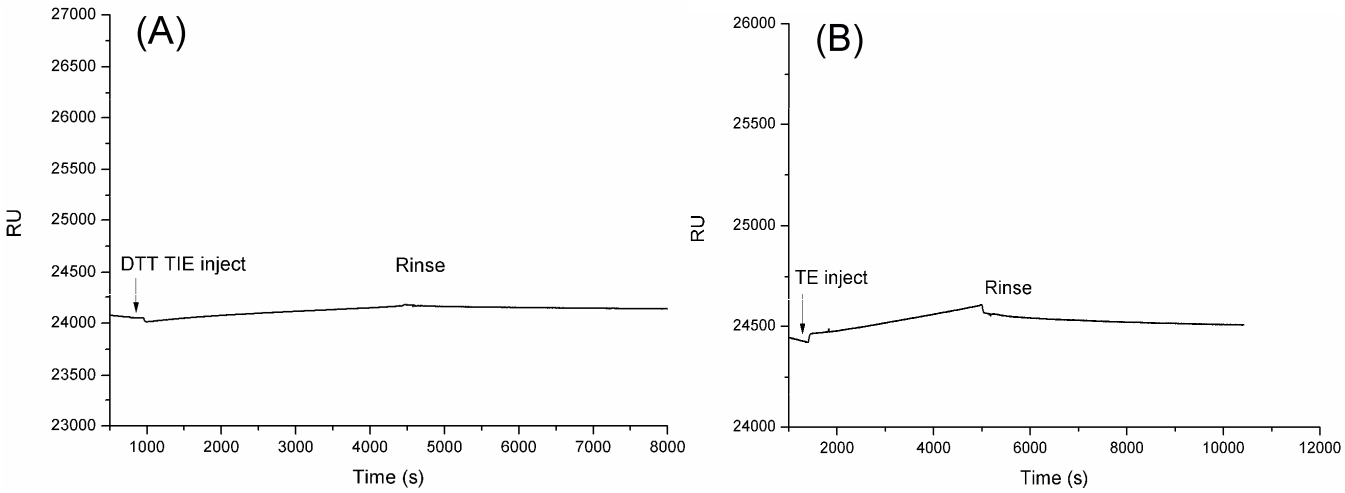

Figure S6. SPR curves for two control experiments for the hydrazine-intein method: (A) Trx-intein-ELP (TIE) cleaved with DTT prior to injection over a 50% hydrazine surface. After cleavage with DTT, the active thioester bond between the C-terminus of Trx and the N-terminus of the intein is no longer available to react with the hydrazine moiety on the surface. (B) Trx-ELP (no intein, TE) injected over a 25% hydrazine surface. The protein concentrations were 0.01 mg/ml, for 100min at 1 µl/min flow rate. ΔRU for both samples was lower than 100 RU.

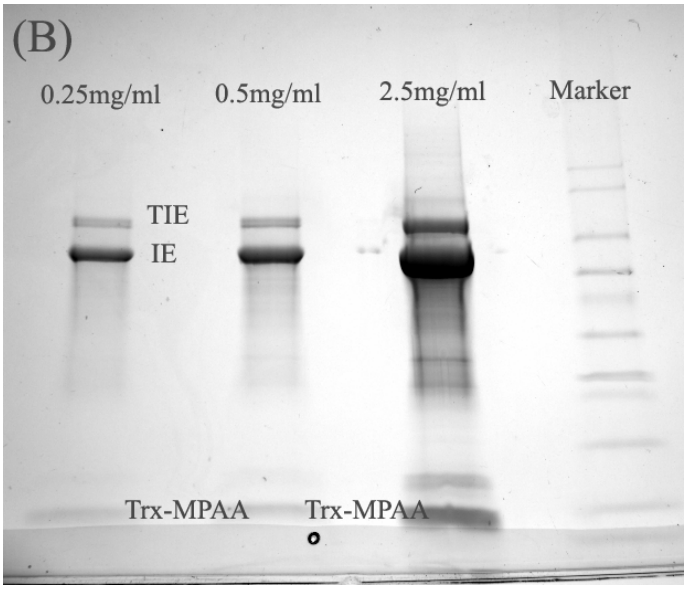

Figure S7. SDS-PAGE gels showing the conversion of Trx-intein-ELP (TIE) to Trx-thioester using (A) DTT (Trx-DTT) and (B) MPAA (Trx-MPAA). In (A), lane 1 corresponds to the fusion protein, showing a band for Trx-intein-ELP and a second band for the *in vivo* cleavage product intein-ELP (IE). Lane 2 corresponds to the fusion protein after the addition of DTT. The disappearance of the Trx-intein ELP band and the appearance of a Trx band suggests that all of the Trx was cleaved from the fusion protein by DTT. Lane 3 corresponds to commercially available Trx, while lanes 4 and 5 correspond to Trx-DTT and intein-ELP, respectively, after separation of the two proteins by ITC. The SDS-PAGE gel shown in (B) was loaded with different concentrations of Trx-intein-ELP after the addition of MPAA. Cleavage efficiencies were estimated at 98% and 67% for DTT and MPAA, respectively, based on band intensities.

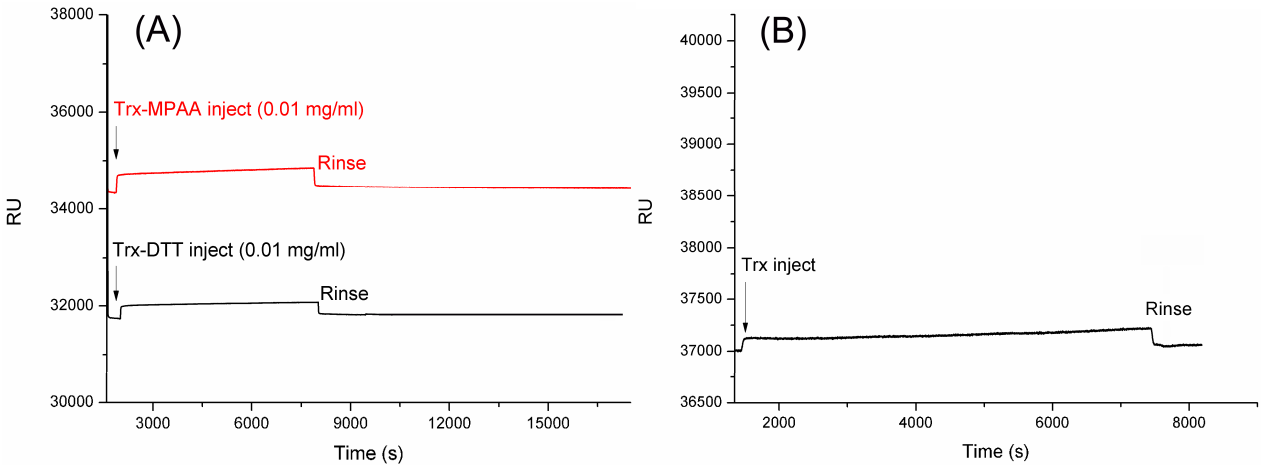

Figure S8. (A) SPR curves showing minimal binding of Trx-DTT and Trx-MPAA to a 50% cysteine surface using protein concentrations of 0.01 mg/ml. (B) Control experiment in which commercial Trx (no thioester functionality) was injected over a 50% cysteine surface.

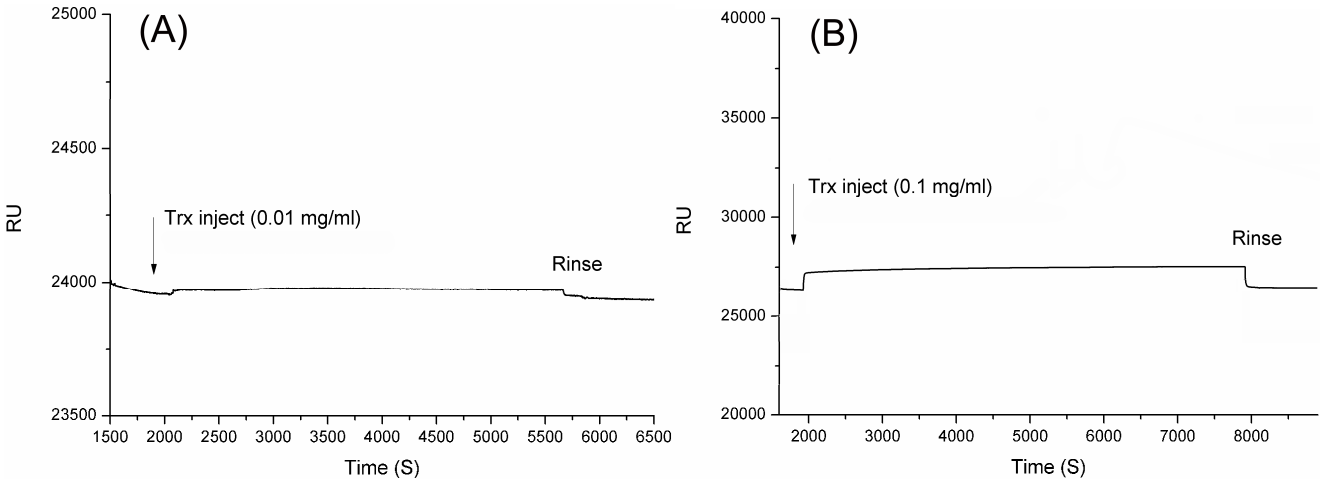

Figure S9. SPR curves showing minimal binding of 0.01 mg/ml (A) and 0.1 mg/ml (B) Trx to a 50% EG6-COOH SAM on gold via EDC/NHS coupling.

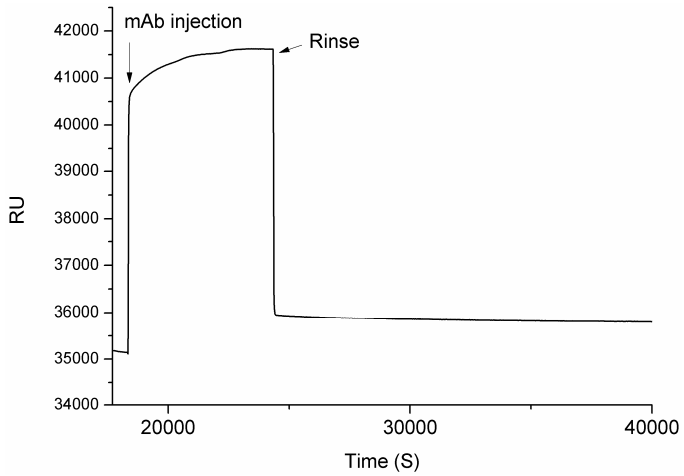

Figure S10. A typical SPR curve of mAb-Trx binding to Trx-thioester immobilized on cysteine surface through conventional EPL. The antibody solution (0.1 mg/ml, 1µl/min, 100µl) was injected subsequently after Trx-MPAA thioester binding finishing over cysteine surface.

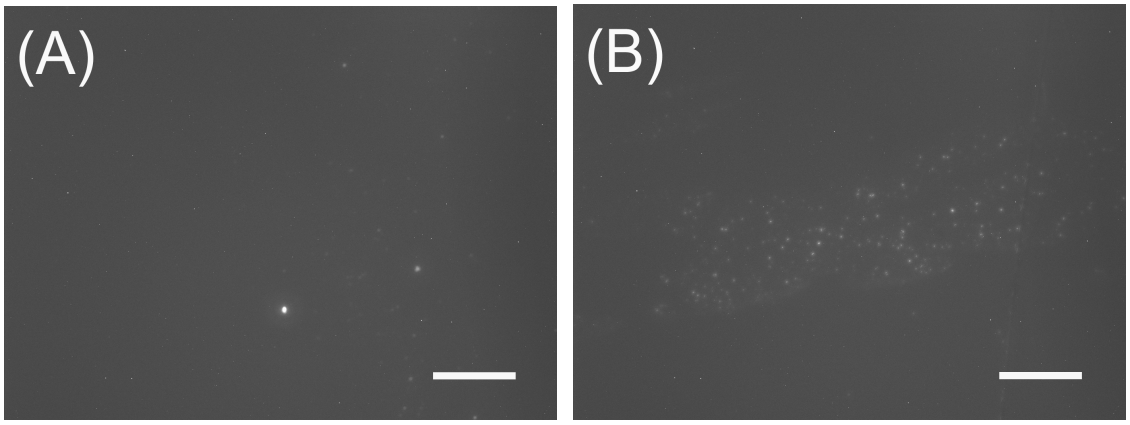

Figure S11. Control experiments: (A) fluorescence microscopy image of a micropatterned hydrazine surface after sequentially incubating in Trx-ELP (no intein), anti-Trx mAb, and Alexa 647-IgG (fluorescently-labeled secondary antibody) solutions under the same conditions as the Trx-intein-ELP experiment; (B) fluorescence microscopy image of micropatterned hydrazine surface after sequentially incubating in a solution of Trx-intein-ELP where the intein was cleaved from the fusion protein by DTT prior to interaction with the surface, anti-Trx mAb, and Alexa 647-IgG solutions under the same conditions as the Trx-intein-ELP experiment. Scale bars are equal to 50 μm.

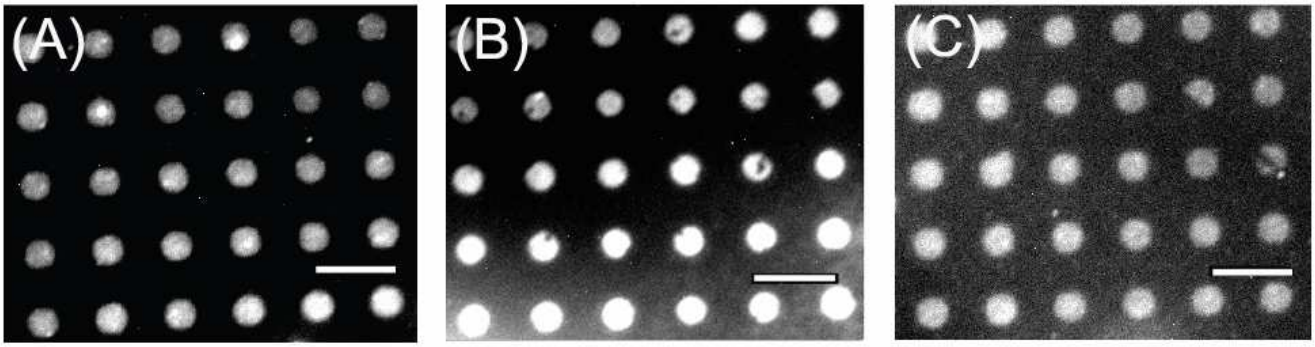

Figure S12. Micropatterning of different concentrations of Trx directly from cell lysate: surfaces patterned with hydrazine SAMs were incubated in *E. coli* lysate that was spiked with (A) 0.1mg/ml (1 μM), (B) 0.01 mg/ml (100 nM), and (C) 0.001 mg/ml (10 nM) Trx-intein-ELP fusion protein. After 2 h incubation at RT, the surfaces were then incubated sequentially in anti-Trx mAb (0.01 mg/ml, RT, 2 h), and Alexa 647-IgG (fluorescently-labeled secondary antibody, 0.01 mg/ml, 37ºC, 2 h) solutions in HBS-EP buffer. The final protein micropattern was viewed under a fluorescence microscope with a Cy5 filter set. The scale bar in all images is 50 μm.

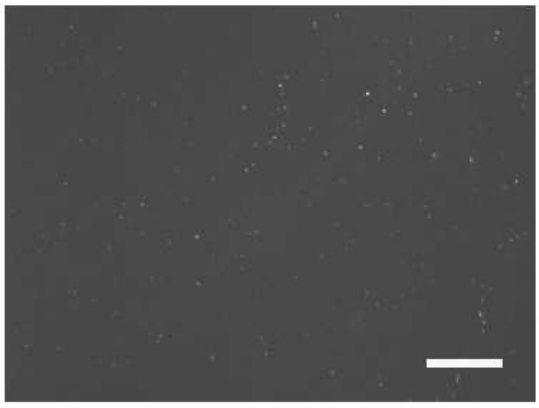

Figure S13. Control experiment: the fluorescence microscopy image of a micropatterned hydrazine surface after sequentially incubating in crude cell lysate with no Trx-intein-ELP, then anti-Trx mAb, and Alexa 647-IgG solutions under the same conditions as the Trx-intein-ELP spiked cell lysate experiment shown in Figure S12. Scale bar is equal to 50 μm.

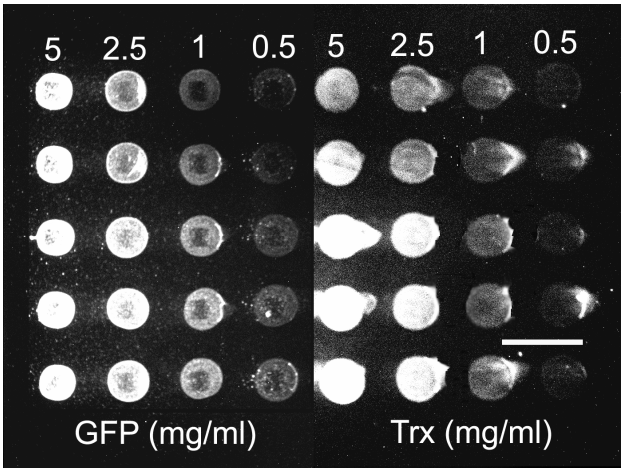

Figure S14. Contact spotting of both GFP-intein-ELP and Trx-intein-ELP on a single hydrazine-functionalized substrate directly from cell lysate at different concentration from 5 mg/ml to 0.5 mg/ml. The final protein micropattern was viewed under a fluorescence microscope with a B function filter set for GFP and a Cy5 filter set for Trx. The scale bar in the image is 500 μm.

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