Electronic supplementary information

Localized molecular automaton for *in situ* visualization of protein with specific chemical modifications

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Experimental procedures

Materials and reagents. Click-iT® L-homopropargylglycine (HPG, for installation of alkyne-modified proteins to cells), Click-iT® tetraacetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz, for the metabolic introduction of azide-modified sialic acid, i.e. azide Sia, onto cell surface), RPMI-1640 (no methionine), fetal bovine serum (FBS), lipofectamine™ 3000 transfection reagent, NuPAGE™ LDS sample buffer (4×), Pierce™ IP lysis buffer, Pierce™ Protein G agarose, SYBR™ gold nucleic acid gel stain (10,000×), Orange DNA loading dye (6×), and Opti-MEM were purchased from Thermo Fisher Scientific Inc. (USA). Ethylenediaminetetraacetic acid (EDTA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), bovine serum albumin (BSA), tris(hydroxymethyl)aminomethane (Tris), copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), (+)-sodium L-ascorbate, tris(2-carboxyethyl) phosphine hydrochloride (TCEP), DBCO-Cy5, azide-PEG_3-biotin, boric acid, methanol, TWEEN® 20, DMSO, Triton X-100 and L-methionine (Met) were purchased from Sigma-Aldrich Inc. (USA). α2-3,6,8,9 Neuraminidase A (NEU), GlycoBuffer (10×), nicking endonuclease (NE, Nt.BbvCI), and CutSmart® buffer (10×) were purchased from New England Biolabs (USA). Tunicamycin (TM), anti-EpCAM antibody, anti-GAPDH antibody, anti-biotin antibody, anti-EGFR antibody (Ab) and Human TROP1 ELISA Kit (EpCAM) were provided by Abcam PLC Co., Ltd. (USA). 2-(4-((Bis((1-(*tert*-butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid (BTTAA) was acquired from Click Chemistry Tools LLC. (USA). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG) was obtained from Santa Cruz Biotechnology Inc. (USA). Phosphate buffer saline (PBS, pH 7.4, containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, 1.41 mM KH_2PO_4), phosphate buffer saline with Ca^{2+} and Mg^{2+} (PBSM, pH 7.4, containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, 1.41 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$), MCF-7 cells, HeLa cells, RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM), trypsin, EpCAM siRNA (sense, 5'-GGUGAAUCCUUGUUUCAUUdTdT-3', antisense, 5'-AAUGAAACAAGGAUUCACCdTdT-3'), and control siRNA (sense, 5'-GUGAGCGUCUAUAUACCAUdTdT-3', and antisense, 5'-AUGGUAUAUAGACGCUCACdTdT-3') were supplied by KeyGen Biotech Co., Ltd. (China). Bicin-choninic acid (BCA) protein assay kit, BSA blocking buffer, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG were purchased from Cwbio Co., Ltd. (China). Sodium dodecyl sulfate (SDS) was obtained from Nanjing Chemical Reagent Co., Ltd. (China). Acryl/bis 30% solution (29:1), RIPA lysis buffer, protease inhibitors (100×), phosphatase inhibitors (100×), penicillin-streptomycin solution (100×), RealBand 3-color broad range protein marker (3.5-245 kDa), ammonium persulfate, Tris-HCl/SDS buffer (4×, pH 6.8), Tris-HCl/SDS buffer (4×, pH 8.8), Tris-glycine gel running buffer (10×, pH 8.3), Western transfer buffer (10×, pH 8.3), TBS buffer (20×, pH 7.4, containing 500 mM Tris-HCl, 2.8 M NaCl, 60 mM KCl), 4% paraformaldehyde (PAF) fix solution, goat serum, epidermal growth factor (EGF) and all the DNA sequences were obtained from Shanghai Sangon Biotech. Inc. (China). AccuWest ECL Blaster Western blotting HRP substrate was obtained from Bioelite Co., Ltd. (China). Magnesium chloride hexahydrate was obtained from Aladdin Co., Ltd. (China). Acetone was obtained from Sinopharm Chemical Reagent Co., Ltd. (China). 2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl (4-nitrophenyl) carbonate was kindly provided by Prof. Xiaojian Wang from Nanjing Tech University. TBE buffer (5×, pH 8.3, containing 445 mM Tris-boric acid and 10 mM EDTA), PBS blocking buffer (PBS containing 5 mM $MgCl_2$ and 10% goat serum), PBS washing buffer (PBS containing 1% FBS and 5 mM $MgCl_2$), PBS binding buffer (PBS containing 5 mM $MgCl_2$) and other aqueous solutions were prepared using ultrapure water (≥ 18 MΩ, Milli-Q, Millipore).

Apparatus. The UV-vis absorption spectra were acquired on a UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu, Japan). The fluorescence spectra were acquired on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence micrographs of cells were acquired on an SP8 STED 3X confocal laser scanning microscopy (CLSM) (Leica, Germany). Cell number was determined with a Countess® II Automated Cell Counter (Thermo Fisher Scientific, USA). BCA measurement was performed on a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, USA). Native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE analysis were performed on an Electrophoresis Analyzer (Bio-Rad, USA) and imaged on a Bio-Rad ChemDoc XRS facility (Bio-Rad, USA). Protein transfer to PVDF membranes was performed on a Trans-Blot Turbo Transfer System (Bio-Rad, USA).

Cell culturing. MCF-7 cells were cultured in RPMI-1640 supplemented with 10% FBS, streptomycin (0.1 mg/mL), and penicillin (0.1 mg/mL). HeLa cells were cultured in DMEM supplemented with 10% FBS, streptomycin (0.1 mg/mL), and penicillin (0.1 mg/mL). Cells were grown in a 37 ºC incubator containing 5% CO_2 and subcultured after reaching confluence.

***In vitro* PAGE analysis.** A PBSM solution containing hairpin DNA **S'-S** or **S'-S-F** was annealed in a thermocycler over a temperature gradient (heated at 95 ºC for 5 min, then cooled to 25 ºC at 0.5 ºC/min) in approximately 3 h and then stored at 4 ºC until use. DNA mixtures containing **H'** and **H**, **P'** and **P-Apt**, or **P-Apt** and **S'-S** were prepared via the same procedure as that for the single-stranded hairpin DNA, respectively.

All the samples were prepared in 1×CutSmart buffer and incubated at 37 ºC for 2 h, and then 10 μL of each sample was mixed with 2 μL loading dye (6×). The mixtures were then characterized by native PAGE (12%). Gels were run in 1× TBE buffer for 70 min at 100 V, and then stained with 1× SYBR™ gold nucleic acid gel dye (diluted from 10,000× dye with 1× TBE buffer) at room temperature (r.t.) on a shaker at about 70 rpm for 30 min, and finally photographed under UV irradiation on a Bio-Rad ChemDoc XRS facility. For samples containing **S'-S-F** or **F-P-Apt**, the gels were imaged under UV irradiation directly without staining.

***In vitro* fluorescence analysis.** The hairpin DNA **Q-S'-S-F** and DNA mixtures containing **Q-H'** and **H-F**, **P'-Q** and **F-P-Apt**, **H'** and **H**, or **P'** and **P-Apt** were thermally annealed by the same procedure as that used for PAGE analysis, respectively. All the samples were prepared in 1×CutSmart buffer and incubated at 37 ºC for 2 h. The fluorescence emission spectra of all the samples were recorded with the excitation set as 494 nm. The excitation and emission slits were both set to 10.0 nm.

CLSM imaging of aptamer binding to EpCAM. MCF-7 (EpCAM-positive) and HeLa (EpCAM-negative) cells were separately seeded on four-well confocal dishes and cultured overnight. After washing three times with PBS washing buffer, cells were blocked with PBS blocking buffer at 4 ºC for 30 min. After washing three times with PBS washing buffer, the cells were incubated respectively with **F-P-Apt** (1.0 μM), annealed **P'•F-P-Apt** (1.5/1.0 μM), and **F-P-Ran** (1.0 μM) in PBS binding buffer at 4 ºC for 30 min. Then the

cells were washed three times with PBS washing buffer and the fluorescence of FAM on cell surface was imaged with CLSM at stationary parameters including the laser intensity, exposure time and objective lens. Under the excitation at 494 nm with white light laser, the emission was collected from 500 to 550 nm. All images were digitized and analyzed by Leica Application Suite X (LAS X) software. In the case of CLSM imaging of mucin 1 (MUC1)-specific aptamer (**Apt_M**) binding to cell surface MUC1, the seeded MCF-7 cells were incubated respectively with **F-Apt_M** (1.0 μM), **S'-S-F-Apt_M** (1.0 μM), and **H-F-Apt_M** (1.0 μM) in PBS binding buffer at 4 °C for 30 min, with other conditions unchanged.

To demonstrate the **P'•P-Apt** was anchored on cell surface in a duplex structure, the seeded MCF-7 cells were subjected to incubation with annealed **P'-Cy3•Cy5-P-Apt** (1.5/1.0 μM) in PBS binding buffer at 4 °C for 30 min. Then the cells were washed and imaged with CLSM. For Cy5, Ex: 640 nm, Em: 650-720 nm; for Cy3, Ex: 550 nm, Em: 555-605 nm.

Incorporation of azide Sia (C_S) on cell surface. MCF-7 cells were seeded on four-well confocal dishes and cultured overnight. Cells were incubated in Met-free RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution (0.1 mg/mL), Ac_4ManNAz (40 μM) and Met (50 μM) for 48 h in a 37 °C CO_2 incubator, followed by three-time washing with PBS washing buffer and 30-min blocking with PBS blocking buffer at 4 °C. In control experiments, Ac_4ManNAz was not added.

CLSM imaging validation of C_S labeling by S'-S-F-DBCO. C_S labeling was achieved by reaction with **S'-S-F-DBCO** through Cu(I)-free click chemistry using **S'-S-F** as control. Cells after incorporation of C_S were subjected to reaction with annealed **S'-S-F-DBCO** (10 μM) or **S'-S-F** (10 μM) in PBS binding buffer at 4 °C for 30 min. Then the cells were washed three times and visualized with CLSM.

CLSM imaging of incorporated C_S after different Ac_4ManNAz incubation time. MCF-7 cells were treated with Ac_4ManNAz (40 μM) for different time (0, 12, 24, 36, 48 h) in a 37 °C CO_2 incubator. This was followed by Cu(I)-free click reaction with **S'-S-F-DBCO** (10 μM) and CLSM imaging.

Incorporation of HPG (C_H) on cell surface. MCF-7 cells were seeded on four-well confocal dishes, cultured overnight, and then incubated in Met-free RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin solution (0.1 mg/mL) for 30 min to deplete endogenous Met. Then cells were incubated in Met-free RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution (0.1 mg/mL), and HPG (50 μM) for 48 h in a 37 °C CO_2 incubator. After three-time washing with PBS washing buffer, the cells were blocked with PBS blocking buffer at 4 °C for 30 min, and ready for the following probe anchoring step. In control experiments, HPG was replaced by Met (50 μM).

CLSM imaging validation of C_H labeling by H-F-N_3, H'•H-F-N_3 or Cy3-H'•H-Cy5-N_3. The binding of **H-F-N_3**, **H'•H-F-N_3** or **Cy3-H'•H-Cy5-N_3** with cell surface C_H was achieved through Cu(I)-catalyzed click chemistry. Cells after HPG incorporation were treated with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), **H-F-N_3** (10 μM) (or annealed **H'•H-F-N_3** (15/10 μM), annealed **Cy3-H'•H-Cy5-N_3** (15/10 μM) or **H-F** (10 μM)), and freshly prepared sodium ascorbate solution (2.5 mM) in PBS binding buffer at r.t. for 5 min. Then the cells were washed three times and imaged with CLSM.

CLSM imaging of incorporated C_H after different HPG incubation time. MCF-7 cells were treated with HPG (50 μM) for different time (0, 12, 24, 36, 48 h) in a 37 °C CO_2 incubator, followed by Cu(I)-catalyzed click reaction with **H-F-N_3** (10 μM) and CLSM imaging.

Demonstration of C_H installation on EpCAM by Western blotting analysis and immunoprecipitation (IP). MCF-7 cells were subjected to HPG incorporation procedure as described before (without blocking step), using Met (50 μM) as control. Then the two samples were lysed with a mixture of cold IP lysis buffer, protease inhibitors and phosphatase inhibitors. The lysates were centrifuged (13,000 rpm) at 4 °C for 20 min and the supernatants were stored at -20 °C until use. Then the protein concentration was determined using BCA protein assay kit. Subsequently, the lysates (equivalent to 1 mg protein each) were incubated in 1 mL IP lysis buffer containing $CuSO_4$ (1 mM), BTTAA (600 μM), azide-PEG_3-biotin (100 μM), and freshly prepared sodium ascorbate solution (2.5 mM) at r.t. for 2 h. After the reaction was terminated by addition of a 4-time volume of pre-chilled acetone, the samples were placed at -20 °C for 30 min and then centrifuged (13,000 rpm) at 4 °C for 5 min to precipitate proteins. The supernatants were discarded, and the pellets were washed twice with pre-chilled methanol, air dried, and resuspended in PBSM containing 1% SDS for further Western blotting and IP analysis.

After determination of the protein concentrations using BCA protein assay kit, the two samples were respectively mixed with LDS sample buffer (4×) (v/v: 3/1) and then heated at 95 °C for 5 min. Subsequently, the proteins were separated by SDS-PAGE, and transferred to PVDF membranes. The PVDF membranes were immersed in BSA blocking buffer, shaken at about 70 rpm for 2 h, and respectively subjected to incubation with anti-biotin and anti-GAPDH antibody in 1× TBST buffer (TBS containing 0.1% Tween® 20) containing 3% BSA at 4 °C overnight. Then the membranes were shaken at r.t. for 30 min and washed three times with 1× TBST for 10 min. Subsequently, the membranes were incubated with secondary HRP-conjugated antibody (diluted with 1× TBST buffer containing 3% BSA) at r.t. for 60 min, and washed three times with 1× TBST for 10 min. Finally, ECL luminescent working solution was evenly placed on the surface of the membranes and the membranes were imaged using a Bio-Rad ChemDoc XRS imaging system.

For IP, the two samples with the same amount of protein were subjected to incubation with anti-EpCAM antibody (1:40) at 4 °C overnight, respectively. Then 100 μL of protein G agarose resin slurry was added to each antigen-antibody complex and incubated for 4 h at r.t. under gentle mixing. Subsequently, the mixtures were centrifuged at 2,500 g for 3 min and washed three times with 0.5 mL of IP lysis buffer. Then the resins were mixed with LDS sample buffer (4×) (v/v: 3/1) and heated at 95 °C for 5 min. The supernatants were collected by centrifugation at 2,500 g for 3 min, and analyzed by Western blotting using anti-biotin antibody and anti-EpCAM antibody.

Incorporation of dual chemical modifications on MCF-7 cells. After being seeded on four-well confocal dishes and cultured overnight, MCF-7 cells were incubated in Met-free RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin solution (0.1 mg/mL) for 30 min to deplete endogenous methionine. Then cells were incubated in Met-free RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution (0.1 mg/mL), Ac_4ManNAz (40 μM) and HPG (50 μM) for 48 h in a 37 °C CO_2 incubator.

After washing with PBS washing buffer for three times, and blocking with PBS blocking buffer at 4 °C for 30 min, the dual-modified cells were ready for further probe anchoring.

Probe anchoring to cell surface. After being incorporated dual chemical modifications, MCF-7 cells were treated with annealed **P'•P-Apt** (1.5/1.0 μM) at 4 °C for 30 min. The cells were then carefully washed three times with PBS washing buffer and treated with annealed **Q-S'-S-F-DBCO** (10 μM) at 4 °C for 30 min. After three times washing with PBS washing buffer, the cells were incubated with TCEP (50 mM) at r.t. for 10 min to quench the unreacted azide, followed by incubation with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), annealed **H'•H-N$_3$** (15/10 μM), and freshly prepared sodium ascorbate solution (2.5 mM) at r.t. for 5 min. The cells were then washed three times with PBS washing buffer and fixed with 4% PAF at r.t. for 15 min.

To demonstrate the successful assembly of each type of probes on cell surface, some of probes were appropriately modified with other probes unchanged. In this context, four sets of experiments were performed:

1) For demonstration of EpCAM binding, **P'•P-Apt** and **Q-S'-S-F-DBCO** were respectively replaced by **P'•F-P-Apt** (1.5/1.0 μM) and **S'-S-DBCO** (10 μM).

2) For C_S tagging, **Q-S'-S-F-DBCO** was replaced by **S'-S-F-DBCO** (10 μM).

3) For HPG tagging, **H'•H-N$_3$** and **Q-S'-S-F-DBCO** were replaced by **H'•H-F-N$_3$** (15/10 μM) and **S'-S-DBCO** (10 μM).

4) For demonstration of the simultaneous anchoring of all three docking probes on cell surface, **P'•Cy5-P-Apt** (1.5/1.0 μM), **S'-S-Cy3-DBCO** (10 μM), and **H'•H-F-N$_3$** (15/10 μM) were used to respectively replace the original probes.

After assembly of the three types of probes (or probe counterparts), the cells were washed and imaged with CLSM.

Execution of automaton I for *in situ* visualization of C_H-installed EpCAM on cell surface. MCF-7 cells after HPG incorporation were treated with annealed **P'-Q•F-P-Apt** (1.5/1.0 μM) at 4 °C for 30 min. After washing, the cells were incubated with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), annealed **H'•H-N$_3$** (15/10 μM), and freshly prepared sodium ascorbate solution (2.5 mM) at r.t. for 5 min, followed by three-time washing and 15-min fixation at r.t. with 4% PAF. After three-time washing, the cells were subjected to incubation with **T** (10 μM) at 37 °C for 2 h in 1×CutSmart buffer. In control experiments, either **H'•H-N$_3$** (15/10 μM) or **T** (10 μM) was absent. Then the cells were washed and imaged with CLSM.

To verify of the dependence of automaton I initiation on the toehold region of **T**, after assembly of **P'-Q•F-P-Apt** and **H'•H-N$_3$** on C_H-installed cells, the cells were subjected to incubation with **T$_{NT}$** (the toehold-removed counterpart strand of **T**, 10 μM) or **T** (10 μM) at 37 °C for 2 h in 1×CutSmart buffer. Then the cells were washed and imaged with CLSM.

The cells after treatment with HPG for different incubating time (0, 12, 24, 36, 48 h) were also analyzed by automaton I to track the influence of HPG incubation time on the automaton output.

Execution of automaton II for *in situ* visualization of EpCAM-carried C_S on cell surface. MCF-7 cells after incorporation of C_S were allowed to incubation with annealed **P'•P-Apt** (1.5/1.0 μM) for 30 min, followed by three-time washing and treatment with **Q-S'-S-F-DBCO** (10 μM) at 4 °C for 30 min. The cells were then washed three times and fixed with 4% PAF at r.t. for 15 min. After three-time washing, the cells underwent toehold-mediated strand displacement and restriction endonuclease cascade reaction by incubation with **H** (10 μM) and NE (600 U/mL) at 37 °C for 2 h in 1×CutSmart buffer. In control experiments, either **P'•P-Apt** (1.5/1.0 μM), **H** (10 μM) or NE (600 U/mL) was absent. Then the cells were washed and imaged with CLSM.

To verify of the dependence of automaton II initiation on the toehold region of **H**, after assembly of **P'•P-Apt** and **Q-S'-S-F-DBCO** on C_S-installed cells, the cells were subjected to incubation with **H$_{NT}$** (the toehold-removed counterpart strand of **H**, 10 μM) or **H** (10 μM) in the presence of NE (600 U/mL) at 37 °C for 2 h in 1×CutSmart buffer. Then the cells were washed and imaged with CLSM.

For cells after treatment with Ac_4ManNAz for different incubating time (0, 12, 24, 36, 48 h), the outputs of automaton II were recorded to track the EpCAM-specific C_S incorporation dynamics.

Execution of automaton III for *in situ* visualization of cell surface EpCAM with dual modifications of interest. To MCF-7 cells that underwent dual chemical modification incorporation and complete probe anchoring procedures, **T** (10 μM) and NE (600 U/mL) were added and allowed to incubate at 37 °C for 2 h in 1×CutSmart buffer. In control experiments, either **P'•P-Apt** (1.5/1.0 μM), **H'•H-N$_3$** (15/10 μM), **T** (10 μM) or NE (600 U/mL) was not added. Then the cells were washed and imaged with CLSM.

To examine the binding stability of the **P'•P-Apt** on the cell surface during automaton execution, a counterpart automaton was executed by replacing **P'•P-Apt** and **Q-S'-S-F-DBCO** with **P'-Q•F-P-Apt** and **S'-S-DBCO**, respectively. In the control experiment, **T** was not added.

Transient transfection of siRNA. MCF-7 cells were seeded on the six-well dishes (1×10^6 cells per well) and cultured at 37 °C overnight. 2.5 μg of EpCAM siRNA or control siRNA (namely siRNA-NC) was added to 125 μL Opti-MEM (without antibiotics and FBS), followed by sufficient mixing. Subsequently, 5 μL lipofectamine™ 3000 was mixed with another 125 μL Opti-MEM. Then the two solutions (each of 125 μL) were mixed thoroughly and incubated at r.t. for 10 min to prepare a 250 μL transfection mixture. After adding the mixture into cell-cultured dishes, the cells were incubated for 6 h and then transferred into 10% FBS-containing RPMI-1640 medium for an additional 42-h incubation at 37 °C.

Western blotting and imaging analysis of EpCAM. After transfecting MCF-7 cells with siRNA or siRNA-NC, the medium was discarded and the cells were gently washed three times with PBSM. Then the cells were lysed with a mixture of cold RIPA lysis buffer, protease inhibitors and phosphatase inhibitors. The lysates were collected by centrifugation (13,000 rpm) at 4 °C for 20 min and the supernatant was stored at -20 °C until use. After determination of protein concentration using BCA protein assay kit, Western blotting analysis of the supernatants was performed using anti-EpCAM antibody and anti-GAPDH antibody, respectively. The transfected MCF-7 cells were also subjected to incubation with **F-P-Apt** (1.0 μM) in PBS binding buffer at 4 °C for 30 min and imaged with CLSM.

Demonstration of the specificity of automaton III.

1) Demonstration of the protein specificity of automaton III by siRNA transfection: After transient transfection of siRNA or siRNA-NC, MCF-7 cells were digested, suspended in RPMI-1640 medium, seeded in four-well confocal dishes, and then cultured overnight. Subsequently, the cells were subjected to the procedures of **incorporation of dual chemical modifications on MCF-7 cells**, **probe anchoring to cell surface**, and finally **execution of automaton III**.

2) Demonstration of the protein specificity of automaton III by using **Q-S'-S-F-Apt$_M$** instead of **Q-S'-S-F-DBCO**. MCF-7 cells after HPG incorporation were treated with annealed **P'•P-Apt** (1.5/1.0 μM) at 4 ºC for 30 min. The cells were then carefully washed three times with PBS washing buffer and treated with annealed **Q-S'-S-F-Apt$_M$** (1.0 μM) at 4 ºC for 30 min. After washing with PBS washing buffer for three times, the cells were incubated with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), annealed **H'•H-N$_3$** (15/10 μM), and freshly prepared sodium ascorbate solution (2.5 mM) at r.t. for 5 min. The cells were then washed three times with PBS washing buffer and fixed with 4% PAF at r.t. for 15 min. **T** (10 μM) and NE (600 U/mL) were then added and allowed to incubate at 37 ºC for 2 h in 1×CutSmart buffer. Then the cells were washed and imaged with CLSM. In control experiments, **T** (10 μM) was not added.

3) Demonstration of the protein specificity of automaton III by using **H'•H-Apt$_M$** instead of **H'•H-N$_3$**. MCF-7 cells after incorporation of C_S were allowed to incubation with annealed **P'•P-Apt** (1.5/1.0 μM) at 4 ºC for 30 min. The cells were then carefully washed three times with PBS washing buffer and treated with annealed **Q-S'-S-F-DBCO** (10 μM) at 4 ºC for 30 min. After washing three times with PBS washing buffer, the cells were treated with annealed **H'•H-Apt$_M$** (1.5/1.0 μM) at 4 ºC for 30 min, washed three times with PBS washing buffer, and fixed with 4% PAF at r.t. for 15 min. Then **T** (10 μM) and NE (600 U/mL) were added and allowed to incubate at 37 ºC for 2 h in 1×CutSmart buffer. Then the cells were washed and imaged with CLSM. In control experiments, **T** (10 μM) was not added.

4) Demonstration of the dependence on HPG installation for executing automaton III by displacing HPG with Met: MCF-7 cells were subjected to procedures of **incorporation of dual chemical modifications** except using Met (50 μM) instead of HPG, **probe anchoring to cell surface**, and finally **execution of automaton III**. In the control sample, HPG was not displaced with Met.

5) Demonstration of the C_S detection specificity of automaton III by NEU cleavage experiment: MCF-7 cells after **incorporation of dual chemical modifications** were subjected to treatment with NEU (0.2 U/mL) in 1× GlycoBuffer at 37 ºC for 1 h. This was followed by the **probe anchoring to cell surface**, and then **execution of automaton III** procedures.

Quantification of azide Sia on EpCAM by IP fluorescence analysis. MCF-7 cells after incorporation of C_S were subjected to treatment with different concentrations of NEU (0, 0.05, 0.1, 0.2 U/mL) in 1× GlycoBuffer at 37 ºC for 1 h. After washing for three times, the cells were blocked and then treated with DBCO-Cy5 (10 μM) at 4 ºC for 30 min to achieve the fluorescence labeling of Sia on the cell surface. After washing, the cells were digested, and suspended in RPMI-1640 medium for cell counting. Then the cells were lysed with a mixture of cold IP lysis buffer, protease inhibitors and phosphatase inhibitors. The lysates were centrifuged (13,000 rpm) at 4 ºC for 20 min and the supernatants were stored at -20 ºC until use. The protein concentration was determined using BCA protein assay kit. The supernatants were then subjected to incubation with anti-EpCAM antibody (1:40) at 4 °C overnight. Then 100 μL of protein G agarose resin slurry was added to each antigen-antibody complex and incubated at r.t. for 4 h under gentle mixing. Subsequently, the mixtures were centrifuged at 2,500 g for 3 min and washed three times with 0.5 mL of IP lysis buffer. Then the resins were mixed with 200 μL of 1% SDS-containing solution and heated at 95 ºC for 5 min. The supernatants were collected by centrifugation at 2,500 g for 3 min and added into a black 96-well plate. Then the emission signals at 670 nm for Cy5 were collected by a spectral scanning multimode reader under 646 nm excitation.

CLSM imaging of azide Sia on HPG-incorporated EpCAM after treatment with different amounts of NEU. MCF-7 cells after **incorporation of dual chemical modifications** were subjected to treatment with different concentrations of NEU (0, 0.05, 0.1, 0.2 U/mL) in 1× GlycoBuffer at 37 ºC for 1 h. This was followed by the **probe anchoring to cell surface**, and then **execution of automaton III** procedures.

ELISA quantification of EpCAM after siRNA transfection. After transfection with different amounts of siRNA (0, 0.625, 1.25, 1.875, 2.5 μg), MCF-7 cells were digested, and suspended in RPMI-1640 medium for cell counting. Then the cells were lysed with a mixture of cold RIPA lysis buffer, protease inhibitors and phosphatase inhibitors. The lysates were collected by centrifugation (13,000 rpm) at 4 ºC for 20 min and the supernatant was stored at -20 ºC until use. Then 100 μL of each TROP1 standard and diluted supernatants were added into appropriate wells and incubated at 4°C overnight with gentle shaking. After washing four times with 1× Wash Buffer, 100 μL of 1× Biotinylated anti-Human TROP1 was added to each well. Then the solutions were incubated at r.t. for 1 h with gentle shaking and washed four times with 1× Wash Buffer. After adding 100 μL of 1× HRP-Streptavidin solution to each well, the solutions were incubated at r.t. for 45 min with gentle shaking and washed four times with 1× Wash Buffer. Then 100 μL of TMB One-Step Substrate Reagent was added to each well. The solutions were incubated at r.t. for 30 min in the dark with gentle shaking. Finally, 50 μL of Stop Solution was added to each well. The absorbance at 450 nm was collected by a spectral scanning multimode reader immediately.

CLSM imaging of dual-labeled EpCAM after transfection with different amounts of siRNA. After transfection with different amounts of siRNA (0, 0.625, 1.25, 1.875, 2.5 μg), MCF-7 cells were digested, suspended in RPMI-1640 medium, seeded in four-well confocal dishes, and then cultured overnight. Subsequently, the cells were subjected to the procedures of **incorporation of dual chemical modifications on MCF-7 cells**, **probe anchoring to cell surface**, and finally **execution of automaton III**.

***In situ* tracking Sia expression variation on C$_H$-installed EpCAM in response to TM or BAG treatment.** MCF-7 cells were subjected to procedure of **incorporation of dual chemical modifications** except adding TM (1.0 μM) or BAG (2.5 mM) in the culture medium. This was followed by the **probe anchoring to cell surface**, and then **execution of automaton III** procedures.

The effect of glycosylation inhibition on the expression level of EpCAM was examined by incubation of the above-mentioned drug-treated cells with **F-P-Apt** (1.0 μM) in PBS binding buffer at 4 ºC for 30 min and imaged with CLSM.

The HPG installation level on MCF-7 cells after drug treatment was also monitored by incubation of the above-mentioned drug-treated cells with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), **H-F-N$_3$** (10 μM), and freshly prepared sodium ascorbate solution (2.5 mM) in PBS binding buffer at r.t. for 5 min, and then imaged with CLSM.

Preparation of Q-S'-S-F-Ab for labeling of phosphotyrosine at Y1068 of EGFR. 2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl (4-nitrophenyl) carbonate (0.065 mmol) in DMSO (2 μL) was added into a mixture of anti-EGFR antibody (20 μL, which specifically binds phosphotyrosine at Y1068 of EGFR), PBSM (64 μL) and DMSO (16 μL). The reaction mixture was stirred at r.t. overnight and then ultrafiltrated using 50 kDa Millipore (10,000 rpm) to obtain the azide-modified anti-EGFR antibody conjugate. Then 12 μL of annealed **Q-S'-S-F-DBCO** (100 μM) was mixed with the anti-EGFR antibody conjugate at r.t. for 2 h. The antibody-DNA conjugate

(**Q-S'-S-F-Ab**) was obtained after removing the unreacted DNA by ultrafiltration using 50 kDa Millipore (10,000 rpm) and stored in PBSM (100 μL).

Execution of automaton IV for *in situ* visualization of phosphorylated and HPG-labeled EGFR in HeLa cells. After HPG incorporation, HeLa cells underwent starvation overnight and then were treated with annealed **P'•P-Apt$_E$** (1.5/1.0 μM) at 4 ºC for 30 min. The cells were then carefully washed three times, followed by incubation with a mixture of $CuSO_4$ (100 μM), BTTAA (600 μM), annealed **H'•H-N$_3$** (15/10 μM), and freshly prepared sodium ascorbate solution (2.5 mM) at r.t. for 5 min. After three-time washing, the cells were incubated with EGF (100 ng/mL) at 37 ºC for 15 min to stimulate the cells. Then the cells were washed three times and fixed with 4% PAF at r.t. for 10 min. After three-time washing, the cells were incubated with PBS containing 5 mM $MgCl_2$ and 0.1% Triton X-100 at r.t. for 10 min. Then the cells were blocked with PBS blocking buffer at 37 ºC for 30 min to prevent the unspecific antibody binding. After washing for three times, the cells were incubated with **Q-S'-S-F-Ab** (5 ng/mL) in 1% BSA in PBST (PBS containing 5 mM $MgCl_2$ and 1 mg/mL Tween® 20) at r.t. for 1 h. After washing, **T** (10 μM) and NE (600 U/mL) were added and allowed to incubate at 37 ºC for 2 h in 1×CutSmart buffer. Then the cells were washed and imaged with CLSM. In control experiments, either **T** (10 μM) was not added or cells were treated with Ab (2 ng/mL) in1% BSA in PBST at r.t. for 1 h before **Q-S'-S-F-Ab** incubation.

Statistical analysis. All data were representative results from at least three independent experiments and mean ± s.d. was shown. Statistical analysis was performed using the Student's *t*-test. ***$p < 0.001$ was considered statistically significant.

Results and discussion

Table S1. DNA sequence information

Name	DNA sequence(5'-3')
T	TGACGAACGAACGACTATAAGTGAAAGGAT
H'	ATCCTTTCACTTATAGTCGTTCGTTCGTCA
H	ATAAATGGAGTGACGAACGAACGACTATTTTTTT
P'	GTTCGTCACTCCATTTATTCCTCA
P-Apt	CGCTGAGGAATAAATGGAGAATGAGTTG**CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG**
S'-S	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGTTTTTTT
H-F	ATAAATGGAGTGACGAACGAACGACTAT-FAM-TTTTTT
H-N_3	ATAAATGGAGTGACGAACGAACGACTATTTTTTT-N_3
H-F-N_3	ATAAATGGAGTGACGAACGAACGACTAT-FAM-TTTTTT-N_3
Q-H'	ATCCTTTCACTT-DABCYL-ATAGTCGTTCGTTCGTCA
F-P-Apt	CGCT-FAM-GAGGAATAAATGGAGAATGAGTTG**CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG**
F-P-Ran	CGCT-FAM-GAGGAATAAATGGAGAATGAGTTGGCTGGTGAAGTGAGGGTCGCGACGAAGAGAAGAGAACGGTGGTGTGGC
P'-Q	GTTCGTCACTCCATTTATTCCTCA-DABCYL
S'-S-F	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT
S'-S-DBCO	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGTTTTTTT-DBCO
S'-S-F-DBCO	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT-DBCO
Q-S'-S-F	DABCYL-ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT
Q-S'-S-F-DBCO	DABCYL-ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT-DBCO
F-Apt_M	FAM-TTT**GCAGTTGATCCTTTGGATACCCTGG**
S'-S-F-Apt_M	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT**GCAGTTGATCCTTTGGATACCCTGG**
Q-S'-S-F-Apt_M	DABCYL-ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-FAM-TTTTTT**GCAGTTGATCCTTTGGATACCCTGG**
H-F-Apt_M	ATAAATGGAGTGACGAACGAACGACTAT-FAM-TTTTTT**GCAGTTGATCCTTTGGATACCCTGG**
H-Apt_M	ATAAATGGAGTGACGAACGAACGACTATTTTTTT**GCAGTTGATCCTTTGGATACCCTGG**
Cy3-H'	Cy3-ATCCTTTCACTTATAGTCGTTCGTTCGTCA
H-Cy5-N_3	ATAAATGGAGTGACGAACGAACGACTAT-Cy5-TTTTTT-N_3
P'-Cy3	GTTCGTCACTCCATTTATTCCTCA-Cy3
Cy5-P-Apt	CGCT-Cy5-GAGGAATAAATGGAGAATGAGTTG**CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG**
S'-S-Cy3-DBCO	ACCACGGTAGTCAGTGTATTCCTCAGCGTGGT-Cy3-TTTTTT-DBCO
T_{NT}	TGACGAACGAACGACTAT
H_{NT}	TGACGAACGAACGACTATTTTTTT
P-Apt_E	CGCTGAGGAATAAATGGAGACTGAGTTG**GCCGTTTCTTCTCTTTCGCTTTTTTTGCTTTTGAGCATG**

Note: The bases in different colors represent individual oligonucleotide domains; **the bold parts** in orange represent the EpCAM-specific aptamer segment (**Apt**); **the bold parts** in fuchsia represent the mucin 1-specific aptamer segment (**Apt_M**); **the bold parts** in brown represent the EGFR-specific aptamer segment (**Apt_E**); the highlighted parts in green represent the fluorophore and quencher moieties; the highlighted parts in yellow represent the nicking restriction site (cleavage between CC and TCAGC); the highlighted parts in blue represent the structural moieties for click reaction. All oligonucleotides were dissolved to approximately 100 μM in PBSM, and the concentrations were determined by UV-vis.

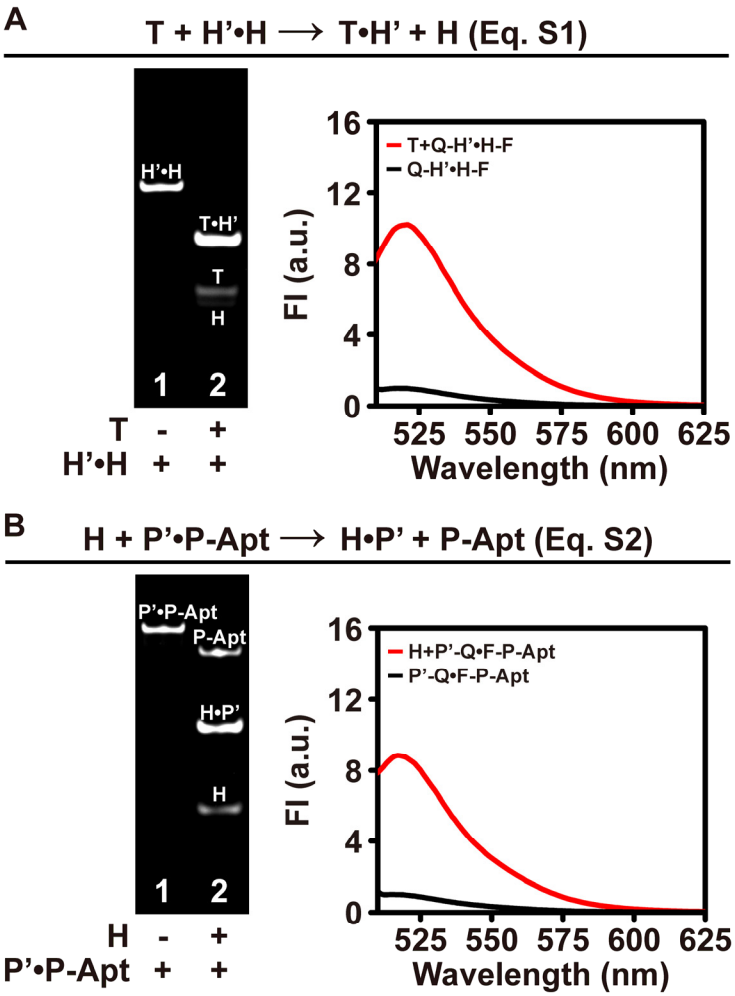

Fig. S1 *In vitro* demonstration of the feasibility of the two toehold-mediated strand displacement reactions exploited by the automaton. (A) The first toehold-mediated strand displacement reaction step. Native PAGE analysis of **H'•H** (450/300 nM, respectively) in the absence or presence of **T** (900 nM), and fluorescence spectra of **Q-H'•H-F** (150/100 nM) with or without **T** (300 nM). The 518 nm peak FI of **Q-H'•H-F** is set to 1. (B) The second toehold-mediated strand displacement reaction step. Native PAGE analysis of **P'•P-Apt** (450/300 nM) in the absence or presence of **H** (900 nM), and fluorescence spectra of **P'-Q•F-P-Apt** (150/100 nM) with or without **H** (300 nM). The 518 nm peak FI of **P'-Q•F-P-Apt** is set to 1. All the systems are prepared in 1× CutSmart buffer and incubated at 37 °C for 2 h.

As expected, the incubation of **T** and **H'•H** led to the disappearance of band for **H'•H** and the appearance of bands for **T•H'** and **H**, demonstrating that **T** can indeed displace and thus release **H** (Fig. S1A, left). For the pair of **H** and **P'•P-Apt**, similar results could be obtained (Fig. S1B, left). For fluorescence verification of the two processes, the two strands in the duplex were labeled with a quencher (**Q**, DABCYL in this work) and a fluorophore (**F**, FAM in this work) respectively. The addition of **T** (or **H**) to **Q-H'•H-F** (or **P'-Q•F-P-Apt**) led to the separation of **Q** and **F**, and a 10-fold (for **T** and **Q-H'•H-F**) and 9-fold (for **H** and **P'-Q•F-P-Apt**) enhancement of fluorescence intensity (FI) at 518 nm could be observed (Fig. S1, right).

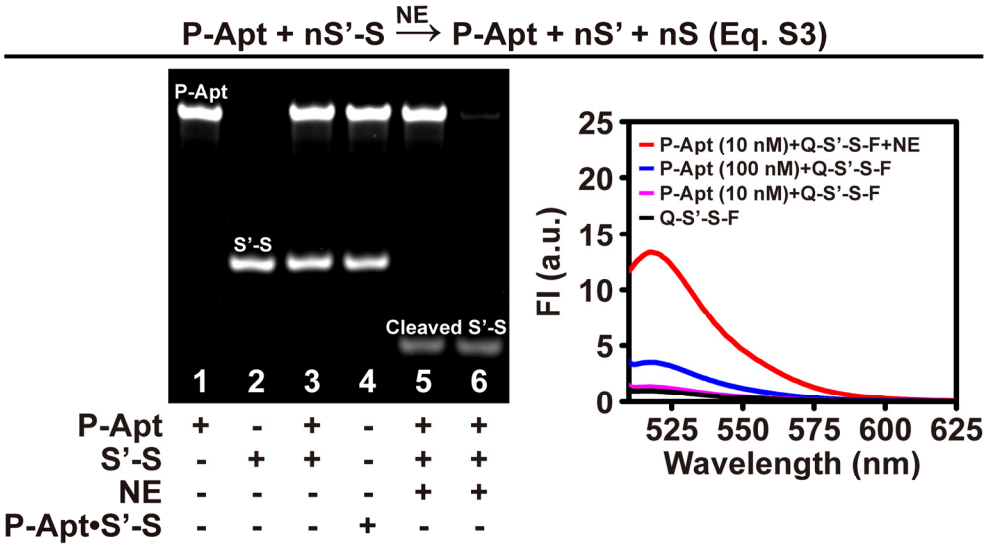

Fig. S2 *In vitro* demonstration of the feasibility of the NE-assisted cyclic nicking reaction exploited by the automaton. Native PAGE analysis of a system containing **P-Apt** (lanes 1, 3, 4, 5: 1 μM; lane 6: 100 nM), **S'-S** (1 μM), and NE (1000 U/mL). Lane 1: **P-Apt**; Lane 2: **S'-S**; Lane 3: **P-Apt** and **S'-S**; Lane 4: thermally annealed **P-Apt** and **S'-S**; Lane 5: **P-Apt**, **S'-S**, and NE with a 1:1 ratio of **P-Apt** to **S'-S**; Lane 6: **P-Apt**, **S'-S** and NE with a 0.1:1 ratio of **P-Apt** to **S'-S**. Fluorescence analysis of a system containing **Q-S'-S-F** (100 nM), **P-Apt** (10 or 100 nM), and NE (100 U/mL). The 518 nm peak FI of **Q-S'-S-F** is set to 1. Both systems are prepared in 1× CutSmart buffer and incubated at 37 °C for 2 h.

The reactions between **P-Apt** and **S'-S** in 1×CutSmart buffer at 37 °C were investigated by native PAGE (Fig. S2, left). In lanes 3 and 4, the bands for **P-Apt** and **S'-S** showed indiscernible change regardless of incubating directly or after thermally annealing, compared with the bands for single **P-Apt** (lane 1) or **S'-S** (lane 2) with the same loading amount. These results indicated that there was little hybridization between **P-Apt** and **S'-S**. Whereas, upon addition of NE, the mixture of **P-Apt** and **S'-S** generated a new band at low molecular weight region corresponding to cleaved fragments, and a largely unchanged **P-Apt** band with complete disappearance of the **S'-S** band (lane 5). This observation suggested that NE can drive the hybridization equilibrium between **P-Apt** and **S'-S** by cleaving **S'-S**, finally rendering the depletion of **S'-S**. The NE-assisted cleavage of **S'-S** was still efficient when the concentration of **P-Apt** was decreased to one-tenth of **S'-S** (lane 6), confirming the recycling usage of **P-Apt** for cleavage of **S'-S** assisted by NE.

The nicking cleavage-facilitated hybridization between **P-Apt** and **S'-S** was also investigated by fluorescence measurement using the pair of **P-Apt** and **Q-S'-S-F**. As illustrated in (Fig. S2 right), the 1:1 incubation of **P-Apt** and **Q-S'-S-F** in the absence of NE generated a fluorescence signal that was 26% of that when complete cleavage of **Q-S'-S-F** was achieved by addition of NE. This result was different from that obtained by PAGE. We speculated that there might be a weak interaction or transient binding between **P-Apt** and **Q-S'-S-F**, leading to a partial opening of the hairpin and separation of **Q** and **F** to some extent, which could only yield a weak FI recovery but are not sufficient to generate a new hybridized band in PAGE experiment (Fig. S2, left, and further demonstrated in Fig. S3). Even though, this possibility would not impair our system, because during the cell anchoring step, **P-Apt** was masked by **P'**.

The recycling capability of **P-Apt** in NE-assisted hybridization process was also evaluated by incubation of **P-Apt** and **Q-S'-S-F** with 0.1:1 ratio (Fig. S2, right). A 13-fold enhancement of FI was obtained upon NE addition, demonstrating the efficiency of using cyclic nicking cleavage reaction for propagating signal to multiple downstream partners.

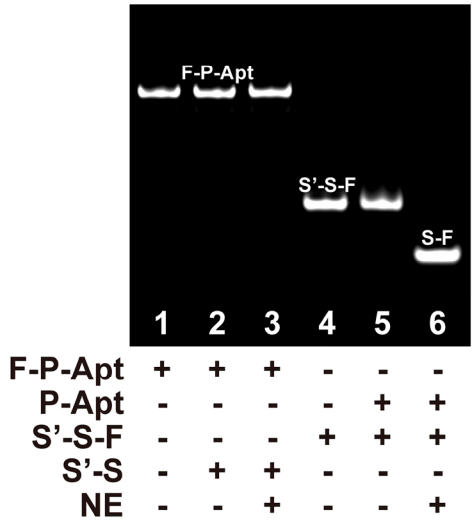

Fig. S3 Complete retention of **F-P-Apt** and full cleavage of **S'-S-F** with the nicking action by NE. Native PAGE analysis of a system containing **F-P-Apt** (5 μM), **P-Apt** (5 μM)**, S'-S-F** (5 μM), **S'-S** (5 μM), and NE (1000 U/mL). Lane 1: **F-P-Apt**; Lane 2: **F-P-Apt** and **S'-S**; Lane 3: **F-P-Apt**, **S'-S**, and NE; Lane 4: **S'-S-F**; Lane 5: **P-Apt** and **S'-S-F**; Lane 6: **P-Apt**, **S'-S-F**, and NE. The fluorescent imaging is achieved directly without staining. Samples are prepared in 1× CutSmart buffer and incubated at 37 °C for 2 h.

To further study the hybridization and nicking cleavage processes, FAM is conjugated to either **P-Apt** (as **F-P-Apt**) or **S'-S** (as **S'-S-F**) for tracking the behavior of each individual strand. Without NE addition, the incubation of pair **F-P-Apt** and **S'-S**, and pair **P-Apt** and **S'-S-F** showed no new bands (lanes 2, 5), demonstrating **P-Apt** and **S'-S** could not effectively hybridize. While upon NE addition, the complete retention of **F-P-Apt** and full cleavage of **S'-S-F** (lanes 3, 6), verified the hybridization-cleavage-dehybridization catalysis process.

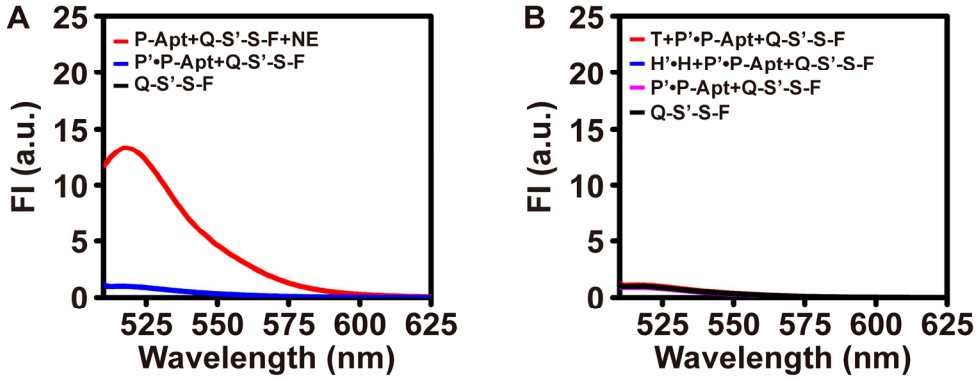

Fig. S4 Control experiments for DNA reactions employed by the automaton. (A) Demonstration of the successful masking of **P-Apt** from hybridization with **Q-S'-S-F** by **P'**. Fluorescence analysis of **Q-S'-S-F** (100 nM) in the absence and presence of **P'•P-Apt** (150/100 nM, with 1:1 molar ratio of **Q-S'-S-F** to **P'•P-Apt**). A sample containing **P-Apt** (10 nM), **Q-S'-S-F** (100 nM), and NE (100 U/mL) that implements full cleavage of **Q-S'-S-F** is also tested for comparison. The 1:1 incubation of **P'•P-Apt** and **Q-S'-S-F** displayed only background level of FI just as **Q-S'-S-F** alone did, excluding the interaction possibility of **P-Apt** and **Q-S'-S-F** before time trigger addition. (B) Controls for Figure 1C. Fluorescence analysis of a system of **T** (90 nM), **H'•H** (45/30 nM), **P'•P-Apt** (15/10 nM), and **Q-S'-S-F** (100 nM). The 518 nm peak FI of **Q-S'-S-F** is set to 1. Samples are prepared in 1× CutSmart buffer and incubated at 37 °C for 2 h.

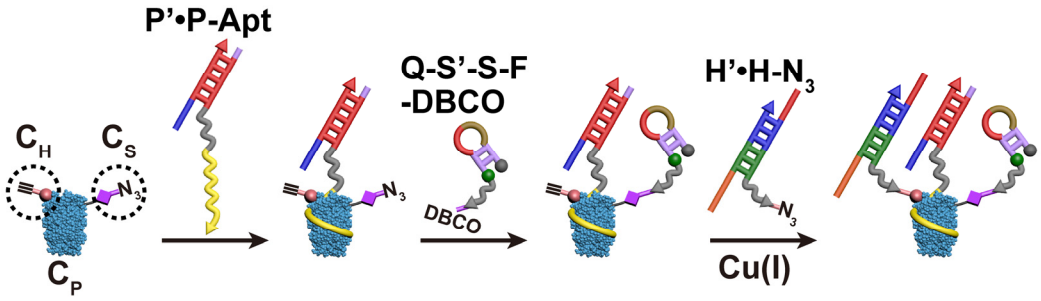

Fig. S5 Scheme of the probe anchoring process. Probes are assembled to cell surface in the order of **P'•P-Apt**, **Q-S'-S-F-DBCO**, and finally **H'•H-N$_3$**. Appropriately modified probes are used to displace the corresponding probes in **Figures S6-S10** and **S12**.

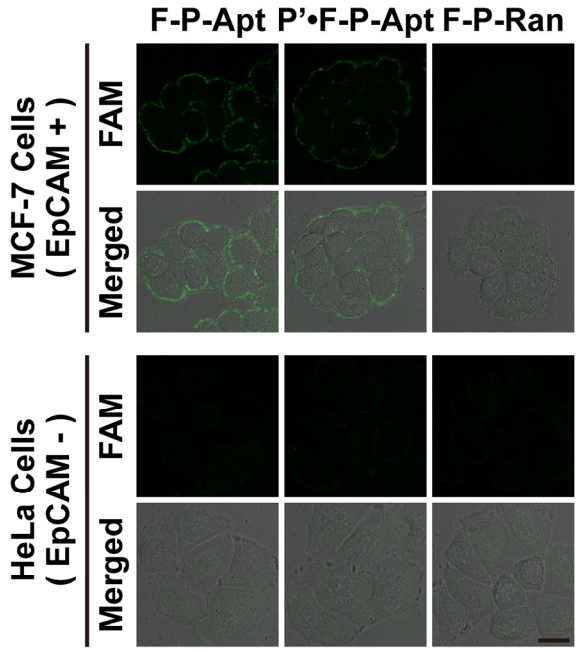

Fig. S6 Demonstration of the specific binding of **F-P-Apt** to EpCAM (i.e. C_P) through **Apt** by CLSM imaging, which is maintained after **P'** hybridization. CLSM images of EpCAM-positive MCF-7 cells and EpCAM-negative HeLa cells after incubation with **F-P-Apt** (1.0 μM), **P'•F-P-Apt** (1.5/1.0 μM), or **F-P-Ran** (1.0 μM) at 4 °C for 30 min. Scale bar: 20 μm.

P'•F-P-Apt and **F-P-Apt** (**F** was conjugated near 5' end of **P** to illustrate probe binding) displayed similar binding signals on the EpCAM-positive cell line, MCF-7,[1] rather than on the negative cell line, HeLa, whereas when displacing Apt of **F-P-Apt** with a random sequence, i.e. using **F-P-Ran**, indiscernible binding could be observed (Fig. S6), thus demonstrating the specific recognition of **F-P-Apt** toward EpCAM, which was still maintained after duplex formation by masking **F-P-Apt** with **P'**.

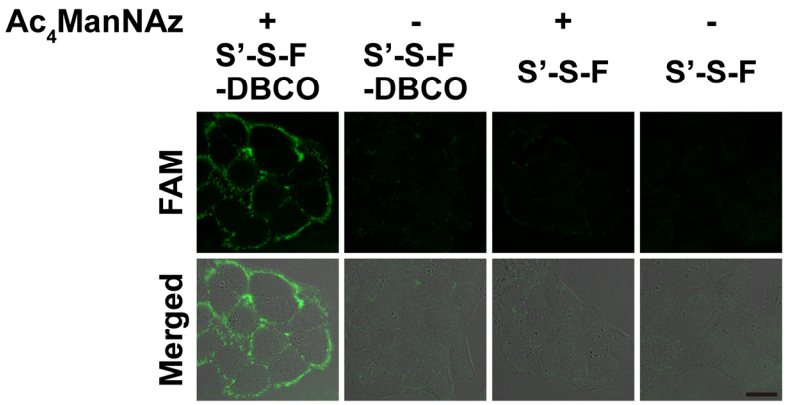

Fig. S7 Demonstration of **S'-S-F-DBCO** tagging to C_S on cell surface. Both a DBCO moiety and metabolic incorporation of azide Sia are required for **S'-S-F-DBCO** binding through Cu(I)-free click reaction. CLSM images of Ac_4ManNAz-treated or untreated MCF-7 cells after incubation with **S'-S-F** (10 μM) or **S'-S-F-DBCO** (10 μM) at 4 °C for 30 min. Scale bar: 20 μm.

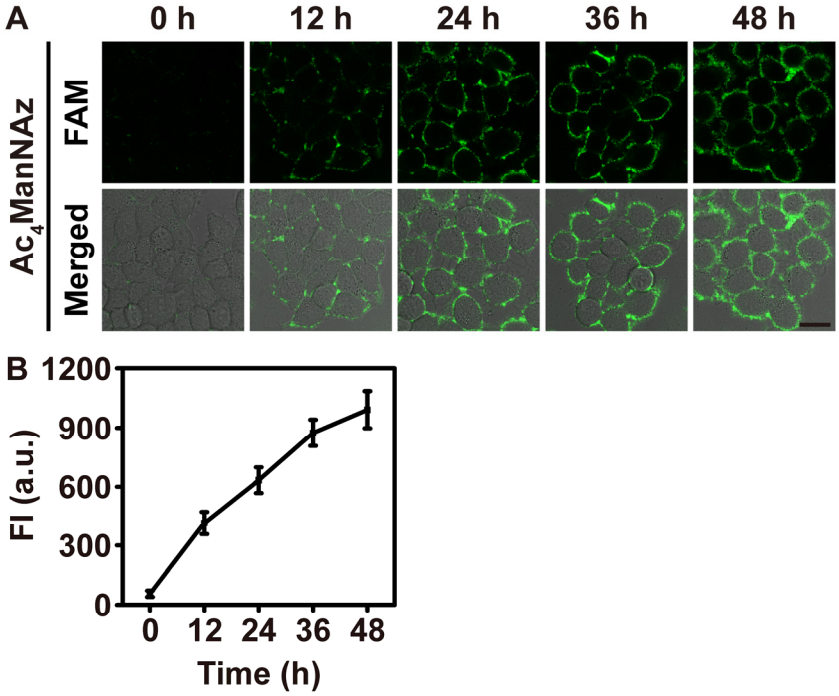

Fig. S8 Demonstration of the time-dependent Ac_4ManNAz incorporation on cell surface. (A) CLSM imaging of MCF-7 cells after incubation with Ac_4ManNAz for different durations of time (0, 12, 24, 36, 48 h), and further treatment with **S'-S-F-DBCO**. Scale bar: 20 μm. (B) Time course of FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells).

The installed C_S on cell surface could be visualized by **S'-S-F-DBCO** via the copper-free click reaction in PBS binding buffer (Fig. S7).[2] While without Ac_4ManNAz incubation or DBCO conjugation, negligible fluorescence could be obtained, thus demonstrating the specificity of **S'-S-F-DBCO** for tagging C_S. The Ac_4ManNAz incorporation is time-dependent as evidenced by increased global fluorescence intensity (referred to as global FI) with longer incubation time of Ac_4ManNAz (Fig. S8), and a 48-h incubation time was chosen for the following experiments.

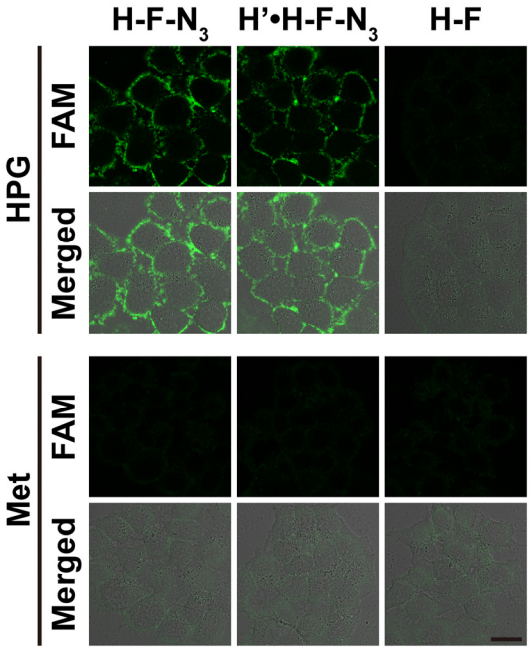

Fig. S9 Demonstration of the **H-F-N₃** labeling to C_H on cell surface. Both an azide moiety and cellular incorporation of HPG are required for **H-F-N₃** binding through Cu(I)-catalyzed click chemistry, which is not affected after **H'** hybridization. CLSM images of MCF-7 cells after treatment with HPG (or Met) and further incubation with **H-F-N₃** (10 μM), **H'•H-F-N₃** (15/10 μM), or **H-F** (10 μM) at r.t. for 5 min. Scale bar: 20 μm.

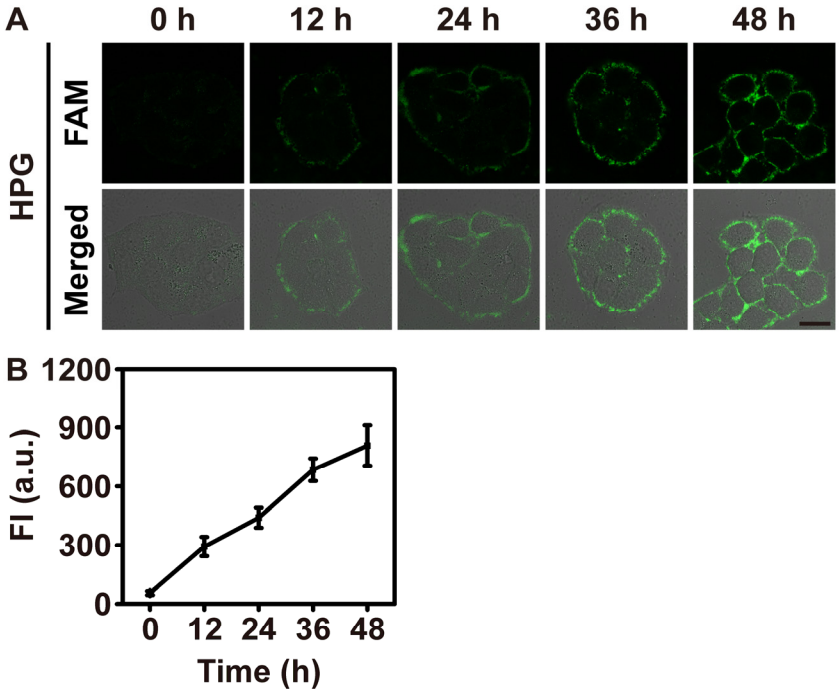

Fig. S10 Demonstration of the time-dependent HPG installation on cell surface. (A) CLSM imaging of MCF-7 cells after incubation with HPG for different durations of time (0, 12, 24, 36, 48 h), and further treatment with **H-F-N₃** mixture. Scale bar: 20 μm. (B) Time course of FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells).

C_H could be lighted up by either **H-F-N₃** or **H'•H-F-N₃** through Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC)[3] (Fig. S9). From the plot of global FI *vs.* HPG incubation time, 48 h was chosen for HPG incubation (Fig. S10), which was the same as that for Ac_4ManNAz incubation.

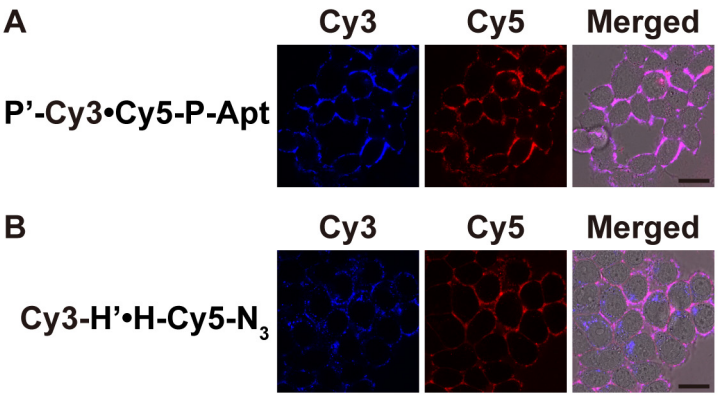

Fig. S11 Demonstration of the duplex **P'-Cy3•Cy5-P-Apt** or **Cy3-H'•H-Cy5-N$_3$** anchoring to cell surface. (A) CLSM imaging of MCF-7 cells after incubation with **P'-Cy3•Cy5-P-Apt** (1.5/1.0 μM). (B) CLSM imaging of C$_H$-installed MCF-7 cells after treatment with **Cy3-H'•H-Cy5-N$_3$** (15/10 μM). Scale bars: 20 μm. The simultaneous observation of the two types of fluorophores at the cell periphery indicated that the two probes were respectively anchored on cell surface in a duplex structure. The weak intracellular Cy3 signal in (B) could be attributed to the endocytosis of the excessive **Cy3-H'** strand, which should not affect the following execution of the automatons.

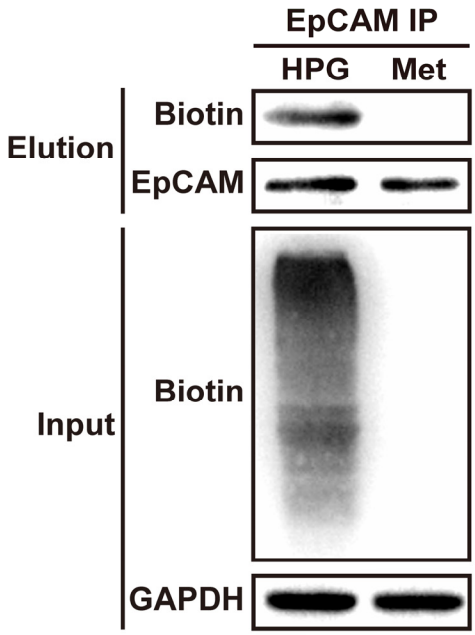

Fig. S12 Verification of HPG incorporation onto cell surface EpCAM. MCF-7 cells are administered with HPG or Met (as a control) for 48 h, lysed and subjected to reaction with azide-PEG$_3$-biotin, followed by immunoprecipitation using anti-EpCAM antibody. Western blotting analysis of the azide-PEG$_3$-biotin treated cell lysates (Input panel) and eluted protein samples (Elution panel) with anti-biotin antibody shows successful incorporation of HPG onto cellular proteins and EpCAM, respectively.

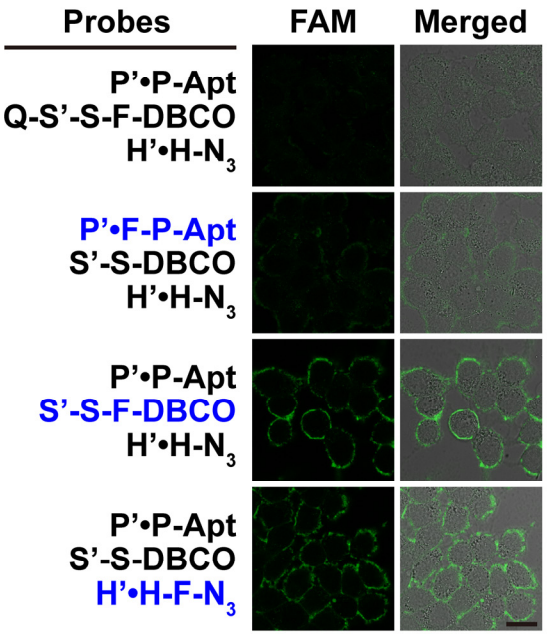

Fig. S13 Exclusion of the possibilities of cross interference between different probe anchoring processes. CLSM images of MCF-7 cells after incorporation of Ac_4ManNAz and HPG, and further treatment with **P'•P-Apt** (or **P'•F-P-Apt**), **Q-S'-S-F-DBCO** (or **S'-S-DBCO** or **S'-S-F-DBCO**), and **H'•H-N_3** (or **H'•H-F-N_3**). Scale bar: 20 μm.

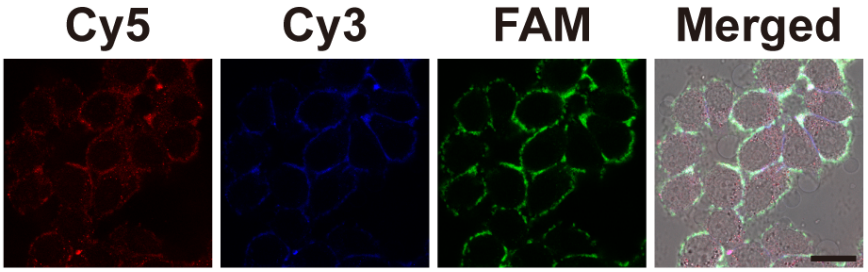

Fig. S14 Validation of the anchoring of all three probes on cell surface by labeling probes with different fluorescence dyes. CLSM imaging of MCF-7 cells after incorporation of Ac_4ManNAz and HPG, and further treatment with **P'•Cy5-P-Apt**, **S'-S-Cy3-DBCO**, and **H'•H-F-N_3**. Scale bar: 20 μm.

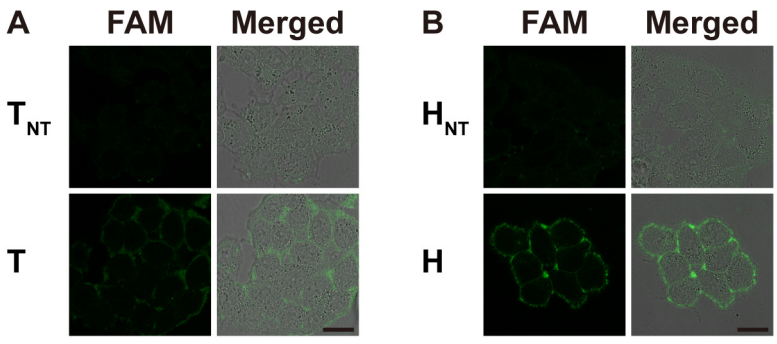

Fig. S15 Demonstration of the dependence of automaton initiation on the toehold region of **T** and **H**, respectively, using toehold-removed **T** (T_{NT}) or **H** (H_{NT}). (A) CLSM imaging of MCF-7 cells after incubation with HPG, and further treatment with **P'-Q•F-P-Apt**, **H'•H-N_3**, and T_{NT} (or **T**). (B) CLSM imaging of MCF-7 cells after incubation with Ac_4ManNAz, and further treatment with **P'•P-Apt**, **Q-S'-S-F-DBCO**, H_{NT} (or **H**), and NE. Scale bars: 20 μm.

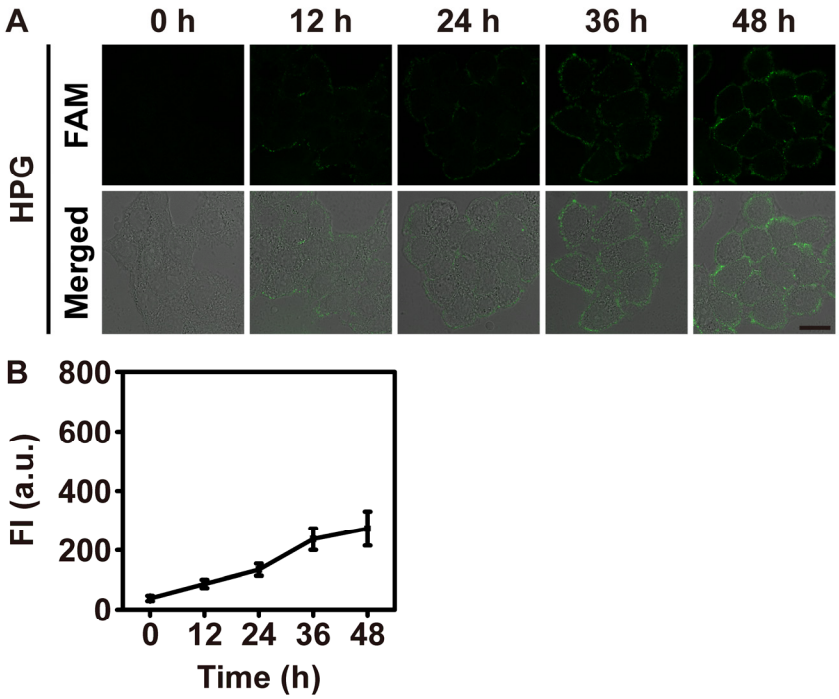

Fig. S16 Tracking of the influence of HPG incorporation level on automaton I output. (A) CLSM imaging of MCF-7 cells after incubation with HPG for different durations of time (0, 12, 24, 36, 48 h), and further treatment with **P'-Q•F-P-Apt**, **H'•H-N$_3$**, and **T**. Scale bar: 20 μm. (B) Time course of FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells).

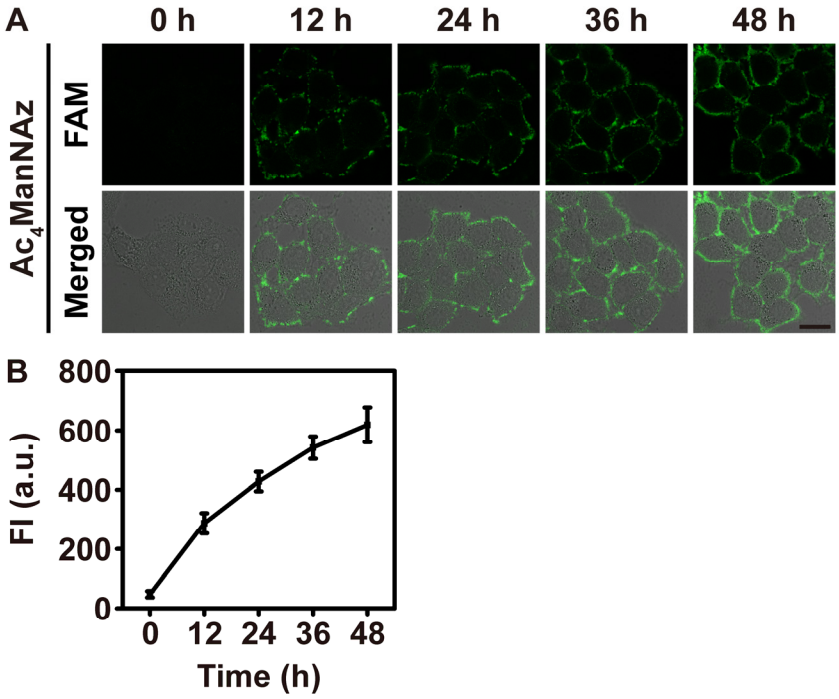

Fig. S17 Tracking of the EpCAM-specific C_S incorporation dynamics using automaton II. (A) CLSM imaging of MCF-7 cells after incubation with Ac_4ManNAz for different durations of time (0, 12, 24, 36, 48 h), and further treatment with **P'•P-Apt**, **Q-S'-S-F-DBCO**, **H**, and NE. Scale bar: 20 μm. (B) Time course of FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells).

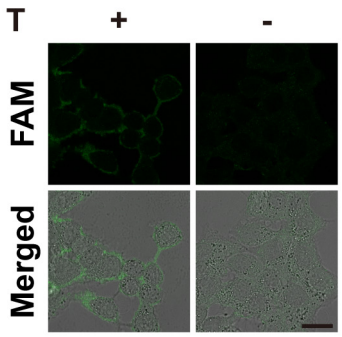

Fig. S18 CLSM imaging validation of the binding stability of the **P'-Q•F-P-Apt** on the cell surface during automaton execution. CLSM imaging of dual-modified MCF-7 cells after treatment with **P'-Q•F-P-Apt**, **S'-S-DBCO** and **H'•H-N_3**, and further incubation with NE in the presence or absence of **T**. Scale bar: 20 μm.

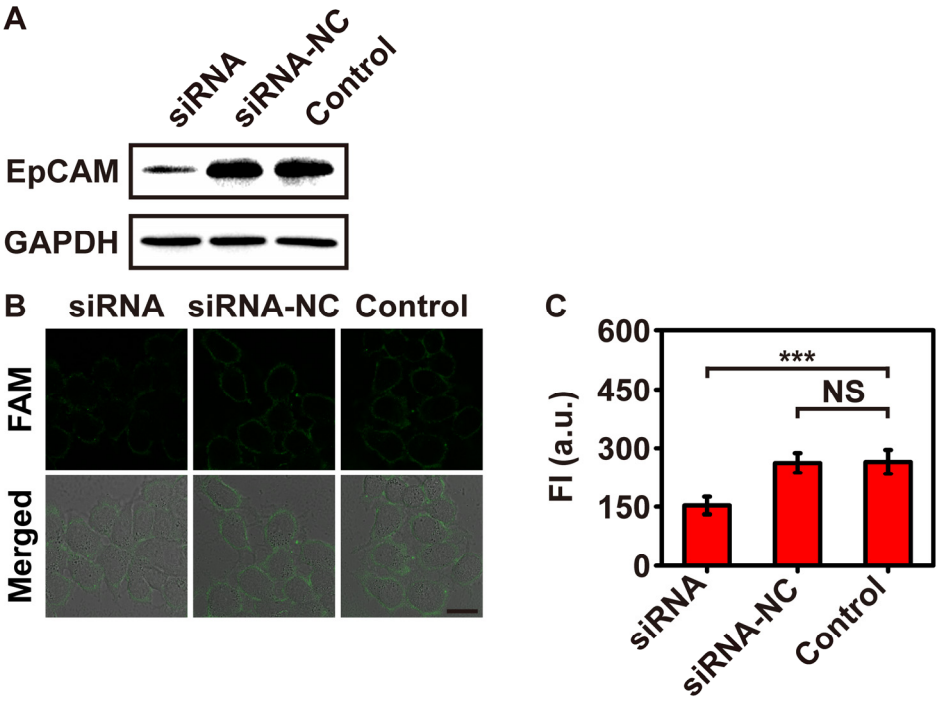

Fig. S19 Confirmation of the decrease of EpCAM expression after transfection with siRNA. (A) Western blotting analysis of EpCAM expression level after transfecting cells with either siRNA or siRNA-NC (control siRNA). Control: no transfection. (B) CLSM imaging of MCF-7 cells after transfection with siRNA or siRNA-NC, and then incubation with **F-P-Apt**. Control: no transfection. Scale bar: 20 μm. (C) FI obtained from (B). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells). Data are statistically analyzed by Student's *t*-test (***$p < 0.001$; NS: not significant).

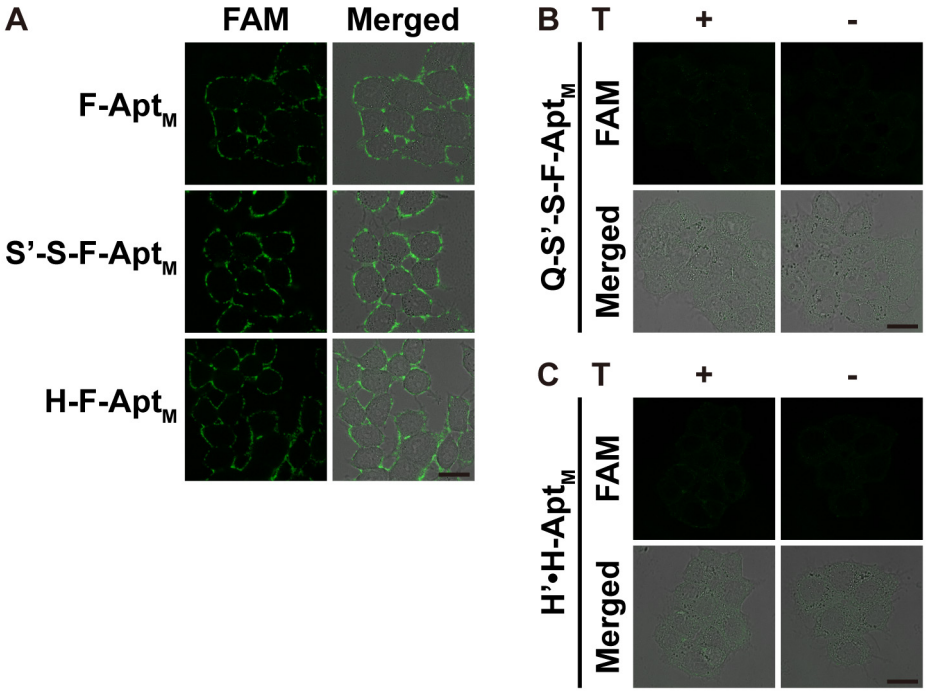

Fig. S20 Demonstration of the protein specificity of automaton III by using **Q-S'-S-F-Apt$_M$** (or **H'•H-Apt$_M$**) instead of **Q-S'-S-F-DBCO** (or **H'•H-N$_3$**). (A) CLSM imaging of MUC1-positive MCF-7 cells after incubation with **F-Apt$_M$**, **S'-S-F-Apt$_M$**, or **H-F-Apt$_M$**. The observed fluorescent signals demonstrated the successful assembly of **F-Apt$_M$**, **S'-S-F-Apt$_M$** and **H-F-Apt$_M$** on MCF-7 cell surface by the recognition of **Apt$_M$** toward MUC1. (B) CLSM imaging of C_H-installed MCF-7 cells after incubation with **P'•P-Apt**, **Q-S'-S-F-Apt$_M$** and **H'•H-N$_3$**, and further treatment with NE in the presence or absence of **T**. (C) CLSM imaging of C_S-installed MCF-7 cells after incubation with **P'•P-Apt**, **Q-S'-S-F-DBCO** and **H'•H-Apt$_M$**, and further treatment with NE in the presence or absence of **T**. Scale bars: 20 μm.

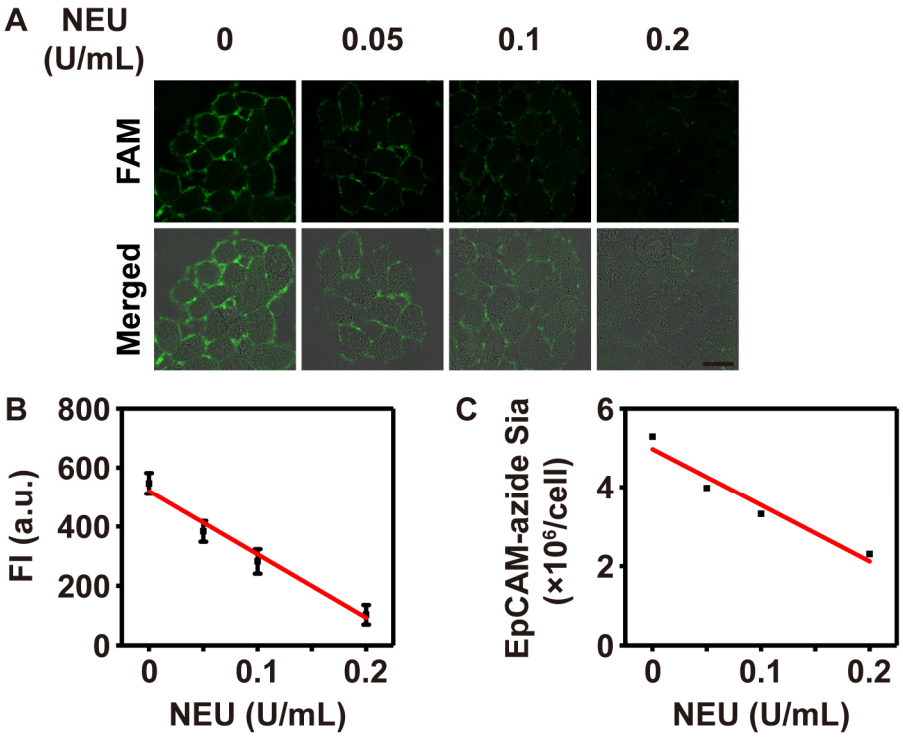

Fig. S21 Demonstration of the relative quantification capability of automaton III for azide Sia on HPG-incorporated EpCAM by treating cells with NEU. (A) CLSM imaging of MCF-7 cells after incubation with Ac_4ManNAz and HPG, cleavage by NEU of different concentrations, and further treatment with **P'•P-Apt**, **Q-S'-S-F-DBCO**, **H'•H-N$_3$**, **T**, and NE. Scale bar: 20 μm. (B) FI obtained from (A) as a function of the concentration of NEU. (C) The amount of EpCAM-bound terminal azide Sia per cell obtained from IP fluorescence analysis as a function of the concentration of NEU. The independent fluorescence measurement of azide Sia in immuneprecipitated EpCAM of different samples confirmed the linear decrease of azide Sia expression on EpCAM due to NEU treatment.

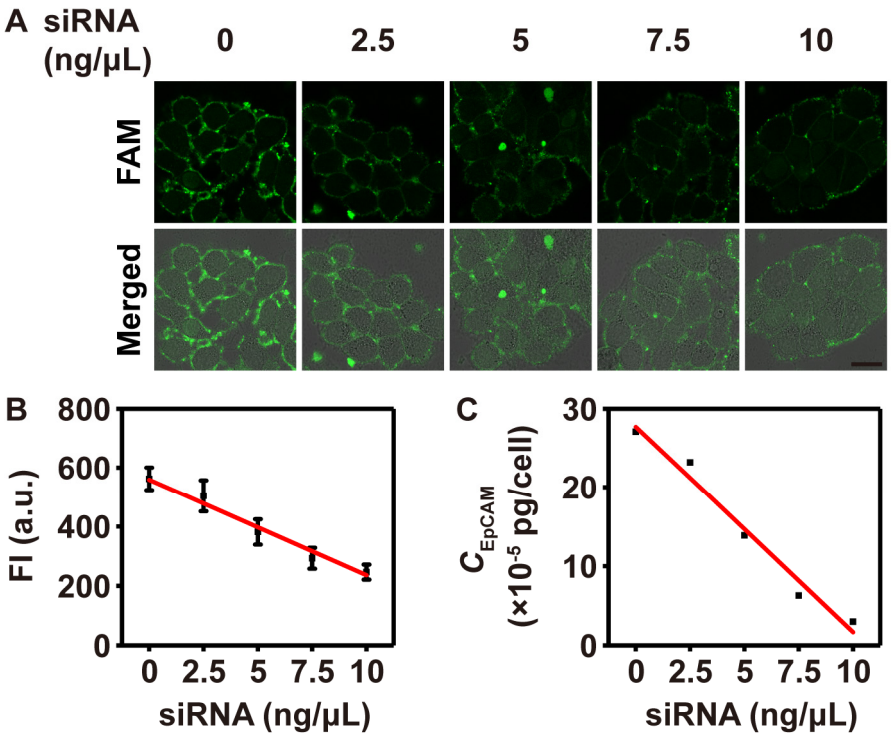

Fig. S22 Demonstration of the relative quantification capability of automaton III for EpCAM with two MOIs by transfecting cells with different amounts of siRNA. (A) CLSM imaging of MCF-7 cells after transfection with different amounts of siRNA, incubation with Ac_4ManNAz and HPG, and further treatment with **P'•P-Apt**, **Q-S'-S-F-DBCO**, **H'•H-N_3**, **T**, and NE. Scale bar: 20 μm. (B) FI obtained from (A) as a function of the concentration of siRNA. (C) EpCAM quantity per cell obtained from enzyme-linked immunosorbent assay (ELISA) as a function of the concentration of siRNA. This experiment verified the linear decreasing of EpCAM expression due to siRNA treatment.

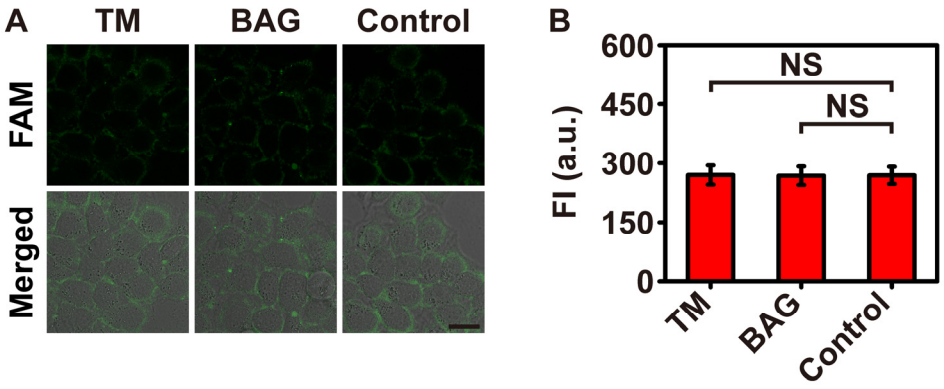

Fig. S23 Examination of the influence of TM or BAG treatments on EpCAM expression of MCF-7 cells. (A) CLSM imaging of MCF-7 cells after treatment with either TM or BAG during Ac_4ManNAz and HPG co-incorporation step and then incubation with **F-P-Apt**. Control: no drug added. Scale bar: 20 μm. (B) FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells). Data are statistically analyzed by Student's *t*-test (NS: not significant).

The statistically insignificant difference between samples demonstrated that EpCAM expression on MCF-7 cells showed little change during TM or BAG treatments.

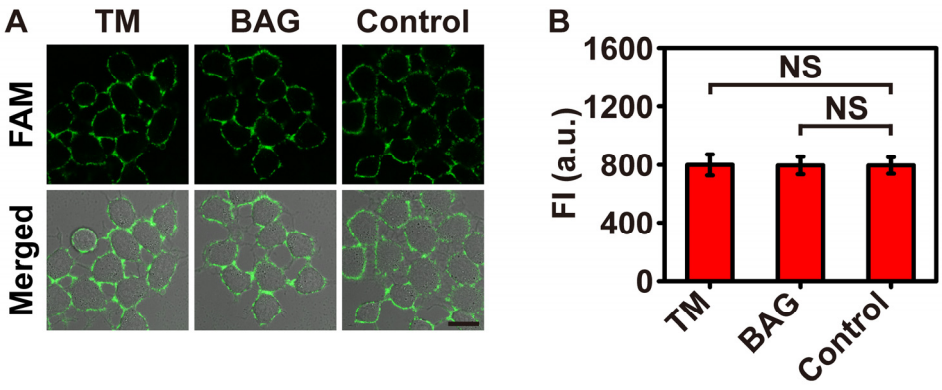

Fig. S24 Examination of the influence of TM or BAG treatments on HPG installation level on MCF-7 cells. (A) CLSM imaging of MCF-7 cells after treatment with either TM or BAG during Ac_4ManNAz and HPG co-incorporation step and then incubation with **H-F-N_3** by Cu(I)-catalyzed azide–alkyne cycloaddition. Control: no drug added. Scale bar: 20 μm. (B) FI obtained from (A). For FI measurement, the data represent mean ± s.d. (n = 3 independent experiments, with a total of 30 analyzed cells). Data are statistically analyzed by Student's *t*-test (NS: not significant).

The indiscernible change of fluorescence signal demonstrated that HPG incorporation levels on MCF-7 cells showed little change after TM or BAG treatments.

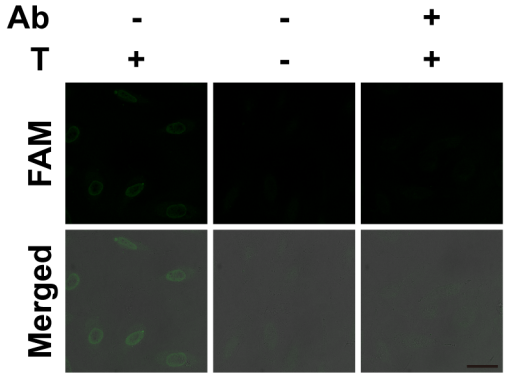

Fig. S25 Execution of automaton IV for *in situ* visualization of phosphorylated and HPG-labeled EGFR in HeLa cells. (Left, middle) CLSM imaging of C_H-installed HeLa cells after incubation with **P'•P-Apt$_E$**, **H'•H-N_3** and **Q-S'-S-F-Ab,** and further treatment with NE in the presence or absence of **T**. (Right) Cells were pre-incubated with Ab before **Q-S'-S-F-Ab** incubation. Scale bar: 40 μm. Without **T** addition or pre-blocking phosphotyrosine sites with Ab led to indiscernible fluorescence, demonstrating that the automaton IV indeed performed as prescribed.

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Author Contributions

L.D., H.J., and L.L. conceived the projects and designed the experiments. L.L., S.L., A.M., G.W. and Y.L. performed the experiments. All authors contributed to the data analysis and manuscript preparation.