

Photostick: a method for selective isolation of target cells from culture

*Miao-Ping Chien, Christopher A. Werley, Samouil L. Farhi, Adam E. Cohen**

Departments of Chemistry and Chemical Biology and Physics, Harvard University, Cambridge, MA 02138

Howard Hughes Medical Institute

cohen@chemistry.harvard.edu

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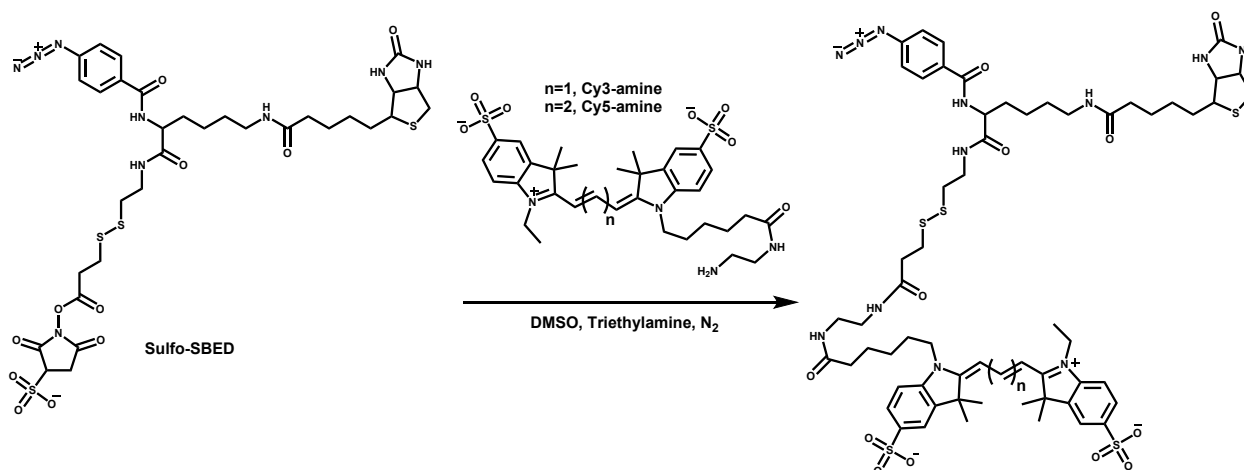
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Detailed Materials and Methods

Synthesis of photoactivatable Cy3-/Cy5-SBED

The two starting materials, Cy3 amine or Cy5 amine (ATT Bioquest) and Sulfo-SBED (Thermo Scientific), were combined in a one-step synthesis where the dye free amine substituted the sulfo-N-Hydroxysuccinimide (NHS) leaving group. We added Sulfo-SBED (0.18 μmol , 1 equ) in DMSO (0.20 mL) to a solution of Cy3- or Cy5-amine (0.22 μmol , 1.2 equ) in DMSO (0.02 mL) with 2 equ of triethylamine (Scheme S1). After stirring for 12 hrs under nitrogen, the product was separated from unreacted dye and triethylamine via dialysis with DMSO in 1,000 MWCO dialysis tubing (Spectra). The solution was dialyzed for 1 day and DMSO solvent was replaced once during the process. After dialysis, the product identity and purity were confirmed using high resolution LC-MS where both products matched the predicted molecular weight to better than 5 ppm (Cy3-SBED: Exp. Mass: 1335.493, $[\text{M}+\text{H}^+]$: 1335.489; Cy5-SBED: Exp. Mass: 1362.509, $[\text{M}+\text{H}^+]$: 1362.503).



Scheme S1. Synthesis of Cy3- or Cy5-SBED. Sulfo-SBED (1 equ), Cy3 amine or Cy5-amine (1.2 equ), and triethylamine (2 equ) reacted in DMSO under nitrogen for 12 hrs. The product was purified by dialysis with DMSO.

Covalent conjugation of fibronectin to glass bottom dish

Glass-bottom dishes (In Vitro Scientific, D35-14-1.5-N) were cleaned and chemically activated by 5 min treatment in a plasma cleaner with low-pressure ambient air. The glass was aldehyde-functionalized with a 1% solution of 11-(Triethoxysilyl) undecanal (Gelest, Inc) in ethanol, which reacted for 1 hour in a nitrogen-purged container. Dishes were rinsed twice with ethanol and once with nanopure water and then cured in a vacuum oven at 65 °C for 1-2 hrs to drive off remaining water or alcohol and complete the glass-silane bond.

Fibronectin (0.1 mg/mL in PBS) was added to the dishes and incubated overnight at 4 °C or at 37°C for 2 hrs, resulting in a covalent imine bond between the surface and free primary amines on the fibronectin.¹ After incubation with fibronectin, dishes were immersed in 0.1% Tween-20

PBS for 10 min followed by rinsing three times with PBS. Completed dishes could either be seeded with cells directly or stored at -80 °C.

MDCK (Madin-Darby canine kidney) cell culture

MDCK epithelial cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin in a 37 °C incubator under 5% CO₂. Cells were grown to 50-70% confluency in 3.5 cm dishes and transfected with a mammalian expression vector coding for YFP under the CMV promoter (pDisplay-AP-YFP) using TransIT-X2 (Mirus). 24-48 hours after transfection, cells were trypsinized and re-plated at a density of ~32,000-35,000 cells/cm² on fibronectin-coated glass bottom dishes (described above). Experiments were performed 12-24 hours after plating on the glass-bottom dishes.

Neuronal cell culture

Sprague Dawley rats were obtained from Taconic Labs. Postnatal day 0 (P0) pups were euthanized and hippocampi were dissected following the procedure in Ref. (2). Briefly, isolated hippocampi were digested with papain and homogenized in Hank's Balanced Salt Solution (HBSS) containing MgCl₂ and kynurenic acid to prevent excitotoxicity. After dissociation, neurons were electroporated (Lonza, Nucleofector electroporation kit) with pLenti-hsyn-Optopatch (1 µg for 1 million neurons) and plated on glass-bottom dishes coated with covalently-bound fibronectin (described above) at a density of ~45,000/cm². Neurons were initially cultured in plating medium [MEM (Life Technologies) containing 10% fetal bovine serum, 0.5% glucose, 10 mM HEPES, 2 mM Glutamax (Life Technologies), 100 mg/L transferrin, insulin, and B27]. After 3 days, the medium was replaced with NbActiv4 (Brainbits, Nb4-500). At 4 days *in vitro* (div) 2 µM AraC was added to suppress further glial growth. At 7-14 div, electroporated neurons were ready for experimentation.

All experimental protocols involving use of animals were approved by the Harvard Institutional Animal Care and Use Committee (IACUC).

Optopatch measurements

Optopatch experiments were conducted on a home-built inverted fluorescence microscope described in Ref. (3). Briefly, illumination was provided by six lasers at 635 nm, 500 mW (Dragon Lasers 635M500), combined in three groups of two. Illumination was coupled into the sample using a custom fused silica prism, without passing through the objective. Fluorescence was collected by the low-magnification objective (Olympus 2× MVX Plan Achromat), passed through an emission filter, and imaged onto a scientific CMOS camera (Hamamatsu Orca Flash 4.0). This microscope imaged a 1.2 × 3.3 mm field of view with 3.25 µm spatial resolution and 2 ms temporal resolution.

Blue illumination for channelrhodopsin stimulation was provided by a 473 nm, 1 W laser (Dragon Lasers), modulated in intensity by an acousto-optic modulator and modulated spatially by a digital micromirror device (DMD, Digital Light Innovations DLI4130 – ALP HS). The DMD was re-imaged onto the sample via the 2× objective. The DMD provided targeted stimulation with 3.5 µm spatial resolution and 0.1 ms temporal resolution. For the Optopatch

measurements, neurons were stimulated with seven, 500 ms duration pulses of blue light. Stimuli lasted 500 ms, and the intensity of successive pulses increased from 0 to 58 mW/cm². Between stimuli, cells were given 5 s recovery in the dark. Fluorescence traces were extracted from the raw movies as described in Ref. 3.

Neuronal electroporation

Neuronal electroporation reagents were purchased from Lonza and the Nucleofector electroporation kit (Lonza) was used following the standard protocol. Briefly, 1.5 µg of pLenti-hsyn-Optopatch plasmid and 1.5 million rat hippocampal neurons were added to 100 µL of Nucleofector solution. The mixture was transferred to an electroporation cuvette and cells were shocked using Nucleofector program G-013. After electroporation, 500 µL of plating culture medium was added to the cuvette and the sample was gently transferred into the prepared fibronectin-conjugated glass-bottomed dishes at a concentration of 45,000 cells/cm². After 2 h of incubation (37°C, 5% CO₂), plates were rinsed to remove unbound neurons and then filled with 1 mL of fresh plating medium. Typical transfection efficiencies were ~50%.

Photostick optics

Photostick experiments at high magnification were performed on a custom-built microscope. Illumination light was provided by either a 637 nm 140 mW Coherent Obis, a 488 nm 100 mW Coherent Obis, or a 405 nm 30 mW Dragon Laser. Laser lines were combined with dichroic mirrors and intensities were modulated using acousto-optical tunable filters (Gooch & Housego). The 488 nm laser line was expanded to illuminate the chip of a DMD (Texas Instruments DLP LightCrafter with DLP 0.3 WVGA chipset) which was subsequently reimaged onto the sample plane. The 637 nm and 488 nm lines were focused at the back focal plane of a LCPlanFI 20x 0.40 NA objective (Olympus). Collimated 405 nm laser light at the back focal plane of the objective was defocused to obtain a 5 µm spot at the sample and was steered in the sample plane using galvo mirrors (Thorlabs GVS202) located in a conjugate plane. Fluorescence light was separated from illumination light using a quad-band dichroic mirror (Semrock #Di01-R405/488/561/635). The fluorescence was then imaged using a custom dual-wavelength imaging system. A rectangular aperture in an image plane set the boundaries of the image. The fluorescence was then split into two channels using a dichroic mirror (Semrock FF662-FDi01) and then recombined on a second dichroic mirror (Semrock FF662-FDi01) and reimaged onto adjacent halves of the chip of a scientific CMOS camera (Hamamatsu Orca Flash 4.0). Red fluorescence was filtered using a HQ700/75m bandpass filter (Chroma). Green and orange fluorescence was filtered using a HQ550/50m bandpass filter (Chroma).

Photostick experiments at low magnification were conducted on a home-built inverted fluorescence microscope described in *Optopatch measurement* section. A 407 nm 200 mW Laser (Lilly Electronics) was modulated spatially by a DMD which was re-imaged onto the sample via the 2x objective. The DMD provided targeted violet stimulation with excitation with 3.25 µm spatial resolution.

Photostick on MDCK cells via Cy3- and Cy5-SBED (Main text, Figure 2)

MDCK cells were seeded at a density of 32,000/cm² on fibronectin-coated glass-bottomed dishes. Immediately prior to the photostick protocol, Cy3-SBED was added to the imaging medium to a final concentration of 4 μM. The cells were illuminated with two squares of 407 nm light (825 J/cm²). After 15 min illumination, the dish was rinsed 3 times with DPBS (PBS without calcium and magnesium) before adding the second photoactivatable crosslinker, Cy5-SBED (4 μM). A rectangular bar was illuminated using the same procedure for another 15 min. The dish was then treated with accutase for 3 min to detach the cells that had not been illuminated. The patterned MDCK cells were imaged using white light trans-illumination and the bound dyes were visualized via fluorescence (Cy3: λ_{ex} = 530 nm, λ_{em} = 574/40 nm BP; Cy5: λ_{ex} = 637 nm, λ_{em} = 665 nm LP).

Photostick on YFP-transfected MDCK cells via Cy5-SBED from a pool of YFP- or non-YFP-expressing cells (Main text, Figure 3A-B)

YFP-transfected MDCK cells were seeded at a density of 32,000/cm² on fibronectin-coated glass-bottomed dishes. This photostick experiment was conducted on a custom-built microscope at high magnification as described above. We recorded a YFP fluorescence image (λ_{ex}: 488 nm, λ_{em}: 525/36nm BP) to identify potential photostick targets. The photochemical crosslinker Cy5-SBED was added to a final concentration of 4 μM. Light at 407 nm was directed exclusively onto a YFP-MDCK cell with a galvanometric mirror pair. Subsequently, the dish was rinsed with DPBS and digested with accutase to detach non-illuminated cells. The remaining adhered cells were lysed with DNA Extract kit (Life Technologies; see below for details) and the lysate was analyzed via PCR (Figure 3G, main text) and gene sequencing. The primers for PCR of YFP were: YFP-primer-fwd: gcaaggcgaggagctgttca; YFP-primer-rev: ccgcttgatagctcgtccatgcc.

Photostick on YFP-transfected MDCK cells via Cy5-SBED from a pool of YFP- or mOrange-expressing cells (Main text, Figure 3C-F)

YFP-transfected MDCK cells were seeded at a density of 32,000/cm² on fibronectin-coated glass-bottomed dishes. This photostick experiment was performed on a home-built inverted fluorescence microscope at low magnification as described above. We recorded a YFP fluorescence image (λ_{ex}: 488 nm, λ_{em}: 525/36nm BP) to identify potential photostick targets. For mOrange fluorescence image, excitation at 535 nm and emission at 575/40nm BP were used. The photochemical crosslinker Cy5-SBED was added to a final concentration of 4 μM. Light at 407 nm was directed exclusively onto three YFP-MDCK cells with a DMD. Subsequently, the dish was rinsed with DPBS and digested with accutase to detach non-illuminated cells. The remaining adhered cells were lysed with DNA Extract kit (Life Technologies; see below for details) and the lysate was analyzed via PCR (Figure 3H, main text) and gene sequencing. The consensus primers for PCR (used in lane1, 3, and 4, Figure 3H, main text) of YFP and mOrange genes were: Con-fwd: ggaattcggcttggggatgccacc; Con-rev: ggcaccacgatgacctctgc. For mOrange specific primers in Lane 2 (Figure 3H, main text) were: mO2-fwd: gtgagcaagggcgaggagaataacat; mO2-rev: ccgcttgtagctcgtccatgc.

Photostick on Optopatch-electroporated neurons via Cy3-SBED (Main text, Figure 4)

Rat hippocampal neurons were electroporated with the Optopatch vector and then seeded on fibronectin-coated glass-bottomed dishes and cultured for 7-14 days. 30 min before Optopatch measurements, 5 μ M all-*trans* retinal was added to culture medium to enhance QuasAr2 fluorescence and voltage sensitivity. Immediately prior to Optopatch measurements, the cellular medium was exchanged to the low auto-fluorescence XC buffer (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 30 mM glucose, pH 7.3). Neuronal action potentials were induced using the stepped stimulus protocol described above. We chose a neuron displaying an unusual rapidly inactivating firing pattern for subsequent Photostick selection. The photo-crosslinker Cy3-SBED was added to a final concentration of 15 μ M and 407 nm light was directed selectively onto the specified neuron (2200 J/cm²). After exposure, the dish was rinsed with XC buffer and non-illuminated cells were detached with accutase. The remaining adhered cells were lysed and the lysate was analyzed via PCR (Figure 4D, main text) and gene sequencing. The primers for PCR of Optopatch were: Optopatch_primer-fwd: atcgcctctgcaggctggttacgac; Optopatch_primer-rev: tcggcaccggcactggg.

Single- or few-cell PCR

After the photostick protocol and accutase development, the dish was rinsed thoroughly with PBS to remove residual non-target cells. Complete removal of non-target cells was verified by examination in the microscope. The target cells were then detached from the dish by incubation with trypsin for ~3 min. The trypsin was then diluted by addition of an equal volume of PBS. The supernatant containing the selected cells was centrifuged at 10,000 rpm for 2 min. After discarding the supernatant, the were lysed with the *DNA Extract All* kit (Life Technologies 4403319), as follows:

- 1) Cells were resuspended with 2 μ L of PBS buffer and gently pipetted up and down a few times.
- 2) 20 μ L of Lysis Solution was added to the cells and reacted at room temperature for 3 min.
- 3) 20 μ L of DNA Stabilizing Solution was added. Samples were either amplified by PCR immediately, or stored at -20 °C before amplification.
- 4) Standard PCR procedures were carried out from the cell lysate.

Supplementary Figures

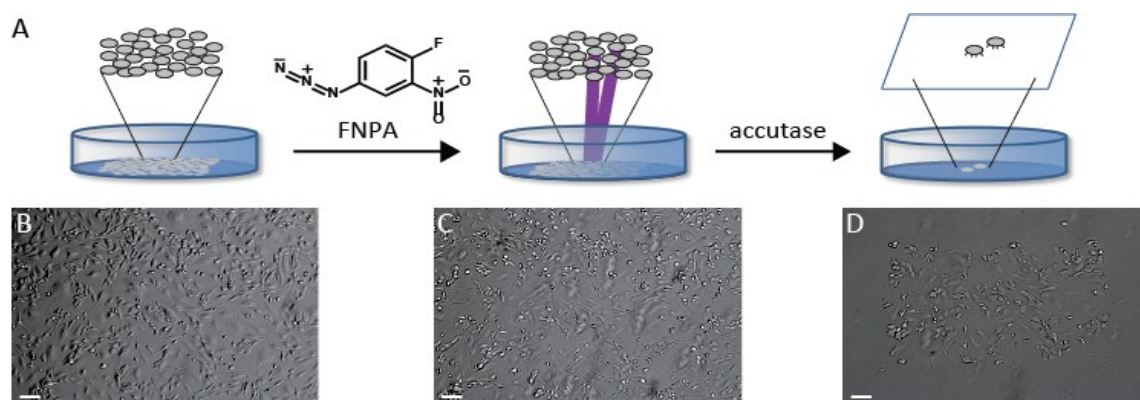


Figure S1. Photosticking MDCK cells with FNPA (4-Fluoro-3-nitrophenyl azide). (A) FNPA ($4 \mu\text{M}$) was added to MDCK cells (B) followed by projecting a square pattern of 407 nm light via DMD onto cells for 15 min ($825 \text{ J}/\text{cm}^2$). Cells were then rinsed three times with DPBS (C) followed by addition of accutase at $37 \text{ }^\circ\text{C}$ for 3 min. A rectangular pattern of MDCK cells can be observed after accutase development (D). Scale bar = $100 \mu\text{m}$.

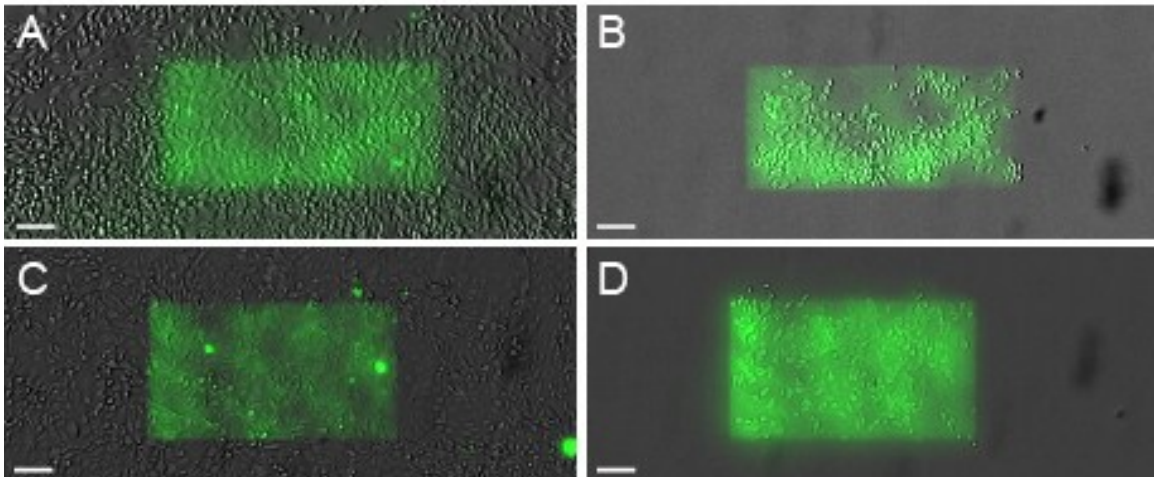
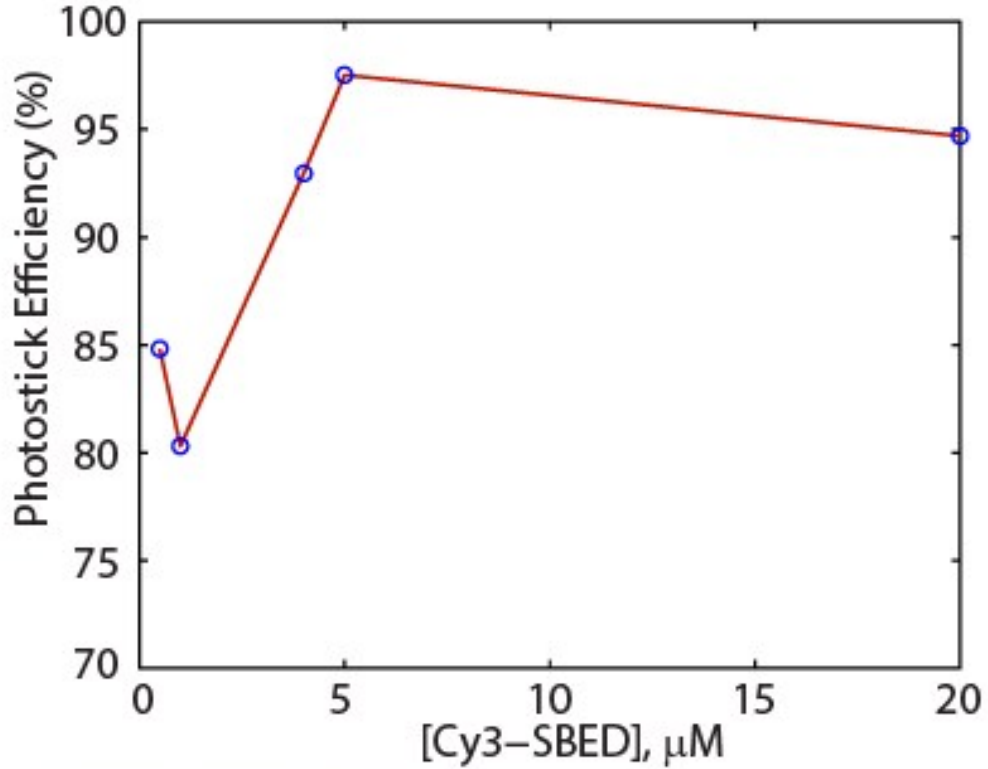


Figure S2. Photosticking efficiency as a function of Cy3-SBED concentration. Cy3-SBED at 0.5 μM , 1 μM , 4 μM , 5 μM , and 20 μM was added to MDCK cells, followed by projecting a square pattern of 407 nm light ($825 \text{ J}/\text{cm}^2$) via DMD onto the cells. Cells were then rinsed three times with DPBS followed by addition of accutase at 37 $^\circ\text{C}$ for 3 min. Cy3-SBED concentration above 4 μM yielded good Photostick efficiency. A-D) Representative data. A) Merged fluorescence and bright field images before accutase treatment with 0.5 μM Cy3-SBED. B) Same sample as (A) after accutase treatment. C,D) Same as (A,B) but with 4 μM Cy3-SBED. Scale bar = 100 μm .

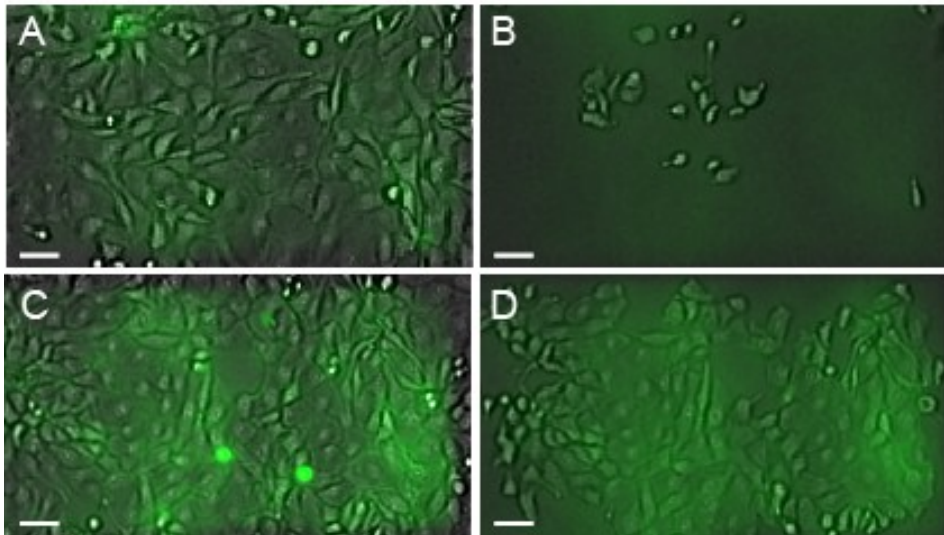
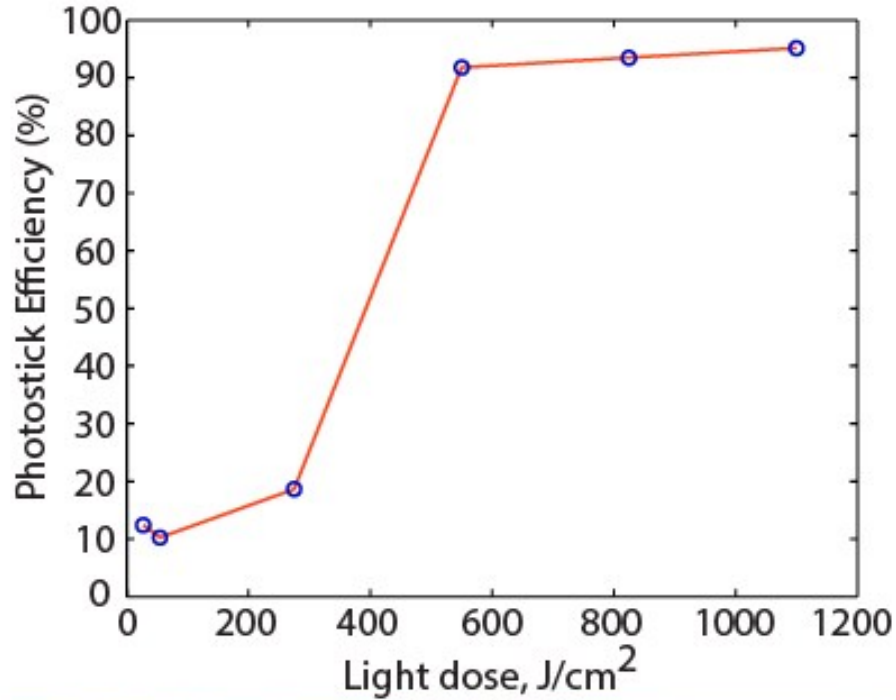


Figure S3. Photosticking efficiency as a function of 407 nm light dose. 4 μM of Cy3-SBED was added to MDCK cells followed by projecting a square pattern of 407 nm light onto the cells with a DMD. After treatment with accutase (37 $^{\circ}\text{C}$ for 3 min), the efficiency was calculated as the number of cells remaining divided by the number of cells illuminated. Illumination light doses above 550 J/cm^2 yielded high photosticking efficiencies. A-D) Representative data. A) Merged fluorescence and bright field images before accutase treatment with illumination dose of 275 J/cm^2 . B) Same sample as (A) after accutase treatment. C,D) Same as (A,B) but with illumination dose of 825 J/cm^2 . Scale bar = 50 μm .

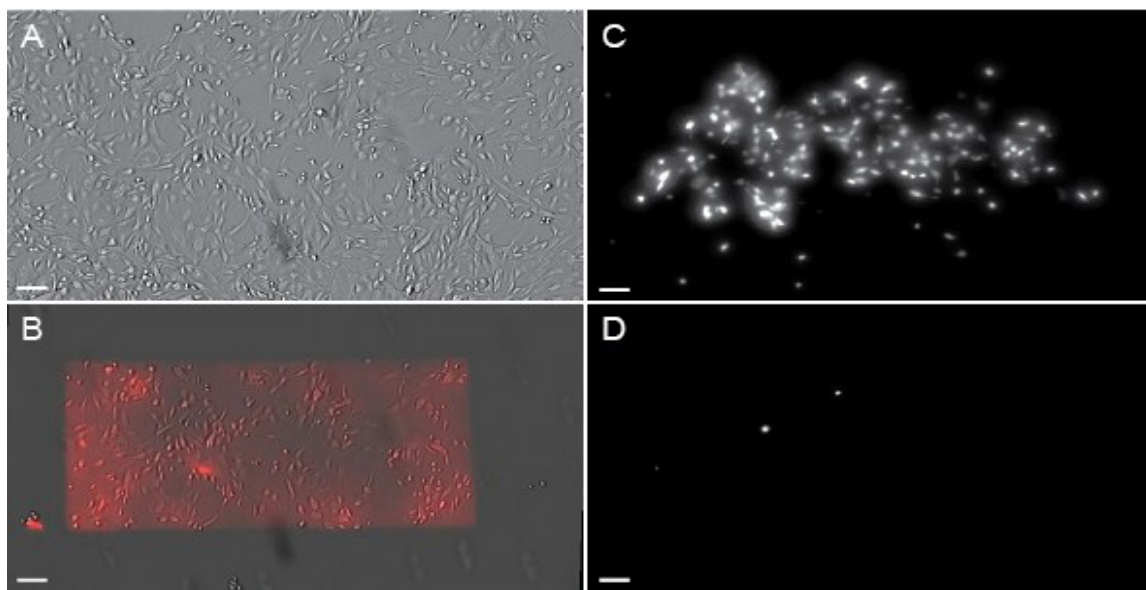


Figure S4. Effect of photostick protocol on cell viability. MDCK cells were subjected to the photostick protocol (illumination dose 825 J/cm^2 , $4 \mu\text{M}$ Cy5-SBED). A) Bright-field image after exposure but before accutase treatment. B) Combined fluorescence and bright-field images after treatment with accutase (3 min, $37 \text{ }^\circ\text{C}$). Cells were then incubated at $37 \text{ }^\circ\text{C}$, 5% CO_2 for 16 hrs before LIVE/DEAD viability staining (Life Technologies, Part Number: L-3224). C) Live cells were stained with green-fluorescent Calcein-AM to indicate intracellular esterase activity. D) Dead cells were stained with red-fluorescent ethidium homodimer-1 (EthD-1) indicating loss of plasma membrane integrity. The survival rate was 98%. Scale bar = $100 \mu\text{m}$.

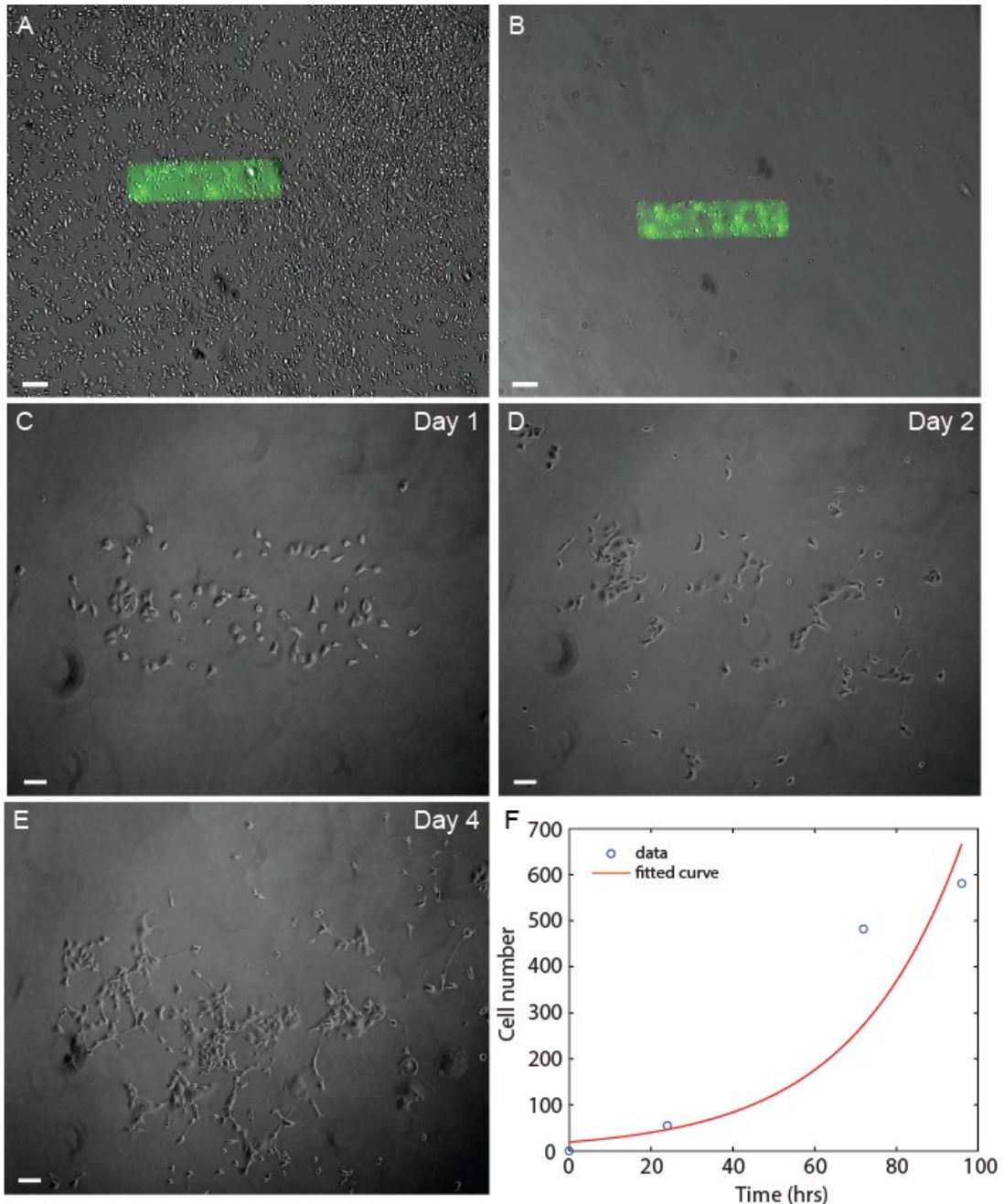


Figure S5. Effect of photostick protocol on MDCK cell growth rate. MDCK cells were treated with 4 μM Cy3-SBED and then exposed to a rectangle of 407 nm light (825 J/cm^2). A) Bright-field image merged with Cy3 image after 407 nm illumination. B) Bright-field image merged with Cy3 image after accutase development. C-E) Bright-field images of MDCK cells after 1, 2 and 4 days showing cell growth and migration. F) The cell doubling time post-photostick is 34 hrs. Scale bar = 200 μm .

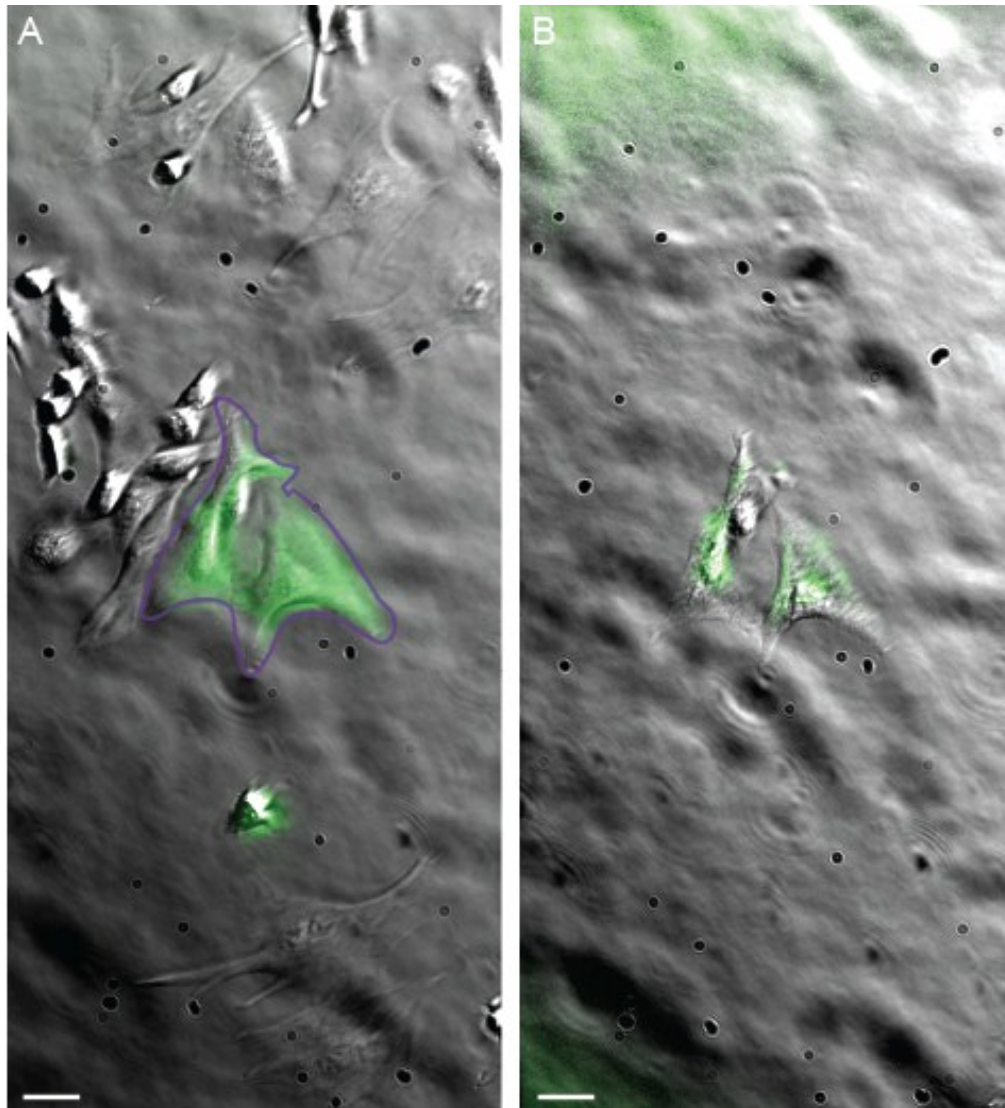


Figure S6. Using photostick to select multiple YFP-MDCK cells. 4 μM of photoactivatable Cy3-SBED was added in the dish and 407 nm light (8200 J/cm^2) was selectively projected onto a small cluster of YFP-MDCK cells using a pair of galvo mirrors. A) Combined bright-field and YFP fluorescence image after 407 nm illumination. Illuminating pattern indicated with purple outline. B) Combined bright-field and YFP fluorescence image after accutase treatment. Scale bar 30 μm .

References

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