Supporting Information for

CRISPR/Cas12a-Responsive Dual-Aptamer DNA Network for Specific Capture and Controllable Release of Circulating Tumor Cells

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Video S5: Video of forming a DNA network in the culture medium and capturing circulating tumor cells.

1. Experimental section

1.1. Materials and reagents

All DNA oligonucleotides (Table S1) were synthesized and purified by Sangon Biotech. Co. Ltd. (Shanghai, China). T4 DNA ligase (400000 U/mL), phi29 DNA polymerase (10000 U/mL), DNase I (2000 U/mL) were obtained from New England Biolabs (NEB, Beijing, China). Deoxyribonucleoside 5′-triphosphate mixture (dNTPs) was obtained from Tiangen Biotech. Co. Ltd. (Beijing, China). crRNA was purchased from GenScript. Biotech. Co. Ltd. (Nanjing, China). Lba Cas12a (cpf1) was obtained from Tolo Biotech. (Shanghai, China). 10 × 2.1 buffer (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 100 μg/mL BSA, pH 7.9) was purchased from New England Biolabs (Beijing, China). CM-DiO (green), CM-DiI (red), CellTracker Green CMFDA (green), and CytoTraceTM Red Fluorescent Probe (red) were purchased from Yeasen (Shanghai, China). Calcein-AM and propidium iodide (PI) were obtained from Meilunbio (Dalian, China). SYBR Green I, GelRed, and normal human serum were purchased from Solarbio (Beijing, China). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY, USA). All other reagents were analytical grade and directly used without further purification. Ultrapure water (resistance ≥ 18.2 MΩ·cm) was used throughout the experiments.

1.2. Apparatus

The cell fluorescence images were captured by confocal laser scanning microscope (Nikon A1R) equipped with 4 ×, 20 ×, and 100 × objective lens. Atomic force microscopy (AFM) characterization was carried out using Bruker Dimension Icon (USA). Scanning electron microscope (SEM) images were measured on JEOL JSM-7500F (Japan). The viscoelastic mechanical properties were investigated by a DHR-2 rheometer (TA Instruments, USA). Flow cytometry results were recorded in FACS Calibur (BD, USA).

Table S1. Sequences of the oligonucleotides used in this work.

Oligonucleotide		Sequences (5' to 3')
Aptamer	sgc8c	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
	FAM-sgc8c	FAM-ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
	sgc4f	ATCACTTATAACGAGTGCGGATGCAAACGCCAGACAGGGGGACAGGAGATAAGTGA
	TAMRA-sgc4f	TAMRA-ATCACTTATAACGAGTGCGGATGCAAACGCCAGACAGGGGGACAGGAGATAAGTGA
	SYL3C	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG
CEM (C1, C2)	Primer1	GTAGGAACATCAAACGACAGCCA
	Padlock1 (sgc8c)	P-TCGTTTGATGTTCCTACTCTAACCGTACAGTATTTTCCCGGCGGCGCAGCAGTTAGATTGGCTG
	Primer2	AAGTGATGGCTGTCGTTTGATGTTCCTA
	Padlock2 (sgc4f)	P-CAGCCATCACTTATCTCCTGTCCCCCTGTCTGGCGTTTGCATCCGCACTCGTTATAAGTGATTAGGAACATCAAACGA
CEM (C2-8c)	Primer2	GTTAGATGGCTGTCGTTTGATGTTCCTA
	Padlock2 (sgc8c)	P-CAGCCATCTAACCGTACAGTATTTTCCCGGCGGCGCAGCAGTTAGATGTAGGAACATCAAACGA
MCF-7 (M1, M2)	Primer1	GTAGGAACATCAAACGACAGCCA
	Padlock1 (sgc8c)	P-TCGTTTGATGTTCCTACTCTAACCGTACAGTATTTTCCCGGCGGCGCAGCAGTTAGATTGGCTG
	Primer2	GTTGGCCTGTGGCTGTCGTTTGATGTTCCTACCACTAC
	Padlock2 (SYL3C)	P-CAGCCACAGGCCAACCCCCCATGACAACGTGGGACAGACGCAACCTCTGTAGTGGTAGGAACATCAAACGA
Padlock1 random (R1)		P-TCGTTTGATGTTCCTACGGTCAGATGAGGAGGGGGACTTAGGACTGGGTTTATGACCTTGGCTG
Padlock2 random (R2)		P-CAGCCATCACTTACCCACCAGCAGTGTATGACCCGTTCGGATCCGAACGGGTCATAGTGTCAGTAGGAACATCAAACGA
CRISPR /Cas12a	crRNA	UAAUUUCUACUAAGUGUAGAUUGAAGUAGAUAUGGCAGCAC

	dsDNA initiator-1	AATATGTCATTATGTGCTGCCATATCTACTTCAGAAACT
	dsDNA initiator-2	AGTTTCTGAAGTAGATATGGCAGCACATAATGACATATT
	dsDNA random-1	AGGTACTGGAGACTGTGTAGAAGCACATATTGTTAAATT
	dsDNA random-2	AATTTAACAATATGTGCTTCTACACAGTCTCCTGTACCT

The blue parts in Padlock strands are complementary to the Primer strands. Padlock strands can form circular RCA templates after treating the Primer/Padlock complex with ligase. The red parts are the templates that are used to produce aptamer sequences.

The two ultra-long RCA chains used for the capture of CEM cells are named C1 and C2, which contain sgc8c aptamer and sgc4f aptamer, respectively. The two ultra-long RCA chains used for the capture of MCF-7 cells are named M1 and M2, which contain sgc8c aptamer and SYL3C aptamer, respectively. The two ultra-long RCA chains contain non-aptamer random sequences named R1 and R2.

1.3. Preparation of DNA Networks

Firstly, circular RCA templates were synthesized. Taking the synthesis of C1, which was used for the capture of CEM cells, as an example, Primer1 (1.5 μM) was first incubated with Padlock1 (sgc8c) (1 μM) in 1 × T4 ligase buffer. Then, the reaction tube was put in a PCR thermal cycler at 95 °C for 5 min and cooled gradually to room temperature. Under the annealing procedure, Primer1 hybridized with the two ends of Padlock1 (sgc8c), which was then ligated with 10 U/μL T4 ligase at 16 °C overnight to obtain a circular template. Secondly, 100 nM circular DNA template, 2 × BSA, 1 mM dNTPs, 50 mM NaCl, 1 × phi29 buffer, and 0.2 U/μL of phi29 polymerase were mixed and incubated at 37 °C for 10 h to obtain long RCA chain C1. In the same way, C2, M1, M2, R1, R2 were prepared. The successful synthesis of above templates was validated through polyacrylamide gel electrophoresis (PAGE) gel. 2 μL 6 × Super GelRed Prestain Loading Buffer (US EVERBRIGHT INC.) was added to the above samples and sufficiently mixed. The samples were analyzed by 10% PAGE in 1 × TAE buffer at a 120 V constant voltage for 50 min. The gel was visualized using a Gel Image

System (Amersham Biosciences).

To prepare the DNA network, C1 and C2 (M1 and M2; R1 and R2) were mixed in a shaker for 5 h at 37 °C and 600 rpm. After being stained with SYBR Green I, the DNA network could be observed under 302 nm UV light. To observe the microstructure, the DNA network needs to be freeze-dried and observed under a confocal laser scanning microscope (Nikon A1R) equipped with 4 × and 20 × objective lens. All rheological experiments were carried out by an AR-G2 rheometer (TA Instruments) using 100 μL DNA network. Time scan tests were carried out with a fixed strain of 1% and a fixed frequency (1 Hz) at 25 °C for 3 min.

1.4. Specific anchoring of aptamers to CEM cells

For the confocal laser scanning microscope analysis, 1×10^5 CEM cells were seeded 24 h in advance. After centrifugation to remove the complete medium, 500 μL of 1 × PBS buffer containing 1 mg/mL BSA, 0.1 mg/mL tRNA, and 100 nM FAM-sgc8c were added and incubated for 30 min at 4 °C. The Cells were washed twice with 500 μL of 1 × PBS buffer containing 1 mg/mL BSA and then suspended in 1 mL of PBS buffer. CEM-targeted anchoring of the sgc4f aptamer can be achieved by simply replacing the FAM-sgc8c with TAMRA-sgc4f. Fluorescence microscopy (Nikon A1R) was applied for cell imaging. The laser excitation wavelength was set at 490 nm and 540 nm for FAM and TAMRA, respectively. The cell images were observed with 100 × oil objective lens.

To verify that the aptamers were indeed anchored on the cell membrane surface, a co-localization experiment with the membrane dye was performed. The steps for anchoring the aptamer to CEM cells were the same as above. In short, CEM cells were incubated with FAM-sgc8c for 30 minutes and washed once with 1 × PBS buffer containing 1 mg/mL BSA. Aptamer-treated CEM cells were then stained with 3 μmol CM-DiI at 37 °C for 15 min. After being washed twice using 1 × PBS buffer, CEM cells were imaged by confocal fluorescence microscopy.

To evaluate the binding performance of aptamers to target cells, various concentrations of FAM-labeled aptamers and 1×10^6 cells were prepared in 200 μL of

working buffer for cell capture and then incubated for 30 min at 4 °C. The working buffer refers to the 1640 medium in the presence of phi29 buffer and T4 ligase buffer. After washing twice with PBS buffer, cells were resuspended in 200 μL of working buffer for flow cytometry analysis by counting 10,000 events (FACS Calibur, BD) for signal recording. The K_d values were obtained by fitting the dependence of fluorescence intensity (Y) on the concentration of aptamers (X) to the equation $Y = B_{max}X/(K_d + X)$ by Origin 8.5 software.

1.5. Capture of CTCs by DNA network

Human acute lymphoblastic leukemia CCRF-CEM (abbreviated as CEM) cells and human Burkitt lymphoma Ramos cells were cultured in RPMI 1640 medium (GIBCO), and human breast cancer MCF-7 cells were cultured in becco's modified Eagle's medium (DMEM). The medium was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin-amphotericin B) at 37 °C in CO_2 incubator. Taking the capture of CEM cells as an example, CEM cells were first obtained by centrifugation at 800 rpm for 3 min. The cell number was counted by a counting chamber before further usage. C1 (stained by SYBR Green I) was incubated with CEM cells (labeled by CM-DiI) in a shaking dry bath at 37 °C for 30 min. Then, C2 was added to the mixture and continued shaking for another 30 minutes. CEM cells and Ramos cells were captured using C1 and C2, and MCF-7 cells were captured using M1 and M2. R1 and R2 with random sequences were used to capture the above three kinds of cells. When testing the cell capture efficiency, the reaction buffer is 1× PBS. When testing the cell capture and release, the reaction buffer is 1640 medium without 10% FBS. The captured cells and DNA network were observed by fluorescence microscopy. The laser excitation wavelength was set at 490 nm and 540 nm for SYBR Green I and CM-DiI, respectively. The cell images were observed with 20 × objective lens. The 3D stacking of cells in the DNA network was obtained by a confocal fluorescence microscope, one layer was scanned at 10 μm, and a total of 15 layers were scanned.

1.6. Evaluation of the cell capture rate and captured cell numbers

To investigate the cell capture rate and captured cell numbers as a function of time, 1×10^5 CEM, Ramos or MCF-7 cells were incubated in 100 μL DNA network. Then, the cells in the supernatant were counted every 10 min. The capture rate was calculated by counting the target cell number in the supernatant before and after capture (N_{before} and N_{after}). The capture rate (R) was calculated as $(N_{before} - N_{after})/N_{before} \times 100\%$. By using different amounts of CEM, MCF-7 and Ramos cells (0.5×10^5, 1×10^5, 2×10^5 and 3×10^5), the capture rate and captured cell numbers were measured in the same way.

1.7. Specific enrichment of CTCs from a cell mixture

CEM (MCF-7) cells stained with CellTracker Green CMFDA (green) and Ramos cells stained with CytoTraceTM Red Fluorescent Probe (red) were mixed at different ratios of 1:1 and 1:10 in 1640 culture media. Taking the enrichment of CEM cells from the mixture of CEM and Ramos cells as an example, C1 was first added to the cell suspension and incubated at 37 °C for 30 min. Then, C2 was added and incubated in a shaking dry bath for another 30 min, allowing DNA network formation. The formed bulk DNA network containing captured cells was transferred into fresh 1640 culture medium and CRISPR/Cas12a solution containing 1 μM dsDNA initiator, 1 μM crRNA, 1 μM Cas12a, 0.1 × NEB buffer 2.1 were added to disassemble the DNA network for 1 h to release the enveloped cells. The released cells were cultured for 48 h, and the cell images were taken under a fluorescence microscope. The fold of enrichment was calculated by comparing the ratio of target cells to non-target cells before and after capture. The capture of MCF-7 cells from the MCF-7 and Ramos mixtures was performed in the same way. When using simulated blood sample for cell enrichment study, the cells were centrifuged and resuspended in normal human serum, and then subjected to cell capture and release as before.

1.8. CRISPR/Cas12a-responsive release of CTCs from DNA network

To investigate CRISPR/Cas12a-responsive disassembly of DNA network, we incubated the DNA network at 37 °C with CRISPR/Cas12a solution containing 1 μM dsDNA initiator, 1 μM crRNA, 1 μM Cas12a, 0.5 × NEB buffer 2.1 for 1 h. The DNA

network was stained by SYBR Green I and the disassembly process was observed under 302 nm ultraviolet light. CRISPR/Cas12a-responsive disassembly of the DNA network could be verified by PAGE analysis in 1 × TAE buffer at a 120 V constant voltage for 50 min.

To investigate CRISPR/Cas12a-responsive cell release from DNA network, the formed bulk DNA network was removed from the supernatant and then transferred to a new tube filled with RPMI 1640 culture medium. The mixture was incubated at 37 °C with CRISPR/Cas12a solution containing 1 μM dsDNA initiator, 1 μM crRNA, 1 μM Cas12a, 0.1 × NEB buffer 2.1 for 1 h. For the cell viability assay, released cells were collected and cultured in a cell culture plate for 48 h and then stained with Calcein-AM and PI to assess the viability of these cells.

Real-time polymerase chain reaction (RT-PCR) was used to detect the mRNA expression of MCF-7 cytokeratin 19 (CK19) and epidermal growth factor receptor (EGFR). Total cellular RNA was extracted by Trizol total RNA extraction kit (Sangon, Shanghai) from pristine and released MCF-7. Total cellular RNA samples were prepared with a BeyoFast™ SYBR Green One-Step qRT-PCR Kit (Beyotime, China) according to the protocol. RT-PCR analysis was performed on an ABI Stepone Plus qPCR instrument. Primers for RT-PCR were as follows:

CK19 forward primer: 5'-GACTACAGCCACTACTACACGACCAT-3';

CK19 reverse primer: 5'-CGCCGTCTTCCTCCATCTCATAGC-3';

EGFR forward primer: 5'-CCAGTGACTGCTGCCACAACCA-3';

EGFR reverse primer: 5'-GAGCGGAATCCACCTCCACACT-3'.

2. Pictures of DNA network under different conditions

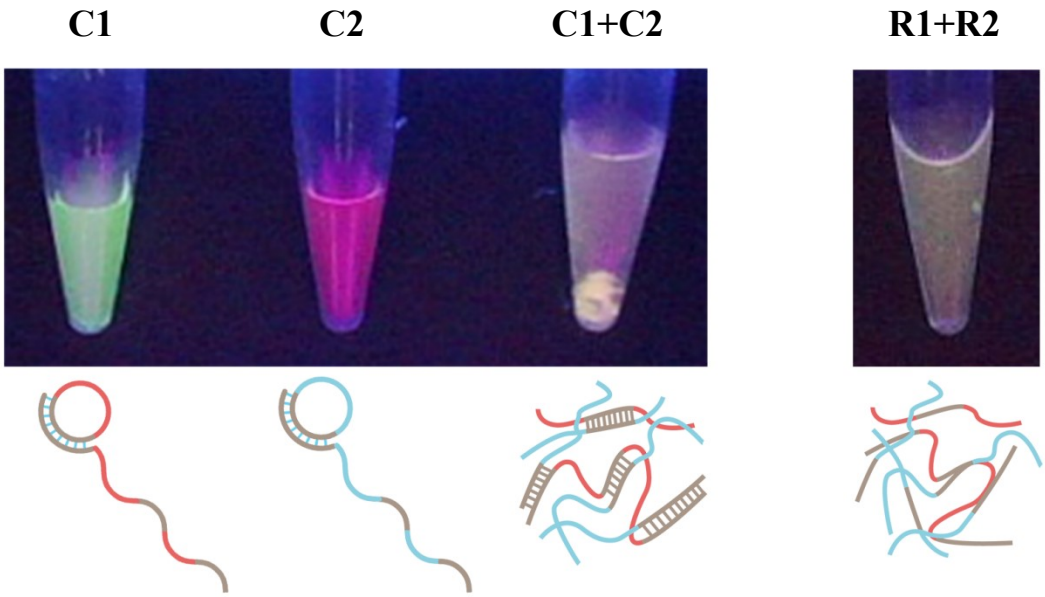

Figure S1. Pictures of DNA network under different conditions. When two RCA chains (*e.g.*, C1 and C2) are mixed, the formation of DNA network can be visualized. When R1 and R2 containing non-complementary random sequences are mixed, the DNA network cannot be formed. The two RCA chains were stained with SYBR Green I and Gel Red, respectively.

3. Confocal laser scanning microscopy images of DNA network

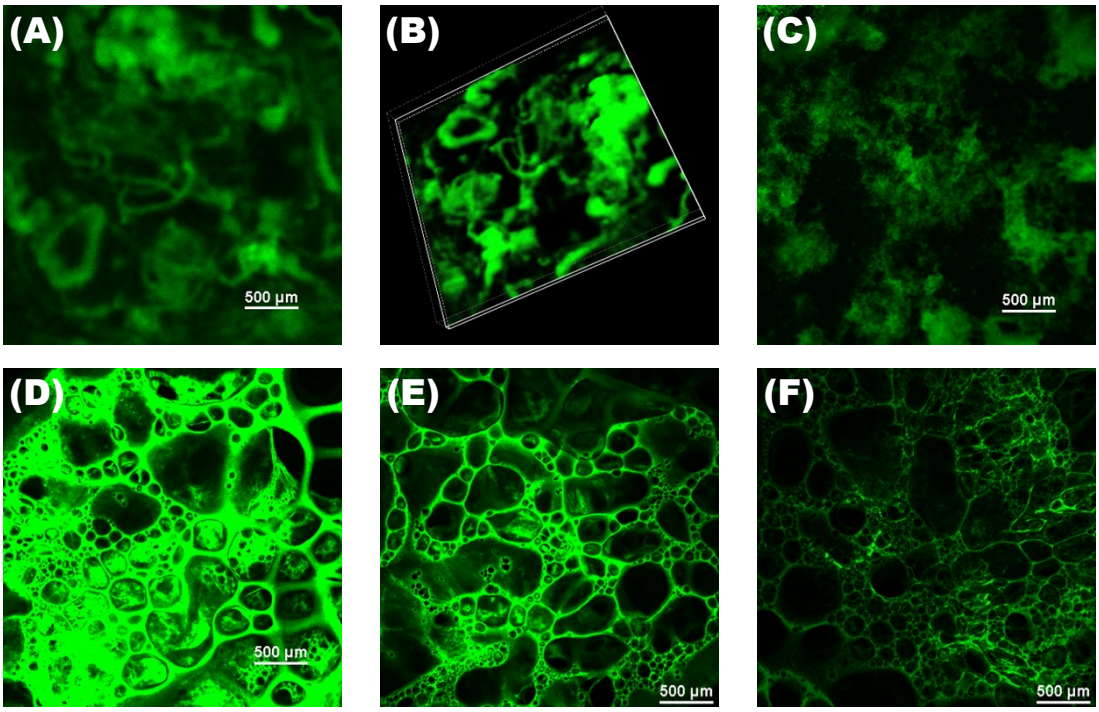

Figure S2. Confocal laser scanning microscopy (CLSM) images of the DNA network (stained with SYBR Green I). The DNA network in (A); (B) and (C) is in an aqueous solution. The DNA network in (D); (E) and (F) has been freeze-dried. The images were observed with 4× dry lens.

4. SEM images of DNA network

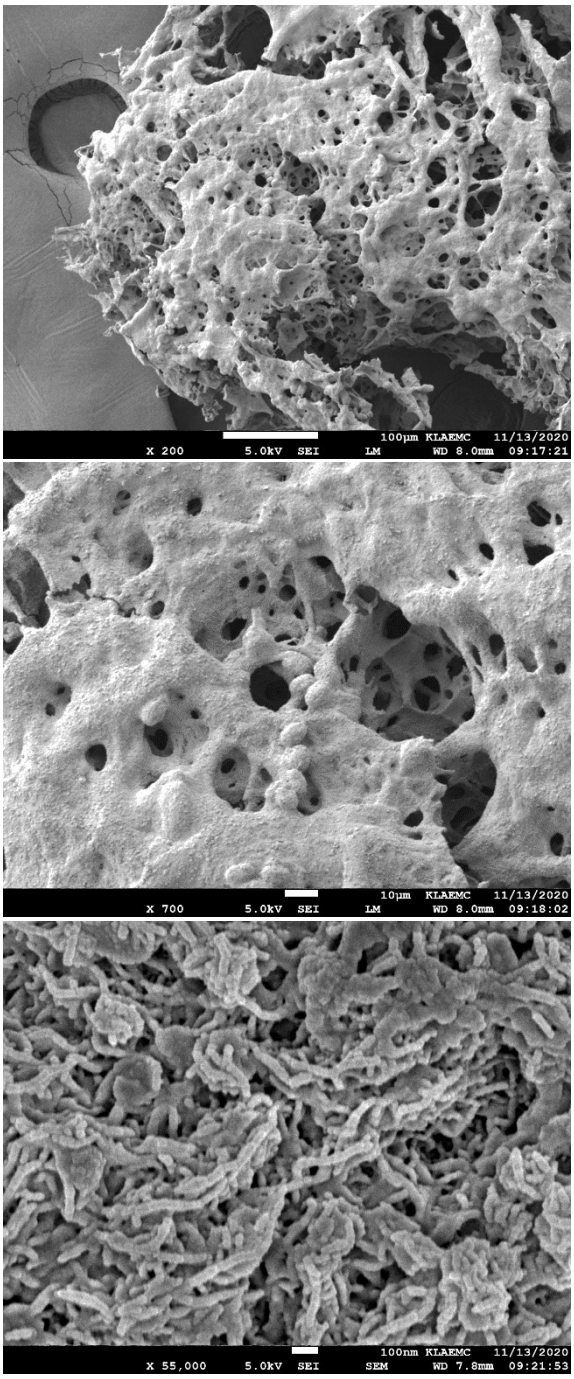

Figure S3. SEM images of the DNA network. The DNA network has been freeze-dried. The images were observed under different magnifications.

5. Specific anchoring of sgc8c on CEM cells

5.1. 3D stack images

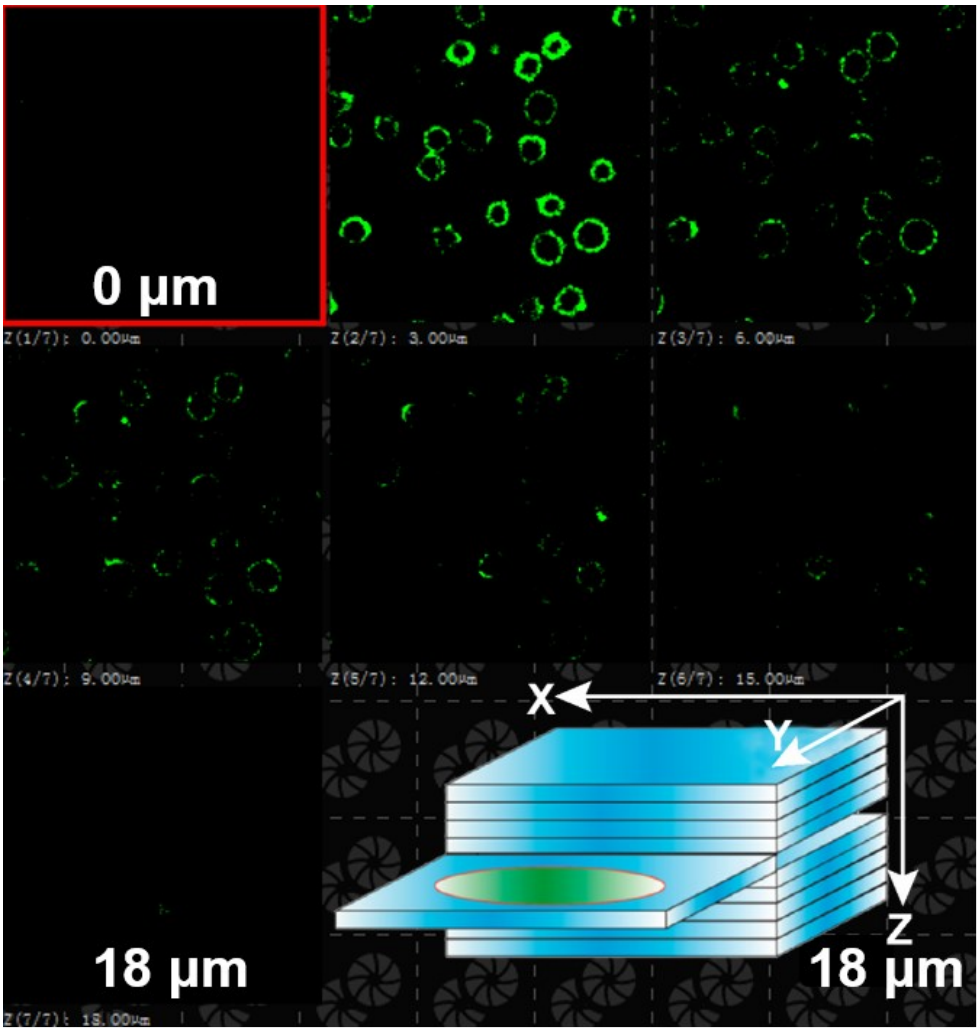

Figure S4. Confocal laser scanning 3D stack images of FAM-sgc8c-anchored CEM cells. CEM cells are scanned every 3 μm for a total of 18 μm

5.2. Colocalization images

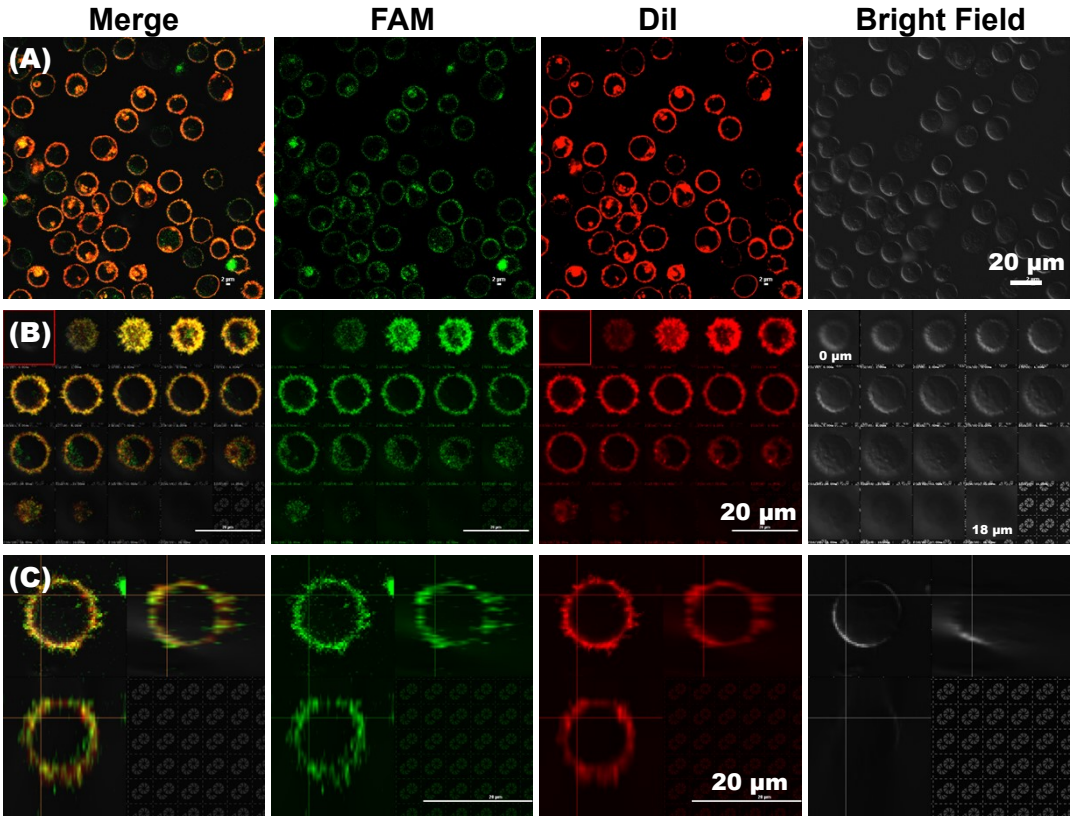

Figure S5. (A) Confocal laser scanning microscopy (CLSM) colocalization images of FAM-sgc8c (green) and membrane localization dye CM-DiI (red). Scale bar = 20 μm. (B, C) CLSM images of one of the enlarged CEM cell. Scale bar = 20 μm.

6. Specific anchoring of sgc4f on CEM cells

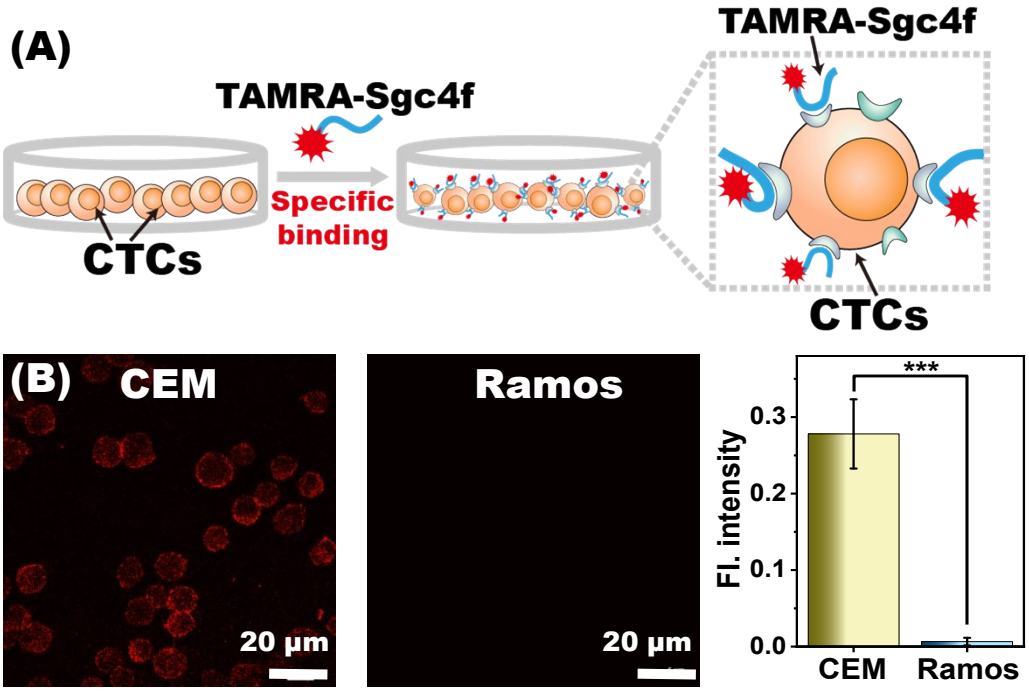

Figure S6. (A) Targeted recognition and binding of TAMRA-sgc4f (red) toward CEM cells. (B) CLSM and fluorescence analysis images demonstrate the specific binding of TAMRA-sgc4f on CEM cells rather than Ramos cells (control). Bars represent mean ± SD (n = 3); statistical analysis was performed using unpaired two-tailed t test; ***, $P < 0.001$.

7. Capture of circulating tumor cells in the DNA network

7.1. 3D stacking CLSM images of CEM cells before and after capture

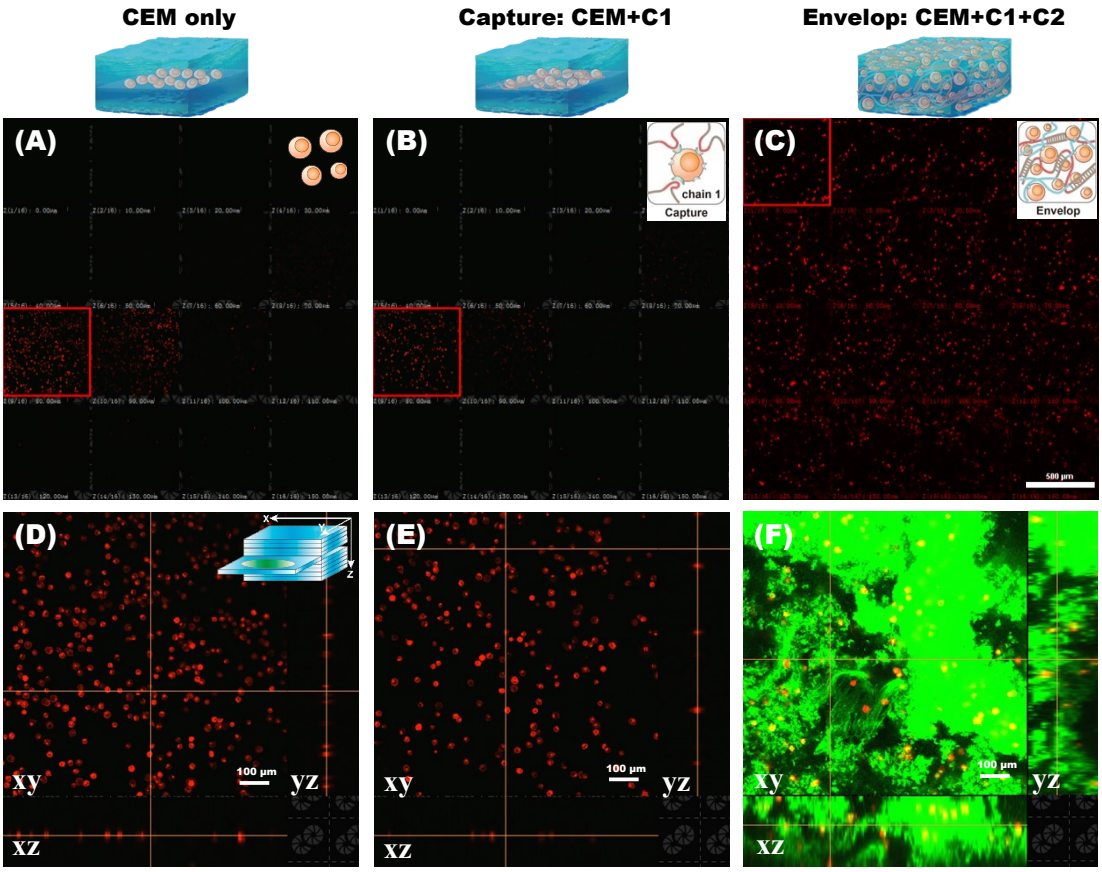

Figure S7. Capture of CTCs in DNA network. (A, D) 3D stacking CLSM images of CEM cells only. (B, E) 3D stacking CLSM image of CEM+C1, showing the anchoring of aptamer units on cells. (C, F) 3D stacking CLSM image of CEM+C1+C2, showing the process of capturing cells. Stack height: 160 μm. CTCs are stained with CM-DiI, red. DNA network are stained with SYBR Green I, green.

7.2. Spatial distribution of CTCs in DNA network

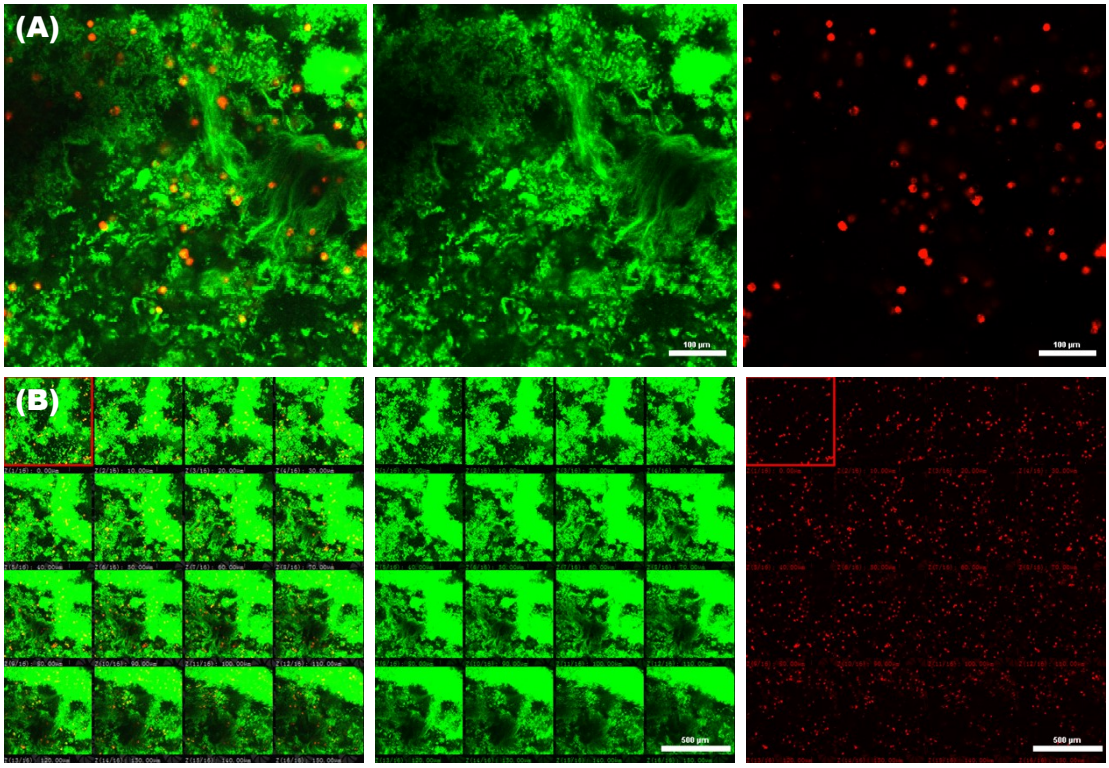

Figure S8. (A) CLSM image of CTCs (stained with CM-DiI, red) enveloped in the DNA network (stained with SYBR Green I, green). (B) 3D stacking images showing the uniform spatial distribution of CTCs in the DNA network.

8. Capture of MCF-7 cells in the DNA network

8.1. Brightfield microscopy characterization of MCF-7 cells capture by different DNA networks

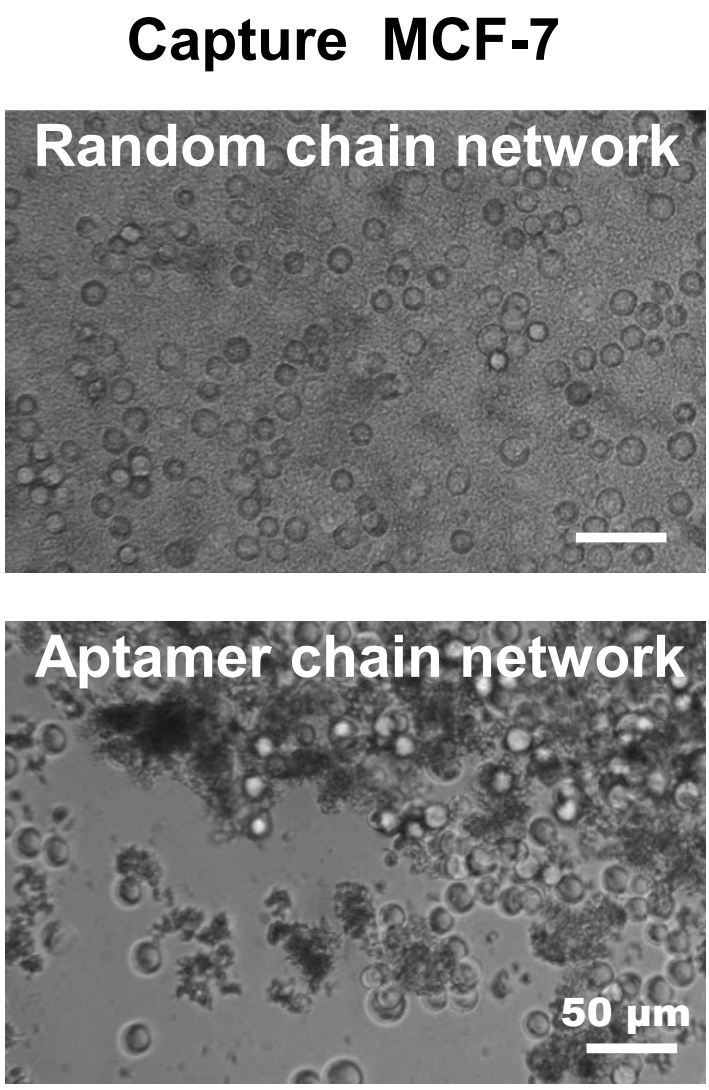

Figure S9. Brightfield microscopy characterization of the capture of MCF-7 cells by a DNA network containing (top) non-aptamer random sequences (R1 and R2) or (bottom) aptamer sequences (M1 and M2). The Cells treated with R1/R2 were evenly dispersed in the DNA network, while the cells treated with M1/M2 were present in tight clusters.

8.2. Binding affinity of SYL3C aptamer to MCF-7 cells

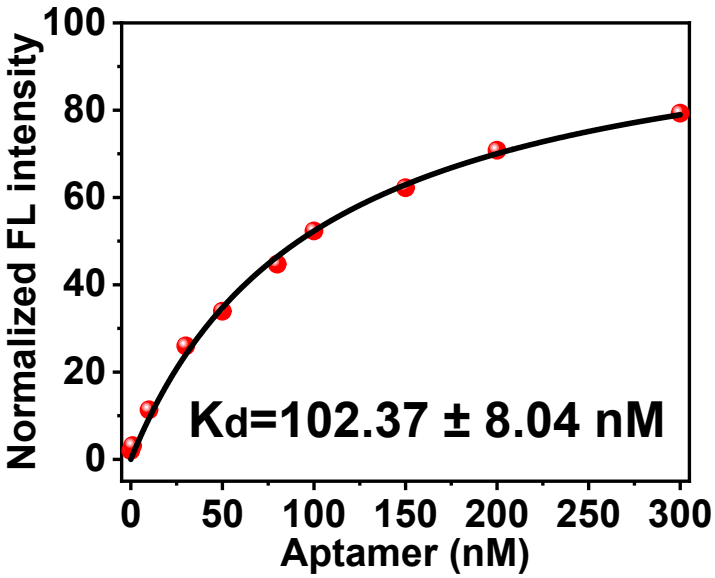

Figure S10. Binding affinity of SYL3C aptamer to MCF-7 cells in the working environment for cell capture.

9. CRISPR/Cas12a-responsive DNA network disassembly and cell release

9.1. PAGE characterization of CRISPR/Cas12a-responsive DNA network disassembly

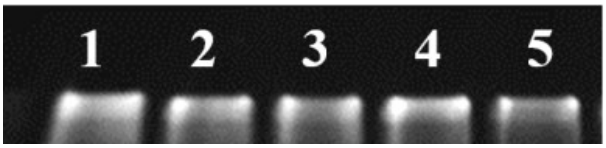

Figure S11. Non-denaturing PAGE characterization of CRISPR/Cas12a-responsive DNA network disassembly under different conditions. Lane 1: DNA network only; lane 2: DNA network + dsDNA + Cas12a; lane 3: DNA network + dsDNA + crRNA; lane 4: DNA network + Cas12a + crRNA; lane 5: DNA network + non-aptamer dsDNA + Cas12a + crRNA.

9.2. Photographs and bright-field microscopy images of cell capture, release and culture

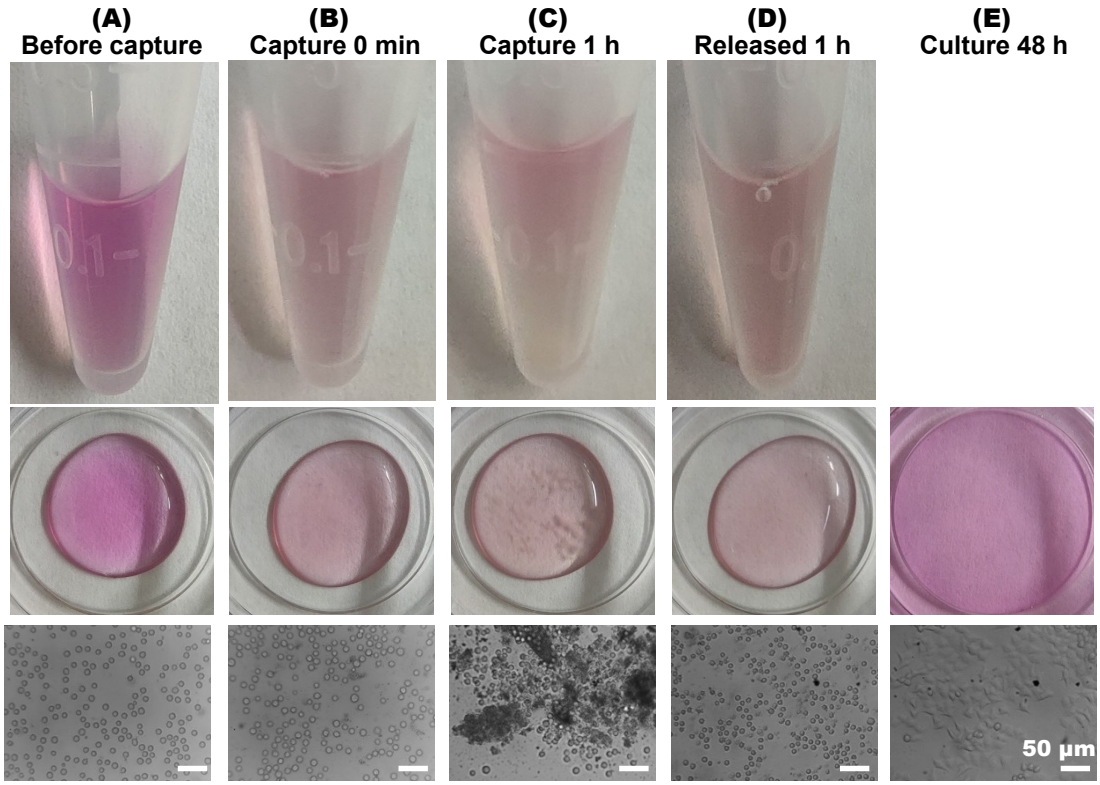

Figure S12. Photographs and bright-field microscopy images of cell capture, release and subsequent culture. (A) Before capture, MCF-7 cells were dispersed in the cell culture medium. (B) MCF-7 cells were still dispersed in the cell culture medium after the initial addition of M1 and M2. (C) 1 h later, DNA network was formed and the cells were enveloped in the DNA network. (D) After incubation of the DNA network with activated CRISPR/Cas12a system for 1 h, the DNA network was decomposed from a solid-state to a solution state, accompanied by the cell release. (E) The released cells were incubated in fresh cell culture medium for another 48 hours. The bottom row is the corresponding bright-field microscope photographs.

9.3. Bright-field microscopy characterization of cell capture, release and culture

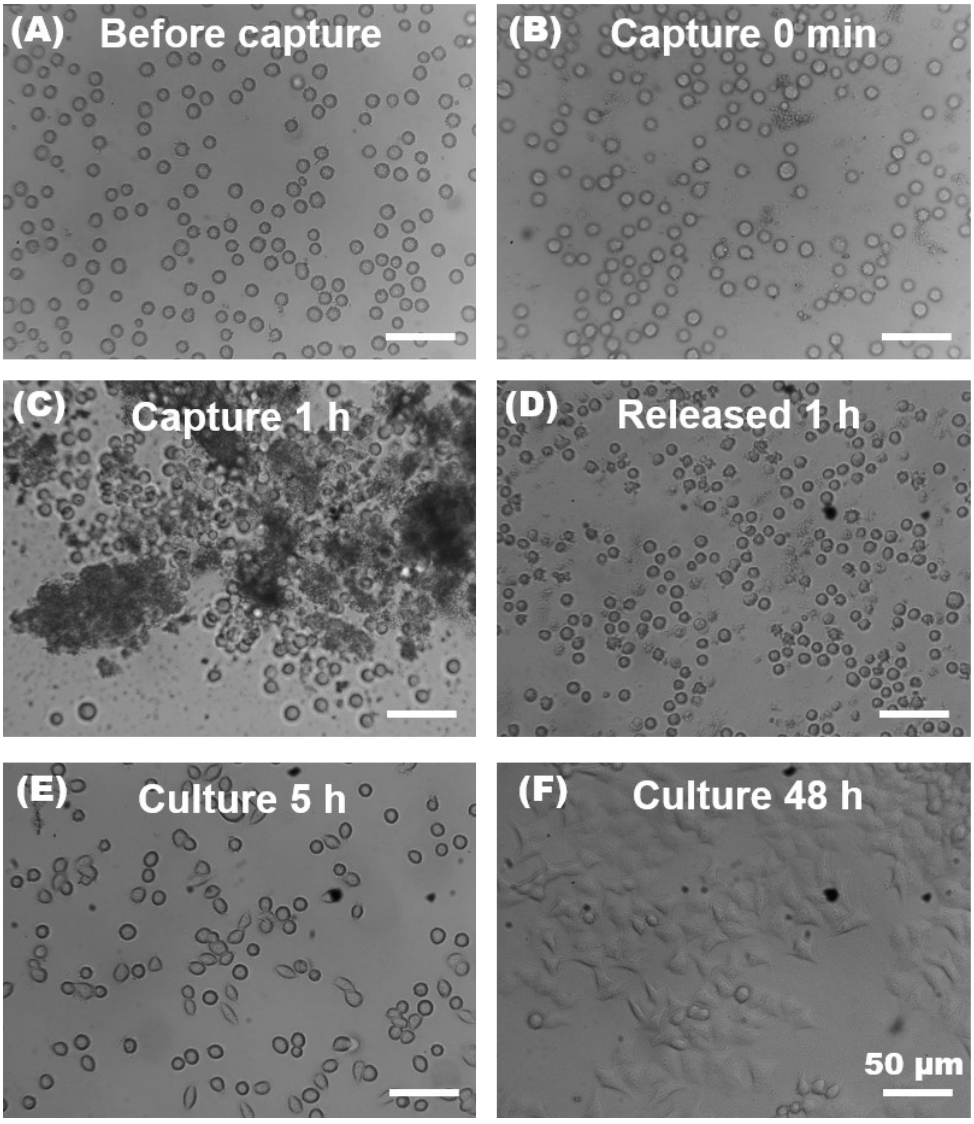

Figure S13. Bright-field microscopy images of capture, release and culture of MCF-7 cells. (A) Before capture, the cells were in monodisperse state. (B). The cells were still in monodisperse state after initial addition of M1 and M2. (C) The cells were encapsulated in DNA network and formed a cluster state after inculbation with M1/M2 for 1 h. (D) The cells returned to monodisperse state after incubation of the DNA network with activated CRISPR/Cas12a system for 1 h. (E) The cells begun to grow adherently after incubation in fresh medium for 5 h). (F) The cells were spindle-shaped and morphologically sound after 48 h of incubation.

9.4. Time-dependent release of captured MCF-7 from DNA network

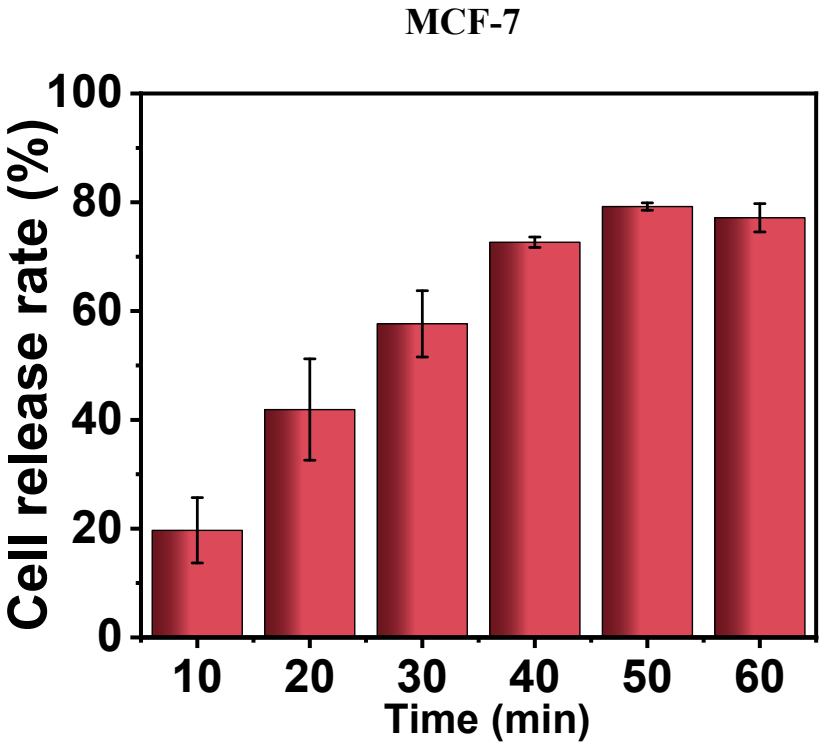

Figure S14. Cell release rate of MCF-7 cells from the DNA network as function of time. Bars represent mean ± SD (n = 3).

9.5. Viability of released CEM cells

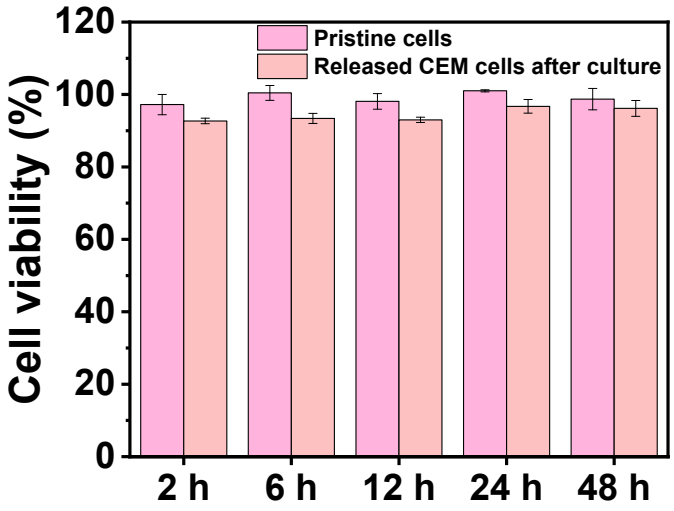

Figure S15. Viabilities of released CEM cells after incubation for different time.

9.6. Culture of released MCF-7 cells

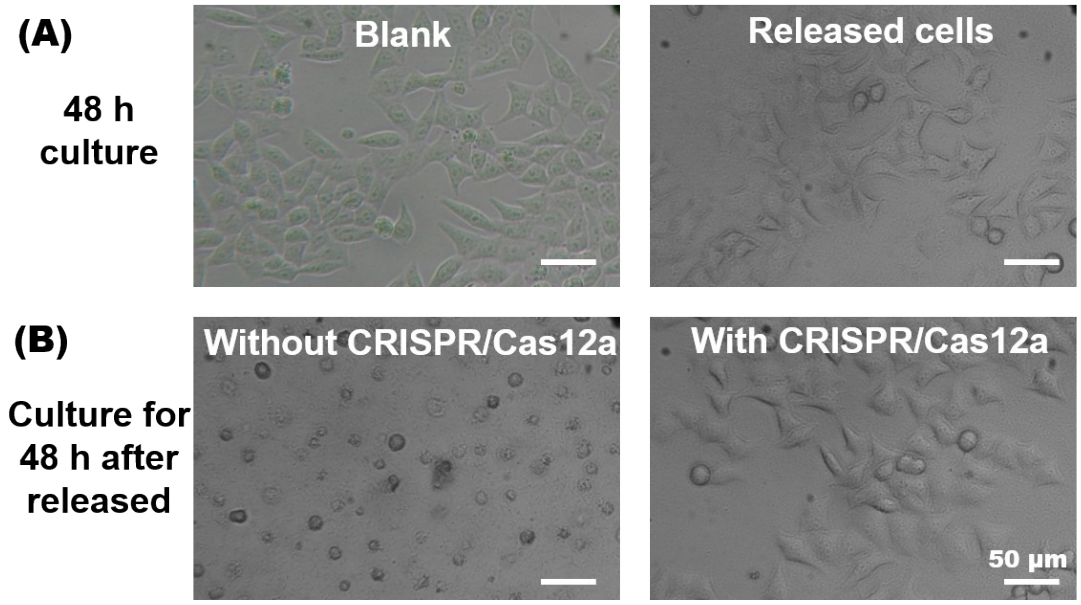

Figure S16. (A) Bright-field microscopy of pristine MCF-7 cells and released MCF-7 cells after culture for 48 h. (B) Bright-field microscopy of captured (without CRISPR/Cas12a treatment) and released (with CRISPR/Cas12a treatment) MCF-7 cells after culture for 48 h.

9.7. Cell viability of released MCF-7 cells

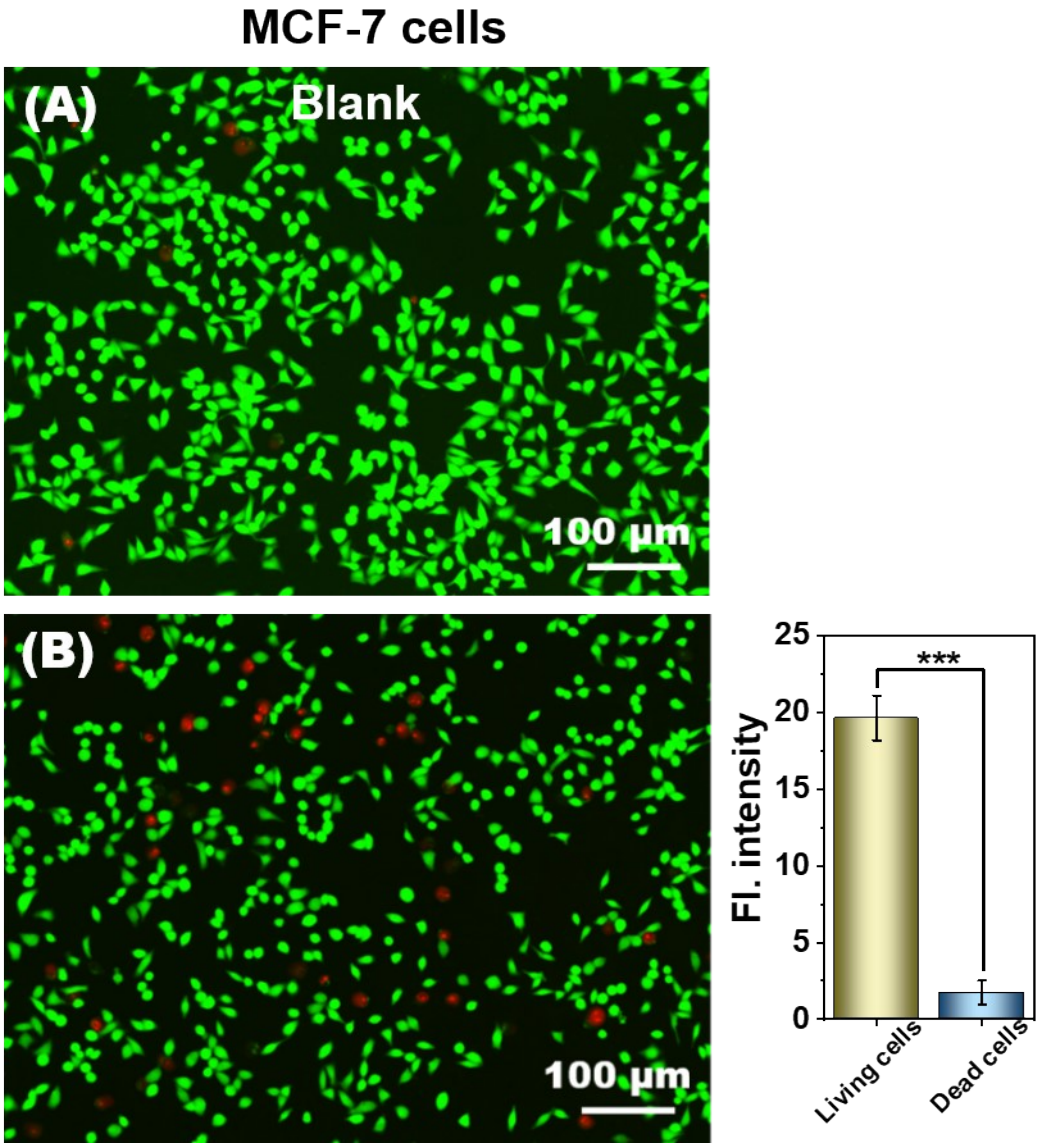

Figure S17. Cell viability comparison pristine and released MCF-7 cells. Living and dead cells were stained with Calcein-AM (green) and propidium iodide (PI, red), respectively. (A) Fluorescence microscopy image of pristine MCF-7 cells without any treatment. (B) Fluorescence microscopy image of released MCF-7 after culture for 48 h. On the right is the average optical densities of the fluorescence images. Bars represent mean ± SD (n = 3); statistical analysis was performed using unpaired two-tailed t test; ***, $P < 0.001$.

10. Evaluation of target cell enrichment from cell mixture

10.1. Enrichment of CEM cells

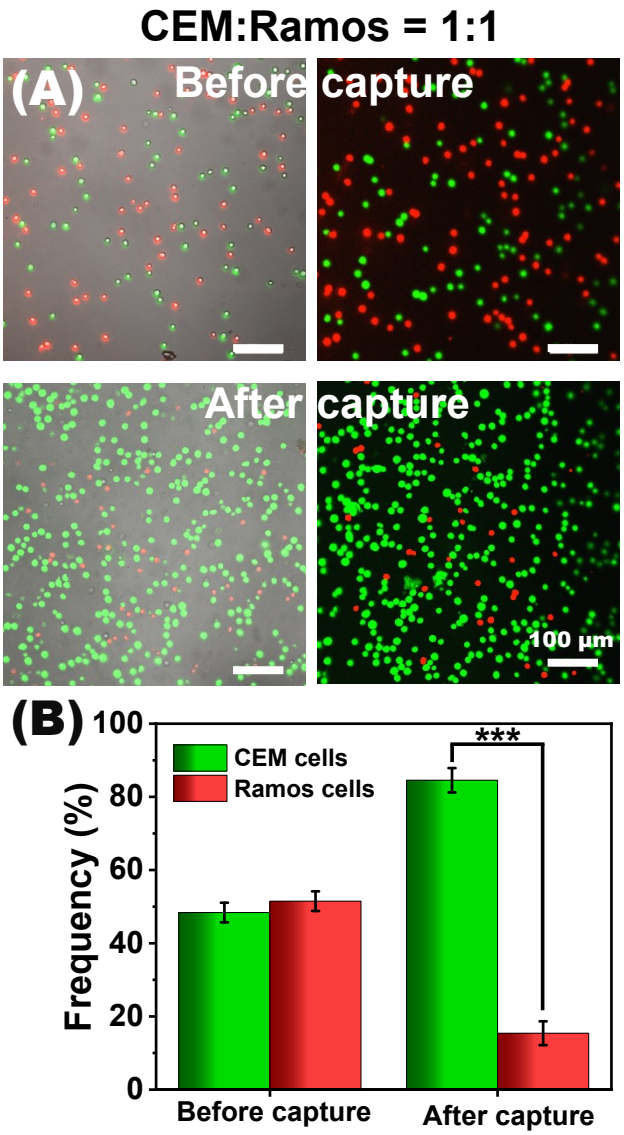

Figure S18. Specific enrichment of CEM cells from cell mixture. (A) Target CEM cells (green) and non-target Ramos cells (red) were mixed at 1:1. After the enrichment, target CEM cells were significantly enriched. The left and right pictures are parallel samples. (B) Statistical analysis shows approximate 6-fold enrichment of target CEM cells. Bars represent mean ± SD (n = 3).

10.2. Enrichment of MCF-7 cells

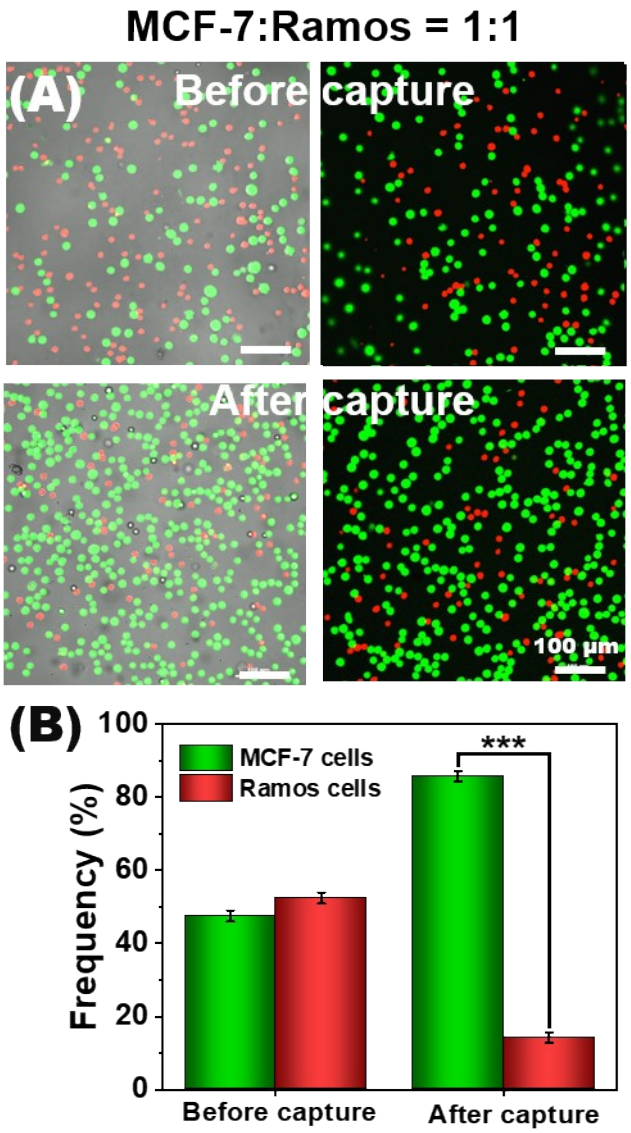

Figure S19. Specific enrichment of MCF-7 cells from cell mixture. (A) Target MCF-7 cells (green) and non-target Ramos cells (red) were mixed at 1:1. After the enrichment, target MCF-7 cells were significantly enriched. The left and right pictures are parallel samples. (B) Statistical analysis shows approximate 6-fold enrichment of target MCF-7 cells. Bars represent mean ± SD (n = 3).

11. Separation and enrichment of CTCs from simulated blood sample

11.1. Specific capture of CTCs from simulated blood sample

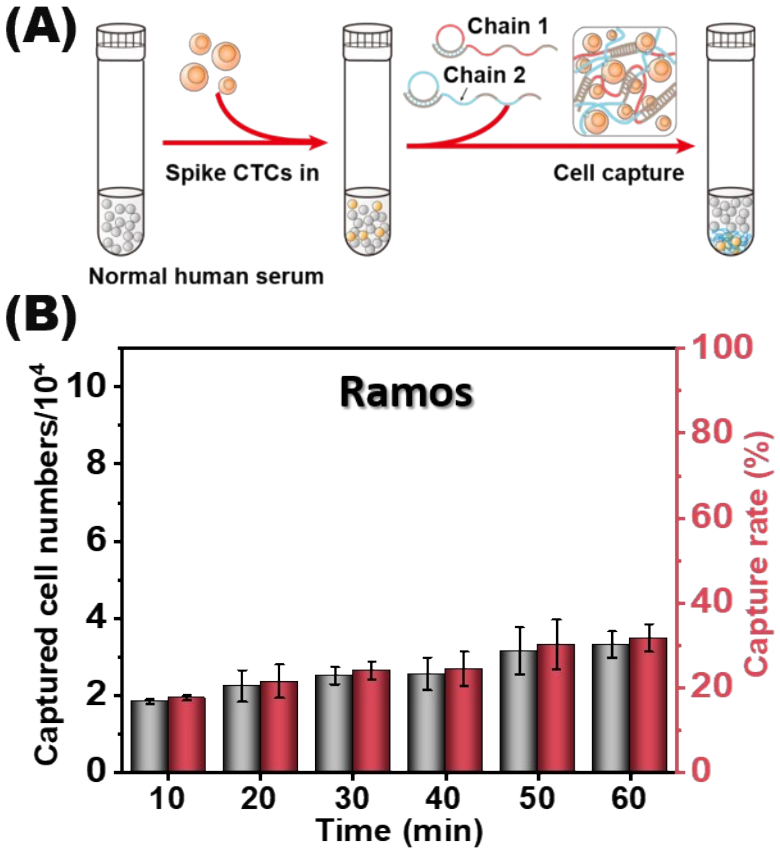

Figure S20. (A) Application of dual-aptamer DNA network for specific capture of CTCs from simulated human blood sample. (B) Time-dependent capture rate and captured cell numbers of Ramos cells from simulated human blood sample. Bars represent mean ± SD (n = 3).

11.2. Enrichment of CTCs from simulated blood sample

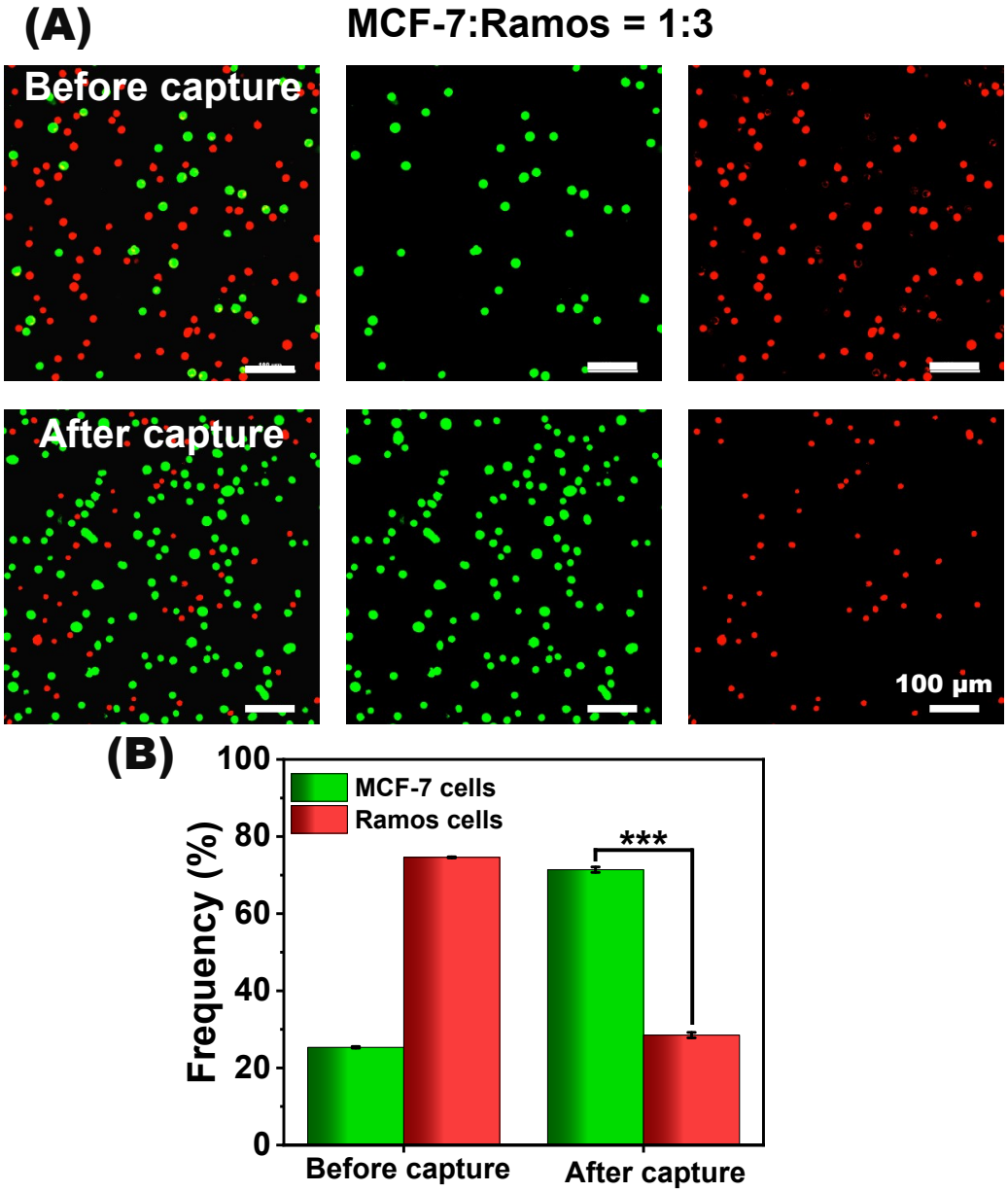

Figure S21. Application of dual-aptamer DNA network for enrichment of CTCs from simulated human blood sample.. (A) Targeted MCF-7 cells (green) and non-target Ramos cells (red) were mixed at 1:3 in normal human serum. After the enrichment, target MCF-7 cells were significantly enriched. (B) Statistical analysis shows approximate 7.5-fold enrichment of target MCF-7 cells.