Supporting Information

Nanowire Substrate-based Laser Scanning Cytometry for Quantitation of Circulating Tumor Cells

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I. Supporting Data

1. Examination of A549 tumor cells captured on QNW arrays using conventional epifluorescence microscopy.

Figure S1 shows optical images together with immunofluorescence-stained images of A549 tumor cells bound to the surface of quartz nanowire (QNW) arrays with low and high magnification, indicating that all of the bound A549 cells were evenly distributed on the surface. To identify captured cells on the QNW substrate, the cells were stained by DRAQ5 (red-635 nm) in PBS in an incubator for 8 min. As shown in Figure S1, it is noticed that the diameters of bound A549 cells on QNW arrays were in the range of 20 to 40 μm. In addition, these fluorescence images correspond to the images using a Genepix microarray scanner 4000B (Molecular Devices, USA) as shown in Figure 2a in main text.

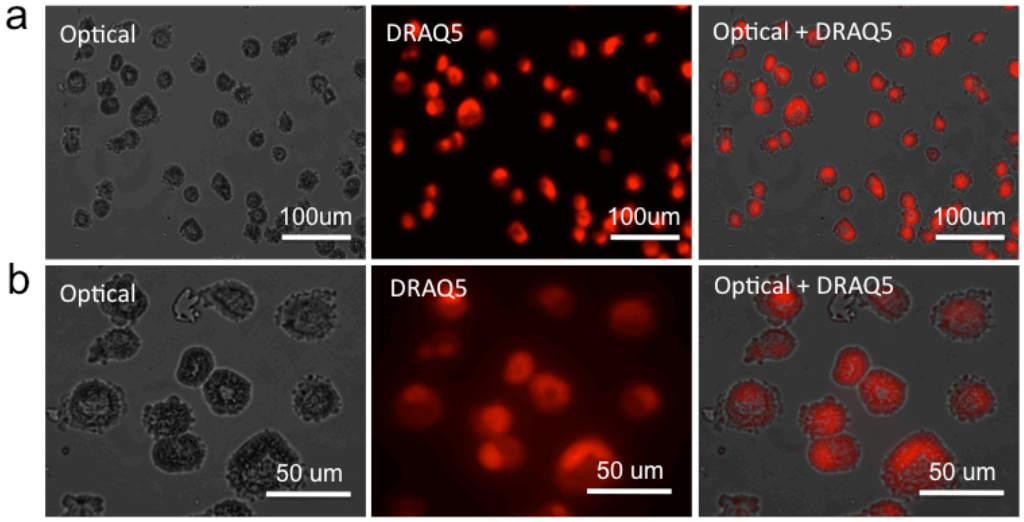


Figure S1: Optical and Fluorescence images of bound A549 cell on QNW arrays. (a) and (b) Low and high magnification optical and fluorescence images of immobilized A549 (human lung carcinoma tumor cells, CCL-185, ATCC, USA) on STR-functionalized QNW arrays, respectively. The captured cells were stained with DRAQ5 (red-635 nm, Cell signaling technology Inc., USA), which stains all nuclei of the cells.

2. Flat glass substrate for tumor cell capture and quantiation using imaging cytometry

The first and second column of Figure S2 show laser scanned (Genepix microarray scanner 4000B) images (white box regions shown in A, B, C, D and E) with 635 nm beam of DRAQ5

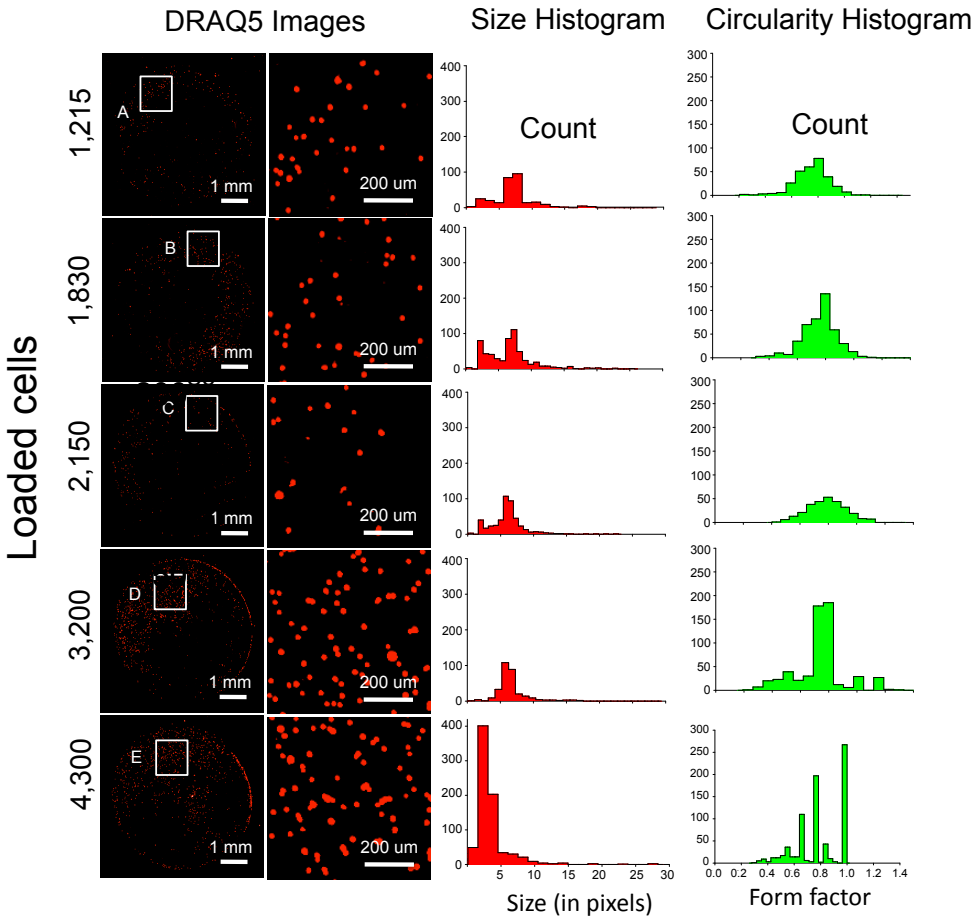


Figure S2: Quantitation of tumor cell capture yield on STR-planar glass. First and second column: low magnification and enlarged images of DRAQ5 nuclear-stained (red-635 nm) and captured human lung tumor cells (A549) on STR-functionalized planar glass substrate for different loaded cell populations in the range of 1,215 to 4,300 cells/well using an automated laser imaging cytometry method. Third and fourth column: size and circularity (form factor) histogram of immobilized A549 cells on STR-functionalized planar glass substrate.

nuclear-stained (in red) and captured human lung tumor cells (A549) on STR-functionalized planar glass substrate for different loaded cell populations in the range of 1,215 to 4,300 cells/well. Figure S2 also reveals that the cells on planar glass substrate are unevenly distributed and bound on the substrate, situating on edge and circumference of PDMS well with a low specific affinity. It was observed that the cell population of surface-bound A549 tumor cells on the STR-functionalized planar glass substrate shows insignificant increase (R^2=0.750) with increasing population of cells loaded into the PDMS wells compared to those on the STR-QNW substrate (R^2=0.938). This provides that the capture yield on STR-planar glass substrate is extremely lower than those on QNW arrays, as shown in Figures 2a-c in main text. The third and fourth column in Figure S2 provide automated processing images to recognize the cell size, shape, and morphological information of immobilized A549 cells on STR-functionalized planar glass substrate. Figure S2 (fourth column) shows the circularity (also known as form factor) histogram of immobilized A549 cells on STR-functionalized QNW substrates, where the form factor is calculated as $\frac{4\pi \times (area)}{(perimeter)^2}$. This indicates that the immobilized cell on the substrate is a perfect circular object if the form factor (ff) equals to 1. It was found that the ffs on flat glass substrate were ~ 0.83 ± 0.12 in the range of 1215 to 4300 cells/well.

3. Laser scanning cytometry analysis of tumor cells captured from mixed cell populations

A549 tumor cells were mixed with human monocytes (U937) to investigate the efficiency and purity of target cells (anti-EpCAM positive A549 tumor cells) from a mixed cell population, where the population of the loaded cells (A549/U937) was to be in the range of 1,400 to 3,000. In the mixture, it is difficult to distinguish between the two cell species under an optical microscope because of their similarity in shape and size even though the size of U937 cells is relatively smaller than that of A549 cells. For this reason, A549 cells (green-DiI-532 nm) and U937 (red-DiD-635 nm) were pre-stained and tagged prior to loading the cells into STR-QNW arrays. This enabled visual tracking of captured target and non-target cells under a fluorescence microscope and microarray scanner. Figure S3 show images of captured cells from the first set of the mixture (A549 green-labeled, U937 red-labeled) on STR-QNW arrays using an automated laser scanning imaging cytometry method. Quantitative analysis of cell size distribution exemplifies the ability of morphometric analysis of all captured cells and apparently these two types of cells do not show significantly difference in cell size. In the case of high-abundance tumor cells, the cell capture purity is ~83.4%.

We also analyzed the distribution of captured cells in the center or at the edge of microwells. The result (see Figure S4) indicates the majority of the non-specific cells were located at the edge due to the ineffectiveness of rinsing and washing steps after the cell capture step.

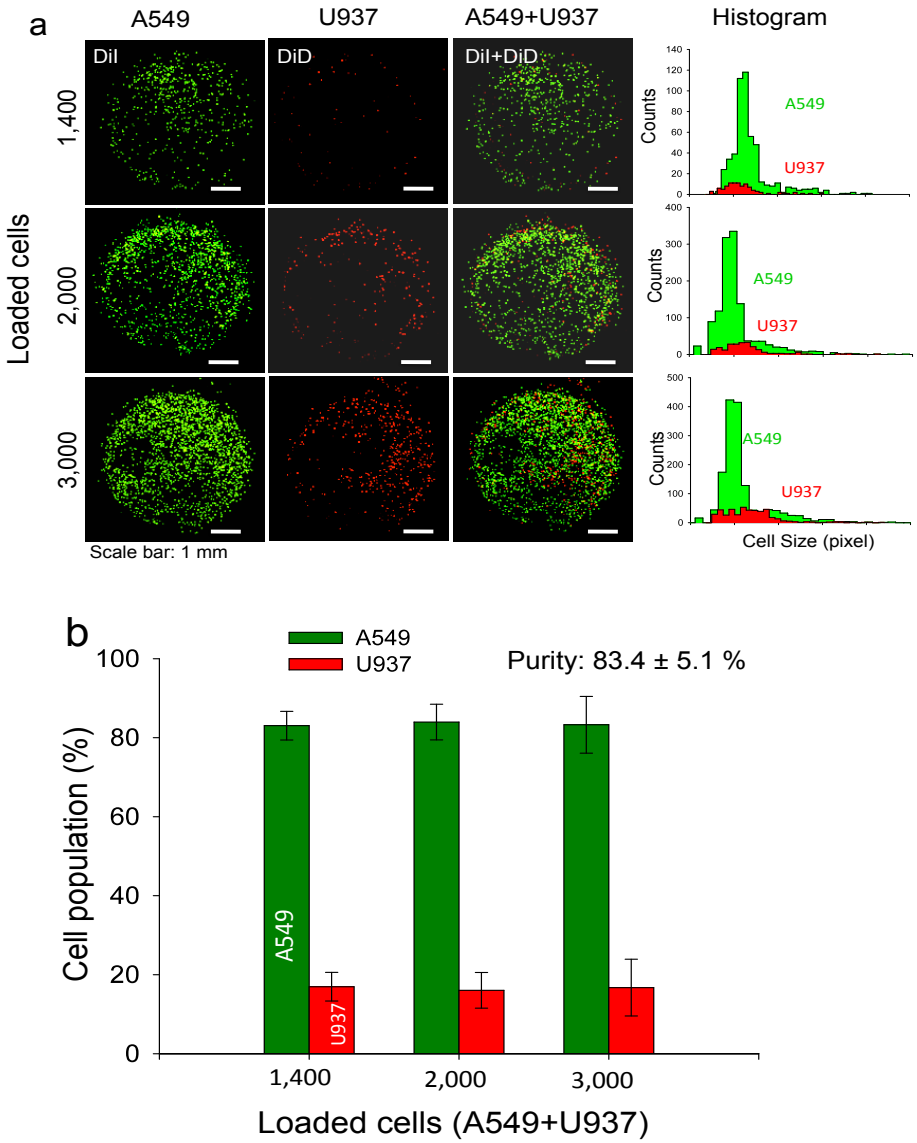


Figure S3. Analysis of lung cancer cells (A549) on the QNW arrays captured from the mixtures of A549/U937 cells. (a) Scanned images of captured cells from the first set of binary mixture (A549 green-labeled, U937 red-labeled) on STR-QNW arrays with size distribution in pixels, respectively. (b) Purity (capture specificity) of the captured cells (A549) on the cell mixtures of A549/U937 bound on STR-QNW arrays as a function of loaded cells in the range of 1,400 to 3,000 cells/well. Each result and error bar represents an average with standard deviation (n=6). Target A549 cells were pre-stained and tagged prior to loading the cells into STR-QNW arrays by 10 μL/mL green-Vybrant DiI (emission 565 nm) while non-target U937 monocytes were stained with 10 μL/mL red-Vybrant DiD (emission 665 nm) solution (Invitrogen, USA).

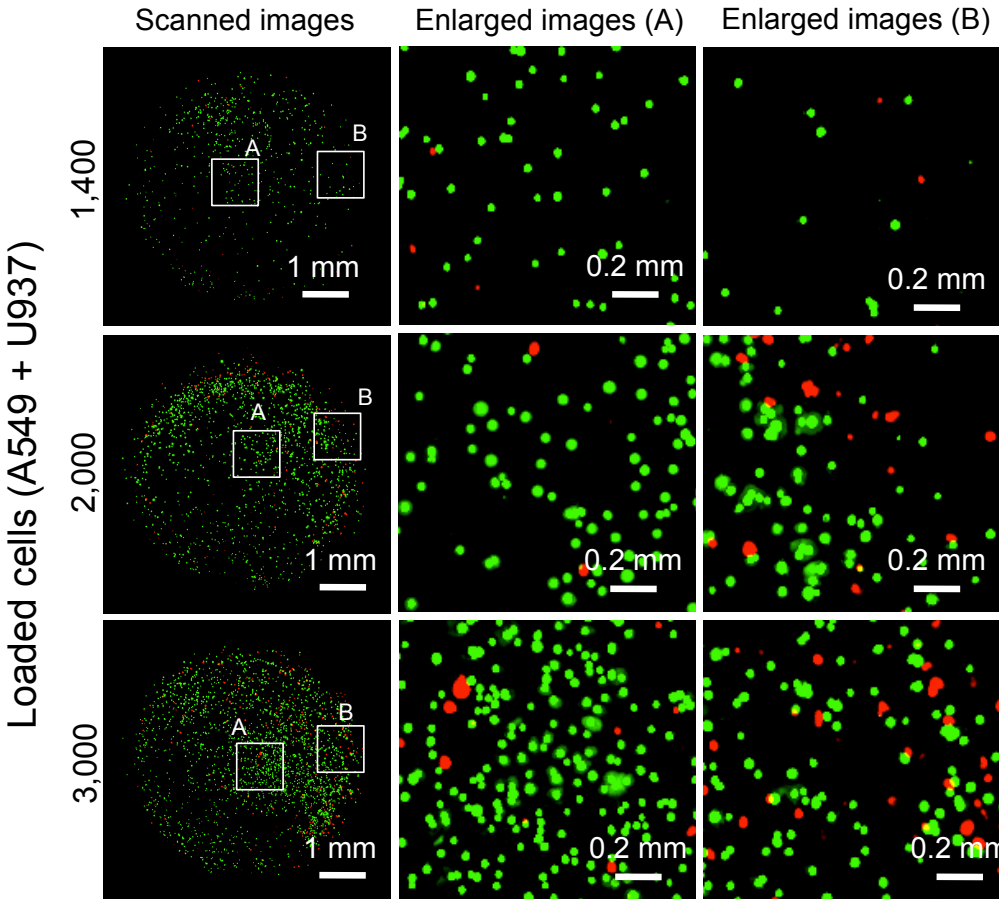


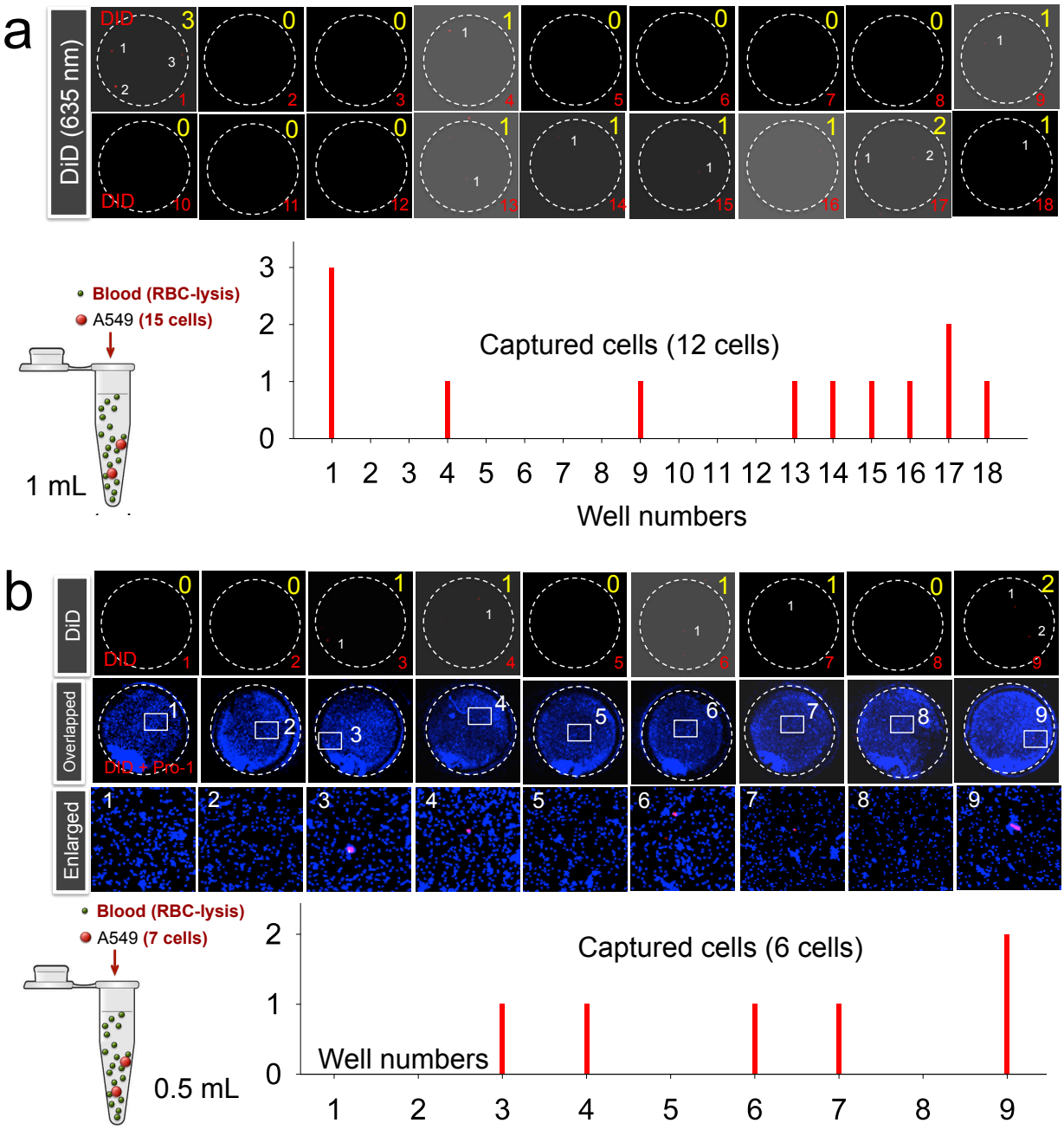
Figure S4. Distribution of non-specific cells captured at different locations of the microwells. Low magnification and enlarged images (white box regions shown in A and B) of captured target A549 cells (human lung tumor cells) and non-target U937 monocytes on STR-functionalized QNW arrays bound with PDMS wells for different loaded cell populations in the range of 1,400 to 3,000 cells/well. A and B denote the locations close to the center or the edge of microwells, respectively.

4. Quantitation of tumor cells from fresh human blood samples

To further verify the potential use in capturing ultra low abundance of CTCs in the settings identical to clinical metastatic cancer patient blood samples, we tested low-abundance tumor cells spiked in peripheral patient blood suspension with total volume of 0.5 ~ 1 mL. For the experiment, 7 or 15 cells of A549 CTC (red-DID+, Emission 665 nm) were spiked into 1mL of primary brain tumor patient bloodsamples(P1 and P2) or healthy volunteers' blood samples (V1 and V2). For patient samples, 0.5 mL (P1) and 1.0 mL (P2) bloods were prepared for the cell suspensions. Additional 0.5 mL blood (NC2) was tested and used for the negative control of CTCs with fluorescence-conjugated anti-Cytokeratin antibody (yellow-555 nm, Cell signaling technology, USA), which is a biomarker for epithelial cells like A549.

First, RBC-lysis solution was added to the peripheral blood sample (10:1 v/v ratio) and incubated for 10 min at room temperature. Then, the supernatant of the solution was completely aspirated after centrifugation at 300xg for 10 min and re-suspended the cell pellet in an DMEM:F12K medium buffer for the cell capture experiment. 0.5 mL and 01.0 mL RBC-lysed cell suspensions containing rare tumor cells (~ 7 or 15), respectively were introduced into the 18 PDMS wells where each PDMS well restrains ~ 60 µL of cell suspension. Prior to the loading the rare CTCs were pre-stained with red-DiD (emission 665 nm) cell-labeling solution (Invitrogen, USA). After fixing the captured cells by 4% PFA in PBS buffer, the cells stained with YO-PRO-1 (emission 491/509 nm, Life technologies, USA) for nucleus satins of the captured cells on substrate for 30 min at room temperature. The CTCs in RBC-lysed peripheral blood suspension from the microarray-scanned images were identifying by considering the cell size, shape, staining color, and intensity of the images. Figures S5a-b show the microarray-scanned images of the captured rare CTCs (red-DiD+) in RBC-lysed brain cancer patient blood samples with the total volume of 1 mL and 0.5 mL, respectively. According to the cell color, size, shape, and intensity of the images transferred from microarray scanner, the captured CTCs were indentified; CTCs show in purple (red-DiD+/blue-YO-PRO-1+) as shown in second and third row images of Figure S5b while non-specific cells appear only in blue (blue-YO-PRO-1+/red-DiD-). Moreover, the captured cells showing in white as shown in Figure S5c has double positive in white (yellow-CK+/red-DiD+), confirming the captured cells to be indeed CTCs bound on the QNW substrate. For the counting the CTCs in microarray-scanned images shown in Figure 5a-c, the part of cells (debris, relatively smaller than CTCs) and non-specific cells (YO-PRO-1+) were left out. As shown in Figure 5 and Figure S5a-b (bar-graphs), the capture efficiency was ~80% for 0.5 ~ 1 mL of RBC-lysed patient blood samples. Thus, this suggests that STR-NW array platform is potentially promising to push the limit of capture to below ~ 10 CTCs in highly abundant hematologic cells in the whole blood. The same samples were also examined using pan-epithelial marker (cytokeratin) that serves as the ultimate solution to detection of rare tumor cells (Figure S6) from clinical cancer patient samples.

Two more whole blood samples were collected from healthy volunteers (V1 and V2). The samples was spiked with ~ 10 CTCs into 1 mL each blood sample. The artificial CTC blood samples were evenly distributed and loaded into 18 QNW array chambers as we did for previous experiments. In Figure S7a, the captured cells showing in purple have double positive in white (blue-CK+/red-DRAQ5+). The average capture efficiency of V2 sample was determined to be ~ 50% as shown in Figure S7b. All the results from fresh human blood samples were quantified and summarized in Figures 4 & 5 (main text).


a
DiD (635 nm)
DND
Blood (RBC-lysis)
A549 (15 cells)
1 mL
Captured cells (12 cells)
Well numbers
b
DiD
Overlapped
Enlarged
DND + Pro-1
Blood (RBC-lysis)
A549 (7 cells)
0.5 mL
Captured cells (6 cells)
Well numbers

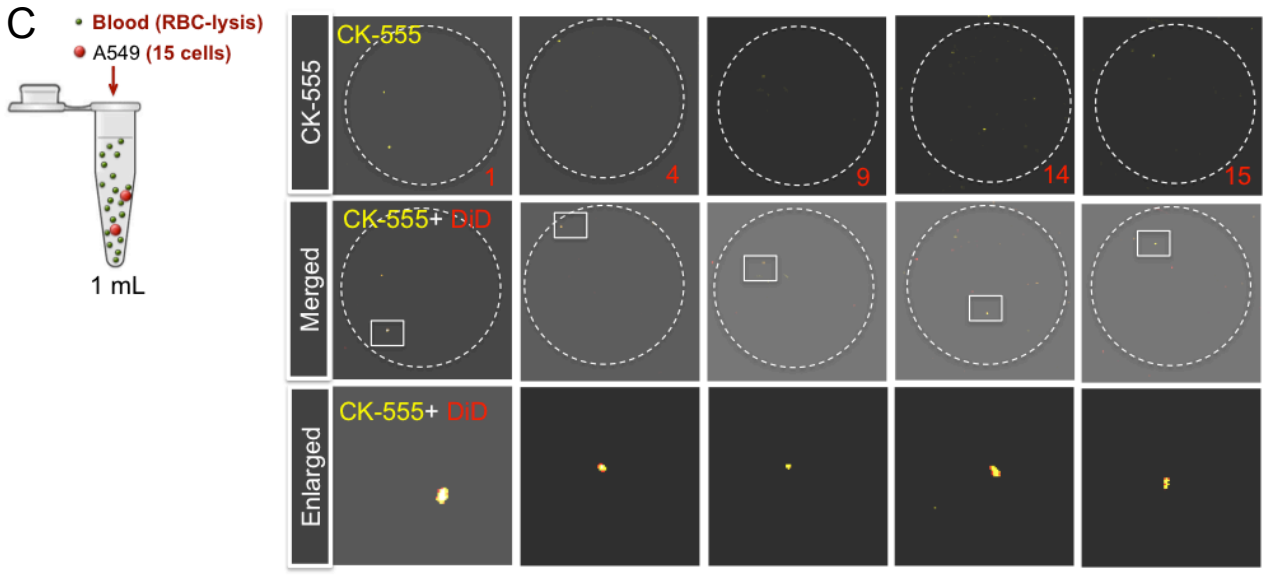


Figure S5. Raw images of the whole nanowire chips for the capture of rare tumor cells captured from fresh human blood samples. (a) and (b) Scanned images of captured rare cells on STR-functionalized NW substrates where only ~15 cells spiked into 1 mL of patient blood (P2) and ~ 7 cells in 0.5 mL, (P1) respectively. Yellow-colored numbers (right-top) for each microarray scanned image denote the number of captured cells for each well while red-colored numbers indicate the well numbers up to 18. Using dual-color imaging, the CTCs shows purple (dual positive, red-DiD+/blue-YO-PRO-1+) while non-specific cells stained only by YO-PRO-1 appear blue (second and third row of top-image in (b)). 15 cells of A549 with final volume of 1 mL were used for the capture experiments. The bar graph are also presented to show the distribution of the captured cells from all the PDMS microwells used this experiment. A representative scanned image, further analyzed from the sample shown in Figure S5(a) using PE-conjugated anti-human cytokeratin. Target A549 cells were stained both with DiD-dye (665 nm, in red) and anti-Cytokeratin (CK)-555 (555 nm in yellow) while non-target cells (peripheral patient blood samples including monocytes and white blood cells were stained with YO-RPO-1 (488 nm, in blue). 15 cells of A549 with final volume of 1 mL were used for the capture experiments (left-bottom). First, second and third row images show the microarray scanned images of CTCs (CK-555+ in yellow), merged (CK-555+/DiD+), and enlarged images (CK-555+/DiD+), respectively for selected scanned wells (1, 4, 9, 14, and 15 well shown in Figure S5a). Red-colored numbers (right-bottom) indicate the well among the 18 wells

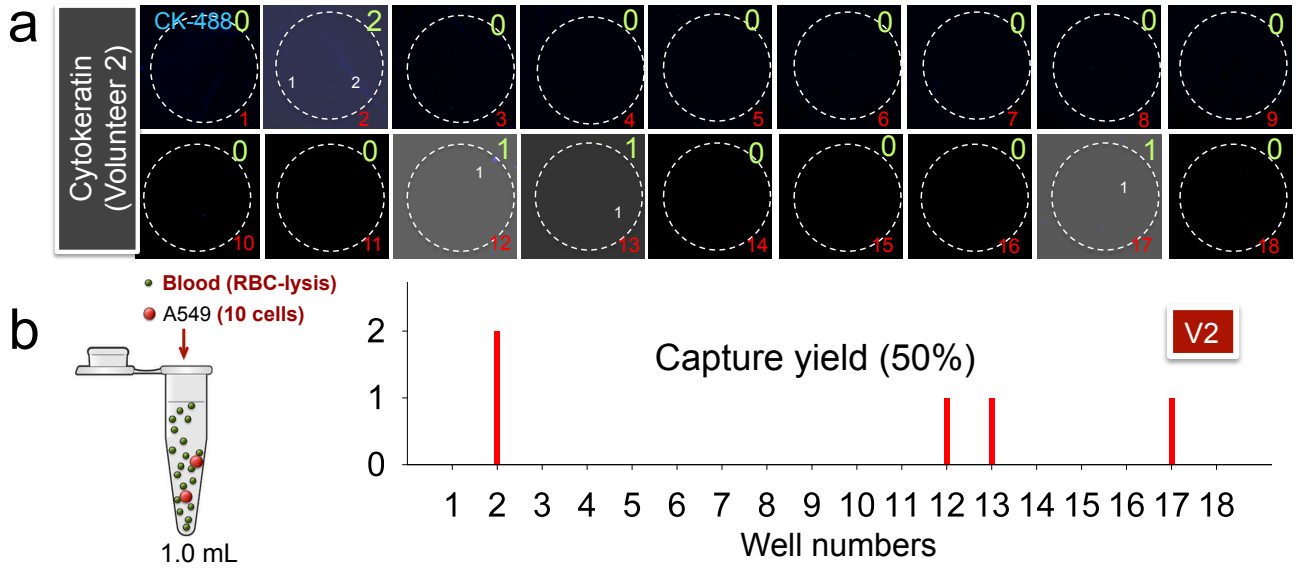


Figure S6. Detection and validation of rare tumor cell captured from the blood sample of a healthy volunteer using pan-epithelial surface marker. (a) Scanned images of captured CTCs on STR-functionalized NW substrates where only ~ 10 cells spiked into peripheral whole blood sample (V2: health volunteer blood sample). The artificial CTC blood samples were evenly distributed and loaded into 18 NW array PDMS wells. (b) Summary of the captured cells for V2 blood sample, showing the capture yield to be ~ 50% in average.

5. Quantitation of tumor cells from negative control blood

To verify the presence of the CTCs in peripheral patient blood samples shown in Figures S5-S6, we prepared additional 0.5 mL blood and used for the negative control (NC2) of CTCs with fluorescence-conjugated anti-Cytokeratin antibody (green-555 nm, Cell signaling technology, USA), which is a biomarker for epithelial cells like A549. In addition, all of the cells including white blood cells (WBC) and monocytes in RBC-lysed patient blood were stained with DRAQ-5 (red-635 nm, Cell signaling technology, USA). Figure S6 shows fluorescence images of white blood cells bound on the QNW substrate from peripheral patient blood samples stained with DRAQ-5 (red-635 nm, first image) and 555 nm-conjugated anti-CK-Abs (second image) with merged image (red-635nm and green-555 nm, third image). As indicated in Figure S7 (second image), no anti-CK positive cells bound to QNW substrate were observed in our peripheral patient blood samples.

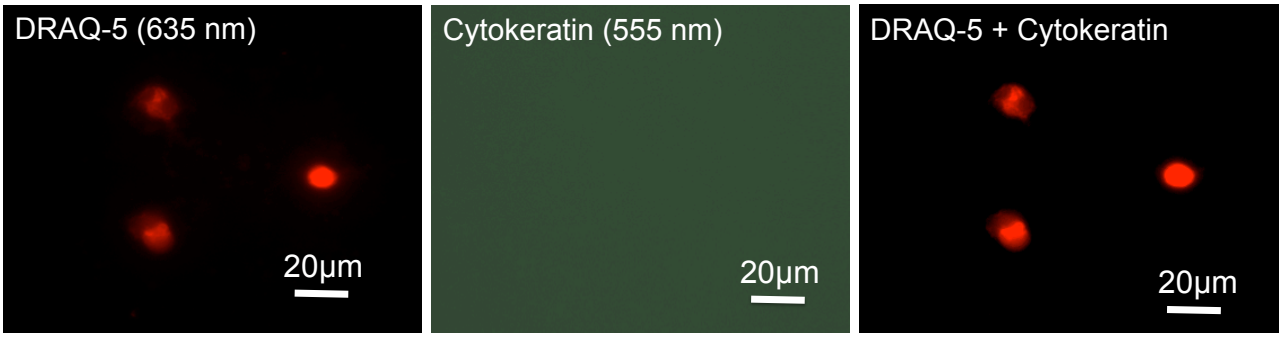


Figure S7. Negative control study – scanned fluorescence image of the nanowire chip to measure brain tumor patient blood samples without spiking tumor cells. Fluorescence images of white blood cells bound on the QNW from peripheral patient blood samples stained with DRAQ-5 (red-635 nm, Cell signaling technology, USA) and anti-Cytokeratin (CK) antibody (green-555 nm, Cell signaling technology, USA), capable of negative or positive CTCs. DRAQ-5 (red-635 nm), shown in red (first image), binds to all nuclei of the cells while Anti-CK-Abs (green-55 nm), shown in green (second image) binds to only CTC in the peripheral patient blood.

6. Effect of non-specific background on the capture of rare tumor cells

We introduced A549 cell suspension with approximately 350 cells into numerous U937 cell suspensions with varying cell densities ranging from 350 cells to 7,000 cells (1:1 up to 1:20 A549/U937 mixtures). While all A549 and U937 mixtures contained the identical number of A549 cells (350 cells), each mixture had the different number of U937 and distinct mixture ratios. We have already shown that the nanowire substrate for our technology exhibited substantially improved tumor cell capture yield than the flat glass substrate (Figure 2b-c in main text). As the ratio of U937 increased, the number of captured U937 has increased. However, despite the increasing number of U937 captured on the platform, the number of captured A549 and tumor cell capture yield remained consistent as shown in Figure S8a-b. More than ~ 89.9% of total loaded A549 cells were consistently captured whereas the capture yield of U937 was not improved as the mixture ratio changed as shown in Figure S8b-c.

We then conducted another quantitative experiment to assess our technology's capture efficiency of A549 tumor cells spiked into peripheral blood mononuclear cells (PBMCs) with cell densities in the order of a million cells (0.5 M and 1.0 M PBMC in the suspension). The fluorescence image generated by implementing laser scanning cytometry (LSC) clearly demonstrated the technology's potential to capture and isolate majority of A549 tumor cells added into a very large number of normal primary cells shown in Figure S9. A549 cells, which emitted red fluorescence, were easily detected and distinguished from non-targeted cells, PBMC, which emitted blue fluorescence. When scanned images of two cell types were merged, the merged

image exhibited numerous, clearly visible red fluorescent dots, which represent captured A549 cells, among significantly more abundant blue fluorescent dots, which represent PBMC. (Figure S9). When about ~400 cells (A549) were added into 0.5 million PBMCs and mixture was loaded into ~9 individual chambers, ~89% of the loaded A539 cells were captured and detected (Figure S9a-b). When the cell density of PBMCs increased to 1 million cells, our technologies have still established persistent detection and high capture efficiency of nearly ~100% (Figure S9b). This observation led us to believe that the NW microarray of our platform mostly retains its capture efficiency of CTCs regardless of the number and proportion of other non-target cells.

The results of these additional experiments demonstrate that the increasing cell densities of non-target primary cells (U937 and PBMCs) shown in Figures S8 and S9 and the varying mixture ratios do not compromise or have adverse effects on our technology's capabilities to successfully capture rare target tumor cells, and to perform rigorous analysis of all individual cancer cells captured on the substrate. The results of this experiment demonstrated that the capture efficiency and detection limit of the target tumor cells by our technology were not adversely affected by increasing number of normal primary cells.

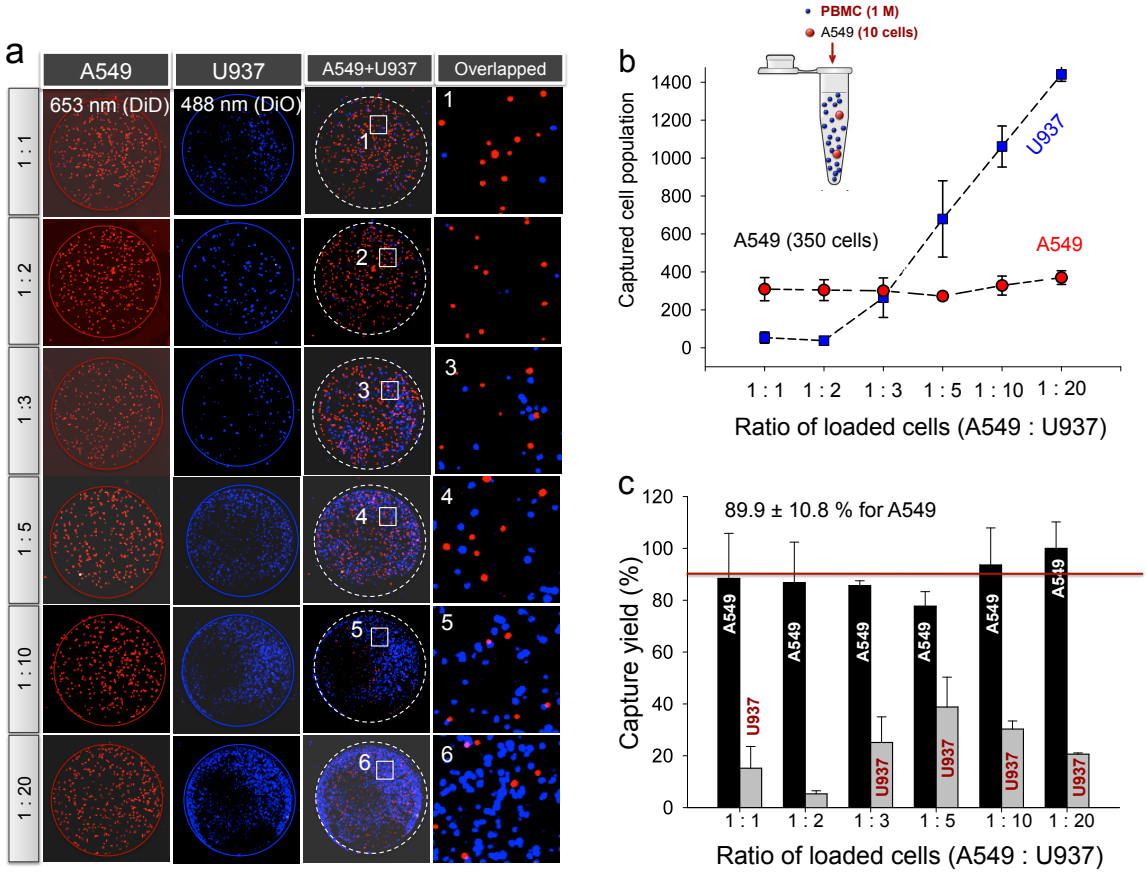


Figure S8: Effect of non-specific background cells on the capture of specific tumor cells (A549). (a) Fluorescence micrographs of QNW substrates on which A549 (target circulating tumor cells) were captured. We varied the mixture ratio of A549 to U937 (normal monocytes) by spiking the fixed number of A549 (~350 cells) into U937 with different cell densities ranging from (350, 700, 1050, 1750, 3500, and 7000 cells). The first column shows the images of captured A549, which were stained with DiD (red-635nm), and the second column shows the images of normal monocytes U937, which were stained with DiO (blue-488nm) and were nonspecifically bound to the substrates. The third and fourth columns show the overlapped and enlarged images of A549 and U937. (b) Quantitative evaluations of the number of target CTCs and non-target normal cells captured on QNW substrates at varying mixture ratio. Although the number of U937 cells captured increased with increasing number of U937 initially loaded, the number of captured A549, the target circulating tumor cells, was not affected by varying number of U937. (c) The capture yield of A549 and U937 when the ratio of loaded cells (A549:U937) was changed. Although the number of U937 cells initially loaded with A549 into QNW substrates significantly increased as we change the ratio of A549 and U937 (1:1, 1:2, 1:3, 1:5, 1:10, 1:20), our QNW substrates showed consistently high CTC-capture performance. The capture yield for A549 was not significantly changed in a response to increasing number of non-target cells that nonspecifically bound to the nanowire cell-capture platform.

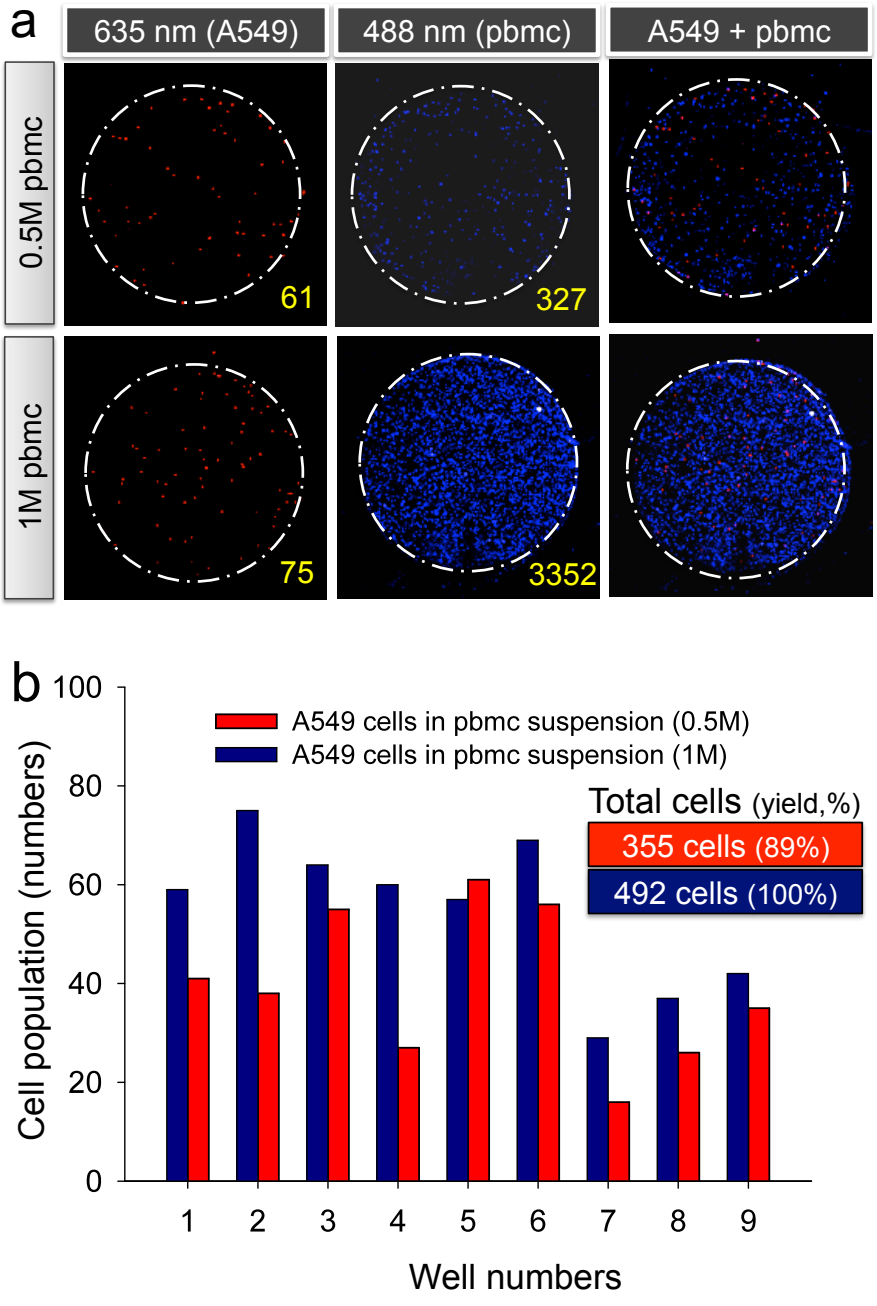


Figure S9: Tumor cell captures efficiency from the mixture of A549 cells and human PBMCs. (a) Fluorescence micrographs of nanowire substrates on which A549 (target circulating tumor cells) were captured in 0.5 and 1.0 M PBMC cell suspension. A549 cells, which emitted red fluorescence, were easily detected and distinguished from while blood cells, U937, which emitted blue fluorescence. When scanned images of two cell types were merged, the merged image exhibited numerous, clearly visible red fluorescent dots, which represent captured A549 cells, among significantly more abundant blue fluorescent dots, which represent U937 cells (When about ~400 cells (A549) were added into 0.5 and 1.0 million PBMCs and mixture was loaded into ~9 individual chambers, ~89% of the loaded A539 cells were captured and detected. (b) The distribution of captured A549 cells of each well, where we used 9 well in total loading ~ 400 cells.

7. Cell morphology and cell signaling from CTCs bound on the NW substrate

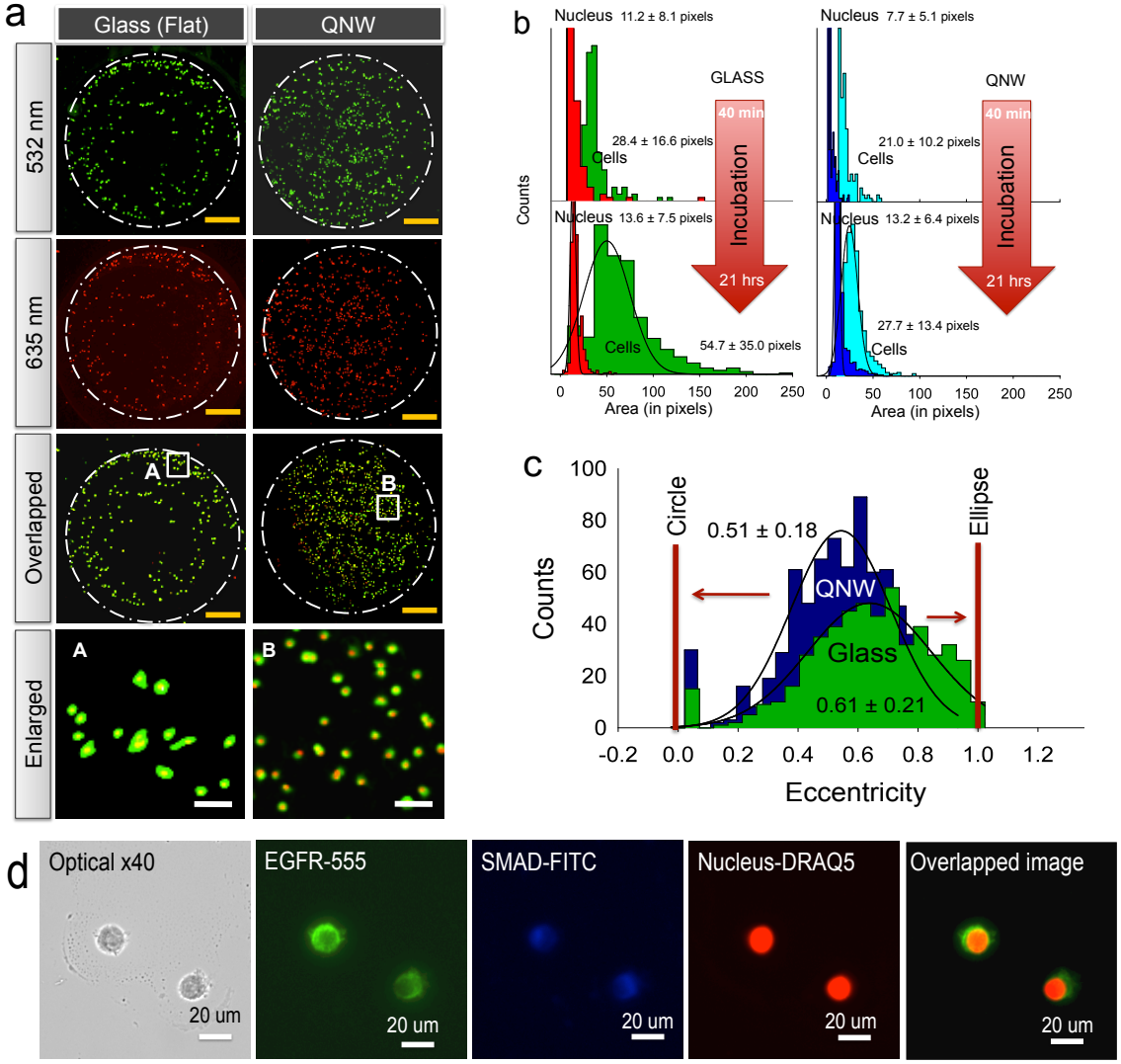


Figure S10: Assessment of physical properties and cellular behaviors of CTCs captured on

glass and QNW substrate. (a) Micrographs of CTC captured on flat (left column) and nanostructured (right column) platform. Quantification and assessment of nanostructure's effects on size, morphology, and growth pattern of captured cells by immunofluorescence staining for cellular cytoplasm (green, 532nm) and nucleus (red, 635nm). Captured CTCs were incubated for 21 hours after their capture, and the image covering the entire substrate area containing captured cells was taken and analyzed through rigorous image processing algorithm. (b) Histogram analysis showed that the cells, which were captured and incubated on flat, glass substrate, exhibited extensive growth and elongation, but CTCs which were captured and incubated on quartz nanowire substrate demonstrated significantly limited growth and elongation. This observation suggested that the interaction of captured cells with nanoscale topography of nanowire substrate inhibited cellular outgrowth activities. (c) Histogram analysis of eccentricity (measurement of cell spreading) showed that the cells on glass substrate displayed more elliptic shape (greater eccentricity) than the cells on quartz nanowire substrate (less eccentricity) that had more rounded shape. This analysis is consistent with results in figure S10b that cells captured on QNW arrays were generally smaller as cellular interaction with nanoscale topology limited cellular growth and elongation. In summary, cells captured and cultured on different substrates developed different morphological characteristics and growth pattern. (d) Immunofluorescence staining of CTC-specific intracellular signaling protein and CTC-specific surface receptor protein. Immunofluorescence staining image demonstrated the significant level of EGFR (surface receptor protein) and Phosphorylated-SMAD (intracellular signaling protein) expression. Nucleus was stained with DRAQ5 to confirm the captured objects as actual CTCs.

8. Non-specific capture of white blood cells (U937) on NW and flat glass substrates

Nanostructured substrate based cell-capture platforms produced significantly improved cell-capture performance than smooth, planar glass substrates. We evaluated capture yield for normal cells (U937; monocytes) on nanowire substrate and planar glass substrate (Figure S11a-c). We found that capture efficiency for normal cells on nanowire substrate was improved over that on smooth glass substrate (10.5% on nanowire vs. 2.4% on glass; Figure S11c and table 1). We believe that nanostructured substrate can capture cells more effectively regardless of their types because distinctive topography of nanostructure promotes cell-nanostructured surface interactions and significantly hinders their movement. However, we think that although nanostructured substrate does not selectively enhance capture efficiency for target CTCs, the captured normal cells would not implicate our platform's excellent capability for enumeration and characterization of captured tumor cells, and its potential as a device for early detection of metastatic cancer development. Progression of metastatic cancer can be diagnosed when blood of a cancer patient contains abnormally high number of circulating tumor cells (detection threshold: <10 CTCs/mL of whole blood).

Table 1. Capture efficiency comparison between NW and Glass substrates

	NW substrate	Glass substrate	
CTCs (A549)	89.2 ± 12.3 %	22.7 ± 3.0 %	Figure 3c
Human monocytes (U937)	10.5 ± 5.1 %	2.4 ± 0.7 %	Supp. 3

- The surface of all samples were functionalized by APTES/GA/STR

Therefore, high capture efficiency and accurate enumeration of captured CTCs are essential for correct diagnosis of metastatic cancer. Because we are only interested in isolating CTCs and assessing their properties and behaviors, we identified and counted only the captured CTCs while all the normal cells that are non-specifically captured on the substrate should be excluded from any counting and analysis. CTCs can be detected based on their distinctive physical properties (e.g. Tumor cells have generally larger nuclei than normal leukocytes).

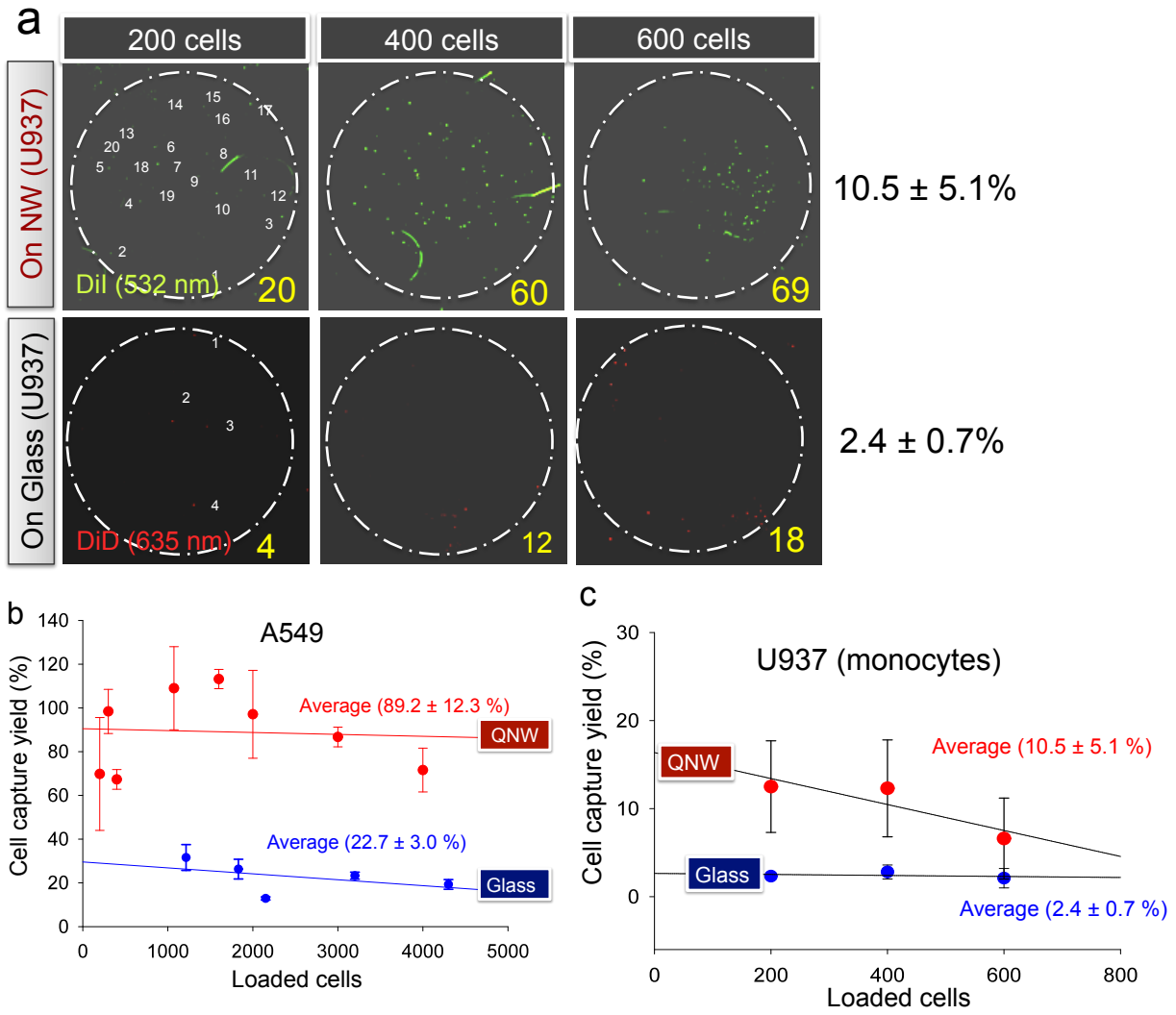


Figure S11: Measurement of capture efficiency of normal cells (U937) on planar glass and NW substrates. (a) U937 cells at different cell numbers (200, 400, 600) were loaded into glass and QNW substrates, and the number of captured U937 on each substrate was determined. The nanowire capture efficiency for normal cells (10.5% ± 5.1%) was determined to be greater than the capture efficiency on the glass (2.4% ± 0.7%). (b) Capture efficiency of CTCs (A549) with the varying loaded cell numbers on glass and nanowire substrates was determined and compared. QNW substrate yielded significantly higher CTC-capture performance (capture yield=89.2%) than flat, glass substrate (capture yield=22.7%). (c) Capture efficiency of normal cells (U937) with the varying loaded cell numbers on glass and nanowire substrates was determined. Although QNW arrays demonstrated higher normal cell-capture performance than glass substrate as QNW did for tumor cells, the U937 capture efficiency on QNW arrays was significantly lower than CTC-capture efficiency.

II. Materials & Method

Nanowire array fabrication. Figures 1a-f show scanning electron microscope (SEM) images and schematic views of each QNW fabrication process. To produce the QNW, the liquid state of colloidal polystyrene (PS) nanoparticles (NPs) monolayer was first deposited on the quartz substrate using a modified self-assembly technique (Figure 1a).[1,2] Then, O_2 plasma etching was performed for 5 s with a mixed gas of O_2/Ar (35/10 sccm), at a pressure of 40 mTorr, a RF power of 100 W, and a bias power of 50 W (Figure 1b). This is employed to secure space between PS NPs by reducing the size of the fabricated monolayer PS on the substrate (Figure 1b). As shown in Figure 1c, Cr metal (25 nm in thickness) was deposited using an e-beam evaporator. PS NPs were thoroughly removed from the quartz (QZ) substrate in N-methyl-2-pyrrolidone (NMP) using ultrasonification. Then, the first RIE process was performed for 40 s (CF_4/Ar=40/5 sccm, RF power of 100 W, and bias power of 50 W). After depositing Ni metal (30 nm) with the e-beam evaporator (Figure 1d), the Cr metal layer was removed through a Cr lift-off process using Cr etchant (CR-7, Cyantek, USA). The second RIE process was performed to fabricate the QNW array for 4 min (SF_6/Ar=20/5 sccm, pressure of 20 mTorr, RF power of 300 W, bias power of 100 W). After RIE was completed, the remaining Ni used as an etch mask for QNW etching was removed with etchant (LCE-12K, Cyantek, USA) (Figure 1e-f).

Nanowire surface functionalization. QNW substrates with nanopillar structures (QNW arrays, 25 mm × 25 mm) were first carefully cleaned with H_2O_2 : H_2SO_4 (1:1) for 10 min to remove all of the organic materials and impurities on the surface. We then washed the substrates using a three-step

cleaning process (acetone, isopropyl alcohol, and distilled water) and dried them with air. The QNW surface was treated with O_2 plasma for 20 s to confer the hydroxyl groups on the surface of the QNW arrays. Our previous study suggested that this treatment plays an important role in streptavidin (STR, Sigma-Aldrich, USA) immobilization on nanotopographical substrates and further improvement of specific lymphocyte capture efficiency because this hydroxyl group allows the QNW arrays to enhance conjugation with 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich, USA).[3-5] The surface of the QNW arrays was modified with 1% (v/v) APTES in ethanol for 30 min at room temperature, resulting in amine group attachment to the surface. The QNW arrays were placed on a hot plate at 120 ^oC for 10 min after rinsing the QNW substrate with ethanol solution for 10 min. The QNW arrays were then reacted with 12.5% (v/v) glutaraldehyde (GA, Sigma-Aldrich, USA) in distilled water for 4 hrs on a 3D-rocker, leading to immobilization of the aldehyde group on APTES coated QNW arrays. After bonding with the PDMS mold (see PDMS well preparation in experimental section), STR was immobilized to GA by incubating the QNW substrate with 50 μg/mL STR solution in phosphate buffered saline (PBS) overnight in an incubator (37^oC, 5%CO_2).

Fabrication of PDMS microwell chip for performing cell capture experiments. Cell-capture microwells with nine circular wells (5 mm in diameter) were fabricated using polydimethylsiloxane (PDMS) elastomer. A mixture of GE RTV 615 PDMS (GE Silicones, USA) prepolymer parts A and B (10:1) was prepared, homogenized, and applied to a blank Petri dish. After degassing for 1 hr, the PDMS mold was cured at about 80 ^oC for 120 min. The solidified PDMS mold was cut to the size of 25 mm × 25 mm and the cell-counting reservoir holes were drilled with a stainless steel hole punch (5mm in diameter). The nine-holed PDMS mold was then bonded thermally to the GA-coated QNW substrates after cleaning with 75% alcohol in an ultrasonic bath. Next, the surface was treated with STR (50 μg/mL) in PBS solution overnight. Prior to loading the tumor cells into the PDMS integrated STR-QNW arrays, the surfaces were rinsed 2-3 times with PBS to flush away unreacted molecules.

Tumor cell capture and quantitation from mixed cell populations. Two cell lines, A549 (human lung carcinoma cell line, CCL-185) and U937 (monocyte, CRL-1593.2), were purchased from American Type Culture Collection (ATCC) in the USA. The peripheral blood mononuclear cells (PBMC) were obtained from Astarte Biologics, LLC in the USA. Biotinylated-epithelial cell adhesion molecule (EpCAM) antibody (anti-human CD326-Ab) was purchased from eBioscience Inc. in the USA. For the single cell capturing/counting experiment, we used A549 cells as a target cell, where the A549 cells were mixed with either U937 or PBMC as non-target cells for the separation experiment in the binary cell mixtures. Prior to introducing the cells into the PDMS wells

on the QNW arrays, the cells (single cell and binary mixtures) were pre-reacted with biotinylated EpCAM-Abs and then stored at 4 ^{o}C for 20 min. A solution of the cells conjugated with biotinylated EpCAM-Abs was pipetted into each of the nine wells. Cell counts were manually performed using a conventional hemocytometer (Hausser Scientific Co. USA) within 10% error. A series of counted and differently diluted cells in culture mediums, RPMI-1640 (500 mL, Invitrogen, USA) for U937 and PBMC and F12K (500 mL, Invitrogen, USA) for A549 cells, with a final volume of about 50 μL for each well was introduced into nine wells with cell populations in the range of 200 to 4,000 cells/well. After incubation at 37 ^{o}C and 5% CO_2 for 40 min, the PDMS wells were washed out 1×PBS with Tween-20 (PBST, KPL Inc., USA) at least three times to remove unbound tumor cells. The captured A549 cells on the QNW substrate were fixed with 4% paraformaldehyde (PFA, Santa Cruz Biotechnology Inc., USA) in PBS for 15 min, and followed by washing out in PBS. The QNW arrays were subsequently treated with 0.2% Triton X-100 (American Bioanalytical Inc., USA) in PBS for 10 min to induce cell permeability and to allow for intra-cell staining. Subsequently, all of the immobilized tumor cells (A549 cell line) were stained with diluted DRAQ5 (Cell signaling technology Inc., USA) 1:1000 (5 μM) in PBS for 8 min in a incubator since DRAQ5 stains all nucleated cells, for identification of captured cells on QNW arrays. For single cell separation from the binary mixtures, A549 cells were pre-stained and tagged prior to loading the cells into STR-QNW arrays by 10 μL/mL green-Vybrant DiI (emission 565 nm) while U937 monocytes and PBMC cells were stained by10 μL/mL red-Vybrant DiD (Emission 665 nm) cell-labeling solutions (Invitrogen, USA), where dyed A549 tumor cells were mixed with U937 and PBMC at a ratio of 1:1, respectively. Then, the stained-QNW arrays with PDMS cell reservoirs were rinsed with PBS at least twice to wash non-specific cells off. The stained STR-QNW arrays were finally given a three-step cleaning process, using PBS, PBS in deionized (DI) water (1:1), and DI water, before a microarray scanner was used to image the captured cells and carry out further analysis.

Whole blood samples from volunteers and brain tumor patients. Whole blood samples were collected from two healthy volunteers according to the institutional review board protocols (HIC 0902004786). Whole blood samples were also obtained from primary brain tumor patients (no metastasis) through the pathology service of Yale-New Haven Hospital. Four whole blood samples from two healthy volunteers and two brain tumor patients (no metastasis) were used and spiked with A549 lung cancer cells for the following experiments.

Tumor cell capture and quantitation from human blood samples. Typically, 3mL of whole blood was drawn from each volunteer or patient using a syringe loaded with anti-coagulant. Then 7, 10 or 15 cells of A549 tumor cells (red-DID+, Emission 665 nm) were spiked into 0.5mL or 1mL of

primary brain tumor patient bloodsamples(P1 and P2) or healthy volunteers' blood samples (V1 and V2). For these samples, additional 0.5 mL of blood (NC) was used for negative control and measured by the same of CTC detection procedure (fluorescence-conjugated anti-cytokeratin antibody, yellow-555 nm, Cell signaling technology, USA). The sample processing is the following. Then, these samples were treated with RBC lysis (remove red blood cells, a standard clinical laboratory medicine procedure). The procedure is the following. First, RBC-lysis solution was added to the spiked whole blood samples (10:1 v/v ratio) and incubated for 10 min at room temperature. Then, all the remaining cells were spun down at 300xg for 10 min and re-suspended in the same volume of buffer for cell capture experiment. Second, 1.0 mL or 0.5 mL of these samples containing rare tumor cells (~ 7 or 15 per sample) was introduced onto the entire nanowire substrate chip, on which there are 18 PDMS wells and each contains ~ 60 µL of sample. Total volume of blood sample analyzed using a nanowire chip is 18x60=1,080 µL or ~1mL. Cells were incubated on the nanowire array for 30-60min for cell capture. For each sample, we also measured 0.5 mL of un-spiked blood for negative control. Third, all the captured cells were fixed by 4% PFA in PBS buffer, stained with Phycoerythrin(PE)-conjugated anti-cytokeratin (ex. 533nm, em. 577nm, invitrogen, USA) and YO-PRO-1 (nuclear dye, ex. 491nm, em. 509 nm, Life technologies, USA) for 30 min at room temperature. Cytokeratin is a biomarker for epithelial cells such as A549. Finally, captured cells will be imaged using the laser scanning cytometry to obtain cell count, as well as cell size, shape, fluorescence intensity of each cell.

Counting and visualizing captured tumor cells. An Axon Genepix microarray scanner 4000B (Molecular Devices, USA) was used to image DRAQ5-stained cells (emitting 635 nm) on the STR-QNW arrays. The microarray scanner contained green (YAG laser-532 nm wavelength) and red (He-Ne laser-635 nm wavelength) channels and was used to scan and visualize square shape STR-QNW arrays (25 mm × 25 mm) with 5 μm resolution. Consequently, the scanned images of the captured tumor cells were visualized with Genepix 6.0 software (Molecular Devices, USA). The visualized cell images exported from the Genepix 6.0 were then transported into CellProfiler™ (http://www.cellprofiler.org) cell image analysis software for further analysis and quantitation of the population of the captured cells.[32] In addition, CellProfiler™ provides additional size and circularity information of the immobilized cells on the STR-QNW substrates. Figure 3a (fifth column) shows the circularity (also known as form factor) histogram of immobilized A549 cells on STR-functionalized QNW substrates, where the form factor is calculated as [4π *(area)/(perimeter)* ^{2}]. The immobilized cell is a perfect circular object if the form factor (ff) equals to 1. As shown in Figure 3a (fifth column), we noticed that the immobilized cells with ff (0.88 ± 0.14) in the range of < 500 cells appear more rounded than those of cells captured in the range > 2,000 cells (ff = 0.67 ±

0.17) on the STR-QNW arrays, suggesting that the morphology of cells captured is highly depending on the cell population immobilized and space for the cell settlement on the substrate in the limited area (19.6 mm^2 circular well).

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