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Simultaneous and Selective Isolation of Multiple Subpopulations of Rare Cells from Peripheral Blood Using Ensemble-decision Aliquot Ranking (eDAR)

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Experimental Section

Microfluidics and Line-confocal Detection Scheme

The design and fabrication of the dual-capture eDAR chips were similar to the procedures described previously.[1] Briefly, the main channel, where the blood sample was introduced, had a height of 50 μm and width of 150 μm; all the other channels were 50-μm tall and 200-μm wide. The two cell trapping areas consisted of 5,000 microslits. Each microslit was 5-μm tall and 5-μm wide. The microslit features were designed using AutoCAD (Autodesk, San Rafael, CA), and written onto a chrome mask (TRICR Corporation, SF, CA). The layer of microslits was fabricated using AZ 1512 as a positive photoresist (Micromanufacturing Facility at the University of Washington), and then etched with an optimized depth in the range of 4.5-5.0 μm using deep reactive ion etching. The second layer was fabricated using SU-8-3050 as a negative photoresist (MicroChem, Newton, MA). After silanization with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Sigma-Aldrich, St. Louis, MO), the silicon master was baked with uncured polydimethylsiloxane (PDMS) for 2 h at 70 °C. The cured PDMS chip was bonded with a piece of cover glass using the standard process of plasma oxidation.

Multicolor line-confocal microscopy was built to rank aliquots into 3 different groups, which had a similar structure reported previously with minor modifications.[2] Briefly, three laser sources with wavelengths at 405 nm, 488 nm, and 633 nm, were shaped and combined using a series of convex lenses, cylindrical lenses, dichroics and mirrors. With a 10X objective, the excitation beam was 250-μm long and 1.5-μm wide with a power at about 1 to 2 mW for each wavelength. Two single-photon counting modules (Excelitas, Canada) were used to detect the fluorescence signals from

the detection window at a frequency of 10,000 Hz. Compared to the solenoid switching time of 1-3 ms, this detection rate is high enough to prevent missing any CTCs in the step of active sorting. An epi-fluorescence imaging scheme was also integrated with the same line-confocal microscope for the downstream imaging of CTCs. This epi-fluorescence microscope was equipped with a 20× objective and was able to collect fluorescence images from five individual color channels: blue (435 to 485 nm for Hoechst or DAPI), green (510 to 540 nm for FITC or Alexa 488), yellow (555 to 605 nm for PE), red (665 to 695 nm for Alexa 647 or APC), and far red (710 to 730 nm for Alexa 700).

Biological Materials and Cell Culture

Three breast cancer cell lines, MCF-7, SKBr-3 and MDA-MB-231 (American Type Culture Collection, ATCC, Manassas, VA), were used to optimize and characterize the dual-capture eDAR platform. These cells were cultured under the same protocol described previously with small modifications. Briefly, MCF-7 and MDA-MB-231 were cultured in Eagle's Minimum Essential Medium (EMEM), and SKBr-3 was cultured in McCoy's 5. The two cell culture media contained 2 mM $_{L}$-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. All the cell-culture ingredients were purchased from ATCC). Healthy donor blood (PlasmaLab International, Everett, WA) were collected in Vacutainer tubes (BD, Franklin Lakes, NJ) containing EDTA to prevent coagulation. Blood samples were stored at 4 °C and analyzed within 24 h.

Dual-capture eDAR experiments

Isoton (Beckman Coulter Inc., Chino, CA) was used as the buffer for all the eDAR experiments unless otherwise specified. When we tested the recovery efficiency, we spiked the same number

of MCF-7 and MDA-MB-231 cells into 1 mL of whole blood sample from healthy donors. To spike 25 or 50 cells precisely, we used a cell spiking method developed in our group [3] to determine precisely the number of cells added to the sample. Traditional methods, such as serial dilution, can be very inaccurate with such a low concentration of spike-in cells due to Poisson distribution. After MCF-7 and MDA-MBA-231 cells were spiked into whole blood, the sample was labeled with anti-EpCAM conjugated with Alexa 488 (BioLegend, San Diego, CA) and anti-EGFR-phycoerythrin (PE) (BioLegend, San Diego, CA) for 30 min at room temperature in the dark. The labeled sample was diluted to 14 mL and then centrifuged to remove the free antibodies. The final volume was always adjusted to be the same as the initial volume. The labeled sample was then injected to the eDAR microchip using a syringe pump at a typical flow rate of 50 μL/min. All the tubing and in-line valves for microfluidics were purchased from IDEX (Oak Harbor, WA). Four pressurized containers controlled by pressure regulators were filled with buffer and connected with the dual-capture eDAR microchip via polytetrafluoroethylene tubes. The pressure drop at the sorting junction was manually adjusted and balanced to ensure the complete hydrodynamic switch of the flow for different microchips. Photon counts from fiber-coupled avalanche photodiodes (Excelitas Technologies. Waltham, MA) were collected by a field-programmable gate array device combined with a compact RIO module purchased from National Instrument (Austin, TX), which also controlled the active sorting steps. The sorting threshold was set to a signal-to-noise ratio that was 7 times the standard deviation of the noise level in each channel to rank the aliquots. Solenoids that were used to switch the blood flow were purchased from the Lee Co. (Lot #: INKA1226212H, Westbrook, CT).

After the two subpopulations of rare cells were trapped onto the two on-chip filtration areas, buffer was flowed through the channels and chambers in less than 5 min. Secondary immunostaining was then performed to label the trapped cells with two groups of markers selectively. Two surface antibodies anti-EpCAM-Alexa 488 and anti-CD45-Alexa 700, were used to label the cells trapped on the EpCAM positive area. Similarly, anti-EGFR-PE and anti-CD45-Alexa 700 was used to label the cells trapped on the EpCAM negative area. After all the surface markers were labeled, cells were fixed with 2% paraformaldehyde (BioLegend, San Diego, CA) and permeabilized with 0.2% saponine (Sigma, St. Louis, MO). Anti-cytokeratin (pan)-Alexa 647 (BioLegend, San Diego, CA) and anti-vimentin-Alexa 647 (Cell Signaling, Danvers, MA) were used to label the two different subpopulations of rare cells. Hoechst or DAPI (Life Technology, Grand Island, NY) was used as the nuclear stain to verify the labeled target was actually a nucleated cell. After staining and washing, we used an epi-fluorescence microscope to image the trapping area, so we could identify the isolated CTCs. The microscope was equipped with a 20× objective and was able to collect fluorescence images from five individual color channels: blue (435 to 485 nm for Hoechst or DAPI), green (510 to 540 nm for FITC or Alexa 488), yellow (555 to 605 nm for PE), red (665 to 695 nm for Alexa 647 or APC), and far red (710 to 730 nm for Alexa 700). The detailed procedures and experimental parameters were provided in our previous work.[1-4]

Reproducibility of the Recovery Efficiency

To test the reproducibility of eDAR, we spiked 20 SKBr-3 cells into 1 mL of eDAR using the capillary-spiking method[5] with slight modifications. The sample was then labeled with anti-EpCAM-PE, and analyzed using eDAR at a flow rate of 50 μL/min. The same experiment was

repeated six times. The recovered number of CTCs was 20, 18, 15, 19, 18, and 18, resulting in an averaged recovery efficiency of 90% with a standard deviation of 7.6%.

References

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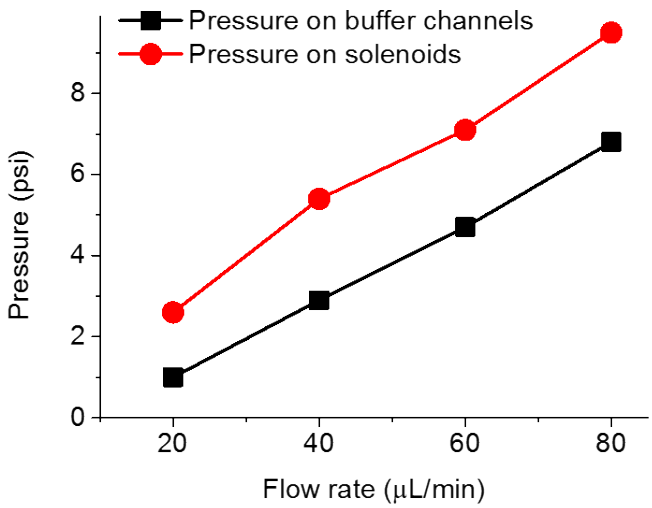

Figure S1 Optimized fluidic pressure for the high-speed sorting step used in the dual-capture eDAR. Because the microchip has a symmetrical design, the pressure values on the two buffer channels (black) were always the same. Similarly, we applied the same pressure on the two solenoid-control channels (red) to ensure the best sorting efficiency and stability.

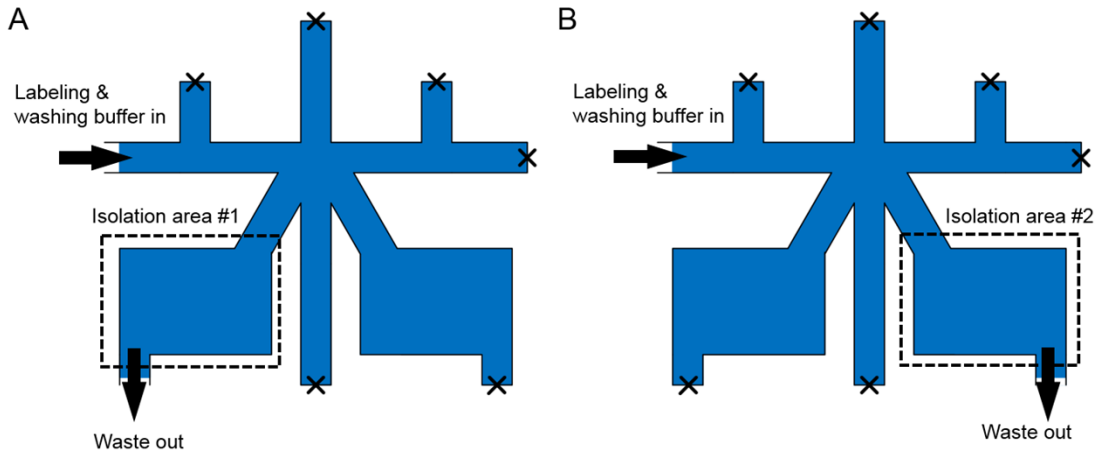

Figure S2 Secondary immunostaining of the captured two subsets of rare cells isolated using dual-capture eDAR. After the isolation, we selectively labeled the two subpopulations with different sets of biomarkers without cross-contamination. For example, if the epithelial CTCs were enriched in isolation area #1, we could inject labeling reagents such as EpCAM, cytokeratin, CD45 and nuclear stain from the left buffer channel, and collect waste from the channel connected to the isolation area #1 (A). Because we closed the flow in all the other channels by turning off the in-line valves on the tubing connected with those channels, this set of biomarkers would never flow into the other isolation area. It was further confirmed by monitoring the fluorescence background of each chamber during the labeling procedure. Similarly, we could manipulate the flow so that the other set of biomarkers, such as EGFR, vimentin, CD45 and nuclear stain, only labeled the mesenchymal CTCs captured in isolation area #2 (B).

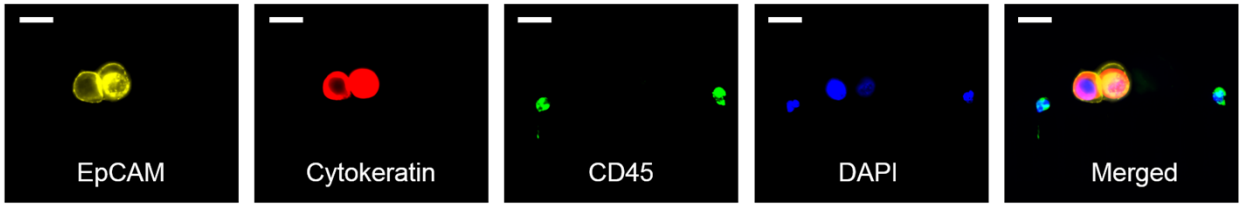

Figure S3. Two CTCs captured in eDAR microchip were stained with PE-anti-EpCAM (yellow), APC-anti-cytokeratin (red), FITC-anti-CD45 (green), and DAPI (blue). In this area, we also observed two WBCs, which were positive to CD45 and DAPI, but negative to EpCAM or cytokeratin. Scale bars are 15 μm.

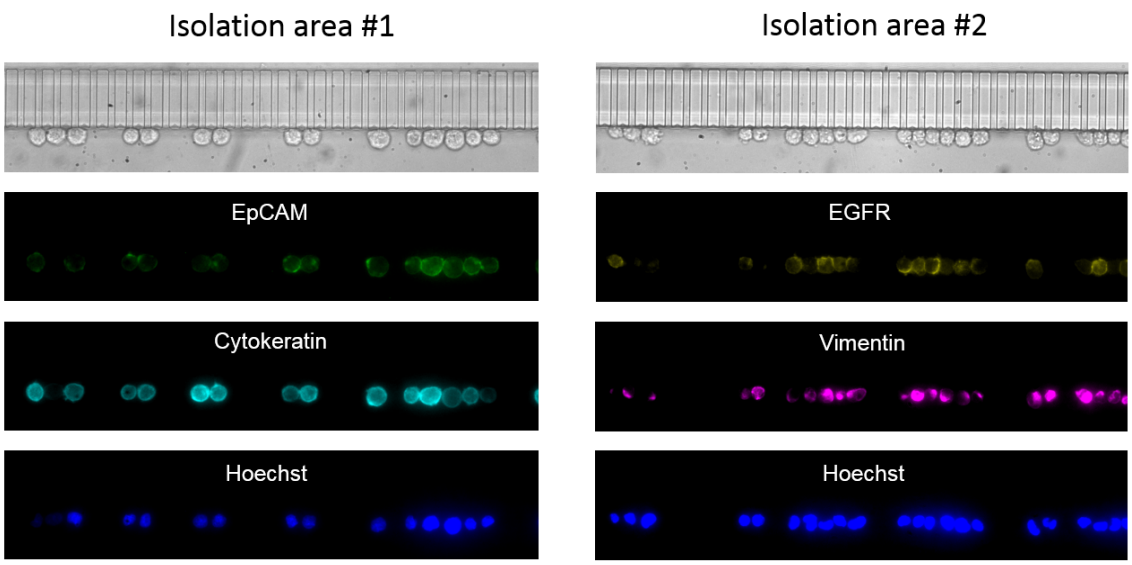

Figure S4 Selectivity of dual-capture eDAR. We spiked 2000 MCF-7 and 2000 MDA-MB-231 cells into 1 mL of diluted blood (50×). We labeled the sample with anti-EpCAM-Alexa 488 and anti-EGFR-PE and carried out dual-capture eDAR on it. After the separation, we first stained the cells in isolation area #1 with anti-EpCAM-Alexa 488, anti-cytokeratin-Alexa 647, and Hoechst; we then stained the cells in isolation area #2 with anti-EGFR-PE, anti-vimentin-Alexa 647, and Hoechst. We did not observe any cells stained with green emission (EpCAM-Alexa 488) in isolation area #2; similarly, there were no cells stained with yellow emission (EGFR-PE) in isolation area #1. Bright field and fluorescence images from other channels also confirmed the very high selectivity of the dual-capture eDAR approach.

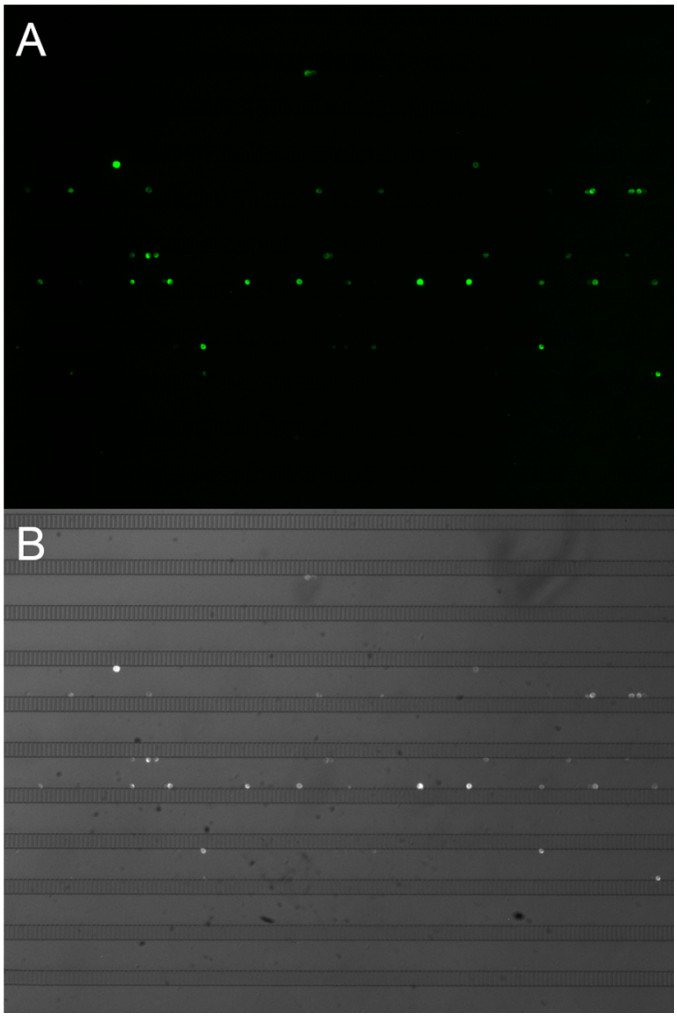

Figure S5 Imaging results of EpCAM^{high} cells trapped on microslits in a single field of view (4x). We spiked 50 MCF-7 and 50 MDA-MB-231 cells into 1 mL of whole blood for this experiment, and recovered 45 cells in the area designed for the subset of EpCAM^{high} CTCs as verified with secondary immunostaining. Because eDAR could trap rare cells along an array of microslits patterned in a small area, the throughput of the enumeration could be very high. In this single field of view, we found 40 cells based on (A) the fluorescent image from EpCAM-Alexa 488, and (B) the bright-field image. We found 43 cells in this area based on the primary labeling, 95% of the recovered EpCAM^{high} cells.

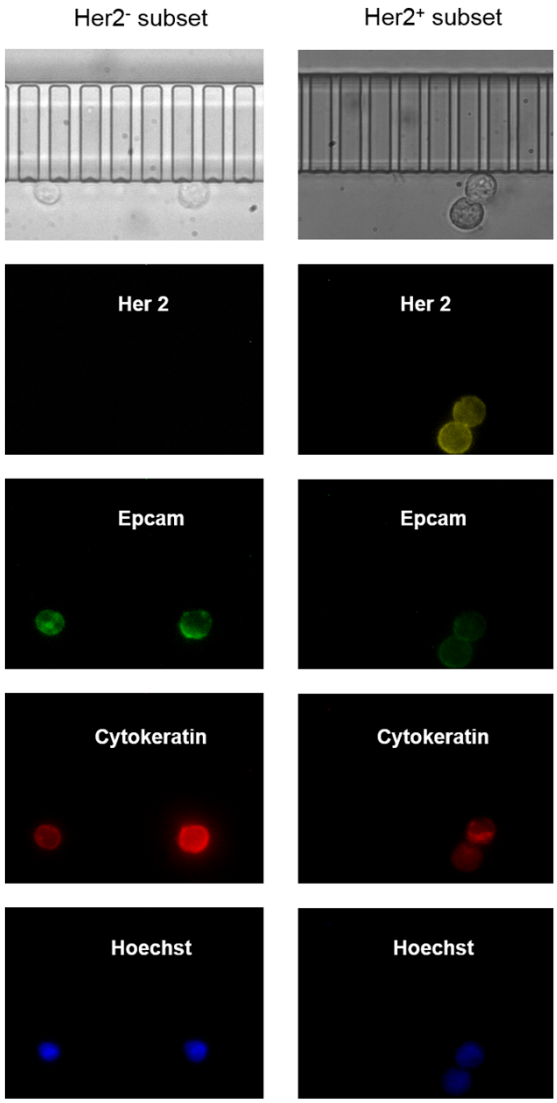

Figure S6 Multicolor fluorescence imaging results for the isolation of $Her2^-$ and $Her2^+$ subsets. We spiked MCF-7 and SKBr-3 cells into 1 mL of whole blood, and analyzed this sample using dual-capture eDAR. The left column shows two cells trapped in the isolation area #1 because of the presence of green fluorescence only (EpCAM-Alexa488). These two cells were further stained with more markers, and showed a clear expression pattern of $EpCAM^+$/ $Her2^-$/ $Cytokeratin^+$/ $Hoechst^+$, which indicates they are actually MCF-7 cells. Similarly, another two cells trapped in the isolation area #2 are $EpCAM^+$/ $Her2^+$/ $Cytokeratin^+$/ $Hoechst^+$, which indicates they are SKBr-3 cells.

Switching events	Average time (ms)	Standard deviation (ms)
Center to left	1.41	0.34
Left to center	2.17	0.36
Center to right	1.51	0.31
Right to center	2.32	0.33

Table S1. Average switching time and the standard deviations for the four types of switching events. These tests were performed in the dual-capture eDAR microchip using whole blood samples from healthy donors. All the switching events were repeated 16 times.