SUPPORTING INFORMATION FOR PUBLICATION

COMBINATION OF MECHANICAL AND MOLECULAR FILTRATION FOR ENHANCED ENRICHMENT OF CIRCULATING TUMOR CELLS

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S-1. Materials and Methods

SI-1A. Materials and reagents

All solutions were prepared with water from a Milli-Q system (resistivity: 18 MΩ cm; Millipore). Phosphate buffered saline (PBS, 1X, pH=7.4, Fisher Scientific), contains 11.9 $10^{-3}$, 137.0 $10^{-3}$ and 2.7 $10^{-3}$ mol L$^{-1}$ of phosphates, NaCl and KCl, respectively. Trypsin-EDTA, sulfuric acid, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES), NaCl, bovine serum albumin (BSA) and Tween 20 were obtained from Sigma-Aldrich. Triton X-100 and paraformaldehyde were purchased from Fisher Scientific. Dulbecco’s modified Eagle medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS) and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies. Antibiotics (penicillin/streptomycin) were obtained from Invitrogen. Anti-EGFR (epidermal growth factor receptor), anti-EpCAM (epithelial cell adhesion molecule), anti-pan cytokeratin-Alexa Fluor 488 and anti-human CD45-phycoerythrin were obtained from R&D systems.

SI-1B. Filter fabrication

The process described in detail elsewhere.$^1$ Briefly, a pillar structure was created by replication of molds obtained by standard photolithography and DRIE. The structure was then closed using a UV curable polymer cover coated on polyethylene terephthalate (PET) carrier to form an enclosed 3D microcavity, which was fully filled by the UV-curable Fluorolink® MD 700 resin (about 10 µL, depending on the surface area of the device and the height of the pillar) thereafter, and cured through UV exposure (2000-EC Series UV curing flood lamp, Dymax). Finally, the blank cover was peeled off, and the molds were bath in acetone during 15-20 minutes, allowing the membranes to self-de-mold from the pillars. Filter pore diameters, determined by microscopy and averaged on 20 pores from three different filters and for each pore size, were 7.9 ± 0.3, 10.0 ± 0.2, 11.7 ± 0.4, 15.0 ± 0.2 and 20.1 ± 0.3 µm.

SI-1C. Cartridge Design

The filtration cartridge used in this work was designed with AutoCAD software (Autodesk Inc.) and 3D printed (Perfactory Micro EDU, Envision Tech). The cartridge is made of two parts in between which the microfilter can be inserted. A small notch (1 mm deep) with the filter shape and size is located at the center of bottom part, on the inside, to allow perfect alignment of the filter with the inlet and outlet. These notches are connected to the inlet and outlet through conical junctions allowing a homogeneous flow to reach the filter surface. Two toric joints, a silicone gasket and a pair of screws and bolts are used to ensure proper sealing.
SI-1C. Design of the filtration cartridge. Screen capture of the AutoCAD design showing the insides of the top and bottom part of the cartridge. The filter is inserted in the notch located on the inside of the bottom part, and the cartridge is closed with the top part and clamp with screws.

SI-1D. Cell Culture

All culture medium and solutions were initially sterile and filtered through a 0.2 μm filter. MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) cell lines were obtained from the American Type Culture Collection (Manassas, VA). 786-O and A-498 renal cells were kindly provided by Dr. Y. Riaz Alhosseini (McGill University, Montreal, Canada). MDA-MB-231, MCF-7 and A-498 cells were cultured in DMEM, supplemented with 10 % FBS and 1 % (v/v) antibiotics (final concentrations of 100 I.U. mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin). 786-O renal cells were cultured in RPMI 1640 medium supplemented with 10 % FBS. All cell cultures were maintained in 5 % CO₂ at 37 °C in 25 cm² flasks (Corning, NY, USA). Almost confluent monolayers (80-90 %) of cells in flasks were harvested through scratching in 2 mL of PBS (for MDA-MB-231 cells) or using diluted trypsin (for MCF-7, 786-O and A-498 cells). 1 mL of the cell suspension was re-suspended in 5 mL of culture media in a new flask while, for spiking experiments, the second milliliter was centrifuged at 4600 rpm for 5 minutes and re-suspended in 1 mL of PBS (density of ≈ 10⁶ cells mL⁻¹). Information regarding the size distribution and the level of expression of EpCAM and EGFR antibodies for all cell lines is provided table 1.
Table 1. Summary of cell types used in this study. * Diameters, measured by microscopy, are reported as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cancer</th>
<th>Diameter* (μm)</th>
<th>EpCAM expression</th>
<th>EGFR expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>14.6 ± 5.1</td>
<td>Low/negative²</td>
<td>High³</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>15.2 ± 4.1</td>
<td>High⁴</td>
<td>Low/negative³</td>
</tr>
<tr>
<td>786-O</td>
<td>Kidney</td>
<td>15.6 ± 5.8</td>
<td>Negative⁵</td>
<td>High⁶,⁷</td>
</tr>
<tr>
<td>A-498</td>
<td>Kidney</td>
<td>15.8 ± 3.8</td>
<td>Negative⁵</td>
<td>High⁶,⁷</td>
</tr>
</tbody>
</table>

SI-1E. Cell Counting

For cell spiking experiments, the cell suspension was first diluted by a factor of 50 to obtain approximately 10-20 cells per microliter. In order to precisely determine the amount of cells within each suspension, 10 μL droplets were placed between a microscope glass slide and a coverslip. The actual number of cells within the 10-μL suspension was manually counted under a microscope. Counting was repeated twice on each slide and averaged on 10 droplets.

SI-1F. Cell enrichment

The sample preparation was directly performed in a syringe. 1.0 mL of blood was diluted with the desired volume of PBS (from 0.0 to 15.0 mL). Once the exact number of (MDA-MB-231, MCF-7, 786-O or A-498) cells in suspension was determined (Supporting information SI-1E), the volume corresponding to the desired number of cells was then spiked into the mixture. After mixing, the syringe was plugged into the cartridge with a polyetheretherketone (Peek) tube (i.d. 0.75 mm, length 10 cm, Sigma Aldrich) at the inlet (top). The desired flow rate (0.1, 0.5, 1.0, 2.0 or 3.0 mL min⁻¹) was applied, making the sample pass through the filter with pore diameters ranging from 8 to 20 μm. Where specified, samples were rinsed with PBS (5.0, 10.0, 20.0, 2 × 5.0 or 3 × 5.0 mL) at the same flow rate as for filtration; in that case, the syringe was re-filled with the chosen volume of PBS.

SI-1G. Cell Staining

Cell staining was performed on the filter, directly in the cartridge after filtration. Before each step described below (from a. to i.), the tubes were unplugged and the outlet was closed with a suitable stopper. Then, 100 μL of appropriate solution was introduced into the cartridge through the inlet with a pipette. After the required incubation time, the cartridge was plugged again to the tubes and a 0.1 mL min⁻¹ air flow was applied to push the solution out.
(a.) Cells fixation with 3.7 % paraformaldehyde (PFA) in PBS for 10 minutes.
(b.) Rinsing twice, 5 min each, in PBS.
(c.) Cells permeabilization with 0.2 % Triton X-100 for 5 min.
(d.) Rinsing twice, 5 min each, in PBS.
(e.) Blocking with 1.0 % BSA in PBS supplemented with 0.1 % Tween 20.
(f.) Cells staining with Anti-Pan-cytokeratin Alexa Fluor 488 (2.0 μg mL\(^{-1}\)) and Anti-Human CD45 Phycoerythrin (PE, 1.0 μg mL\(^{-1}\)), diluted in PBS, for 1 hour.
(g.) Rinsing with PBS.
(h.) Nucleus staining with 4’,6-diamidino-2-phenylindole (DAPI, 0.1 μg mL\(^{-1}\)).
(i.) Rinsing with PBS.
SI-2. Effect of the flow rate on purity

The effect of flow rate on the number of white blood cells (WBCs) remaining on the filter was estimated directly after filtration (no rinse). For these experiments, 101.3 ± 3.3 MDA-MB-231 cells were spiked in 1.0 mL of blood, and samples, diluted with 6.0 mL of PBS, were filtered through 8 μm diameter pores at different flow rates (0.1, 0.5, 1.0, 2.0 or 3.0 mL min⁻¹). Fluorescence images were acquired after cell staining.

As expected, the number of WBCs seems to decrease when the flow rate increases (Figure SI-2A). However, for some flow rate conditions, that number can reach high values (< 1000), preventing the direct count of WBCs. In order to overcome this issue, the surface of the filter covered by fluorescently labeled WBCs was estimated using ImageJ software (Wayne Rasband). All images were acquired with the same exposure time (3 seconds) allowing the direct comparison of their fluorescence intensity. For each filter, 10 images were collected and converted to 8 bit greyscale images. The level of fluorescence intensity of WBCs was used as threshold value (fixed for all images and all conditions) to separate background (areas of the filter with intensity lower than the threshold) and areas covered by WBCs (intensity higher than the threshold).

The percentage of the filter surface covered by WBCs, averaged on the 10 images per filter and on three samples is plotted in figure SI-2B. In good agreement with the qualitative observation (Figure SI-2A), the surface covered by WBCs decreases from 29 ± 4 % at 0.1 mL min⁻¹ to 8 ± 3 % at 3.0 mL min⁻¹, indicating that higher flow rate improves purity.
Figure SI-2. WBCs captured on filter as function of flow rate. (A) Representative images of the filter surface covered by fluorescently labeled WBCs (anti-CD45-Phycoerythrin) for various flow rates. (B) Percentage of the filter surface covered by WBCs, averaged on 10 images per filter and on three samples. 101.3 ± 3.3 MDA-MB-231 cells were spiked in 1.0 mL of blood and diluted with 6.0 mL of PBS. Samples were filtered through filters with 8 μm diameter pores at 0.1, 0.5, 1.0, 2.0 or 3.0 mL min⁻¹. The error bars correspond to the standard deviation of three independent experiments.
SI.3. Effect of the number of cells spiked in blood

Circulating tumor cells (CTCs) are typically in the range of 1 to 10 per milliliter of blood. However, depending on the type and stage of cancer, this count can strongly vary. For instance, up to 115 and 224 CTCs per milliliter have been detected in metastatic breast and gastric cancer patients respectively. The influence of that number on capture efficiency was therefore investigated on a large range, by spiking 6.1 ± 1.5, 26.6 ± 1.4 or 101.1 ± 5.1 MDA-MB-231 cells in 1.0 mL of blood diluted with 6.0 mL of PBS. Samples were filtered through 8, 10, 12, 15 or 20 μm diameter pores at 1.0 mL min⁻¹. Fluorescence images were recorded after immunostaining.

As previously observed, for each number of MDA-MB-231 cells spiked in blood, efficiency clearly decreases when pore size increases. For instance, 61 ± 10 % of MDA-MB-231 were captured with 8 μm diameter pores and 6.1 cells spiked in blood, and this value decreases down to 38 ± 10 when filters with 20 μm diameter pores are used (Figure S1-3).

For each single pore size, efficiency values obtained for various number of MDA-MB-231 spiked in blood (6.1 ± 1.5, 26.6 ± 1.4 or 101.1 ± 5.1 MDA-MB-231 cells) were compared using Fisher test. In all cases, p values are higher than 0.05, indicating that the number of MDA-MB-231 initially spiked in blood has no effect on efficiency.

Moreover, one can notice that the errors bars (standard deviations of three independent experiments) are in the same range for a single number of MDA-MB-231 cells spiked in blood but clearly decreases when the number of MDA-MB-231 cells spiked in blood increases. This is due to the fact that missing a cell will impact more on the efficiency for small number of cells. Indeed, one missed cell when spiking about six MDA-MB-231 cells, corresponds to about 16 % of error, while when spiking about a hundred cells, it approximately corresponds to 1 %.
Figure SI-3. Effect of the number of MDA-MB-231 spiked in blood on efficiency. ▲ 6.1 ± 1.5, ▼ 26.6 ± 1.4 or □ 101.1 ± 5.1 MDA-MB-231 cells were spiked in 1.0 mL of blood and diluted with 6.0 mL of PBS. Samples were filtered at 1.0 mL min⁻¹ through filters with 8, 10, 12, 15 or 20 μm diameter pores. The error bars correspond to the standard deviation of three independent experiments.
SI.4. Filter functionalization

The performance of various treatments of the filter surface for antibody functionalization was evaluated using fluorescently labeled antibody (FITC-labeled anti-rabbit antibodies). Following each surface treatment described in Table SI-4, 100 μL of the antibody solution (2 μg mL⁻¹) was added on the filter surface for three hours at 4 °C to avoid evaporation. The filter was then rinsed with PBS, deionized water, and dried under a nitrogen stream.

<table>
<thead>
<tr>
<th>Labels in figure 4A (main paper)</th>
<th>Surface treatment before antibody incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>Pristine filter</td>
</tr>
<tr>
<td>O₃ + EDC/NHS</td>
<td>Ozone treatment (20 minutes in an ozone generator, OzoMax, Inc., Shefford, Quebec, Canada) + 20 minutes incubation in EDC/NHS (0.05 mol L⁻¹)</td>
</tr>
<tr>
<td>O₂ plasma + EDC/NHS</td>
<td>Oxygen plasma (2 minutes, 150 W, O₂ pressure of 200 mTorr, Plasmalab 80 Plus, Oxford Instruments, Bristol, United Kingdom) + 20 minutes incubation in EDC/NHS (0.05 mol L⁻¹)</td>
</tr>
<tr>
<td>O₃ + CNBr</td>
<td>Ozone treatment (20 minutes in an ozone generator) + 20 minutes incubation in CNBr (0.5 mol L⁻¹ in nitric acid).</td>
</tr>
<tr>
<td>O₂ plasma + CNBr</td>
<td>Oxygen plasma treatment (2 minutes, 150 W, O₂ pressure of 200 mTorr) + 20 minutes incubation in CNBr (0.5 mol L⁻¹ in nitric acid).</td>
</tr>
</tbody>
</table>

Table SI-4. Surface treatments studied to optimize filter surface functionalization. EDC: 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, NHS: N-hydroxysuccinimide, CNBr: Cyanogen bromide.

The presence of antibodies on the surface was directly evidenced using fluorescence microscopy. For each condition, fluorescence images were collected before and after functionalization with 3 seconds exposure. The mean fluorescence intensity was determined from four randomly chosen areas of the filter. Finally, the variation between the fluorescence intensity of the pristine filter and that obtained after functionalization (Δfluo), averaged on three independent experiments, is plotted in figure 4A of the main paper.

Briefly, the highest variation of fluorescence intensity was obtained after oxygen plasma followed by EDC/NHS based-chemistry. Therefore, in order to obtain the highest antibody density, filters were functionalized after O₂ plasma treatment and EDC/NHS chemistry. For cells experiments, same protocol was used and antibody concentrations were optimized (Figure 4B and C of the main paper).
REFERENCES