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Supporting Information

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Micromachine-Enabled Capture and Isolation of Cancer Cells in Complex Media**

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Supporting Information

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Experimental Section

Reagents and solutions:

6-Mercaptohexanol (MCH), 11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and sodium cholate were purchased from Aldrich. Mouse anti-carcinoembryonic antigen (CEA), Clone Col-1 (18-0057) (anti-CEA), and Sybr Green II nucleus staining dye, and 4′,6-diamidino-2-phenylindole (DAPI, nucleus staining) were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA), human serum (from human male AB plasma), KCl, Na_2HPO_4, $K_2HPO_4.3H_2O$ and NaCl were purchased from Sigma. Reagents were used without any further purification. Experiments were carried out at room temperature.

For tissue culture, 10% fetal bovine serum was acquired from Hyclone (Logan, UT), sodium bicarbonate was obtained from Cellgro (Herndon, VA), RPMI culture medium, penicillin/streptomycin, sodium pyruvate, L-glutamine, and MEM non-essential amino acids were purchased from Invitrogen (Carlsbad, CA), and EDTA powders were bought from Fisher Scientific.

Other solutions employed were: Phosphate buffered saline 1×PBS buffer (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.4) and 1% BSA solution in the same PBS buffer, each prepared in deionized water. All chemicals used were of analytical-grade reagents, and deionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm).

Microrockets fabrication:

The Ti/Fe/Au/Pt microtube rockets were prepared by modifying previously reported lithographic protocols.[1] Briefly, a positive photoresist (Microposit S1827, Microchem, Newton, MA) which serves as a sacrificial layer, was spin-coated on a silicon wafer at 3000 rpm for 60 seconds. The coated-wafer was baked at 115 °C for 60 seconds and exposed to UV light with an MA6 mask aligner for 35 seconds to create pre-defined patterns. Exposed patterns were developed using a MF-321 developer for 90 seconds and thoroughly washed with DI water. Metallic layers of Ti: 10 nm, Fe: 15 nm, Au: 5 nm and Pt: 10 nm were deposited sequentially using an e-beam evaporator under high vacuum conditions ($<10^{-4}$ Pa). The e-beam substrate holder was tilted to 50° in order to asymmetrically deposit metals on the patterns. Upon selective removal of the exposed photoresist layer using MF-1165 (Rohm & Haas, Marlborough, MA), the prestressed metallic layers self-assemble into microtubes. The microrockets were washed and stored in isopropanol before undergoing critical-point drying to maintain structural integrity. A thin (~60 nm) gold layer was sputtered onto the rolled-up microtubes to facilitate surface functionalization with the antibody receptor through the assembly of alkanethiols.

Microrockets modification:

The external gold surface of the microrockets was modified by an overnight immersion in a binary mixture of 2.5 mM of MUA and 7.5 mM of MCH in absolute ethanol. After washing with ultrapure water, the microrockets modified with the resulting mixed monolayer were treated with a solution of NHS (20 mM) and EDC (10 mM) in ultrapure water for 30 min, followed by an 1 h immersion in a solution of 1×PBS buffer (pH 7.4) containing 2 μg/ml of anti-CEA mAb. The remaining reactive groups of the activated monolayer were blocked with 1 M ethanolamine (pH 8.5) for 30 min. The microrockets were subsequently immersed in a 1 % (w/v) solution of BSA in 1×PBS buffer (pH 7.4) for 1 h. Finally, the modified microrockets were washed for 60 s with ultrapure water and resuspended in 1×PBS buffer (pH 7.4). All incubation steps were carried out at room temperature followed by immersion in ultrapure water for 1 min.

'Control' microrockets (without the mAb) were prepared using the same protocol (with the SAM assembly, activation and blocking steps) but omitting the addition of the anti-CEA mAb and carrying out the corresponding incubation in buffer (without mAb). It is important to note that the anti-CEA mAb-modified microrockets can be used to capture and transport CEA+ cells if they are stored (up to 2 weeks) in 1× PBS buffer (pH 7.4) at 4ºC. Also worth noting is that the Fe layer of the microrocket is very susceptible to the presence of HCl salt in EDC during activation of the –COOH of the monolayer which can render them non-magnetic. In order to maintain the magnetic property of the microrockets, at least 3 times more Fe layer is required. This allows for effective propulsion and proper navigation of modified microrockets even in complex biological media.

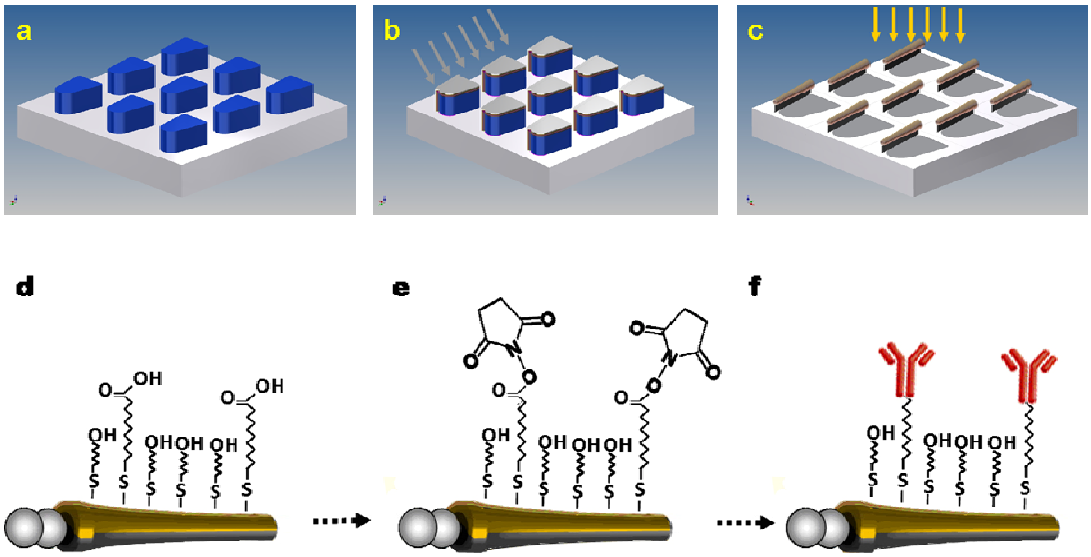

Figure S1. Schematics showing fabrication of microrockets using standard photolithography (a-c) and chemical procedures to modify the microrockets with mAb (d-f). a) Exposed photoresist layer revealing 'microrocket' patterns on silicon wafer, b) multilayer metal evaporation at 50° angle creates strain for

roll-up process, c) sputtering of Au layer on rolled-up microtube for subsequent chemical modification. Surface functionalization of the microrocket with the anti-CEA mAb receptor. Steps involved: d) self-assembling of MUA/MCH (2.5/7.5 mM) binary monolayer; e) conversion of the carboxylic terminal groups of the MUA to amine-reactive esters by the EDC and NHS coupling agents; f) reaction of NHS-ester groups with the primary amines of the anti-CEA mAb to yield stable amide bonds.

Preparation of Suspended Cancer Cells:

The human pancreatic CEA-positive BxPC-3 (CEA+ cells) and the CEA-negative XPA-3 (CEA- cells) cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf albumin, penicillin/streptomycin, L-glutamine, MEM nonessential amino acids, sodium bicarbonate, and sodium pyruvate. Both cell lines were cultured at 37°C with 5% CO_2. To prepare the cells in suspension, each cell line was detached following 20 min incubation in PBS (without Ca/Mg) containing 15 mM of EDTA. The cells were then pelleted and resuspended in PBS with Ca/Mg. The viability of the cells was confirmed using a trypan blue dye exclusion assay. Prior to their fluorescent imaging, the BxPC-3 and XPA-3 cells were nuclear stained in 1× Sybr Green II and DAPI solution, respectively.

Identification and Isolation of Cancer Cells:

Microrockets functionalized with the anti-CEA mAb were isolated from the substrate surface and suspended in 1×PBS buffer (pH 7.4). A mixture of microrockets (1 µl) and sodium cholate (1%(w/v), 3 µl) was added to a freshly cleaned glass slide. To this, 5 µl of suspended cancer cells and 3 µl of H_2O_2 were added (final peroxide concentration, 7.5%(w/v)). Microrockets traveling along the bottom (glass) surface experienced an additional frictional force. Such microrockets were used in control and mixture experiments to increase the interaction time between the microrocket and CEA-cells. The microrockets were magnetically guided towards the CEA+ and CEA- cells to study and identify the cell-microrocket interaction which was monitored using a Nikon Eclipse TE2000S fluorescence microscope. Videos were captured using CoolSNAP HQ^2 camera, 20× objective (unless mentioned otherwise) and acquired at the frame rate of 10 using the Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA). Snap shot images in static cell-capture studies on mixture samples were taken with a DeltaVision deconvolution microscope.

Microrocket Cellular Transport Calculations:

A nanovehicle with a large towing force is an important selection criterion for the cellular transport. Although many catalytic nanomotors have shown the ability to carry objects up to 5 µm in diameter,[2] there is no report of utilizing such nanomotors for cell (diameter 16 µm) manipulation in viscous physiological media. Using Stokes' law (Equation 1), we can estimate the force necessary to counter the

drag force ($\mathbf{F_d}$) that a cell would experience at a constant velocity of one body length per second in the working solution:

$$\mathbf{F_d} = 6\pi\mu r v \qquad (1)$$

where μ is the solution viscosity, r is the cell radius and v is the linear velocity of the cell (16 μm/s). Calculations using these values reveal that the following forces are needed to carry such a large cell at a reasonable speed in the different media (Table S1).

Table S1. Forces necessary to carry a 16 μm diameter cell in different media at 1 body-length/s.

Medium	μ* cP	F_d, pN
Fuel solution containing 7.5% (w/v) H_2O_2 in 1× PBS, 1% (w/v) sodium cholate	1.05	2.5
Fuel solution containing 7.5% (w/v) H_2O_2 in 25% (v/v) human serum, 1% (w/v) sodium cholate	1.14	2.8
Human blood	5.0	12.1

* All viscosity values were taken assuming a room temperature of 20ºC.

In order to determine the propulsive force of our microrocket, we used Stokes' drag law for a cylinder[3] as shown in Equation 2, assuming that the microrocket experiences drag as a solid cylinder (as the fluid can not freely flow through the oxygen-bubble containing microrocket and negating the slight 2-4 degree angle along the microrocket, i.e., approaching a cylindrical shape).

$$\mathbf{F_d} = \frac{2\pi\mu L}{\ln\left(\frac{2L}{R}\right) - 0.72} v \qquad (2)$$

where R and L are the radius and the length of the microrockets (2.5 and 60 μm, respectively).

Although such modified microrockets have a sufficient force to transport these large cells, it is important that they do not apply a large shear force which could prevent binding from occurring or disturb the viability of the captured cells. The shear stress (τ_s) exerted as a result of microrocket interaction with the cell can be calculated based on the following equation 3:

$$\tau_s = \frac{\mathbf{F_d}}{A} \qquad (3)$$

where A represents the interaction area. Values of the drag force applied by the anti-CEA mAb-modified microrocket to the carried cell are summarized in Table S2.

Table S2. Drag forces and shear stress applied to the carried cell as a function of the speed of the anti-CEA mAb-modified microrocket in different working media.

Medium	v, μm/s	F_d, pN	τ_s, dyn/cm^2
Fuel solution containing 7.5% (w/v) H_2O_2 in 1× PBS, 1% (w/v) sodium cholate	150	18.1	2.3
Fuel solution containing 7.5% (w/v) H_2O_2 in 25% (v/v) human serum, 1% (w/v) sodium cholate	100	13.3	1.7
Fuel solution containing 7.5% (w/v) H_2O_2 in 1× PBS, 1% (w/v) sodium cholate*	45	5.7	0.7

* anti-CEA mAb-modified microrocket moving in the bottom plane.

The interaction area was estimated as 2/3 of the average cell diameter for the width, and ½ of the microrockets circumference as the height (7.85 μm). Such estimates are based on visual observations which show a slight cell deformation around the microrocket as it passes. Therefore, the average contact area is 78.5 pm^2 and the shear stress is directly proportional to the force and ultimately to the microrocket speed. The values obtained for the shear stresses in PBS and human serum (Table S2) are similar to that obtained for cell capture using microchip technology.[4] The lower shear stress (0.7 dyn/cm^2) induced by the anti-CEA mAb-modified microrocket in the control experiments ensures an increase in binding affinity between the anti-CEA mAb-modified microrocket and the CEA on the cell surface during pick up. Once the cancer cell was captured by the anti-CEA mAb-modified microrocket, the interaction is strong enough for retaining the cell upon experiencing larger shear stresses.

Cancer cells viability under experimental conditions:

Trypan blue exclusion test was used to determine the number of viable cells present in a cell suspension after exposure to the conditions used in the capture and transport experiments. The cancer cells were incubated in PBS solutions containing 1% of sodium cholate and various concentrations of the peroxide fuel. After different incubation times the cell suspension was mixed with the dye and then it was examined under a microscope to determine whether the cells take up or exclude the dye (Figure S2). The results of the viability are shown in Table S3. It should be noted that under all conditions assayed dead cells retain spherical shape (no cell lysis).

Table S3. Viability of CEA+ pancreatic cancer cells in the presence of PBS solution containing 1% of sodium cholate and various concentrations of H_2O_2 .

H_2O_2 concentration, % (w/v)	Viability Window
8	50% viable, 10 min
5	50% viable, 22 min
2	> 90% viable, > 1 h
1	> 90% viable, > 1 h

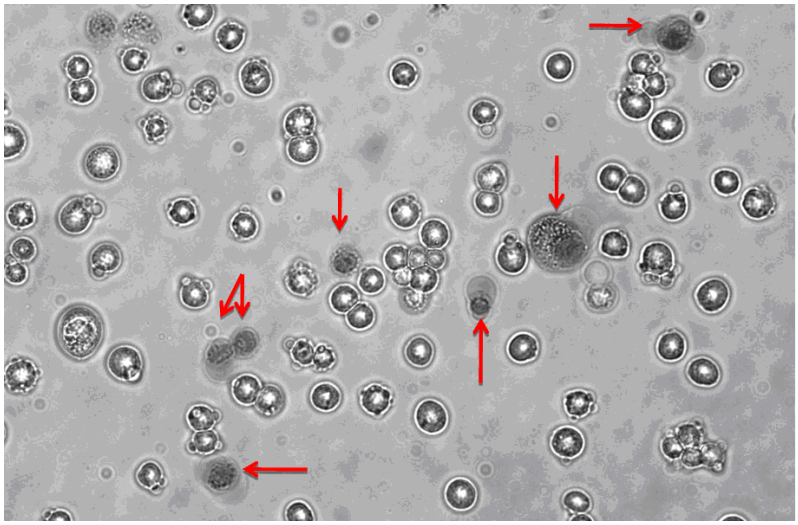

Figure S2. CEA+ cells' viability in PBS containing 2% (w/v) H_2O_2 and 1% (w/v) sodium cholate after 45 min of incubation. Red arrows indicate trypan blue stained dead cells.

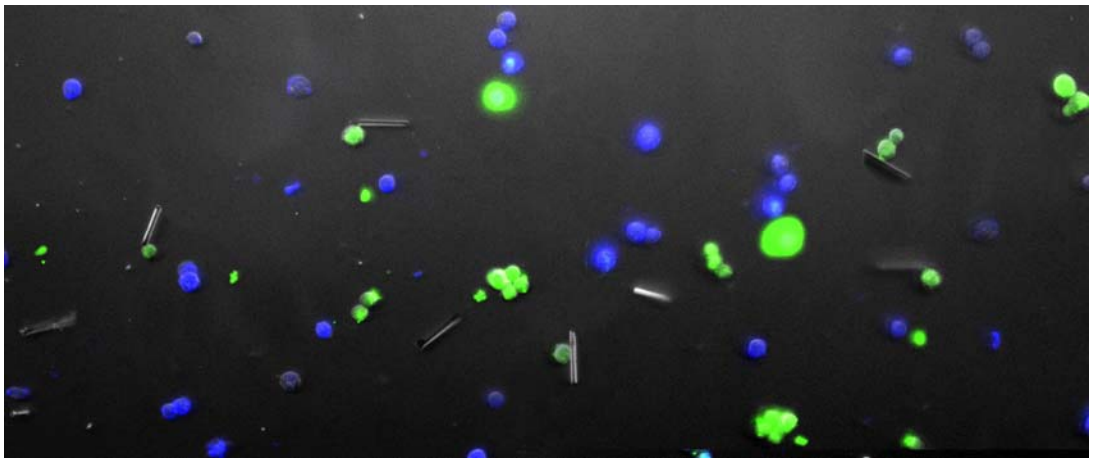

Figure S3. Complied snap shot images of anti-CEA mAb-modified microrockets after incubation in a mixture containing both CEA+ (green stained) and CEA- (blue stained) cancer cells.

References:

[1] Y. Mei, G. Huang, A. A. Solovev, E. Bermúdez Ureña, I. Mönch, F. Ding, T. Reindl, R. K. Y. Fu, P. K. Chu, O. G. Schmidt, Adv. *Mater.* **2008**, *20*, 4085–4090.

[2] A. A. Solovev, S. Sanchez, M. Pumera, Y. F. Mei, O. G. Schmidt, *Adv. Func. Mater.* **2010**, *20*, 2430-2435.

[3] W. F. Paxton, A. Sen, T. E. Mallouk, *Chem. Eur. J.* **2005**, *11*, 6462-6470.

[4] S. Nagrath, L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G. Tompkins, D. A. Haber, M. Toner, *Nature* **2007**, *450*, 1235-1239.

Complete references corresponding to those abridged in the manuscript (10 or more authors):

[2] S. Nagrath, L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G. Tompkins, D. A. Haber, M. Toner, *Nature* **2007**, *450*, 1235-1239.

[3] A. A. Adams, P. I. Okagbare, J. Feng, M. L. Hupert, D. Patterson, J. Göttert, R. L. McCarley, D. Nikitopoulos, M. C. Murphy, S. A. Soper, *J. Am. Chem. Soc.* **2008**, *130*, 8633-8641.

[7] D. Kagan, R. Laocharoensuk, M. Zimmerman, C. Clawson, S. Balasubramanian, D. Kang, D. Bishop, S. Sattayasamitsathit, L. Zhang, J. Wang, *Small* **2010**, *6*, 2741-2747.

[10] a) Y. Mei, G. Huang, A. A. Solovev, E. Bermúdez Ureña, I. Mönch, F. Ding, T. Reindl, R. K. Y. Fu, P. K. Chu, O. G. Schmidt, Adv. *Mater.* **2008**, *20*, 4085–4090.