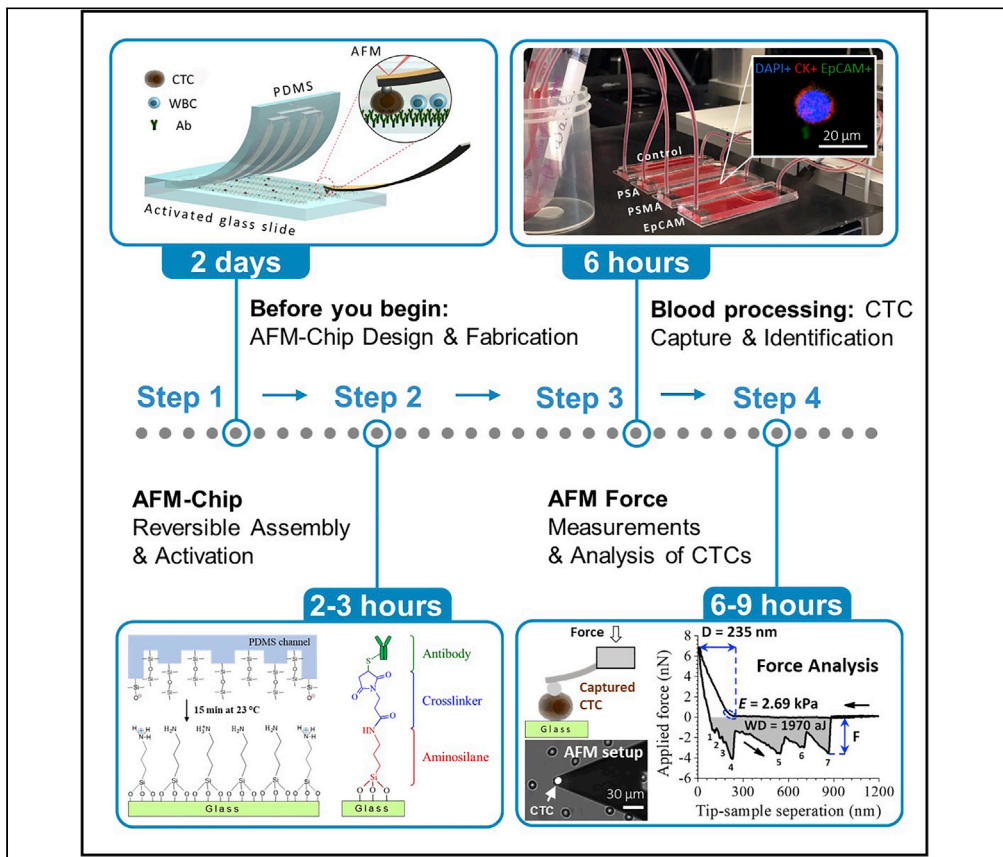


Protocol

Characterizing circulating tumor cells using affinity-based microfluidic capture and AFM-based biomechanics



Elasticity and bio-adhesiveness of circulating tumor cells (CTCs) are important biomarkers of cancer. CTCs are rare in blood, thus their capture and atomic force microscopy (AFM)-based biomechanical characterization require the use of multifunctional microfluidic device. Here, we describe procedures for fabrication of such device, AFM-Chip, and give details on its use in affinity-based CTC capture and integration with AFM via reversible physical assembly. In the AFM-Chip, CTC capture is efficient, and transition to AFM characterization is with minimal cell loss.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Muhammedin
 Deliorman, Ayoub
 Glia, Mohammad A.
 Qasaimeh

maq4@nyu.edu

Highlights

Microfluidic device for isolation of CTCs from whole blood samples of cancer patients

Reversible physical assembly in microfluidics enables seamless integration with AFM

AFM-based biomechanics of CTCs for biophysical phenotyping

AFM-Chip for liquid biopsy and biomechanical characterization of cancer cells

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Protocol

Characterizing circulating tumor cells using affinity-based microfluidic capture and AFM-based biomechanics

Muhammedin Deliorman,¹ Ayoub Glia,¹ and Mohammad A. Qasaimeh^{1,2,3,4,*}¹Division of Engineering, New York University Abu Dhabi (NYUAD), P.O. Box 129188, Abu Dhabi, UAE²Tandon School of Engineering, New York University, Brooklyn, NY 11201, USA³Technical contact⁴Lead contact*Correspondence: maq4@nyu.edu<https://doi.org/10.1016/j.xpro.2022.101433>

SUMMARY

Elasticity and bio-adhesiveness of circulating tumor cells (CTCs) are important biomarkers of cancer. CTCs are rare in blood, thus their capture and atomic force microscopy (AFM)-based biomechanical characterization require use of multi-functional microfluidic device. Here, we describe procedures for fabrication of such device, AFM-Chip, and give details on its use in affinity-based CTC capture, and integration with AFM via reversible physical assembly. In the AFM-Chip, CTC capture is efficient, and transition to AFM characterization is seamless with minimal cell loss.

For complete details on the use and execution of this protocol, please refer to Deliorman et al. (2020).

BEFORE YOU BEGIN

The protocol below describes detailed procedures for the design, fabrication, assembly, and use of the AFM-Chip. The AFM-Chip (Figure 1A) was developed as a multifunctional microfluidic device to allow multiplex capture of CTCs in whole blood samples of prostate cancer patients by antibody-antigen interactions, and characterization of their elasticity and bio-adhesiveness using AFM procedures (Deliorman et al., 2020). Therefore, the timings reported throughout the protocol are based on simultaneously processing blood in 3 AFM-Chips for each patient's sample: first targeting the capture of prostate CTCs through their epithelial cell adhesion molecule (EpCAM), second through their prostate specific membrane antigen (PSMA), and third through their prostate specific antigen (PSA). However, the protocol can also be successfully employed to other forms of cancer such as breast, pancreatic, and colorectal by simply modifying the capture antibody layer. As a result, the timings should be expected to differ depending on experimental conditions. Moreover, for own safety, it is important to wear proper personal protective equipment (gloves, a lab coat, goggles) at all times during the experiments, especially when handling chemicals and blood samples.

The AFM-Chip, adapted from our developed microfluidic device (Qasaimeh et al., 2017), comprises large array of microfluidic channels to achieve high throughput (Figure 1B), thus reducing the blood processing time; and herringbone (HB) elements (Figure 1C) to serve as a fluidic chaotic mixer for enhanced cell-substrate interactions, thus improving the CTC capture yield within the channels. Moreover, in the AFM-Chip, the assembly occurs via physical polydimethylsiloxane (PDMS)-to-glass bonding using silane molecules (Figure 1D). Such bondage is stable, creating firm, high-performance microfluidic channels that could withstand up to 150 $\mu\text{L}/\text{min}$ flow rates with no leakage of blood; and reversible, enabling—thanks to highly hydrophobic nature of silanized glass



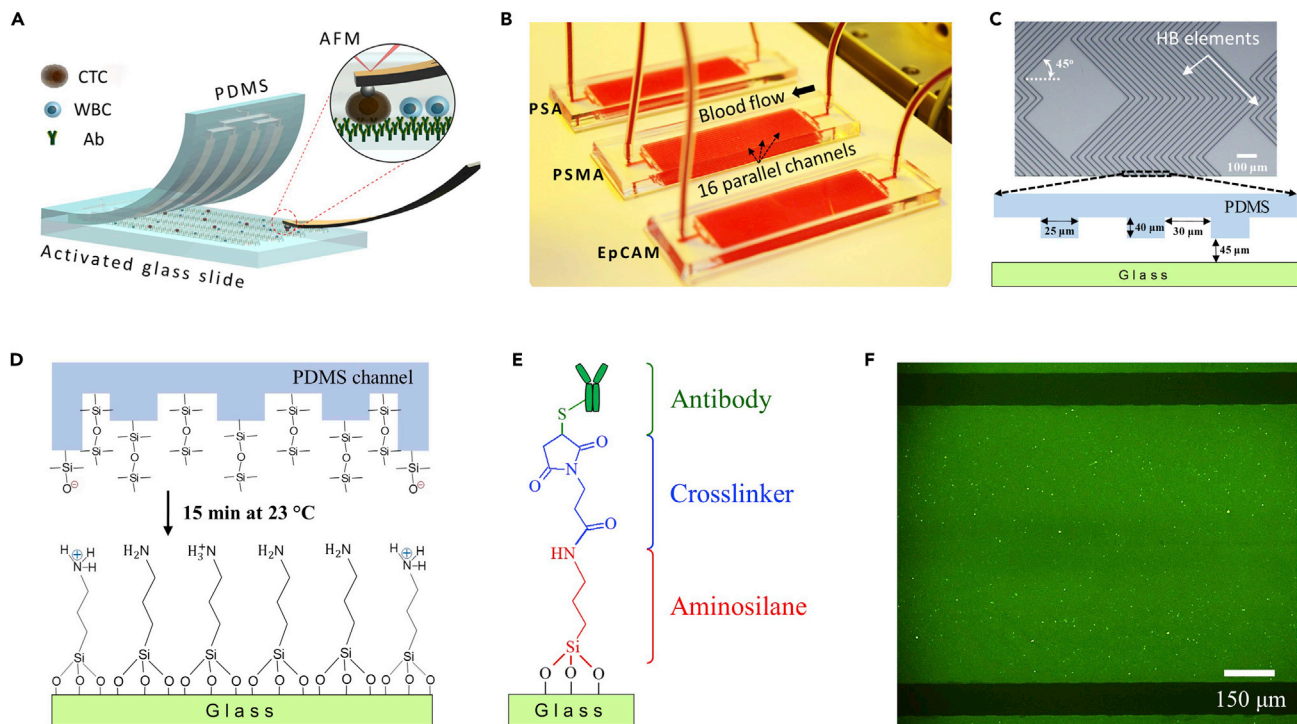


Figure 1. The AFM-Chip microfluidic technology for affinity-based multiplex capture of prostate CTCs and their subsequent nanomechanical characterization using AFM

(A) Following specific capture of CTCs on antibody (Ab) activated glass slide, AFM measurements are performed after peeling off the PDMS chip from the capture glass slide.

(B) Three AFM-Chips used to multiplex capture prostate cancer CTCs through their EpCAM, PSMA, and PSA markers. The PDMS-to-glass reversible physical bonding is firm to prevent leakage of blood during sample pumping.

(C) Micrograph (top) and cross-sectional schematics (bottom) show the geometry, periodicity, and size of HB elements residing on top of the PDMS channels.

(D) Schematic featuring the mechanism of reversible physical bonding between PDMS chip and APTES-silanzed glass substrate via stable NH–O hydrogen bonds.

(E) After microfluidic device assembly, linker chemistry steps involve the reaction of APTES silane molecules with a modular crosslinker BMPS to covalently immobilize antibodies on the device glass substrate.

(F) The fluorescence micrograph of the green area within the microfluidic channel indicates that anti-EpCAM antibodies have been successfully immobilized with high density and the desired orientation (activity). The accessibility of the antigen binding sites of anti-EpCAM antibodies was evaluated by labeling them with NHS-fluorescein. Images and schematics in (A–F) reused with permission from [Deliorman et al. \(2020\)](#).

substrate—opportunities for conducting fundamental studies on intact captured CTCs by simply peeling off the PDMS post-capture of cells.

The reversible AFM-Chip assembly further allows the use of intermediate linker molecules for site-specific covalent immobilization of antibodies exclusively on glass surfaces (Figure 1E). These molecules, if properly deposited, serve as (a) flexible site-specific linkers between the silanzed glass surface and the antibodies, and (b) physical spacers which provide freedom of motion to the tethered antibodies, thus facilitating antibody-antigen interactions. This provides a high density of oriented (active) antibodies (Figure 1F), thus improving the specific capture of CTCs and significantly minimizing the capture of other blood cells. Moreover, the antibody loading capacity in AFM-Chip is independent of the choice of antibodies due to highly site-specific coupling between intermediate linker molecules and antibodies. This gives a great flexibility to target different surface markers of CTCs for their specific capture. Noteworthy, as opposed to other affinity-based microfluidic devices ([Cho et al., 2018](#)) where CTCs are captured on all walls of the microfluidic device (and thus statistically reducing their capture numbers on glass surfaces alone), in the AFM-Chip CTCs are exclusively captured on the glass capture surfaces due to selective reaction of linker molecules with antibodies,

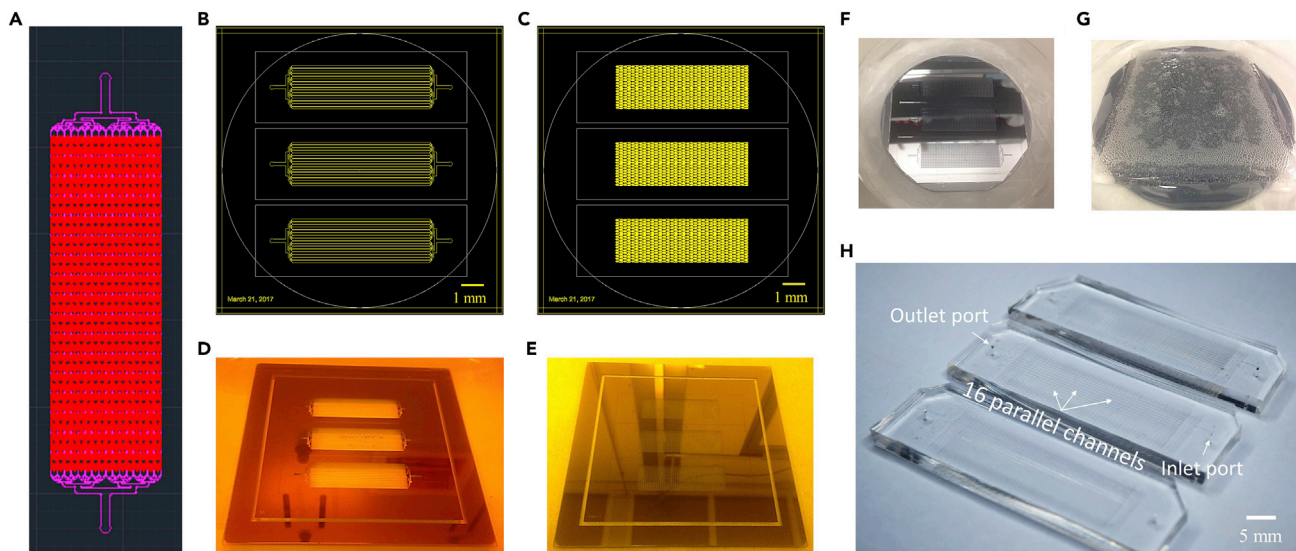


Figure 2. Methods of AFM-Chip for design, fabrication, and PDMS replica molding

(A) A CAD design of 16 parallel microfluidic channels incorporated with HB elements (red) whose inlet and outlet ports are common (purple).

(B and C) The mask designs of microfluidic channels and HB elements in the AFM-Chip, respectively, generated separately from the AFM-Chip CAD design.

(D and E) Micrographs show the hardmask blanks after the designs of microfluidic channels and HB elements were transferred using a direct laser writer.

(F) Micrograph shows a silicon wafer (master mold) with 3 AFM-Chips prepared following sequential double coating of high-viscosity negative resist using a standard photolithography protocol: first for the microfluidic channels and the second for the HB elements. The wafer is firmly taped on a Petri dish bottom for follow up PDMS replica molding.

(G) Micrograph shows the master wafer immediately after pouring and covering it with PDMS mixture for mold formation. The mixture is freed from air bubbles under vacuum pressure and further solidified in oven.

(H) Micrograph shows 3 typical PDMS replica molds (75 mm × 25 mm) of AFM-Chip with punched inlet and outlet ports.

while their capture on PDMS walls is minimal and mainly as a result of non-specific cell-surface interactions. This brings advantage in terms of higher sample size (i.e., number of available captured CTCs) when glass slides are transferred to AFM stage.

All combined, the AFM-Chip is simple, robust, and reliable to use. It provides efficient and reproducible multiplex capture of prostate cancer CTCs and detailed characterization of their biomechanical properties.

Institutional permissions

Approval for the blood procedures described in this protocol was obtained from NYUAD's Institutional Review Board (IRB) and local ethics committee.

AFM-Chip design

⌚ Timing: 1 day

1. Use computer-aided design software (e.g., AutoCAD) to draw a microfluidic chip with 16 channels, each 900 μm wide, 85 μm deep, and 15 mm long, and HB elements, each 25 μm wide and 40 μm deep, oriented at 45° with respect to longitudinal axes of the channels, and with 30 μm gaps in between them (Figure 2A).

Mask fabrication

⌚ Timing: 4 h

△ **CRITICAL:** Conduct the mask fabrications in a fully equipped clean room to prevent contamination. Handle all chemicals in a fume hood.

2. Use mask design software (e.g., Layout Editor, AutoCAD) to prepare photolithography mask designs for microfluidic channels and HB elements (Figures 2B and 2C).

Note: The timing for this step is excluded from overall timing of this section. There are several guided protocols available online (e.g., <https://www.layouteditor.org>) on how to use the Layout Editor, and can be taken as reference during the mask design process to minimize the timing.

3. Proceed with mask fabrications of microfluidic channels and HB elements.
 - a. Mask fabrication of microfluidic channels (Figure 2D).
 - i. Transfer a 150 mm mask blank on stage of a direct laser writer (e.g., Heidelberg DWL 66+), and use the mask design to generate the microfluidic channels on the mask blank.

Note: Set the optimum laser exposure parameters (laser power and intensity, focus offset, filter) depending on the instrument used. For example, based on Heidelberg DWL 66+ Standard Operating Procedure, in our experiments these values read as 120 mW for the laser power, 25 mW/cm² for the laser intensity, 0% for the focus offset, and 25% for the filter.

▣ **Pause point:** With direct laser writers, it is typical to follow standard operating procedures of the instrument. As such, these instruments utilize acousto-optic modulators to adjust the laser intensity, acousto-optic deflectors to scan the laser beam, and pneumatic and optical sensors to adjust the focus offset. Moreover, the mask blank is held on the stage of a laser writer by vacuum, and when it moves in the x-y plane during the laser exposure, its position is monitored by an interferometer system.

△ **CRITICAL:** Mask blank is pre-coated with a layer of 540 nm thick AZ1500 resist and needs to be handled under a yellow light.

- ii. Immerse the patterned mask in a 1:5 (v/v) mixture of AZ351B developer and DI water and gently shake it for about 1 min. Re-immerses it in a DI water and shake it for another 20 s to stop the reaction of the developer. Rinse the mask thoroughly with DI water and dry it with nitrogen gas.

△ **CRITICAL:** Use upright light microscope (e.g., Nikon LV100) to visually verify that no defects are present on the generated patterns.

- iii. Immerse the mask in a chromium etchant and shake it gently for about 1 min. Re-immerses it in a DI water and shake it for another 20 s to stop the reaction of the etching. Rinse the mask thoroughly with DI water and dry it with nitrogen gas.

△ **CRITICAL:** Use upright light microscope (e.g., Nikon LV100) to visually verify that the resist has been completely removed in the generated patterns.

- b. Mask fabrication of HB elements (Figure 2E).
 - i. Proceed with mask fabrication of HB elements following steps i–iii.

Master mold fabrication and storage

⌚ Timing: 4 h

△ **CRITICAL:** Conduct the master mold fabrication in a fully equipped clean room to prevent contamination. Handle all chemicals in a fume hood.

4. Proceed with transferring microfluidic channels and HB elements from the masks into a 100 mm silicon wafer (master mold) with sequential double coating of high-viscosity negative resist SU8 2050 using a standard photolithography protocol (Wolfe et al., 2010; Khoo et al., 2018): first for the microfluidic channels and the second for the HB elements.
 - a. Transfer of microfluidic channels.
 - i. Place the wafer in a glass ware and ultrasonically clean it in DI water and in ethanol for 3 min to remove large particles from its surface. Rinse it thoroughly with ethanol, and dry it with nitrogen gas.
 - ii. Bake the wafer at 200°C for 10 min on a hot plate to remove water residues from its surface.
 - iii. Place the wafer on a vacuum chuck of a spin coater (e.g., Laurell WS650) and dispense 4 mL of SU8 2050 at its center.
 - iv. Spin coat the wafer at 500 rpm for 5 s, then at 2200 rpm for 30 s to spread the resist across the entire wafer.
 - v. Soft bake the wafer on a hot plate at 65°C for 3 min, and then at 95°C for 8 min to remove residual solvent from the resist layer.

△ **CRITICAL:** Cool the wafer slowly to 23°C. This is to minimize the possibility of thermally cracking the SU8 film.

- vi. Place the wafer on a vacuum chuck of spin coater (e.g., Laurell WS650) and spin it at 1000 rpm. While wafer is spinning, spray acetone on its bottom edge to eliminate resist beading on the wafer edge.
 - vii. Align the mask and wafer on the stage of mask aligner (e.g., Karl Suss MA8). Expose the wafer to UV light through the mask at 120 mJ/cm² energy for 20 s to yield the desired microfluidic channel designs on the wafer.
 - viii. Post bake the wafer on a hot plate at 65°C for 2 min, and then at 95°C for 7 min to increase the durability of the mold. Cool it slowly to 23°C.
 - ix. Immerse the wafer in SU8 developer solution for 5 min, followed by spin development at 200 rpm for 1 min.
 - x. Rinse the wafer thoroughly with DI water and isopropanol, and dry it with nitrogen gas.

△ **CRITICAL:** After master development, use an upright stereo microscope (e.g., Nikon LV100) to visually verify the developed channels and HB elements within them; and a profilometer (e.g., Bruker Dektak XT-A) to measure the dimensions of the developed structures.

- b. Transfer of HB elements.
 - i. Repeat steps v–x for transferring HB elements from masks to silicon wafer.

△ **CRITICAL:** It is important that the mask of HB elements is well-aligned with the microfluidic channels on the wafer in step v. A misalignment will result in improper transfer of HB elements into microfluidic channels.

5. Storage of master mold.

Firmly tape all the edges of the master wafer on a 150 mm Petri dish bottom (Figure 2F). Seal the Petri dish with parafilm, and store the wafer in a vacuum desiccator.

▮ **Pause point:** The master wafer can be kept in the desiccator for extended periods and reused multiple times.

PDMS replica molding

⌚ Timing: 4 h

6. Use a plastic cup to mix the PDMS base elastomer (Sylgard 184) and the curing agent at a 10:1 (w/w) ratio. Stir the mixture homogeneously for 5–10 min using a plastic stirrer.

⚠ **CRITICAL:** If PDMS is not mixed thoroughly or the amount of catalyst is not adequate, PDMS will not cure completely and will leave residuals on the master wafer.

7. Slowly pour the mixture over the master wafer till 5–10 mm thickness to form a PDMS replica mold (Figure 2G).
8. Place the wafer in a vacuum desiccator for 1 h to remove the air bubbles generated within the PDMS during the mixing step.
9. Cure the PDMS mold in a 60°C dry oven for at least 2 h. When fully cured, the PDMS mixture will solidify.
10. After the PDMS is cured, remove the mold from the oven, allow it to cool down to 23°C, and carefully cut it along the edges of master wafer. When cutting, keep the pressure minimum to prevent wafer from breaking.
11. Slowly peel off the PDMS. Leave the master wafer inside Petri dish with surrounding PDMS and store it safely for future PDMS molding.
12. Cut the PDMS mold into 75 mm × 25 mm chips using a razor blade and remove all uneven edges (Figure 2H). When cutting, ensure that microfluidic channels of each chips are well-centered with respect to chip edges.

⚠ **CRITICAL:** Observe the channels and HB elements of PDMS chips under upright light microscope (e.g., Nikon LV100). Discard the ones with distorted features.

13. Punch the inlet and outlet ports of the PDMS chips straight down using a 2 mm biopsy punch. Ensure that the punched holes are completely free of PDMS (Figure 2H).
14. Place the PDMS chips in a glass dish with channel surfaces facing up. Ultrasonically clean them in DI water and in ethanol for 3 min to remove large particles from the channel surfaces. Cover the glass dish with aluminum foil when cleaning.
15. Hold the PDMS chips using tweezers, wash them thoroughly with ethanol, and dry them with nitrogen gas.
16. Place the cleaned PDMS chips in a Petri dish with channels surfacing upward. Seal the Petri dish with parafilm, and store the PDMS chips in a vacuum desiccator.

Optional: Cover the top surfaces of PDMS chips with clear plastic tape (e.g., scotch tape). Although this is very common approach, it is not recommended prior to follow up chemical modifications due to residues that tape may leave on chip surfaces.

Glass cleaning and silanization

⌚ Timing: 3 h

⚠ **CRITICAL:** Handle all chemicals in a fume hood.

⏸ **Pause point:** All glass cleaning and silanization/annealing steps must be carried in clean glass crystallizing dishes covered with aluminum foil. Glass slides (75 mm × 25 mm) must be handled with tweezers.

17. Leave a small cross mark on far end corner of one of the surfaces on glass slides using a diamond point marker.

△ CRITICAL: Ensure that marked surfaces always face up during cleaning and silanization/annealing steps.

18. Place the glass slides in a glass dish and ultrasonically clean them in DI water and ethanol for 3 min to remove large particles from their surfaces. Dry the slides with nitrogen gas.
19. Clean the glass slides under UV-ozone for 30 min to remove the overlayer (~100 nm) of environmental carbon contamination (C-C) from their surfaces, and treat the native surface oxide layer of the glass to generate a high density of hydroxyl (-OH) groups.

Note: The hydroxyl groups are anchoring points for covalently bound silane molecules (i.e., the first layer of the multilayer tether molecule structure), and thus their presence is important for the stability of follow-up surface modifications.

Note: A wide variety of cleaning methods for native glass oxide surfaces (e.g., SiO₂) are described in the literature, where the common procedures include UV-ozone, wet chemical, and plasma cleaning. These cleaning procedures aim to reduce the overlayer of environmental carbon contamination from the glass surfaces so that the native oxide layer of ~2 nm thickness is exposed to air.

Note: The initial formation of hydroxyl groups on the glass surface then occurs through hydrolysis of Si-O groups by water molecules in the air (Grundner and Jacob, 1986). The hydroxyl groups further act as a preferential adsorption site for water molecules, thus breaking up neighboring Si-O groups (Grundner and Jacob, 1986). As a result, high density of hydroxyl groups on the glass surface is generated.

▣ Pause point: A small but persistent amount of carbon will always be present on the glass surfaces as an indication of environmental contamination. Exposure to air makes it impossible to prevent such trace contamination.

20. Soak the glass slides in an anhydrous toluene solution containing 0.5% wt. APTES molecules for 45 min at 23°C.

Note: The APTES molecule has three ethoxy (-OCH₂CH₃) groups attached to a silicon atom (Figure 3A). Through hydrolysis, one of these ethoxy groups is cleaved to allow the Si on the APTES to form a covalent bond with a hydroxyl (-OH) group on the glass surface to produce a stable -Si-O-Si- linkage.

Note: The reactive amine (-NH₂) group at the opposite end of the APTES can interact with an appropriate linker molecule to facilitate the attachment of an antibody to a flexible tether molecule. Upon deposition, therefore, it is desirable to have these amine groups directed away from the surface (see troubleshooting 3).

△ CRITICAL: Silanization step must be conducted under fume hood. Toluene rapidly produces toxic concentrations at 23°C, therefore direct inhalation must be avoided.

21. After silanization, immerse the glass slides in ethanol and sonicate for 3 min to remove loosely bound molecules.
22. Wash thoroughly the glass slides with ethanol and dry them with nitrogen gas.
23. Place the glass slides in a 100°C dry oven for 30 min to allow APTES annealing.

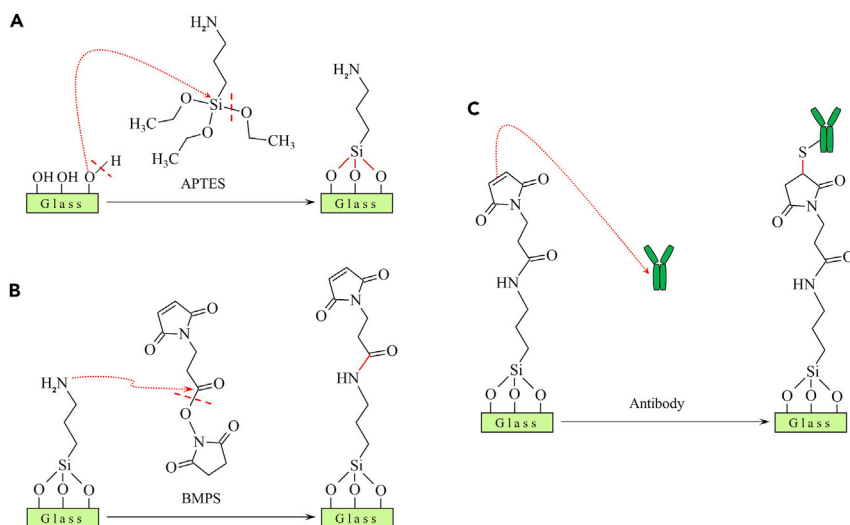


Figure 3. Elementary steps associated with the linker chemistry of immobilizing antibodies exclusively on glass surface of AFM-Chip

(A) Coupling reaction of APTES with an amine terminus on cleaned glass surface.

(B) Coupling reaction of BMPS on amine-terminated glass surface.

(C) Coupling reaction of an antibody on maleimide-activated glass surface. The solid red lines in (A–C) represent newly formed bonds during these reactions.

Note: Annealing silanized surfaces improves the silanization because of heat promoting the conversion of protonated groups ($-\text{NH}_3^+$) to reactive amino groups, and heat liberating the loosely bound protonated groups through evaporation.

⚠ **CRITICAL:** Longer annealing times and annealing at higher temperatures could cause reduction of the amine groups through oxidation.

24. Place the silanized glass slides in a Petri dish with channels surfacing upward. Seal the Petri dish with parafilm, and store the glass slides in a vacuum desiccator.

⏸ **Pause point:** The glass slides can be kept in desiccator for up to 2 weeks without jeopardizing the stability of silane molecules.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Sterile Dulbecco's phosphate buffered saline (PBS, pH 7.2–7.6)	Sigma-Aldrich	59331C
Deionized (DI) water	Milli-Q	ZR0Q008WW
3-aminopropyltriethoxysilane (APTES)	Sigma-Aldrich	440140
N-β-Maleimidopropylsuccinimide ester (BMPS)	Fisher Scientific	22298
Anhydrous toluene	Sigma-Aldrich	244511
Absolute ethanol	Merck	100983
Absolute isopropanol	Merck	109634
Acetone	Merck	100014
SU8 2050 photoresist	MicroChem	n/a
SU8 developer	MicroChem	n/a
AZ351B developer	AZ Electronics	n/a

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium etchant	MicroChem	n/a
Sylgard 184 silicone elastomer kit	Sigma-Aldrich	1317318
5/6-carboxyfluorescein succinimidyl ester (NHS-fluorescein)	Fisher Scientific	46409
Dimethylformamide (DMF)	Sigma-Aldrich	227056
Bovine serum albumin (BSA)	Fisher Scientific	BP9700100
Antibodies		
Anti-human monoclonal 9C4 antibodies (EpCAM)	Santa Cruz	sc-21792
Anti-human monoclonal YPSMA-1 antibodies (PSA)	Santa Cruz	sc-7638
Anti-human monoclonal C-19 antibodies (PSMA)	Santa Cruz	sc-59674
Anti-human monoclonal PSMA antibodies (Alexa Fluor 488)	Santa Cruz	sc-516606
Anti-human monoclonal CD45 antibodies (Alexa Fluor 647)	Santa Cruz	sc-1178
Anti-human monoclonal CK5 antibodies (Alexa 647)	Abcam	ab28106
4',6-diamidino-2-phenylindole (DAPI) dye	Abcam	ab228549
Other		
Pre-cleaned glass slides (75 mm × 25 mm × 1 mm)	J. Melvin Freed	301M
Sterile 1.5 mL Eppendorf tubes	Sigma-Aldrich	T9661
Sterile 150 mm Petri dishes	Falcon	351007
Sterile 200 μ L pipette tips	Fisher Scientific	10619331
Sterile 50 mL centrifuge tubes	Fisher Scientific	14-432-22
20–200 μ L adjustable-volume pipette	Fisher Scientific	14-388-100
1 mL plastic transfer pipettes	Fisher Scientific	13-711-9AM
Spatula	Fisher Scientific	13-820-056
Weighing paper	Fisher Scientific	09-898-12A
Microbalance	METTLER TOLEDO	ME54
Vortex mixer	Fisher Scientific	02-215-365
Ultrasonic cleaner	Branson	CPX3800H
Benchtop ultraviolet (UV)-ozone cleaner	Novascan	PSD-UV
Dry oven	DAIHAN	DH20030300P2132
Vacuum desiccator	Sigma-Aldrich	Z119016
Timer	Sunnex	360594
Parafilm	VWR	HS234526C
Aluminum foil	VWR	89079-069
Razor blade	VWR	55411-050
2 mm biopsy punch	VWR	21909-132
Wafer-handling tweezers	Fisher Scientific	17-467-334
Disposable plastic cups and stirrers	Fisher Scientific	S04201
Disposable 10 mL plastic syringes	VWR	76124-650
Disposable 38.1 mm needles	VWR	BD305185
Polyethylene tubes with 0.8 mm inner diameters	Ismatec	MF0001
Polyethylene tubes with 4.00 mm inner diameters	Ismatec	SC0462
1 mL glass syringes with tubing connectors	CETONI GmbH	M1101000109
180 mL glass crystallizing dishes	Sigma-Aldrich	CLS314070
10 mL glass vials	Fisher Scientific	02-912-376
Fume hood	Labconco	2246700
Class II laminar air flow biosafety cabinet	NuAire	NU-437S
Liquid nitrogen tank	Arpege	40
Low pressure syringe pump (neMESYS)	CETONI GmbH	290n
Digital rocker	Thermo Scientific	15218844
Ethylenediaminetetraacetic acid (EDTA) tubes	VWR	95057-231
Blood transportation box	Hach	49-12300
Gel refrigerant packs	VWR	10029-374
AFM colloidal tips	NanoAndMore	CP-PNPL-SiO-C-5
Silicon wafers	Wafer World	5142
Hardmask blanks	CS Technology	160708-11

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hot plate	Thermo Scientific	88880002
Spin coater (WS650)	Laurell	N/A
Direct laser writer (DWL 66+)	Heidelberg	N/A
Mask aligner (MA8)	Karl Suss	N/A
Profilometer (Dektek XT-A)	Bruker	N/A
Upright light microscope (LV100)	Nikon	N/A
Inverted fluorescence microscope (Ti)	Nikon	N/A
Atomic force microscope (LS-AFM)	AFM Workshop	N/A
Clear plastic tape	Fisher Scientific	NC0013582
Stirring bottle (neMIX)	CETONI	A3927000008
AFM liquid sample heater	AFM Workshop	N/A
Software and Algorithms		
CAD software (AutoCAD)	This paper	autodesk.com
Mask design software (Layout Editor)	This paper	layouteditor.com
Image processing software (GIMP)	This paper	gimp.org
Image processing software (ImageJ)	This paper	imagej.nih.gov
Statistical computing and graphics software (MATLAB)	This paper	mathworks.com
G'MIC color transfer filter	This paper	gmic.eu

STEP-BY-STEP METHOD DETAILS

Reversible AFM-Chip assembly

⌚ Timing: 30 min

This step will produce reversible AFM-Chip assembly to be used for further antibody immobilization on glass surface.

1. Bring the cleaned PDMS chips (channel sides facing down) and APTES-silanized glass slides (crossed sides facing up) into contact at 23°C and under atmospheric pressure to enable reversible AFM-Chip assembly.

Note: The reversible AFM-Chip assembly occurs through physical bondage of silanols (Si–O[−]) on PDMS with protonated nitrogens on silanized glass slide via NH–O hydrogen bonds (Figure 1D).

⚠ **CRITICAL:** Misalignment in this step may cause the channels to leak or not to fit into glass slide properly.

2. Gently press on the PDMS chips to make sure that the surfaces of the AFM-Chip are well-bonded.

⚠ **CRITICAL:** Avoid pressure on the channel areas, which could collapse channels and damage the structure of HB elements.

3. Keep the AFM-Chips in a vacuum desiccator for 15 min to allow the trapped gas bubbles present between the surfaces to escape passively.

Antibody immobilization on glass

⌚ Timing: 2 h

This step will decorate the glass slides with antibodies at a high density while preserving their activity.

△ **CRITICAL:** Handle all chemicals in a fume hood.

▮▮ **Pause point:** After each step below, droplets of PBS need to be pipetted on the inlet and outlet ports of the AFM-Chips to prevent any liquid evaporation within the channels during incubation times. Post incubations, AFM-Chip channels need to be manually flushed with 100 μ L PBS using syringes so that unbound molecules/antibodies are removed.

4. Prepare a 3 mg/mL solution of BMPS in PBS in a glass vial. Mix the solution using a vortex mixer for 5 s.

▮▮ **Pause point:** Compared to other solvents (e.g., toluene, methanol, acetonitrile), BMPS is not completely dissolved in PBS because of its non-polar nature.

5. Fill a glass syringe with 100 μ L BMPS solution (3 mg/mL in PBS) and manually pass it through channels of AFM-Chip using polyethylene tubes. Incubate the silanized glass surfaces for 30 min at 23°C.

Note: BMPS is an intermediate linker molecule that provides an excellent route to covalently tether antibodies to silanized glass surfaces in an oriented fashion and at high concentrations through site-specific coupling (Figure 3B): succinimide group to couple with the reactive silane amino groups through an amide (N–C=O) linkage, and maleimide group to couple with the sulfhydryl groups of antibodies through a thiol (C–S) linkage.

Note: Due to its short spacer-arm length of 5.9 Å in comparison with a typical antibody size of \sim 10 nm, BMPS forms a rigid covalent bridge between the amine-terminated APTES on the surface and the sulfhydryl groups on antibodies.

6. Dilute concentrated antibody solutions (EpCAM, PSMA, and PSA) to 10 μ g/mL using PBS in Eppendorf tubes under biosafety cabinet. Mix the solution using a vortex mixer for 5 s.

△ **CRITICAL:** For multiplex capture of prostate cancer CTCs, repeat steps 6 and 7 three times, each time using separate AFM-Chips, to immobilize anti-EpCAM, anti-PSMA, and anti-PSA antibodies.

Note: Most antibodies are expected to be stable when stored in PBS (pH 7.2–7.6) for long time (weeks). Therefore, the excess antibody solutions can be stored at 4°C and reused multiple times when needed.

Note: 10 μ g/mL antibody concentration used in functionalizing the AFM-Chip is sufficient to fully cover the glass surface within its 16 channels in an oriented fashion. Here, the high density and activity of antibodies aim to increase the CTC capture yield, while minimizing the nonspecific binding of other cells and thus increasing the CTC purity rate. However, it should also be noted that proper antibody concentration is subject to characterization and biological testing.

7. Fill a glass syringe with 100 μ L antibody solution per AFM-Chip and manually pass it through their channels using polyethylene tubes. Incubate the maleimide-activated surfaces for 45 min at 23°C.

Note: During antibody immobilization, the pH of PBS is expected to be within the physiological range (7.2–7.6), thus providing stability to antibodies throughout their incubation time.

Note: The specific binding of antibodies is only on the glass substrate through maleimide interaction (Figure 3C). The top and side walls of the channels are expected to have minimal amount of antibodies physically adsorbed through non-specific interactions.

8. Prepare 1% (v/v) BSA solution in PBS in a 50 mL centrifuge tube. Transfer it to a 100 μ L syringe and manually pass it through AFM-Chip channels using polyethylene tubes. Allow for 20 min to block the non-specific binding sites on microfluidic channel surfaces.

Note: BSA is used to block the remaining nonspecific binding sites on the substrate surface, primarily available due to protonated amines formed during the APTES silanization process.

9. Store the AFM-Chips at 23°C until the cell capture experiments.

Antibody coverage characterization

⌚ **Timing:** 2 h

This step will verify that immobilized antibodies maintain a high binding efficiency with antigens involving both arms of the Y-shaped antibodies.

⚠ CRITICAL: Handle all chemicals in a fume hood.

⏸ Pause point: This section is optional. However, antibody coverage characterization is recommended to verify the chemisorption and orientation of each antibody functional group to maleimide-activated glass substrate as control.

10. Manually pass 1 mg NHS-fluorescein in 400 μ L of DMF through AFM-Chip channels using a syringe and allow incubation for 60 min in the dark at 23°C.

Note: Succinimide groups of NHS-fluorescein bind specifically with the N-terminal groups of antibodies, which are located at the ends of their Fab arms.

11. Manually flush the channels with 100 μ L PBS using a syringe to remove unbound dyes. Detach the glass substrate from the PDMS and immerse them in Petri dishes containing dye-free PBS.
12. Carry out imaging of glass slides with inverted fluorescence microscope (e.g., Nikon Ti) using a fluorescein isothiocyanate (FITC) filter cube and through a 10 \times air objective (Figure 1F).
13. Calculate the corrected total fluorescence of images following an algorithm that scales and shifts each pixel value of the input image to match its mean and standard deviation to those of target images (corrected).

Note: In processing the fluorescence images, use a G'MIC color transfer filter to transfer the color characteristics of one image to another, and analyze images in ImageJ to calculate the corrected total fluorescence of images using

$$\text{IntDen} = (\text{Area}) \times (\text{MGV}_{\text{Sample}})$$

where IntDen (Integrated Density) = a measure of "grayness" on an individual selection, Area = area of interest, MGV_{Sample} (Mean Gray Value of the sample) = the sum of the gray values of all the pixels in the selection divided by the number of pixels. With this, the corrected total fluorescence (CTF) is calculated using.

$$\text{CTF} = \text{IntDen} - (\text{Area} \times \text{MGV}_{\text{Background}}) = (\text{Area}) \times (\text{MGV}_{\text{Sample}} - \text{MGV}_{\text{Background}}).$$

Blood processing using AFM-Chip

⌚ **Timing:** 5 h

This step will demonstrate capturing clinical CTCs from prostate cancer patients' whole blood samples.

▣ **Pause point:** Obtain immunization against blood-borne diseases, including Hepatitis B and Tetanus. Obtain IRB ethical approvals from your institution and the collaborating hospital, and signed consents from volunteering human subjects after explaining the study. Collection and use of blood must conform to all relevant governmental and institutional regulations and IRB guidelines.

△ **CRITICAL:** Treat all blood as potentially infectious. In case of blood spill, ensure that the infected surface is thoroughly wiped with ethanol. After experiments, properly dispose the glass slides along with all other experimental components used in the capture/characterization of CTCs.

△ **CRITICAL:** Collect the blood samples in EDTA tubes (Figure 4A) and process them within 5–6 h after collection. Place the EDTA tubes on digital rocker at a slow mode while performing cell capture experiments.

Note: For multiplex capture of prostate cancer CTCs, repeat the below steps 14–22 at once using AFM-Chips activated with anti-EpCAM, anti-PSMA, and anti-PSA antibodies.

14. Fill a glass syringe with 100 μ L BSA solution in PBS (1%, v/v). Inject the BSA through the polyethylene tubes to prevent cell from sticking on the tubes' inner walls.
15. Transfer 1 mL of whole blood from EDTA tubes into syringes of choice (we recommend disposable plastic syringes) under biosafety cabinet using a disposable needle (Figure 4B). [Troubleshooting 1](#).
16. Connect polyethylene tubes to the syringe and inject the blood slowly until it reaches the other end of the tubes.
17. Place antibody activated AFM-Chips on a leveled stage (Figure 4C). Mount the syringes to a syringe pump (e.g., CETONI neMESYS) and connect them to the AFM-Chips using the polyethylene tubes. Connect the exit ports of the AFM-Chips to a waste container through another set of polyethylene tubes (Figure 4D). [Troubleshooting 2](#).
18. Adjust the flow rate of a syringe pump to 20 μ L/min and inject the blood completely into microfluidic channels.
19. Replace the empty blood syringes with glass syringes filled with 100 μ L of PBS. Wash channels at 20 μ L/min flow rate for 5–10 min to remove the blood with any unattached cells from the channels.
20. Replace the empty PBS syringes with glass syringes filled with 100 μ L of anti-PSMA antibodies conjugated to Alexa Fluor 488 (green) (1:10 by volume concentration in PBS). Pass the antibodies through channels at 20 μ L/min flow rate for 5–10 min and allow incubation for 20 min in the dark to immunostain captured CTCs.
21. Wash the channels with 100 μ L of PBS at 20 μ L/min flow rate for 5–10 min to remove any excess staining antibodies.
22. For purity evaluation, repeat the steps 19–21 with 100 μ L of anti-CD45 antibodies conjugated to Alexa Fluor 647 (red) (1:10 by volume concentration in PBS) to immunostain nonspecifically bound white blood cells (WBCs).

▣ **Pause point:** Different staining antibodies can be mixed for staining the different cell types at the same time/run; however, this may cause cross-staining and thus compromise specificity.

23. Follow with fluorescence imaging of stained cells as below.

CTC capture and purity evaluation

⌚ **Timing:** 1 h

This step will identify the captured CTCs and WBCs in the background.

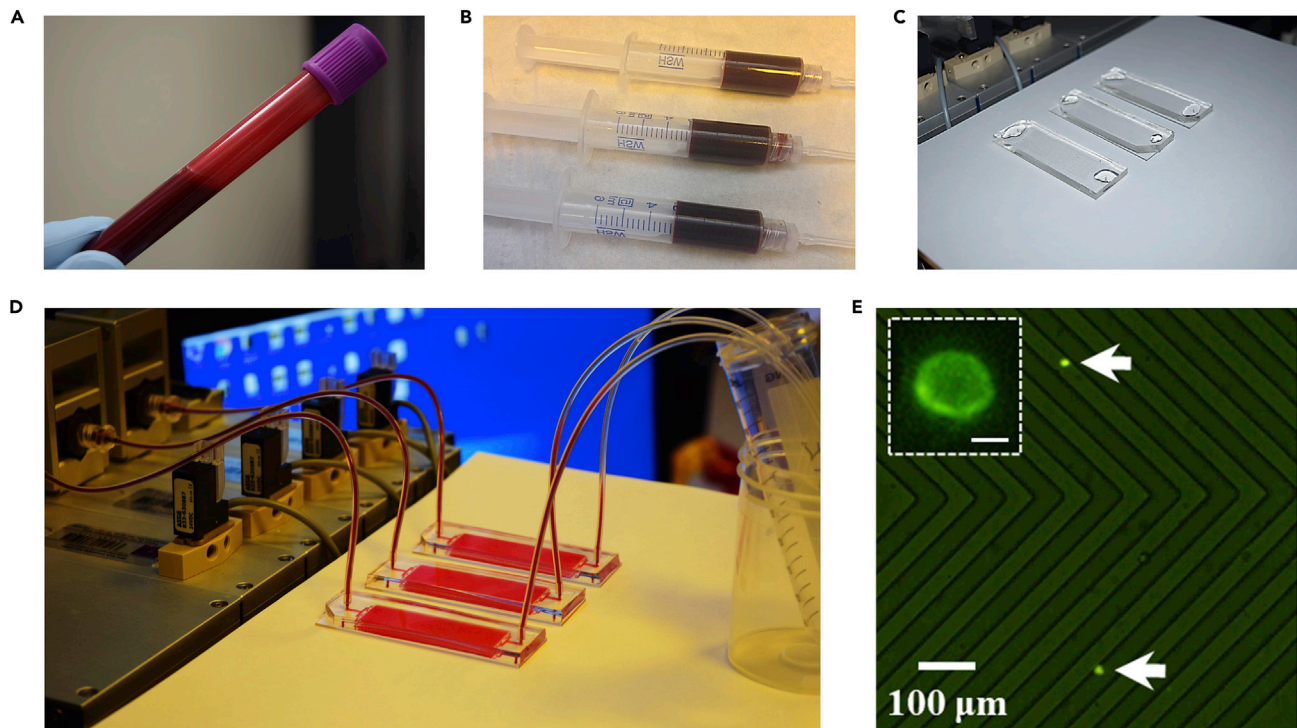


Figure 4. Methods of AFM-Chip for processing blood within 5 h of blood draw

(A and B) A whole blood sample of a prostate cancer patient collected in EDTA tube and transferred to disposable syringes, respectively. Note that trapped air bubbles in the syringes should be removed.

(C and D) Micrographs show the three antibody activated AFM-Chips along with the experimental setup for the multiplex capture of CTCs of prostate cancer from whole blood. Note that in the experimental setup, blood simultaneously passes through the devices to target the EpCAM, PSMA, and PSA markers of the CTCs.

(E) Once captured, antigen-specific immunomarkers are used for the identification of CTCs (green dots pointed by arrows). A magnified single captured intact CTC is shown in the inset (Scale bar: 8 μm). Images in (E) reused with permission from [Deliorman et al. \(2020\)](#).

24. Investigate the CTC capture by manually counting captured CTCs with inverted fluorescence microscope in the dark using FITC filter cube and through 20 \times air objectives (Figure 4E). [Troubleshooting 3](#).
25. Investigate the isolation purity by counting nonspecifically bound WBCs with inverted fluorescence microscope in the dark using tetramethylrhodamine (TRITC) filter cube and through a 20 \times air objective.
26. Calculate the purity of the device as the ratio of captured CTCs to the total number of bound cells including WBCs.

AFM force measurements

⌚ Timing: 4 h

This step will provide detailed AFM force procedures on single captured CTCs.

⏸ **Pause point:** Perform the AFM measurements using commercially available system (e.g., LS-AFM, AFM Workshop). Ideally, the AFM paired with an inverted fluorescence microscope and enclosed in vibration enclosure will suit best for identifying immunomarked CTCs and achieving a high degree of sound absorptions during the sensitive force measurements.

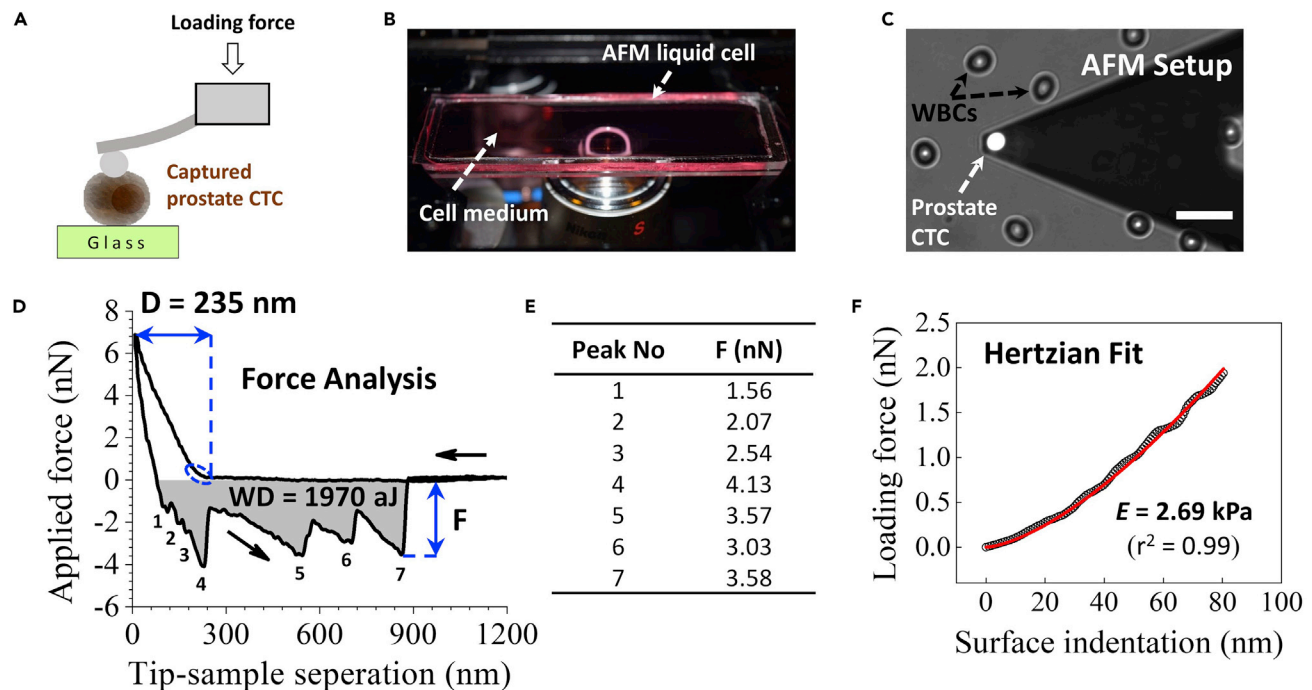


Figure 5. AFM setup and force measurements of prostate cancer CTCs captured using AFM-Chip

(A) Cartoon shows an AFM tip engaged with a single captured intact prostate CTC.
 (B) After peeling off the PDMS from glass slide, custom-made AFM liquid cell was used to confine the cell medium.
 (C) Micrograph shows an AFM tip positioned above the center of a stained prostate CTC for follow up force measurements (Scale bar: 30 μm).
 (D) Typical force curve demonstrates the interaction between the AFM tip and the CTC, where tip's approach to and retraction from the cell surface is shown by black arrow. After initial tip-cell surface contact, the cell experiences an amount of surface deformation "D" due to the applied constant loading force. During tip retraction, the cell experiences a number of adhesion forces "F" (peaks 1–7 in the retraction process) due to its interactions with the AFM tip surface. In the process, the work of detachment "WD" (shaded gray area below the zero-force line) indicates the amount of work needed to completely detach the tip from the cell surface.
 (E) The measured adhesion values are tabulated for all peaks in (D).
 (F) After the initial tip-cell surface contact, force data (black circles) were fitted (red line) to surface indentation (dashed blue ellipse in (D)) to estimate the local Young's modulus "E". Images and schematics in (A–F) reused with permission from [Deliorman et al. \(2020\)](#).

27. Slowly peel off the PDMS in the AFM-Chips and transfer the glass slides with immunostained captured CTCs onto AFM stage for their nanomechanical characterization ([Figure 5A](#)). [Troubleshooting 4](#).
28. Use a custom-made PDMS gasket as the "AFM liquid cell" to hold the cell medium in the glass slides ([Figure 5B](#)). [Troubleshooting 5](#).

Note: Cell medium choice can range from PBS to cell culture medium of choice (e.g., DMEM, RPMI) to help in maintaining the viability of the CTCs.

29. Mount a 6 μm -diameter colloidal tip with a nominal spring constant of 0.06 N/m on AFM liquid tip holder.

Pause point: Probing CTCs with AFM in liquid could be very challenging because of cell softness. To minimize the cellular damage, cantilevers with spring constants comparable to softness of the cells are preferred in the AFM measurements, which typically range from 0.01 N/m to 2 N/m. The shape and size of the tip is another factor that needs an attention. Colloidal tips with sizes comparable to the average size of mammalian cells are preferred to minimize the interference caused by the electrostatic interactions between the cantilever and the cell surface, which could result in inconsistencies among the adhesion measurements.

Note: Potential tip substitutions, such as high aspect ratio tips (e.g., tips with 10:1 height-to-width ratio) would also result in reliable force collection, both quality and accuracy wise, provided that there are no irregularities in the attached tips and tips are not contaminated.

30. Determine the cantilever spring constants from the power spectral density of the thermal noise fluctuations.
31. Define a clean (cell-free) area on the glass surface and approach the tip to engage with the surface. Calibrate the sensitivity of the photodiode by indenting the tip against the glass surface. [Troubleshooting 6](#).
32. Disengage the tip and position it above the center of a single captured intact CTC using the AFM integrated camera ([Figure 5C](#)). Approach the tip to engage with the cell surface.
33. Perform the force measurements either in single force mode or in force volume mode. In the latter, repeat the tip approach-retraction process over a $10\ \mu\text{m}^2$ cell surface area at a resolution of 8×8 pixels. [Troubleshooting 7](#).
34. Set the trigger force (i.e., applied constant force) to 6–12 nN, reflecting cell surface indentations of ~ 0.2 – $0.4\ \mu\text{m}$. Set the tip approach and retraction velocities to $4\ \mu\text{m/s}$. [Troubleshooting 8](#).

Note: In typical AFM force measurements involving cells, it is critical to define a “proper” trigger force by which the cells will be pushed against the tip following initial tip-cell surface contact. The trigger (applied) force is experimentally determined on the cell prior to collecting force data and kept constant during subsequent AFM measurements with attention to not deform the cell above levels where tip can burst or dislocate it.

35. Repeat steps 29–31 until all captured CTCs are characterized.
36. After AFM measurements, further fix the captured CTCs and WBCs (background) using 4% (v/v) para-formaldehyde for 10 min, and permeabilize them using 0.25% (v/v) Triton X-10 for 10 min. Afterwards, immunostain the cells for DAPI (blue) and CK (red) in the dark for 30 min to verify, with inverted fluorescence microscope using DAPI and TRITC filter cubes and through a $60\times$ air objective, that captured CTCs are DAPI+/CK+/EpCAM+ ([Figure 6A](#)) and WBCs are DAPI+/CD45+/EpCAM- ([Figure 6B](#)).

Optional: Measure the cytoplasmic-to-nuclear area ratio (CK+/DAPI+) of the CTCs using ImageJ.

AFM force analysis

⌚ **Timing:** 6 h

This step will guide with analysis of AFM force curves.

▮▮ Pause point: One way of analyzing AFM force curves is through extracting raw data as ASCII files using the AFM instrument software. Then, numerical computing environments, such as MATLAB, can be utilized to process the extracted raw data. For our raw AFM data, we utilized an in-house software (specifically developed for LS-AFM system of AFM Workshop) to extract the information on the elasticity, bio-adhesiveness, deformation, and work of detachment.

37. Exclude the force curves with unclear approaches and/or retraction curves from the analysis.
38. For the cell surface deformation measurements, measure the distance from cell contact point till trigger force is reached ([Figure 5D](#)).
39. For the adhesion measurements, define the maximum adhesion forces from each data point in the retraction curves and assign as minima if they were less than their 5 nearest points to the right and left. Choose 10 pN as the cut-off force value due to experimental noise during measurements ([Figures 5D and 5E](#)).

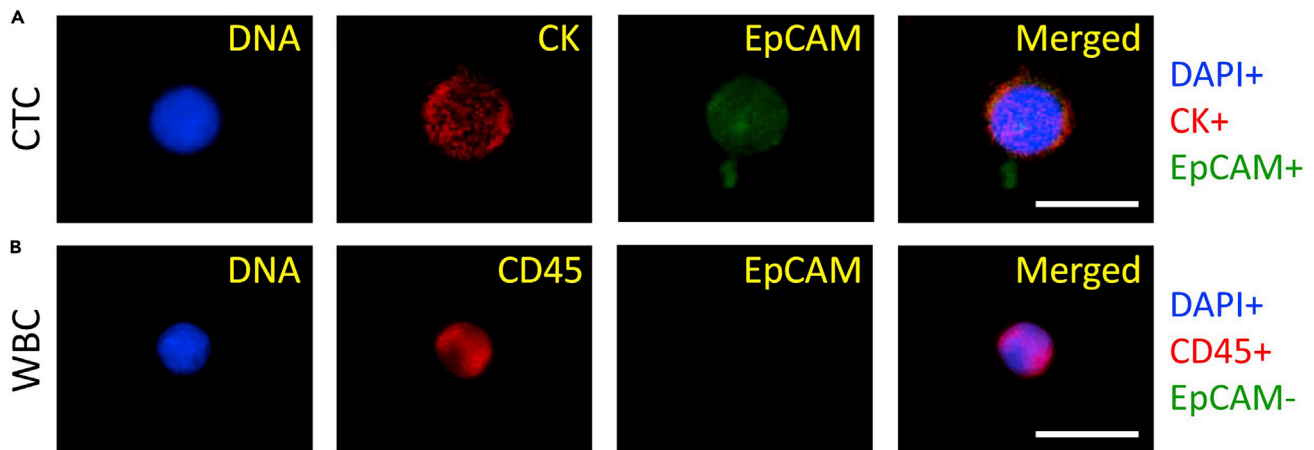


Figure 6. Identification of captured CTCs and nonspecifically bound WBCs

(A and B) Fluorescent images of (A) a captured CTC and (B) a bound WBC. Fluorescent images reveals the immunostaining of cell nuclei (blue, DAPI+ for CTC and WBC), cytoplasmic cytokeratin (red, CK+ for CTC), CD45 (red, CD45+ for WBC) and EpCAM membrane expression (green, EpCAM+ for CTC and EpCAM- for WBC). Scale bars: 20 μm . Images in (A and B) reused with permission from [Deliorman et al. \(2020\)](#).

40. For the work of detachment measurements, use a trapezoidal integral to calculate the area confined between the zero-force line and the retraction curve ([Figure 5D](#)).
41. For the elasticity measurements, use the classical Hertz model of contact mechanics. Choose 100 nm fit range when applying the model ([Figure 5F](#)).

Note: According to the model, the loading force (F_L) applied by the AFM tip required to indent a distance (δ) into the cell surface is given by

$$F_L = \frac{4}{3} \frac{E}{(1 - \nu^2)} R^{1/2} \delta^{3/2}$$

where E is the elasticity modulus of the cell, R is the radius of the AFM tip, and ν is the Poisson ratio of the cell, which was set to 0.5 assuming cell's incompressibility.

EXPECTED OUTCOMES

The multifunctional AFM-Chip described in this protocol is suitable for multiplex capture of CTCs in whole blood samples of prostate cancer patients ([Figures 4 and 6](#)). After peeling off the PDMS chip from the glass slide, immunostained captured intact CTCs residing on activated glass substrates can be efficiently transferred onto an AFM stage for high-resolution biomechanics measurements ([Figures 5A–5C](#)). Here, the antibody-antigen bonding would allow CTCs to remain firmly attached to the glass substrate during the disassembly of the device, whereas the highly hydrophobic nature of the APTES-silanized glass substrate (through C–Si alkyl substituents) would provide a protective shield for the captured CTCs. After the AFM measurements, the collected force data can be quantified for Young's modulus (elasticity), deformation, adhesion forces, and work of detachment ([Figures 5D–5F](#)) following procedures outlined in this protocol. It is expected that the captured EpCAM+ cells are CTCs when they are positive for nuclear (DAPI+) and cytoplasmic cytokeratin (CK+) staining ([Figure 6A](#)). White blood cells (WBCs) in the background, on the other hand, are not expected to show a positive response to immunostaining EpCAM (EpCAM-), but positive for the nucleus (DAPI+) and transmembrane marker CD45 (CD45+) ([Figure 6B](#)). It is also expected that the analysis of biomechanical properties of CTCs would show differences depending on the metastatic potential of analyzed CTCs.

LIMITATIONS

Below we describe in detail the limitations of the AFM-Chip, both inherent and related to its functioning. Some of the AFM-Chip limitations can be overcome, for example by employing 3D printing for cost- and time-effective master mold fabrication, fabricating larger channels for increased throughput, and exposing blood to longer capture path by sequentially connecting three AFM-Chips. Recently, on the other hand, we proposed more effective way of multiplex capturing CTCs from whole blood samples of cancer patients by introducing the HB-MFP (Glia et al., 2021). The HB-MFP, which is acronym for herringbone-microfluidic probe, is a fully 3D printed "open microfluidic" device, that allows for multiplex capture of CTCs in a single run, high throughput by processing larger blood volumes for higher CTC capture, complete cell recovery by minimizing cell losses and damage, and seamless external access to captured cells.

Limitation 1

For high throughput, the multiplex capture of CTCs in this method is only feasible by simultaneously processing blood in separate AFM-Chips. As such, the large array of channels (typically 16, each 900 μm wide) in the AFM-Chip are designed to permit high throughput under optimized conditions, with up to 1.2 mL of whole blood processing per 1 h. Patterning individual channels with antibody cocktails is doable but not practical due to multiple reasons: First, it will reduce the effective capture area for each antibody set within the channels. Second, additional PDMS replica mold with an array of multiple capillaries will be needed to covalently immobilize different antibodies on repeated patterns of stripes. Third, enlarging the width of microfluidic channels to accommodate patterns of different antibodies may result on channels' collapse due to the soft structural nature of PDMS. Therefore, in case of prostate cancer CTCs, three AFM Chips are needed to capture CTCs while targeting three different cell surface receptors during the same experiment: EpCAM, PSMA, and PSA as shown in [Figure 1B](#).

Limitation 2

There are inherent limitations of AFM-Chip which mainly stem from its fabrication method that is very time consuming, expensive, and labor intensive due to photo- and soft-lithography procedures; the narrow channel dimensions that limit the throughput, make the channels susceptible to clogging, jeopardize cell integrity and viability, and prevent capturing clusters of CTCs due to high shear stresses; the dependency on distinct CTC surface antigens that limits the enrichment of CTCs due to highly heterogeneous subpopulations; and the extra steps needed to detach bound CTCs from glass for further genetic analysis, which limits the cell recovery due to their fragile nature.

Limitation 3

In AFM-Chip, the transition from cell capture to cell characterization using AFM can be susceptible to cell loss due to manually peeling off the PDMS.

TROUBLESHOOTING

Problem 1

Cells are sedimented in the syringe or connecting tubes during processing the blood (major step 15).

Potential solution

Position the syringe vertically to prevent cells from sedimenting inside of it. Use a stirring bottle between the syringe and the AFM-Chip to prevent cells from sedimenting inside the connecting tubes.

Problem 2

Air bubbles are trapped in the process of filling the syringe with blood (major step 17).

Potential solution

Slowly discharge the air bubbles with the syringe end pointing upward.

Problem 3

No CTC capture is observed in AFM-Chip ([before you begin](#) step 20, major step 24).

Potential solution

Follow with antibody coverage characterization using NHS-fluorescein to verify that antibodies are densely present on glass in oriented fashion. Then check the capture efficiency of the AFM Chip using model human cancer cell lines (e.g., prostate PC3, breast MCF-7) immunostained and spiked in blood.

Also note that, in the deposition of an antibody-binding layer, the initial step of forming amine-terminated surfaces on the glass substrate is the most critical step. Thus, the ultimate requirement is to have a silanized glass surface which is stable in an aqueous environment for a long period of time and has as many reactive amine groups as possible. In the presence of water, however, these amine groups have a tendency to interact with surface hydroxyl groups and neighboring silane molecules via covalent, hydrogen, van der Waals and electrostatic interactions. This causes a polymerization of the APTES both in the vertical and the horizontal directions. In the process, the amine moieties tend to incorporate protons from the environment, forming protonated groups. As a result, the polymerization of APTES molecules is problematic during the preparation of silane films, since the availability and reactivity of the surface amine groups are crucial to the construction of the bioactive channels presented in this protocol. To prevent such polymerization, strictness is needed when following the protocol of depositing APTES molecules onto glass surfaces.

Problem 4

The transition from CTC capture to single-cell characterization in the AFM-Chip is susceptible to cell loss (major step 27).

Potential solution

This is associated with the shearing involved in manually peeling off the PDMS layer. This can be minimized by slowly peeling off the PDMS starting from its inlet port toward its outlet port.

Problem 5

The glass surface is drying after peeling off the PDMS chip (major step 28).

Potential solution

During the peeling off PDMS, the hydrophobic nature of the APTES-silanized glass substrate will confine certain amount of liquid within the channel regions. However, this liquid will be prone to evaporation if kept exposed to air even for as short as few minutes (>3 min). Therefore, it is critical that after removing PDMS, the AFM liquid cell is placed immediately and the glass slide is filled with cell medium afterward to prevent its surface from drying.

Problem 6

The tip is not engaging with the glass and/or cell surface (major step 31).

Potential solution

Ensure that the tip is attached to the cantilever for proper engagement with the glass and/or cell surface. This could be verified by electron microscope images of the tips prior to experiments.

Problem 7

Attached CTCs are changing shape during the AFM measurements as observed with inverted microscope camera (major step 33).

Potential solution

Conducting an AFM experiment will be a time consuming process. Therefore, if CTCs are kept for considerable time (e.g., 30-60 min) out of their physiological environment they may burst. To avoid this

uncertainty, the AFM measurements need to be carried out using a custom-made AFM setup (i.e., in a modified cell culture medium that maintains the cell medium pH at 7.2 along by using a liquid sample heater that maintains the cell medium temperature at 37°C (Xie et al., 2018; Glia et al., 2020).

Problem 8

The generated force curves are with unclear approaches and/or retraction curves (major step 34).

Potential solution

Reason for the failure of obtaining force curves with clear approach and retraction curves will be most likely due to either tip contamination or irregularities in the silica beads. Therefore, for reliable data collection in the former the tip needs to be replaced with a new one. While in the latter, the tip may need to be carefully investigated (e.g., using electron microscope) for the shape of the beads prior to measurements.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mohammad A. Qasaimeh (maq4@nyu.edu).

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

The published article includes all datasets and code generated and analyzed for this study.

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AUTHOR CONTRIBUTIONS

M.D. and M.A.Q. conceived the idea and M.D. executed the study. M.D. and A.G. prepared the figures and wrote the manuscript. M.A.Q. critically revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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