Supplementary Information for:

Single Cell mRNA Cytometry via Sequence-Specific Nanoparticle Clustering and Trapping

Mahmoud Labib[†], Reza M. Mohamadi[†], Mahla Poudineh[†], Sharif U. Ahmed[†], Ivaylo Ivanov[†], Ching-lung Huang[†], Maral Moosavi[†], Edward H. Sargent[§], Shana O. Kelley[†,‡,‖,*]

[†] Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ontario M5S 3M2, Canada, [‡]Institute for Biomedical and Biomaterials Engineering, University of Toronto, Toronto, Ontario M5S 3G4, Canada, [§]Department of Electrical & Computer Engineering, University of Toronto, Toronto, Ontario M5S 1A8, Canada, [‖]Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

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Chip fabrication. Chips were fabricated using Poly(dimethoxysilane) (PDMS, Dow Chemical, US) soft-lithography. Masters were fabricated on silicon substrates and patterned in SU-8 3050 (Microchem, US). PDMS replicas were poured on masters and baked at 67 C for 45 min. After peeling the replicas, holes were pierced to connect the tubing. PDMS replicas were attached to no. 1 glass cover slips using a 30s plasma treatment and left to bond overnight. This step allows for enhancing the bonding and making it irreversible by oxidizing both the replica and the cover in plasma discharge. Afterward, the silicon tubing was attached to the inlet and outlet of the device. Prior to use, devices were conditioned with 1% Pluronic F68 (Sigma-Aldrich, US) in phosphate-buffered saline (PBS) for 1 h, to reduce the nonspecific adsorption. Each device was sandwiched between two arrays of N52 Nd FeB magnets (K&J Magnetics, US, 1.5 mm by 8 mm) with alternating polarity. A syringe pump (Chemyx, US) was used for the duration of the cell capture process.

Cell culture. VCaP cells (ATCC CRL-2876) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002). PC3 cells were cultured in F-12K Medium (ATCC 30-2004). LNCaP cells were cultured in RPMI-1640 medium (ATCC 30-2001). All media were supplemented with 10% FBS and 1% penicillin-streptomycin and cells were cultured at 37 °C and 5% CO_2 in T75 flasks. Cells were harvested when they reached more than 70-80% confluence. Cell detachment from the culture dishes was performed using 1 mL of 0.25% (w/v) Trypsin-0.53 mM EDTA solution for 3 min at 37 ºC. The cells were then filtered using a 40 µm BD falcon cell strainer (Becton, Dickinson and Company, US).

Preparation of the magnetic nanoparticles labeled with capture probes. Briefly, 100 µL of 20 µM of the antisense oligonucleotide solution in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, US) containing 1 mM dithiothreitol (DTT, Sigma-Aldrich, US), were heated for 5 min at 60 ºC for deaggregation. Afterward, the solution was transferred to a microtitre plate and incubated with 1.5 µL of 10 mg mL^{-1} streptavidin-coated magnetic nanoparticles (100nm, Chemicell, US) for 30 min at room temp. Subsequently, the magnetic nanoparticles-labeled capture probes (MNPs-CPs) were pelleted using a magnetic-ring stand (Thermofisher Scientific, US) and washed three times with DPBS/DTT solution.

Cellular mRNA analysis. Prostate cancer cell lines (200 cells in 100 µL DPBS) were fixed with 100 µL of 8% paraformaldehyde (PFA, Sigma-Aldrich, US) solution in DPBS/DTT for 15 min at 37 ºC. After centrifugation, the cells were incubated with 100 µL of 0.3% Triton X-100 (TX-100, Sigma-Aldrich, US) in DPBS/DTT for 10 min at room temp. Then, 100 µL of MNPs-labeled CP1 and MNPs-labeled CP2 in DPBS/DTT (prepared in the previous step) were added and the suspension was gently shaken for 3 h at room temp. A control experiment was carried out in which the cells were gently shaken with the MNPs-labeled nonspecific binary probe (NSP) for 3 h at room temp, subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were gently shaken with MNPs-anti-EpCAM for 3 h at room temp. Finally, the cells were loaded into the microfluidic device at a flow rate of 600 µL h^{-1}.

Cell staining and imaging. Captured cells were counted using fluorescence microscopy. Prior to staining, captured cells were fixed inside the chip using 100 µL of 4% PFA in DPBS/DTT followed by 100 µL of 0.2% TX-100 in DPBS/DTT for permeabilization. Captured cells were

immunostained with a mixture of 3% allophycocyanin-labeled anti-cytokeratin antibody (APC-CK, GTX80205, Genetex, US), 3% APC-labeled anti-EpCAM antibody (APC-EpCAM, Miltenyi Biotec Inc., US), and 3% alexafluor 488-labeled anti-CD45 antibody (AF488-CD45, MHCD4520, Invitrogen, US) in 100 µL PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich, US) and 0.1% Tween-20 (Sigma-Aldrich, US). Immunostaining was carried out for 60 min at a flow rate of 100 µL h^{-1}. After washing with 0.1% Tween-20 in PBS, the cells were stained with 1 drop of 4',6-diamidino-2-phenylindole (DAPI Prolong Gold nuclear stain, Invitrogen, US) in 100 µL PBS for 10 min at a flow rate of 600 µL h^{-1}. After staining, the cells were washed with 0.1% Tween-20 in PBS, and stored at 4 ºC. Finally, chips were scanned using a Nikon Ti-E Eclipse microscope with an automated stage controller and a CMOS Camera (Andor Neo). The blue channel was used for DAPI staining, with a typical exposure time of 10–20 ms. The green channel was used for the AF488-CD45 staining, with a typical exposure time of 40–60 ms. The red channel was used for the APC-CK and APC-EpCAM staining, with a typical exposure time of 200–300 ms. The exposure time was set individually for each chip and kept constant in the course of scanning. The imaging was qualitative in nature and hence the variation of exposure time between chips did not affect the results. Cells were counted by overlaying the bright field, red, blue, and green fluorescent images.

Calculation of capture fraction and expression index. The mRNA capture fraction is calculated from formula **1**:

$$\text{mRNA capture fraction} = (N_{CP} - N_{NSP}) / N_{Ab} \qquad (\mathbf{1})$$

N_{CP} denotes the number of cancer cells captured using the capture probe, N_{NSP} represents the number of cells captured by the nonspecific probe, and N_{Ab} is the total number of cells in the sample captured by anti-EpCAM. The percentage of cells captured in each zone is multiplied by the mRNA capture fraction to demonstrate the distribution of cell populations bearing different mRNA expression levels and generate a normal distribution fit from which the average capture zone ($Zone_{Ave}$) is determined.

A unique mRNA expression index (EI_{mRNA}) can then be calculated from formula **2**:

$$EI_{mRNA} = (\text{mRNA capture fraction}) / Zone_{Ave} * 10 \qquad (\mathbf{2})$$

Dynamic light scattering (DLS). DLS experiments were carried out using Zeta sizer Nano series (Malvern Instruments, UK), to prove the formation of MNP clusters upon hybridization between MNPs-labeled binary probe and target mRNA. Prior to analysis, MNPs-labeled CP1-TMPRSS2/ERG (15 µg) and MNPs-labeled TMPRSS2/ERG-CP2 (15 µg) were incubated with 1 pM synthetic TMPRSS2/ERG in DPBS/DTT for 3 h at room temp. A control experiment was carried in which the target was incubated with individual capture probes (CP1 or CP2).

Transmission electron microscopy (TEM). PC3 cells (10 000 cells in 100 µL) were fixed with 100 µL of 8% PFA in DPBS/DTT for 15 min at 37 ºC. After centrifugation, the cells were incubated with 100 µL of 0.3% TX-100 in DPBS/DTT for 10 min at room temp. Afterward, the cells were gently shaken with 100 µL of DPBS/DTT containing either MNPs-labeled CP1-survivin, MNPs-labeled survivin-CP2, or a mixture of MNPs-labeled CP1 and CP2-survivin in DPBS/DTT for 3 h

at room temp. The cells were centrifuged for 5 min at 8000 r.p.m. and the supernatant was discarded. The cells were fixed with a solution of 4% PFA and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temp. After washing three times with the same buffer, the cells were post-stained with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temp. After washing two times with the same buffer, the cells were dehydrated with 25% ethanol (2 changes in 15 min), 50% ethanol (2 changes in 20 min), 70% ethanol (2 changes in 30 min), 90% ethanol (2 changes in 45 min), and 100% ethanol (3 changes in 60 min). The cells were gently shaken with a mixture of EPON resin and 100% ethanol (1:2) for 2 h, then (2:1) for 3 h, and finally with 100% EPON resin overnight at room temp. The next day, the resin was removed and the cells were gently shaken with fresh EPON resin for 2 h at room temp then the resin was allowed to polymerize in plastic dishes for 48 h at 40 ºC. The samples were sliced into 70−90 µm sections with an ultracut microtome then loaded onto carbon-coated copper grids and left to dry at room temp. TEM (Hitachi H-7000) equipped with XR60 CCD camera was used to examine the morphology of the cells and internalized MNPs.

Transfection of PC3 cells. Before transfection, PC3 cells (3×10^4/well) were grown at a 65% to 75% density overnight in a 6-well plate. After 24 h, the cells were washed with DPBS twice and transfected with 50 µM LY2181308[1] in 2 mL serum-free F-12K medium containing 40 µL lipofectin reagent (Thermofisher Scientific, US) for 6 h at 37 ºC. A control experiment was carried out in which the cells were incubated with 2 mL serum-free F-12K medium containing 40 µL lipofectin reagent. The medium was removed after 6 h and the cells were incubated with F-12K medium containing 10% FBS for 48 h at 37 ºC.

Flow cytometric analysis survivin expression in PC3 cells. Flow cytometry was used to analyze the level of survivin protein in PC3 cells before and after silencing the *survivin* gene with LY2181308 siRNA. Briefly, PC3 cells (200,000 cells) were incubated with the blocking buffer (1% BSA in PBS) for 30 min at room temp. Afterward, the cells were fixed 4% PFA and permeabilized with 0.2% TX-100. The cells were incubated with 10 µL of 100 µg mL^{-1} of DL555-labeled anti-survivin antibody (Novus biologicals, US) for 1 h at room temp. Mouse IgG (Abcam, US) was used as a negative control at the assay conditions. Subsequently, samples were injected into FACSCanto flow cytometer (BD Biosciences, US) and measurements were plotted as histograms. Absorbance values were normalized to unstained control. A total of 10,000 cells were analyzed per cell line.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cultured cells by Triazol reagent (Invitrogen, US) and used for RT-qPCR as described previously.[2] The isolated RNA was used for cDNA synthesis using the first strand DNA synthesis kit (Invitrogen, US), which contained random hexamer primers and Superscript III reverse transcriptase, according to the manufacturer's protocol. A comparative Ct experiment was performed on ViiA™ 7 real-time PCR (Life Technologies, US). The following TaqMan probes were used: TMP/ERG (TMP-ERG_CDU62RE), BIRC5 (Hs04194392_s1), AR-FL (AR-FL_CDRWEKJ), AR-V7 (Hs04260217_m1), and TBP (Hs99999910_m1) as a housekeeping gene control. The assay was carried out in triplicates using 10 ng cDNA for each sample in a 96-well plate. The 10µL reaction mix consisted of 5 µL 2X TaqMan gene expression master mix (Life Technologies,

US), 0.5 µL of 20X assay, 3.5 µL water and 1 µL of 10 ng μL^{-1} cDNA. Cycling conditions for the qPCR were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

Cellular determination of survivin mRNA in blood spiked with PC3 cells. PC3 cells (5, 10, 25, 50, 100, 500, 1000) were spiked into 1 mL of blood, each-at-a-time. The mononuclear cells were isolated using Ficoll method and were subsequently suspended in 250 µL of 2% FBS solution in PBS. Anti-CD15 antibody was modified with MNPs by incubating 100 µL of 40 µg μL^{-1} biotin-tagged anti-CD15 antibody (Abcam, US) in PBS with 6 µL of 10 mg mL^{-1} streptavidin-coated MNPs for 30 min at room temp. The modified MNPs were pelleted using a magnetic-ring stand and washed three times with PBS. The beads were gently shaken with the cell suspension for 30 min at room temp. The captured leukocytes were pelleted using a magnetic separation rack (Thermofisher Scientific, US) and the supernatant was collected for further analysis. The supernatant was incubated with 250 µL of 8% PFA in DPBS/DTT for 15 min at 37 ºC. After centrifugation, the cells were incubated with 100 µL of 0.3% TX-100 in DPBS/DTT for 10 min at room temp. The cells were gently shaken with 100 µL of DPBS/DTT containing MNPs-labeled CP1-survivin and MNPs-labeled survivin-CP2 in DPBS/DTT for 3 h at room temp. A control experiment was carried out in which the cells were gently shaken with the MNPs-labeled NSP for 3 h at room temp, subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were gently shaken with MNPs-anti-EpCAM for 3 h at room temp. The cells were loaded into the 6-zone microfluidic device at a flow rate of 600 µL h^{-1} and subsequently stained with APC-labeled anti-EpCAM, APC-labeled anti-CK antibodies, AF488-labeled anti-CD45 antibody, and DAPI.

Fluorescence in situ hybridization-flow cytometry (FISH-flow) analysis of survivin mRNA in PC3 cells. PC3 cells (25, 50, 100, 500, 1000, 5000, 10000) were spiked into either 1 mL of buffer or 1 mL of blood, each-at-a-time. The mononuclear cells were isolated using Ficoll method and were subsequently suspended in 250 µL of 2% FBS solution in PBS. Anti-CD15 antibody was modified with MNPs by incubating 100 µL of 40 µg μL^{-1} biotin-tagged anti-CD15 antibody in PBS with 6 µL of 10 mg mL^{-1} streptavidin-coated MNPs for 30 min at room temp. The modified MNPs were pelleted using a magnetic-ring stand and washed three times with PBS. The beads were gently shaken with the cell suspension for 30 min at room temp. The captured leukocytes were pelleted using a magnetic separation rack and the supernatant was collected for further analysis. The supernatant was incubated with 250 µL of 8% PFA in DPBS/DTT for 15 min at 37 ºC. After centrifugation, the cells were incubated with 0.2% Tween 20 in DPBS/DTT for 30 min at room temp. The cells were then incubated with 100 µL of 10 µM of AF488-Survivin-CP1, CP2-Survivin-AF488, AF488-Survivin-CD1, and AF488-Survivin-CD2 overnight at room temp. The cells were washed with 0.2 mg mL^{-1} RNase-free BSA in nuclease-free water. After centrifugation, the cells were suspended into DPBS/DTT. Subsequently, samples were injected into FACSCanto flow cytometer and measurements were plotted as histograms. Absorbance values were normalized to unstained control.

FISH analysis of survivin mRNA in PC3 cells subsequent to single-cell mRNA cytometry. PC3 (100 cells in 100 µL DPBS) were fixed with 100 µL of 8% paraformaldehyde (PFA, Sigma-Aldrich, US) solution in DPBS/DTT for 15 min at 37 ºC. After centrifugation, the cells were incubated with the cells were incubated with 0.2% Tween 20 in DPBS/DTT for 30 min at room

temp. Then, 100 µL of AF488-Survivin-CP1-MNPs and MNPs-CP2-Survivin-AF488 in DPBS/DTT were added and the suspension was gently shaken for 3 h at room temp. The cells were loaded into the microfluidic device at a flow rate of 600 µL h^{-1}. Prior to staining, captured cells were fixed inside the chip using 100 µL of 4% PFA in DPBS/DTT. Captured cells were immunostained with a mixture of 3% allophycocyanin-labeled anti-cytokeratin antibody and 3% APC-labeled anti-EpCAM antibody in 100 µL DPBS/DTT containing 1% RNase-free BSA and 0.1% Tween-20 (Sigma-Aldrich, US). Immunostaining was carried out for 60 min at a flow rate of 100 µL h^{-1}. The cells were imaged with a confocal microscope. The cell fluorescence was measured for cells captured in each zone within the microfluidic device using Image J. The corrected total cell fluorescence (CTCF) was calculated from the following formula:

CTCF = Integrated density – (area of selected cell * mean fluorescence of background readings)

Cellular determination of TMPRSS2/ERG and AR-V7 mRNAs in patient blood samples. Metastatic castration-resistant prostate cancer (CRPC) patients were recruited from the Princess Margaret Hospital according to the University of Toronto Research Ethics Board approval protocol. All patients were enrolled subsequent to informed consent. Sixteen milliliters of peripheral blood samples were collected from CRPC patients in CellSearch tubes containing EDTA. All the samples were analyzed within 24 h after collection. A set of patient samples (n=4) were analyzed to determine whether the approach would be suitable for the analysis of CTCs mRNA. Sixteen milliliters of blood were split into four tubes to be further utilized for the determination of N_{CP}, N_{NSP}, and N_{Ab} using the microfluidic approach and for RT-qPCR analysis. The mononuclear cells were isolated using Ficoll method and were subsequently suspended in 250 µL of 2% FBS solution in PBS. Anti-CD15 MNPs were gently shaken with the cell suspension for 30 min at room temp. The captured leukocytes were pelleted using a magnetic separation rack and the supernatant was collected for further analysis. In the first tube, the supernatant was incubated with 250 µL of 8% PFA in DPBS/DTT for 15 min at 37 ºC. After centrifugation, 100 µL of 0.3% TX-100 in DPBS/DTT were added and the suspension was incubated for 10 min at room temp. For analysis of TMPRSS2/ERG mRNA, the cells were gently shaken with 100 µL of DPBS/DTT containing MNPs-labeled CP1-TMP/ERG and MNPs-labeled TMP/ERG-CP2 for 3 h at room temp. For analysis of AR-V7 mRNA, the cells were gently shaken with 100 µL of DPBS/DTT containing MNPs-labeled CP1-AR-V7 and MNPs-labeled AR-V7-CP2 for 3 h at room temp. In the second tube, a control experiment was carried out in which the cells were gently shaken with the MNPs-labeled nonspecific binary probe (NSP) for 3 h at room temp, subsequent to cell fixation and permeabilization. In the third tube, another control experiment was carried out in which the cells were gently shaken with MNPs-anti-EpCAM (without prior fixation or permeabilization) for 3 h at room temp. The cells were loaded into the 6-zone microfluidic device at a flow rate of 600 µL h^{-1} and subsequently stained with APC-labeled anti-EpCAM, APC-labeled anti-CK antibodies, AF488-labeled anti-CD45 antibody, and DAPI. In the fourth tube, the cells were gently shaken with MNPs-anti-EpCAM (without fixation or permeabilization) for 1 h at room temp. The cells were loaded into a cell-extraction microfluidic device[3] at a flow rate of 8 mL h^{-1}. After washing, the Tygon tubing connecting the zones were cut and the cells were gently pipetted out the device and stored at −80 ºC before RT-qPCR analysis.

Table 1. Sequences of capture probes (Integrated DNA Technologies, US)

Nucleic acid	Sequence
CP1-TMPRSS2/ERG	5' GAT AAG GCT TCC TGC CGC GC 3'–Biotin–(TEG)
TMPRSS2/ERG-CP2	Biotin–(TEG) –5' CAA CGA CTG GTC CTC ACT CA 3'
TMPRSS2/ERG (synthetic)	5' GCG CGG CAG GAA GCC TTA TCA GTT GTG AGT GAG GAC CAG TCG TTG 3'
Ctrl-TMPRSS2/ERG	5' GTT GCT GAC CAG GAG TGA GTG TTG ACT ATT CCG AAG GAC GGC GCG 3'
CP1-survivin	5' CAG TTC TTG AAT GTA GAG AT 3'–Biotin–(TEG)
Survivin-CP2	Biotin–(TEG) –5' GCA GGC GCA GCC CTC CAA GA 3'
AF488-Survivin-CP1	AF488–5' CAG TTC TTG AAT GTA GAG AT 3'–Biotin–(TEG)
CP2-Survivin-AF488	Biotin–(TEG) –5' GCA GGC GCA GCC CTC CAA GA 3'–AF488
AF488-Survivin-CD1	5' GGT CCT TGA GAA AGG GCT GC 3'–AF488
AF488-Survivin-CD2	5' AAC GGG TCC CGC GAT TCA AA 3'–AF488
CP1-AR-FL	5' TGC TTT CAT GCA CAG GAA TT 3'–Biotin–(TEG)
AR-FL-CP2	Biotin–(TEG) –5' CTG GAA TAA TGC TGA AGA GT 3'
CP1-AR-V7	5' CTG ATG AAG AGA AGC ATG TG 3'–Biotin–(TEG)
AR-V7-CP2	Biotin–(TEG) –5' TGG GAG AAG AAT GAG AGG CT 3'
LY2181308	5' TGT GCT ATT CTG TGA ATT 3'
TMPRSS2/ERG Forward primer	FAM–5' CAG GAG GCG GAG GCG GA 3'–MGB NFQ
TMPRSS2/ERG Reverse primer	FAM–5' GGC GTT GTA GCT GGG GGT GAG 3'–MGB NFQ
Survivin Forward primer	FAM–5' CTT TCT CAA GGA CCA CCG CAT CT 3'–MGB NFQ
Survivin Reverse primer	FAM–5' GCA CTT TCT CCG CAG TTT CCT C 3'–MGB NFQ
AR-FL Forward primer	FAM–5' GGA ATT CCT GTG CAT GAA AGC 3'–MGB NFQ
AR-FL Reverse primer	FAM–5' CGA TCG AGT TCC TTG ATG TAG TTC 3'–MGB NFQ
AR-V7 Forward primer	FAM–5' CTT GTC GTC TTC GGA AAT GTT ATG 3'–MGB NFQ
AR-V7 Reverse primer	FAM–5' CTT TCT TCA GGG TCT GGT CAT T 3'–MGB NFQ
TBP Forward primer	FAM–5' CGG CTG TTT AAC TTC GCT TC 3'–MGB NFQ
TBP Reverse primer	FAM–5' CAC ACG CCA AGA AAC AGT GA 3'–MGB NFQ
GAPDH Forward primer	FAM–5' GAG TCA ACG GAT TTG GTC GT 3'–MGB NFQ
GAPDH Reverse primer	FAM–5' GAC AAG CTT CCC GTT CTC AG 3'–MGB NFQ

Table 2. T-test analysis results for the capture efficiency of PC3 cells using CP1 and CP2 shown in Figure 1E

	CP1	*CP2*
Mean	26	23.5
Variance	32	12.5
Observations	3	3
Unpaired t test		
P value		0.55
Are means significantly different? (P < 0.05)		No
One- or two-tailed P value?		Two-tailed
t, df		t=0.65, df=4
Standard error of difference		3.85

Table 3. T-test analysis results for the capture efficiency of PC3 cells using CP2 and CP1+CP2 shown in Figure 1E

	CP2	*CP1+CP2*
Mean	23.5	81.5
Variance	12.5	24.5
Observations	3	3
Unpaired t test		
P value		>0.0001
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=16.52, df=4
Standard error of difference		3.51

Table 4. T-test analysis results for the capture efficiency of PC3 cells using CP1 and CP1+CP2 shown in Figure 1E

	CP1	*CP1+CP2*
Mean	26	81.5
Variance	32	24.5
Observations	3	3
Unpaired t test		
P value		>0.0001
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=12.79, df=4
Standard error of difference		4.34

Table 5. T-test analysis results for the hydrodynamic radius after incubation of TMPRSS2/ERG and CP1 and CP2 shown in Figure 1F

	CP1	*CP2*
Mean	137.9	151.9
Variance	112.5	706.88
Observations	3	3
Unpaired t test		
P value		0.44
Are means significantly different? (P < 0.05)		No
One- or two-tailed P value?		Two-tailed
t, df		t=0.85, df=4
Standard error of difference		16.53

Table 6. T-test analysis results for the hydrodynamic radius after incubation of TMPRSS2/ERG and CP2 and CP1+CP2 shown in Figure 1F

	CP2	*CP1+CP2*
Mean	151.9	1797
Variance	706.88	54450
Observations	3	3
Unpaired t test		
P value		0.0003
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=12.13, df=4
Standard error of difference		135.59

Table 7. T-test analysis results for the hydrodynamic radius after incubation of TMPRSS2/ERG and CP1 and CP1+CP2 shown in Figure 1F

	CP1	*CP1+CP2*
Mean	137.9	1797
Variance	112.5	54450
Observations	3	3
Unpaired t test		
P value		0.0004
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=11.09, df=4
Standard error of difference		149.56

Table 8. T-test analysis results for the survivin mRNA expression indices of PC3 and LNCaP cells shown in Figure 2D

	PC3	*LNCaP*
Mean	4.37	3.48
Variance	0.35	0.41
Observations	3	3
Unpaired t test		
P value		0.11
Are means significantly different? (P < 0.05)		No
One- or two-tailed P value?		Two-tailed
t, df		t=2.08, df=4
Standard error of difference		0.43

Table 9. T-test analysis results for the survivin mRNA expression indices of LNCaP and VCaP cells shown in Figure 2D

	LNCaP	*VCaP*
Mean	3.48	0.85
Variance	0.41	0.13
Observations	3	3
Unpaired t test		
P value		0.0014
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=7.88, df=4
Standard error of difference		0.33

Table 10. T-test analysis results for the survivin mRNA expression indices of PC3 and VCaP cells shown in Figure 2D

	PC3	*VCaP*
Mean	4.37	0.85
Variance	0.35	0.13
Observations	3	3
Unpaired t test		
P value		0.0009
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=8.83, df=4
Standard error of difference		0.4

Table 11. T-test analysis results for the survivin mRNA expression indices of PC3 before and after silencing the *survivin* gene shown in Figure 2F

	PC3	*VCaP*
Mean	4.3	1.41
Variance	0.13	0.24
Observations	3	3
Unpaired t test		
P value		0.0012
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=8.24, df=4
Standard error of difference		0.35

Table 12. T-test analysis results for the AR-FL mRNA expression indices of PC3 and LNCaP cells shown in Figure 3D

	PC3	*LNCaP*
Mean	0.18	2.28
Variance	0.02	0.15
Observations	3	3
Unpaired t test		
P value		0.0009
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=8.96, df=4
Standard error of difference		0.23

Table 13. T-test analysis results for the AR-FL mRNA expression indices of LNCaP and VCaP cells shown in Figure 3D

	LNCaP	*VCaP*
Mean	2.28	5.49
Variance	0.15	2.08
Observations	3	3
Unpaired t test		
P value		0.0203
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=3.73, df=4
Standard error of difference		0.86

Table 14. T-test analysis results for the AR-FL mRNA expression indices of PC3 and VCaP cells shown in Figure 3D

	PC3	*VCaP*
Mean	0.18	5.49
Variance	0.02	2.08
Observations	3	3
Unpaired t test		
P value		0.0032
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=6.35, df=4
Standard error of difference		0.84

Table 15. T-test analysis results for the AR-V7 mRNA expression indices of PC3 and LNCaP cells shown in Figure 3D

	PC3	*LNCaP*
Mean	0.1	0.28
Variance	0.12	0.05
Observations	3	3
Unpaired t test		
P value		0.48
Are means significantly different? (P < 0.05)		No
One- or two-tailed P value?		Two-tailed
t, df		t=0.77, df=4
Standard error of difference		0.24

Table 16. T-test analysis results for the AR-V7 mRNA expression indices of LNCaP and VCaP cells shown in Figure 3D

	LNCaP	*VCaP*
Mean	0.28	2.11
Variance	0.05	0.8
Observations	3	3
Unpaired t test		
P value		0.0265
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=3.43, df=4
Standard error of difference		0.53

Table 17. T-test analysis results for the AR-V7 mRNA expression indices of PC3 and VCaP cells shown in Figure 3D

	PC3	*VCaP*
Mean	0.1	2.11
Variance	0.12	0.8
Observations	3	3
Unpaired t test		
P value		0.0219
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=3.64, df=4
Standard error of difference		0.55

Table 18. T-test analysis results for the TMPRSS2/ERG mRNA expression indices of PC3 and LNCaP cells shown in Figure 3D

	PC3	*LNCaP*
Mean	0.31	0.63
Variance	0.18	0.24
Observations	3	3
Unpaired t test		
P value		0.44
Are means significantly different? (P < 0.05)		No
One- or two-tailed P value?		Two-tailed
t, df		t=0.85, df=4
Standard error of difference		0.37

Table 19. T-test analysis results for the TMPRSS2/ERG mRNA expression indices of LNCaP and VCaP cells shown in Figure 3D

	LNCaP	*VCaP*
Mean	0.63	2.89
Variance	0.24	0.1
Observations	3	3
Unpaired t test		
P value		0.0025
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=6.73, df=4
Standard error of difference		0.34

Table 20. T-test analysis results for the TMPRSS2/ERG mRNA expression indices of PC3 and VCaP cells shown in Figure 3D

	PC3	*VCaP*
Mean	0.31	2.89
Variance	0.18	0.1
Observations	3	3
Unpaired t test		
P value		0.001
Are means significantly different? (P < 0.05)		Yes
One- or two-tailed P value?		Two-tailed
t, df		t=8.51, df=4
Standard error of difference		0.3

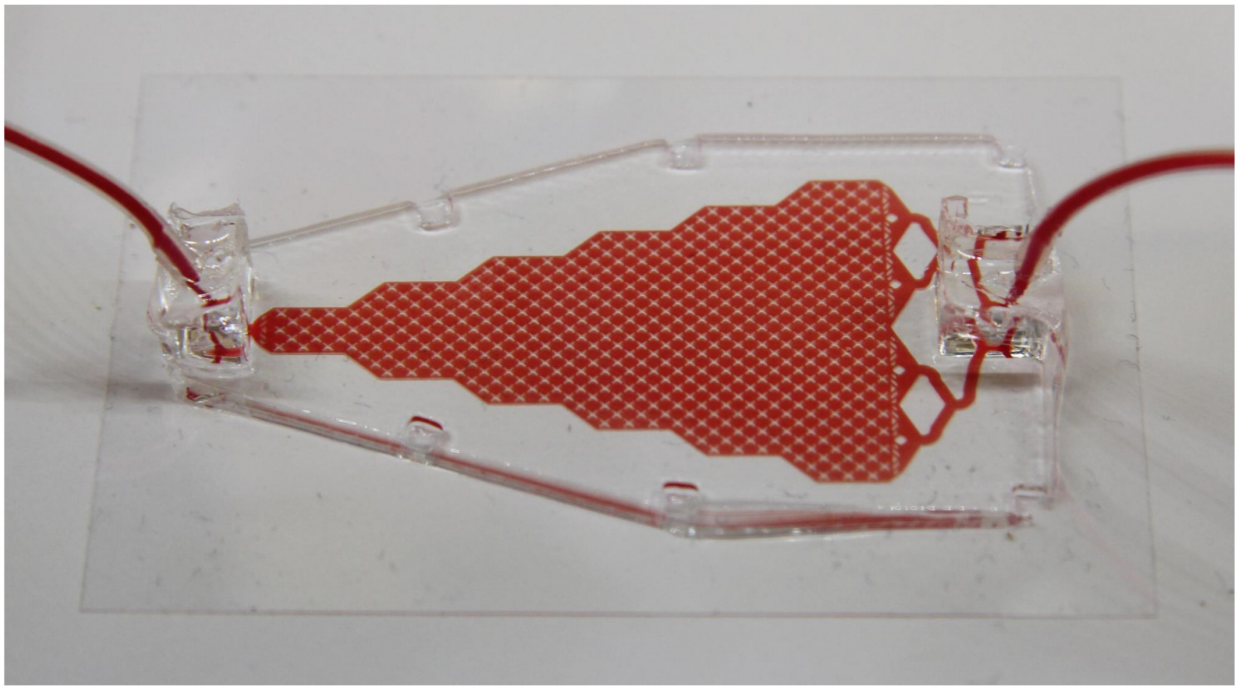

Figure 1. Device used for cellular mRNA determination. Two arrays of magnets were positioned on upper and lower sides of the device to capture cells containing trapped clusters of magnetic nanoparticles.

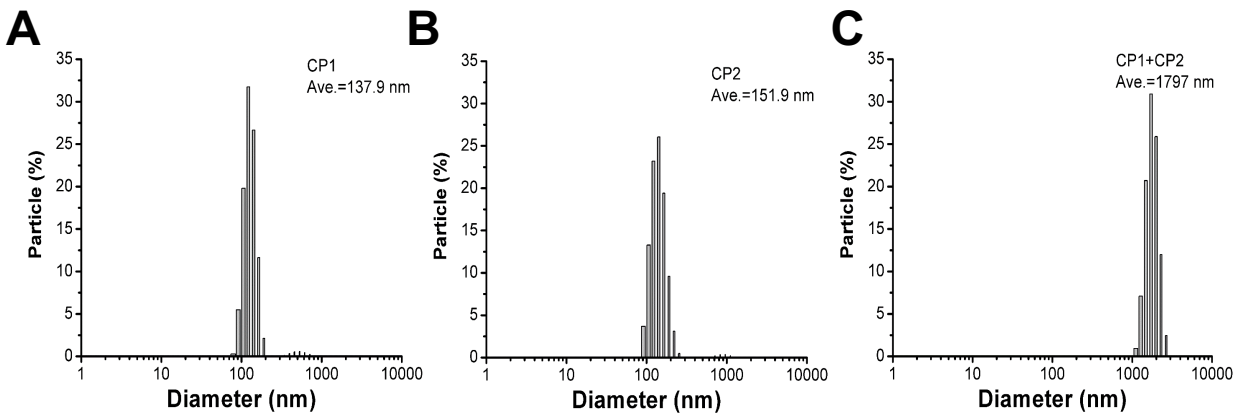

Figure 2. Dynamic light scattering data for capture probe 1 and 2 incubated with a model target sequence. A synthetic TMPRSS2/ERG was incubated with (A) CP1, (B) CP2, and (C) CP1+CP2.

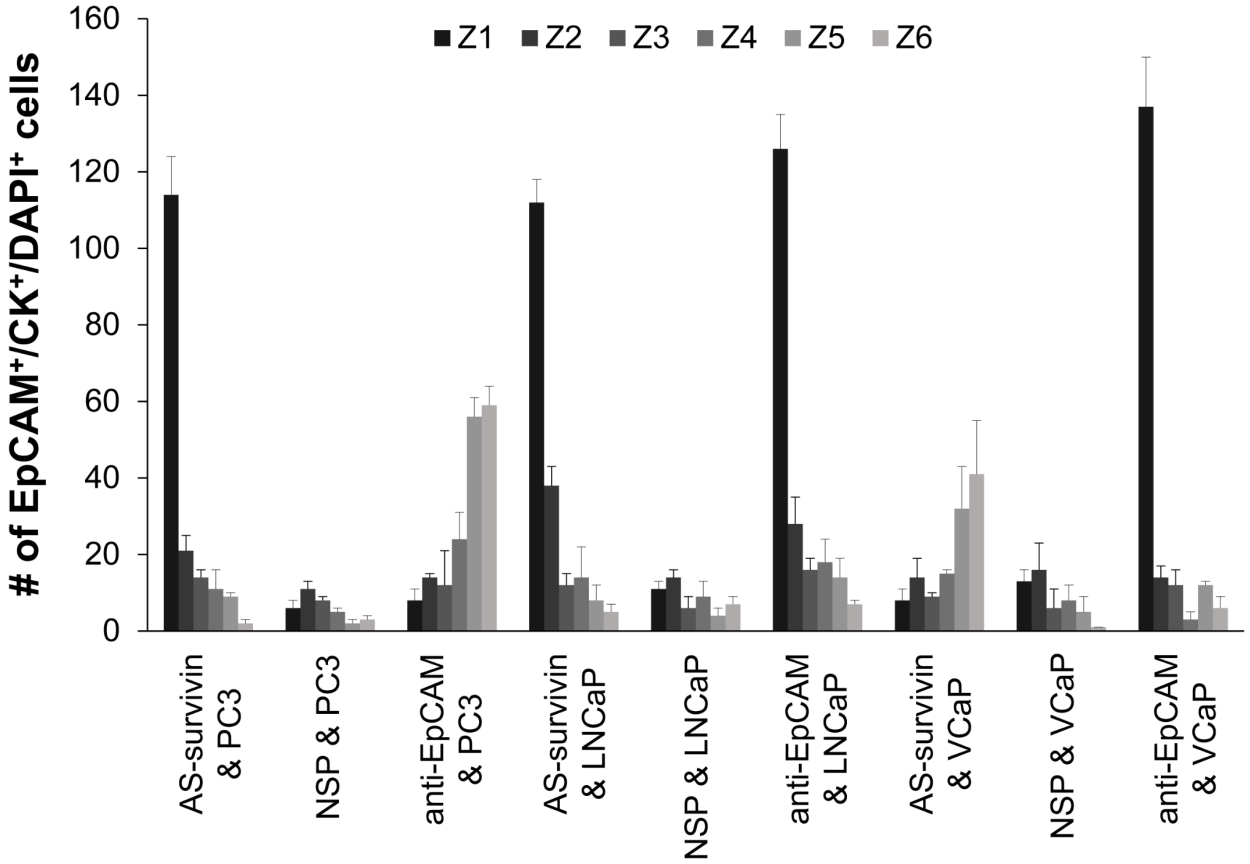

Figure 3. Raw data for single-cell mRNA cytometry targeting the survivin mRNA in cancer cell lines tested in Figure 2. Analysis of survivin mRNA in PC3, LNCaP, and VCaP. Three prostate cancer cell lines (200 cells) were fixed with 4% PFA and permeabilized with 0.3% TX-100. The cells were incubated with two MNPs-tagged DNA probes complementary to the target survivin mRNA (AS-survivin). A control experiment was carried out in which the cells were incubated with MNPs-tagged nonspecific binary probe (NSP), subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were incubated with MNPs-tagged anti-EpCAM. Two hundred cells were used in these trails. The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, stained with APC-labeled anti-CK, APC-labeled anti-EpCAM, and DAPI. Only $CK^+/EpCAM^+/DAPI^+$ cells were counted.

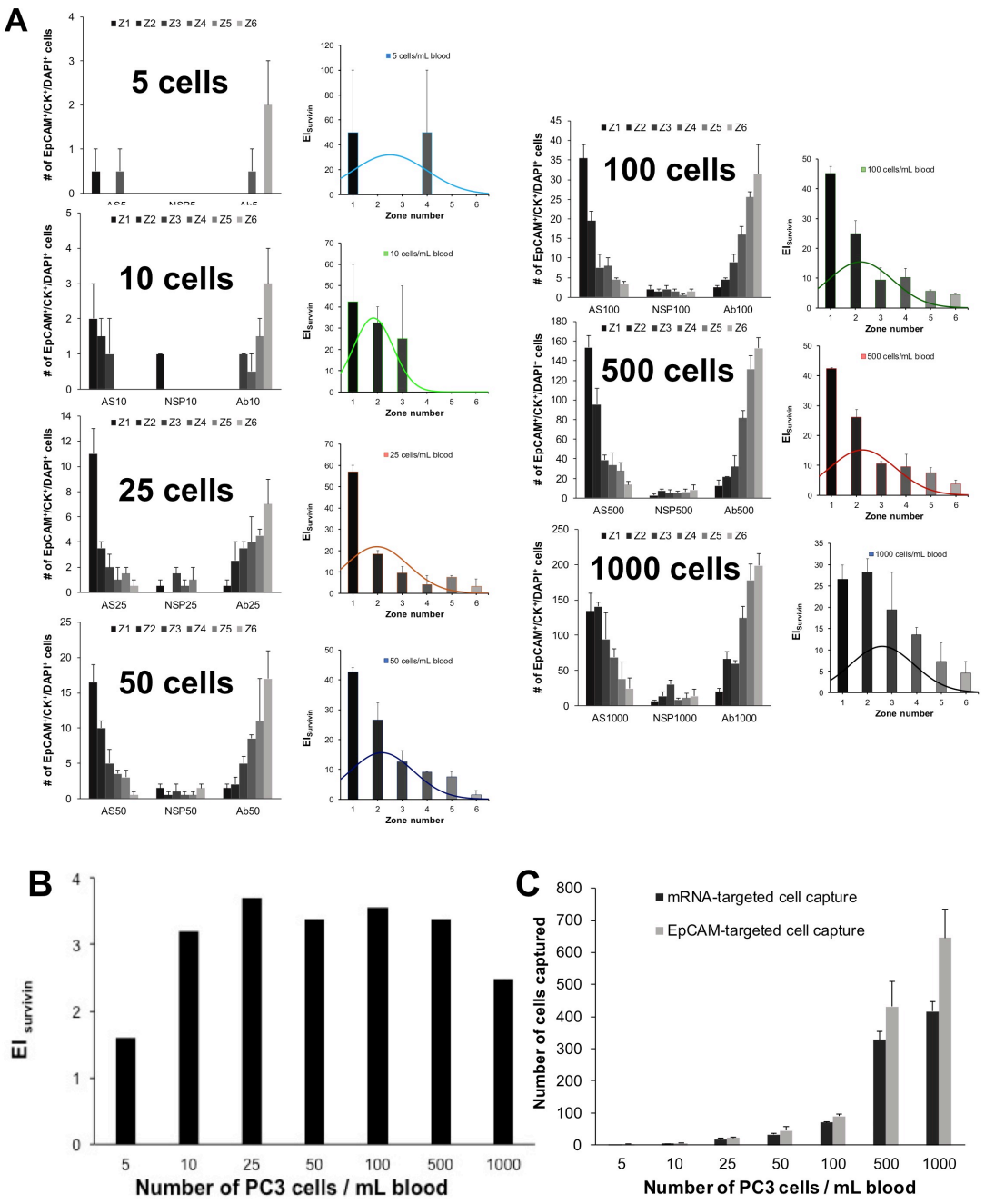

Figure 4. Sensitivity and dynamic range of the mRNA analysis approach in whole blood. A. The sensitivity of the approach was tested by spiking different number of PC3 cells into 1 mL of blood. The spiked blood samples were depleted of RBCs and WBCs prior to analysis using the Ficoll method and MNPs labeled anti-CD15, respectively. **B.** Variation of expression index values with the number of cells in a sample. EI values are consistent when the cell number is between 10 and 500. At low cell counts, not sufficient zonal information is available, and at high cell numbers the early zones of the device become saturated. **C.** mRNA analysis is performed alongside EpCAM-based capture. In all experiments, capture of cells is performed using anti-EpCAM as well as mRNA capture probes.

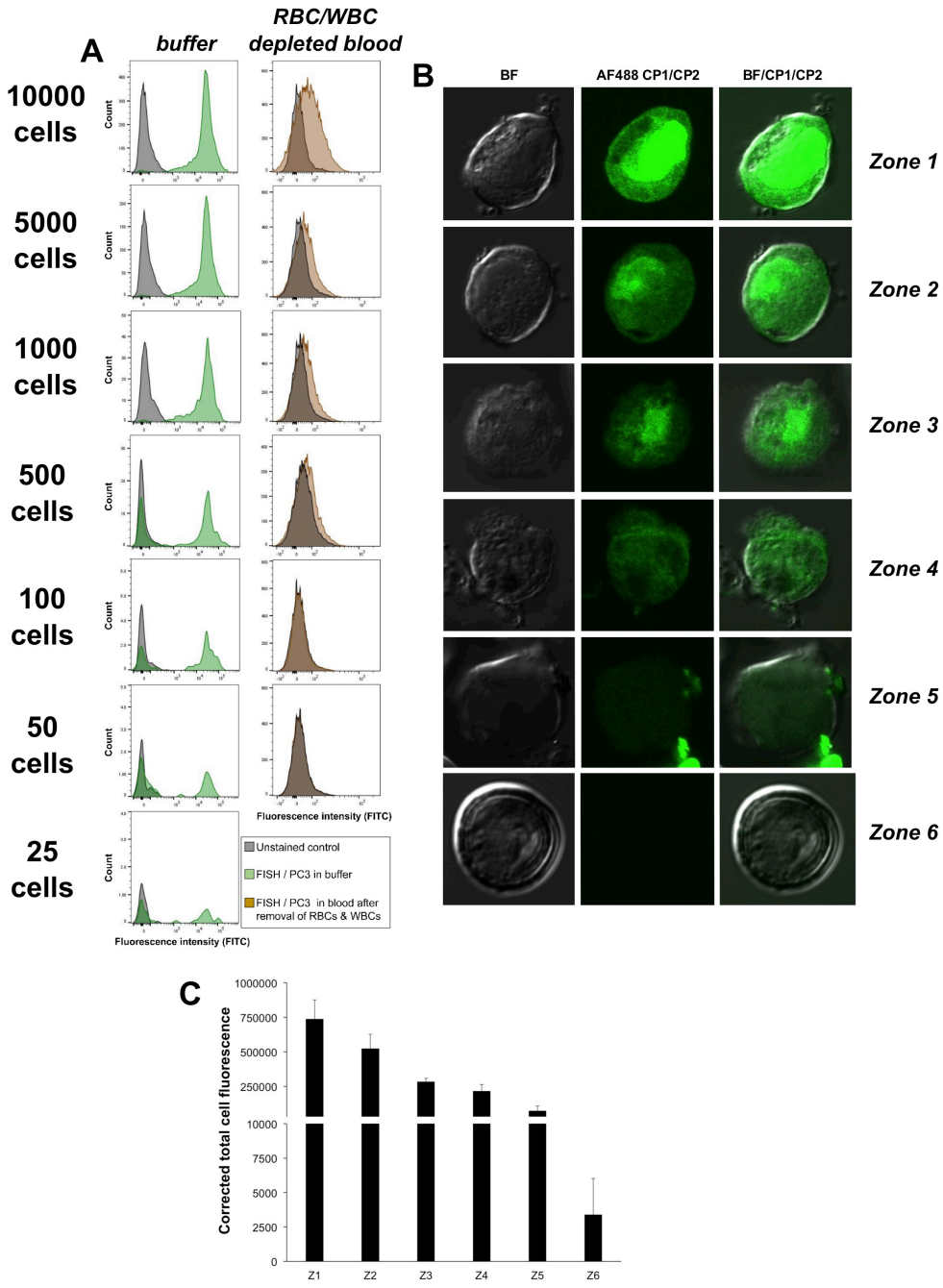

Figure 5. mRNA expression analysis using flow cytometry and RNA FISH. A. PC3 cells were spiked into buffer and blood and stained with fluorescent capture probes targeted towards the survivin mRNA. Cells were detected in the buffer solution at low cell counts, but even when RBCs/WBCs were removed from the whole blood, high cell counts were needed for cells to be detectable. **B.** RNA FISH measurements were carried out on captured PC3 cells using capture probes dually labeled with fluorescent dyes and magnetic nanoparticles. The fluorescence of the cells decreases as the capture zone increases, providing independent verification that the cells are captured as described. **C.** Quantitation of cellular fluorescence for captured cells as a function of zone number. Values are corrected for background fluorescence.

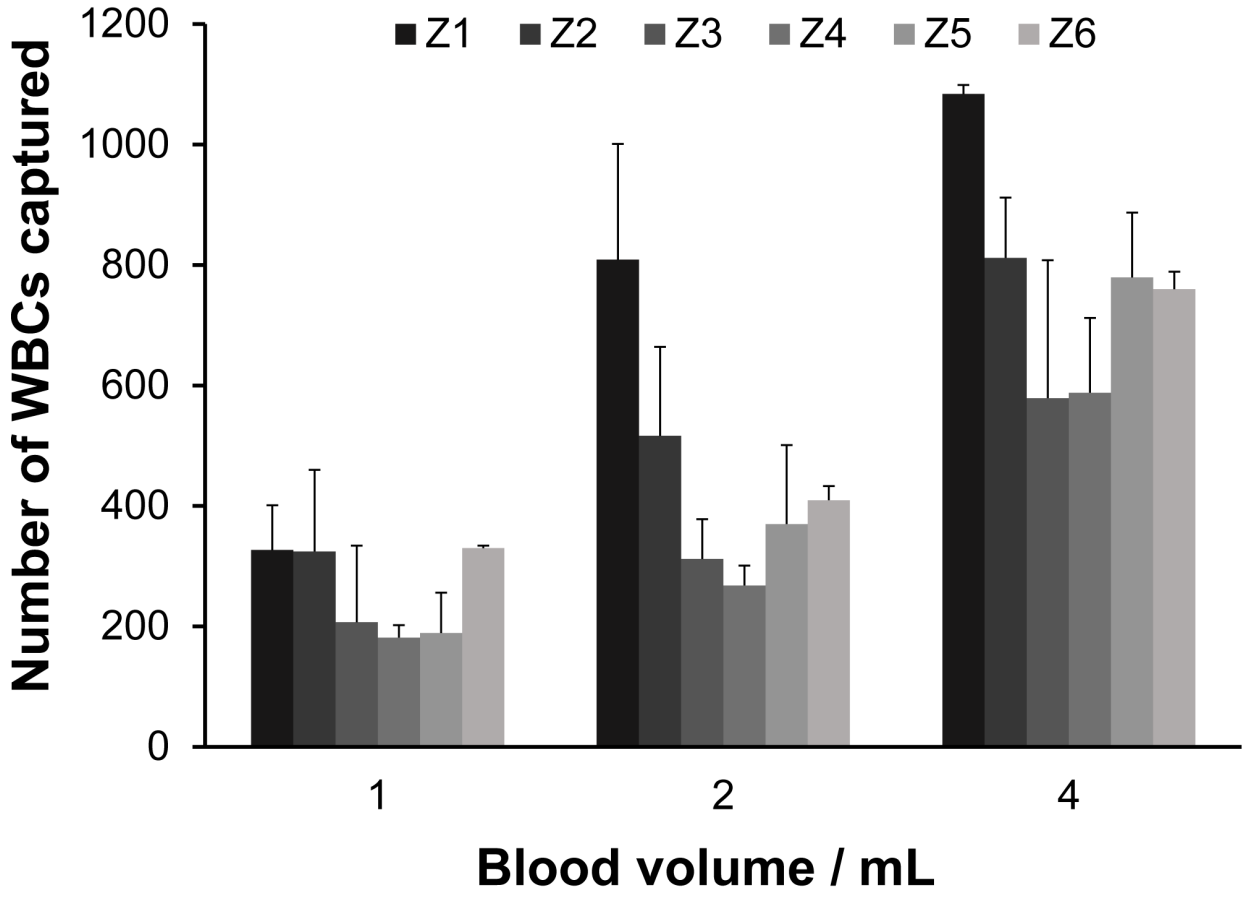

Figure 6. Purity of the cancer cells captured within the mRNA cytometry device. PC3 cells were spiked into 1, 2, and 4 mL of blood. RBCs were removed using the Ficoll method and WBCs are depleted using MNPs-tagged anti-CD15 antibody. After fixation with 4% PFA and permeabilization with 0.3% TX-100, the cells were incubated with two MNPs-tagged DNA probes complementary to the target survivin mRNA (AS-survivin). The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, stained with APC-labeled anti-CK, APC-labeled anti-EpCAM, DL555-labeled anti-survivin, and DAPI. Only $DAPI^{+}/CD45^{+}$ cells were counted to determine the number of WBCs non-specifically bound to each zone in the device.

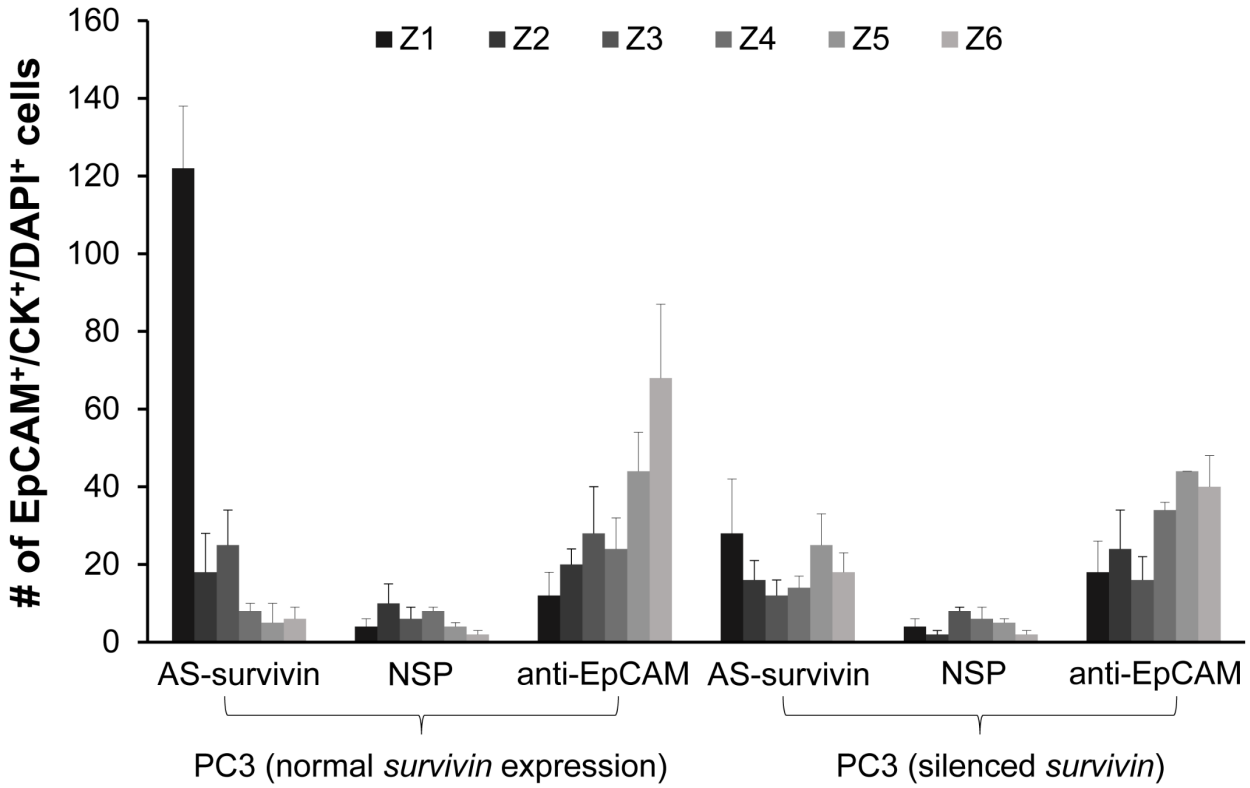

Figure 7. Raw data for mRNA cytometry targeting survivin mRNA in PC3 cells before and after silencing the *survivin* gene with LY2181308 siRNA. PC3 cells (200 cells), either with normal *survivin* expression or after *survivin* silencing, were fixed with 4% PFA and permeabilized with 0.3% TX-100. The cells were subsequently incubated with two MNPs-tagged DNA probes complementary to the target survivin mRNA (AS-survivin). A control experiment was carried in which the cells were incubated with a MNPs-tagged non-specific binary probe (NSP) subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were incubated with MNPs-tagged anti-EpCAM. The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, immunostained with APC-labeled anti-CK, APC-labeled anti-EpCAM, DL555-labeled anti-survivin, antibodies specific to two apoptosis markers including AF488-labeled anti-PARP and AF488-labeled anti-caspase 3, and DAPI. Only $CK^+/EpCAM^+/DAPI^+$ cells were counted. The number of cells captured with EpCAM with and without survivin knockdown were 196±23 and 176±6, respectively. The number of cells captured with and without survivin knockdown using mRNA capture were 184±9 and 113±23, respectively.

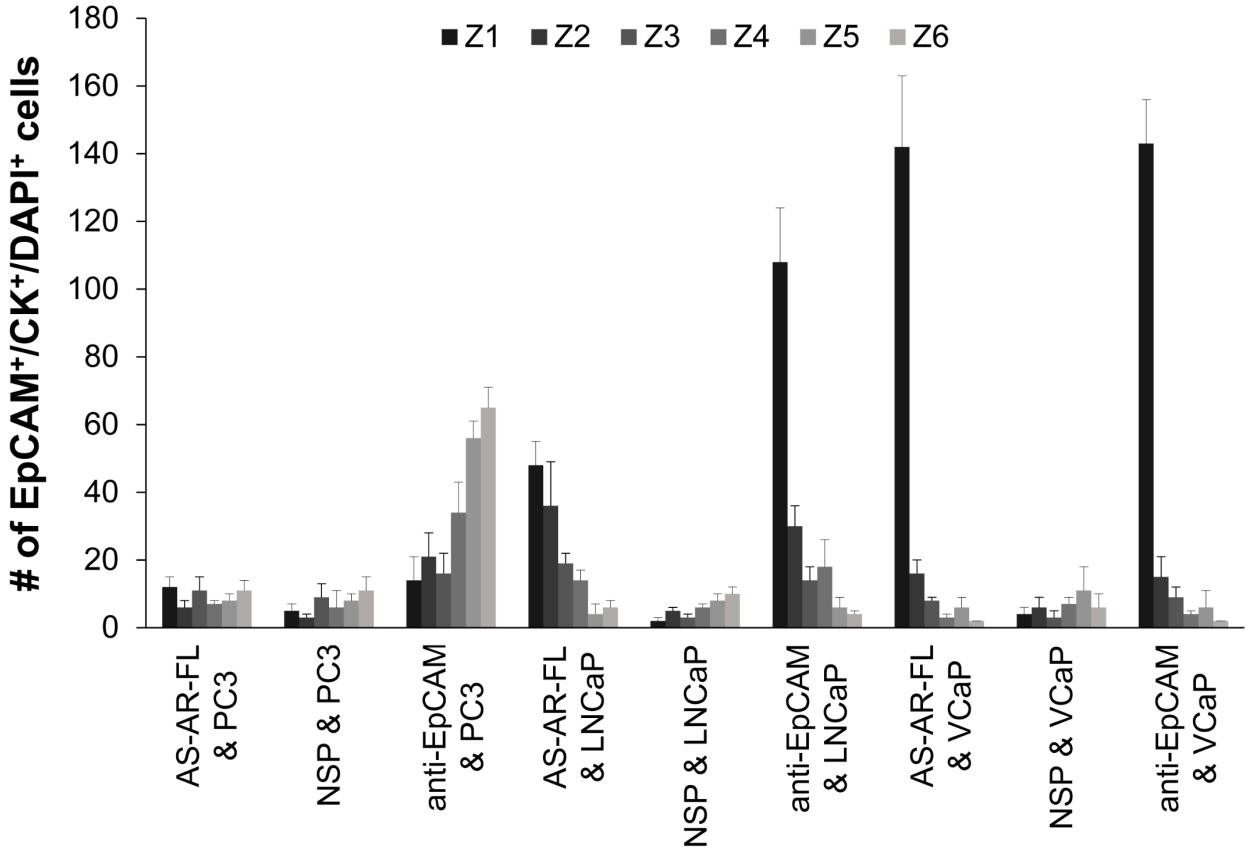

Figure 8. Raw data for single-cell mRNA cytometry targeting the full-length androgen receptor mRNA. Three prostate cancer cell lines (200 cells) were fixed with 4% PFA and permeabilized with 0.3% TX-100. The cells were incubated with two MNPs-tagged DNA probes complementary to the target AR-FL mRNA (AS-AR-FL). A control experiment was carried out in which the cells were incubated with MNPs-tagged nonspecific binary probe (NSP), subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were incubated with MNPs-tagged anti-EpCAM. Two hundred cells were used in these trails. The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, stained with APC-labeled anti-CK, APC-labeled anti-EpCAM, and DAPI. Only $CK^+/EpCAM^+/DAPI^+$ cells were counted.

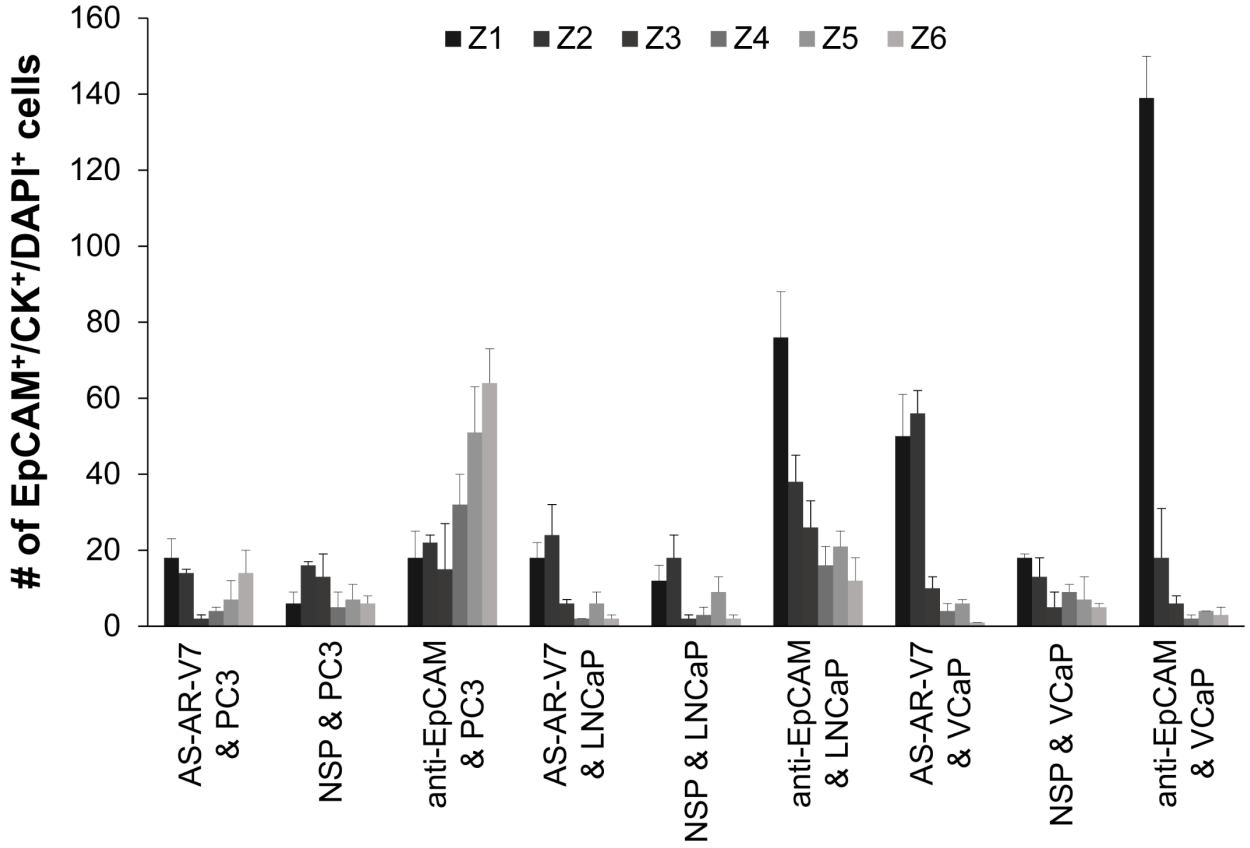

Figure 9. Raw data for single-cell mRNA cytometry targeting the androgen receptor splice variant mRNA (AR-V7). Three prostate cancer cell lines (200 cells) were fixed with 4% PFA and permeabilized with 0.3% TX-100. The cells were incubated with two MNPs-tagged DNA probes complementary to the target AR-V7 mRNA (AS-AR-V7). A control experiment was carried out in which the cells were incubated with MNPs-tagged nonspecific binary probe (NSP), subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were incubated with MNPs-tagged anti-EpCAM. Two hundred cells were used in these trails. The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, stained with APC-labeled anti-CK, APC-labeled anti-EpCAM, and DAPI. Only $CK^+/EpCAM^+/DAPI^+$ cells were counted.

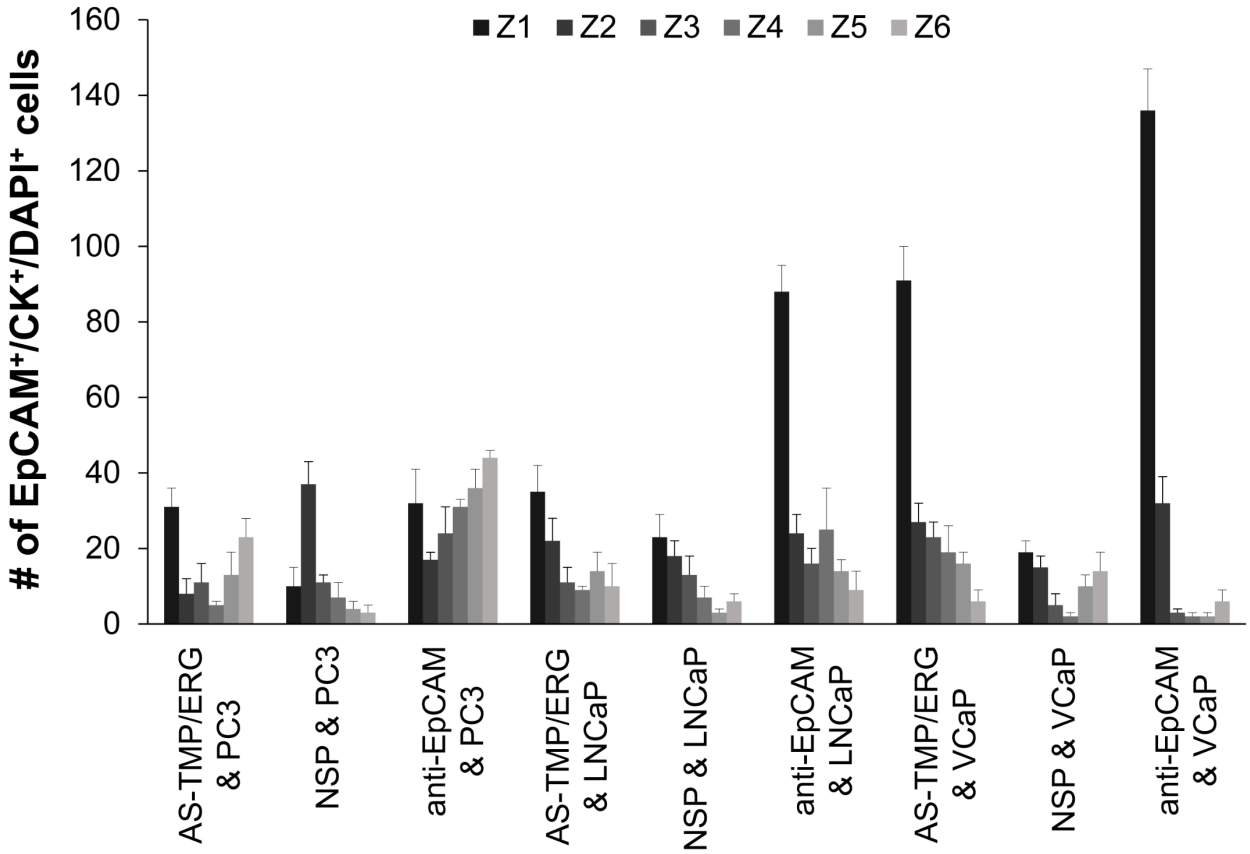

Figure 10. Raw data for single-cell mRNA cytometry targeting the TMPRSS2/ERG mRNA in prostate cancer cell lines. Three prostate cancer cell lines (200 cells) were fixed with 4% PFA and permeabilized with 0.3% TX-100. The cells were incubated with two MNPs-tagged DNA probes complementary to the target mRNA (AS-TMP/ERG). A control experiment was carried out in which the cells were incubated with MNPs-tagged nonspecific binary probe (NSP), subsequent to cell fixation and permeabilization. Another control experiment was carried out in which the cells were incubated with MNPs-tagged anti-EpCAM. Two hundred cells were used in these trails. The cells were loaded into the microfluidic device at a flow rate of 600 μL h^{-1}, stained with APC-labeled anti-CK, APC-labeled anti-EpCAM, and DAPI. Only $CK^+/EpCAM^+/DAPI^+$ cells were counted.

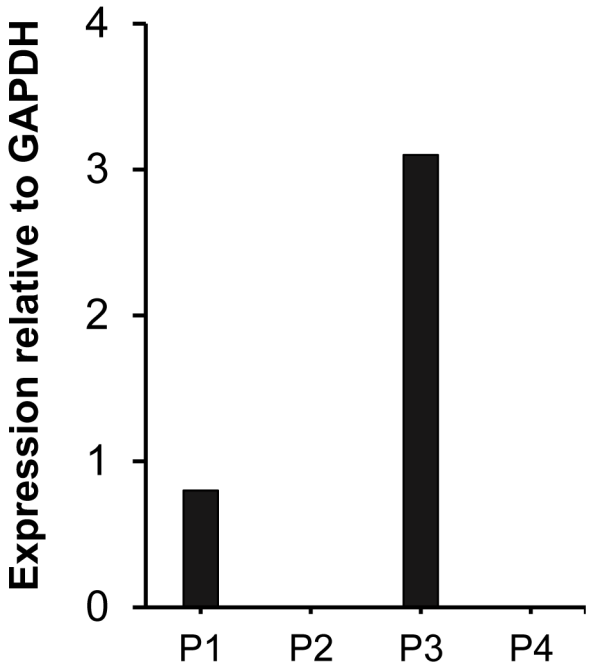

Figure 11. RT-qPCR analysis of TMPRSS2/ERG mRNA in CRPC patient's blood. Analysis was carried out subsequent to RBCs and WBCs depletion.

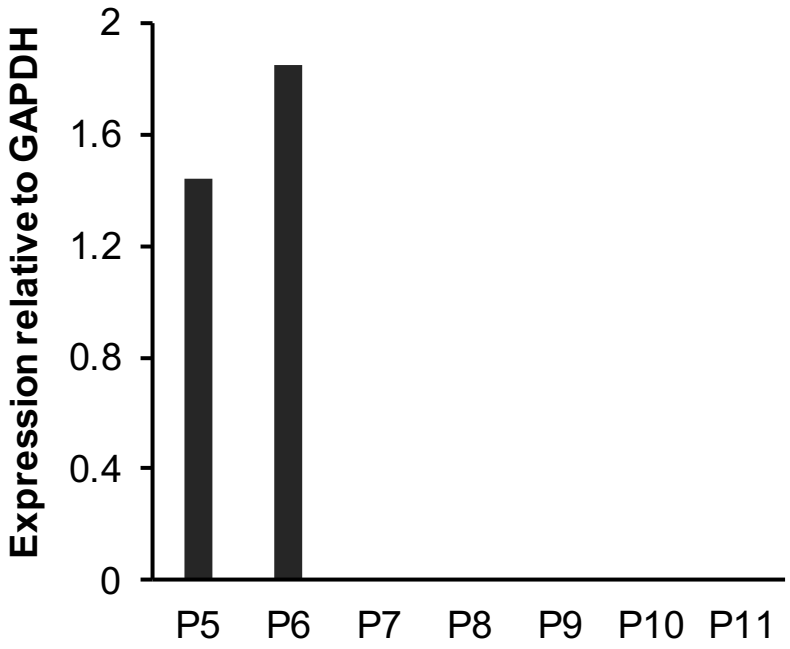

Figure 12. RT-qPCR analysis of AR-V7 mRNA in CRPC patient's blood. Analysis was carried out subsequent to RBCs and WBCs depletion.

Simulations: cell capture in mRNA profiling device

Cell capture occurs when the magnetic force acting on the cell counterbalances the drag force caused by the flow. The magnetic force acting on cells tagged with magnetic nano-beads can be calculated according to formula (I):

$$\vec{\boldsymbol{F}}_m = N_b V_m \frac{\Delta\chi_{bead}}{\mu_0}(\vec{\boldsymbol{B}} \cdot \nabla)\vec{\boldsymbol{B}} \qquad \text{(I)}$$

Where N_b is the number of beads per cell, V_m is the bead volume, $\Delta\chi_{bead}$ [unitless] represents the difference between the magnetic susceptibility of the bead and the medium, μ_0 [H/m] is the permeability of free space ($4\pi\times10^{-7}$ H/m), and B [T] is the applied magnetic field. Here we used magnetic beads with diameter of 100 nm.

Stokes' law can be used to determine the transverse drag force acting on a cell, neglecting wall effects at low Reynolds numbers, according to formula (II):

$$\vec{\boldsymbol{F}}_d = -6\pi\eta r\vec{v} \qquad \text{(II)}$$

Where r [m] is the cell radius (10 μm), η [Pa×s] is the dynamic viscosity of the medium (0.001 Pa×s), and v [m/s] is the velocity of the cell.

We designed a microfluidic device in which the linear velocity varies along the length of the device. In this design, the device features six zones with different average linear flow velocities (1x, 0.47x, 0.31x, 0.23x. 0.18x, 0.15x) to facilitate capturing cells with different magnetic content. Cells with high magnetic content are captured in the first zone, whereas cells with medium to low magnetic content are captured in later zones. Magnetic and flow field simulations were used to determine the positions in which cells with variable levels of magnetic content are captured (Supplementary Figure 1 and 2). The simulations were carried out using COMSOL Multiphysics software (Comsol Inc., US) in order to compare the magnitude of the magnetic force at each zone within the device with the drag force opposing cell capture.

Any region is considered a “capture zone” when the magnitude of magnetic force acting on the cell is comparable to the drag force. The capture zone diameters for cells with high and low magnetic content are shown in Supplementary Figure 2 at an optimized flow rate of 600 uL h^{-1}.

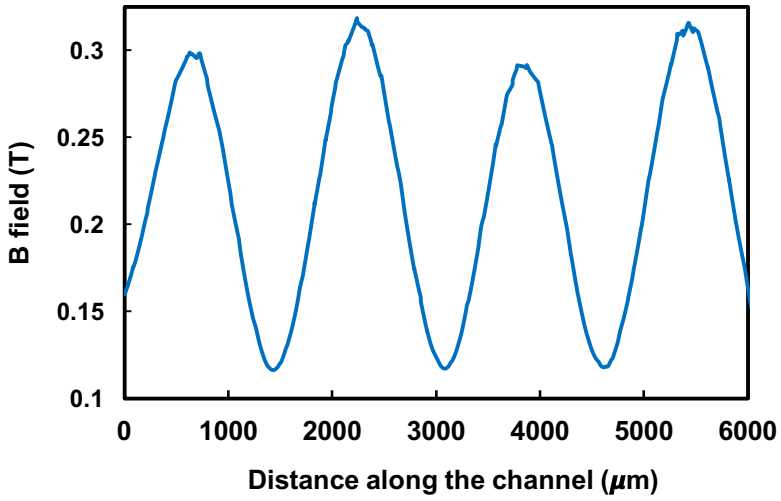

Figure 13. The magnetic field inside the channel as a function of distance along the channel and at a height of 10 μm.

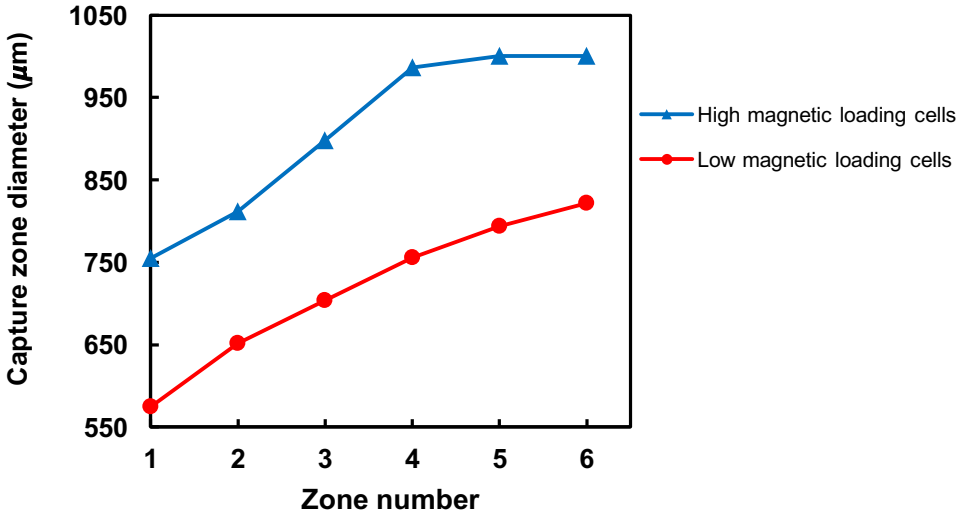

Figure 14. Capture zone diameter versus zone number at a height of 10 μm. The diameter of the capture region increases with decreasing the average flow velocity along the device.

In order to quantify the capture probability of cells, we assumed that cells would be captured when they flow within the capture radius along the device, whereas cells flowing away from the capture radius will continue their journey along the device. We then determined the percentage of the cells that traversed the device within a given radial distance from the centre of an X-structure. Additional flow simulations were carried out using a series of concentric control surfaces for various radial positions from the centre of the X-structures. The positive volume flux crossing the capture zone was determined by integrating the dot product of the velocity vector at the surface with the surface unit normal vector over the capture zone area. We essentially calculated the amount of fluid changeover at a given radial position from the center of an X-structure. The positive volume flux crossing the capture zone of cells having different levels of magnetic content (numbers of magnetic nanoparticles = 10^5, 10^4, 10^3, 10^2, and 10) at different zones were calculated to determine the capture probability of cells within the device (Supplementary Figure 3). It is worth to mention that all simulations were carried out at the optimal flow rate of 600 µL h^{-1}.

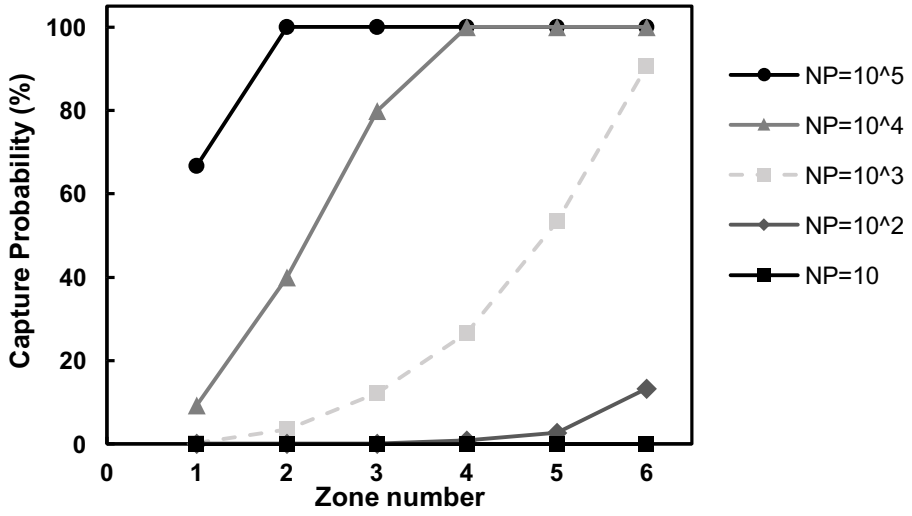

Figure 15. Capture probability of cells having different levels of magnetic content within the mRNA cytometry device at flow rate of 600 µL h^{-1}.

It is evident from Figure 3 that cells with different levels of magnetic loading, and thus different levels of mRNA expression, are captured within different zones of the device.

In order to show the capability of the device for capturing cells with low magnetic loading, we simulated the cell capture inside the device for cells having 100 magnetic nano-particles at low

flow rate (50 μL h^{-1}). As shown in Supplementary Figure 4, the device was able to retain low magnetic loading cells with a probability of 100% when the flow rate was reduced to 50 μL h^{-1}.

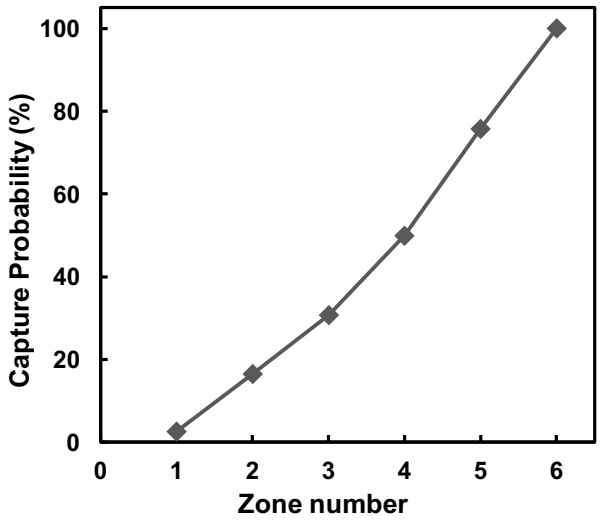

Figure 16. Capture probability of cells having low levels of magnetic loading within the mRNA cytometry device when the flow rate is reduced to 50 uL h^{-1}.

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3. Besant, J. D.; Mohamadi, R. M.; Aldridge, P. M.; Li, Y.; Sargent, E. H.; Kelley, S. O., Velocity valleys enable efficient capture and spatial sorting of nanoparticle-bound cancer cells. *Nanoscale* **2015,** *7*, 6278-6285.