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Supplementary Information for

Functional profiling of circulating tumor cells with an integrated vortex capture and single-cell protease activity assay

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Supplementary Information Text

Materials and Methods

Immunostaining

Prior to CTC isolation, we stain whole blood samples directly in EDTA collection tubes to identify subpopulation of cells. For every 6mL of blood, we use 10µL CD45-PE (BD Bioscience cat # 555483), 10µL CD66c-PE (eBioscience cat # 12-0667-41), 5µL CD31-PE (catalog # ab30349),10µL PSMA-APC (Miltenyi Biotec cat # 130-106-609), and 6µL of 50µg/mL Hoechst (Thermo Fisher H3570). All antibodies target surface epitopes, such that membrane permeabilization is not necessary for access to each protein. Fig. S 11 shows the positive PSMA stain on LnCaP cells without permeabilization. We add the antibodies to 6mL of whole blood in the collection tube protected from light at room temperature for 30 minutes.

Vortex device operation

After staining the blood, we dilute it 20X in filtered PBS immediately before processing through the vortexHE chip. The vortexHE chip isolates CTCs from diluted whole blood at 2.6 ml/min. During CTC isolation, the wash buffer and substrate runs at 0.3 ml/min and the sample runs at 2 ml/min. The wash and substrate need to be on infuse mode to prevent blood backflow into the solutions. These flow rates were optimized for 20mL BD plastic pack syringes, processed on a Harvard apparatus syringe pump (cat # 71-2001).

After CTC trapping in vortices, a solution exchange to PBS washes out background molecules. While the wash buffer runs at 2.4 ml/min the sample withdraws simultaneously at a low flow rate of 0.1ml/min. The sample withdraw prevents remaining blood from infusing in the chip and contaminating the signal. After a 1 minute wash, we stop the wash buffer flow, and switch the substrate flow to 2.7 ml/min; this second solution change introduces the substrate. After 4 seconds the vortices fill with the substrate. We determined the time required to completely fill the vortices from high speed imaging of trypan blue as a contrast agent in the vortex chambers. The substrate is a peptide sequence FRET (Fluorescence Resonance Energy Transfer) based broad spectrum MMP substrate from AAT Bioquest (cat # 13510); it is specific for 11 different MMPs. We used a 0.5% dilution in RPMI base media (Invitrogen cat #11875119) of the stock substrate. For all MMP9 specific experiments, we used a different peptide FRET substrate from Biozyme (cat # PEPDAB052m001) at 10µM dilution in RPMI.

Once the substrate replaces all the solution around the cells, we divert captured cells to the second outlet of the pinch valve (Takasago, part # PM 0815W24V). leading to a droplet generator. The valve switches the flow towards the droplet generator at time 0, and the fluid splits between the forward vortex exit direction and the disengaged sample syringe. We can tune the ratio of flow split by reducing the resistance in the sample syringe tubing. By using a large lumen tubing for the sample, we can rapidly slow flow towards the vortex exit by driving a larger portion of the flow towards the sample syringe. The pinch valve has two input tubes, one is normally pinched in the off position while the other is open. The input of the droplet generator connects to the normally off tube, while the second tube goes to the waste outlet. When the valve receives 24V input, the switch alters pinch position, such that the output from the Vortex chip goes to the droplet generator, and the waste tube is pinched off. The vortices dissipate when we release the actuation on the sample syringe and lower the substrate flow to 0.05 ml/min. The release step runs for 10-15 seconds, while the cells get encapsulated into droplets. The step emulsifier is highly parallelized with 100 channels, which allows it to operate at very

high flow rates. Initially the droplet generator is 50% full with 0.5% pico-surf (Sphere Fluidics, cat # SF-000149) in Novec 7500 (3M, cat # 9802122937), and held at a 90-degree angle to allow droplets to move away from the inlet region and allow more droplets to form. After encapsulation, the droplet generator is filled completely with the 0.5% pico-surf in Novec 7500 and put in a horizontal position. This allow the droplets to form a monolayer. The sequence of steps used is shown in Figure 1.

The reaction between protease and substrate occurs inside the droplets for 3 hours. Following incubation, we analyze the fluorescent intensity in each droplet using a fluorescent microscope or an imaging flow cytometer. We identify the intensity in droplets which also contain high signal for fluorescent labels targeting cellular biomarkers of each encapsulated cell. This approach allows us to investigate single-cell secretions of proteases from CTCs with high purity and low noise, making it amenable to other single-cell secretions and genomic and proteomic analyses with a seamless workflow. Imaging for all cell line experiments was done using the Axio Observer Z1 Zeiss fluorescence microscope, and imaging for all clinical samples was done using a Nikon Ti-E fluorescence microscope. Both microscopes used Nikon CoolSnap HQ2 cameras. The substrate fluoresces in the FITC channel and was imaged with a 400ms exposure. An image processing algorithm was developed in MATLAB to detect the boundary of the droplets in brightfield and calculate the intensity of the secreted MMPs in the FITC channel. Droplets with specific cells in them were identified before calculating the fluorescent intensity. The SNAIL transfected cells contained GFP, therefore the cell area was masked and not included in calculating the fluorescent intensity of the droplets.

Cell culture conditions

Cancer cell lines A549, HCC827, H1703, and LnCaP were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin- glutamine (P/S) at 37°C with 5% CO_2. VCaP cells were grown in DMEM (Thermo Fisher cat# MT-10-013-CM), 10% FBS, and 1% P/S. PC3 cells were grown in F-12K (Invitrogen cat # 21127022), 10% FBS, and 1% P/S. Endothelial cells were grown in MCDB131 complete growth media (VEC Technologies cat # MCDB131C). Once the cells were semi confluent, they were lifted from their adherent layer using 0.25% Trypsin EDTA (Gibco) and 3 minutes incubation at 37°C. The trypsin was neutralized with media and cells were spun down at 2300 rpm for 5 minutes, VCaP and endothelial cells were spun at 300g for 5 min. The trypsin and media were removed, and the cells were suspended back in media before processing.

Cell line spiking in PBS

Cells were suspended in media and stained for viability with 1μM calcein red-orange (Thermo Fisher cat # C34851). Cell concentration was determined by hemocytometer and diluted to 100 cells per ml in PBS and 5ml of this cell solution was processed. Cells in which calcein-red viability dye leaked out and remained in the encapsulating droplet were considered permeabilized during processing and were not included in our analysis.

Cell treatment with histamine

A confluent flask of endothelial cells was grown in vascular basal media (Vascular Cell Basal Medium (ATCC PCS-100-030) for 16 hours. Then cells were put in suspension and processed for encapsulation. Cells were encapsulated with 10μM histamine that was added to the diluted substrate solution.

Cell treatment with monensin and brefeldin

A semiconfluent flask of PC3 cells were used. Protein Transport Inhibitor Cocktail (eBioscience cat # 00-4980-03) is a cocktail of monensin and brefeldin A. The stock solution of 500X was diluted to 2X and added to the growth media. The cells were incubated in 37ºC with 5%CO_2 for 15 hours before the cells were lifted and encapsulated into droplets with the same concentration of the transport inhibitor dissolved along with the substrate solution.

Device fabrication

Devices were made with the polymer polydimethylsiloxane (PDMS) using replica molding. The vortexHE device is fabricated using methods described previously (1). The droplet generator is fabricated using double layer photolithography. The mold structure was fabricated on a 4 inch silicon wafer (University Wafer Inc.) by photolithograpy. For the first layer containing the channels, KMPR 1050 (Microchem) was spin coated with spin speed of 3500 rpm, ramped at 300 rpm/s for 30s for 50µm heights and 30µm widths and 1000 µm lengths. Devices were soft baked for 15 minutes at 100ºC and cooled for 5 minutes. The exposure time was 120s at 8.5W power. They were post exposure baked for 3 minutes at 100ºC. The devices were cooled for 5 minutes on a metal cooling bench. The second layer of KMPR was spun at 900 rpm ramped at 300 rpm/s for 30s, soft baked for 20 minutes. The wafer was cooled for 5 minutes and a third layer of KMPR was poured and the previous step was repeated. The wafer was then protected from light and cooled for at least 15 hours at room temperature. The masks were aligned using alignment marks and the wafer was exposed for 200s. The post exposure bake was done for 10min. The wafer was cooled for 5 minutes and developed using SU-8 developer. The container with the wafer and developer was put in a sonicator for 2 minutes, removed and unexposed photoresist was removed by agitation. The reservoirs had a height 500 µm – 1000 µm. Device features were measured with a Dektak profilometer. The PDMS droplet generator device was made with Sylgard 184 Elastomer (Dow Corning Corporation) with a cross-linker to polymer ratio of 1:10, and cured at 60°C for 21 hours. The devices were cut from the mold, and entry ports were punched using a 1.5mm biopsy needle (Integra Miltex cat # 33-38). The PDMS layer and a glass slide (VWR International, LLC) were O_2 plasma treated (Oxford Technics RIE) (for 30 seconds, at 500 mTorr, 80 W power before being bonded together to enclose the microchannels.

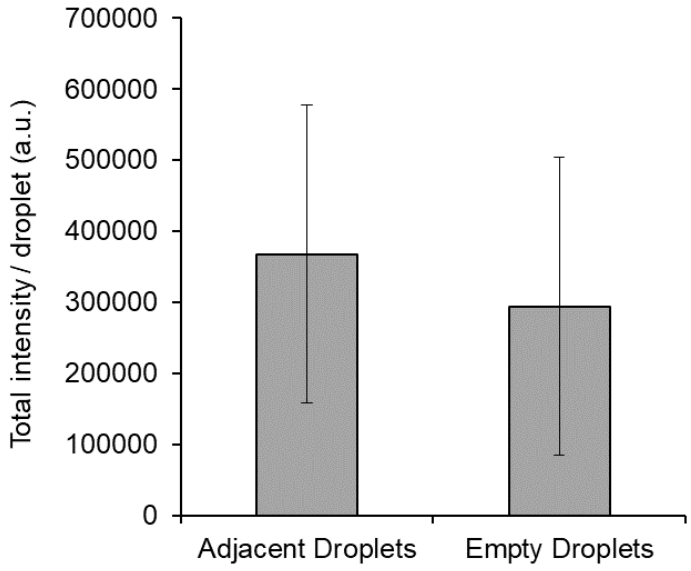

Fig. S1. In order to study the transport of cleaved peptide substrate between adjacent drops we compared the range of signal within the three hour incubation period between adjacent droplets surrounding a droplet containing an A549 cell and empty droplets. Empty droplets used for comparison only had the substrate and no cells in any droplets around them. The mean signal is within a standard deviation of each set.

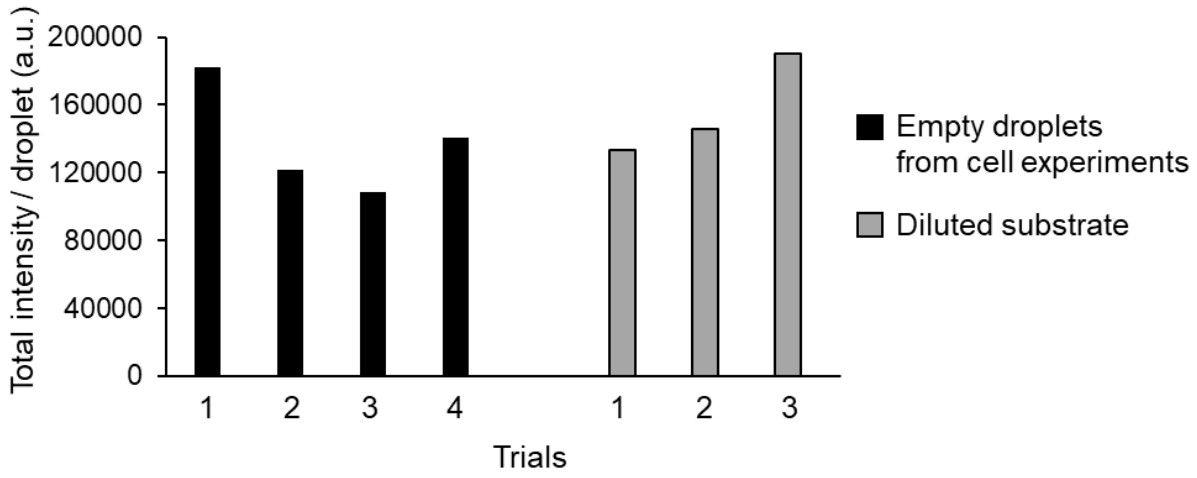

Fig. S2. The baseline values are calculated from empty droplets. Empty droplets from experiments with cells have similar levels as droplets from control experiments with only diluted substrate. Intensity values of droplets with only substrate and no MMPs (grey) are comparable to those with potential MMPs from contaminating media or serum or neighboring drops containing secreting cells (black).

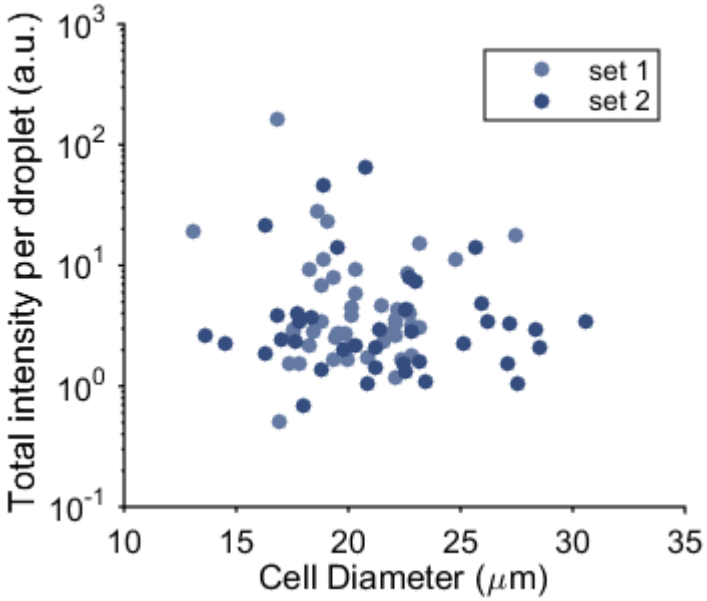

Fig. S3. Cell diameter versus MMP activity for 85 single droplet-encapsulated A549 cells shows no statistically significant correlation between these two parameters. Set 1 and set 2 are two different trials of experiment with A549 cells. The correlation coefficient is -0.11 and -0.22, p = 0.5, 0.15 for set 1 and 2 respectively.

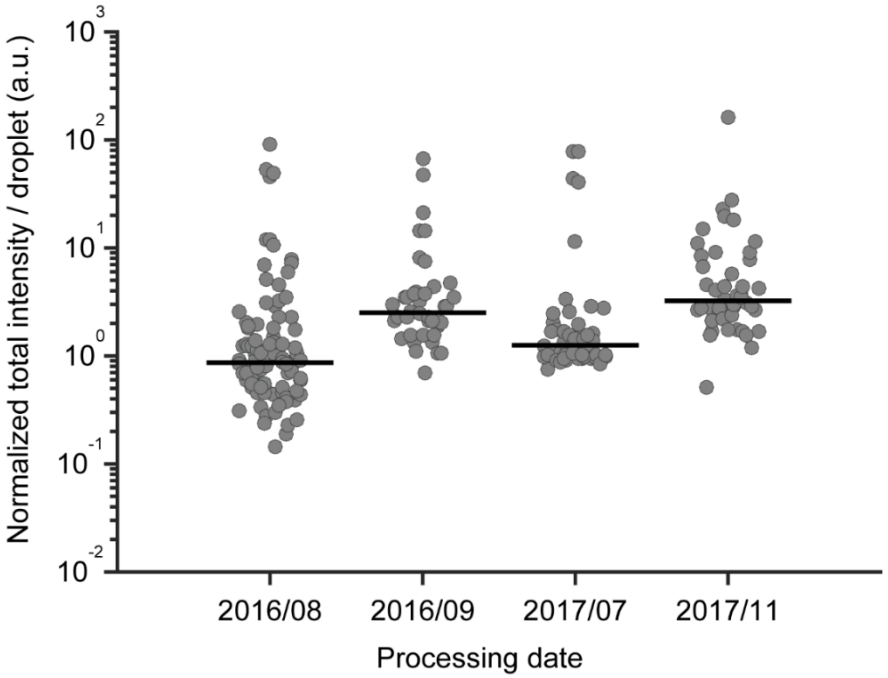

Fig. S4. Assay repeatability. The A549 secretion assays show comparable variance across different days and batches of the MMP substrate. The droplets having lower than “1” normalized values reflects the baseline variance in intensity across droplets, even in empty drops.

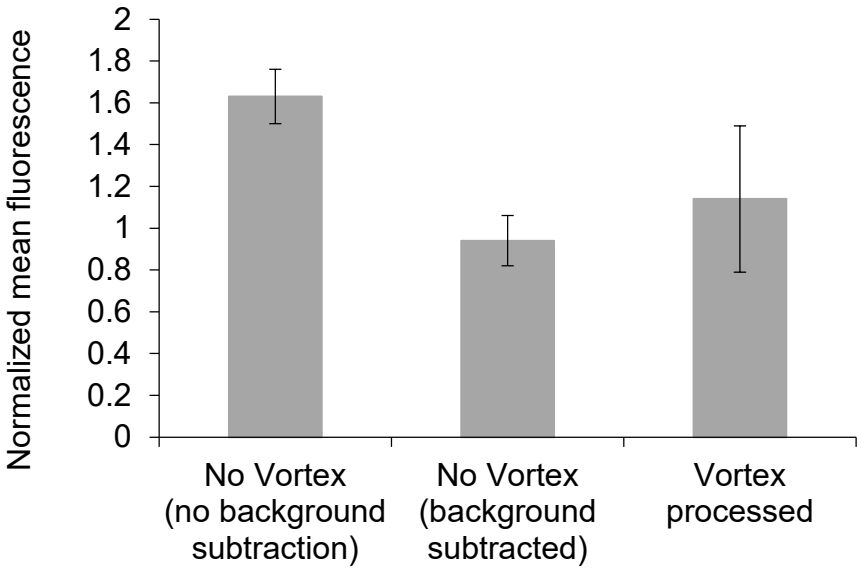

Fig. S5. Bulk measurement of MMP secretion from cells. PC3 cells were grown to 70% confluency and lifted using accutase. Cells were processed through the vortex device and approximately 850 – 1000 cells were collected in 96 well plates. The same number of (vortex unprocessed) cells from the same stock were added to a well plate. The unprocessed cells have a large amount of background signal from secretions of other cells in the stock solution. In order to estimate the stock solution background, we centrifuged the cells and took an equal volume aliquot of the supernatant and added to a separate well with the diluted substrate. We allowed the MMPs to react for 3 hours and then measured MMP activity. After the background subtraction, there is no significant difference in normalized MMP activity per cell between the vortex processed and unprocessed cells. The p value when comparing fluorescence values between no vortex processing with background subtraction and vortex processed samples is 0.4. Error bars represent a standard deviation.

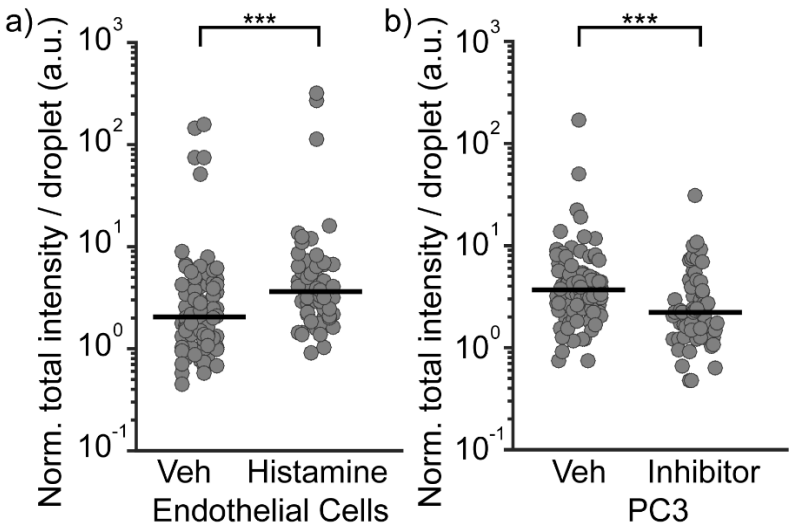

Fig. S6. The assay can correctly detect modulation of MMP secretion from cells. (a) Histamine is shown to upregulate MMP secretion when exposed to endothelial cells and leads to increased signal compared to vehicle control (Veh) in our assay. (b) PC3 prostate cancer cells were exposed to secretion inhibitors brefeldin and monensin which sequester proteins in the golgi apparatus and prevent them from being secreted. As expected, there is a decrease in measured intensity indicating a decrease in MMP secretion in the presence of the inhibitors. *** $p < 0.001$.

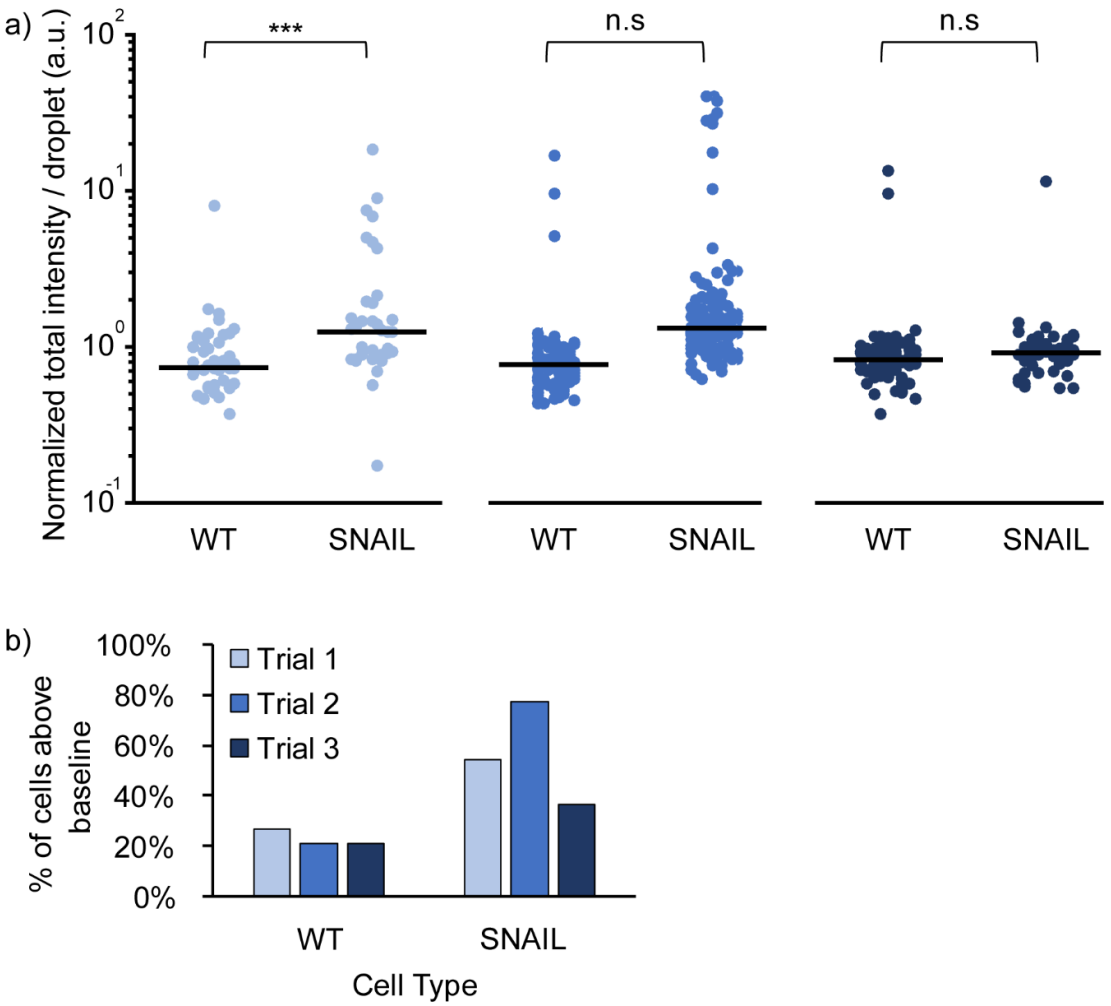

Fig. S7. a) We studied the effects on MMP9 secretion in wild-type A549 lung cancer cells (WT) and A549 cells overexpressing SNAIL, a transcription factor involved in epithelial to mesenchymal transition (EMT). There is a significant increase in signal from a peptide substrate cleaved by a broad spectrum of MMPs in SNAIL overexpressing cells, indicating these cells secrete higher levels of MMPs, although not MMP9. b) Percentage of SNAIL overexpressing cells secreting above baseline levels of MMP-9 is higher than the percentage of wild-type cells secreting above baseline levels. *** $p<0.001$

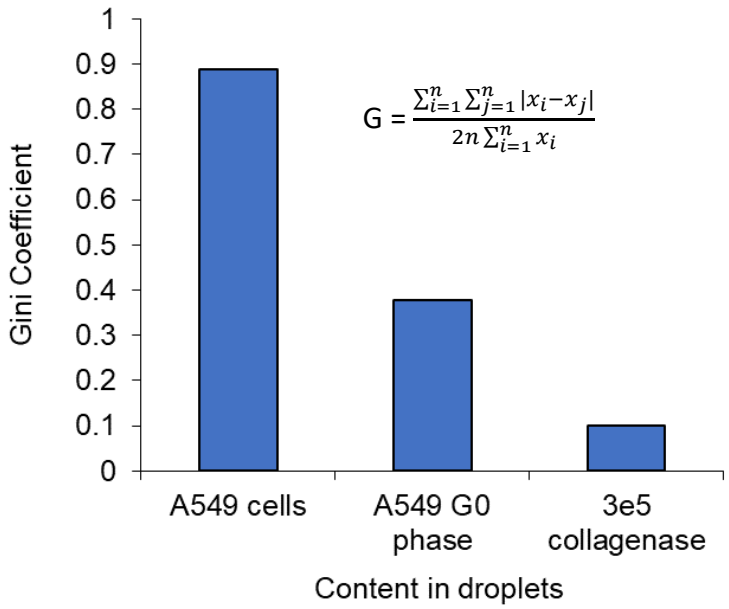

Fig. S8. The variation in signal intensity observed decreases as the cells/solution used to generate the signal becomes less heterogenous. Here we quantify the dispersion with the Gini coefficient. The coefficient varies from 0 (most uniform) to 1 (most extreme). The index quantifies the spread in the distance between each point. It is more robust than variance when data sets are not normally distributed with a number of outliers and has been used in the past to identify rare cells in studies quantifying single cell gene expression (2). The signal from the A549 cells has a higher heterogeneity than that from cells synchronized to G0 quiescent phase. By performing cell cycle synchronization to quiescent G0 phase (via serum starvation), we were able to reduce the amount of MMP secretion in serum starved cells ($p<0.001$). Cellular activity decreases during the G0 phase, thus secretory function becomes minimal in the majority of the cells. However, other types of cell cycle synchronization using S-Trityl-L-Cysteine and double thymidine block did not reduce the heterogeneity in MMP secretion. These results indicate that cell cycle may not be a major contributing factor to MMP secretion heterogeneity. The heterogeneity is least when a known amount of collagenase is loaded into the droplets for the reaction with substrate. This experiment shows that the heterogeneity we observe is due to biological differences across individual cells and not intrinsic variability of the assay.

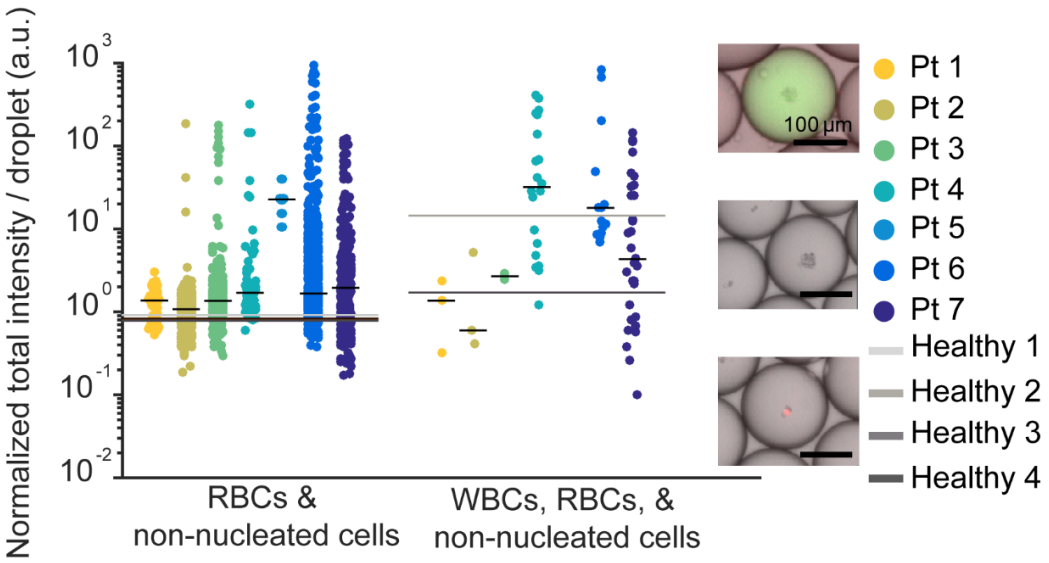

Fig. S9. In addition to CTCs and WBCs a number of RBCs or other non-nucleated cells (which may be clusters of platelets) were trapped in droplets due to a small amount of dead volume in the connections to the pinch valve. The median level of MMP-generated signal from these cell types were higher in prostate cancer patient samples than in healthy controls.

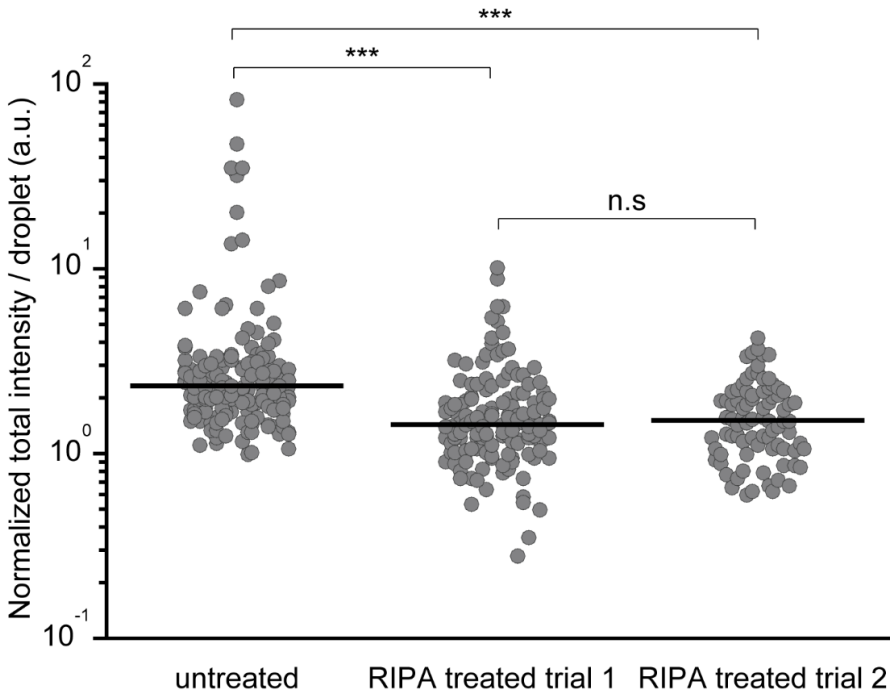

Fig. S10. Cells were treated with 10% RIPA buffer while trapped in the vortex. The shear force from the vortex and lysis agent porated the cells. The SDS in RIPA buffer leads to solvation of membrane lipids, membrane proteins, and loss of internal proteins and other soluble molecules. These “ghost” cells were incubated with the same concentration of substrate as other experiments and propidium iodide (PI). Due to membrane poration, PI molecules entered the cell and stained the nuclear DNA. These ghost cells were observed to generate relatively low levels of MMP signal which is significantly different from untreated cells and comparable to the signal levels of empty droplets, suggesting signal in patient samples is due to active secretion of functional enzyme from live cells. *** $p<0.001$.

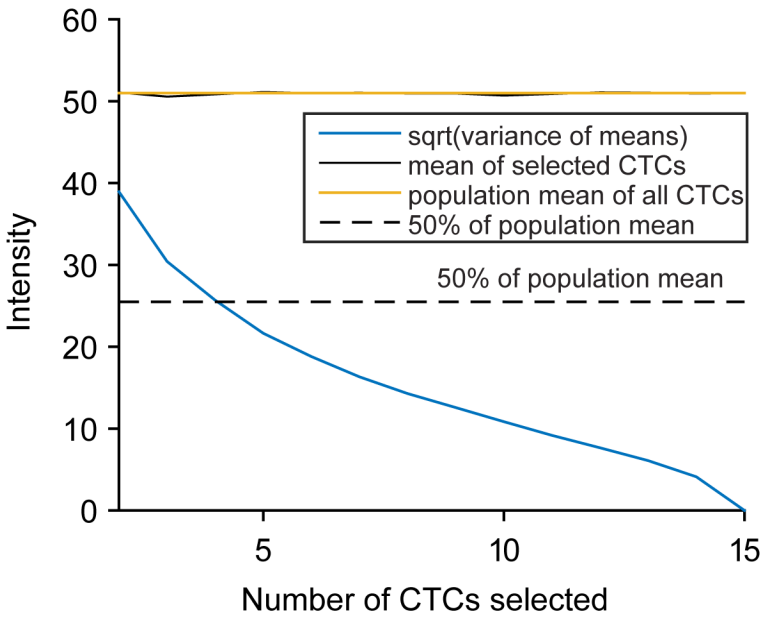

Fig. S11. To explore the concept of how many CTCs are needed to obtain an accurate signal, we selected patient 7 – the sample with the highest number of CTCs to simulate how changing the number of CTCs varies the mean MMP activity values. Repeated random sampling (6000 times) over a range of CTCs show the lowest number that would accurately represent the mean of the entire set. As expected when sampling multiple times per CTC number, the mean for the selected CTC number (2 to 15) fluctuates around the population mean, and the standard deviation of the mean with multiple samplings decreases with increasing number of CTCs sampled. We found that randomly selecting 5 or more CTCs yielded a standard deviation less than 50% the population mean. Understanding that in our data we see a >2-fold difference in MMP activity between CTCs and WBCs from a patient, we could set a lower cut-off that having at least 5 CTCs would provide a relatively good representation of the entire population.

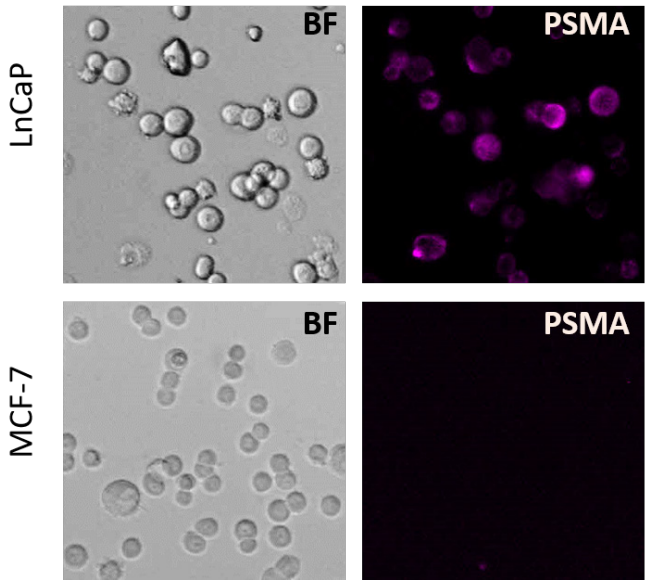

Fig. S12. Immunostaining validation for Prostate Specific Membrane Antigen. PSMA positive cells (LnCaP) were used for a positive control and breast cancer cells MCF-7 were used as a negative control. Adherent cells were lifted from plates using Accutase (Thermo Fisher Scientific cat # A11105-01) to preserve the membrane proteins. Cells were stained with 10µl of anti-PSMA-APC (Miltenyi Biotec cat # 130-106-609) in 1mL of PBS suspension for 30 min and imaged as shown. Cell staining in blood was also verified. 1000 LnCaP cells were spiked into 6mL of healthy blood. 10µl of anti-PSMA-APC was added to the sample and incubated for 30 min. The cells were isolated from blood using the VortexHE device as mentioned in the methods section and imaged with a fluorescence microscope.

Table S1. Blood samples from seven prostate cancer patients were assayed. The clinical state of each patient is reported here.

Patient ID ranked MMP level low-high	Clinical Information: all patients with mCRPC	PSA	No. of metastases
1	Initial clinical response to PI3K inhibitor followed by progressive disease with rising PSA and new bone metastases.	8.8	2
2	Radiographic progression, BET inhibitor ~3 wks prior to blood draw. PSA response to bromodomain inhibitor.	96.9	0
3	Radiographic progression. PSA increasing but relatively low (lower PSA value may represent a more poorly differentiated cancer) Germline BRCA2 mutation, Responding to olapraib (PARP inhibitor) with decreased pain and size of metastases.	0.03	0
4	State of response to treatment; stable disease on abiraterone/prednisone	2.4	0
5	Rising PSA and radiographic progression; followed by PSA response to chemotherapy with docetaxel.	120	multiple
6	Radiographic progression in bones and lymph nodes: currently deceased	50.8	2
7	Radiographic progression in bones; initial response to abiraterone acetate/prednisone followed by clinical and PSA progression.	267.7	1

Movie S1. Particles remain trapped during solution exchange under continuous flow. Trypan blue was used as the new solution exchange reagent to view flow conditions. The newly introduced reagent initially follows the main streamlines in the center and gradually diffuses into and fills the reservoirs in about 4 seconds. 20μm beads were initially trapped in plain PBS, they remained trapped after trypan blue was infused for 4 seconds.

Movie S2. High throughput droplet generation using the parallelized step emulsifier. The step emulsifier is parallelized to 100 channels, that each are 30μm wide and 50μm high. The inline connected droplet generator splits the flow from the vortex device and encapsulates the released cells into uniform microdroplets without any manual transfer steps. It generates about 2,200 droplets per second. The droplets float away from the generation region due to buoyancy differences with the oil and form a monolayer on top of the reservoir.

Movie S3. Time lapse of cell secretion over time. A549 cells were stained with calcein and encapsulated by SPEC. Two cells in droplets were imaged every 5 minutes for 25 minutes. The differences in their rate of secretion can be observed by the differences in fluorescent signal generated when the substrate is cleaved.

Supplementary References

1. Sollier E, et al. (2014) Size-selective collection of circulating tumor cells using Vortex technology. *Lab Chip* 14(1):63–77.

2. Jiang L, Chen H, Pinello L, Yuan G-C (2016) GiniClust: detecting rare cell types from single-cell gene expression data with Gini index. *Genome Biol* 17(1):144.