

Supporting Information

Label-free Counting of Affinity-Enriched Circulating Tumor Cells (CTCs) using a Thermoplastic Micro-Coulter Counter (μ CC)

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Chemicals and materials

Ag/AgCl paste (electrically conductive medical electrode ink) was purchased from Creative Materials Inc, Ayer, MA. Sylgard 184 silicone elastomer base and the curing agent were obtained from Dow Corning, Midland, MI. SU-8 2025 was purchased from MicroChem Corp., Westborough, MA. Silicon wafers (4") were purchased from University Wafer, South Boston, MA. Human whole blood was ordered from Bioreclamation IVT, Westbury, NY and healthy blood samples were secured from the KU Biospecimen Repository Core Facility according to an approved IRB (Kansas University Medical Center, Kansas City, KS). All blood samples were collected into EDTA tubes and vacutainers (Becton-Dickinson, Franklin Lakes, NJ). SKBR3, and RPMI 8226 cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA. Polystyrene beads of 10 μm , 15 μm , and 20 μm in diameter were purchased from Phosphorex Inc., Hopkinton, MA. 2-(4-morpholino)-ethane sulfonic acid (MES) buffer and phosphate buffered saline (PBS) was purchased from Life Technologies, Carlsbad, CA. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was obtained from Sigma-Aldrich, St. Louis, MO. Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) were ordered from Thermo Fisher Scientific, Carlsbad, CA. Uracil Specific Excision Reagent (USER) enzyme and the Cut smart buffer were purchased from New England BioLabs Inc, Ipswich, MA. Single-stranded oligonucleotide heterobifunctional linkers with an internal dU residue was obtained from Integrated DNA Technologies, Coralville, IA. Mouse anti-human EpCAM (clone 158210) was purchased from R&D Systems, Minneapolis, MN. DAPI II stain (Vysis) was purchased from Abbott Laboratories Limited, Abbott Park, IL. Live Green (Calcien AM) of LIVE/DEAD™ Cell Imaging Kit (488/570) was purchased from Thermo Fisher Scientific, Carlsbad, CA.

Assembly of PDMS μCC

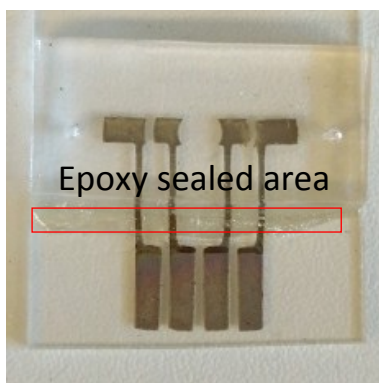


Figure S1. Picture of a PDMS/glass μCC showing the epoxy resin sealing the area around the edge of PDMS-electrode lead interface.

Experimental setup for μCC sensor

In-house-built electronics were used for signal generation and data acquisition. As described in the manuscript, the μCC consisted of 4 macroscale electrodes; the outer 2 electrodes were used for signal generation and the inner 2 electrodes were used as sensing electrodes. The excitation signal was a $\pm 7\text{ V}$ / 50 kHz sine wave and the voltage changes due to a resistance change in the micro-aperture was measured using the 2 inner sensing electrodes. Real-time automatic background correction was performed by the electronics package during data acquisition.

Different gain values for the electrical signals were used for different sensitivity requirements. Figure S1 shows the experimental setup for characterization of the μ CC device.

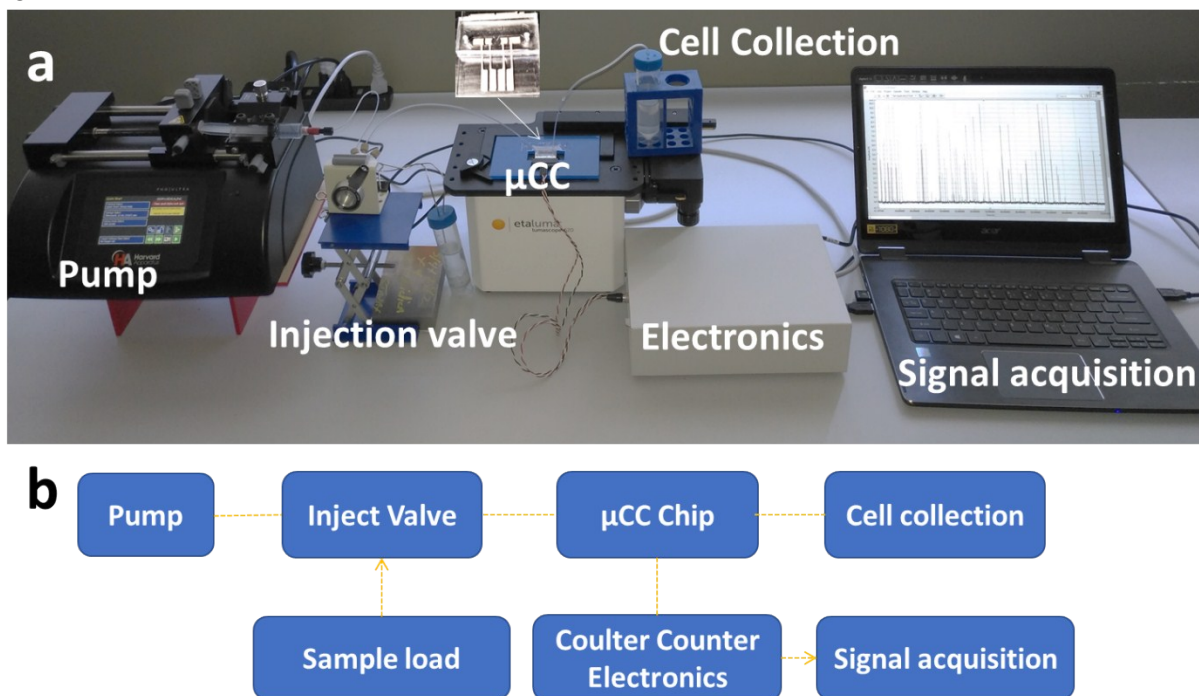


Figure S2. a) Experimental setup for μ CC chip characterization. b) Schematic illustration of the connection of each unit in the experimental setup.

In this experimental setup, a syringe pump was used to maintain flow through the measurement system, a 6-port manual rotary injector was used to introduce samples into the μ CC, and the μ CC chip was connected to this rotary injector. The μ CC could also be connected directly to the CTC chip to allow for enumeration of the CTCs following selection and release from this chip. The μ CC chip was also connected to the electronics package, which was used to transfer data to the data acquisition/analysis computer.

Cell culture and staining

RPMI 8226 (multiple myeloma cell line, non-adherent) cancer cell line was cultured using RPMI 1640 with 2.5 mM L-glutamine media supplemented with 10% FBS (GIBCO). SKBR3 (breast cancer cell line, adherent) cells were cultured in 1x McCoy/ 10% FBS. SKBR3 cells were stripped from the culture flask using 0.05% Trypsin with 0.53 mM EDTA. All cell lines were cultured at 37°C under a 5% CO₂ atmosphere. Harvested cells were kept in media in 15 mL Eppendorf tubes on a rocker to provide uniform suspension of the cells and were used within 5 h of harvest.

Cells were pre-stained with Live Green (Calciem AM) or other stains after collection into microwells. Cells collected into microwells were spun down for 5 min at 400g, PBS buffer was carefully removed, and cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and cell nuclei were stained with 0.01% DAPI II. Wells were imaged using a Zeiss Axiovert 200M microscope equipped with a 10× objective, an XBO 75 W lamp, DAPI/FITC/Cy3/Cy5 filter sets (Omega Optical), a Cascade 1K EMCCD (Photometrics) camera, and a MAC 5000 stage (Ludl Electronic Products), all of which were computer-controlled via

Micro-Manager. Each well was imaged via Micro-Manager's grid collection software, and the image sets were stitched and analyzed via a custom ImageJ macro, which identified nuclei and provided event counts.

Fabrication of CTC enrichment microfluidic device

Detailed information regarding fabrication and the subsequent antibody attachment to the plastic surface of the CTC enrichment chip, oligonucleotide linker sequences, and protocols for CTC enrichment and release was provided in our previous publications.¹⁻⁸ Briefly, CTC enrichment chips were fabricated in COC (TOPAS 6013S-04, TOPAS Advanced Polymers) using hot embossing from brass molds. Each chip possessed an array of 50 high aspect ratio sinusoidal microfluidic channels with geometry optimized for cell capture. The sinusoidal microchannels were 25 μm wide, 150 μm deep, and 30 mm long. The inlet and outlet channels of the CTC enrichment chip were attached to silica capillary tubing to allow for connection to a syringe pump for introduction of sample or wash buffer into the chip, and for connection to the μCC . Microfluidic channels were UV/O₃ activated for 10 min at 22 mW/cm² using a commercial UVO-Cleaner (Model 18, Jelight Company), which produced surface-bound carboxylic acid moieties. Following UV/O₃ activation, chips were flooded with a solution of 20 mg/mL EDC in PBS buffer and incubated at room temperature for 20 min. EDC solution was removed using an air filled syringe and immediately after that, a 40 μM solution of a ssDNA linker in PBS buffer (pH 7.4) was introduced into the device and allowed to incubate for 2 h at room temperature or overnight at 4°C to covalently attach the ssDNA linker at its 5'-terminus to the activated COC surface. After the reaction was complete, the microfluidic chip was rinsed with 100 μL of PBS (pH 7.4) and 300 mM DTT solution in carbonate buffer (pH 9) that was infused into the microfluidic chip for 20 min to reduce the 3'-disulfide group into a reactive sulfhydryl moiety (-SH). The microfluidic chip was rinsed with 100 μL of PBS (pH 7.4) and immediately, an aliquot of the modified anti-EpCAM-SMCC antibody was introduced (1.0 mg/mL) into the microchannels. The reaction was allowed to proceed for 30 min at room temperature followed by 2 h at 4°C.

Integration of μCC device with the CTC enrichment chip

As a proof-of-concept for the integration of the μCC device with a CTC enrichment chip, whole blood from healthy donors was seeded with CTCs surrogates (SKBR3 cells) and infused at 25 $\mu\text{L}/\text{min}$ through the CTC enrichment chip modified with anti-EpCAM monoclonal antibodies. During the CTC enrichment process, the μCC device was not turned on and signal acquisition was not initiated. The detector was turned after the CTC release step.

Reproducibility of μCC devices

Device-to-device reproducibility of the μCC was tested by counting SKBR3 cells using different μCC units that were connected in series. Data for 10 experiments are summarized in Figure S2. Similar patterns of signals were observed from each μCC device indicating high reproducibility of manufacturing the μCC sensor.

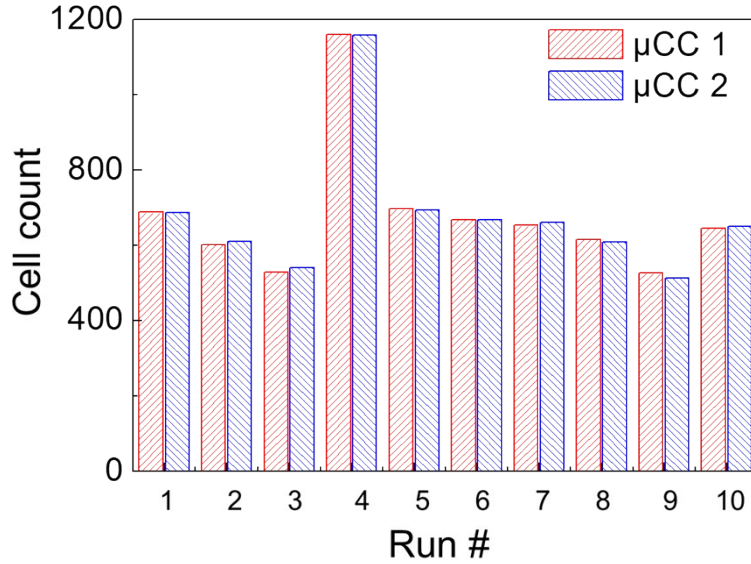


Figure S3. Repetitive μ CC measurements of SKBR3 cells using two different μ CC units connected in series.

Influence of blood on μ CC chip performance

To test whether blood passing through the μ CC chip would affect its performance, the μ CC chip was connected with the CTC enrichment chip and whole blood from healthy donors seeded with SKBR3 cells was pumped through the system at a flow rate of 25 μ L/min. Following blood infusion, both chips were washed with 2 mL of 0.5% BSA/PBS at 55 μ L/min. Resistive pulse traces for SKBR3 cells before and after passing whole blood through the μ CC showed no differences in their signal amplitudes (average signal amplitude was 4.18 ± 2.11 V before blood pass and 4.28 ± 2.16 V after blood pass). Furthermore, statistical data analysis was performed for the resistive pulse amplitude distributions of the SKBR3 cells as shown in Figure 5b. Both data sets were non-normal according to the Shapiro Wilk test of normality. Wilcoxon rank sum showed a p-value of 0.6468 between before and after the blood passed through the μ CC. This experiment indicated that the μ CC performance was not influenced by blood flow through it.

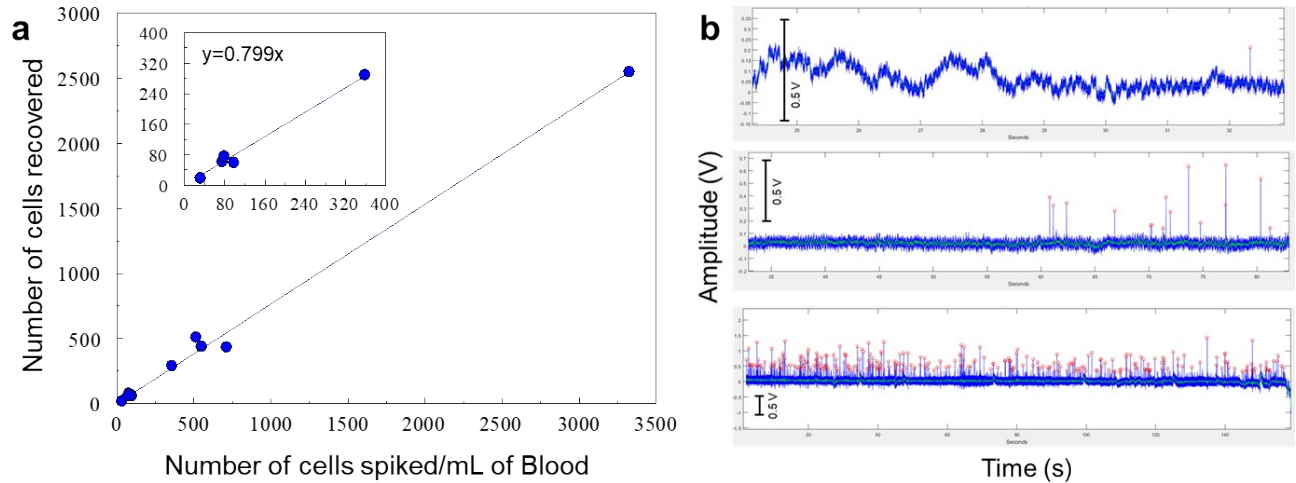


Figure S4. (A) Recovery of SKBR3 cell line from blood and (B) ability of the μ Coulter Counter to enumerate wide range of cell numbers. Examples here are shown for 1, 14, and 289 SKBR3 cell(s).

Cell recovery using CTC chip and counting wide range of cell events.

Recovery of SKBR3 cell line was evaluated in the range of ~10 and ~3,300 cells spiked per mL of healthy donor blood. The slope of the line in Figure S4A is indicative of the recovery of 79.9% in the tested dynamic range of cells numbers. The μ Coulter Counter has the ability to identify single cell events, as shown in Figure S4B, for 1, 14, and 289 cells.

Matlab Script for μ CC Signal Processing

```
clear
clc
close all
in_ext = '.txt';
out_ext = '-result.txt';
fil_ext = '-filtered.txt';

pb = 0.05; %time width of background
ps = 0.00015; %time width of peaks
fpr = 0.004; %false positive rate / quantile of noise estimation
ts = 5; %select noise window start time
te = 15; %select noise window end time

File_List = dir(['*',in_ext]);
todo = {};
for file_num = 1:length(File_List)
    filename = File_List(file_num).name;
    dotplace = strfind(filename, '.');
    trimmed_filename = filename(1:dotplace-1);
    fprintf('%32s',trimmed_filename);
    if exist([trimmed_filename,out_ext])==2
        fprintf('\t done');
    else
        fprintf('\t new');
        todo{end+1} = trimmed_filename;
    end
    fprintf('\n');
end

for file_num = 1:length(todo)
    filename = todo{file_num}
    display('loading file');
    tic
    data = load([filename,in_ext]);
    toc
    time = data(:,1)';
    signal = data(:,2)';

    %     crap = abs(signal)>9;
    %     time(crap)=[];
    %     signal(crap)=[];

    clear data;
    dt = time(2)-time(1);
    nb = ceil(pb/dt/2)*2;
    ns = ceil(ps/dt)*2;

    x = -nb/2+0.5:1:nb/2-0.5;
```

```

fb = pdf('normal',x,0,nb/8);
fb = fb/sum(fb);

x = -ns/2+0.5:1:ns/2-0.5;
fs = pdf('normal',x,0,ns/8);
fs = fs/sum(fs);

display('Subtracting Baseline');
baseline = conv(signal,fb,'same');
baseline = baseline(nb/2:end-nb/2);
time = time(nb/2:end-nb/2);
signal = signal(nb/2:end-nb/2);

display('Smoothing away noise');
smoothed = conv(signal-baseline,fs,'same');
smoothed = smoothed(ns/2:end-ns/2);
time = time(ns/2:end-ns/2);
signal = signal(ns/2:end-ns/2);
baseline = baseline(ns/2:end-ns/2);

noise = smoothed(find(time>=ts & time <=te));
stdr = std(noise);
thresh = 5*stdr;%threshold = x * noise std

threshold = thresh*ones(size(smoothed));
rel = signal-baseline;

[pks,locs,w,p]=
findpeaks(smoothed,time,'MinPeakHeight',thresh,'WidthReference','halfheight',
'Annotate','extents');
%find peaks with that drop at least = threshold V on either side before
the signal attains a higher value

wms =w.*1000;%peak width in ms
a = [locs;wms;p];
b = size(locs,2)

idx = a(2,:)<0.8 & a(3,*)>0.2;%filter out peak width >= 0.8 ms and peak
height <= 0.2 V
c = a(:,idx);
d = size (c,2)
tnew = c(1,:);
wnew = c(2,:);
pnew = c(3,:);

display('Plotting signal and threshold');
figure(1)
clf
subplot(2,1,1);

findpeaks(smoothed,time,'MinPeakHeight',thresh,'WidthReference','halfheight',
'Annotate','extents')
mod_filename = filename;
unders = strfind(filename,'_');
mod_filename(unders)='-';xlabel('Seconds')
title(mod_filename)

```

```

hold on
plot(time,threshold,'m-');
hold on
plot(c(1,:),c(3,:),'ro');

subplot(2,1,2);
plot(time,signal,'b')
xlabel('Seconds')
title('Raw Signal')

drawnow;
print('-dpng','-r200',[filename,'.png']);

fid=fopen([filename,out_ext],'wt');
formatSpec = 'total peaks: %d threshold: %d\n';
fprintf(fid,formatSpec,b,thresh);
fprintf(fid,'time_s\twidths_ms\thights_V\n');
fprintf(fid,'%05.2f\t%6.4f\t%6.4f\n',a);
fclose(fid);

fid=fopen([filename,fil_ext],'wt');
formatSpec = 'total peaks: %d threshold: %d\n';
fprintf(fid,formatSpec,d,thresh);
fprintf(fid,'time_s\twidths_ms\thights_V\n');
fprintf(fid,'%05.2f\t%6.4f\t%6.4f\n',c);
fclose(fid);

display('Plotting histograms');
figure(2)
subplot(2,1,1);
edges = [0:0.02:1];
h = histogram(wms,edges);
h.Normalization = 'Probability'
title(mod_filename)
xlabel('Peak Widths /ms')
ylabel('Normalized Frequency')

subplot(2,1,2);
edges = [0:0.1:10];
h = histogram(p,edges);
h.Normalization = 'Probability'
xlabel('Peak Height /V')
ylabel('Normalized Frequency')

filenamew = strcat(filename,'_a');
drawnow;
print('-dpng','-r200',[filenamew,'.png']);

figure(3)
subplot(2,1,1);
edges = [0:0.02:1];
h = histogram(wnew,edges);
h.Normalization = 'Probability'
title(mod_filename)
xlabel('Peak Widths /ms')

```



```

ylabel('Normalized Frequency')

subplot(2,1,2);
edges = [0:0.1:10];
h = histogram(pnew,edges);
h.Normalization = 'Probability'
xlabel('Peak Height /V')
ylabel('Normalized Frequency')

filenamew = strcat(filename, '_b');
drawnow;
print('-dpng', '-r200', [filenamew, '.png']);

display('Plotting distribution');
figure(4)
scatter(p,wms,10, 'filled')
axis([0,10,0,2])
title(mod_filename)
xlabel('Peak Height /V')
ylabel('Peak Widths /ms')

filenamedis = strcat(filename, '_distr');
drawnow;
print('-dpng', '-r200', [filenamedis, '.png']);

figure(5)
scatter(pnew,wnew,10, 'filled')
axis([0,10,0,2])
title(mod_filename)
xlabel('Peak Height /V')
ylabel('Peak Widths /ms')

filenamedis = strcat(filename, '_distrb');
drawnow;
print('-dpng', '-r200', [filenamedis, '.png']);

end
didisplay('end')

```

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