Supporting Material for:

Microfluidic concentration and separation of circulating tumor cell clusters from large blood volumes

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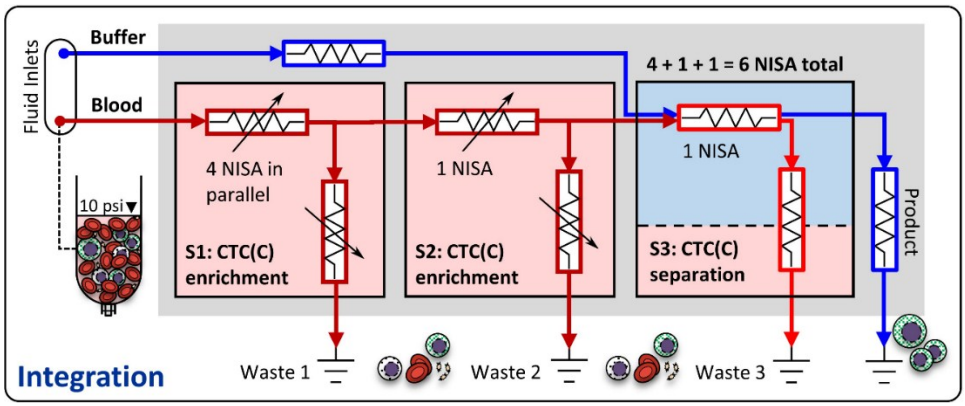
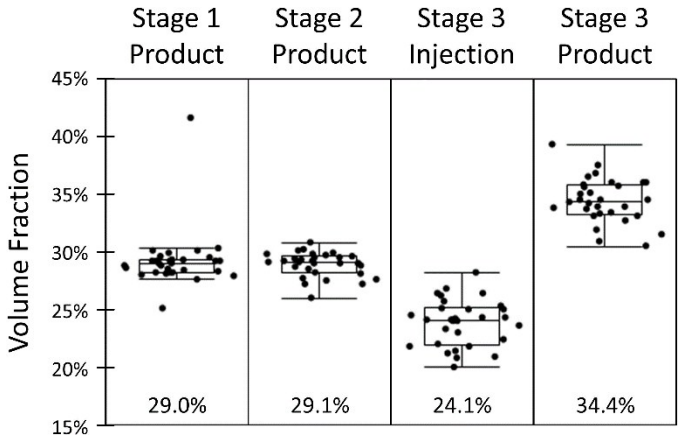
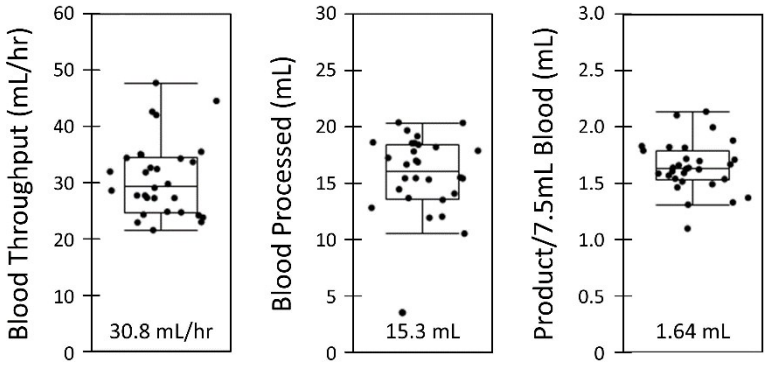
Fluidic performance. To ensure proper function of the integrated 3-stage NISA device, it is necessary to control the volumetric splits at each joining or splitting of flow. Therefore, all flow rates have been controlled passively by the inclusion of fluidic resistances connecting the three NISA stages as depicted in **Suppl. Fig. 1**. In stages 1-2, where 100% sample is injected across four lanes in each NISA device, the minimum product fraction is the fraction of flow in the product lane (25%) minus the fluid shift fraction (3.6%), or 21.4%. However, this would be very risky, so the target was raised to >25% which is met in all cases in **Suppl. Fig. 2** (first two plots). In stage 3, a buffer co-flow joins the stage 2 product stream. To keep a full lane of buffer between sample and product (as in **Fig. 1D**), sample injection fraction was tuned to <30% (sample width broadens due to its ~2× higher viscosity relative to buffer). Product fraction was required to be >29% in stage 3 (but less than 40% to avoid skimming blood cells). With the exception of one run where the stage 1 product fraction rose >40%, volumetric splits fell within tightly distributed clusters. These experiments were run with an average of 15 mL whole blood diluted to 20% hematocrit by addition of buffer (1% w/v F127 in PBS). Blood throughput, blood volume processed, and the volume of product per nominal tube of blood (7.5 mL) are each given in **Suppl. Fig. 3** for the same set of experiments. To push speed and volume further, whole blood was pushed through a single NISA module (1/4 of the chip) with separation seen in **Suppl. Fig. 4**.



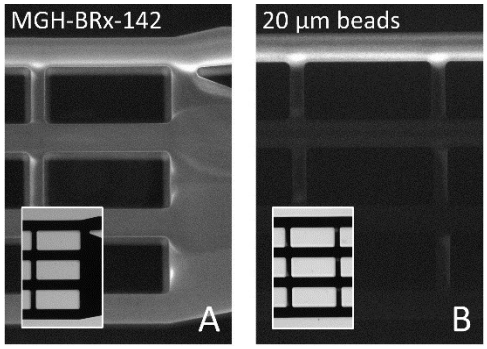
Supplementary Figure 1: Integration of six NISA-XL devices into one CTC(C) sorter. The PDMS device of **Fig. 1B** is constructed from three interconnected stages. Four 100% injection NISA devices (stage 1, **S1**) feed one 100% injection NISA device (**S2**) which feeds one NISA device with buffer co-flow (**S3**). Input (blood and buffer), output (wastes 1-3 and product), and internal flow splits (stages 1-3 product, stage 3 injection) are controlled by a network of channel resistances. A lumped resistance model is shown.



Supplementary Figure 2: Reliability of flow control (integrated three-stage device). Volumetric flow rate ratios are given at each location of flow split (stage 1-3 products) and joining (stage 3 injection) for 10 psi input pressure. Data are from 30 experiments processing an average of 15.3 mL whole blood diluted to 20% hematocrit samples. Note that the product lane in stages 1-2 holds 25% of the flow, but the third stage product lane holds a bit more due to the viscosity mismatch between buffer and sample, so it required a somewhat higher product fraction.

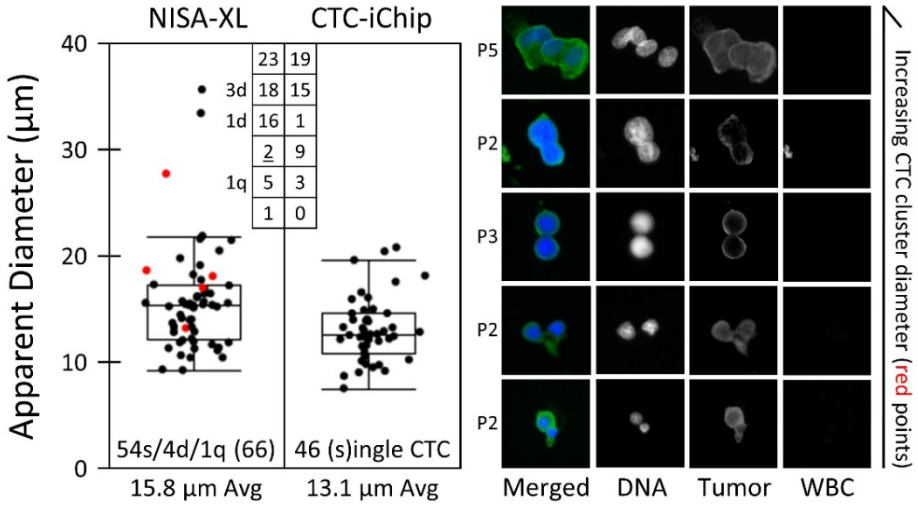


Supplementary Figure 3: Blood throughput (left), total blood processed (middle), and product volume per nominal tube of blood (right). Plots and mean values are for 30 runs at 10 psi input pressure to integrated three-stage device using EDTA-collected blood. Sample input was 20% hematocrit.



Supplementary Figure 4: Single array (just 25% of the chip) processing whole blood. **A** 47% HCT blood is running at 2mL/min. Spiked CTC(C)s concentrate in the product lane as seen in the fluorescence image (bright field inset at lower left). **B** To push processing volume, 20 mL of 35% HCT blood was run four times at 4 mL/min.

CTC/cluster isolation in clinical samples. Spiked CTCs are different than CTCs and clusters present within patient blood samples. In particular, it has been shown that patient CTCs are in general smaller than many cultured CTCs used in spiking experiments [1] and they are often apoptotic. Therefore, it is informative to measure the fraction of primary CTCs which could be isolated by the 3-stage NISA-XL chip of **Fig. 1B**. To this end, blood samples from six melanoma patients were processed by NISA-XL (18 mL blood per patient). To isolate smaller CTCs that were missed by size sorting alone, NISA-XL waste fractions were processed by the CTC-iChip [1] using negative selection (CD45, CD16, and CD66b targeted magnetic beads). Immune fluorescence (IF) imaging at 10× was then used to find putative CTC(C)s similar to our prior work [1]. The results are reported in **Suppl. Fig. 5**, where a total of 66 CTCs were found by the NISA-XL chip (including 4 CTC doublets and one four-cell CTC cluster), and 46 were found by the CTC-iChip, all of which were single CTCs. In total, this amounts to 1.0 CTCs per mL blood, similar to our prior results in melanoma [1]. Notably, 11% of all putative CTCs were in CTC clusters, and CTC clusters were found in 3/6 patients. In all, 59% of CTCs were found by the integrated NISA chip (54% of single CTCs and all clustered CTCs), with the remaining single CTCs being found in the CTC-iChip product. This dataset includes an experiment where bubbles were observed in the stage 3 NISA device (18% yield of single CTCs in patient 4). These fractions will change with patient cohort, and some CTCs or clusters in the NISA-XL waste may be missed by the CTC-iChip. Yet because many patient-derived CTCs overlap in size with WBCs [1], one would expect to lose a fraction by size sorting alone, consistent with these results.



Supplementary Figure 5: Isolation of putative CTC(C)s from melanoma patients. Six samples (mean: 18 mL blood) were processed by NISA-XL. Waste was processed by CTC-iChip (negative selection). Products were fixed, plated, and stained for IF imaging at 10× to find putative CTCs and clusters. Apparent cell size (at 10×) is plotted at left with total CTCs at bottom for singles (s), doublets (d) and quadruplets (q). Total CTCs per patient are enumerated in the 6×2 embedded table between the two plots (underlined entry denotes run where bubbles were seen in stage 3 NISA). Rescans (at 60×) of clusters (red points) are at right (40 µm boxes).

Melanoma CTC immunofluorescence. Products collected from NISA-XL or CTC-iChip were fixed (0.5% PFA, 10 min), plated on slides using the *Shandon EZ megafunnel* (*Thermo Fisher*) and *Cytospin cytocentrifuge* (2000 rpm, 5min), then permeabilized (0.3% Triton X-100, 45 min). Tyramide signal amplification (TSA staining system, *Akoya Biosciences*) was used to amplify multi-color IF, where unconjugated primary antibody was bound to target antigens, and secondary antibodies conjugated to horseradish peroxidase were then added to bind the primaries (incubation with TSA reagent adds the fluorophore). In this manner, leukocytes and CTC(C)s were labeled with a red exclusion channel (AlexaFluor 594) and green inclusion channel (AlexaFluor 488) respectively. Specifically, CD45 was used to identify WBCs (primary: 3H1363 clone, *Santa Cruz*; secondary: polyclonal, *Jackson ImmunoResearch*). Three melanoma cell targets were pooled in green to identify CTCs: premelanosome protein (PMEL), tyrosinase, and chondroitin sulfate proteoglycan 4 (CSPG4 or NG2). PMEL is one of the structural components in the premelanosome (primary: HMB45 clone, *Dako*; secondary: polyclonal, *Jackson ImmunoResearch*). Tyrosinase is an enzyme regulating tyrosine metabolism in melanin synthesis (primary: T311 clone, *Dako*; secondary: polyclonal, *Jackson ImmunoResearch*). CSPG4 has been associated with melanoma [2] (primary: LHM-2 clone, *R&D Systems*; secondary: polyclonal, *Jackson ImmunoResearch*). Finally, DNA was labeled with DAPI. Putative CTCs were identified at 10× magnification as inclusion-positive, exclusion-negative, nucleated events, and clusters were rescanned at 60× using multi-spectral imaging (Vectra, *PerkinElmer*) to obtain images at right in **Suppl. Fig. 5**. The measured diameter of each CTC and cluster was computed

from a drawn mask of the merged 10× image (ImageJ) by finding the diameter of a circle that has the same total area. It is important to note, however, that after plating and staining, putative CTC(C) geometry can be different than diameter under flow. Nevertheless, we see a difference in average size (**Suppl. Fig. 5**, lower left).

References (supplementary)

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