Electronic Supplementary Information

Automated 3D-Printed Unibody Immunoarray for Chemiluminescence Detection of Cancer Biomarker Proteins

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Assay sequence using the Chemyx programmable pump

1. Antibody array with printed capture antibodies were loaded into the detection chamber of the fluidic device.
2. Reagents including 115 µL biotinylated detection antibodies premixed with streptavidin polyHRP, 125 µL wash buffer, and 125 µL chemiluminescence reagent were loaded/pipetted into their designated reservoirs.
3. 10 µL of protein standards or 500X diluted patient samples were then loaded into the same reservoir containing the biotinylated detection antibodies and streptavidin polyHRP mixture. The reagents filled device was then placed into the G:Box bioimaging system.
4. 50 µL min^{-1} for 6.5 mins to allow the mixture of sample, biotinylated detection antibodies (Ab_2) and streptavidin polyHRP to mix efficiently inside the mixer network, allowing the polyHRP bound detection antibodies to capture the proteins of interest.
5. When the resulting mixture arrived at the detection chamber housing the antibody array after 6.5 mins, the flow was stopped for 15 min allowing the capture antibodies (Ab_1) on the antibody array to capture the PolyHRP-Ab_2-protein conjugate from the mixture from step 1.
6. Flow rate was then increased to 125 µL min^{-1} for 5.5 mins allowing the wash buffer to wash off excess sample components from the Ab_1 array and the chemiluminescent reagents to fill the detection chamber. The signal was then immediately measured by a CCD camera in G:Box bioimaging system and integrated for 60 s.

Table S1. Comparison of traditional ELISA to the automated CL immunoarray in current work

	Traditional ELISA		3D-printed Chemiluminescence array (current work)
	PSA[1]	PF4[2]	
Cost	US$ 9.00 per replicate for 2 proteins		< US$ 1.00 per replicate for 2 proteins
Time	4.5 hours for each protein		30 mins for 2 or more proteins
Automation	possible with expensive robotics		Yes
Multiplexed	No		Yes, >2
LOD	8 pg mL^{-1}	20 pg mL^{-1}	0.5 pg mL^{-1}
Specificity	No cross-reactivity with cytokines*	No cross-reactivity with cytokines*	No cross-reactivity with biomarkers tested (< 5%)
Dynamic range	10 to 1500 pg mL^{-1}	20 to 15000 pg mL^{-1}	0.5 to 5000 pg mL^{-1} for PSA 0.5 to 10000 pg mL^{-1} for PF-4
Precision	CV < ±10%	CV < ±10%	CV < ±8%

Table 2. Comparison of stereolithography and fused deposition modelling 3D-printing

Method[3, 4]	Principle	Material	Transparency	Resolution / Layer height / Surface roughness (µm)	Cost (USD)
SLA (Formlab)	Layer-by-layer UV curing of photoresin	Acrylate based resin with photo-initiators	Yes, >90% optical clear materials	50 to 250 / 5 to 10 / 8	Formlab 1+: 2999
FDM	Layer-by-layer extrusion of molten thermos plastics	Poly Lactic acid, polycarbonate, acrylonitrile butadiene styrene	Possible, with less number of printed layers	250 / 100 / <1	Makerbot Replicator: 2000

Mixing of reagents within the complete assembled fluidic device

The mixing ability of the passive mixer is visualized by using dye solutions. The steps of adding dye solutions into the reservoirs stimulate the actual assay protocol, in which the 115 µL of biotinylated detection antibodies premixed with streptavidin polyHRP is first loaded into the reservoir 1 followed by the addition of 10 µL of protein standards or 500-fold diluted patient samples into the same reservoir.

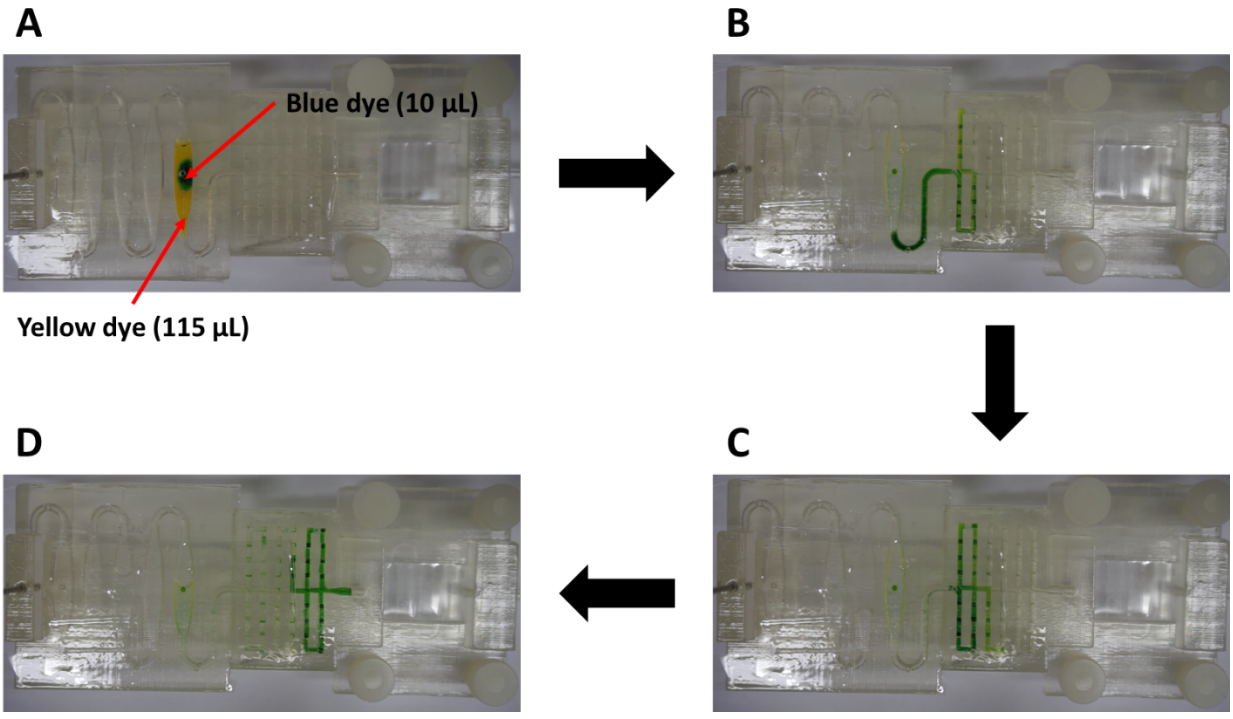

Figure S1. Mixing in the passive mixer. (A) First, 115 µL of yellow dye was loaded into the first reservoir followed by the addition of 10 µL of blue dye into the same reservoir. (B) and (C) The flow was started at 50 µL min^{-1} forcing the dye solutions to enter the mixer. (D) Efficient mixing is demonstrated by the homogenous green dye solution.

Optimization of mixer network

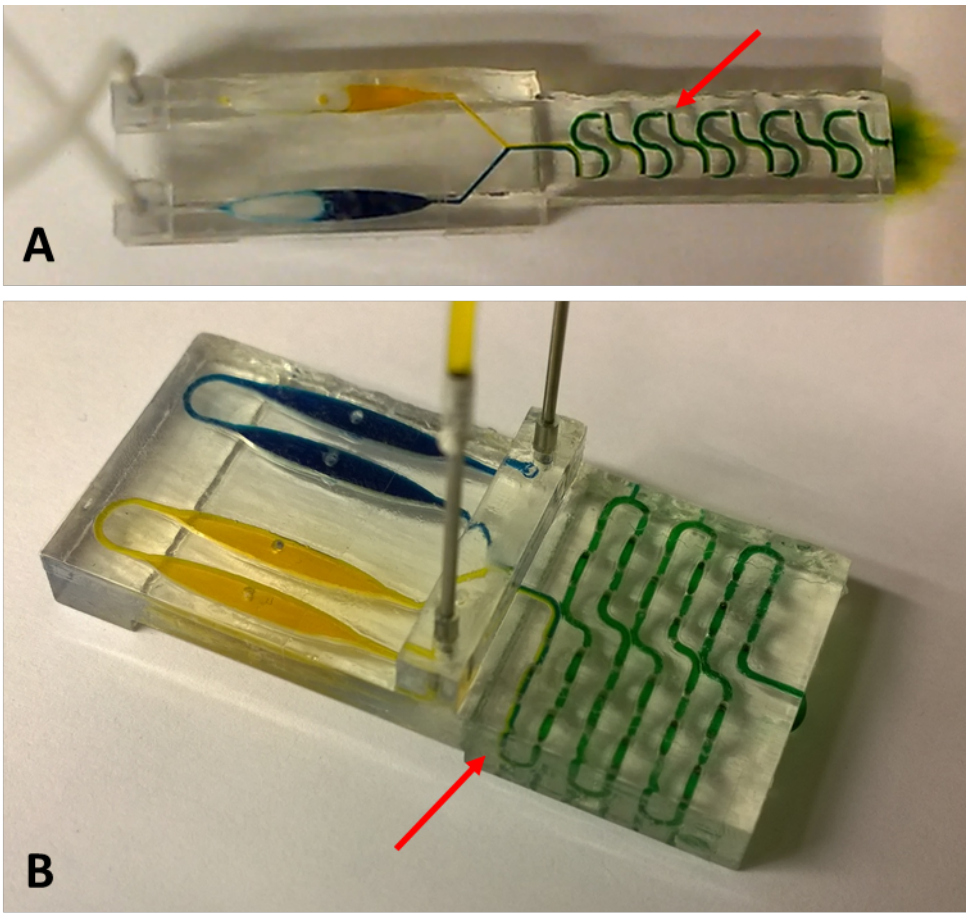

Figure S2. Examples are tested mixer channel geometries. (A) A planar serpentine design with circular channels. (B) A mixer with similar geometry as the final design equipped with circular turns. Red arrow indicates the mixing of blue and yellow dyes.

Both planar (a) and non-planar (b) designs were tested. Examples of the tested designs are shown here with the planar design consisted of a modified serpentine planar channel to change the flow directions. As shown in figure S3a, there was still a separation of the blue and yellow dye until near the mid-section of the mixer. Figure 3b represents a non-planar protoype with a similar geometry with the final design but with circular turns as compared to sharp 90˚ turns in the final design. A separation of the blue and yellow can still be seen 10^{th} turn of the mixer. The dyes began to mix at the 3^{rd} turn in the final design. With sharp 90˚ turns and increased number of turns, the final presents the best mixer of the prototypes tested.

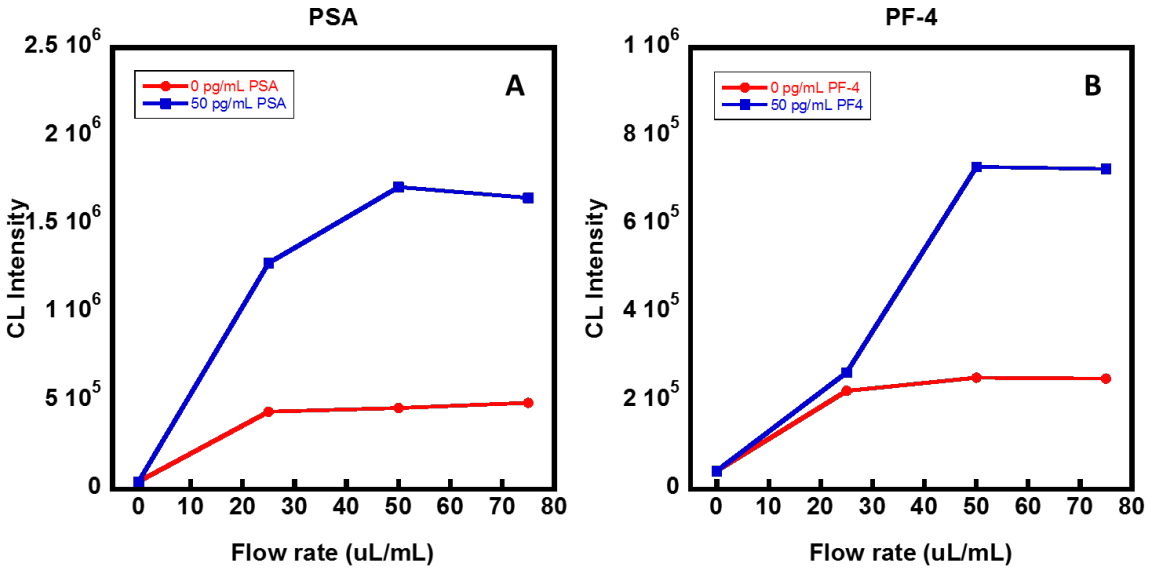

Figure S3. Optimization of flow rate for (A) PSA and (B) PF4.

Flow rate was optimized by monitoring the change in CL intensity of a fixed concentration of analyte proteins at flow rates of 25, 50, and 75 µL min^{-1} when mixing in the mixer network. These flow rates allow the design requirement of ≤30 min assay time to be achieved. A zero protein control was also run to establish a baseline. With both PSA and PF4, the optimal flow rate of 50 µL min^{-1} were chosen given the highest signal-to-noise ratios for both proteins.

Stability of capture antibody array

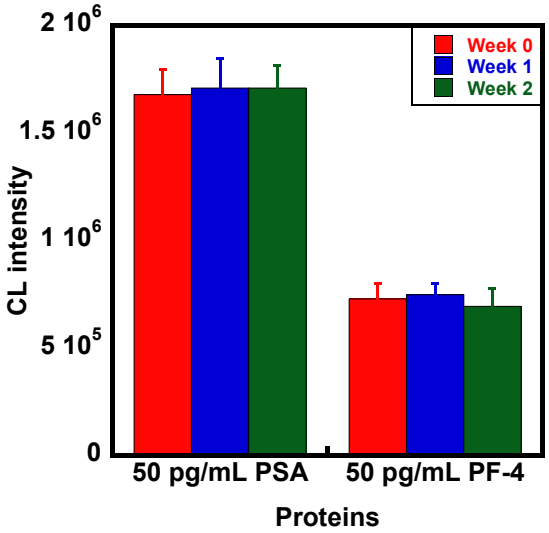

Figure S4. Stability of the capture antibody array (n = 3). Signal responses were measured immediately after antibody immobilization, 7 and 14 days at -20 ˚C after immobilization and casein blocking with very little change.

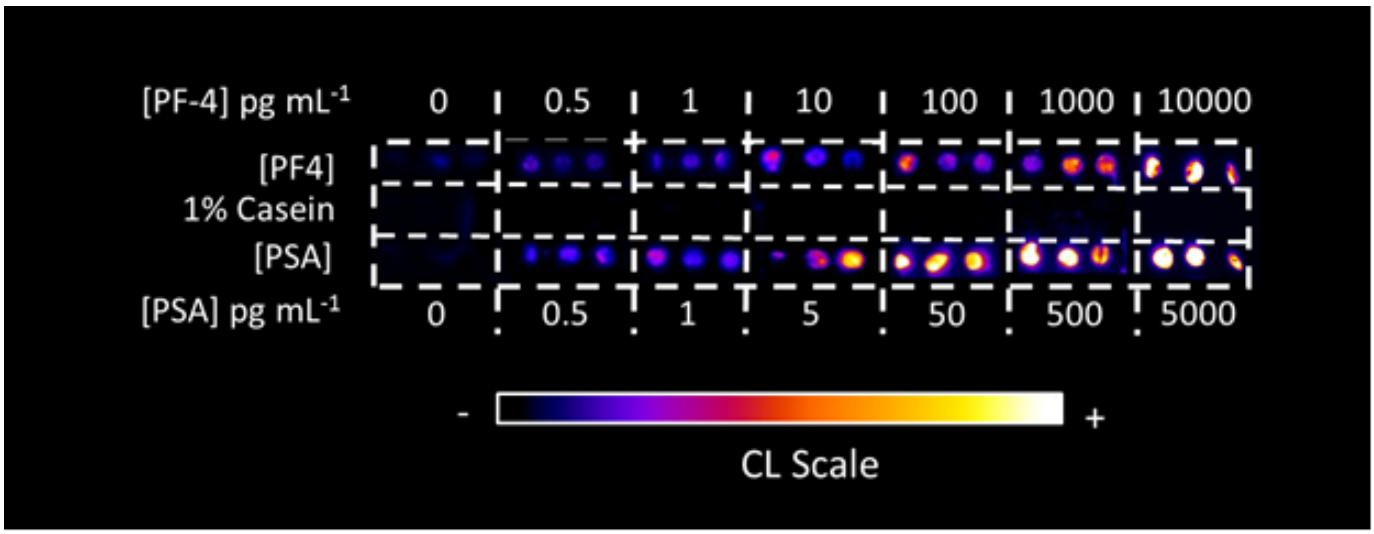

Figure S5. Re-colorized CL output obtained from the automated assay performed in the 3D mixer device. Each concentration was measured in triplicate.

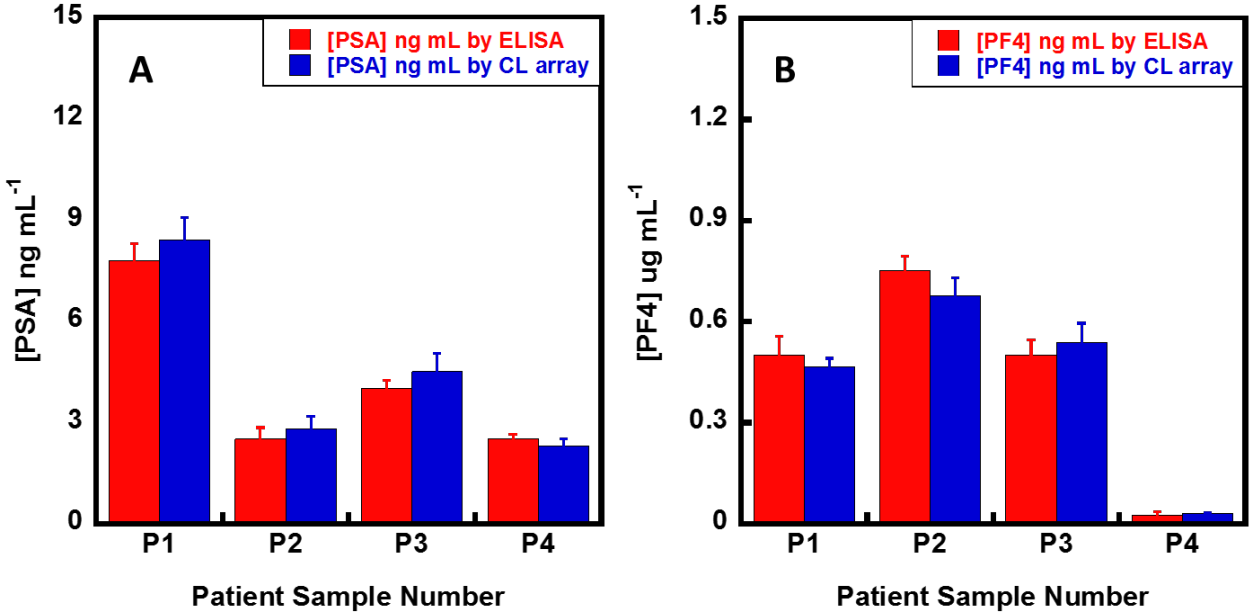

Figure S6. Comparison of human serum sample assay results using CL immunoarrays with those of single-protein ELISA for (A) PSA and (B) PF-4. Sample P1-P3 are from prostate cancer patients and P4 is from cancer-free patient. Error bars are standard deviations for the CL arrays (n=3) and ELISA (n=3).

References

1 Sigma Alrich PSA ELISA kit (RAB0031) - certificate of analysis.
2 Sigma Alrich PF-4 ELISA kit (RAB0402) - certificate of analysis.
3 G. W. Bishop, J. E. Satterwhite-Warden, K. Kadimisetty, J. F. Rusling, Nanotechnology, 2016, 27, 284002.
4 B. C. Gross, J. L. Erkal, S. Y. Lockwood, C. Chen, D. M. Spence, Anal. Chem., 2014, 86, 3240– 3253.