

**Analytical and Bioanalytical Chemistry**

**Electronic Supplementary Material**

**Ultrasensitive microfluidic array for serum pro-inflammatory cytokines and C-reactive protein to assess oral mucositis risk in cancer patients**

Colleen E. Krause, Brunah A. Otieno, Gregory W. Bishop, Gayatri Phadke, Linda Choquette, Rajesh V. Lalla, Douglas E. Peterson, James F. Rusling

## Detailed Instrumentation and Methods

### Chemicals

Screen-printed carbon arrays with 8 sensors (700  $\mu\text{m}$  dia.) were from Kanichi Research, Ltd. L-Glutathione reduced (GSH, 99%), gold (III) chloride trihydrate ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ , 99.9%), sodium borohydride (99%), poly(diallyldimethylammonium chloride) (PDDA, MW 100,000-200,000, 20%), 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), Tween-20, hydroquinone (HQ,  $\geq 99\%$ ), lyophilized 99% bovine serum albumin (BSA), and calf serum were from Sigma-Aldrich. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 30%) was from Fisher. The polydimethylsiloxane (PDMS) kit was from Dow Corning. Buffer pH 7.4 phosphate saline (PBS) was 0.01 M in phosphate, 0.14 M NaCl, 2.7 mM KCl. Streptavidin-coated 1  $\mu\text{m}$  diameter magnetic beads (SAMP) were from Life Technologies. All solutions were prepared with water purified by a Hydro water purification system to 18  $\text{M}\Omega \cdot \text{cm}$ .

### Antibodies and Proteins

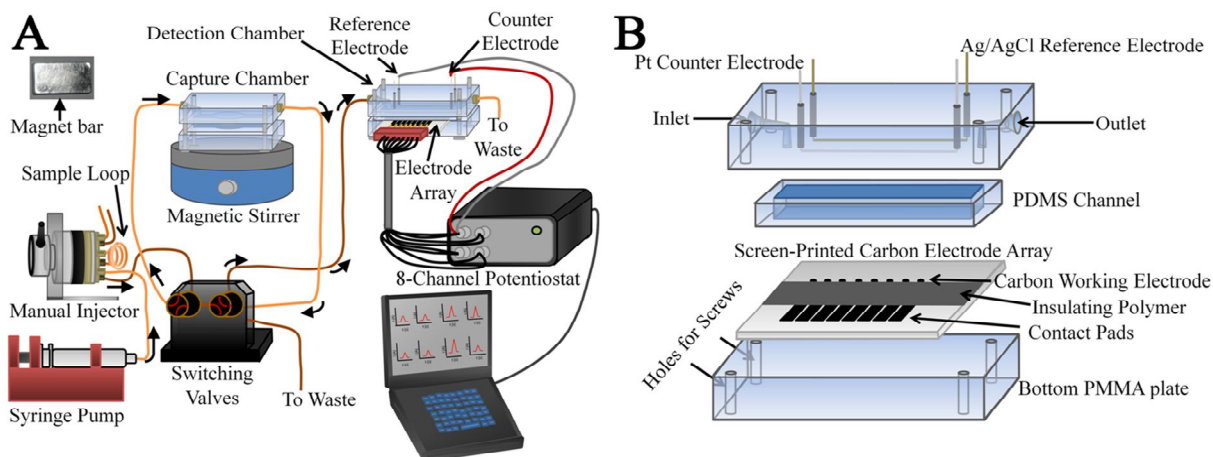
Tumor Necrosis Factor (TNF- $\alpha$ ) DuoSet (catalog # DY210), Human C-Reactive Protein (CRP) DuoSet (catalog # DY1707), and Interleukin-1 $\beta$  (IL-1 $\beta$ ) DuoSet (catalog # DY201) were from R&D systems, Inc. Human Interleukin-6 (IL-6) standard ELISA kit (catalog # 900-K16) was from PeproTech. Lyophilized 99% bovine serum albumin (BSA) and calf serum were from Sigma-Aldrich. ELISA kits were from R&D systems with detection limits of 0.7  $\text{pg mL}^{-1}$  for IL-6, 5.5  $\text{pg mL}^{-1}$  for TNF- $\alpha$ , <1  $\text{pg mL}^{-1}$  for IL-1 $\beta$  and 22  $\text{pg mL}^{-1}$  for CRP. Serum samples with concentrations below the detection limit of the ELISA kits were further analyzed using a high sensitivity magnetic bead-based multiplex assay from Millipore Corporation (HSCYTMAG-60SK).

### Human Serum Samples

Human serum samples were collected from patients undergoing high-dose radiation therapy for H&N cancer at the Neag Comprehensive Cancer Center, University of Connecticut Health Center (UCHC). The study protocol was IRB approved and written Informed Consent of participants was obtained prior to sample collection. All samples were stored at or below  $-80^\circ\text{C}$  until use. Serum samples were assayed directly and in selected cases spiked with biomarker proteins for validation tests.

### Instrumentation

The online microfluidic device features a syringe pump (Harvard, no. 704504), injector valve (Rhedodyne, 9725i), capture chamber containing a tiny magnetic stir bar, a switching valve to change the direction of fluid flow, and a detection chamber that housed sensors, reference and counter electrodes (Fig. S1)[1]. The capture chamber features a layer of molded, flexible PDMS, contained between two poly(methylmethacrylate) (PMMA) plates to form an oval cylindrical channel 1.5 mm wide and  $100 \pm 2$   $\mu\text{L}$  in volume that houses a tiny magnetic stir bar. The top PMMA plate featured two female ports (4 mm diameter) for screwing in standard male plastic fittings (1.5 mm i.d., Upchurch) to hold 0.2 mm i.d. PEEK connecting tubing for an inlet and outlet. The detection chamber (Fig. S1B) features a layer of molded, flexible PDMS sandwiched between two PMMA plates to form a rectangular channel 1.5 mm wide and 2.8 cm long and  $63 \pm 2$   $\mu\text{L}$  in volume. The top PMMA plate again features the two female ports (4 mm diameter) and in addition is equipped with holes for Ag/AgCl reference electrode (0.6 mm dia.) and Pt counter electrode (0.2 mm dia.). The syringe pump was connected to two switch valves through a sample injector using 0.2 mm i.d. tubing. The two valves are used to either direct flow to waste or to the detection chamber.



**Fig. S1** Illustrated representations of the (A) on-line capture setup featuring syringe pump, a manual injector, sample loop, capture chamber on-top a magnetic stirrer, switching valves, detection chamber, 8-channel potentiostat and computer read-out; (B) the detection chamber that is used to house the 8-electrode array and both the Pt counter and Ag/AgCl reference electrodes

### Array Fabrication

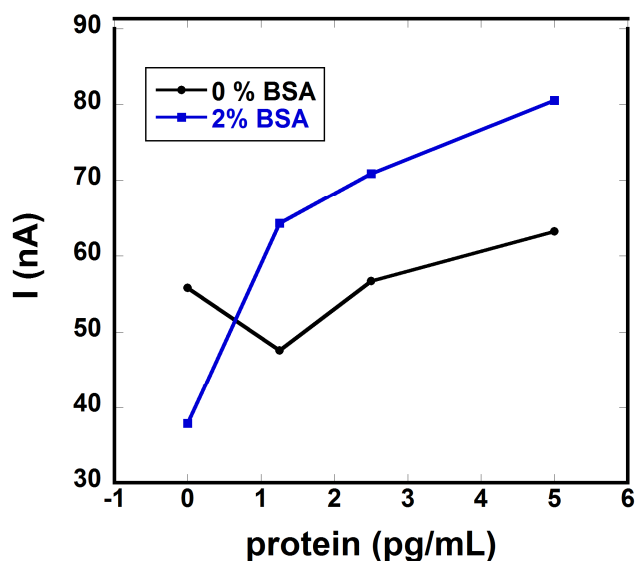
Screen printed carbon sensors in the array (main paper, Fig. 1 panel IIA) were coated with sequential layers of the polycation PDDA and 5 nm glutathione-coated gold nanoparticles (GSH-AuNPs) using layer-by-layer electrostatic adsorption as previously reported [2]. Surface carboxyl groups on the GSH-AuNP layer were activated by freshly prepared EDC and NHSS to attach primary antibodies ( $Ab_1$ ) to array elements through amidization [3, 4]. The arrays were then washed and incubated with 2% BSA in PBS for 1 hr to block non-specific binding (NSB). For amperometric measurements, the  $Ab_1$ -modified array was housed in a detection chamber that consisted of a microfluidic channel placed above the 8-sensors and fitted with reference and counter wire electrodes (Fig. S1B). A new AuNP-antibody array was used for each assay. The arrays modified with capture antibody were found to be stable up to 9 days after modification with minimal change in the response signal (Fig. S4).

### Preparation of $Ab_2$ -magnetic particle-HRP bioconjugates

Both biotinylated secondary antibodies ( $Ab_2$ ) and biotinylated horseradish peroxidase (HRP) labels were attached to 1  $\mu\text{m}$  diameter streptavidin-coated superparamagnetic beads as previously reported [4]. Briefly, 10  $\mu\text{L}$  of magnetic particles ( $10 \text{ mg mL}^{-1}$ ) were washed three times with 0.1% BSA in PBS pH 7.4 and then reconstituted with 40  $\mu\text{L}$  of 0.1% BSA in PBS pH 7.4. 40  $\mu\text{L}$  of biotinylated-HRP ( $2.5 \text{ mg mL}^{-1}$ ) and 20  $\mu\text{L}$  of the optimal concentration for biotinylated- $Ab_2$  (Fig. S3), were simultaneously added to the magnetic bead particle dispersion. The reaction proceeded at 37°C for 25 mins for the conjugation to occur. Following the conjugation, the beads were magnetically separated using an Invitrogen DynaMag-spin magnet, and subsequently washed 3 times with 0.1% BSA in PBS pH 7.4 to remove any non-specifically bound  $Ab_2$  and HRP. The conjugate was then reconstituted with 100  $\mu\text{L}$  of 0.1% BSA in PBS and stored at 4°C. The magnetic bead conjugates were stable for up to 14 days at 4°C [4]. The number of  $Ab_2$  per magnetic particle was estimated to be 40,000 ( $\pm 5,000$ ) using a bicinchoninic acid assay (BCA) kit [5]. In addition the amounts of HRP per magnetic particle were estimated to be 300,000 ( $\pm 27,000$ ) following the 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic acid (ABTS) assay [6].

### Effect of blocking agent

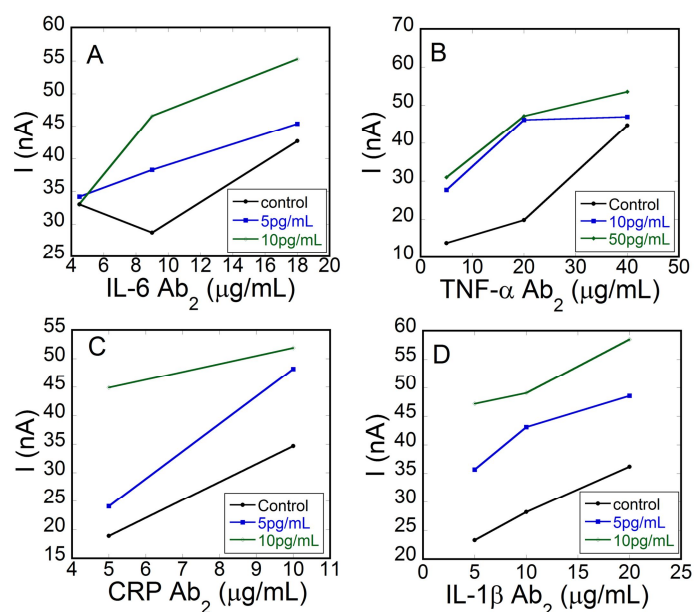
Background signals in our assays result from a combination of direct reduction of hydrogen peroxide and NSB of the labeled bioconjugate beads on the sensors. The BSA blocking agent was employed to prevent NSB of macromolecules in the sample that may interfere with the assay, and binding of labeled- $Ab_2$  on non-antigen sites on the sensor. In absence of BSA, signals arising from NSB generate large background currents that can not be differentiated from those originating from antigen-antibody binding, and are not proportional to concentration of the antigen (Fig. S2).



**Fig. S2** The Current response for sensors with/without 2% BSA blocking agent

### Optimization of Secondary Antibody

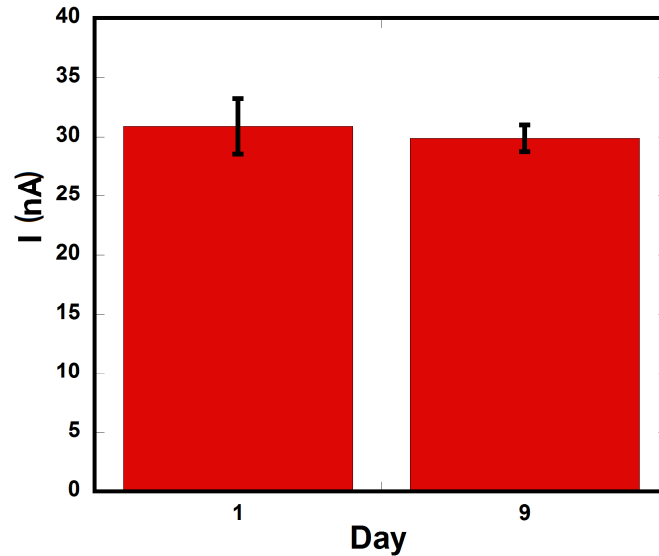
In order to establish optimal conditions for the immunoassay as well as to improve both the sensitivity and signal to noise ratio, secondary antibody concentrations were varied on the magnetic bead conjugates. Optimal secondary antibody concentrations were obtained using a consistent primary antibody ( $Ab_1$ ) concentration of  $100 \mu\text{g/mL}$  and employing standard concentrations of 0, 5, 10, and  $50 \text{ pg mL}^{-1}$  for IL-6, TNF- $\alpha$ , CRP, and IL-1 $\beta$ . The greatest signal difference between control and sample concentration indicated the optimal secondary antibody concentration to be  $9 \mu\text{g mL}^{-1}$  for IL-6,  $20 \mu\text{g mL}^{-1}$  for TNF- $\alpha$ ,  $10 \mu\text{g mL}^{-1}$  for CRP, and  $10 \mu\text{g mL}^{-1}$  for IL-1 $\beta$  (Fig. S3).



**Fig. S3** Optimization of secondary antibody ( $Ab_2$ ) using a consistent primary antibody ( $Ab_1$ ) concentration of  $100 \mu\text{g mL}^{-1}$  and employing standard concentration of 0, 5, 10, and  $50 \text{ pg mL}^{-1}$  for (A) IL-6, (B) TNF- $\alpha$ , (C) CRP, (D) IL-1 $\beta$ . Optimal concentrations were determined to be  $9 \mu\text{g mL}^{-1}$  (IL-6),  $20 \mu\text{g mL}^{-1}$  (TNF- $\alpha$ ),  $10 \mu\text{g mL}^{-1}$  (CRP), and  $10 \mu\text{g mL}^{-1}$  (IL-1 $\beta$ )

### Stability of Electrode Arrays modified with capture antibodies

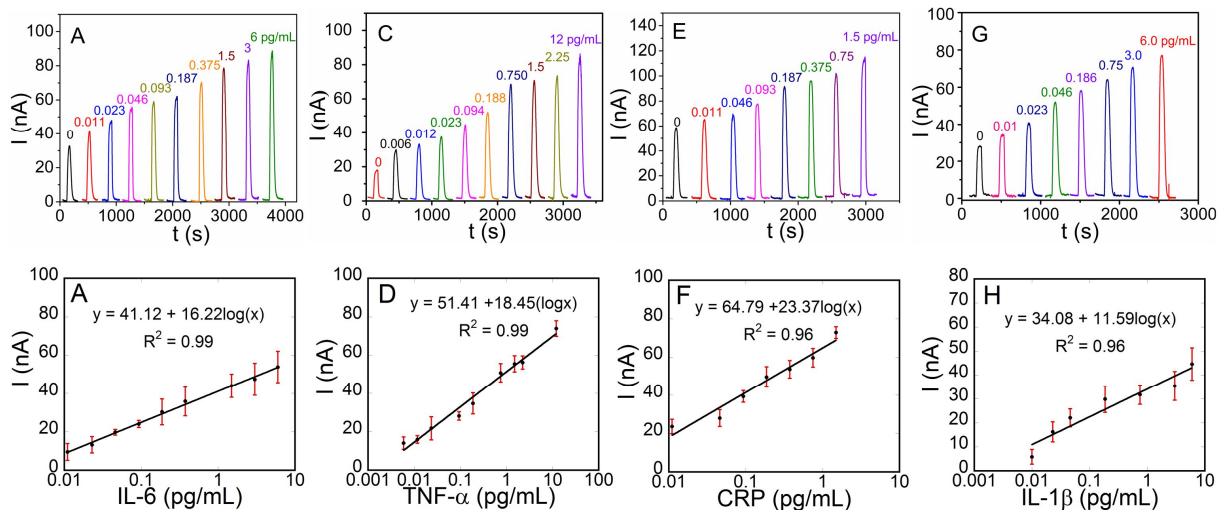
The stability of the capture antibody modified arrays was investigated up to 9 days after modification and found to be stable with minimal change in the response (Fig. S4).



**Fig. S4** Stability of the array sensor (n=8 electrodes) modified with capture antibodies. Signal responses were measured at day 1 and day 9 with very little change

### Single Protein Biomarker Detection

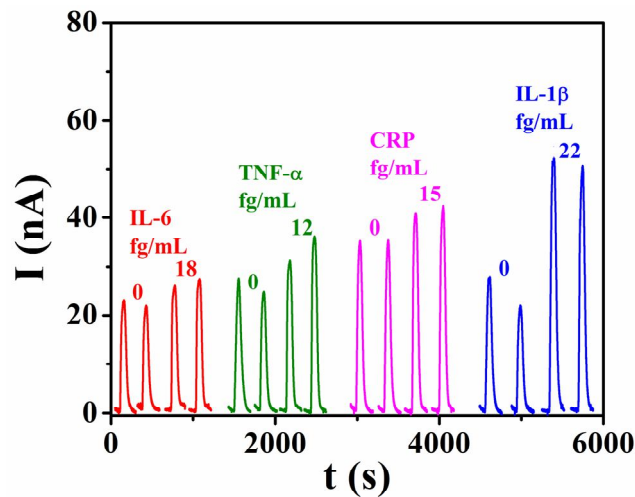
Representative amperometric responses for single biomarker protein detection (Fig. S5 A,C,E,G) show high sensitivity over at least 3 decades of concentration from  $\text{pg mL}^{-1}$  to the low  $\text{fg mL}^{-1}$  range. Amperometric signals were linear with respect to log protein concentration from  $\text{fg mL}^{-1}$  to low  $\text{pg mL}^{-1}$  with  $R^2$  values of  $0.98 (\pm 0.02)$  (Fig. S6 B,D,F,H). Relative standard deviations (n = 8) for single biomarkers were  $8 (\pm 3)\%$ . Detection limits (DLs) measured as three times the standard deviation above that of the control were  $11 \text{ fg mL}^{-1}$  for IL-6,  $6 \text{ fg mL}^{-1}$  for TNF- $\alpha$ ,  $11 \text{ fg mL}^{-1}$  for CRP and  $10 \text{ fg mL}^{-1}$  for IL-1 $\beta$ .



**Fig S5** Immunoassay calibration peaks for individual biomarker proteins in 5-fold diluted calf serum for (A) IL-6, (C) TNF- $\alpha$ , (E) CRP and (G) IL-1 $\beta$  developed by injecting a mixture of 1 mM HQ and 0.1 mM  $\text{H}_2\text{O}_2$  at -0.2 V vs. Ag/AgCl and the corresponding calibration plots for (B) IL-6, (D) TNF- $\alpha$ , (F) CRP and (H) IL- $\beta$

### Specificity of Multiplex Detection

Prior to multiplex detection, the specificity for each of the selected protein biomarkers was established on a single array for both a control, and sample (Fig. S6). The control array featured full immunoassay procedures without protein analytes. The sample array consisted of a standard mixture of 18 fg mL<sup>-1</sup> IL-6, 12 fg mL<sup>-1</sup> TNF- $\alpha$ , 15 fg mL<sup>-1</sup> CRP, and 22 fg mL<sup>-1</sup> IL-1 $\beta$ . Current peaks for control is a combination of residual non-specific binding from the bead conjugates (Ab<sub>2</sub>-MP-HRP) and the direct reduction of hydrogen peroxide. Fig. S6 demonstrates selective reproducible peaks with high signal/ noise ratios.

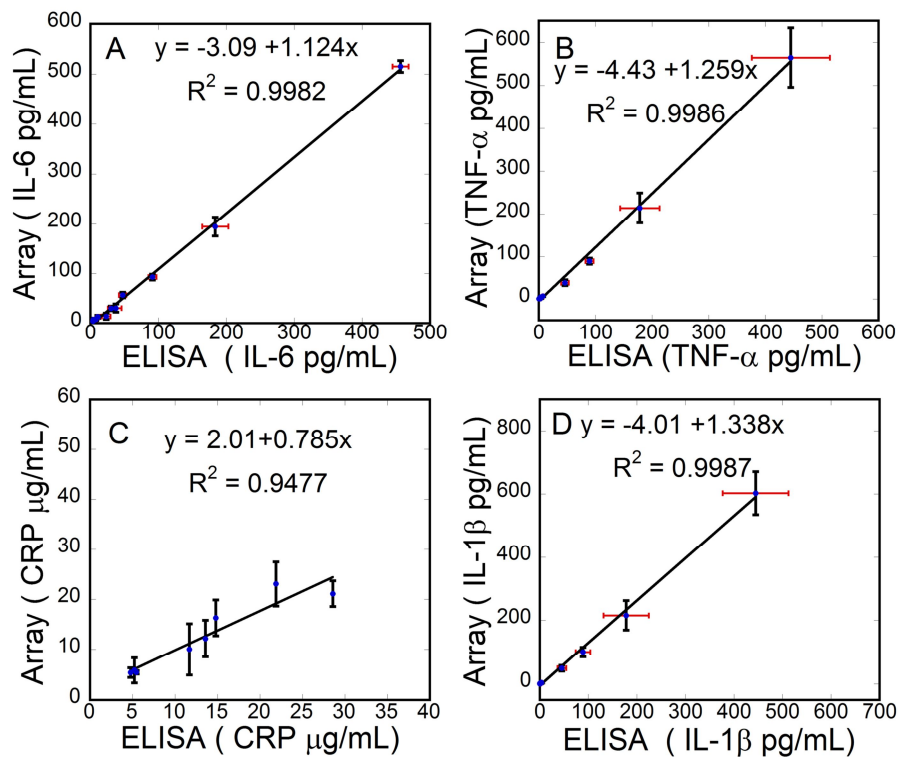


**Fig. S6** Duplicate responses measured simultaneously for control mixture of 0 fg mL<sup>-1</sup> for IL-6, TNF- $\alpha$ , CRP and IL-1 $\beta$ , and standard mixture of 18 fg mL<sup>-1</sup> IL-6, 12 fg mL<sup>-1</sup> TNF- $\alpha$ , 15 fg mL<sup>-1</sup> CRP, and 22 fg mL<sup>-1</sup> IL-1 $\beta$ , illustrating reproducibility and selectivity

### Assay validation with Human Serum Samples from Head & Neck (H&N) Cancer Patients

Serum levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  are in the pg mL<sup>-1</sup> range while CRP is in the  $\mu$ g mL<sup>-1</sup> range in oral cancer patients [7-9]. Reported mean serum levels of healthy individuals for IL-6, IL-1 $\beta$ , and TNF- $\alpha$  are 10-30 pg mL<sup>-1</sup>, and 30  $\mu$ g mL<sup>-1</sup> for CRP [7]. Serum levels before radiation therapy in patients with oral cancer were reported to increase to 20-190 pg mL<sup>-1</sup> for IL-6, IL-1 $\beta$  and TNF- $\alpha$ , and  $\geq$ 30  $\mu$ g mL<sup>-1</sup> for CRP [7].

On-line capture microfluidic array results were compared to standard ELISA results using human serum samples in patients with oral cancer (Fig. 3, main paper). Four patient samples were spiked with 50, 100, 200, and 500 pg mL<sup>-1</sup> standards for IL-6, TNF- $\alpha$ , and IL-1 $\beta$  to ascertain analytical recovery of the assay and extend the linear ranges of correlation plots (Fig. S7). Good correlation of our results with those obtained from standard single-protein ELISA measurements are illustrated in Table 3 with slopes approaching 1 and intercepts near 0.



**Fig. S7** Linear correlation plots of immunoarray results against those from individual ELISAs for human serum samples from cancer patients for (A) IL-6, (B) TNF- $\alpha$ , (C) CRP, and (D) IL-1 $\beta$ . ELISA assays for CRP (C) represent a single trial

**Table S1** Detection limits and sensitivities of the microfluidic immunoarray for single and multiplexed detection of the analyte proteins

<b>Proteins</b>	<b>Single Detection (DL fg/mL)</b>	<b>Multiplexed Detection (DL fg/mL)</b>	<b>Sensitivity (Single) (nA/cm<sup>2</sup>)/(mL/pg)</b>	<b>Sensitivity (Multiplexed) (nA/cm<sup>2</sup>)/(mL/pg)</b>
<b>IL-6</b>	<b>11</b>	<b>18</b>	<b>6.41</b>	<b>5.35</b>
<b>TNF-<math>\alpha</math></b>	<b>6</b>	<b>10</b>	<b>7.29</b>	<b>5.27</b>
<b>IL1-<math>\beta</math></b>	<b>10</b>	<b>40</b>	<b>4.58</b>	<b>4.18</b>
<b>CRP</b>	<b>11</b>	<b>15</b>	<b>9.23</b>	<b>7.07</b>

**Table S2** Data on the human serum samples from H&N cancer patients undergoing radiation treatment

Patient Samples	Visit	OMAS	WHO	Average Pain	Worst Pain
1	2	1.63	2	4	10
2	2	0.56	2	4	4
3	2	1.67	3	5	7
4	3	1.78	3	6	8
5	3	1	2	1	1
6	3	2.44	3	1	6
7	3	1.33	4	2	3
8	4	NA	NA	NA	NA
9	4	NA	NA	NA	NA
10	4	NA	NA	NA	NA

Key: Visit 2 – Day 14 of radiation, Visit 3 – Day 35 of radiation, Visit 4 – 21 days post-radiation (no scales obtained at the visit). Oral Mucositis assessment scores (OMAS) showing site scoring of ulceration and erythema with maximum score of 5 (severe mucositis). World Health Organization (WHO) oral toxicity scale; 0 (no mucositis), 1 (erythema of oral mucosa and pain), 2 (Ulceration present, can eat solid foods), 3 (Ulceration present, diet limited to liquids) and 4 (Ulceration present, no alimentation possible). Pain scores (0-10 numerical scale); 0 (none) and 10 (most severe). Pain scores– average and worst pain experienced by the patient since the last visit (one score for the entire period of 1-5 days between visits).

**Table S3** Slopes and intercepts of microfluidic immunoassay vs. ELISA correlation plots for the human serum samples from patients with H&N cancer

Proteins	Slope	Intercept	R <sup>2</sup>
IL-6	1.12 ± 0.014	-3.09 ± 1.84	0.998
TNF- $\alpha$	1.26 ± 0.019	-4.43 ± 2.51	0.999
IL1- $\beta$	1.34 ± 0.022	-4.01 ± 3.09	0.999
CRP	0.78 ± 0.11	2.00 ± 1.67	0.948

## References

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