Supporting information for the paper:

Multi-Parameter Determination of TNFα, PCT and CRP for Point-of-Care Testing

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Experimental

S1. Chemicals and proteins

Buffer salts (sodium hydrogen carbonate, sodium chloride, sodium phosphate, disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate), and Tween 20 were purchased from Merck (Darmstadt, Germany). LowCross-Buffer® was obtained from Candor Bioscience GmbH, Wangen im Allgäu, Germany.

HEPES buffer, bovine serum albumin fraction V (BSA, powder), human serum albumin (HSA, 97-99%, lyophilized powder), and polyethylene glycol (average MW 400) were bought from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Human recombinant procalcitonin (hrPCT, $MW_{recomb\ PCT}$=17,130 D; 114 amino acids fragment (3-116) with an amino-terminal hexahistidine tag) was purchased from Prospec-Tany Technogene Ltd., Rehovot, Israel.

Mouse anti-CRP mAbs C5 and C7, mouse anti-TNFα mAbs B-C7 and B-F7, CRP (≥95% pure, 21,000 D, from human fluids, Biodesign, Saco, ME, USA), and human recombinant TNFα (hrTNFα, predicted $MW_{recomb\ TNF\alpha}$=17,352 D, e-Bioscience, San Diego, CA, USA) were purchased from EXBIO Praha, Czech Republic. Rat anti-PCT mAbs PROC1 3G3 and PROC4 6B2 are in-house clones.[S1] In addition, detection mAbs C7, B-C7, and PROC4 6B2 were labeled with DY647 by EXBIO Praha, Vestec, Czech Republic.

S2. Materials and Instruments

Demineralized water was prepared by a Milli-Q (MQ) filtration system (Millipore, Eschborn, Germany) and was used for the preparation of all standard solutions and buffers.

One-way chips with PMMA (polymethyl methacrylate) prisms (11x23 mm) and double-sided tape were obtained from pes diagnosesysteme, Leipzig-Markkleeberg, Germany. This tape (shaded area, see Fig. 1, communication) left a fluidic channel open that had a capillary aperture of 50 µm, and it was 6 mm wide and 14 mm long) Spotter BioRobotics, UK, now Genomic

Solutions, Ann Arbor, MI, USA, was used for spotting the dots on the surface of these PMMA prisms. During the spotting process, the prism was hold by an especially designed holder, which was crafted by Fraunhofer Institute of Physical Measurement Techniques, Freiburg, Germany. The dots were spotted in diagonal (Fig. 1, communication) and had a distance of 4 mm to each other.

The optical immunosensor, including software, laptop, and squeezing mashine - the latter being used for mounting the PMMA prisms to the one-way chip - were obtained from Siemens, Munich, Germany.

S3. Optimization of spotting conditions for the capture antibodies

The three capture mAbs were diluted in separate vials using different spotting buffers with the following compositions:

- 4 mM PBS with 0.05% (v/v) Tween 20
- 4 mM PBS with 0.1% (v/v) Tween 20
- 4 mM PBS with 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA
- 4 mM PBS with 0.05% (v/v) PEG400.

Each dot was spotted ten times, which resulted in about 500 nL of solution on each spot of the surface of the PMMA prism. The spots were put on the chip diagonally (Figure 1 a) to permit an unimpaired access of the flow and reagents to the different mAbs during the succeeding assay procedure. This allowed also the spotting of the same capture mAbs (with different spotting buffers) on the same chip; no cross talk or influence between the immunoreagents was noticeable; only the corresponding pairs were binding to each other.

After all spots were added, the prisms were stored in the refrigerator (4°C). The next day, they were mounted to the sensor chip prior to the measurements and were used for the analysis of standard solutions or samples.

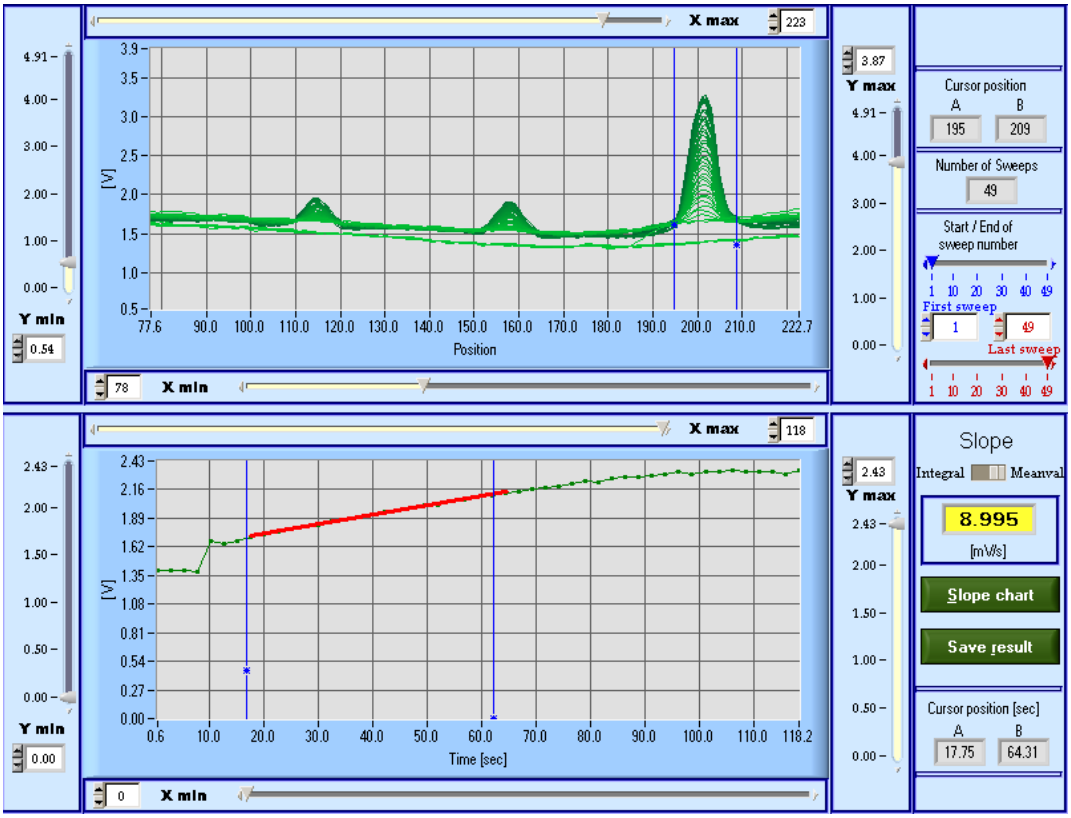
S4. Measurements with the optical immunosensor

The optical immunosensor consists of a bench-top optical read-out-device, which is controlled by a laptop. The disposable single-use low-cost sensor chip (75x24x10 mm) has an optical PMMA prism (11x23 mm,additional dimension, see section S2) and includes a fluidic system and a piston pump for the controlled transport of the fluids. The valve and the pump were interfaced with a cock drive and a plunger, the latter being a part of the bench-top device. The technology is based on evanescent field excitation of fluorescent markers and has been described earlier.[S2,S3] The combination of both fluorescence measurements and evanescent wave excitation provides extremely sensitive detection, thus avoiding washing or separation steps. The system enables measurements of any parameters carried out by means of receptor-ligand binding technology, where one of the components is labeled with a fluorescent dye.[S3-S5]

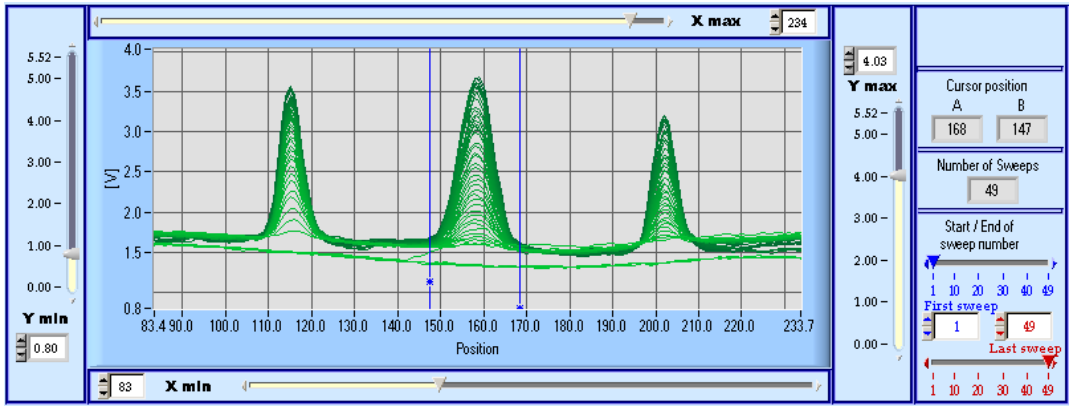
The PMMA prism was the surface for the immobilization of the three different specific capture mAbs for TNFα (B-F7), PCT (PROC1 3G3), and CRP (C5) (Fig. 1a and 1b, step 1, communication). After the immobilization via adsorption, the optical prism was attached to the carrier with a double-sided tape that had a capillary aperture of 50 µm, and which defined the flow channel for the fluidics. The flow channel had an inlet and outlet for the fluid. The immunochemical analysis started with a preincubation step for 20 min at 27 °C of the mixture of all three analytes at definded concentrations (or serum sample) with the specific fluorophore-labeled mAbs for TNFα, PCT and CRP, namely anti-TNFα B-C7-DY647, anti-PCT PROC4 6B2-DY647, and anti-CRP C7-DY647 (Fig. 1b, step 2, communication). Two hundred and eighty µL of this mixture were pipetted into the opening of the sensor chip, and the measurement was started and commenced in accordance with the software settings made previously on the computer. All steps were controlled by these settings, including the defined movements of the cock drive and the plunger. The mixture was pumped through the capillary aperture over the surface of the prism with a constant flow rate of 0.85 µL s^{-1} for 120 s, which refers to 102 µL. One the surface the immobilized capture antibodies were positioned (Figure 1b, steps 3-4, communication). The small capillary aperture ensured that the binding of the fluorophore-labeled mAbs-analyte complex to the immobilized capture mAbs was only insignificantly limited by diffusion processes. The light of a laser beam (λ_{ex} 635 nm) was used for the excitation of the fluorophore (Figure 1b, step 5a, communication). An evanescent field was hereby generated by total internal reflection of the beam at the interface between the high-refractive PMMA prism and the low-refractive liquid sample inside the flow channel. Only the fluorescence dye of the bound mAbs was excited to fluoresce due to the low penetration depth of the evanescent field of approximately 150 nm. A photomultiplier tube (PMT) was used for detection (λ_{em} 675 nm; Figure 1b, step 5b). The whole system was temperature-controlled with an operating temperature at 35.5°C. After about 2 min the measurement was complete, and the instrument indicated the end. Data were then transmitted from the bench-top device to the computer via the serial interface RS 232 for further evaluation. The results were recorded via the software Aqua-Optosensor, version 1.00 (Siemens AG, Munich, Germany). For evaluation of the results the position of the peak must be localized by setting cursor positions A and B to the beginning and the end of the peak, resp**ectively** (Figure S1 a, top right). Cursor position [s] A and B were set to maximize the period of time in which the slope was linear. The values were displayed in mV s^{-1}. The change in time of the fluorescence drawn from the evaluation is directly proportional to the binding concentration of the fluorophore-labeled mAb to the immobilized mAb (sandwich formation via the parameter) and therefore directly proportional to the concentration of the parameter in the standard or sample (Figures S1 a and b).

Theoretically up tp 12 spots could be spotted to the prism surface. The problem though is the software of the measurement instrument for the scanning of the signal. It does not allow the signal measurements when the spots are above (vertical) of each other. So therefore - for another

application - we spotted to a maximum of 6 spots in diagonal orientation on the chip. This number of parameters was not needed for this application though.



a)



b)

Figure S1. Original signals on the screen of the laptop. Parameters from left to right: hrTNFα, hrPCT, and CRP. (a) Concentrations of the different parameters were for hrTNFα 100 µg L^{-1}, hrPCT 915 µg L^{-1}, and CRP 4000 µg L^{-1}. (b) Concentrations were for hrTNFα 2000 µg L^{-1}, hrPCT 2500 µg L^{-1}, and CRP 3000 µg L^{-1}.

S5. Optimization of conditions for immunosensor measurements

As described above (see S4), a preincubation of the three parameters (with different concentrations, see below) together with their corresponding DY647-labeled mAbs took place for 20 min at 27°C. Solutions were mixed and shaken during this step. We have also tried 30 min (in

previous experiments and publications), but here 20 min was sufficient and therefore chosen during these measurements. The temperature was selected to have a constant temperature guaranteed above the room temperature (at least 5 °C). These parameters also showed good results in other experiments (with other antibodies and assays), and therefore we decided to use these conditions. The optimized mAb-DY647 conjugate concentrations were for anti-TNFα mAb B-C7-DY647 2 µg mL^{-1}, for anti-PCT mAb PROC4 6B2-DY647 3 µg mL^{-1}, and for anti-CRP mAb C7-DY647 5 µg mL^{-1}. These mAbs were diluted in the corresponding buffer used during the procedure (for example 10 mM HEPES/2.5 mM $CaCl_2$ with 0.05% (v/v) Tween 20, and 50% (v/v) LowCross-Buffer). LowCross-Buffer was originally designed for this sensor system and was always included in the different buffer compositions tested. The background signal (see Fig. S1) was lowered with the addition of LowCross, thus enabling to set the cursor positions for the determination of the slope of the peak correct (Fig. S1a).

Standard concentrations utilized for setting up the standard curve of each parameter varied and ranged for hrTNFα from 10 to 2000 µg L^{-1}, for hrPCT from 50 (or 100) to 3660 µg L^{-1}, and for CRP from 1265 to 6000 µg L^{-1}. The zero standard (0 µg L^{-1}) was again the corresponding buffer which was used during the procedure and was identical to the buffer composition of all standards. Usually, two chips were measured for one set of concentrations of the parameters. High concentrations of one parameter did not have an influence on the concentrations of the other parameters.

S6. Data processing

Curve fitting of standard curves was performed with SigmaPlot™ (Systat Software, Inc., Chicago, IL, USA) using the 4-parameter fit according to the following equation:

$$y = \frac{A - D}{1 + (\frac{x}{C})^B} + D$$

where A is the y-value corresponding to the asymptote at low values of the x-axis and D is the y-value corresponding to the asymptote at high values of the x-axis. The coefficient C is the x-value corresponding to the midpoint between A and D (e.g. µg L^{-1}; test midpoint (IC_{50})). The coefficient B describes how rapidly the curve makes its transition from the asymptotes in the centre of the curve.

Standard curves were then normalized to %control values according to the formula:

Control (%) = (S / S_0) x 100,

where S is the slope value for each standard, and S_0 is the slope value of the zero standard (buffer composition).

From these normalized standard curves, the working range of the assays was determined from 90% to 10% control for hrPCT and CRP, and from 80% to 10% control for hrTNFα.

S7. Measurement of human serum samples

Human serum samples were drawn from healthy volunteers (Sj07, Sj10, and Sj11; University Hospital of Graz, Austria) and had been analyzed for PCT and CRP by laboratory analysis. For the analysis of PCT the Brahms luminescence immunoassay (Brahms, Hennigsdorf, Germany) in the sandwich format (working range 0.1-500 ng mL^{-1}) was used. CRP was measured by immunoturbidometry (working range 1-228 mg mL^{-1}) on a Roche/Hitachi modular automated analyzer (Tina-quant® CRP (Latex) assay, Cobas (Roche Diagostics, Penzberg, Germany). Here anti-CRP mAbs are bound to latex particle and the sample is added. The antigen-antibody complex (agglutination) is then measured by turbidometry.

Samples analyzed with the immunosensor they were measured in duplicate - unspiked and spiked with different concentrations of the parameters. Serum was diluted 1:10 in the same buffer composition as the standards (for example in 10 mM HEPES/2.5 mM $CaCl_2$ with 0.05% (v/v) Tween 20 and 50% (v/v) LowCross-Buffer).

Supporting Information References

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