Supporting Information

AC Electroosmosis-Enhanced Nanoplasmofluidic Detection of Ultralow-Concentration Cytokine

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Device layout

**Figure S1.** ACE-LSPR optofluidic device layout. (a) Photo image of the ACE-LSPR optofluidic device chip. The scale bar is 1 cm. (b) Device layout showing the design incorporating three parallel meandering AuNR patterns (yellow) and six parallel sample loading/detection channels made of PDMS (blue). Inset: x10 darkfield image of the sensing spot formed at the intersection between the AuNR lines and the PDMS channels. Ref: ACEO-Decoupled LSPR Biosensor.

Figure S1 shows the detailed device layout, which consists of coplanar rectangular Pt microelectrodes, three parallel meandering lines of gold nanorods (AuNRs) (depicted in yellow), and six parallel sample loading/detection channels of PDMS (depicted in blue) on the same glass substrate. Each intersection between the AuNR lines and the horizontal sample loading/detection channels yields a sensing spot, which has an ACEO-coupled LSPR biosensor region between the microelectrodes and two ACEO-decoupled LSPR biosensor regions 200 μm away from the
microelectrodes. The ACEO-decoupled LSPR biosensor regions are used as references in control experiment (depict as Ref in Figure S1b inset). Three identical sets of the sensing spot were constructed within each sample loading/detection channel on the same chip for triplicate measurements.

**Gold nanorod (AuNR) Properties**

The AuNRs were purchased from NanoSeedz™, Hong Kong (product # NR-40-650). Each AuNR is 40 nm in diameter and 84 nm in length. The particle shape and size were chosen to yield high sensitivity and sufficient binding sites. The AuNRs were synthesized by a seed-mediated method and were stabilized by cetyltrimethylammonium bromide (CTAB). The measured ensemble-average scattering longitudinal plasmon resonance of these particles in water is at \( \lambda = 655 \) nm.

**Device fabrication**

Figure S2 shows the schematic of the ACE-LSPR device fabrication process. It started with deposition of parallel coplanar Cr/Pt (60 nm) plate microelectrodes (50 um wide, 25 um gap) on glass substrates (Figure S2a). A glass substrate was first treated with Piranha solution ((H2SO4:H2O2 = 3:1 v/v), rinsed thoroughly with DI water, and air dried before use. A layer of positive photoresist (SPR 220 3.0) was spin-coated on the glass substrate and photolithographically patterned using a darkfield photomask. An E-beam evaporation method (EnerJet Evaporator) was used to deposit a 10 nm-thick layer of chromium as the adhesion layer and then a 50 nm-thick layer of platinum on top of it. The Cr/Pt layer was subsequently lifted off using acetone and ultrasonication, which formed the microelectrode patterns. The Cr/Pt microelectrode-patterned glass substrate was then thoroughly rinsed with D.I. water and dried with \( \text{N}_2 \).
Figure S2. Schematic of the device fabrication process which includes: (a) Pt/Cr microelectrode patterning by sputtering and liftoff processes; (b) AuNRs deposition by O\textsubscript{2} plasma treatment, microfluidic patterning, and thermal annealing; (c) Bioconjugation of AuNR sensor sites with anti-IL-1\textbeta with EDC/sulfo-NHS linking using 16-Mercaptohexadecanoic acid.

The next step involved deposition and patterning of gold nanorods (AuNRs) to form the three parallel meandering sensor lines on the glass substrate using a microfluidic patterning technique (Figure S2b).\textsuperscript{2} The substrate was again Piranha cleaned (10 min) and then treated with O\textsubscript{2} plasma. The O\textsubscript{2} plasma treatment generates a negatively charged surface as a result of the dissociation of hydroxyl groups, which enables the glass substrate to attract the positively charged, CTAB stabilized AuNRs suspended in a colloidal solution onto its surface. Immediately after plasma treating, a soft-lithographically molded PDMS microfluidic channel layer was placed on the substrate with the straight-line segments of its meandering-shaped channels aligned to the microelectrodes under a long working-distance stereomicroscope. The AuNR containing colloidal
solution with a carefully determined particle concentration was loaded into the microfluidic channels from their inlets in both directions. A uniform monolayer of AnNR with an average inter-particle distance > 200 nm was settled on the substrate (Figure 1f in the main text), which was followed by a thermal annealing step\(^3\) (120°C, 1h) to increase the binding strength between the AuNRs and the substrate. The straight-line segments of the meandering channels formed the AuNR sensor lines.

The third step was biofunctionalization of the AuNR patterns with antibodies (Figure S2c). After removing the aforementioned PDMS layer, the ACE-LSPR biosensor glass substrate was rinsed with pure ethanol. An O\(_2\) plasma descum process was used to clean the substrate surface and to remove the excess CTAB. The substrate was then immersed in 0.5mM of 16-Mercaptohexadecanoic acid (MHA) dissolved in ethanol overnight to form a uniformly coated self-assemble monolayer (SAM). Finally, the substrate was rinsed with ethanol and DI water, and the chip was covered with another soft-lithographically molded PDMS layer with straight sample loading/detection channels (400 \(\mu\)m in width and 80 \(\mu\)m in height). These channels were aligned orthogonal to the directions of the microelectrodes and the AuNR sensor lines. A solution of 0.4M EDC and 0.1M sulfo-NHS was loaded into the microchannel and incubated for 40min to activate the carboxylic acid groups of MHA. Rinsed with DI water, the chip was incubated for 1.5h by loading 50ug/ml anti-IL1β solution and then passivated by SuperBlock™ (PBS) Blocking Buffer (ThermoFisher Scientific) for 30min, and finally became ready for the real-time LSPR biofunctional nanoparticle imaging study.
Experimental setup and ACE-LSPR imaging technique

Figure S3. (a) Schematic of the dark-field microscopy setup used in this study. (b) Illustration of the ACE-LSPR biofunctional nanoparticle imaging process. (c) Photo image of the ACE-LSPR device mounted on the motorized X-Y stage of the dark-field microscopy setup.

Figure S3 shows the dark-field microscopy setup used for ACE-LSPR biofunctional nanoparticle imaging and signal detection. The molecular binding on the AuNR surface induces a red-shift of scattering spectrum due to the change of local refractive index as well as an increase of the particle scattering cross-section. The electron multiplying charge coupled device (EMCCD) was implemented to quantify the signal intensity enhancement here by coupling with a proper band-pass filter (680nm/13nm). We also used a fiber spectrometer to check the extinction spectrum of the entire AuNR substrate and roughly monitored the spectral shift at each bio-conjugation step. The ACE-LSPR biofunctional nanoparticle imaging process involved mounting the ACE-LSPR device chip on a motorized X-Y stage (ProScanIII, Prior Scientific, Rockland, MA), loading a
sample of 5 μL into each microfluidic channel by a syringe pump, and then performing time-lapse recording and automated image scanning across the entire sensing spots within the same channel. The optical intensity signal was averaged over the sensor surfaces, each holding a large number of AuNR biosensor nanoparticles, using a customized MATLAB code developed in our lab.

**Modeling of electrical double layer**

Modeling the ACEO effect accompanying our device operation is a key task of the theoretical part of this study. First, the long coplanar plate electrodes immersed in aqueous electrolyte were modeled as a series of distributed capacitors associated with the electrical double layer and semicircular resistors due to the electrolyte. The potential drop across the electrical double layer is given by

\[
\Delta \phi_{EDL} = \frac{V_0}{2 + i\omega \pi (\varepsilon / \sigma) \kappa x}
\]

where \(V_0\) and \(\omega\) are the peak value and frequency of the applied AC bias, respectively, \(\varepsilon\) and \(\sigma\) are the permittivity and conductivity of the electrolyte, respectively, \(\kappa\) is the reciprocal Debye length, which is the function of the ionic strength of the solution, and \(x\) is the distance from the center between the two coplanar microelectrodes. This equation is valid when \(V_0\) is small enough to avoid an additional electrochemical effect and \(\omega\) is much smaller than the relaxation frequency of the solution. Since the thickness of the electrical double layer is small compared to the microfluidic channel height, a linear relationship was assumed between the surface ion charge density \(\sigma_s\) and the potential drop across the diffuse layer \(\Delta \phi_{EDL}\) as \(\sigma_s = \varepsilon \kappa \Delta \phi_{EDL}\). The electrical force acting on the fluid was derived by Coulomb’s law, which is opposed by the viscous force of the fluid as
\[ F_E = E_t\rho_e dy = -\mu \left( \frac{d^2 u_{ACEO}}{dy^2} \right) dy \] (2),

where \( E_t \) is the tangential component of the electric field, \( \rho_e \) is the charge density, \( \mu \) is the dynamic viscosity of the solution. Integrating Equation (2) from infinity (\( y = \infty \)) to the electrode surface (\( y = 0 \)) and combine the surface charge expression, we derived the AC electroosmosis velocity \( u_{ACEO} \) as shown in the main text. It should be noted that although a more complicated electrical double layer modeling may be needed especially for high ion concentration conditions, this simple circuit model fairly well predicts the experimentally observed flow velocity fields in the solution, and thus is sufficient for providing insights into the physical phenomena governing the ACE-LSPR device performance.

Flow visualization

AuNRs (l=84 nm, d=40 nm) were used as tracer particles to visualize the flow patterns within the fabricated device. The particle motions were observed using the above-described dark-field microscope (Figure S3) and recorded by the EMCCD camera (Supporting Video 1). We selected the operating voltage and frequency in a buffer solution with varying ion concentration such that the fastest particle motions were visually observed from the dark-field image. The buffer solution we used were 0.001x phosphate-buffered saline (PBS) and 50% DI diluted human serum matrix (HSM). The colloidal medium suspending gold nanoparticles was first centrifuged in a tube. The original medium was collected from the tube using a pipette and replaced by the PBS solution. The gold nanoparticles in the PBS solution were loaded into the microfluidic channels of the ACE-LSPR device. The gold nanoparticles are much smaller than fluorescent latex spheres normally used in conventional particle image velocimetry for flow visualization. The small mass of the nanoparticles together with the non-fluorescence detection approach has the potential to visualize
even weak flow patterns in a nanofluidic environment without photobleaching. Supporting Video 1 shows nanoparticle motions resulting from a vortex flow pattern whose center of circulation is close to the electrode edge as predicted by our theoretical calculation. As the buffer was switched from PBS to HSM, the speed of the nanoparticle motions significantly decreased due to an increase of the ion concentration in the medium. The video also shows flow patterns at different frequencies. The fastest nanoparticle motions were observed at 1 kHz in HSM, thus suggesting that it is close to the optimum ACEO bias frequency. Note that some aggregation of nanoparticles due to the positive dielectrophoretic effect was visualized at the edge of the electrodes under condition $V_0 = 2V$ at $f=1$ kHz. This dielectrophoretic effect could cause non-specific adsorption of free background proteins occurred in serum.

2D micron resolution particle image velocimetry (μ-PIV) analysis of the visualized flow

This study developed a flow visualization technique to characterize ACEO velocity fields experimentally within the ACE-LSPR device based on 2D micron resolution particle image velocimetry (μ-PIV) analysis. An open source MATLAB toolbox\textsuperscript{5} was utilized to perform image processing and fluid velocity field measurement. We captured a video series of the gold nanorods moving in the 0.001x PBS buffer ($\sigma = 1.59$ mS/m) at $V_0 = 1$ V and 16.67 frame/s with the frequency varied using darkfield microscopy. Since the video were taken from the top view, the focal plane was fixed at 40 $\mu$m above the microelectrode surface to ensure the image quality and unidirectional flow. Some image preprocessings were first applied to enhance the image contrast, filter the background noise, and remove the very bright particle spots. A region of interest (ROI) was then selected around the electrode surface as depicted by the blue dotted line in Figure S4a. A mask image was placed at the center of the microelectrodes so that the out of plane flow will not be quantified. Cross-correlation functions were built up based on a fast Fourier transform (FFT)
algorithm. Lastly, the velocity field at the frame rate was visualized with arrows as shown in Figure S4a. Figure S4b shows the theoretical and experimental ACEO velocity values at $x = 17.5 \, \mu m$ from the center of the microelectrodes at frequency 200Hz. The scattered dots represent experimental values obtained by averaging the tracing particles’ $x$-direction velocity along the red dotted line (shown in Figure S4a) at $V_0 = 1V$ while the curve represents an simulation obtained at $V_0 = 0.613V$. Here, anticipating experimental uncertainties associated with our electronics setup, the value of $V_0$ in the simulation was slightly corrected to be lower to match the theoretical ACEO velocity value with the one measured at the operational frequency of 200Hz. Note that the bell-shaped simulation velocity is slightly narrower than the experimental results. A similar discrepancy was also found by N.G. Green et al.,\textsuperscript{6} which is likely to be caused by the simplified linear assumption in the electrical double layer model as mentioned above and the deviation of the relaxation time $\tau = \varepsilon/\sigma$ associated with the fabricated electrodes from the theoretical value. Nonetheless, combining the $\mu$-PIV measurement and the theoretical mass transfer calculation allows us to accurately estimate the upper bound for the ACEO enhancement of biosensing.
Figure S4. (a) Fluid velocity field measured by 2D micron resolution particle image velocimetry (μ-PIV) analysis. Images of the gold nanorods moving in the 0.001x PBS buffer ($\sigma = 1.59$ mS/m) were captured at 16.67 frame/s using darkfield microscopy with a focal plane at 40 μm above the microelectrode surface. (b) Theoretical and experimental ACEO velocity at $x = 17.5$ μm from the center of the microelectrodes at 200Hz. The scattered dots represent experimental values obtained by averaging the tracing particles’ x-direction velocity values along the red dotted line in (a) at $V_0 = 1$V. The curve represents simulation results at a corrected value of $V_0 = 0.613$V.

Parameter Selection

Table S1 summarizes key parameters used for our simulation and device design. The dissociation constant $k_D$ was estimated based on published cytokine binding kinetics.$^{7-8}$ Note that the electrode thickness was carefully selected to be 60 nm in our device, higher than AuNR’s diameter (40nm) to ensure the binding is well-protected from the movement of the electrical double layer. Previous study$^9$ suggests that too high a flow velocity on the electrode surface at a high voltage may pull the antigen out of the bond, thus causing a negative effect.

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<th>Simulation Parameter</th>
<th>Design Parameter</th>
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<td>$k_D$ (M)$^{10-11}$</td>
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**Table S1.** Simulation and design parameters selected for the ACE-LSPR device.

**Two-tailed heteroscedastic t test for 5min initial slope analysis**

The initial slope analysis for analyte binding curves under ACEO operation (ACE-LSPR binding curves) provides an effective and advantageous way to reduce the sampling-to-answer time for assays using label-free biosensors.$^{10}$ Plotting the initial slope value as a function of the IL-1β concentration yielded a calibration curve showing a similar trend to the conventional method analyzing the signal intensity at the end point of the analyte binding assay. We found out that the selection of the time period from the starting point of the ACE-LSPR binding curve for which the initial slope value was extracted (initial slope extraction time) strongly affected the extraction of the calibration curve. To determine the minimum initial slope extraction time required for reliable calibration curve extraction, we performed a two-tailed heteroscedastic t-test. This analysis involved comparing the initial slope values obtained for the extraction time of 1, 2, 3, …, 7 min (shown in Figure 4a in the main text) with the one obtained for the extraction time of 8 min, as shown by Figure S5. Here, fitting to the ACE-LSPR binding curve data within the first 8 min was found to yield the same initial slope value as fitting to the whole binding curve covering the entire incubation period of 15 min. Thus, the analysis took the value for the initial slope extraction time
of 8 min as the reference. All the initial slope values obtained for time periods longer than 5 min show statistically insignificant differences (P>0.2) while these values were distinct from those obtained for time periods shorter than 3 min (P<0.05). Thus, we concluded that the incubation time of 5 min was long enough to yield an ACE-LSPR binding curve for which we can obtain consistent standard curve data. The initial slope analysis for the 5 min incubation allows us to perform the rapid analyte quantification for patient samples.

Figure S5. Initial slope values obtained from analyte binding curves under ACEO for the initial slope extraction time of 1 to 8 min. The time period of 5 min was determined as the minimum period to obtain the calibration curve. P values were calculated between the initial slope values for 1 to 7 min and the value for 8 min for the IL-1β concentration at 100 pg/mL, 500 pg/mL, and 1 ng/mL. *, p-value < 0.05; **, p-value < 0.01; NS, no significant difference.

ELISA correlation test

The Invitrogen™ 96-well Human IL-1β ELISA Kit was used to quantify the level of human IL-1β in the same patient serum sample as we used for our ACE-LSPR assay. For the both tests, two identical sets of serum sample were prepared during each freeze-thaw cycle. The patient serum samples were diluted two times by DI water, and the standard curves were obtained using 50%
diluted heat inactivated and charcoal stripped human serum matrix (EMD Millipore) spiked by known concentrations of IL-1β. Figure S6 shows an excellent correlation between data obtained from the ACE-LSPR assay and ELISA for both spiked-in serum samples and patient samples for the IL-1β concentration ranging from 1pg/ml to 1000pg/ml.

**Figure S6.** Correlation between data obtained from the ACE-LSPR and ELISA for both the spiked-in serum samples and patient samples with the IL-1β concentration ranging from 1 to 1000 pg/mL.

**REFERENCES**