Supplementary Information

Introducing AVAC as an Ultra-Sensitive Platform with Broad Dynamical Range for High-Throughput Multiplexed Biomarker Detection Using Digital Counting of Plasmonic Nanoparticles

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1. 2D-histograms and example dark-field images for the p24 and IL-6 studies

For the two studies “Ultra-Sensitive Detection of HIV p24 Protein” and “Precise Quantification of Interleukin-6 (IL-6)” in the publication, AVAC measurements were performed as described in the “AVAC measurements and analysis” section in Materials. 13×13 images were taken per well, of which the central 9×9 images were used to define the particle classification and to derive the monomer counts.

The two-dimensional histograms (blue color vs. brightness) were calculated for the whole plate, see Figure S1 below, and the particle classification was automatically derived.

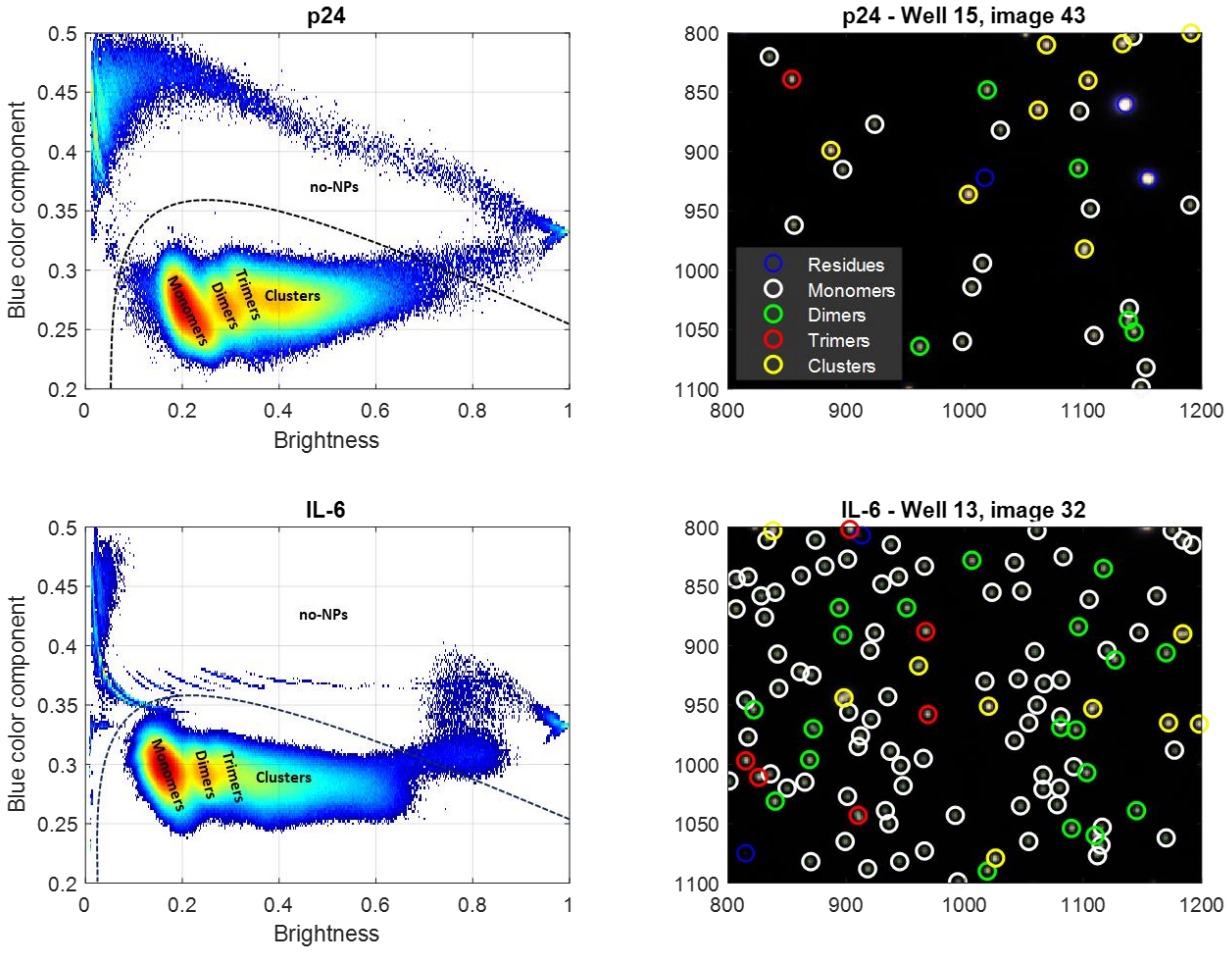

Figure S1: Two-dimensional histograms (left) and zooms of example AVAC images (right), for the p24 (top) and the IL-6 study (bottom).

For the classification, the nanoparticles were first separated from the other particles (“no-NPs”, “Residues”), as indicated by the dotted black line in the histograms. Second, the nanoparticle distribution was classified into monomers, dimers, trimers, and larger agglomerations (“clusters”).

Application of the classification criteria resulted in each individual particle being assigned to one of the classes. This classification is illustrated by zooms of two sample AVAC images shown in Figure S1 (right):

Here, the residues (=non-nanoparticles) are marked with thin blue circles, and the nanoparticles with thick circles, the colors indicating the agglomeration state.

Only the monomer counts (particles marked with white circles in the images) were used as a measure of biomarker concentration, to ensure the highest possible specificity.

2. Dilution linearity experiment

A dilution linearity experiment was performed to validate the performance of the assay diluent. A high-concentration sample was used to prepare a dilution series in a low-concentration sample, to maintain the characteristics of the original matrix; the low-concentration sample used was below the LoD of the analyte. The GNP counts obtained from the serial dilutions of the high-concentration sample were interpolated in the calibration curves (obtained from spiked samples in assay diluent) to calculate the concentration. The correlation between the expected and observed concentrations is shown in Figure S2.

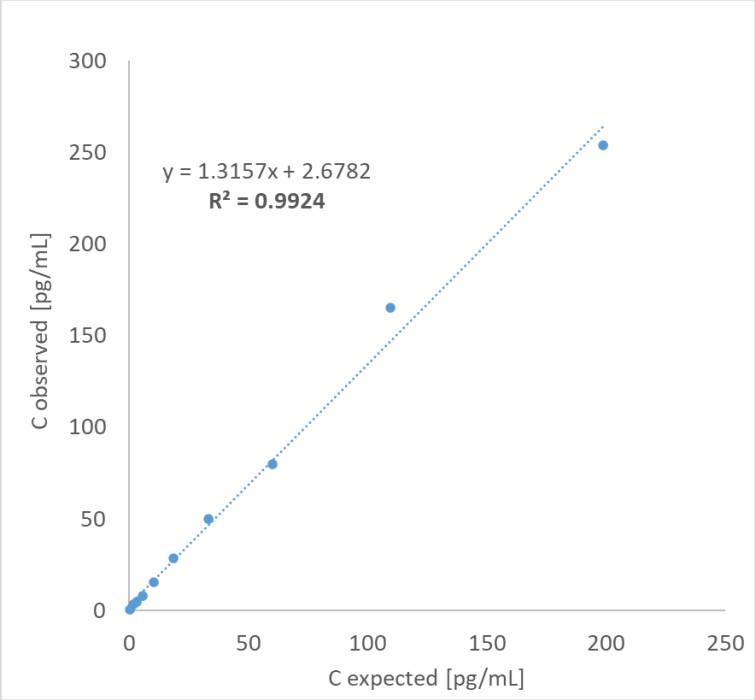

Figure S2: Correlation between expected and observed concentrations.

The data demonstrates that the AVAC technology maintains consistent sensitivity and specificity in both pure and complex matrices. In particular, the real-time response for a pure sample (spiked sample in assay diluent) showed a strong correlation ($R^2 > 0.99$) with the results obtained from the serum sample, confirming the robustness of the approach and the minimal impact of potential matrix effects from human serum.

3. Spatial multiplexing

In order to capture biomarkers in the triplex configuration, capture antibodies were spatially multiplexed on silicon wafers. This process involved subdividing the substrate corresponding to a single sample (a "well") into three equal areas, as illustrated in Figure S3.

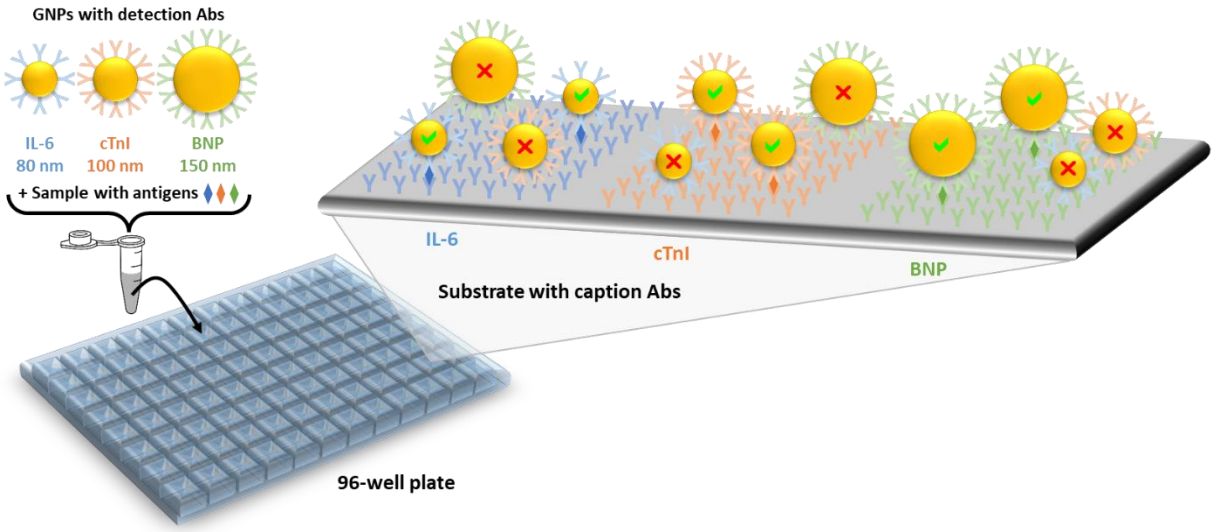

Figure S3: Spatial multiplexing. The substrate area of each of the 96 wells (see bottom left) is subdivided into three regions (top right), each one covered with one of the three capture antibodies (Abs). Each sample is mixed with the three types of GNPs (top left), and incubated in one of the wells.

Each of these areas was then covered with one of the three capture antibodies, with the antibodies against IL-6, cTnI, and BNP shown from left to right in the schematics.

The sample, which contained a mixture of all three biomarkers, was then combined with the three types of AuNPs, each of which had been functionalized with one of the detection antibodies (80 nm for IL-6, 100 nm for cTnI, and 150 nm for BNP). The mixture was incubated in the well, resulting in both the three intended sandwich complexes (marked with a green tick, e.g., IL-6 captured by IL-6 antibodies and labeled with 80 nm AuNPs) and six potential unspecific binding events (marked with a red cross, e.g., a 150 nm AuNP for BNP binding to an IL-6 detection antibody).

The analysis results demonstrate that the three types of AuNPs can be distinguished based on their characteristic brightness and color (see Figure 4A in the manuscript). In each of the three areas per well, only the number of the corresponding type of AuNP was used to derive the biomarker concentration. For example, only 80 nm AuNPs were used in the IL-6 area.

This approach allowed incubation of each sample with all three GNP types at once, as illustrated in the schematics, while at the same time minimizing unspecific cross-reactions, which otherwise would have caused an increase of the background signal, resulting in a deterioration of the specificity and thus of the accuracy of quantification.

4. Concentration dependence of monoplex and triplex assays

The concentration dependencies for the three biomarkers IL-6, TnI, and BNP were measured both in the standard monoplex configuration, and as a triplex assay as described in the previous section. From the replicates of each biomarker concentration, the mean values and their standard deviations were calculated, and fitted with a 5PL function, see Figure S4.

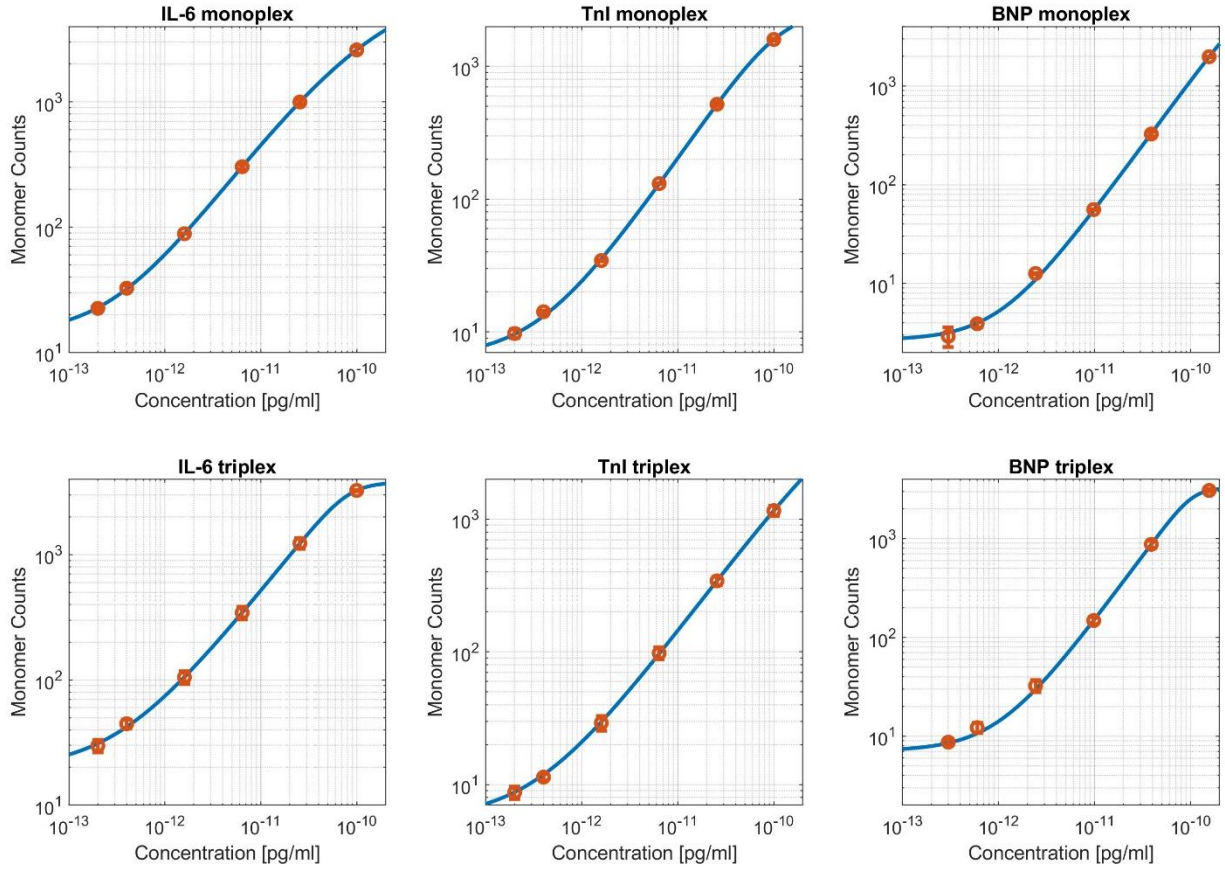

Figure S4: Concentration dependencies for the three biomarkers IL-6, TnI, and BNP (from left to right), as used in a monoplex (top) and a triplex (bottom) assay. The red circles and errorbars indicate the mean of the signal and its standard deviation obtained from the replicates per concentration; the blue line is the result of the fit of a 5PL curve to the experimental data.