Supporting Information

Multiplexed Salivary Protein Profiling for Patients with Respiratory Diseases using Fiber-Optic Bundles and Fluorescent Antibody-Based Microarrays

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Experimental Section

Microsphere Encoding and Microsphere Coupling with Capture Antibodies

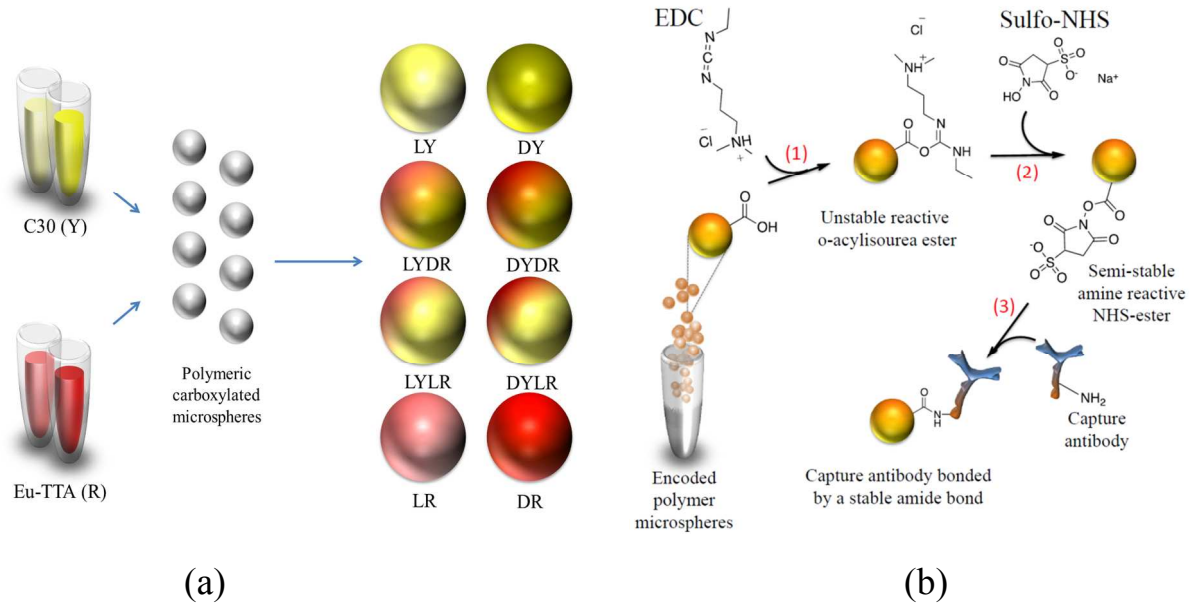

Figure S1. Illustration showing the microsphere encoding and coupling process. (a): Microspheres were internally encoded with Eu-TTA and C30 dyes to generate eight different encodings (seven were used in this study). Y; yellow; R: red; LY: light yellow; DY: dark yellow; LR: light red; DR: dark red. (b): Encoded microspheres were coupled with capture antibodies for different target proteins through surface carboxyl groups using the EDC and sulfo-NHS coupling method.

Image Analysis

Fluorescent images from three channels showing a small portion of a fiber-optic bundle are shown in Figure S2a-c. The images were analyzed using a custom-designed algorithm in Matlab R2009b. The analysis employed information from the Eu-TTA and C30 images (Figure S2a and Figure S2b, respectively) to decode the microspheres, and to calculate the fluorescent intensities of the different microspheres in the signal image (Figure S2c). Briefly, the automatic analysis process is described below:

- *Step 1: Filtering the Interested Pixels in the Eu-TTA image*
  Pixels in a user-defined intensity range in the Eu-TTA image were filtered (red dots in Figure S2d). All areas containing these pixels were put into a watch list for future processes.
- *Step 2: Classifying Interested Microspheres in the Eu-TTA image*
  The criterion for differentiating whether an area in the watch list would be retained or not was based on its shape. If an area contained a "hollow" center, then it implied that the interested area was actually not from an interested microsphere, but from some irrelevant microsphere(s) due to light attenuation. After classification, hollowed areas in the Eu-TTA image were automatically removed from the watch list. All retained areas were kept in the watch list and re-built as microspheres with a fixed diameter (red dots in Figure S2e).
- *Step 3: Refining the Interested Microspheres in Cou image*
  All microspheres from the watch list were further filtered based upon the responses in the corresponding C30 image. The microsphere would be retained only if the C30 intensities of all its pixels fall in the user-defined range (red dots in Figure S2f).
- *Step 4: Calculating Signal Strength*
  The intensities for all the pixels in a certain microsphere were averaged to obtain the signal of that microsphere. The signal of this microsphere type was calculated by the trimean of all microspheres in the watch list and calculated via the following equation, where $Signal_{VEGF}$ and $Signal_{Control}$ referred to the trimean intensity of the VEGF microspheres and the control microspheres, respectively.

$$Signal_{Normalized} = Signal_{VEGF} - Signal_{Control,}$$

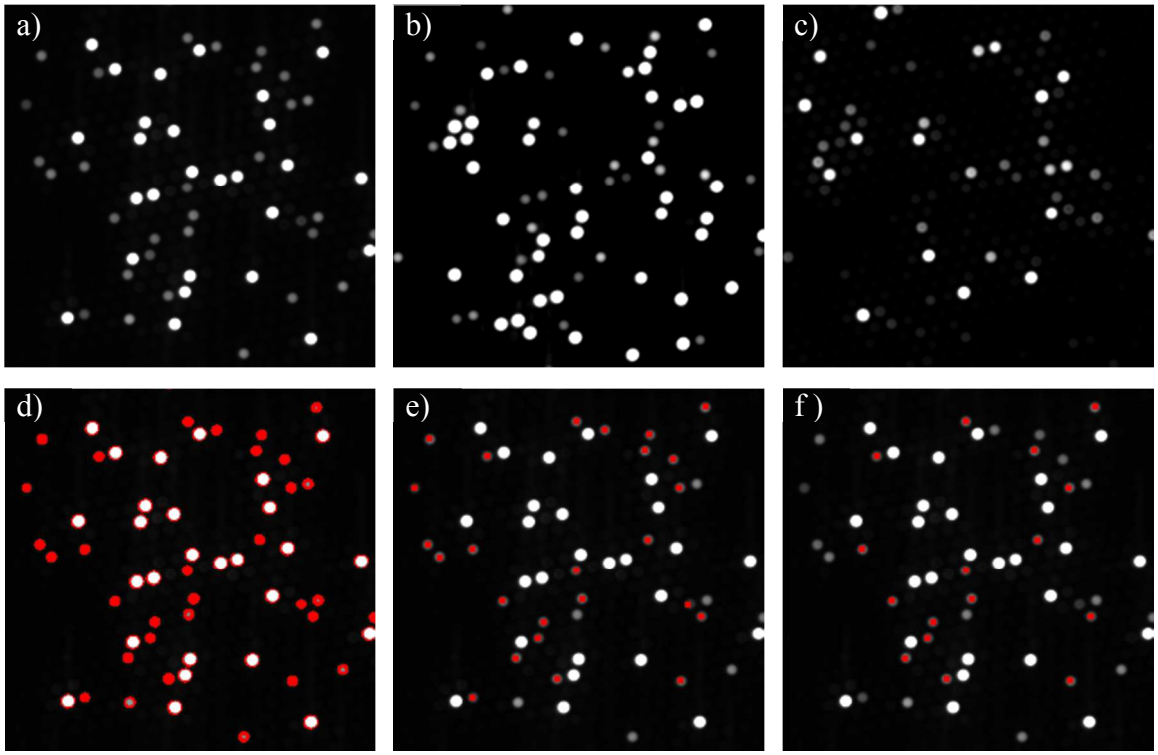

Figure S2. (a) Eu-TTA encoding image, (b) C30 encoding image, (c) signal image from the RPE channel, (d) areas in the watch list after step 1, (e) microspheres in the watch list after step 2, (f) microspheres in the watch list after step 3.

Results and discussion

Reproducibility of the Coupling Protocol

To evaluate the reproducibility of the coupling protocol, three batches of MMP-9 microspheres with different encodings were coupled by different researchers on different days using the exact same protocol. The responses from different batches of microspheres were tested by using multiplexed detection of 10 ng/mL MMP-9. The results from three independent tests are listed in Table S1. No statistical variance was observed between different batches of microspheres.

Table S1. Reproducibility of the coupling protocol. All responses were shown in arbitrary units.

Researcher	Coupling Date	Test 1	Test 2	Test 3	Average	Std
SN	01/12/2011	773.8	690.1	756.8	740.2	44.2
SN	01/26/2011	791.9	721.8	867.0	793.6	72.6
EBP	02/05/2011	787.9	686.0	940.6	804.8	128.1

Controlling the Signal Responses

The signal responses of the microspheres could be adjusted by controlling the amount of antibodies added in the antibody coupling solution. Based on the equation suggested by the manufacturer, 3.0 μg of IgG antibody is required to form a monolayer on 1 mg of 4.5 μm microspheres; this antibody concentration is henceforth named as 1×. To evaluate the responses of the microspheres modified with different amounts of antibodies, microspheres with different encodings were coupled with antibody amounts of 1.5 μg (0.5×), 3 μg (1×), 6 μg (2×), 15 μg (5×), and 30 μg (10×). The coupled microspheres were mixed and tested by both recombinant protein standards and human saliva samples using the multiplexed detection. The average results from four independent tests are shown in Figure S3. The signal intensities increased when more antibodies were added into the coupling solution. This strategy would be useful for multiplexed detection of proteins with wide ranges of concentrations.

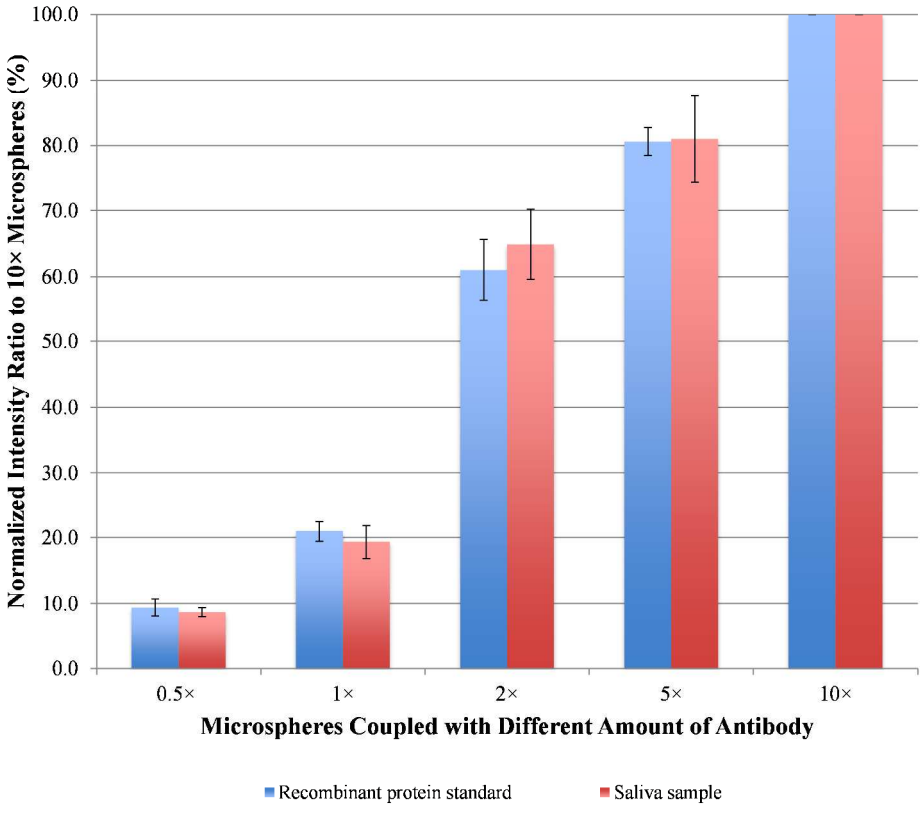

Figure S3. Normalized responses on microspheres coupled with different amounts of antibodies. Microspheres coupled with different amounts of antibodies were mixed and tested using recombinant protein standards and saliva samples. The responses from different microspheres were normalized to the signal from the "10× microspheres", the averages and standard deviations from four independent tests were shown..

Array Characterization

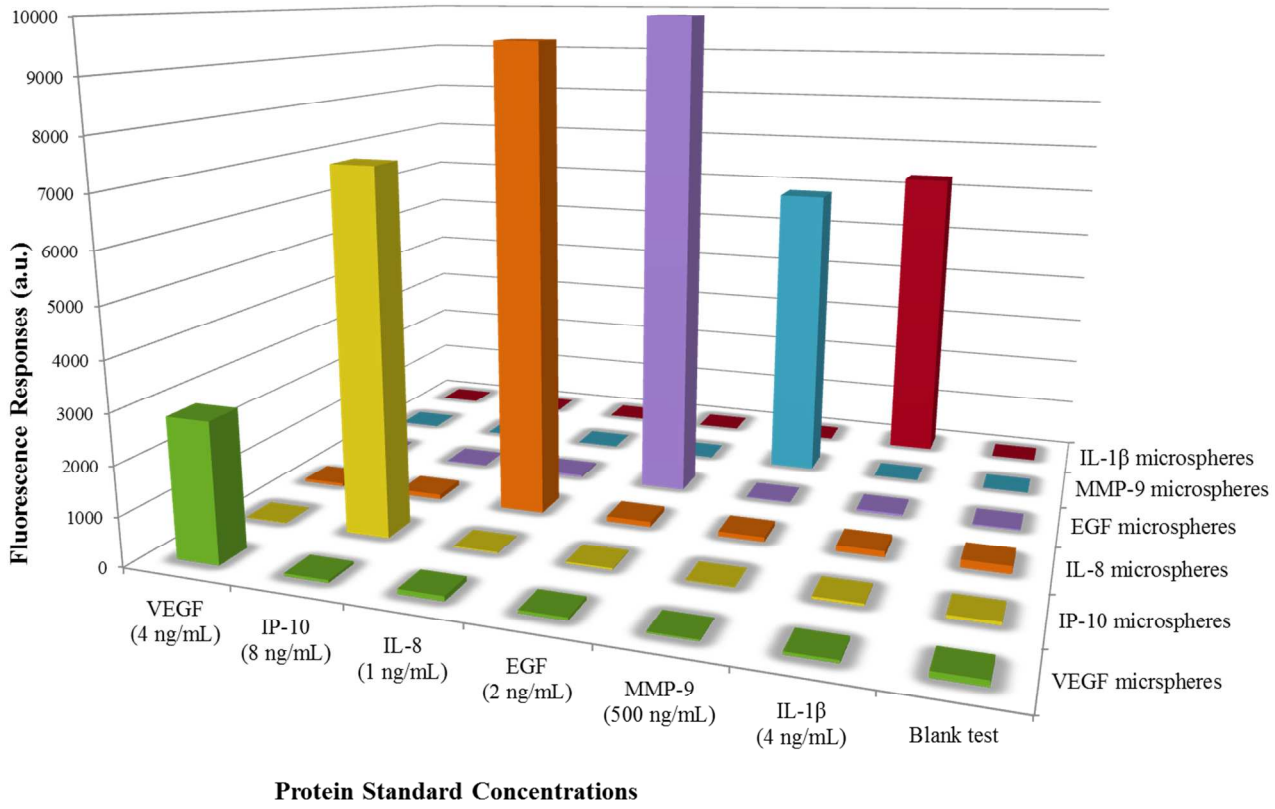

Figure S4. Results for both the cross-reactivity and blank experiments. A mixture of seven microspheres was sequentially incubated with a single protein, a mixture of all six detection antibodies, and SARPE in each experiment. The signal intensities of different microsphere types (shown in different colors) from all experiments are shown. The average responses from six blank tests (using PBSS buffer) are listed on the right as reference.