Multiplexed Sandwich Immunoassays using Electrochemiluminescence Imaging Resolved at the Single Bead Level

Frédérique Deiss[†], Christopher N. LaFratta[§], Matthew Symer[§], Timothy M. Blicharz[§], Neso Sojic[†,*], David R. Walt[§,*]

Supporting information

Preparation of the microstructured electrode

Hexagonal fiber-optic bundles 1.5 mm in diameter containing ~50 000 individual 3.1 μm fibers were custom-drawn by Schott (Southbridge, MA). They were polished using three lapping papers successively (40M, 12M, 0.3M). The microwells were formed by wet etching one face in a 0.025 M hydrochloric acid for 80 s and then rinsing it in water. Gold was sputtered under argon with two 30 s deposition steps using a Polaron SC502 at 7.10^{-1} mbar (~18 mA). To electrically connect the array a silver-coated copper wire was wound around the gold-coated fiber and fixed in place with silver paint (G302, Agar Scientific). Finally, one or two layer of black nail varnish was used to insulate the connection.

Preparation of the internally-encoded antibody-coated microspheres (3.1 µm)

See reference 1 in the communication.

Solvent and buffer

Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS) concentrate (10×) and tris-buffered saline (TBS) with Tween 20 were purchased from Sigma-Aldrich. Distilled deionized water (Milli-Q Water Purification system Millipore, Billerica, MA) was used for reconstituting reagents. TBS StartingBlock, PBS StartingBlock, and PBS Protein-Free blocking buffers were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Bioveris Assay buffer (pH=7.4), the ECL reading buffer containing tri-*n*-propylamine was bought from Bioveris.

Modification of streptavidin with $Ru(bpy)_3^{2+}$ complex (SA-Ru)

Streptavidin and Bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium N-succinimidyl ester-bis(hexafluorophosphate) were obtained from Sigma-Aldrich. 1 mg streptavidin was dissolved in 1mL of distilled deionized water. Then 100 µL of this solution was added in 810 µL of distilled water and 90 µL of PBS(10x) and mixed with 1 mg of the ruthenium complex, which was first dissolved in 100 µL of DMSO. This mixture was incubated 2 hours at 4 °C. Then it was purified by dialysis in PBS (1x). Slide-A-Lyzer Dialysis Cassettes 10k molecular weight cut off were bought from Thermo Scientific.

Immunoassay

Antibodies specific for human vascular endothelial growth factor (VEGF), interleukin 8 (IL-8) and tissue inhibitors of metalloproteinase 1 (TIMP-1); biotinylated detection antibodies complementary to each cytokine; and purified recombinant proteins were obtained from R&D Systems, Inc. (Minneapolis, MN).
Antigen storage aliquots were prepared in PBS 1x/BSA 0.1% and detection antibody storage aliquots were prepared in TBS StartingBlock.

First the array was blocked for 30 min. in 50 µL of PBS Protein-Free blocking buffer to avoid non-specific adsorption. The array was washed. Every washing step was done by dipping the array face of the optical fiber bundle in 100 µL of TBS with 1% Tween 20.1 µL of a cocktail of the three types of microspheres in PBS is dried on the array. The array was washed. Incubation for 2 hours in the antigen sample solution (dilute to the appropriate concentration with PBS StartingBlock). The array was washed. Incubation for 30 min. in 50 µL of the detection antibodies solution (3µg/mL for each type of antibody, in PBS StartingBlock). The array was washed.
A fluorescence image for mapping was then acquired. Incubation for 2 min. in the SA-Ru solution diluted to 20 µg/mL in PBS StartingBlock. The array was washed twice. ECL image was then acquired.

Luminescence Observation

An Olympus IX81 inverted microscope equipped with an Electron Multiplying Charge Coupled Device (EM-CCD) Camera (Hamamatsu, 9100-13) was used for imaging. The objective was an Olympus 60× 1.2 NA water immersion.

Photoluminescence was acquired during 2 s with a mercury arc lamp and a filter set from Chroma with excitation filter centered at 450 nm (FWHM 50 nm), emission filter centered at 630 nm (FWHM 60 nm) and a long pass dichroic mirror at 480 nm which corresponds to the Europium complex and the Ruthenium complex. For these images the camera was in standard mode.
For the electrochemical set-up, the working electrode was the optical fiber bundle, the counter electrode a platinum wire and the reference electrode an Ag/AgCl/KCl(3M) electrode. A cyclic voltammogram was performed with an Autolab PGSTAT30 potentiostat in 50 µL of BioVeris Assay Buffer, from 0 to 1.4 V vs. Ag/AgCl/KCl at 50mV/s.
The luminescence generated was acquired during 12 s started at 0.8 V vs. Ag/AgCl/KCl with the camera in the EMCCD mode, photon imaging mode #1, and with the gain set to maximum. The location of each bead type was determined according to the fluorescence image map. The ECL intensities of the pixels for each bead in the map were averaged to give a mean intensity value for a given type of bead. The cross-reactivity plot has been made using this method of quantification.

Note on the microspheres distribution
Comparing Figure 2A and 2C one notices that not all the beads are in wells and some have disappeared after the fluorescence imaging. This type of error occurs because some beads, less than a few percent, are not properly seated in the wells and shift when put in different solutions. This problem can be solved by more aggressive washing following bead deposition to remove loosely bound beads, which will be statistically inconsequential because the majority of beads are secured in the wells. To verify that it was beads moving and not spurious ECL, a photoluminescence image was taken after incubation in the SA-Ru solution which records the resulting photoluminescence of both the Europium complex and the Ruthenium complex.

Complete Ref. 3: Thorleifsson, G.; Walters, G. B.; Gudbjartsson, D. F.; Steinthorsdottir, V.; Sulem, P.; Helgadottir, A.; Styrkarsdottir, U.; Gretarsdottir, S.; Thorlacius, S.; Jonsdottir, I.; Jonsdottir, T.; Olafsdottir, E. J.; Olafsdottir, G. H.; Jonsson, T.; Jonsson, F.; Borch-Johnsen, K.; Hansen, T.; Andersen, G.; Jorgensen, T.; Lauritzen, T.; Aben, K. K.; Verbeek, A. L. M.; Roeleveld, N.; Kampman, E.; Yanek, L. R.; Becker, L. C.; Tryggvadottir, L.; Rafnar, T.; Becker, D. M.; Gulcher, J.; Kiemeney, L. A.; Pedersen, O.; Kong, A.; Thorsteinsdottir, U.; Stefansson, K., *Nat Genet* **2009**, 41, (1), 18-24.