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

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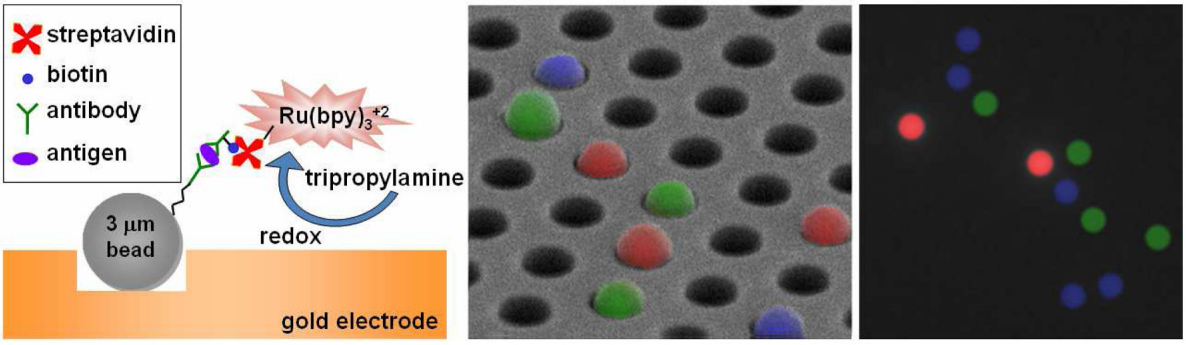
Abstract



A new class of bead-based microarray that uses electrogenerated chemiluminescence (ECL) as a readout mechanism to detect multiple antigens simultaneously is presented. This platform demonstrates the possibility of performing highly multiplexed assays using ECL because all the individual sensing beads in the array are simultaneously imaged and individually resolved by ECL. Duplex and triplex assay results are demonstrated as well as a cross reactivity study.

We report here the design and implementation of a new class of sensing microarray that uses electrogenerated chemiluminescence (ECL) as a readout mechanism to detect multiple antigens simultaneously. The approach involves attaching specific receptors to microbeads and leads to localization of all the ECL reagents on the microbeads where analyte has bound. The method enables multiplexed assays because all the individual sensing beads in the array are simultaneously imaged. Duplex and triplex assay results are demonstrated as well as a cross reactivity study.

We have previously reported multiplexed immunoassays using fluorescence as a readout mechanism.[1,2] Fluorescence is widely used for nucleic acid microarrays, which have been applied to genome-wide association studies where over 300,000 single nucleotide polymorphisms can be simultaneously analyzed.[3] While fluorescence assays are common[4], fluorescent labels suffer from photobleaching, furthermore, spectral overlap of the reporter

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dyes may limit the degree of multiplexing while luminescent impurities can interfere with the interpretation of results. Here we demonstrate ECL-based multiplexed imaging as an alternative to fluorescence because it is an extremely sensitive technique.[5,6]

ECL is a controllable form of chemiluminescence where light emission is initiated by an electron-transfer reaction occurring at an electrode surface. The most common system used for analytical purposes consists of the luminophore label $Ru(bpy)_3^{2+}$, or one of its derivatives, with tri-*n*-propylamine (TPrA) as a co-reactant.[5] The ECL mechanism involving TPrA with dissolved $Ru(bpy)_3^{2+}$ or with the ruthenium complex immobilized onto a bead is an active area of investigation.[7,8] A microbead-based ECL system was initially commercialized by IGEN[9] (technology later acquired by Roche Diagnostics Corp.) for a number of assays with a particular focus on immunoassays. This assay uses magnetic microbeads decorated with probe molecules, such as antibodies or nucleic acids, to capture analyte molecules in a sample. The microbeads are collected onto an electrode using a magnet. Analyte presence is measured by attaching a detection molecule, such as an antibody conjugated to a $Ru(bpy)_3^{2+}$ analog, and generating then ECL in a TPrA solution. The luminescence is collected with a photomultiplier tube. The light emission for a single analyte is therefore integrated from many thousands of microbeads. This system is not readily multiplexed because beads for different analytes are indistinguishable.

Multiplexed ECL assays have recently been commercialized by Meso Scale Diagnostics using specially made microtiter plates containing carbon electrodes in each well. Each electrode is coated with capture molecules to immobilize different analytes in a sample. Following attachment of a $Ru(bpy)_3^{2+}$ label, ECL intensity is measured from each well[10] but the ECL signal still originates from an effectively macroscopic surface.

In this report, we describe a bead-based platform that exploits ECL to detect three antigens simultaneously by individually imaging fluorescently-encoded microbeads located in a microwell array. This approach should enable the analysis of dozens of analytes simultaneously.

The multiplexed ECL platform consists of 3.1 μm polystyrene microspheres loaded into the wells of an electrode, prepared from etched fiber optic bundles[1] coated with gold (Figure 1). The surfaces of the microspheres were modified with capture antibodies. Microspheres with different antibodies are internally encoded with different concentrations of a europium (Eu^{3+}) dye to allow them to be distinguished from one another in the array. We pooled three bead types and allowed the beads to self-assemble into the wells on the electrode surface. The bead-based assay was performed by incubating the array first in a sample containing antigen and then in a solution of biotinylated detection antibody. The final step was to attach the ECL label by exposing the array to a solution containing a streptavidin-modified $Ru(bpy)_3^{2+}$ complex (SA-Ru). The $Ru(bpy)_3^{2+}$ label should bind only to beads where antigen is present, and can be detected by performing a cyclic voltammogram while viewing ECL from the array with a microscope. An EM-CCD camera was used to image the ECL emitted by each bead in the array.

Figure 2A shows an array with three types of beads for the antigens VEGF, IL-8, and TIMP-1. The false-color image (Figure 2B) corresponds to the three levels of Eu^{3+} fluorescence used to encode the three different bead specificities and was taken before exposure to the SA-Ru solution. The ECL image (Figure 2C) shows luminescence originating only from the IL-8 specific beads because only IL-8 was present in the sample. Well-separated ECL spots of micrometer dimensions reveal the locations of individual anti-IL-8 labeled beads. One can observe that the entire bead emits ECL light rather than only the small region in contact with the gold coated wells. This emission pattern suggests the ECL mechanism is predominantly

via the "revisited route", where TPrA radicals diffuse and react with immobilized Ru $(bpy)_3^{2+}$. Emission by the "catalytic route" may also occur at the beadelectrode interface.[7] This experiment demonstrates that the beads are individually readable in an array format using ECL imaging.

The cross reactivity plot in Figure 2D shows the results of three experiments where all three beads types were present on the array but the sample solution contained only one type of antigen. The average ECL signal from each bead type has been normalized to the brightest bead type in each experiment. Figure 2D highlights that cross-reactivity is negligible for these three antigens.

This platform demonstrates the possibility of performing highly multiplexed assays using ECL because the ECL emission from individual sensing beads is imaged. This capability was further demonstrated by exposing the array to sample solutions containing two or three antigens and simultaneously detecting them. The results of these experiments are displayed in Figure 3. The concentrations of the antigens in these studies are moderately high and were chosen only as a proof of principle demonstration.

In conclusion, we have demonstrated the ability to use ECL imaging as an alternative readout mechanism to detect multiple antigens in a microarray format. To our knowledge, these results represent the first time that individual sensing beads have been imaged by ECL in a multiplexed sandwich immunoassay. This new multiplexed ECL platform could be extended for the simultaneous analysis of dozens of analytes and for DNA assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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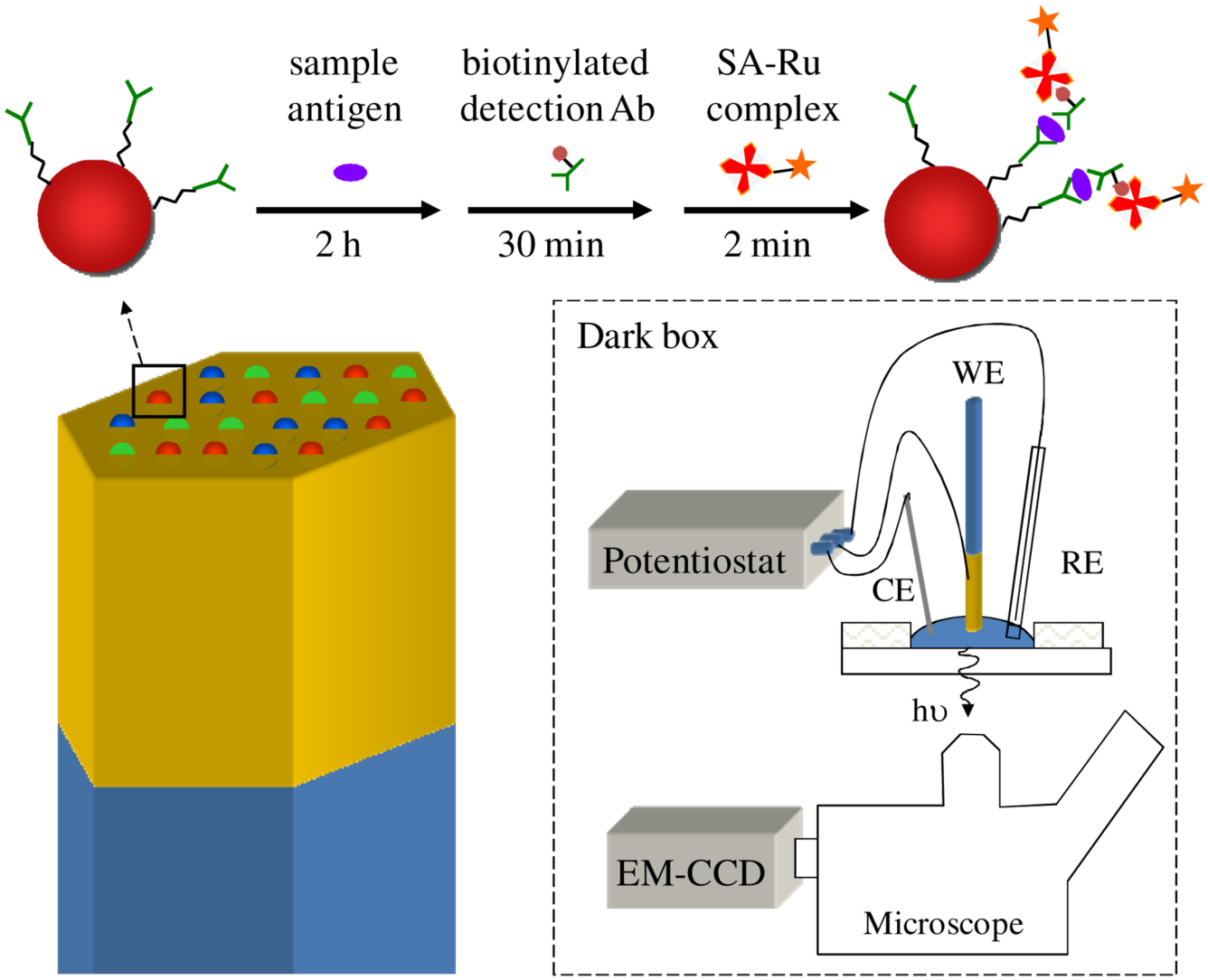

Figure 1.

(Top) A sandwich immunoassay is performed by exposing antibody-functionalized microbeads to three solutions: (1) an antigen containing sample, (2) biotinylated detection antibodies, and (3) streptavidin modified with a $Ru(bpy)_3^{2+}$ complex (SA-Ru). The beads are housed in microwells created from an etched gold-coated fiber-optic bundle. The gold coated fiber bundle acts as the working electrode (WE) for ECL. CE and RE refer to counter electrode and reference electrode.

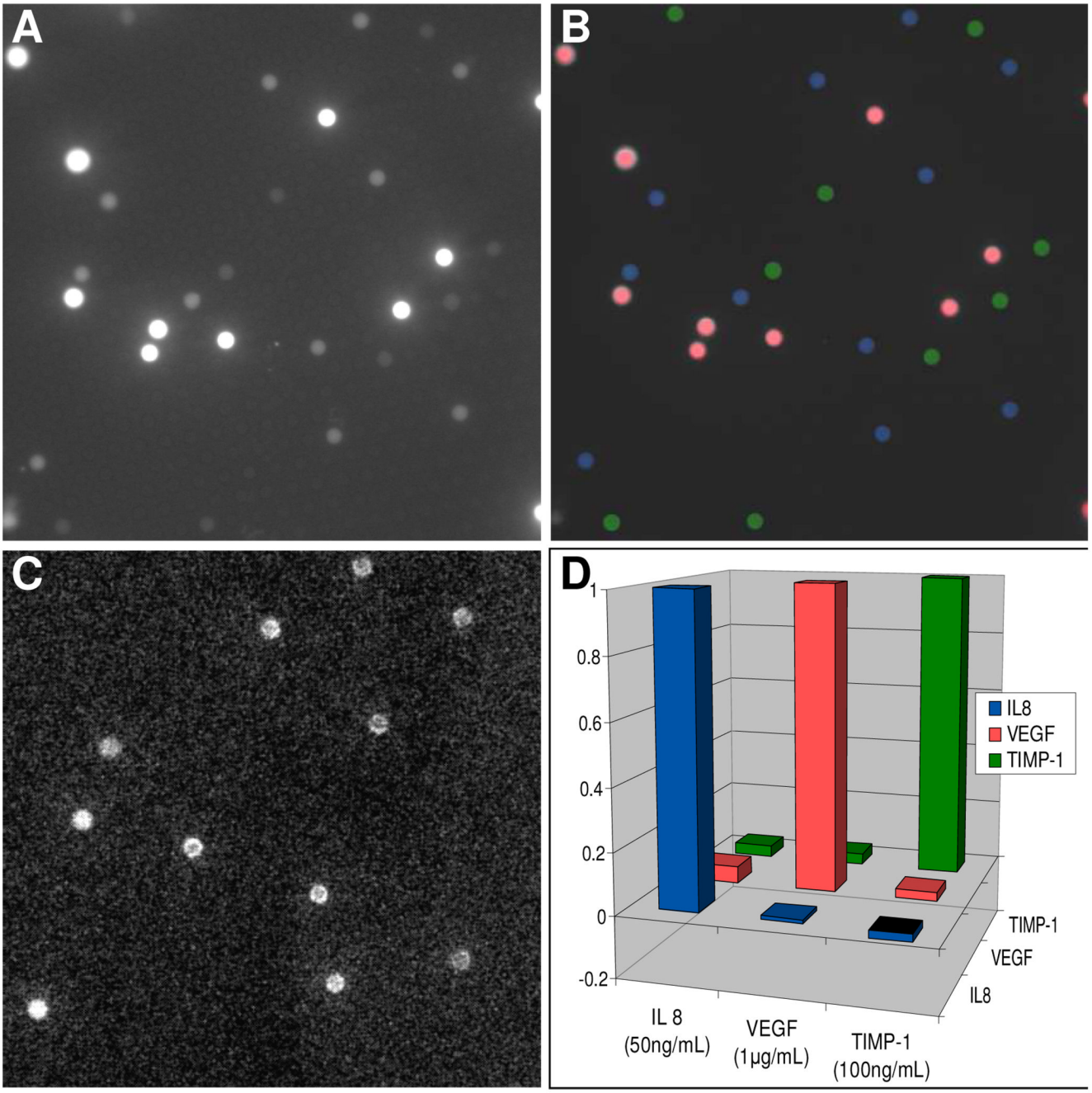

Figure 2.

(A) Fluorescence image showing the presence of anti-VEGF, IL-8, and TIMP-1 modified 3.1 µm beads, which are internally encoded with high, intermediate, and low amounts of Eu^{3+} dye, respectively. (B) False-color overlay of the region of interest (ROI) defined in (A) identifying anti-IL-8 (blue), VEGF (red), and TIMP-1 (green) beads. (C) ECL image of the same ROI following exposure to 50 ng/mL of IL-8. Note that only the blue beads give an ECL signal. (D) Normalized cross-reactivity plot for three experiments where a single antigen was presented to an array containing all three bead types. ECL images were acquired over a 12 s exposure time while the potential was scanned from 0.8 V to 1.4 V vs. Ag/AgCl/KCl in a 100 mM TPrA solution.

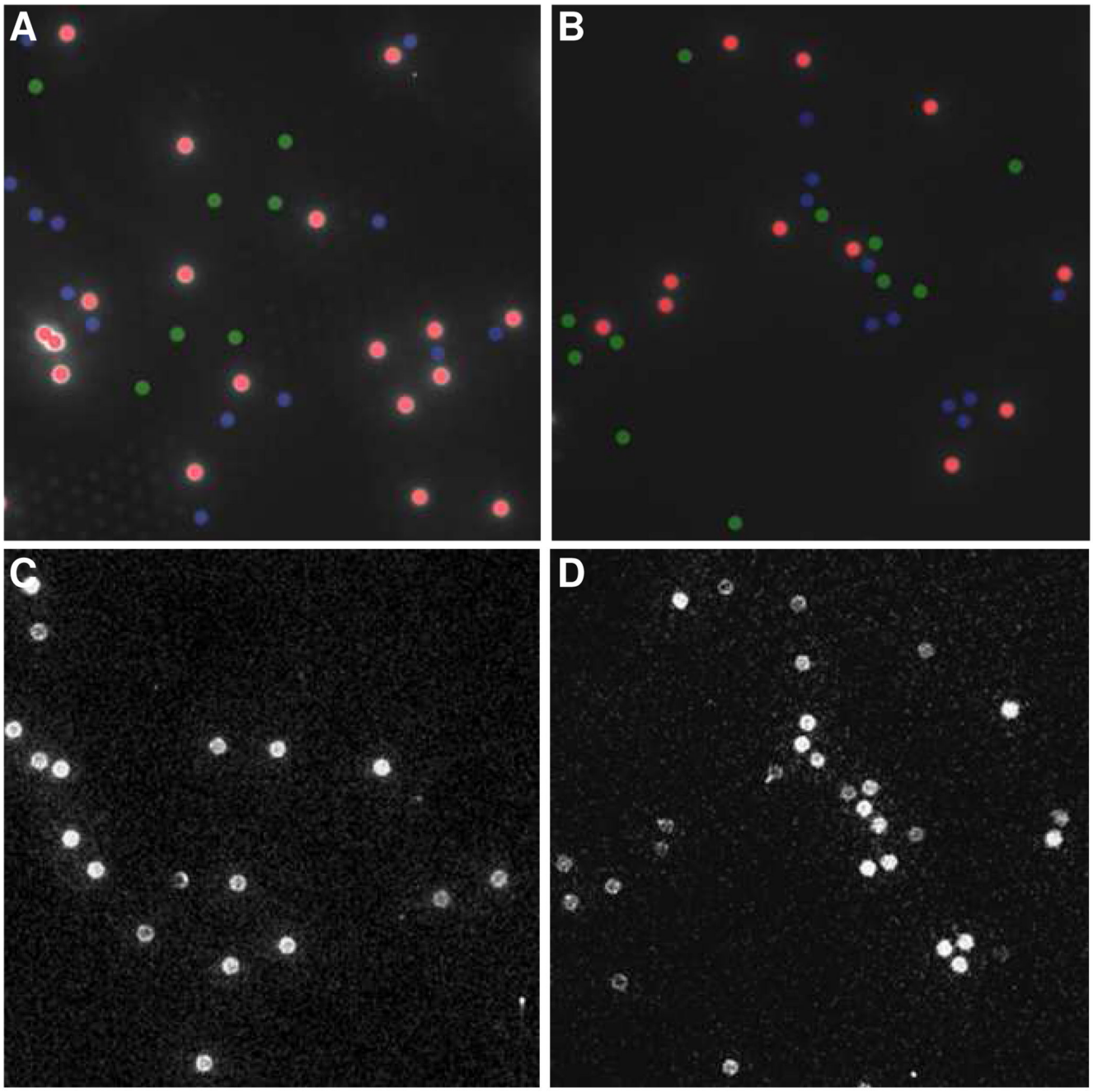

Figure 3.
(A, B) Fluorescence images of two arrays containing anti-VEGF, IL-8, and TIMP-1 modified beads (false-color overlay with color scheme same as for Figure 2). (C) ECL image of the array in (A) following exposure to a sample solution containing both [IL-8] = 50 ng/mL and [TIMP-1] = 1.5 μg/mL. (D) ECL image of the array in (B) following exposure to a sample solution containing [IL-8] = 50 ng/mL, [TIMP-1] = 1.5 μg/mL, and [VEGF] = 1.5 μg/mL