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Accelerated Articles

Chemiluminescence Detection for Hybridization Assays on the Flow-Thru Chip, a Three-Dimensional Microchannel Biochip

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Chemiluminescence (CL) detection is seldom used in two-dimensional solid support microarray platforms because adequate sensitivity and spatial resolution is difficult to achieve. The three-dimensional ordered microchannels of the Flow-thru Chip increase both the sensitivity and spatial resolution required for quantitative CL measurements on microarrays. Enzyme-catalyzed CL reactions for the detection of hybridizations on microchannel glass were imaged using a CCD camera. Signal uniformity, sensitivity, and dynamic range of the detection method were determined. The relative standard deviation of signal intensities across an array of 64 spots was 8.1%. A detection limit of 250 amol of target with a linear dynamic range of 3 orders of magnitude was obtained for a 3-h assay. Similar to two-color fluorescence measurements, multiple enzyme labels were employed to demonstrate two-channel chemiluminescence. A unique method for measuring the relaxation time of a chemiluminescent species is also described.

While fluorescence is typically used in microarray technologies, novel detection strategies such as chemiluminescence (CL) imaging provide an alternative detection method on multiplexed array-based systems. An attractive feature of CL is the inherently low background. Unlike fluorescence, where an excitation source is needed and nonspecific radiation can be produced, photons are generated only where the reactants are present. Consequently, in fluorescence imaging, some amount of signal can be generated in areas of the microarray where the fluorophore is not present. In CL imaging, however, nonspecific radiation is significantly reduced. Warmup and drift of the light source and interference from light scattering present in fluorescence methods are absent in CL, making the background component much lower. CL imaging of microarrays can be accomplished using much simpler instrumentation than the fluorescence counterparts. CL systems generally include only an imaging device such as a CCD and lack the excitation light source and wavelength selection optics of fluorescence systems.

CL is a process in which light is emitted from an excited-state product of a chemical reaction. The analytical performance of CL detection techniques is controlled by the efficiency of the reaction and the luminescent characteristics of the light-emitting species. A general reaction scheme for CL is shown below:

$$\text{catalyst} + \text{substrate} \leftrightarrow \text{catalyst}-\text{substrate complex} \quad (1)$$

$$\text{catalyst}-\text{substrate complex} \leftrightarrow \text{catalyst} + \text{converted substrate} \quad (2)$$

$$\text{converted substrate} \rightarrow \text{excited-state product} \quad (3)$$

$$\text{excited-state product} \rightarrow \text{product} + \text{light} \quad (4)$$

The signal intensity is the product of the catalytic turnover of the substrate (eqs 1 and 2) and the lifetime of the converted substrate (eq 3). The lifetime of the excited-state product (eq 4) is extremely short in comparison to the other steps and has no effect of the observed kinetics.[1] When the first-order reaction in eq 3 is slow, the CL reaction is termed a "glow" reaction. When the reaction is fast, it is termed a "flash" reaction. Glow reactions are characterized by a converted substrate formation period on the order of minutes and persistence of light emission at full intensity for several hours. Flash reactions are characterized by a formation period and light emission on the order of seconds. In terms of imaging techniques, signal duration allows the collection of many photons of light over an extended period of time, allowing low assay detection limits. However, the kinetics of the reaction

(1) Schaap, A. P. http://www.lumigen.com/CLAssays.html, 1998; Vol. 2001.

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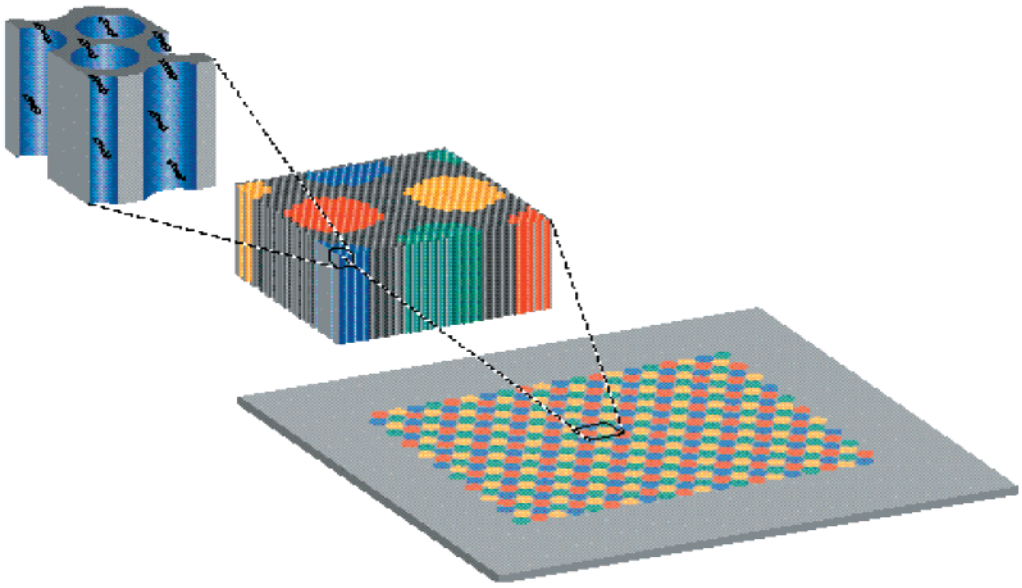

Figure 1. Conceptual schematic of a microchannel glass array. The chip is composed of an ordered array of microchannels that connect the planar surfaces. Analyte-specific reagents, or probes, are deposited on the chip in spots. Each spot incorporates several individual channels.

contribute to diffusion of the light-emitting species and thus lower resolution of the image. Flash CL reactions minimize diffusion, enhancing resolution and image analysis.

Few examples of CL detection on two-dimensional solid support microarray platforms appear in the literature. A reason may be that adequate sensitivity and spatial resolution is difficult to achieve.[2−4] Unlike fluorescence techniques where the fluorophore is bound to the surface, the light-emitting species in CL detection is not surface bound and freely diffuses in solution. In CL, the light-emitting species can therefore be excited, diffuse some distance, and emit light in a different region of the array, thus limiting the spatial resolution of the image. Although CL is widely used to image membrane-based assays with physical dimensions that are large on the molecular diffusion length scale, CL-based systems for imaging microarrays have yet to be put into practice.

Sensitive assays that utilize CL have been developed for a wide variety of applications.[5−7] Systems using the highly efficient CL reactions of 1,2-dioxetanes and luminol catalyzed by alkaline phosphatase and horseradish peroxidase have been developed,[8−11] including immunoassays[12−14] and membrane hybridizations.[15−18] We have developed a microarray platform in which molecular interactions occur within the three-dimensional volume of ordered microchannels rather than at two-dimensional surfaces.[19] The three-dimensional, ordered geometry of microchannel glass (MG) allows for sensitive and expeditious chemiluminescence detection of hybridization assays by localizing the light-emitting species within the microchannels. As illustrated in Figure 1, microchannels of the chip connect the upper and lower faces such that fluid can flow through the chip. The MG geometry permits dynamic hybridization assays, where solutions containing analytes can uniformly flow through the chip. MG chips are fabricated by depositing analyte-specific reagents, or probes, into groups of microchannels. Examples of probes that could be implemented on MG chips include small molecules, nucleic acids (RNA, DNA), proteins (ligand receptors, antibodies, enzymes), and cells. In the case of DNA analysis, as described herein, the probe molecules are single-stranded nucleic acids. Advantages for developing MG biochips include the following: (1) improved responsiveness and dynamic range; (2) reduced assay times; and (3) more uniform probe deposition and higher array densities. The additional surface area gained by adding depth to the chip allows for larger amounts of probe immobilization, thus increasing the binding capacity for target. The larger the amount of probe immobilized within a spot, the greater the responsiveness to target and the more signal per unit concentration. By physically confining the probe and target

(2) Akhavan-Tafti, H.; Reddy, L. V.; Siripurapu, S.; Schoenfelner, B. A.; Handley, R. S.; Schaap, A. P. *Clin. Chem.* **1998**, *44*, 2065−2066.
(3) Kasai, S.; Zhou, H.; Matsue, T. *Chem. Lett.* **2000**, 200−201.
(4) Roda, A.; Pasini, P.; Musiani, M.; Girotti, S.; Baraldini, M.; Carrea, G.; Suozzi, A. *Anal. Chem.* **1996**, *68*, 1073−1080.
(5) Kricka, L. J.; Stanley, P., E.; Thorpe, G. H. G.; Whitehead, T. P. *Analytical Applications of Bioluminescence and Chemiluminescence.*; Academic Press: London, 1984.
(6) Baeyens, W. R. G.; De Keukeleire, D.; Korkidis, K. *Luminescence Techniques in Chemical and Biochemical Analysis.*; Marcel Dekker: New York, 1990.
(7) Garcia-Campana, A. M.; Baeyens, W. R. G. *Chemiluminescence in Analytical Chemistry.*; Marcel Dekker: New York, 2001.
(8) Thorpe, G. H. G.; Kricka, L. J. In *Bioluminescence and Chemiluminescence: New Perspectives.*; Scholmerich, J., Andreesen, R., Kapp, A., Ernst, M., Woods, W. G., Eds.; Wiley: Chichester, U.K., 1987; pp 199−208.
(9) Thorpe, G. H. G.; Kricka, L. J. *Methods Enzyomol.* **1986**, *133*, 311−354.
(10) Beck, S.; Koster, H. *Anal. Chem.* **1990**, *62*, 2258−2270.
(11) Bronstein, I.; Edwards, B.; Voyta, J. C. *J. Biolumin. Chemilumin.* **1989**, *4*, 99−111.
(12) Kricka, L. J.; Stott, R. A. W.; Thorpe, G. H. G. *Complementary Immunoassays.*; Wiley: Chichester, England, 1988.
(13) Bronstein, I.; Voyta, J. C.; Lazzari, K. G.; Murphy, O.; Edwards, B.; Kricka, L. J. *BioTechniques* **1990**, *8*, 310−314.
(14) Weeks, I. *Chemiluminescence Immunoassay*; Elsevier: New York, 1992.
(15) Kai, M.; Kazuko, O.; Kuroda, N.; Nakashima, K. In *Chemiluminescence in Analytical Chemistry*; Garcia-Campana, A. M., Baeyens, W. R. G., Eds.; Marcel Dekker: New York, 2001; pp 551−566.
(16) Bronstein, I.; Voyta, J. C.; Edwards, B. *Anal. Biochem.* **1989**, *180*, 95−98.
(17) Knight, D. P.; Simmonds, A. C.; Schaap, A. P.; Akhavan-Tafti, H.; Bray, M. A. W. *Anal. Biochem.* **1990**, *185*, 353−358.
(18) Roda, A.; Pasini, P.; Musiani, M.; Baraldini, M. *Methods Enzyomol.* **2000**, *305*, 120−132.
(19) Steel, A.; Torres, M.; Hartwell, J.; Yu, Y.-Y.; Ting, N.; Hoke, G.; Yang, H. In *Microarray Biochip Technology*; Schena, M., Ed.; 2000 BioTechniques Books: Natick, MA, 2000; pp 87−117.

into a reaction volume that is small on the molecular diffusion length scale, reaction kinetics are enhanced.[20] The MG chip allows for enhanced probe immobilization and more uniform spot shape, facilitated by slower evaporation of deposition solution within the microchannels.[21]

Contrary to membrane studies that utilize glow CL reactions in a static environment, continuous flow of flash CL substrate through the chip has been implemented herein during image acquisition. Continuous flow of a flash CL substrate provides enhanced signal intensities per unit time over a static experiment. By combining flash CL reaction kinetics and the MG geometry, diffusion of the light-emitting species is minimized and adequate spatial resolution of the microarray is conserved. Continuously flowing CL substrate through the chip during image acquisition provides attomole detection limits in exposure times of seconds.

In this report, we demonstrate the analytical utility of chemiluminescence detection in an ordered three-dimensional microarray format. Signal intensity and uniformity is investigated by hybridization of a single target to arrays prepared on MG and flat glass substrates. The sensitivity and dynamic range of the technique is is demonstrated through hybridization experiments utilizing three test targets at a range of concentrations. Similar to two-color fluorescence measurements, the use of multiple enzyme−substrate combinations for two-channel chemiluminescence is demonstrated. A novel method for measuring the relaxation time of a CL species is also described.

EXPERIMENTAL SECTION

Chip Preparation. The preparation of MG chips has been previously described.[19,22] In brief, the 0.5-mm-thick microchannel wafer (Galileo Electro-Optics Corp., Stubridge, MA) was cut into 1-cm^2 chips. Individual microchannels of the MG chip have a volume of 39 pL and a radius of 5 μm. The chips were cleaned by degreasing in organic solvent and surface activated by sonication in 1.0 N HNO_3 for 10 min. The chips were blotted dry and sonicated in absolute ethanol for 10 min and then blotted dry and sonicated in deionized water for 10 min. The chips were then dried at 85 °C for 4 h.

Silanization. The chips were immersed in a 2.5% (3-mercaptopropyl)trimethoxysilane (Sigma, St. Louis, MO) in anhydrous toluene (Sigma) for 1 h. Following silanization, the glass chips were sonicated in anhydrous toluene for 10 min, blotted dry, and sonicated in a 50:50 anhydrous toluene/anhydrous ethanol solution for 10 min. Finally, the chips were sonicated in anhydrous ethanol for 10 min, blotted dry, and baked in an 85 °C oven overnight prior to spotting.

Probe Immobilization. The sequences for probes P1−P4 and the targets T1−T4 are detailed in Table 1, where an aminohexyldeoxythymidylate modifier on the 5′ end was used for probe immobilization to the chip surface and a biotin phosphoramidite on the 5′ end of the target sequences was used for streptavidin−horseradish peroxidase attachment. 5′ Label targets with fluorescein isothiocyanate were used during the two channel experiments. The complementary pairs P1−T1, P2−T2, P3−T3, and P4−T4 are representative of the genes GAPDH, IL2, β-actin, and TNF-α, respectively. The probes were purchased from Research Genetics (Huntsville, AL) and the targets were from Operon (Alameda, CA). The probes were reacted with the heterobifunctional cross-linker sulfo-γ-maleimidobutyrloxysuccinimide (s-GMBS; Pierce, Rockford, IL) in 1× saline sodium citrate buffer. The reaction mixture was 30 μM probe to 300 μM s-GMBS. The reaction was allowed to proceed for 1 h at room temperature. A 5-nL aliquot of the reaction mixture was spotted onto the silanized chips using a Packard BioChip piezoelectric spotter (Packard, Meriden, CT). Arrays (8 × 8) of P2 were spotted for the signal intensity and uniformity experiments, with center-to-center spacing of 500 μm. The sensitivity and dynamic range experiments used 4 × 4 arrays with each probe (P1−P4) spotted in quadruplicate, with 400-μm spacing. Spotted chips were allowed to dry in a dehumidified, low-light box for 24 h prior to immersion in a blocking agent.

Table 1. Probe and Target Sequences Used for Hybridization Experiments

name[a]	sequence
probe 1	5′-CCT CTG ACT TCA ACA GCG ACA CT-3′
target 1	3′-GGA GAC TGA AGT TGT CGC TGT GGG TG-5′
probe 2	5′-CAC CAG GAT GCT CAC ATT TAA GTT-3′
target 2	3′-GTG GTC CTA CGA GTG TAA ATT C-5′
probe 3	5′-TCC TCC TGA GCG CAA GTA CTC-3′
target 3	3′-AGG AGG ACT CGC GTT CAT GAG-3′
probe 4	5′-CCC TGG TAT GAG CCC ATC TAT C-3′
target 4	3′-ACC ATA CTC GGG TAG ATA G-3′

[a] The probes contained a C6 dT amino linker at the 5′ end for surface immobilization. The targets contained a biotin label at the 5′ end for subsequent streptavidin−horseradish peroxidase staining for indirect detection of hybridization by chemiluminescence.

Chip Blocking. All chips were incubated in 250 μL of an aqueous solution of 0.1% poly(vinylpyrrolidone) (PVP) and 0.1% Ficoll for 15 min, blotted dry, and baked at 85 °C for 1 h. Blocked chips were stored in a dehumidified, low-light chamber until use.

Hybridization Assay. The chip cartridge was manufactured from anodized aluminum with a single inlet and outlet that are connected to an external fluid delivery system via a pin and septum. The chip−cartridge assembly contained a glass window for imaging and was sealed by use of Viton gaskets. Prior to hybridization, the interior of the cartridge is blocked using a 50-μL injection of 16% goat serum (Sigma-Aldrich, St. Louis, MO) solution in a 1× saline sodium phosphate edta (SSPE) solution by a closed-loop recirculation method for 5 min at a rate of 500μL min^{-1}. Total volume of the closed loop was 830 μL. The cartridge was then flushed with 1× SSPE for 5 min at 1000 μL min^{-1}. A 50-μL aliquot of target solution was introduced to the system and recirculated for 3 h at a rate of 500 μL min^{-1}. Again, the cartridge was flushed with 1× SSPE for 5 min at 1000 μL min^{-1}. Another goat serum block/rinse was performed before streptavidin staining. A 50-μL aliquot of a 10 μg mL^{-1} streptavidin−horseradish peroxidase (Pierce) was then injected into the system and recirculated for 5 min at 500 μL min^{-1}. For the two-channel experiments, another staining step was performed with a 50 μL aliquot of a 50 μg mL^{-1} solution of alkaline phosphatase anti-fluorescein (Vector Labs, Burlingame, CA). Hybridization assays were performed at room temperature, 20−23 °C.

(20) Chan, V.; Graves, D. J.; McKenzie, S. E. *Biophys. J.* **1995**, *69*,. 2243−2255.
(21) It should be noted that we refer to the surface-immobilized oligonucleotide as the probe and the solution-phase nucleic acid as the target.
(22) Benoit, V.; Steel, A.; Torres, M.; Yu, Y.-Y.; Yang, H.; Cooper, J. *Anal. Chem.* **2001**, *73*, 2412−2420.

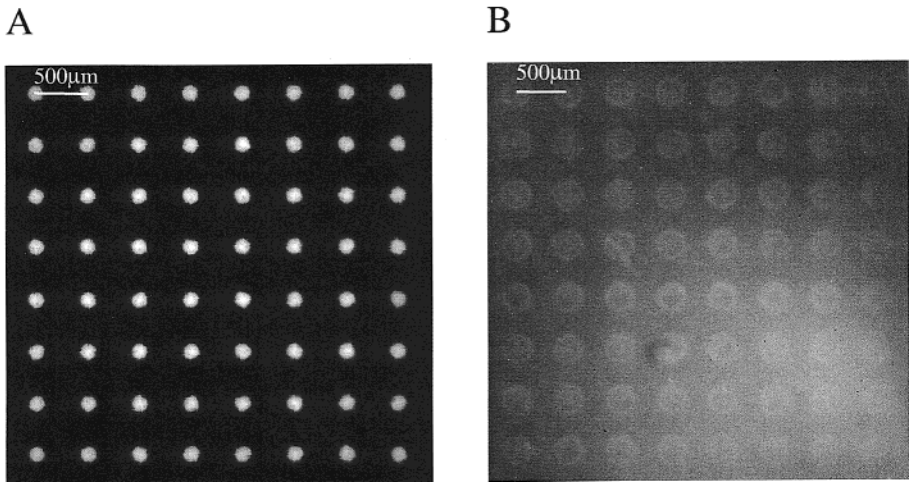

Figure 2. Chemiluminescence detection of hybridization assays on microchannel glass (A) and flat glass (B) microarrays. The microchannel glass chip yielded signal intensities 96 times greater than the flat glass array, consistent with the 101-fold increase in available surface area for probe immobilization. The relative standard deviation of spot intensities for the microchannel glass and flat glass arrays were 8.1 and 25.3%, respectively. The contrast for each image was adjusted for optimal viewing.

Detection. Chips were imaged after hybridizations using an ORCA-ER CCD (Hamamatsu Photonics K. K., Hamamatsu City, Japan) fitted with a VZM 300i c-mount video lens (Edmund Industrial Optics, Barrington, NJ) set at 1.5× zoom. SimplePCI imaging software (C-Imaging, Cranberry Township, PA) was used for image capture and analysis. Continuous flow of chemiluminescent substrate was delivered during image capture using a syringe pump at a rate of 600 μL min^{-1}. Exposure times ranged from 0.1 to 60 s. The CL substrate was allowed to flow though the chip for 10 s prior to image capture to ensure stable luminescent intensity. For the experiments that used streptavidin−horseradish peroxidase, Super Signal West Femto Maximum Sensitivity Substrate (Pierce) was used, and for the alkaline phosphatase anti-fluorescein, APS-5 (Lumigen, Southfield, MI) was employed.

RESULTS AND DISCUSSION

One challenge in using CL for detection of hybridization assays in a solid support microarray format is the mobility of light-emitting species. In most membrane CL imaging systems, the catalyst is immobilized by complexation to labeled material on the solid support and the reactive reagents are in solution.[23] For CL systems with long converted substrate lifetimes, migration prior to light emission ultimately decreases the resolution of the measurement; however, the steady-state region of the intensity−time profile is relatively long so low detection limits are attainable with extended film exposure.

Flash CL emission, however, is desired for CCD imaging of solid support microarrays. Mass transfer of converted substrate is minimized, and the detection is complete within seconds rather than minutes to hours. An enzyme−substrate combination that has sufficient reaction kinetics and quantum efficiency is the luminol/H_2O_2 reaction catalyzed by horseradish peroxidase. Slower reaction kinetics of some 1,2-dioxetanes catalyzed by alkaline phosphatase limit their use in the system described herein. Other enzyme−substrate combinations that yield kinetically fast reactions have also been employed in this system.

Signal Intensity and Uniformity. An 8 × 8 array using P2 was spotted with 500 μm center-to-center spacing on both the MG and a flat glass substrate. The flat glass surface was designed to allow fluid flow over the array in a recirculating flow hybridization system. After hybridization and staining with streptavidin−horseradish peroxidase, chips were imaged under continuous flow of a luminol-based CL substrate, shown in Figure 2. Fluid flow during image capture of the MG chip was designed to flow from the surface of the chip closest to the camera, through the chip, and then out to waste through an exit port. Since the fluid does not penetrate the flat glass array, the fluid flow was from the top left of the image to the bottom right of the image and then out to exit. Although the same volume (5 nL) of probe solution was spotted on both chip surfaces, the spots on the flat chip have a diameter 60% larger than those on the MG chip due to capillary wetting of the microchannels. This effect affords increased spot density at similar dimensions on MG chips as compared to flat glass. Quantitative signal comparisons were made by normalizing the relative spot area (pixels on the CCD camera) and exposure time for the two chip substrates. The signal intensity per pixel per unit time over background was calculated (CPS, counts pixel^{-1} second^{-1}) for each spot and averaged across the array. The MG chip resulted in a CPS = 4130 ± 333, whereas the flat glass array yielded a CPS = 43 ± 11. The increase in surface area provided by the MG array is given by

$$S_{MG}/S_{FG} = 1 + k(2h/r) \quad (5)$$

where S_{MG} and S_{FG} represent the available surface area on the MG and flat glass chips, respectively, k is the open area fraction, h is the chip thickness, and r is the radius of the individual microchannels in the chip. For the experiment shown in Figure 2, $k = 0.5$, $h = 500$ μm, and $r = 5$ μm, which yields a 101-fold increase in the surface area of the MG chip relative to the flat glass array. The hybridization assay on the MG chip yielded

(23) Keller, G. H.; Manak, M. M. *DNA Probes: Background, Applications, Procedures*; Stockton Press: New York, 1993.

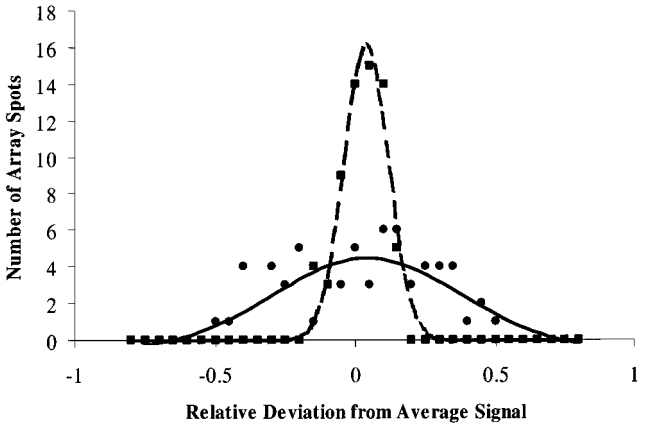

Figure 3. Histogram of the chemiluminescence signal uniformity on microchannel glass (■) and flat glass (●) arrays. The lines represent a Guassian fit to the data.

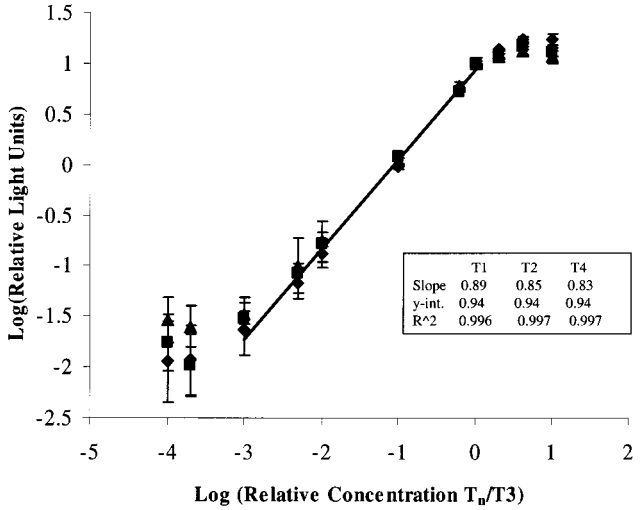

Figure 4. Calibration curve for chemiluminescence detection of oligonucleotide hybridizations on microchannel glass. Target solutions of T1 (◆), T2 (■), and T4 (▲) were hybridized at varying concentrations and the signal intensities were normalized to 5 nM T3.

signals 96 times higher than the flat glass surface, consistent with the expected 101-fold increase. The correlation of the observed and calculated surface area enhancement demonstrates that the CL-imaging system is able to capture photon emission from the entire depth of the chip.

Signal uniformity was evaluated in terms of intensity variation across entire arrays for both the flat glass and the MG chip. The relative deviation from the average CPS for each array spot was plotted in a histogram, shown in Figure 3. The smooth and dashed lines represent a Guassian fit to the data. Based on the smaller width at half-peak maximum, the MG chip provides a significantly more uniform CL signal as compared to a flat glass array. The relative standard deviation of the CPS for the MG chip and flat glass were 8.1 and 25.3%, respectively.

Sensitivity and Dynamic Range. MG chips with 4 × 4 arrays using P1−P4 were spotted and target solutions of T1−T4 were prepared. For this study, the concentration of T3 was held constant at 5 nM while the other three target concentrations were varied. The target solutions were recirculated through the chip for a 3-h hybridization assay. By confining the target molecules within the microchannels of the chip, mass transport distances are greatly reduced compared to distances at flat glass substrates, and the rate of hybridization is increased. Using dynamic hybridization on the MG chip, signal increases linearly with time, until saturation occurs, so that increased sensitivity can be attained in the assay by increasing the hybridization period. The signal response relative to T3 was evaluated for concentrations ranging from 500 fM to 50 nM. The results ($n = 3$ at each concentration) are shown in Figure 4, where the test target signal relative to the control target is plotted as a function of relative concentration. The linear portion of these curves range over 3 orders of magnitude with the detection limit of 5 pM. Based on a 50-μL injection of target solution, the detection limit is 1.5×10^8 molecules, or 250 amol. The detection limit is defined as the lowest concentration of target that produced a signal at least 3 times greater than background noise and was collinear with higher concentration signal responses. Although concentrations lower than 250 amol registered as detectable signal, the response was above linearity for higher concentration targets. The slopes of the calibration curves range from 0.83 to 0.89, where a theoretical slope of 1.00 describes a unit change in response for a unit change in concentration. Slopes less than unity may result from multiple biotins binding to a single streptavidin during the staining step of the assay. R^2 values ranged from 0.996 to 0.997 for the curves, which demonstrates the high degree of linearity throughout the dynamic range of the technique. Longer assays could have been employed for these experiments in an effort to lower the detection limit; however, shorter assays are preferred and standard 3-h hybridizations were performed.

The curves plateau on either end of the linear range. The low end of the curve deviates from linearity at the analytical detection limit of the imaging optics. At high concentration, the curve plateau is presumably due to saturation of enzyme within the spot. Saturation packing of SA−HRP can be approximated using known dimensions of the streptavidin conjugate. The diameter and height of a single channel of the MG chip are 10 and 500 μm, respectively, which yields a surface area of 1.57×10^{10} nm^2/channel. The probe density is $\sim 1 \times 10^{13}$ molecules/cm^2;[19,24] therefore, the maximum number of probes within one channel as 1.57×10^9 molecules/channel. The footprint of a streptavidin molecule, smaller than the SA−HRP conjugate, is ~20 nm^2/molecule.[25] The size of streptavidin limits the number of enzymes to 7.8×10^8 molecules/channel. Therefore, the maximum number of enzymes is less than the capacity to capture targets. Furthermore, if a streptavidin molecule, which contains four biotin binding sites, binds more than one biotin, the signal will not be linearly proportional to the amount of hybridization events. At higher target concentrations, we observed deviations from linearity in the calibration curves above 5 nM. For quantitative analysis, the detection technique must be performed below enzyme saturation levels.

Two-Channel Chemiluminescence. Similar to two-color fluorescence detection techniques, multiple enzyme−substrate combinations can be used to conduct two-channel CL measurements. To demonstrate, biotin- and FITC-labeled targets to the same probe were hybridized simultaneously to a MG chip. The biotin-labeled targets were stained with SA−HRP and the FITC-

(24) Halliwell, C. M.; Cass, A. E. G. *Anal. Chem.* **2001**, *73*, 2476−2483.
(25) Spinke, J.; Liley, M.; Schmitt, F. J.; Ander, H. J.; Angermaier, L.; Knoll, W. *J. Chem. Phys.* **1993**, *99*, 7012−7019.

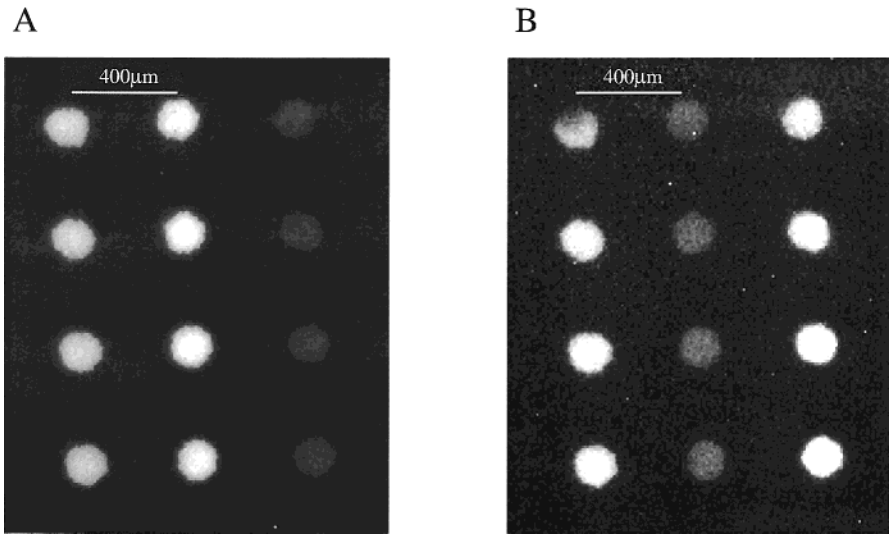

Figure 5. Two-channel chemiluminescence. Channel A is a luminol-based reaction catalyzed by horseradish peroxidase, and channel B is an acridan phosphate-based substrate catalyzed by alkaline phosphatase. The images are of equal integration time; however, the contrast of each has been adjusted for optimal viewing. Column 1 in both images represent a 1:1 ratio of biotin- to fluorescein-labeled targets. Columns 2 and 3 represent 3:1 and 1:1 ratios, respectively. Streptavidin−horseradish peroxidase and anti-fluorescein−alkaline phosphatase were used for staining and indirect detection of hybridization.

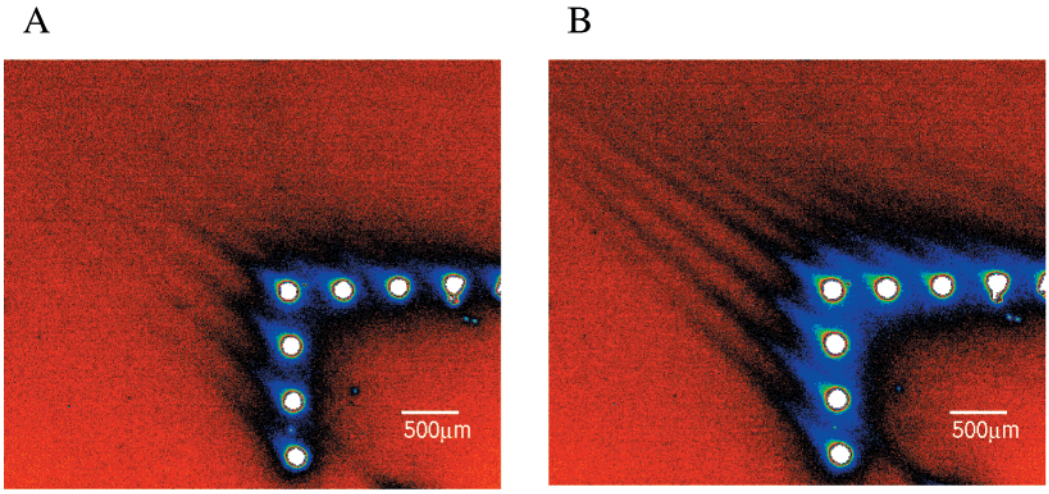

Figure 6. Chemiluminescence relaxation time measurements. By varying the flow rate of chemiluminescent substrate through the chip and measuring the length of an emission tail from spots, it is possible to determine a relaxation time for the emitting species. Image A was captured during 0.2 $cm^3\ s^{-1}$ flow. Image B was captured during 0.4 $cm^3\ s^{-1}$ flow.

labeled targets were stained with anti-fluorescein alkaline phosphatase (AP). Images were captured in succession for the HRP and AP channels by continuous flow of a luminol-based chemiluminescent substrate followed by a buffer rinse, and then a second image was captured using an acridan phosphate-based substrate, as shown in Figure 5. The concentration of targets complimentary to the probe in column 1 had a 1:1 ratio of biotin/FITC. Columns 2 and 3 had 3:1 and 1:3 ratios, respectively. Image analysis yielded a 3.7-fold increase in signal for column 2 and a 0.19-fold decrease in column 3, differing slightly from the 3- and 0.33-fold theoretical changes.

The affinity constant of streptavidin/biotin complex is 10^{13}–$10^{15}\ M^{-1}$,[26] whereas the affinity constant for an anti-fluorescein monoclonal antibody is $10^{10}\ M^{-1}$.[27] This difference in affinity could account for the skewing in signals generated. Also, the differences in catalytic turnover of the substrate (eqs1 and 2) and the lifetime of the converted substrate (eq 3) may effect the results.

CL Relaxation time. When the direction of CL substrate flow is reversed, such that the substrate flows from underneath the chip to the top (the camera side) of the chip, tails off the highly concentrated array spots are observed, as seen in Figure 6. Tails are a result of CL substrate flow toward the cartridge exit after being excited by the peroxidase immobilized in the microchannels. The length of the tails is a function of the flow rate of substrate through the chip; faster flow rates yield longer tails. By varying the flow rate of chemiluminescent substrate through the chip and

(26) Jung, L. S.; Nelson, K. E.; Stayton, P. S.; Campbell, C. T. *Langmuir* **2000**, *16*, 9421−9432.

(27) Denzin, L. K.; Whitlow, M.; Voss, E. W., Jr. *J. Biol. Chem.* **1991**, *266*, 14095−14103.

Table 2. Relaxation Time of the Luminol-Based Reactions at Three Different Flow Rates[a]

flow rate ($cm^3\ s^{-1}$)	tail length (cm)	τ (s)
1.67×10^{-3}	0.11 ± 0.01	36 ± 3
3.33×10^{-3}	0.21 ± 0.01	35 ± 2
6.67×10^{-3}	0.43 ± 0.03	35 ± 2

[a] The open area of the microchannel glass chip was 0.55 for these experiments.

measuring the length of an emission tail from spots, it is possible to determine a relaxation time for the emitting species by the following calculation:

$$\tau = l/(\Phi/A) \tag{6}$$

where l is the tail length (cm), Φ is the flow rate ($cm^3\ s^{-1}$), A is the open area of the chip (cm), and τ (s) is the relaxation time of the light-emitting species. Table 2 shows the relaxation time of the luminol-based reactions at three different flow rates.

Based on the tail length from the spots in Figure 6, the relaxation time for the luminol reagent used herein is roughly 36 s. For a first-order reaction, the relaxation time is inversely proportional to the reaction rate. The experiment presented here would describe the reaction rate of eqs 1−3 for the luminol reagent as $2.8 \times 10^{-2}\ s^{-1}$. We are not aware of literature values for the relaxation time or the reaction rate for this luminol reagent.

CONCLUSION

Although adequate sensitivity and spatial resolution is difficult to achieve on flat glass microarrays, the three-dimensional ordered microchannels of the Flow-thru Chip localize the light-emitting species and provide an unique system for CL detection in a microarray platform. The three-dimensional nature of the MG chip provides 100 times more signal than flat glass arrays based on the additional surface area. The sensitivity and dynamic range of CL imaging on the MG chip proves to be an attractive alternative to fluorescence detection method in a microarray format. A detection limit of 250 amol was realized with a linear response over 3 orders of magnitude of target concentration. Two-channel CL detection of a microarray was demonstrated, similar to two-color fluorescence measurements. A unique aspect of the detection method on the MG chip could potentially be useful for the study of CL substrate reaction kinetics and kinetic-based determinations for various analytes.

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