Chemiluminescence Imaging of Localized Enzymes by Scanning Chemiluminescence Microscopy

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A novel scanning chemiluminescence microscopy (SCLM) was used for imaging localized horse radish peroxidase (HRP) or glucose oxidase (GOD) immobilized at a solid substrate. SCLM equips a scanning capillary tip (diameter, 1 μm) for injecting a small amount of luminol onto the substrate to generate localized chemiluminescence. The chemiluminescense induced by the enzyme-catalyzed reaction was detected with a photon-counter. Two-demensional mapping of the photon-counting intensity against the tip position gave images indicating the activity of immobilized HRP and GOD.

Detection of chemiluminescence with photon-counters and image-intensitied CCD cameras has been popular for imaging and trace analysis biomolecules at solid substrates.[1-7] One of the most notable drawbacks of the CCD camera-based imaging in solution is that it detects undesired chemiluminescence from the solution and increases the background level, which results in lowering the contrast and sensitivity in imaging of the target molecules on the solid substrate.[6] Another drawback is that the chemiluminescence-generating species added to the solution might induce undesired chemical reactions with molecules at the substrates.[7] We report here a novel scanning chemiluminescence microscopy (SCLM) for imaging of localized chemiluminescence from microspots of immobilized horse radish peroxidase (HRP) or glucose oxidase (GOD) at a glass substrate. The present system induces a chemiluminescence-generating reaction in a limited area and, therefore, the interfering chemiluminescence and undesired chemical reaction can be avoided. To our knowledge, the present SCLM is the first scanning probe microscopy with a scanning capillary for injection of chemical species to obtain images of localized chemiluminescence.

The basic principle of SCLM is illustrated in Figure 1. The SCLM equips a scanning glass capillary (inner diameter about 1 μm) filled with a solution containing chemiluminescence-generating chemicals. The capillary was attached to a high-precision motor-driven XYZ stage (Chuo Seiki, M9103) placed on an inverted microscope (Nikon, TMD 200). The inner solution was injected onto the substrate dipped in a 0.2 M phosphate buffer solution by using a microinjector (Narishige, IM-300). The chemiluminescence was detected by a photon- counter (Hamamatsu Photonics, H5920-01) fixed at a side port of the microscope. The XYZ stage was controlled by a notebook computer through a GPIB interface (Interface, AZI-3506). The acquisition of photon counting data was also done by the computer through a pulse counting board (Hamamatsu Photonics M3949). The photon counting image of the substrate was obtained by mapping the photon counting data against the position of the capillary tip.

The immobilization of HRP or GOD onto a glass substrate was carried out as follows. A clean glass substrate was dipped into a 10 mM (3-aminopropyl)triethoxysilane/benzene solution for 8 h, followed by thorough rinse with water under supersonication. A very small droplet (approximately 15 pL) of a $2x10^3$ unit/mL HRP or $1x10^3$ unit/mL GOD solution containing 1% (v/v) glutaraldehyde/water solution was spotted on the aminosilanized substrate using the SCLM system and allowed to be polymerized at the substrate. The radius of the enzymes immobilized area was approximately 25 μm. The absolute amount (based on activity in solution) of HRP immobilized area was $3.6x10^{-5}$ unit and that of GOD was $3.9x10^{-5}$ unit. For the SCLM investigation of the immobilized HRP, we injected a solution containing 5.0 mM sodium luminol (NaLH)/25 mM H_2O_2 from the capillary tip at 78 pL/s. HRP catalyzes the oxidation of LH^- by H_2O_2 to generate chemiluminescence. Figure 2 shows chemiluminescence intensity as a function of distance

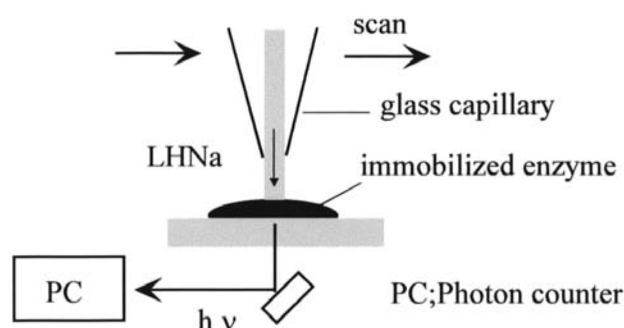

Figure 1. Schematic drawing of the operating principle of scanning chemiluminescence microscopy(SCLM).

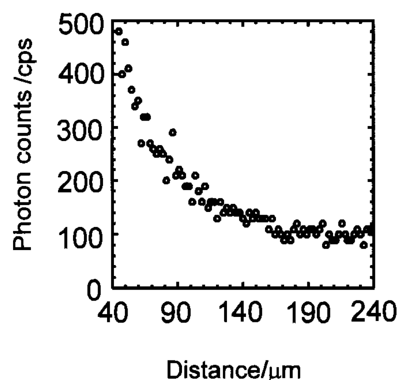

Figure 2. Chemiluminescence intensity as a function of distance between the tip and HRP-immobilized substrate .Solution inside the capillary, 5.0 mM NaLH +25 mM H_2O_2. Injection rate,78 pL/s. Scan rate, 2.4 μm/s. Inner diameter of capillary,1 μm.

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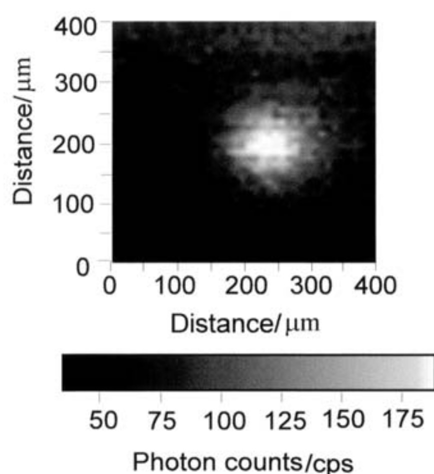

Figure 3. SCLM image of HRP-immobilized substrate in a 0.2 M phosphate buffer solution (pH 8.7). Solution inside the capillary, 5.0 mM NaLH+25 mMH_2O_2. Injection rate,78 pL/s. Scan rate, 2.4 μm/s. Inner diameter of capillary,1 μm. Tip height,40 μm.

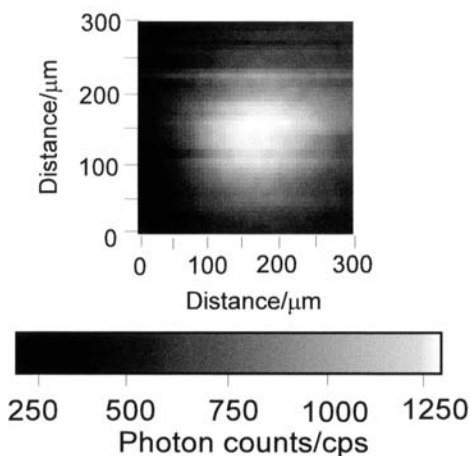

Figure 4. SCLM image of GOD-immobilized substrate in a 0.2 M phosphate buffer solution containing 24 mM glucose (pH 8.7). Solution inside the capillary,5.0 mM NaLH+271 Unit/mL HRP. Injection rate,78 pL/s. Scan rate, 4.9 μm/s. Inner diameter of capillary,1 μm. Tip height,30 μm.

between the tip and HRP-immobilized substrate. Chemiluminescence intensity decreased sharply with increasing the distance and reached a background level when the distance was above 150 μm. No chemiluminescence above the background level was observed at the substrate without immobilized HRP. Chemiluminescence intensity was influenced by the tip height, enzyme concentration, capillary size, injection speed, and NaLH concentration.

Figure 3 shows an SCLM image of a substrate with an HRP immobilized area ($3.6x10^{-5}$ unit/immobilized area). The HRP immobilized area was displayed as a bright area with high chemiluminescence intensity. The size of the bright area corresponds with the size of the HRP immobilized area (radius, 25 μm). In this measurement, the capillary tip was scanned at 4.9 μm/s in a constant height mode (substrate-tip distance, 40 μm). The total volume of the capillary solution injected into the outside medium is approximately 500 nL and the final concentrations of NaLH and H_2O_2 in the outside solution are 250 nM and 1.25 μM, respectively. These final concentrations of NaLH and H_2O_2 are low enough not to increase the background chemiluminescence intensity above the detection level.

The SCLM system can be applied for imaging a microspot of immobilized GOD. The immobilized GOD catalyzes the oxidation of glucose by oxygen to form H_2O_2 which oxidizes LHNa in the presence of HRP to generate chemiluminescence. Figure 4 shows an SCLM image of a GOD immobilized area ($3.9\ x10^{-5}$ unit/immobilized area) on a glass substrate. In this case, the solution inside the scanning capillary (4.9 μm/s) was 5.0 mM NaLH and 271 unit/mL HRP and it was injected continuously at 78 pL/s. The intensity of the chemiluminescence is primarily influenced by the localized concentration of H_2O_2 which is delivered by the catalytic reaction of the immobilized GOD and, therefore, the image indicates the catalytic activity of the immobilized enzyme. It should be noted that the size of the high intensity area does not indicate the size of the immobilized GOD area since the size depends on the diffusion region of the enzymatically-generated H_2O_2 over the GOD immobilized area.

In summary, we have developed a novel SCLM system in which a scanning capillary tip injected chemical species onto a solid substrate to generate localized chemiluminescence. The chemiluminescence was detected with a photon-counter to give a two-dimensional image. This system has been used for imaging the localized enzyme activity of immobilized HRP and GOD. Since the chemiluminescence-generating reaction proceeds only in a limited area, undesired chemiluminescence and side reactions can be eliminated in the present system.

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