Enhanced sensitivity and spatial resolution for *in vivo* imaging with low-level light-emitting probes by use of biocompatible chemical agents

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We describe a technique that uses biocompatible chemical agents to enhance both the sensitivity and the resolution of *in vivo* imaging with low-level light-emitting probes. We demonstrate experimentally, with chemiluminescence (CL) imaging *in vitro* as an example, that the detected intensity of CL from treated 3-mm-thick skin tissue is approximately fivefold stronger than that from untreated skin. The spatial resolution correspondingly increases approximately threefold.

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In vivo imaging by use of light that is generated within biological tissue and transmitted through superficial tissue is an exciting approach for the study of biological processes. This approach offers the advantage of noninvasive *in vivo* assessment for molecular and cellular events that are often the targets of a specific biological process or therapy. This nascent area is rapidly gaining acceptance in such diverse fields as cancer biology, immunology, gene therapy, microbiology, tissue injury, and energy metabolism studies. Some examples of such *in vivo* imaging techniques include (1) Bioluminescence imaging. Because some biomolecules are luminescent proteins and reporter genes that encode bioluminescent proteins, they have been used to create internal biological light in the study of small animal models for human biology and diseases.[1] With these light-emitting probes, scientists are able to carry out gene expression monitoring,[2] metabolic mapping[3] (including cancer heterogeneity and treatment response), and immune cell trafficking.[4] (2) Chemiluminescence (CL) imaging. With some CL agents, for example, *Cypridina* luminescent analog (CLA) and its derivatives, free radical formation in several important processes such as tissue injury, photodynamic and sonodynamic therapy of cancer, and ultraviolet-ray irradiation can be imaged *in vivo* in real time.[5,6] (3) Imaging with sonoluminescence[7,8] and ultraweak spontaneous photon emission.[9] The latter has helped researchers to monitor brain activity, cerebral energy metabolism, and oxidative stress.

One of the major problems facing the aforementioned *in vivo* imaging techniques is the limited sensitivity and spatial resolution because the internally generated light by emitting probes has to transmit through superficial biological tissue that is generally highly scattering and absorptive in nature. Multiple scattering and absorption are responsible for the eventual decay of light as it travels through tissue; this ultimately limits the light-delivering efficiency to the imaging device. Modeling photon diffusion through tissue indicates that approximately 10^6 bioluminescent cells are required to generate signals that are detectable through 2 cm of tissue, on the assumption that there are 30 photons/cell/s at a 650-nm wavelength.[10] Therefore, for applications in which relatively weak light sources are to be imaged, for example, in the diagnosis of certain diseases at an early stage, it is essential to find ways to enhance both the imaging sensitivity and the spatial resolution.

The administration of hyperosmotic and biocompatible chemical agents to tissue has been successfully reported to be capable of reducing light scattering, thereby enhancing the penetration of light through tissue. A series of studies have shown that osmotically active agents effectively alter the optical properties of *in vitro* and *in vivo* tissues.[11–17] Interrogation of tissue with agents such as glycerol,[12,13] propylene glycol,[14] glucose,[15,16] and dextran[17] causes opaque biological tissue, for example, skin, sclera, and aorta, to become optically transparent because of the refractive-index matching environment within tissue. However, such an effective approach has not yet been adopted for *in vivo* imaging techniques with low-level light-emitting probes.

In this Letter we demonstrate experimentally, for the first time to our knowledge, that a significant enhancement of both the sensitivity and the spatial resolution for low-level light-imaging techniques can be achieved by topical application of a biocompatible hyperosmotic agent. We used CL measurements as an example in the experiments. The CL sources covered with skin tissue applied with a glycerol solution were imaged with a cooled CCD imaging system to show the capability of this method.

Figure 1 is a schematic of the experimental system that we used. The CL light was detected with a cooled CCD detector (Starlight SLX8, UK) whose spectra response ranged from 300 to 800 nm with a peak at approximately 500 nm and an average quantum

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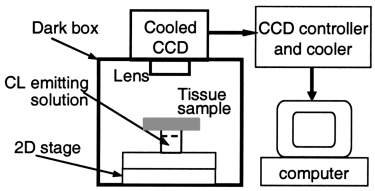

Fig. 1. Schematic of the experimental system.

efficiency of 60%. The CCD surface was cooled to −30 °C to reduce the thermal noise down to 0.003 e/pixel/s. The control and power module was used to control the CCD cooling and data buffering and transferring to a computer where the digitized image was produced by PIX-L8 software. The CL intensity was represented by kilocounts per second (kcounts/s).

We used a luminol–hydrogen peroxide (H_2O_2)–horseradish peroxidase (HRP) CL emitting system[18] as the low-level light sources, and the glycerol solution as the tissue-clearing agent. All the agents were purchased from Sigma UK, Ltd. Following Ref. 18, the final concentrations of luminol, H_2O_2, and HRP were 3.3 mM, 1 mM, and 10 U/ml, respectively. The concentration of glycerol was chosen to be 50% according to a previous study.[13] The fresh porcine skin was chosen as the tissue sample for demonstration. The specimens were cut into 3-mm-thick pieces. Glycerol solution was topically administered to the skin surface and allowed 30 min for absorption before the experiments.

The two identical square CL sources shown in Fig. 2(a) were used as the targets for imaging. The photograph of skin tissue sample, which was taken under dim white-light illumination, is shown in Fig. 2(b), where the dark line divides the image into two halves with the left half from the untreated skin and the right half from the skin treated with 50% glycerol for 30 min. Note that in Fig. 2(b) we created the nonuniformity, particularly the brightness at the bottom, by shining an external dim light that was not directly at the top where the CCD detector was located, i.e., the illumination was at an angle. Figure 2(c) shows the images of two square CL sources covered by the skin tissue sample. The exposure time for Fig. 2(c) was 60 s. It can be clearly seen that the CL imaging from the sample at the right-hand side is much brighter than that at left, indicating that applying skin tissue with a glycerol solution greatly enhances the CL imaging capability from highly scattering biological tissue. To illustrate this improvement quantitatively, the CL intensity as a function of position across the center of an object is plotted in Fig. 2(d). After background subtraction, the average intensity of the left image is approximately 8.5 ± 0.5 kcounts/s, whereas that of the right image is approximately 40 ± 2.3 kcounts/s.

To investigate enhancement of the spatial resolution for CL imaging with the tissue-clearing technique, we used two movable CL light sources with the same intensity as shown in Fig. 2(a). The distance between the two light sources was Δx, which was varied from 1 to 4 mm. Figures 3(a) and 3(b) show one-dimensional CL intensities across the centers of two sources obtained for various distances Δx covered by the intact and treated tissue samples, respectively. In Fig. 3(a) we show that a conventional CL imaging technique can barely resolve two objects separated by less than 3 mm mainly because of the significant scattering nature of the intact tissue. However, the resolution increased to 1 mm after we applied the tissue by use of the glycerol solution, as shown in Fig. 3(b). The spatial resolution was limited by the significant amount of scattering property of the tissue. The application of hyperosmotic and biocompatible chemical agents to tissue is an effective method to reduce light scattering in tissue.[11] We believe that the clearing effect of a hyperosmotic agent is caused by refractive-index matching between agents and tissue fluid in two ways. The first is simply by refractive-index matching of the chemical agent with the main tissue constituents and components. The second is by dehydration caused by the osmotic characteristics of the agents, which results

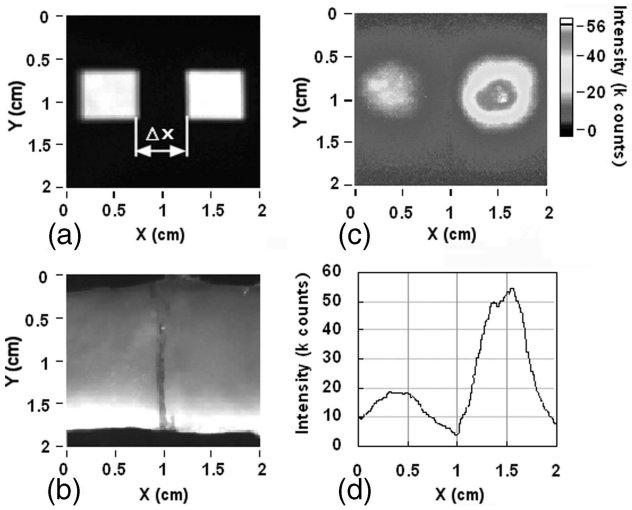

Fig. 2. (a) Two identical square CL sources. (b) Photograph of the tissue sample. The central dark line divides the sample into intact (left) and treated (right) parts. (c) Imaging of the CL sources in (a) covered by the sample with an exposure time of 60 s. (d) The CL intensity as a function of positions across the center in (c).

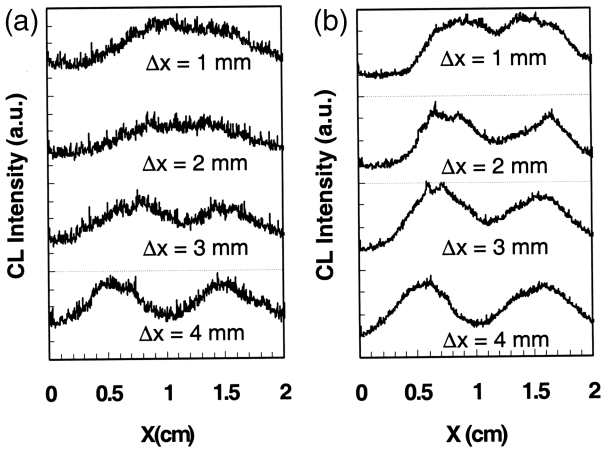

Fig. 3. One-dimensional CL intensities across the centers of two movable sources, shown in Fig. 2(a), for various distances of Δx covered by (a) intact and (b) treated tissue samples.

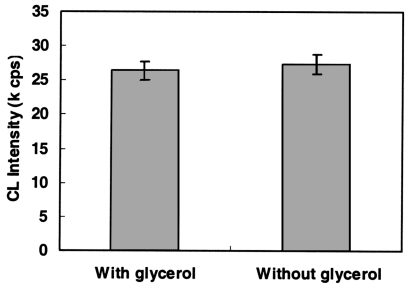

Fig. 4. CL intensities of the reaction solution of the luminol–H_2O_2–HRP mixture with and without glycerol at a final concentration of 5%.

in water loss from the interstitial space, thus leading to a concentration increase of glycosamino proteins within tissue that gives rise to an increased refractive index of the background medium.[11–15]

Low-level light emission often originates from some biochemical reactions. It is thus important to assess whether the hyperosmotic agents used for optical clearing in the current study affect the efficiency of the CL reaction. Toward this end we performed another set of experiments to measure the CL intensities of the reaction solution of the luminol–H_2O_2–HRP system with and without glycerol at the final concentration of 5%. Figure 4 shows such results with an exposure time of 5 s, where it can be seen that luminol solutions with or without glycerol deliver almost the same CL intensity of approximately 27 kcounts/s ($P > 0.05$). This indicates that the addition of a glycerol agent does not affect the CL emitting efficiency in this study. Nevertheless, for applications other than luminol CL, this prerequisite needs to be tested further although glycerol is a small molecule with stable chemical reactivity.

In the current experiments, the CL of luminol is a broadband violet-blue emission with a center wavelength of 425 nm.[18] When light in this region travels in tissue, absorption plays a more important role than does scattering. Because optical clearing is believed to reduce light scattering rather than to reduce absorption in tissue, the sensitivity and resolution enhancement of low-level light imaging by optical clearing is more significant when the emission has a longer wavelength. For example, in cases such as use of a firefly luciferin–luciferase system to emit red light with a peak at 600 nm[1–4,10] and fluoresceinyl CLA CL at 532 nm,[5,6] an optical clearing method is expected to be more effective to enhance the sensitivity and spatial resolution.

In conclusion, we used CL measurements as an example to demonstrate a significant improvement of both sensitivity and spatial resolution by use of tissue clearing with the application of biocompatible chemical agents. The tissue-clearing agents did not affect the CL emitting efficiency, at least in our study. This method could have potential applications for *in vivo* imaging with other low-level light sources such as bioluminescence, sonoluminescence, and ultraweak photon emission.

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