Supplementary Information

Bioluminescence-activated deep-tissue photodynamic therapy of cancer

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Supplementary Information

Photomedicine Various light-based technologies have been developed for diagnosis and treatment. Fluorescence imaging has shown great promise in the early detection of diseases because of its high sensitivity and molecular specificity in vivo (Ntziachristos et al., 2005; Weissleder and Mahmood, 2001). Optically controlled treatment methods, such as photodynamic therapy (PDT) (Castano et al., 2006; Hamblin and Hasan, 2004; von Maltzahn et al., 2009), low-level light therapy (Huang et al., 2009), controlled drug release (Yavuz et al., 2009), and photochemical tissue bonding (Kamegaya et al., 2005), utilize the photothermal and photochemical effects of light on cells and molecules. These techniques are rapidly emerging, while PDT in particular is widely used in ophthalmology and oncology (Agostinis et al., 2011). In addition, new ground-breaking tools, such as optogenetics (Boyden et al., 2005) and light-controlled synthetic biology (Ye et al., 2011), continue to emerge. Fiber-optic catheters can bring a light source closer to the tissue in the body; however, delivering the light further into the deeper target remains a challenge. To date, the clinical applications of light-based techniques have been limited to the superficial tissues in the skin or eye or to the epithelial surfaces of internal organs.

Light penetration in tissue Figure 1 illustrates the problem of light penetration. When light is applied to the skin topically, its penetration is limited to the epidermis and upper dermis layers (Salomatina et al., 2006). At a typical optical dose (180 J/cm²), bleaching of the dye was observed only in the superficial layer (<1.5 mm), indicating that sufficient optical intensity was achieved only at the top surface of the skin. Increasing the light intensity is not possible, as it can cause thermal damage in the superficial tissue. Increasing the illumination time is not practical either. This problem of penetration depth is a serious limitation for many optical techniques. For example, photochemical tissue bonding is a promising method for scar-less wound closure (Yang et al., 2012), but its application to full-thickness (5-20 mm) skin excisions is currently not possible due to shallow illumination depths.

Optical attenuation in tissue Optical penetration in tissue is fundamentally limited by absorption. Scattering contributes to illumination loss in two ways: by increasing the absorption through the increase of the propagation length in tissue and also by causing the beam to diverge. In general, light intensity decreases exponentially with depth. In the case of uniform illumination over a wide area (>10 cm²), the attenuation can be expressed as:

\[ I(z) = I_0 \times \exp(-\mu_{\text{eff}} z) \]  

(1)
where $I_0$ is the input intensity. Here, the effective attenuation coefficient $\mu_{\text{eff}}$ is given by:

$$\mu_{\text{eff}} = [3\mu_s \ast (\mu_s + \mu_a)]^{1/2}, \quad (2)$$

where $\mu_s$ is the reduced scattering coefficient, and $\mu_a$ is the absorption coefficient of the tissue. At a wavelength ($\lambda$) of 480 nm, $\mu_{\text{eff}} = \sim 20$ cm$^{-1}$, and the total attenuation is $4 \times 10^{-9}$ cm$^{-1}$ or 84 dB/cm. That is, to deliver one photon to $z=1$ cm, it is necessary to illuminate the surface with as many as 200 million photons. The attenuation generally decreases at longer wavelengths (e.g., 30-60 dB/cm at $\lambda=650$ nm, see Table 1) (Cheong et al., 1990). For point sources, such as fiber-optic illumination, the optical intensity tends to decrease more rapidly with depth due to beam divergence. The experiments and the Monte Carlo simulation show that the attenuation is 18-23 dB at $z=1$ mm and 28-33 dB at 2 mm for $\lambda=480$-650 nm in various tissues (Yizhar et al., 2011).

$$
\begin{array}{|c|c|c|}
\hline
\text{\lambda (nm)} & \mu_a (\text{cm}^{-1}) & \mu_s (\text{cm}^{-1}) \\
\hline
480 & 5-10 & 15-40 \\
650 & 1-3 & 10-35 \\
\hline
\end{array}
$$

Table 1. Optical scattering and absorption in a typical soft tissue.

Bioluminescence resonance energy transfer (BRET) Bioluminescence is a naturally occurring form of chemiluminescence in many organisms, such as the jellyfish, sea pansy, and firefly. A small-molecule substrate luciferin is oxidized in the presence of the enzyme luciferase to produce oxyluciferin and a photon (Wilson and Hastings, 1998). Bioluminescence imaging has been widely used for visualizing gene expression (Contag and Bachmann, 2002) and disease progression (Ntziachristos et al., 2005). In many organisms, light is emitted via bioluminescence resonance energy transfer (BRET). The green light emission observed in the jellyfish Aequorea victoria is a result of the non-radiative energy transfer from the aequorin to green fluorescent protein (GFP) (Shimomura, 2009). Renilla reniformis, a coral, emits bright green light by energy transfer from the interaction of luciferase (RLuc) and coelenterazine (Shimomura and Johnson, 1978) with a dimer GFP. Efficient energy transfer from RLuc to quantum dots (So et al., 2006) and from RLuc to EYFP (Hoshino et al., 2007) were demonstrated. BRET has been exploited for Ca$^{2+}$ sensing (Martin et al., 2007), protein folding (Angers et al., 2000), and protein-protein interactions (Pfleger and Eidne, 2006).

Reference


Supplementary Fig. 1: Energy diagram of the sequential Förster resonance energy transfer (FRET) from an activated coelenterazine (CTZ) to quantum dot (Q-dot or QD) to Chlorin e6, and to oxygen molecule. The end products are reactive oxygen species (ROS), through two types of processes, both of which generate cytotoxicity.

Supplementary Fig. 2: TEM images. (a) Luc-QD conjugates. The hydrodynamic diameter of the conjugate was measured to be about 20 nm. (b) Luc-QD conjugates attached to the external surface of the lipid bilayers of exosomes. (c) Cells incubated with Luc-QD for 20 min. (d) Control cells without Luc-QD.
**Supplementary Fig. 3:** Time-lapse confocal fluorescence images of Luc-QD, showing the time-dependent accumulation of the BL source at the external surface of the cellular membrane. Scale bars, 50 µm.

![Time-lapse confocal fluorescence images of Luc-QD](image1)

**Supplementary Fig. 4:** A schematic of the setup to measure the total amount of (a) laser absorption and (b) bioluminescence radiation. For the laser measurement, the incident power was 2.2 mW, and the difference in transmission through the cell culture before and after incubation with Ce6 was about 13 µW. In bioluminescence measurement, the collection efficiency is determined by the active area of the power meter head and the distance of the photodetector from the cell culture.

![Schematic setup to measure total amount of laser absorption and bioluminescence radiation](image2)

\[
\text{Collection eff.} = \frac{\text{Detector area}}{4\pi d^2}
\]
Supplementary Fig. 5: Measured bioluminescence from Luc-QD in the cell culture media. Blue circles: the measured data, Green squares: an exponential fit. Red circles: a fit with a theoretical curve derived from the rate equations. In this model, the BL intensity is proportional to the product of the number of Luc-QD ($N_1$) and CTZ ($N_2$), which is described by the first equation in the inset. Both Luc-QD and CTZ were assumed to be degraded as they react to produce bioluminescence, as indicated by the second and third equations. Solutions of the equations predict a $1/t$-type decay of both Luc-QD and CTZ. Therefore, the BL intensity is given by the last equation in the inset. If the degradation of Luc-QD is neglected and only CTZ is depleted, the BL intensity should follow an exponential decay. The measured data are fit best with the $1/t^2$-type decay initially in the first 1-2 minutes after the injection of CTZ, suggesting a rapid consumption of Luc-QD. Later after 3 min, however, the decay seems to follow an exponential decay, indicating the remaining Luc-QD stay functional for up to several minutes.
Supplementary Fig. 6: MTT assay of B16F10 cells treated with BL-PDT and laser-PDT.

Supplementary Fig. 7: BL-PDT on B16F10-GFP melanoma cells. (a) Time-lapse confocal fluorescence images during BL-PDT. The decreases of GFP signals, accompanied by changes of cellular morphology, in both cases indicate cellular damages. The GFP signals in control cells showed no changes (not shown here). The red fluorescence increases over time during BL-PDT as the Luc-QD conjugates in the medium accumulate around the cells at the bottom of the culture well plate. (b) GFP signals during laser-PDT. Control cells without laser illumination showed no changes in GFP fluorescence intensity.
Supplementary Fig. 8: Confocal images of LLC cells after treatments. PI-positive nuclei are surrounded by large Luc-QD (i, ii), and PI-negative nuclei are with no or less Luc-QD (iii and iv).
Supplementary Fig. 9: Confocal fluorescence images of Single-Oxygen-Sensor-Green (SOSG) post CTZ injection into CT26 cells in vitro. (a) At 0 min, SOSG are located in the culture medium outside the cells in a non-fluorescent form. At 3 min, SOSG have entered some of the cells through their damaged membranes and turned to fluorescent forms by reaction with singlet oxygen. At 10 min, most cells show green fluorescence in their cytoplasm. Scale bars, 100 µm. (b) Time-lapse confocal images of SOSG in another experiment, taken with a time interval of 1 min.
Supplementary Fig. 10: (a) Immunohistochemistry (IHC) image of an untreated tumor stained for CD31. (b) IHC images of a BL-PDT treated tumor, stained for CD31 (green) and apoptosis marker, APR-648 (white). (c) IHC for DAPI, CD31, Caspase-3 and APR-648. (d) The vascular density estimated by the CD31 signals integrated over the APR-648 positive region, indicating a significant vascular disruption by BL-PDT. *** p<0.001.
Supplementary Fig. 11: Bioluminescence images showing the draining of Luc-QD and CTZ to the popliteal LN.

Supplementary Fig. 12: Bioluminescence images showing the draining of Luc-QD and CTZ to the lumbar LN (arrows).
Supplementary Fig. 13: Lung samples excised from the animals at day 25 after implantation of LLC tumors in the footpad. Top, from animals treated with BL-PDT at day 11; Bottom, untreated animal group.
Supplementary Fig. 14: Reduced lymphatic drainage to the sentinel lymph node, observed four days after BL-PDT in a CT-26 bearing mouse.