

Real-Time Tracking and In Vivo Visualization of β -Galactosidase Activity in Colorectal Tumor with a Ratiometric Near-Infrared Fluorescent Probe

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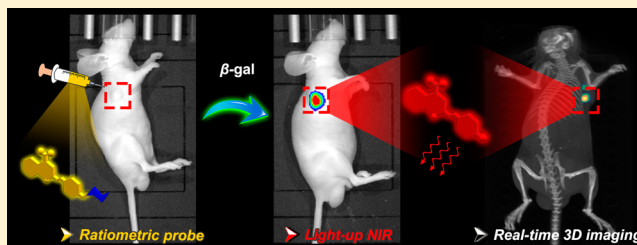
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Supporting Information

ABSTRACT: Development of “smart” noninvasive bioimaging probes for trapping specific enzyme activities is highly desirable for cancer therapy in vivo. Given that β -galactosidase (β -gal) is an important biomarker for cell senescence and primary ovarian cancers, we design an enzyme-activatable ratiometric near-infrared (NIR) probe (DCM- β gal) for the real-time fluorescent quantification and trapping of β -gal activity in vivo and in situ. DCM- β gal manifests significantly ratiometric and turn-on NIR fluorescent signals simultaneously in response to β -gal concentration, which makes it favorable for monitoring dynamic β -gal activity in vivo with self-calibration in fluorescent mode. We exemplify DCM- β gal for the ratiometric tracking of endogenously overexpressed β -gal distribution in living 293T cells via the *lacZ* gene transfection method and OVCAR-3 cells, and further realize real-time in vivo bioimaging of β -gal activity in colorectal tumor-bearing nude mice. Advantages of our system include light-up ratiometric NIR fluorescence with large Stokes shift, high photostability, and pH independency under the physiological range, allowing for the in vivo real-time evaluation of β -gal activity at the tumor site with high-resolution three-dimensional bioimaging for the first time. Our work provides a potential tool for in vivo real-time tracking enzyme activity in preclinical applications.



INTRODUCTION

Molecular bioimaging of enzyme activity in vivo is rapidly emerging as a powerful strategy for accurate disease diagnostics.^{1–6} The development of “smart” noninvasive imaging reagents for the determination of specific enzyme activity in vivo is critically required for cancer diagnosis.^{7–15} However, during the onset and progression of cancer, the dynamic living system complexity makes it difficult to track and visualize in vivo enzyme activity. As a typical enzyme, β -galactosidase (β -gal) has been demonstrated as an important biomarker for cell senescence and primary ovarian cancers.^{16–19} Much effort has been devoted to treating β -gal as an enzymatic target with fluorescent probes to visualize its activity in preclinical diagnosis.^{20–25} However, owing to the limited indirect prediction via histology or in vitro experiments, the currently fluorescent β -gal probes are not capable of precisely tracking in vivo real-time enzyme activity required for cancer diagnosis.

One of the major obstacles encountered with fluorescence imaging of enzyme activity in vivo is the strong intrinsic autofluorescence background from living tissues, which significantly compromises the accuracy of measurement under

physiological conditions. Near-infrared (NIR) fluorescence imaging is an accessible tool for noninvasive in vivo visualization of mammalian tissues with decreased autofluorescence, low light scattering, and high penetration depth.^{26–34} Weissleder and co-workers designed DDAOG emitting in the NIR region for in vivo detection of β -gal activity,³⁵ but severe cross-talk between the excitation and emission spectra weakened the ability to capture valid signals. Moreover, in contrast with the single-wavelength fluorescence-intensity based systems, ratiometric fluorescence probes are of considerable practical advantage due to enhanced signal-to-background ratio, in which the detectable ratio signal can be obtained by two independent read-out channels of activated versus unreacted probes, resulting in improved and reliable signal quantification.^{36–40}

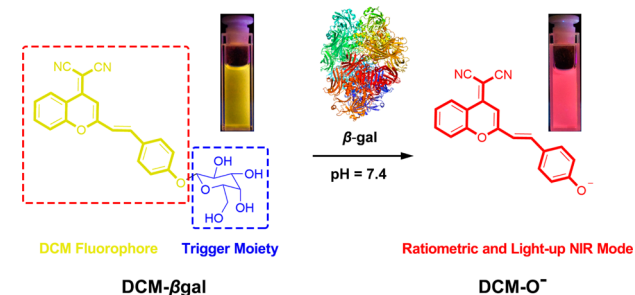
The aforementioned concerns encourage us to create a novel fluorescent probe for tracking the in vivo behavior of the important β -gal enzyme and clarifying its potential role in tumor diagnosis. Our strategy is based on grafting an enzyme-

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active trigger onto a controllable emissive, bright, and photostable NIR chromophore. Controllable emission with large spectral shifts via modulation of the electron-donor ability of novel NIR-active fluorophores is critical and required for excellent imaging contrast agents. Herein, we report on a β -gal-targeting ratiometric and light-up NIR fluorescent probe (DCM- β gal, Scheme 1), in which the dicyanomethylene-4H-

Scheme 1. Proposed Sensing Mechanism for β -Gal Enzymatic Activation of DCM- β gal



pyran (DCM) chromophore is utilized as a NIR fluorescence reporter^{41–45} and a β -gal cleavable unit is utilized as the enzyme-active trigger. As expected, the emission wavelength is distinctly changed upon removal of the β -gal-responsive group (the enzyme-triggered moiety, Scheme 1), resulting in a new

broad emission band in the NIR region. The system enables the direct and accurate monitoring of intracellular endogenous β -gal distribution in transfection living cells with a high signal-to-noise ratio. That in turn allows for real-time in vivo bioimaging of β -gal activity in colorectal tumor-bearing nude mice. Probe DCM- β gal possesses several striking characteristics, such as ratiometric fluorescence activation with a light-up NIR emission and high photostability in physiological range. To the best of our knowledge, DCM- β gal is the first light-up NIR fluorescence probe for the in vivo and in situ tracking of β -gal activity in tumor-bearing nude mice using high-resolution three-dimensional fluorescence imaging.

RESULTS AND DISCUSSION

Design and Synthesis. NIR-active fluorophores are favored for in vivo optical imaging contrasts. As well-known laser dyes, DCM derivatives could perform controllable emission in the NIR region with large spectral shifts via tuning electron-donor ability,^{41–46} which offers such critical features as excellent imaging contrasts. Specifically, DCM-OH has favorable donor- π -acceptor (D- π -A) characteristics for sensor design, with a phenolic group for regulating electron-donating capability. The emission wavelength could be distinctly changed upon removing the substituted group (for instance, the enzyme-triggered moiety, Scheme 1). In this regard, we designed probe DCM- β gal by grafting a β -gal

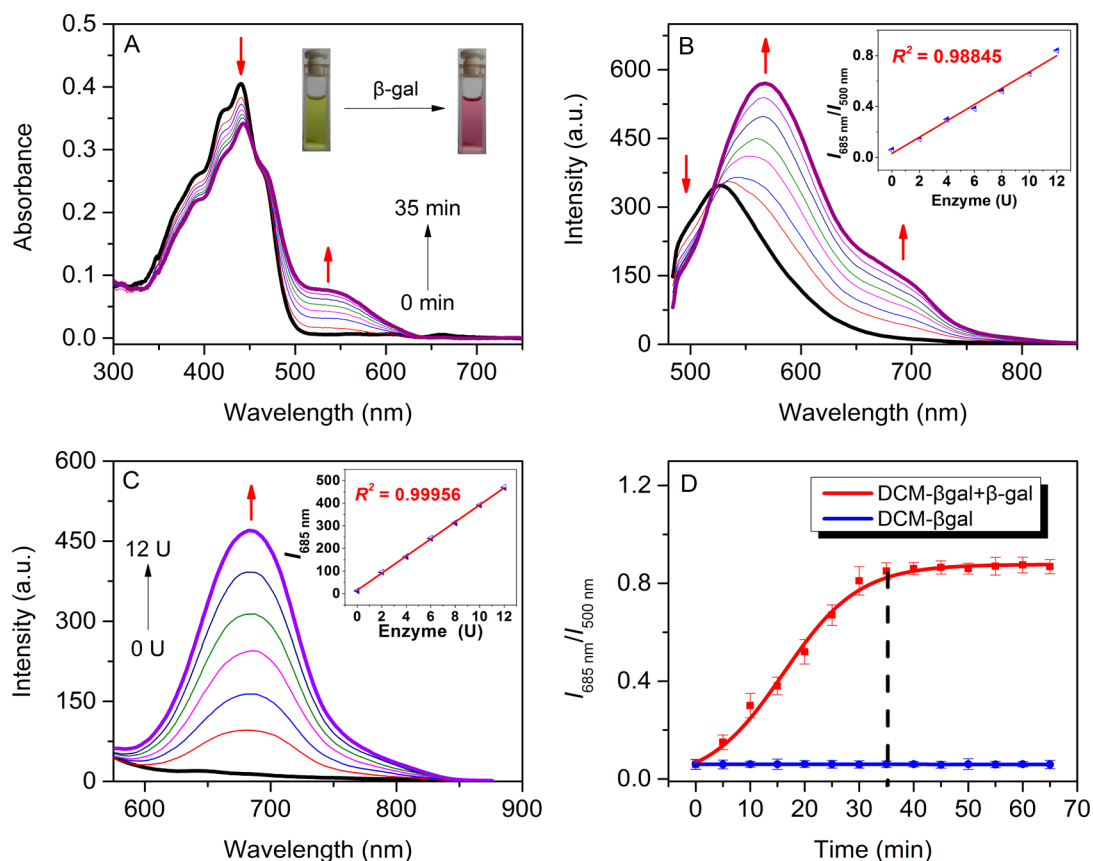


Figure 1. Spectral profiles of DCM- β gal (10 μ M) incubation with β -gal (12 U) in aqueous solution (phosphate-buffered saline (PBS)/dimethyl sulfoxide (DMSO) = 7:3 v/v, pH = 7.4, 37 $^{\circ}$ C). (A) Time dependence of absorption spectra. Inset: images before and after treatment with β -gal. (B) Time dependence of emission spectra (0–35 min), $\lambda_{\text{ex}} = 450$ nm. Inset: the relationship between $I_{685\text{nm}}/I_{500\text{nm}}$ and β -gal concentration. (C) Emission spectra of DCM- β gal upon addition of β -gal (0–12 U), $\lambda_{\text{ex}} = 535$ nm. Inset: the relationship between $I_{685\text{nm}}$ and β -gal concentration. (D) Time dependence of $I_{685\text{nm}}/I_{500\text{nm}}$ for DCM- β gal in the presence (red) and absence (blue) of β -gal, $\lambda_{\text{ex}} = 450$ nm.

activatable unit onto the DCM-OH moiety. The synthetic route for the probe is depicted in the Supporting Information. DCM- β gal was finally synthesized with a yield of 33% under mild conditions, which was fully characterized by ^1H and ^{13}C NMR and high-resolution mass spectrometry (HRMS) (Supporting Information).

Spectroscopic Properties and Optical Response to β -Gal. To test the validity of this probe, the spectral properties of DCM- β gal were investigated with β -gal in physiological buffer solution. DCM- β gal shows a typical broad absorption band at 440 nm from its intrinsic intramolecular charge transfer (ICT). Upon titration with 12 U β -gal, the absorption peak of DCM- β gal at 440 nm decreased, and a concomitant new absorption peak appeared at \sim 535 nm (Figure 1A), which is in perfect accordance with the absorption of DCM-O $^-$ (Figures S1 and S2). The well-defined isosbestic point at 450 nm clearly demonstrates the coexistence of DCM- β gal and the cleavage product (DCM-O $^-$). The color change from faint yellow to rose red allows the colorimetric detection of β -gal using the naked eye, which is invoked by a large red-shift of ca. 100 nm in the absorption spectra.

As expected, the emission profiles of DCM- β gal are correlated to those of the absorption profiles in the presence of β -gal. When excited at the isosbestic point of 450 nm, an obvious ratiometric fluorescent signal ($I_{685\text{nm}}/I_{500\text{nm}}$) was observed. As shown in Figure 1B, a new broad band falling into the emission range of 550–800 nm sharply increased, along with a decrease in the emission band at 500 nm. Upon excitation of the new absorption peak at 535 nm, a remarkable NIR fluorescence enhancement was observed with a peak at 685 nm (Figure 1C and Table S1). Clearly, the new, distinct large red-shifted fluorescence response confirms that β -gal-mediated hydrolysis of DCM- β gal liberates the oxygen atom as a strong electron donor in the D- π -A structure, thereby increasing ICT and shifting the emission maximum to the NIR region.

Figure 1D depicts the ratiometric fluorescent signal ($I_{685\text{nm}}/I_{500\text{nm}}$) as a function of time. It takes \sim 35 min to reach a plateau, which is also directly reflected by the fluorescence response at 685 nm (Figure S3). The kinetic results imply that the fluorescent response can be utilized for the rapid detection of β -gal, which is much faster than that of the previously reported fludeoxyglucose (FDG) (3 h).⁴⁷ Therefore, in our subsequent experimental assays, the detection limit was set at 35 min. Specifically, when the probe was incubated with 12 U β -gal for ca. 35 min, a 14-fold increase was observed in the fluorescence ratio of $I_{685\text{nm}}/I_{500\text{nm}}$ (Figure 1B), or a 34-fold fluorescence enhancement at 685 nm was observed (Figure 1C). Notably, the plots of the $I_{685\text{nm}}/I_{500\text{nm}}$ ratio and $I_{685\text{nm}}$ against the concentrations of β -gal ranging from 0 to 12 U both display a good linear relationship (insets of parts B and C of Figure 1, respectively). Hence, the linear curve of the $I_{685\text{nm}}/I_{500\text{nm}}$ ratio allows for the convenient quantitative detection of β -gal over this concentration range. The Michaelis constant (K_m) of DCM- β gal in the β -gal-catalyzed reaction was calculated to be 60.1 μM (Table S2), which was much lower than that of commercial X-gal (260.6 μM). Obviously, DCM- β gal shows higher affinity to β -gal than to X-gal. Furthermore, derived from the change in concentration-dependent ratio, the detection limit of DCM- β gal was calculated as 1.7×10^{-4} U mL^{-1} , indicative of ultrasensitivity to changes in β -gal expression (Figure S4). Consequently, DCM- β gal enables the fast and quantitative ratiometric fluorescence detection of β -gal.

Sensing Mechanism. For gaining insight into the activation of DCM- β gal with enzyme, a series of experiments were also carried out. The high-performance liquid chromatography (HPLC) results confirmed that DCM- β gal is a substrate of β -gal, which exclusively generates DCM-O $^-$ (Figure S5). In the electrospray ionization (ESI)-MS spectra of DCM- β gal with β -gal, the peaks of DCM- β gal and DCM-O $^-$ were found at m/z 497.1441 and 311.0697 (Figure S6), respectively. In fact, the aforementioned spectra from the reaction of DCM- β gal with β -gal resemble those of DCM-O $^-$, which also strongly supports the fact that the enzyme-triggered cleavage reaction causes the release of free DCM-O $^-$. All these observations confirm that DCM- β gal can be hydrolyzed by breaking the C–O bond upon enzyme-reactive reaction with β -gal to release an electron-rich aglycon, DCM-O $^-$, which emits in the NIR region and possesses a distinct ratiometric fluorescent signal with a large spectral shift.

To test the interference with other biological analytes, the reactivity of DCM- β gal toward a variety of enzyme species, amino acids, and biomolecules was examined. There were only subtle changes in fluorescence ratio upon the addition of 100 equiv of cellulase, reductase, lysozyme, esterase, cysteine, homocysteine, glutathione, dithiothreitol, hydrogen peroxide, and hydrogen sulfide. However, only when treated with β -gal was the large and distinct enhancement of fluorescence ratio ($I_{685\text{nm}}/I_{500\text{nm}}$) observed (Figure 2A), accompanied by an obvious color change from faint yellow to rose red (Figure 2B), which corresponds to the evolution of DCM-O $^-$. These results demonstrate the excellent selectivity of DCM- β gal toward β -gal over other competitive analytes, which is required in order to achieve accurate detection under practical applications.

Photostability. The high photostability of DCM- β gal and DCM-OH is highly desirable to perform long-time in vivo tracking of enzyme activity in preclinical applications. Here the photostability of DCM- β gal and DCM-OH was evaluated by time-course fluorescence measurements upon sustained illumination in aqueous solution. The commercially FDA-approved NIR dye ICG was chosen as a control. After exposure to high-density light (Hamamatsu, LC8 Lightnigcure, 300 W) for \sim 60 s, the fluorescence intensity of ICG was reduced to a minimum value, indicating that ICG was almost completely decomposed; however, 90% of the DCM- β gal and DCM-OH fluorescence intensity remained under the same conditions. Indeed, the fluorescence half-life of the DCM- β gal was 35-fold longer than that of ICG (Figure 2C). The photostabilities of both DCM- β gal and DCM-OH are much better than that of ICG, which is a critical factor for the long-time tracking and bioimaging in vivo.

pH Profiles of DCM- β gal and DCM-OH. pH is an important factor on the photophysical properties of the sensing probe. We subsequently evaluated the pH dependence of the emission profiles of DCM-OH and DCM- β gal (Figure S7). There was negligible fluorescence change at 685 nm for DCM- β gal during the pH range from 3.5 to 9.8 upon excitation at 535 nm. Obviously, the fluorescence of DCM- β gal remains silent over the physiological pH range. More excitingly, the hydrolytic product of DCM- β gal can only be produced at the optimal physiological pH range. That is, the $\text{p}K_a$ of DCM-OH was measured to be ca. 7.1, which allows DCM- β gal to favorably exploit the physiological conditions of living systems.

Ratiometric Imaging of Endogenous β -Gal in Living Cells. We further explored whether DCM- β gal could be used for the ratiometric tracking and imaging of endogenous β -gal

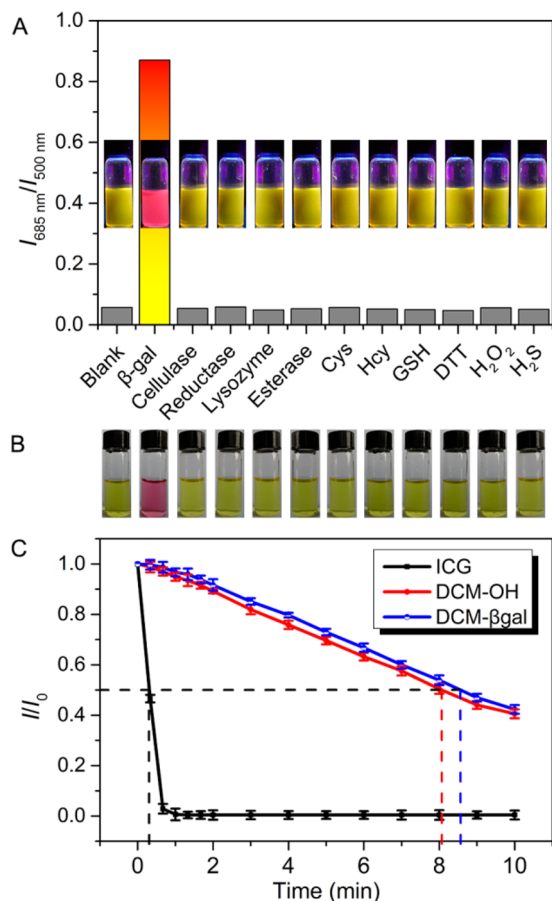


Figure 2. (A) Fluorescence ratio ($I_{685\text{nm}}/I_{500\text{nm}}$) responses and (B) color changes of DCM- β -gal (10 μM) to various analytes in aqueous system (PBS/DMSO = 7:3 v:v; pH = 7.4, 37 $^\circ\text{C}$). Bars correspond to the fluorescence ratio ($I_{685\text{nm}}/I_{500\text{nm}}$) of DCM- β -gal with 100 equiv of analytes, $\lambda_{\text{ex}} = 450\text{ nm}$. (C) Time-dependent fluorescence intensity of ICG (10 μM , monitored at 812 nm, and $\lambda_{\text{ex}} = 780\text{ nm}$), DCM-OH (10 μM , monitored at 565 nm, and $\lambda_{\text{ex}} = 450\text{ nm}$), and DCM- β -gal (10 μM , monitored at 525 nm, and $\lambda_{\text{ex}} = 450\text{ nm}$) under sustained illumination.

activity in living cells. As a highly efficient and facile transfection process, human embryonic kidney cells (293T cells) were chosen as model cell lines. To generate endogenous β -gal in living cells, *lacZ* gene was introduced into 293T cells by employing a gene transfection method to overexpress β -gal. The MTT assay results indicate that both DCM- β -gal and its hydrolysate DCM-OH have minimal toxicity and enjoy superior biocompatibility toward cultured cell lines (Figure S8). After incubation with DCM- β -gal (10 μM) at 37 $^\circ\text{C}$ for 30 min, the *lacZ*(-) 293T cells without overexpressed β -gal exhibited both bright fluorescence in the green channel and little fluorescence in the red channel (Figure 3B and C), which corresponds to the two peaks (525 and 685 nm) in the emission spectra of DCM- β -gal. In contrast, the *lacZ*(+) 293T cells with overexpressed β -gal exhibited a decreased fluorescence in the green channel and a concomitant increase in the red channel (Figure 3I and J), corresponding to formation of DCM-O. The ratiometric imaging of endogenous β -gal activity was constructed from red and green channels ($F_{\text{red}}/F_{\text{green}}$; Figure 3D and K). The average emission ratios $F_{\text{red}}/F_{\text{green}}$ of the *lacZ*(-) and *lacZ*(+) 293T cells were calculated to be 0.43 and 2.20, respectively. An ~ 5 -fold increase in ratio for the *lacZ*(+) compared to *lacZ*(-) 293T cells was observed (Figure

S9). Therefore, we have successfully demonstrated the quantitative ratiometric imaging of endogenous β -gal activity.

Moreover, upon excitation at 560 nm, a clear fluorescence image was captured in the overexpressed *lacZ*(+) 293T cells in the NIR region due to the liberation of DCM-O by intracellular β -gal, while a fluorescent signal was barely observed for the *lacZ*(-) 293T cells (Figure 3F and M). Moreover, OVCAR-3 from human ovarian cancer patients that has demonstrated overexpression of endogenous β -gal^{19,25,48} was also chosen as another cell lines model. As expected, the same as in living 293T cells via the *lacZ* gene transfection method, we also clearly observed the quantitative ratiometric and light-up NIR imaging of endogenous β -gal activity in OVCAR-3 cells (Figure 3O–U). As shown in Figure 3A1, the light-up fluorescence signals were sharply decreased in the presence of a competitive β -gal inhibitor (D-galactose).⁴⁹ These results further confirmed that DCM- β -gal could monitor endogenous β -gal activity. Notably, because the incubation of DCM- β -gal was processed without any other complex loading techniques, DCM- β -gal has been demonstrated to be highly cell membrane-permeable and photostable. These results suggested that DCM- β -gal can be specifically activated in β -gal-expressing cells, thereby providing a ratiometric and light-up NIR readout for the in situ quantitative tracking and visualization of endogenous β -gal activity in living cells.

Real-time In Vivo Imaging of β -Gal Activity in Tumors.

The current fluorescent probes for imaging of β -gal activity are not very suitable for in vivo experiments because their emissions are not located in NIR region,^{20–23} and thus fail to penetrate deeper tissue and are complicated by intrinsic autofluorescence background signals. Recently, Urano and co-workers have made important progress by developing HMRef- β Gal for in vivo fluorescence endoscopy of β -gal activity.²⁵ Inspired by the distinct “light-up” NIR emission, we next examined its capability for real-time in vivo visualization of β -gal activity in tumors. A commercial tumor-targeting reagent (avidin- β -gal) was initially applied to localize β -gal to tumor growth of human colorectal cancer LoVo cell xenografts in vivo by intravenous injection^{50,51} because avidin has high affinity for lectins on the surface of LoVo cells.^{52,53} We next examined whether avidin- β -gal could be targeted to tumor cells in vivo by intravenous injection. After injection for 18 h, the main internal organs were obtained by anatomy and stained with X-gal (0.04 mg mL⁻¹), a commercial detection reagent for β -gal. After incubation for another 2.5 h, only in the disseminated tumor was a blue precipitate observed, suggesting that avidin- β -gal can be efficiently targeted to LoVo tumor cells in vivo without loss of the enzymatic activity (Figure 4). The mice bearing subcutaneously implanted tumors were administered DCM- β -gal by intravenous or intratumoral injection and then scanned at different time points with an IVIS Lumina Kinetic series III imaging system.

Owing to amplified fluorescence signals by enzymatic turnover and particularly low background interference in the NIR region, the remarkable NIR fluorescent signal was observed in the tumor site after in situ injection of DCM- β -gal (0.0375 mg kg⁻¹, Figure 5A) for β -gal upregulating mice induced by intravenous injection of avidin- β -gal (100 μg). It should be noted that, after merely 5 min post injection, the NIR fluorescence signal was already clearly appreciated in tumor, indicative of the rapid activation of DCM- β -gal, and the fluorescence intensity gradually increased, reached a maximum level after 3 h post injection, then slowly decayed as the time

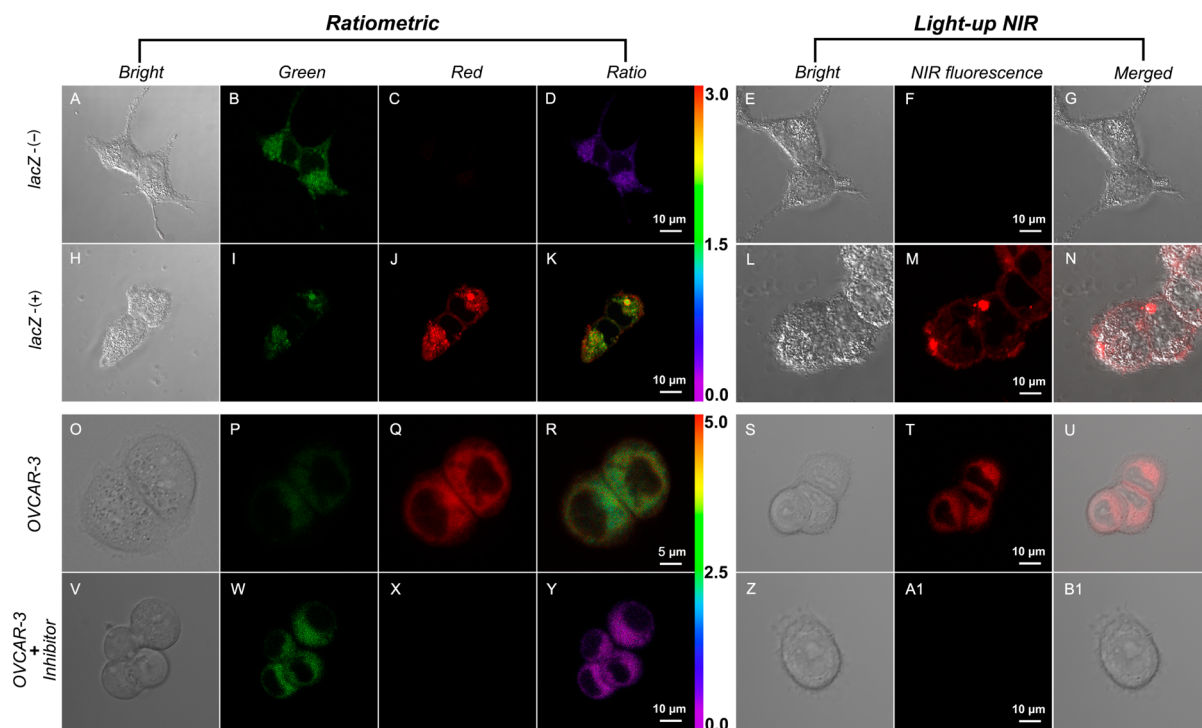


Figure 3. Confocal and ratiometric images of 293T and OVCAR-3 cells incubated with DCM- β gal (10 μ M) for 30 min: (A–G) *lacZ*(–) 293T cells without overexpressed β -gal, (H–N) *lacZ*(+) 293T cells with overexpressed β -gal, (O–U) OVCAR-3 cells, and (V–B1) OVCAR-3 cells pretreated with inhibitor for 30 min. Green channel obtained from 490 to 530 nm, red channel obtained from 650 to 720 nm, and ratiometric images generated from red and green channel, $\lambda_{\text{ex}} = 404$ nm. NIR fluorescence images obtained from 605 to 725 nm, $\lambda_{\text{ex}} = 560$ nm. The ratiometric images were obtained by the image analysis software Image Pro-plus 6.0.

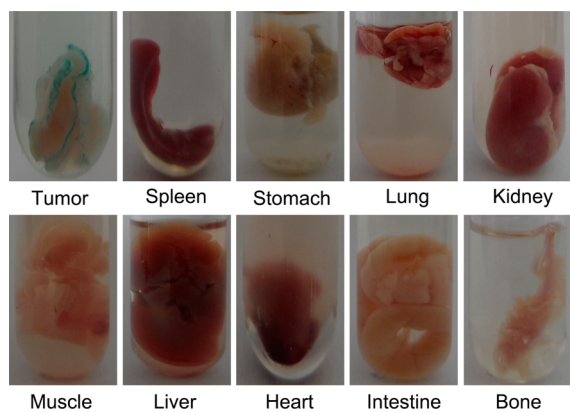


Figure 4. Biodistribution of avidin- β -gal targeting to LoVo tumor-bearing mice in vivo. Avidin- β -gal (100 μ g) in PBS was intravenously injected into LoVo-implanted mice, and the mice were sacrificed after 18 h. The main internal organs (tumor, spleen, stomach, lung, kidney, muscle, liver, heart, intestine, and bone) were removed and stained with X-gal (0.04 mg mL⁻¹) for another 2.5 h.

increased. In comparison, for the untreated tumor-bearing mouse, there is minimal light-up fluorescence under the same conditions (Figure 5B). To further confirm that the NIR fluorescent signal comes from the cleavage of DCM- β gal by β -gal in the tumor, we also recorded the ex vivo fluorescence images of tumor and other normal organs collected immediately after sacrificing the mice with upregulating β -gal at 3 h post injection. Consistent with the in vivo results, only the tumor was fluorescent (Figure 5C). As expected, the fluorescence images of other normal organs and tumor gained

from the control mice displayed no fluorescence signal (Figure 5D).

Real-time three-dimensional imaging is a powerful tool for accurate disease diagnostics, particular for suspicious lesions with high spatiotemporal precision. The preferable characteristics of DCM- β gal with light-up NIR performance and rapid response to β -gal activity (5 min) at the lesion site make it suitable to perform real-time three-dimensional imaging. After postinjection of the probe into the mice containing abundant β -gal, we obtained the real-time, high-resolution three-dimensional fluorescence image with an IVIS Spectrum CT imaging system. Observed from different perspectives in real time, the tumor site was precisely located in vivo and in situ with the help of NIR light-up fluorescence signals (Figure 5E and Movie 1 in the Supporting Information). By contrast, in the control experiments, there was no fluorescence signal located in tumors (Figure S10 and Movie 2 in the Supporting Information). For the first time, we exemplify the in situ three-dimensional fluorescence imaging to visualize real-time in vivo β -gal activity in tumors.

CONCLUSIONS

We have developed a ratiometric and light-up NIR fluorescent probe DCM- β gal, composed of the DCM scaffold as a typical NIR fluorescence reporter and a β -gal cleavable unit as the enzyme-active trigger. The probe has higher photostability than commercial ICG and displays pH independency over the physiological range. DCM- β gal displays higher affinity for β -gal than commercial X-gal and much faster response to β -gal than the previously reported FDG. More importantly, this probe was successfully applied for in situ and in vivo visualization of β -gal activity in human colorectal tumor in mice model, as well as

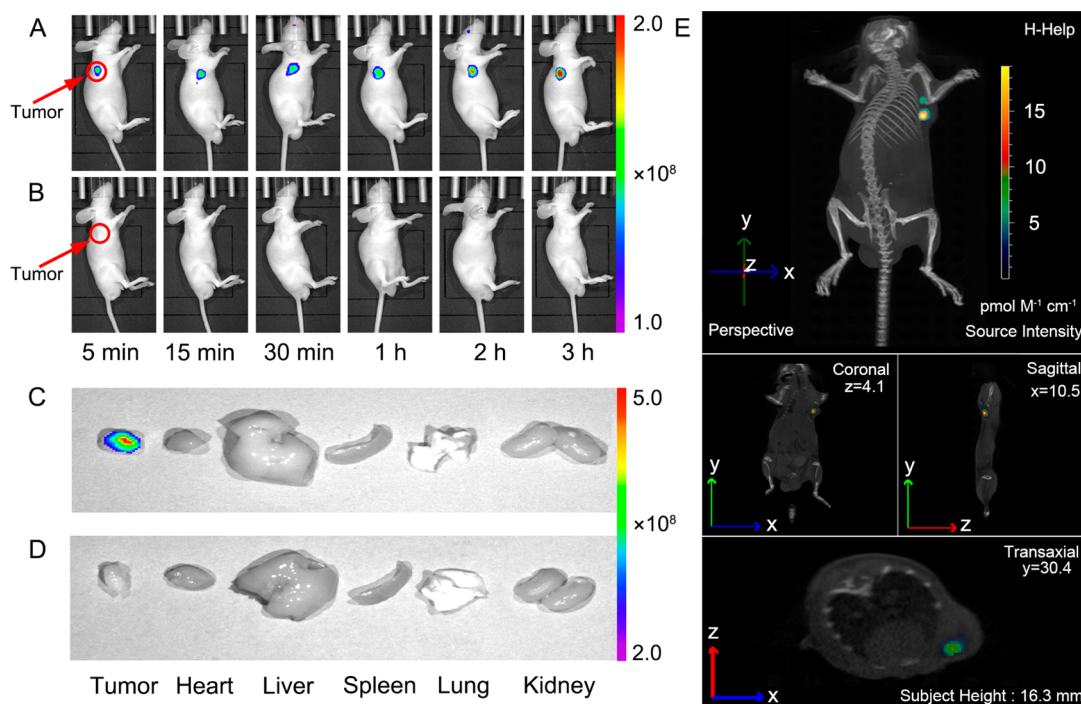


Figure 5. (A and B) In vivo imaging of β -gal activity in tumor-bearing nude mice after tumor injection, (C and D) fluorescence images of the main internal organs after anatomy, and (E) three-dimensional in vivo imaging of β -gal activity in tumor-bearing nude mice after tumor injection of DCM- β gal for 3 h. (A, C, and E) Avidin- β -gal (100 μ g) in PBS was intravenously injected into LoVo-implanted mice, and after 18 h DCM- β gal was then injected into the mice. (B and D) Tumor-bearing mice were not pretreated with avidin- β -gal before injection of DCM- β gal acting as the control.

real-time trapping of intracellular endogenous β -gal distribution in overexpressed living 293T cells via the *lacZ* gene transfection method and OVCAR-3 cells. For the first time, in vivo real-time capture of β -gal activity at tumor site with high-resolution three-dimensional view was performed. In light of its simplicity, sensitivity, and biocompatibility, this ratiometric and light-up enzyme-activatable NIR fluorescent probe provides an accessible tool for the unprecedented quantification and trapping of β -gal activity in vivo for human colorectal cancer diagnosis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01705.

More detailed experimental procedures, characterizations, supplementary optical spectra, and figures (PDF) Movie showing real-time, high-resolution three-dimensional fluorescence image with an IVIS Spectrum CT imaging system after postinjection of the probe into the mice containing abundant β -gal (AVI)

Movie showing results of control experiments, where there was no fluorescence signal located in the tumors (AVI)

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Notes

The authors declare no competing financial interest.

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