

## REVIEW ARTICLE

# In vivo optical imaging of cancer cell function and tumor microenvironment

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In vivo optical imaging using fluorescence and bioluminescence is superior to other methods in terms of spatiotemporal resolution and specificity, and represents a new technology for comprehensively studying living organisms in a less invasive way. Nowadays, it is an indispensable technology for studying many aspects of cancer biology, including dynamic invasion and metastasis. In observations of fluorescence or bioluminescence signals in a living body, various problems were caused by optical characteristics such as absorption and scattering and, therefore, observation of deep tissue was difficult. Recent developments in techniques for observation of the deep tissues of living animals overcame this difficulty by improving bioluminescent proteins, fluorescent proteins, and fluorescent dyes, as well as detection technologies such as two-photon excitation microscopy. In the present review, we introduce these technological developments and in vivo application of bioluminescence and fluorescence imaging, and discuss future perspectives on the use of in vivo optical imaging technology in cancer research.

**KEYWORDS**

bioluminescence imaging, fluorescence imaging, metastasis, tumor microenvironment, two-photon excitation microscopy

## 1 | INTRODUCTION

In vivo imaging technology is a method for non-invasively visualizing and analyzing various biological phenomena occurring within a living body. In recent years, image diagnostic technology capable of molecular-level imaging has developed dramatically in the medical field.<sup>1-3</sup> In vivo imaging technology has contributed greatly to the diagnosis and treatment of various diseases, including cancer and cranial nerve diseases, and the medical system is rapidly changing as a consequence of these advances. In contrast, in basic research fields such as cell biology, it has become possible to miniaturize image diagnostic instruments used in clinical fields and apply them to studies of experimental animals. It is now possible to observe cancer cell

dynamics and brain cell functions in the laboratory in living animals. However, these instruments are still large and expensive for use in laboratories. Because PET requires a cyclotron for preparing radioactive isotope probes, facilities capable of using this technique are limited. MRI has the disadvantage of low sensitivity. In addition, because spatial resolution of PET (1-2 mm), MRI (0.025-0.1 mm) and CT (0.025-0.1 mm) is low, it remains difficult to use these devices to carry out real-time observation of specific molecules in living cells or animals in the laboratory.<sup>1-3</sup>

Bioluminescence imaging, using the luminescent enzyme luciferase, and fluorescence imaging, using fluorescent proteins and dyes, are collectively referred to as optical imaging. These techniques have already been developed for in vitro and ex vivo applications in

**Abbreviations:** BMP, bone morphogenetic protein; Cdk2, cyclin dependent kinase; CEA, carcinoembryonic antigen; CT, computed tomography; FRET, Förster (or fluorescence) resonance energy transfer; Fucci, fluorescent ubiquitination-based cell-cycle indicator; ICG, indocyanine green; MVD, microvascular density; Raichu, Ras and interacting protein chimeric unit; RFP, red fluorescent protein; TGF- $\beta$ , transforming growth factor beta; VEGF, vascular endothelial growth factor.

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molecular and cellular biology. Although in vivo optical imaging has not yet been used clinically, it is attracting attention as a new research tool based on its speed, convenience, and versatility. Following the development of new bioluminescence and fluorescent probes and further improvement of the performance of instruments such as microscopes, various vital phenomena, including cancer, can be visualized using in vivo optical imaging technology.<sup>3-5</sup> Because in vivo optical imaging does not use special equipment such as a cyclotron or a large machine, it is possible to conduct experiments in a variety of research settings. In vivo bioluminescence imaging has a low background in living bodies and high sensitivity, and it is easy to detect signals in deep tissues of small living animals; consequently, it is useful for cancer metastasis research. Unlike in vivo luminescence imaging, in vivo fluorescence imaging does not require giving luciferin, a substrate for luciferase, and is therefore superior in terms of convenience and economy. However, it is difficult to use in vivo fluorescence imaging to detect a signal in the deep part of the tissue because the background in living bodies is high and the sensitivity is low. To address these problems, studies of in vivo fluorescence imaging using wavelengths in the near-infrared region are underway. Probes with these longer wavelengths have low background in living bodies and are less influenced by absorption and scattering of hemoglobin, making it possible to observe even deeper into the tissue. Furthermore, as a result of recent advancements in adjustable wavelength technology, it has become possible to use fluorescent proteins and dyes of various wavelengths, and applications such as multi-dimensional analysis are expanding. Moreover, multi-photon excitation fluorescence microscopy of non-linear optics enables deep-tissue observation of living animals with reduced influence of scattering.

## 2 | NECESSITY FOR OPTICAL IMAGING IN CANCER RESEARCH

Along with the development in recent years of molecular biology and cell biology, cancer research has made remarkable achievements.<sup>6,7</sup> Analysis of cancer-related genes and proteins has progressed at the molecular level, and our understanding of the biological characteristics of cancer has grown. However, cancer has an extremely complicated mechanism, and complete understanding of cancer malignancy, including aspects such as cancer cell invasion and metastasis, remains to be elucidated. Recently, the importance of cancer stem cells in malignant cancer and resistance to treatment has attracted attention. Moreover, it has become clear that the tumor microenvironment, including blood vessels and interstitial carcinoma, is a complex entity that provides important support to cancer cells. Therefore, to comprehensively understand cancer stem cells and tumor microenvironments, we must acquire not only in vitro information but also information on individual cells and biomolecules in vivo in terms of time and space and we need to know whether to take functional dynamics. Among the various imaging modalities, optical imaging is a

promising method for analyzing cancer cell function and the tumor microenvironment in vivo.

## 3 | VISUALIZATION OF CANCER CELL MOVEMENTS BY IN VIVO BIOLUMINESCENCE IMAGING

Luciferases derived from firefly and *Renilla* have been widely used in reporter assays to measure the expression of target genes in cell culture. Cells that stably express luciferase protein as a result of introduction of a luciferase gene produce biopermeable luminescence after giving a substrate such as D-luciferin or coelenterazine.<sup>8-10</sup> With the development of ultra-sensitive charge-coupled device (CCD) cameras, it has become possible to detect a trace amount of photons emitted within an animal's body. In addition, the data can be digitized by software and presented as real-time image data. As a result, it is possible to detect photons emitted by cancer cells in living animals and to monitor cancer cells in living bodies. For example, when cancer cells constitutively expressing *Renilla*-derived luciferase are transplanted into mice, and the substrate coelenterazine is given, the movement of cancer cells can be seen in the living animals.<sup>8</sup>

## 4 | VISUALIZATION OF CANCER CELL SIGNALING BY IN VIVO BIOLUMINESCENCE IMAGING

Signal transduction (transcriptional activity) can be visualized using a promoter reporter linking a luciferase gene downstream of a signal responsive promoter. For example, when cancer cells expressing firefly-derived luciferase under the control of TGF- $\beta$ -responsive promoter are transplanted into mice, and the substrate D-luciferin is given, TGF- $\beta$  signaling in cancer cells can be seen in the living animals.<sup>8</sup> We succeeded in observing TGF- $\beta$  and BMP signaling in bone-metastasized breast cancer cells in living mice by bioluminescence imaging. Furthermore, protein stability can be monitored using a fusion protein in which luciferase is linked to a protein of interest. For example, Zhang and colleagues succeeded in visualizing Cdk2 activity in cancer cells in living mice by transplanting cancer cells stably expressing a fusion protein of luciferase and p27, and found that in vivo accumulation of p27 is induced by giving a Cdk2 inhibitor.<sup>11</sup> Thus, bioluminescence imaging is a promising method for visualizing and analyzing cancer cell functions in living animals.

## 5 | VISUALIZATION OF CANCER CELL MOVEMENT BY IN VIVO FLUORESCENCE IMAGING

Although spatial resolution of bioluminescence and fluorescence is depth-dependent, it is superior in comparison with other imaging methods such as PET, MRI and CT. Particularly, fluorescence imaging

has superior spatial and temporal resolution in comparison with bioluminescence imaging, and this technique can be used to trace single cells in real time.<sup>3,4</sup> The spatial resolution of the two-photon excitation microscope is submicron level and the spatial resolution of the super resolution microscope is several nm beyond the diffraction limit. Hoffman and Yang developed the skin-flap method to visualize cancer cells moving in blood vessels in real time.<sup>12</sup> In this approach, the authors prepared HT1080 human fibrosarcoma cells expressing GFP in the cell nucleus and RFP in the cytoplasm (dual-color HT1080 cells), and s.c. transplanted these cells into blood vessels of immunodeficient mice. When the skin-flap method was applied in combination with an IV100 intravital laser scanning microscope (Olympus Corp., Tokyo, Japan), an in vivo fluorescence microscope, it was possible to observe cancer cell movement in blood vessels in real time (Figure 1). With this method, not only the movement of cancer cells in blood vessels, but also complex metastasis processes such as intravasation of cancer cells in primary tumors and extravasation of cancer cells in metastatic lesions, can be detected in real time, providing a great deal of information about the mechanisms of cancer metastasis.

## 6 | VISUALIZATION OF CANCER CELL SIGNALING BY IN VIVO FLUORESCENCE IMAGING

One feature of in vivo optical imaging is that functional molecular probes are easy to prepare. Consequently, not only cell morphology and movement, but also intracellular functions such as signal transduction, can be visualized. For intracellular signal transduction, FRET<sup>13-15</sup> or bimolecular fluorescence complementation (BiFC)<sup>16,17</sup> can be used to image activation and interactions of intracellular signaling molecules. Mochizuki and colleagues developed FRET-based molecular probes such as Raichu, which monitors the activation of Ras superfamily G protein, and observed gene activity in cancer cells in real time.<sup>18</sup> Moreover, imaging GFP expression under the control of the promoters of certain signaling target molecules allows us to visualize signal transduction in cancer cells. In addition, using transgenic mice harboring the aforementioned reporter genes, signal transduction of cells in the tumor microenvironment can be visualized. Using a reporter transgenic mouse linked to the GFP gene downstream of the VEGF promoter, Brown and colleagues showed

that the promoter activity of VEGF is high in the tumor microenvironment.<sup>19</sup> This observation suggests that the tumor promotes the production of VEGF in surrounding cells, resulting in angiogenesis. Thus, fluorescence imaging is a promising method for visualizing and analyzing cancer cell signaling in living animals.

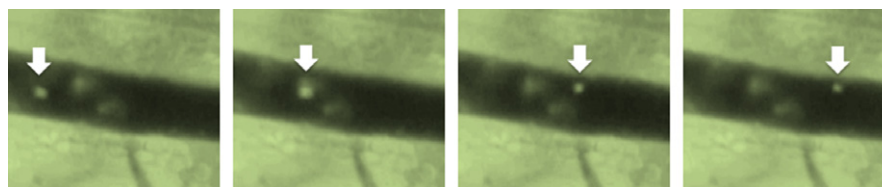
## 7 | VISUALIZATION OF CANCER CELL CYCLE BY IN VIVO FLUORESCENCE IMAGING

In 2008, Dr Sakaue-Sawano and our group developed a fluorescence imaging technique, Fucci, that visualizes progression of the cell cycle in real time.<sup>20</sup> Fucci is an advanced fluorescence imaging technique that takes advantage of the fact that the expression of cell cycle-related proteins is strictly controlled by the ubiquitin-proteasome system during cell proliferation. With Fucci, it is possible to analyze life-cycle phenomena related to the cell cycle, such as development, differentiation, regeneration, and carcinogenesis, in living cells and in animals.<sup>20-23</sup>

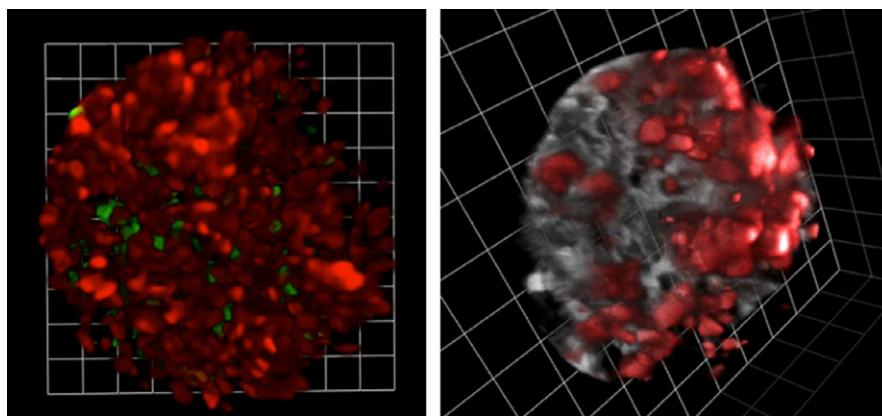
For example, transplanting Fucci-expressing cancer cells into mice allows analysis of the cell cycle of cancer cells in real time and in 3 dimensions. For example, we succeeded in imaging the cell-cycle progression of cancer cells (red in G1 phase, green in S/G2/M phase) in 3 dimensions using the bone metastasis model of Fucci-expressing breast cancer cells (Figure 2). In contrast, transgenic mice expressing Fucci systemically have been generated and applied to research in developmental biology and brain science. Furthermore, it is possible to apply the Fucci technology to laboratory animals other than mice, including zebrafish, which are both genetically tractable and optically transparent. With these Fucci technologies, molecularly targeted therapeutic agents are being developed that use the cell cycle as an indicator.

## 8 | VISUALIZATION OF TUMOR ANGIOGENESIS BY IN VIVO FLUORESCENCE IMAGING

Cancer growth and metastasis are closely related to angiogenesis, and angiogenesis inhibitors have anticancer activity. Angiogenesis is often evaluated in vivo by measuring MVD by immunohistochemical



**FIGURE 1** Visualization of cancer cells flowing in blood vessels by in vivo fluorescence imaging. After transplanting dual-color HT1080 cells into subcutaneous vessels of living mice, single cancer cell (arrow) migrating in blood vessels was imaged in real time using IV100 intravital laser scanning microscope (Olympus, Tokyo, Japan)



**FIGURE 2** In vivo imaging of cell-cycle progression of human breast cancer in a bone metastasis mouse model. Fluorescent ubiquitination-based cell-cycle indicator (Fucci)-expressing highly bone metastatic human breast cancer MDA-231-D cells were injected into the left cardiac ventricle of a female nude mouse. Six weeks after inoculation, cell cycle of a bone metastasis site was visualized using IV100 intravital laser scanning microscope (Olympus, Tokyo, Japan). (Left panel) Nuclei in red indicate G1 or G0 phase, whereas nuclei in green indicate S/G2/M phase. (Right panel) Bone stained by OsteoSense 750 (PerkinElmer, Waltham, MA, USA) is indicated by white

staining for CD34, a vascular endothelial cell marker. However, because animals must be killed to collect tissue samples for MVD, it is impossible to make sequential observations. Therefore, for studies of individual differences in tumor angiogenesis, large numbers of animals are required to achieve sufficient statistical power.

We attempted to sequentially visualize tumor angiogenesis in tumor-bearing mice in vivo, using a near-infrared fluorescence probe. For this purpose, nude mice transplanted with HT1080 cells were treated with a near-infrared probe, AngioSense 750 (PerkinElmer, Waltham, MA, USA), which labels blood vessels, and tumor angiogenesis was observed in vivo using the OV100 and IV100 imaging systems (Olympus Corp.).<sup>24</sup> Tumor blood vessel analysis using an in vivo fluorescence imaging system can evaluate changes over time in the same individual, allowing highly reliable data to be obtained. In addition, because this approach decreases the number of animals necessary for statistical analysis, it is beneficial for animal welfare, as well as being an effective tool for evaluating angiogenesis inhibitors and other drugs.

## 9 | VISUALIZATION OF ENZYME ACTIVITY BY IN VIVO FLUORESCENCE IMAGING

Proteases play important roles during cancer invasion and metastasis. When cancer cells invade, various proteases are secreted and activated to degrade the ECM. Various protease-activatable in vivo fluorescence imaging agents are available. For instance, MMPsense (PerkinElmer) is a fluorescent probe that visualizes the protease activity of MMP-2, -3, -9, and -13 by fluorescence-quenching effect. Using this probe, it is possible to image protease activity around tumors in living mice.<sup>25</sup> Furthermore, by combining this probe with cancer cells expressing GFP and AngioSense, tumors, tumor blood vessels, and enzyme activity can be simultaneously visualized and analyzed. Thus, fluorescence imaging is a promising method for

visualizing and analyzing the interactions between cancer cells and the tumor microenvironment in living animals.

## 10 | VISUALIZATION OF IN VIVO DEEP TISSUE BY MULTI-PHOTON EXCITED FLUORESCENCE MICROSCOPY

Multi-photon excited fluorescence microscopy is one of the most promising candidates for achieving deep-tissue fluorescence imaging in living animals.<sup>25-28</sup> In single-photon absorption in conventional laser light microscopy, a fluorescent molecule absorbs a photon that has energy equal to the energy difference between the ground state and the first excited state of the molecule. In multi-photon excitation fluorescence microscopy, a fluorescent molecule simultaneously absorbs two photons with approximately twice the wavelength and half the excitation energy of the aforementioned single photon. This is called a two-photon excitation process, and is comparatively very rare; consequently, in multi-photon excitation fluorescence microscopy, excitation of the fluorescent molecule occurs at the focal point only.

The advantage of two-photon excitation is that, because long-wavelength light is used, the influence of scattering is small, and it is possible to excite the fluorescent molecule at the focal position only, allowing high-resolution imaging in the deep part of living tissue. In the fields of neuroscience and immunology, research using two-photon excitation microscopy has been conducted widely for more than 10 years. Applications such as imaging of cerebral neocortical pyramidal cells,<sup>29</sup> dynamics of lymph node immune cells,<sup>30</sup> and kinetics of bone marrow osteoclast precursor cells<sup>31</sup> have been reported in living animals. Our group carried out fluorescence imaging of the cerebral neocortex of a living animal using a two-photon excitation fluorescence microscope, at a depth of approximately 1 mm from the brain surface, spanning from the



cell bodies of pyramidal cell layer V to the upper and lower basal dendrites (Figure 3).

## 11 | CLINICAL APPLICATION OF IN VIVO FLUORESCENCE IMAGING

Spatial and temporal resolutions of fluorescence imaging technology are superior to those of CT, MRI, and PET. Consequently, these methods are expected to be useful as tools for localization of intra-operative cancer in laparoscopic surgery. ICG, a liver function test reagent, has already been applied as a fluorescent probe for navigation surgery, allowing illumination of blood vessels, bile ducts, and sentinel lymph nodes in surgery and brain surgery operations. Because such a near-infrared fluorescent imaging technique excites the fluorescent substance with long-wavelength light, its biological tissue permeability is superior to that of visible light, and it is therefore more suitable for observation of deep tissue in a living body. However, in *in vivo* imaging by conventional fluorescence macro-microscopy using near-infrared light, the signal to noise ratio of the image is low as a result of autofluorescence and high background from non-specifically bound fluorescent probe.

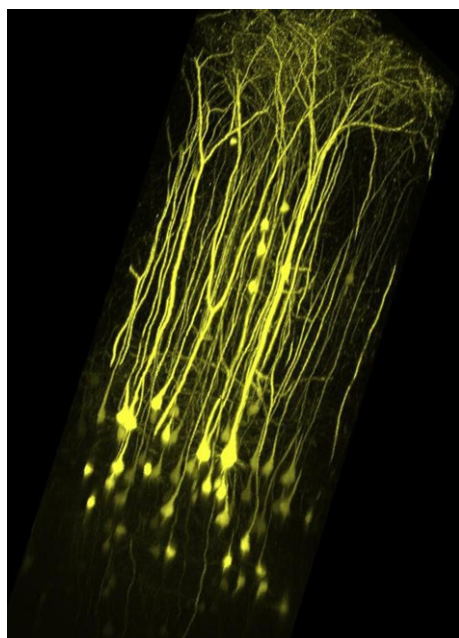
To overcome these problems of near-infrared fluorescence imaging, we attempted to develop a biomarker fluorescence imaging technique by two-photon excitation microscopy.<sup>32</sup> For this purpose, we used fluorescently labeled antibodies against cancer-specific antigens as fluorescent probes and observed cancer cells with a two-

photon excitation microscope *in vivo*. Specifically, human cancer cells expressing CEA, a malignant marker for cancer, were s.c. transplanted into immunodeficient mice, and fluorescently labeled anti-CEA antibody was given (Figure 4). In the image of cancer tissue obtained on the two-photon excitation microscope, cancer cells are displayed in the same way as in a stained image of a pathological section. A method that can diagnose without resection is referred to as "optical biopsy".<sup>33</sup> By combining two-photon excitation microscopy and cancer-specific fluorescent probes, optical biopsy can be realized.

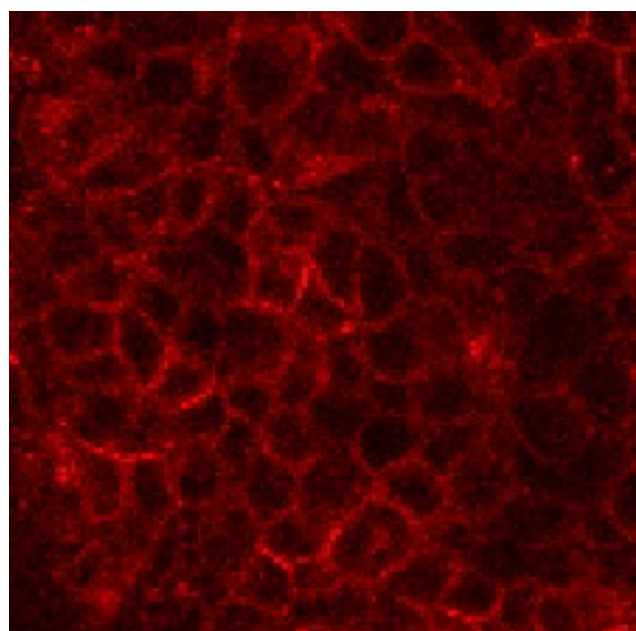
## 12 | CONCLUSION AND PERSPECTIVES

*In vivo* optical imaging is a promising technique for studying complex biology and various disease processes, including cancer, *in vivo*. In particular, *in vivo* fluorescence imaging using various fluorophores and/or fluorescent proteins, in conjunction with the appropriate type of microscopy, allows *in vivo* visualization of cancer cell behavior and function, as well as the tumor microenvironment.<sup>34</sup> Using two-photon excitation microscopy, it will be possible to diagnose cancer *in vivo* without biopsy (optical biopsy).

However, for the cancer fluorescence imaging technique described above, it is necessary to ensure the safety and effectiveness of the fluorescent probes that recognize cancer before they can be applied to humans. One potential solution for this problem is unstained imaging using autofluorescence, second harmonic



**FIGURE 3** *In vivo* fluorescence imaging of mouse brain using a two-photon excitation microscope. Cerebral neocortex of a H-line transgenic mouse was imaged using a two-photon excitation microscope. Enhanced yellow fluorescent protein fluorescence was detected from layers deeper than 1 mm beneath the brain surface in an anesthetized mouse



**FIGURE 4** *In vivo* fluorescence imaging of human cancer cells in a model mouse using a two-photon excitation microscope. Human gastric cancer MKN45 cells were inoculated into immunodeficient mice, and *in vivo* fluorescence imaging of the tumor mass was carried out using a two-photon excitation microscope, 24 h after injection of Alexa Fluor 594-conjugated anti-CEA antibody

generation and third harmonic generation.<sup>35–37</sup> Further development of unstained imaging methods will provide additional insights into the applications of in vivo imaging in humans.

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There are many important papers in this field and, for reasons of space, we have not been able to mention all of them. We apologize to those investigators whose papers could not be cited. This work was supported by JSPS KAKENHI Grant Number JP15H04962, MEXT/JSPS KAKENHI Grant Number JP16H06280 "Advanced Bioimaging Support", JP15H05952 "Resonance Bio" and the Research Program of "Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials" in "Network Joint Research Center for Materials and Devices" to TI, and JSPS KAKENHI Grant Number JP16K18578 to TS.

## CONFLICT OF INTEREST

Research finding: Takeshi Imamura from SONY Corporation and Chugai Corporation.

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