In vivo molecular and single cell imaging

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Molecular imaging is used to improve the disease diagnosis, prognosis, monitoring of treatment in living subjects. Numerous molecular targets have been developed for various cellular and molecular processes in genetic, metabolic, proteomic, and cellular biologic level. Molecular imaging modalities such as Optical Imaging, Magnetic Resonance Imaging (MRI), Positron Emission Computed Tomography (SPECT), and Computed Tomography (CT) can be used to visualize anatomic, genetic, biochemical, and physiologic changes in vivo. For in vivo cell imaging, certain cells such as cancer cells, immune cells, stem cells could be labeled by direct and indirect labeling methods to monitor cell migration, cell activity, and cell effects in cell-based therapy. In case of cancer, it could be used to investigate biological processes such as cancer metastasis and to analyze the drug treatment process. In addition, transplanted stem cells and immune cells in cell-based therapy could be visualized and tracked to confirm the fate, activity, and function of cells. In conventional molecular imaging, cells can be monitored in vivo in bulk non-invasively with optical imaging, MRI, PET, and SPECT imaging. However, single cell imaging in vivo has been a great challenge due to an extremely high sensitive detection of single cell. Recently, there has been great attention for in vivo single cell imaging due to the development of single cell study. In vivo single imaging could analyze the survival or death, movement direction, and characteristics of a single cell in live subjects. In this article, we reviewed basic principle of in vivo molecular imaging and introduced recent studies for in vivo single cell imaging based on the concept of in vivo molecular imaging.

INTRODUCTION

Molecular Imaging is a growing biomedical field focusing on the visualization, characterization, and quantification of biological processes in living subjects. Molecular imaging is used to improve the disease diagnosis, prognosis, monitoring of treatment in living subjects (1, 2). Current research in molecular imaging require a multidisciplinary approach for various research fields such as molecular biology, chemistry, medical physics, engineering, biomedical imaging, and computer science. Various molecular images allow us to visualize cellular and subcellular processes with genetic, metabolic, proteomic, and cellular biologic imaging in vitro, in vivo, and even patients (1, 2).

Molecular images such as Optical Imaging, Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT), and Computed Tomography (CT) could visualize anatomic, genetic, biochemical, and physiologic changes (Fig. 1). For molecular imaging, numerous molecular targets have been developed for cellular and molecular processes in genetic, metabolic, proteomic, and cellular biologic level. As strategies for molecular imaging, there are direct and indirect labeling methods to monitor cells in small animal (Fig. 2). Direct cell labeling methods usually use a target-specific probe which could be interacted with a specific target in cells. As molecular imaging probe, nanoparticles, antibodies, peptides and aptamers could be synthesized with other materials for imaging signals such as fluorescence dye and radioisotopes. For indirect labeling, reporter gene-based imaging system in target cells could be used to monitor gene expression. The reporter gene could encode detectable proteins based on the specific promoter, and the reporter proteins with specific imaging signals could provide indirect information for reporter gene expression. Reporter gene imaging systems were used in optical, nuclear medicine, and magnetic resonance imaging. Through these labeling methods, the ultimate goal of molecular imaging can diagnose diseases at the cellular level and noninvasively monitor the biochemical processes of cells in real time. In cell-based therapeutics, identifying migration pathways of cells are important issues and essential for in vivo application (3, 4). Various cell-based therapies using immune cells and stem cells focus on their location and biodistribution in vivo, their effects, and the presence or absence of toxicity in vivo (5, 6).
addition, suppression of cancer cells through cell-based therapies requires monitoring and quantification of cells in vivo. In vivo cell imaging through molecular imaging modalities could be useful tools for disease diagnosis and treatment monitoring methods for various diseases (3, 4, 7). In the process of treating cardiovascular diseases using stem cells, cell tracking could be used for the cell activity. For these reasons, in vivo cell imaging could be considered importantly for in vivo application. In terms of in vivo cell imaging, single cell imaging and tracking have the greatest advantages to identify individual characteristics of each cell in the body (8-10). With real-time single cell imaging in vivo, specific characteristics of various single cells can be identified, and it could be possible to broaden the research direction of cell-based therapy in the future. This review describes various studies for in vivo molecular imaging tools with their advantages and disadvantages and we would like to review the recent research related to single cell imaging and discuss the future directions of in vivo single cell imaging.

**IN VIVO MOLECULAR IMAGING**

1. OPTICAL IMAGING

Fluorescence imaging and bioluminescence imaging are types of optical imaging that generates light from different sources (1, 2). Fluorescence imaging uses a fluorescent protein (fluorophore) which is excited by an external light source. In bioluminescence imaging, luciferase enzyme converts luciferin substrate to oxyluciferin in the presence of oxygen, magnesium, and ATP, producing emitted visible light. Luciferase mainly used as a bioluminescence reporter gene including Firefly luciferase (Fluc), Renilla luciferase (Rluc) and gauussia luciferase (Gluc). During imaging acquisition, fluorescence imaging required specific optical filters for both excitation and emission wavelengths but bioluminescence imaging only required emission filter. Cooled charge coupled device (CCD) camera reduces thermal noise and increase sensitivity. Both fluorescence and bioluminescence are powerful imaging modalities’ to visualize molecular processes in vitro and in vivo, because these modalities are simple, cheap, and convenient comparing to other imaging modalities (3, 4). For fluorescence imaging, it doesn’t require a substrate and has a short acquisition time that can acquire images within seconds (Table 1). However, fluorescence imaging requires external light source, causing phototoxicity and photobleaching. In addition, fluorescence imaging has some limitations for in vivo application due to autofluorescence and imaging depth. Bioluminescence imaging have the advantages for high sensitivity and specificity, high signal-to-noise ratio, and no phototoxicity (Table 1). But bioluminescence imaging also has the limited imaging depth like fluorescence imaging and requires a substrate for imaging. In addition, disadvantages of optical images are the limited quantification for imaging, and poor spatial resolution due to scatter (11).

Optical imaging could be used for in vivo cell imaging. Unlike various molecular imaging methods such as nuclear medicine imaging, fluorescence imaging uses fluorescent proteins without a reporter probe to monitor gene expression, cellular localization, and protein-protein interaction (12). GFPs have been widely used to visualize the specific protein

Table 1. The properties of molecular imaging modalities

| Imaging modalities | Penetration | Sensitivity (mol/L) | Resolution (mm) | Time    |
|--------------------|-------------|---------------------|-----------------|---------|
| Fluorescence       | Poor        | $10^{-9}$-$10^{-12}$ | 0.2-2 mm        | Sec-min |
| Bioluminescence    | Fair        | $10^{-15}$-$10^{-17}$ | 0.2-2 mm        | Min     |
| PET                | Good        | $10^{-11}$-$10^{-12}$ | 1-2 mm          | Min     |
| SPECT              | Good        | $10^{-10}$-$10^{-11}$ | 1-2 mm          | Min     |
| MRI                | Excellent   | $10^{-3}$-$10^{-5}$  | 10-100 um       | Min-hr  |

![In vivo molecular imaging is useful for single cell imaging](Seongje_Hong_et_al.png)

![Seongje_Hong_et_al.png](http://bmbreports.org)
distribution in cells, as well as to analyze the biological response of cells. Various types of engineered fluorescent proteins such as enhanced GFPs (eGFPs), Red Fluorescent Proteins (RFPs), mCherry, and tdTomato have been developed to improve the fluorescence brightness and photostability (12). In addition, fluorescent proteins with long emission wavelengths such as NIR-I (700-1,000 nm) or NIR-II (1,000-1,700 nm) have been recently developed for research and clinical studies. NIR fluorescence imaging has an advantage to visualize deep-tissue structure in vivo and to monitor surgical margins intraoperatively in clinical application (13-15). Some studies were conducted with fluorescence imaging to visualize cancer metastasis, cancer cell division, apoptosis, and cell cycle in vivo (16). By injecting various fluorescence-tagged tumor cells in the body, tumor metastases could be monitored in specific organs in the body (17, 18). Other study showed that Cancer stem-like cells (CSCs) and non-stem cancer cells (NSCCs) were labeled with each fluorophore and injected into the subcutaneous and spleen of nude mice for in vivo cell imaging (19). The survival rate, tumorigenesis, and metastasis of CSCs against chemical drugs were studied in vivo. By expressing fluorescence proteins in HT-1080 fibrosarcoma cells, the nuclear-cytoplasmic ratio was visualized (20). For cell cycle study, the method using the fluorescence ubiquitination cell cycle indicator (Fucci) is used to observe changes in the expression of fluorescent proteins depending on the cell cycle (21, 22). Fluorescence imaging are expected to be continuously studied and developed to analyze cell differentiation, mobility, and characteristics for in vivo application.

Various studies for in vivo imaging have been conducted using bioluminescence imaging (1, 2). Originally, in vivo bioluminescence imaging was developed in bacterial injection models (23). In this study, Salmonella typhimurium was marked with bioluminescence protein, which is a bacterial luciferase, and localization of bioluminescent Salmonella was detected in animals. For in vivo cell imaging, various kinds of cells are commonly labeled with bioluminescence proteins such as Fluc and Gluc (3, 4). Bioluminescence imaging are widely used for in vivo whole-body imaging than fluorescence imaging due to high sensitivity and low background signals (Table 1). The study showed that doxorubicin-resistant breast cancer cell lines were tagged with the renilla luciferase (RLuc), and Natural Killer cells were also tagged with the enhanced firefly luciferase (effluc) (24). In addition, the expression of specific genes in the brain have been studied using bioluminescence imaging (25). Other in vivo study was conducted to quantify the level of specific gene expression by expressing Alzheimer’s-inducing tau mutations with luciferase (26). There was study proliferation, migration and differentiation on neural stem cells using bioluminescence imaging (27). Bioluminescence Resonance Energy Transfer (BRET) is a method to study protein-protein interaction in living cells, based on the non-radioactive energy transfer exhibited by luminescence donors (28). Fluorescence resonance energy transfer (FRET) is also used for protein-protein interaction, but there are some limitations due to the emission wavelength and autofluorescence in FRET. On the other hand, BRET’s luminescence donor could emit light through the action with the substrate without phototoxicity or autofluorescence. However, the low signal output of luminescence donors reduces the resolution of BRET, and various luminescence donors have been developed to overcome this limitation (29). For example, NanoLuc (Nluc) improves the resolution of BRET with 150-fold higher signal intensity compared to Rluc or Fluc (30, 31). It could be applicable for various fields such as protein-protein interaction, gene regulation, protein stability and imaging through NanoBRET. Studies for transmembrane receptors are being conducted using BRET (32). Ligands that specifically bind to G-Protein Coupled Receptors (GPCRs) are analyzed using BRET as biosensors (33). In addition, the BRET-based GPCR constitutive sensor was investigated for conformational changes.

2. MAGNETIC RESONANCE IMAGING

MRI is a non-invasive imaging modality that uses for disease diagnosis, detection, and treatment monitoring (1, 2). MRI detects changes in the rotational axis of protons in the water and uses strong magnets in the body to align protons (3, 4). A radio frequency stimulates the protons and the protons spin out of equilibrium. When a radio frequency is turned off, the MRI sensor detects the energy which is released from the protons realigned by the magnetic field. The amount of energy varies depending on the chemical properties of the molecule and the environment, and substances such as contrast agents and superparamagnetic iron oxide (SPIO) nanoparticles are used to increase signal intensity. MRI has the advantage of acquiring anatomical and physiological information with high spatial resolution (Table 1). However, MRI has a low sensitivity for imaging agents and molecular reactions. In addition, it requires highly trained person and high costs for imaging acquisition. For in vivo studies, MRI imaging is used for cell monitoring in cell-based therapies. The reporter genes used in MRI contains a cellular receptor, an enzyme coding gene, and an endogenous reporter gene (34). Transferrin receptor is a commonly used as MRI reporter gene expressed on the cell surface membrane of the transfected cells, resulting in decreased T2 signaling (34, 35). Tyrosinase (TYR) is an enzyme that produces melanin to chelate paramagnetic ions, inducing a high MR signal (36). β-galactosidase is an enzyme coding gene to produce strong T1 contrast with a contrast agent such as EgdavMoe (37). Ferritin is an endogenous reporter gene that binds and stores iron (34, 38, 39). For in vivo cell imaging, SPIO nanoparticles have been widely used in MRI (40-42). The stem cells labeled with SPIO were monitored by MRI for diagnosis and therapies. Recent study used SPIO for MRI imaging of hepatocellular carcinoma, showing excellent targeting and transfection ability for liver cancer (43).

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3. NUCLEAR MEDICINE IMAGING
Many radionuclides for nuclear medicine imaging have been used for a diagnosis and treatment of patients (1, 2). The principle of nuclear medicine imaging is that radiation emitted from radioactive materials is detected by external detectors (3, 4). For PET imaging, a radioisotope is labeled with PET carriers, emitting a positively charged electron. This positron interacts with the electron, causing two photons in opposite directions by an annihilation reaction. The PET scanner identifies the pair of these photons, and the computer reconstructs the data through a specific algorithm (44). PET has advantages which are highly sensitive and quantitative, detecting the molecular probes of picomolar concentration (Table 1). SPECT is an imaging modality for detecting and imaging radiation emitted from gamma-emitting isotopes (1, 2). The SPECT detector identifies gamma rays, and three-dimensional images are reconstructed. Because gamma-emitting isotopes occur in all directions, collimators are used to achieve a specific range of resolution. Nuclear medicine imaging provides anatomic and functional information invasively (3, 4). Nuclear medicine imaging has several advantages for high sensitivity, high quantification, no attenuation problems, and easy translation from animal to human. Currently, there has been many approved imaging agents for clinical use and developing various novel agents in research fields. However, nuclear medicine imaging has several disadvantages of poor spatial resolution and limited signal to noise ratio and it requires high operating costs and health risk by radiation exposure. To improve spatial resolution, imaging instruments for small animal such as micro-PET have been developed for the basic research. For in vivo cell imaging, reporter gene systems were used with nuclear medicine imaging. The herpes simplex virus type 1–thymidine kinase (HSV1-TK) is a reporter gene commonly used for PET imaging (1, 2). The HSV1-TK enzyme can phosphorylate the radiolabeled substrate such as pyrimidine nucleoside and purine analogs. The study imaged the cytotoxic T lymphocytes by expressing the HSV1-TK reporter gene (45). Dopaamine 2 receptor (D2R) is used as a nuclear medicine reporter gene to combine the Dopaamine 2 receptor ligand such as 18F-fluoroethylspiperone (46). Sodium iodide symporter (NIS) is the cell membrane protein used to uptake radionuclides such as 123I and 131I (47). Somatostatin receptor (SSTR) reporter gene is a membrane receptors which uses PET tracers such as 68Ga-DOTATOC for the diagnosis of neuroendocrine tumors and other various types of tumors (48). Estrogen receptor is a reporter gene for a estrogen receptor ligand (49). Human Norepinephrine transporter (NET) has been recently reported using the elevated AkaLumine, showing higher signals in bioluminescence image and permeability to the blood-brain barrier (BBB) than D-luciferin (57). In this study, in vivo bioluminescence signals could be confirmed even in a single cell, detecting the signals of cells in the lung of mice. For this experiment, the signal levels for D-luciferin/Fluc and AkaLumine/Akaluc in cells were compared (8–10). As a result, AkaLumine/Akaluc combination showed ~52 fold stronger signals than D-luciferin/Fluc in vivo. Through these high signals, it was possible to visualize single cell in the body of mouse with optical imaging methods. A HeLa/Akaluc single cell was injected into a mouse and the signals of a HeLa/Akaluc single cell was then confirmed in bioluminescence imaging. The results showed positive signals in 2 out of 12 mice and it could be inferred that a single cell in 10 mice just passed through the lungs showing no signals. In addition, the experiments showed the stability of this molecule by penetrating the BBB on the brain. A single cell imaging with AkaLumine/Akaluc combination could allow real-time monitoring of single cell. Since AkaLumine could be used to monitor a

C2Am in mouse lymphoma models and in human colorectal xenografts (54).

IN VIVO SINGLE CELL IMAGING
In vivo single cell imaging could be studied to identify the migration, characteristics, differentiation of single cells and effects of drug treatment. The size of single cells was generally known from 1 to 100 um, depending on the cell types. Since the size of single cells is extremely small comparing to the body, it’s very challenging to track a single cell in vivo (8-10). Although in vivo molecular imaging is useful tools to monitor the distribution of cells in the body, it is generally possible to image the cells in bulk non-invasively, not to individual single cells. Recently, there has been great attention for in vivo single cell imaging due to the development of single cell study (55). If it is possible to track a single cell in vivo, it could be helpful to investigate various studies with oncology, immunology, and stem cell biology. Here, we would like to review the recent research for in vivo single cell imaging (Fig. 3).

1. OPTICAL IMAGING
The reason that optical imaging has limitations in vivo is that the emission maximum of D-luciferin is 562 nm, showing a low access to deep tissue. To overcome these limitations, bioluminescence imaging using the near-infrared (NIR) has been developed. AkaLumine is a luciferin analogue in which the aromatic structure of D-luciferin was replaced with a benzothiazole moiety, generating NIR bioluminescence for sensitive deep-tissue imaging. The emission maximum of AkaLumine is 675 nm. AkaLimine could react with Fluc emitting the NIR wavelength light. This methods could solve the limitations of deep tissue in bioluminescence imaging for various animal study (56). The single cell imaging in vivo has been recently reported using the elevated AkaLumine, showing higher signals in bioluminescence image and permeability to the blood-brain barrier (BBB) than D-luciferin (57). In this study, in vivo bioluminescence signals could be confirmed even in a single cell, detecting the signals of cells in the lung of mice. For this experiment, the signal levels for D-luciferin/Fluc and AkaLumine/Akaluc in cells were compared in vivo. As a result, AkaLumine/Akaluc combination showed ~52 fold stronger signals than D-luciferin/Fluc in vivo. Through these high signals, it was possible to visualize single cell in the body of mouse with optical imaging methods. A HeLa/Akaluc single cell was injected into a mouse and the signals of a HeLa/Akaluc single cell was then confirmed in bioluminescence imaging. The results showed positive signals in 2 out of 12 mice and it could be inferred that a single cell in 10 mice just passed through the lungs showing no signals. In addition, the experiments showed the stability of this molecule by penetrating the BBB on the brain. A single cell imaging with AkaLumine/Akaluc combination could allow real-time monitoring of single cell. Since AkaLumine could be used to monitor a
single cell, it would be a promising approach for in vivo cell tracking in the future.

2. MAGNETIC RESONANCE IMAGING

MRI has sensitivity for molecular probes in the range of micromolar to millimolar concentrations. Various studies for cell imaging have been performed by MRI (58). The showed that primary mouse hepatocytes were labeled with iron oxide particles and fluorescent agents and transplanted them into the mouse spleen (59). Since hepatocytes can migrate from the spleen to the liver as a single cell level, in vivo MRI could detect a black contrast area from a single cell in the livers. In addition, high-resolution MRI could be possible to image single cells in vivo by labeling cells with micron-sized iron oxide (MPIO) (60). In this study, MPIO could be taken up by a various cell types and the best strategies to label cells, because it could uptake approximately ~3 fold higher than nanometer-sized particles. Therefore, it could suggest various research directions for in vivo single cell imaging using MRI. On the other hand, the study could monitor the metastasis of single breast cancer to the brain in vivo using MRI (61). In this study, MDA-MB-231BR cells labeled with MPIO were injected through the left ventricle of a heart in mouse. Over the time, the micro-metastasis of labeled cells was visualized on the brain in single cell level using MRI, showing the tumor formation on the brain from day 28 to day 33. These above research results showed that MRI could be a useful tools to monitor single cells and investigate the micro-metastasis of a single cancer cell in vivo. Therefore, the technology for diagnosing initial tumor metastasis in vivo would be developed in single cell level and various research directions could be suggested in the future. However, MRI imaging have some limitations for in vivo real-time imaging. Since single cell imaging in in vivo MRI could be only possible at uniform-background anatomical organs such as brain and MRI doesn’t have the sufficient temporal resolution to track the moving cells in vivo, some improvement could be required in the future.

3. INTRAVITAL MICROSCOPY IMAGING

Intravital microscopy (IVM) is a tool that can visualize several biological processes in live animals (62). With the development of intravital microscopy, biological research has been investigated to image in vivo subcellular structures. Single-cell pharmacokinetics imaging (SCPKI) could be used for the high resolution and temporal imaging of single cells in vivo (63). In this study, high-resolution imaging with intravital microscopy was used to measure drug kinetics through in vivo single cell imaging. A fluorescent derivative of a PARP inhibitor has been synthesized to measure drug distribution. The distribution of drugs could be visualized and analyzed over time according to the location of a single cell in vivo. In addition, it was possible...
to measure the proportion of cells which are receiving sub-therapeutic drugs or are not taking drugs. This quantitative data could be used to predict the effects of drugs in the body. Multiphoton intravitral microscopy (MP-IVM) is a method that enables dynamic monitoring of cells under various physiologic conditions in living animals (64). MP-IVM uses a longer wavelength (700 nm and greater) than confocal techniques to penetrate more deep tissue, and could be used to monitor the pharmacological action of drugs in vivo (65). The MP-IVM obtains high-contrast cellular-level images from thick opaque specimens with minimized toxicity and photobleaching, and enables long-term imaging of biological processes in laboratory animals (66). MP-IVM was used to monitor leukocytes in vivo (67). In this study, the spatiotemporal dynamics of immune cells were visualized in mouse model of mandibular draining lymph nodes using MP-IVM. Through this, the pathogenesis of immune and inflammatory diseases in specific sites can be studied. Advances in these studies would allow pharmacokinetic and pharmacodynamic imaging to be used for the therapeutic efficacy in vivo. It also could have the potential on cell biology to investigate mechanisms for the physiological and pathological characteristics. However, since IVM methods can analyze the characteristics of single cell on shallow tissues, not on the whole-body, it would be considered for in vivo whole-body experiments (68).

4. PHOTOACOUSTIC IMAGING

Photoacoustic (PAT) imaging is a molecular imaging tool that could image organs in a living subject with high contrast and high spatial resolution (69). PAT uses the photoacoustic effect, and the photoacoustic energy is converted from absorbed light energy. PAT has the advantage for the deep tissue penetration and high spatial resolution compared with pure optical imaging (70). The photoacoustic imaging was investigated for in vivo studies related to Red Blood Cells (RBCs). As oxygen transporters, RBCs play an important role in vivo, but the technology for in vivo imaging of single RBCs is lack currently. Single-RBC photoacoustic flowoxigraphy (FOG) was developed for in vivo study of oxygen metabolism in RBC (71). In this study, real-time spectral imaging of RBCs could be used in vivo. A single RBC FOG could image single erythrocyte oxygenation on the brain of a mouse. In addition, this study showed a strong correlation between single-erythrocyte oxygenation and neurostimulation, and it was a novel approach to confirm the various effects through single-erythrocytes in the brain. Single-RBC FOG has the advantage for not using a contrast agent which may affect the experimental results. This single-RBC FOG imaging modality could bring some benefits to investigate an early diagnosis and treatment of tumors. With development of technologies, various studies related to tissue oxygenation could be conducted in the future.

5. NUCLEAR MEDICINE IMAGING

Among molecular imaging modalities, PET has the highest sensitivity to detect the picomolar level of molecular targets. It has been considered as the most promising method imaging modality for in vivo single cell tracking. Originally, the concept of tracking in PET was used to measure the dynamics of powder and fluid flows in chemical engineering (72). Then a mathematical frame was developed to tracking the radioactive sources and radiolabeled cells (73). In addition, single cell tracking performance was validated in simulation and phantom studies using radioactive droplets (74). Recent research showed in vivo real-time single cell tracking in whole-body animals with PET (10). In this study, human breast cancer cells were labeled with mesoporous silica nanoparticles (MSNs) highly concentrating the 68Ga radioisotope. The labeled single cells were injected through the tail vein of mice, and real-time single cell tracking was performed with PET in vivo. For real-time single cell tracking, a novel single cell tracking algorithm was required with low temporal resolution, because a temporal resolution of normal PET is several minutes to hour. In previous study, a novel trajectory reconstruction algorithm was developed for single cell tracking, showing a low temporal resolution about 10 ms (73). To track a single cell in vivo through this algorithm, the high radioactive single cells in which minimum radioactivity is > 20 Bq should be developed. Due to the superior properties such as a high surface area, MSNs could ferry radioisotopes efficiently. In conclusion, this PET imaging study revealed in vivo real-time position of the injected single cells, inferring the movement speed and stationary site in the body through imaging processing. This in vivo single cell tracking methods could be used to investigate the early stage of metastasis mechanism in cancer biology, and to study the kinetics of cell tracking on cell-based therapy such as regenerative therapy and immunotherapy. According to the recent development of computer science, the imaging analysis techniques were improved through imaging processing with deep learning in artificial intelligence. In addition, with the continuous development of PET scanners and single cell biology, in vivo single cell tracking techniques would be very promising and powerful tools for research fields and clinical use.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Blasberg RG and Gelovani-Tjuvajev J (2002) In vivo molecular-genetic Imaging. J Cell Biochem 39, 172-183.
2. Kang JH and Chung JK (2008) Molecular-genetic imaging based on reporter gene expression. J Nuc Med 49, 1645-
3. Kircher MF, Grambihler SS and Grimm J (2011) Noninvasive cell-tracking methods. Nat Rev Clin Oncol 8, 677-688
4. Youn H and Hong KJ (2012) In vivo noninvasive small animal molecular imaging. Osong Public Health Res Perspec 3, 48-59
5. Sahaapathy V, Mentarm J, Jacob PM and Kumar S (2015) Noninvasive optical imaging and in vivo cell tracking of indocyanine green labeled human stem cells transplanted at superficial or in-depth tissue of SCID mice. Stem Cell Int 2015, 606415
6. Peruelas I, Mazzolini G, Boín JF et al. (2005) Positron emission tomography imaging of adenovirus-mediated transgene expression in liver cancer patients. Gastroenterology 128, 1787-1795
7. Rogers WJ, Meyer CH and Kramer C (2006) Technology insight: in vivo cell tracking by use of MRI. Nat Clin Pract Cardiovasc Med 3, 554-562
8. Kwak YH, Hong SM and Park SS (2010) A single cell tracking system in real-time. Cell Immuuno 265, 44-49
9. Carlson AL, Fujiksi J, Wu J et al. (2013) Tracking single cells in live animals using a photoconvertible near-infrared cell membrane label. PLoS One 8, e69257
10. Jung KO, Kim TJ, Yu JH et al. (2020) Whole-body tracking of single cells via positron emission tomography. Nat Biomed Eng 4, 835-844
11. Herschman HR (2004). Noninvasive imaging of reporter gene expression in living subjects. Adv Cancer Res 92, 29-80
12. Shaner NC, Steinbach PA and Tsien RY (2005) A guide to choosing fluorescent proteins. Nat Methods 2, 905-909
13. Pan K, Deng H, Hu S et al. (2020) Real-time surveillance of surgical margins via ICG-based near-infrared fluorescence imaging in patients with OSCC. World J Surg Oncol 18, 96
14. Chiu CH, Chao YK, Liu YH et al. (2016) Clinical use of near-infrared fluorescence imaging with indocyanine green in thoracic surgery: a literature review. J Thorac Dis 8, S744-S748
15. Kosaka N, Ogawa M, Choyke PL and Kobayashi H (2009) Clinical implications of near-infrared fluorescence imaging in cancer. Future Oncol 5, 1501-1511
16. Hoffman RM (2015) Application of GFP imaging in cancer. Lab Invest 95, 432-452
17. Yang M, Baranov E, Jiang P et al. (2000) Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. Proc Natl Acad Sci USA 97, 1206-1211
18. Bouvet M, Wang J, Nardin SR et al. (2002) Real-time optical imaging of primary tumor growth and multiple metastatic events in a pancreatic cancer orthotopic model. Cancer Res 62, 1534-1540
19. Sueitsugu A, Osawa Y, Nagaki M et al. (2010) Simultaneous color-coded imaging to distinguish cancer “stem-like” and non-stem cells in the same tumor. J Cell Biochem 111, 1035-1341
20. Sueitsugu A, Jiang P, Moriwaki H, Saji S, Bouvet M and Hoffman RM (2013) Imaging nuclear - cytoplasm dynamics of cancer cells in the intravascular niche of live mice. Anticancer Res 33, 4229-4236
21. Yano S, Zhang Y, Zhao M et al. (2014) Tumor-targeting Salmonella typhimurium A1-R decoys quiescent cancer cells to cycle as visualized by Fucci imaging and become sensitive to chemotherapy. Cell Cycle 13, 3958-3963
22. Yano S, Mwia S, Miz S et al. (2014) Invading cancer cells are predominantly in G0/G1 resulting in chemoresistance demonstrated by real-time Fucci imaging. Cell Cycle 13, 953-960
23. Contag CH, Contag PR, Mullins J, Spilman SD, Stevenson DK and Benaron DA (1995) Photonic detection of bacterial pathogens in living hosts. Mol Microbiol 18, 593-603
24. Kwak YH, Hong SM and Park SS (2010) A single cell tracking system in real-time. Cell Immuuno 265, 44-49
25. Reumers V, Deroose CM, Krylyshkina O et al. (2008) Noninvasive and quantitative monitoring of adult neuronal stem cell migration in mouse brain using bioluminescence imaging. Stem Cells 26, 2382-2390
26. Prinz A, Diskar M and Herberg FW (2006) Application of bioluminescence resonance energy transfer (BRET) for biomolecular interaction studies. ChemBioChem 7, 1607-1612
27. Kobayashi H, Picard LP, Schöneegge AM and Bouvier M (2019) Bioluminescence resonance energy transfer-based imaging of protein-protein interactions in living cells. Nat Protoc 4, 1084-1107
28. Dale NC, Johnstone EK, White CW and Pfleger KD (2019) NanoBRET: the bright future of proximity-based assays. Front Bioeng Biotechnol 7, 56
29. England CG, Ehlerding EB and Cai W (2016) NanoLuc: a small luciferase is brightening up the field of bioluminescence. Bioconjug Chem 27, 1175-1187
30. El Khamlichi C, Reverchon-Assadi F, Hervouet-Coste N, Reumers V, Deroose CM, Krylyshkina O et al. (2008) Bioluminescence resonance energy transfer-based imaging of protein-protein interactions in living cells. Nat Protoc 4, 1084-1107
31. Prinz A, Diskar M and Herberg FW (2006) Application of bioluminescence resonance energy transfer (BRET) for biomolecular interaction studies. ChemBioChem 7, 1007-1012
32. Reumers V, Deroose CM, Krylyshkina O et al. (2008) Noninvasive and quantitative monitoring of adult neuronal stem cell migration in mouse brain using bioluminescence imaging. Stem Cells 26, 2382-2390
33. Picard LP, Schöneegge AM and Bouvier M (2018) Bioluminescence resonance energy transfer-based biosensors allow monitoring of ligand-and transducer-mediated GPCR conformational changes. Commun Biol 1, 2017-8034
34. Yang C, Tian R, Liu T and Liu G (2016) Reporter gene expression in living subjects. Adv Drug Deliv Rev 80, S744-S748
35. Zhang R, Feng G, Zhang C, Cai X, Cheng X and Liu B (2016) Tyrosinase as a multifunctional reporter gene for Photoacoustic/MRI/PET triple modality molecular imaging. Sci Rep 6, 2017-8034
36. Qin C, Cheng K, Chen K et al. (2013) Noninvasive cell-tracking methods. Nat Rev Clin Oncol 8, 677-688
4. Youn H and Hong KJ (2012) In vivo noninvasive small animal molecular imaging. Osong Public Health Res Perspect 3, 48-59
5. Sahaapathy V, Mentarm J, Jacob PM and Kumar S (2015) Noninvasive optical imaging and in vivo cell tracking of indocyanine green labeled human stem cells transplanted at superficial or in-depth tissue of SCID mice. Stem Cell Int 2015, 606415
6. Peruelas I, Mazzolini G, Boín JF et al. (2005) Positron emission tomography imaging of adenovirus-mediated transgene expression in liver cancer patients. Gastroenterology 128, 1787-1795
7. Rogers WJ, Meyer CH and Kramer C (2006) Technology insight: in vivo cell tracking by use of MRI. Nat Clin Pract Cardiovasc Med 3, 554-562
8. Kwak YH, Hong SM and Park SS (2010) A single cell tracking system in real-time. Cell Immuuno 265, 44-49
9. Carlson AL, Fujiksi J, Wu J et al. (2013) Tracking single cells in live animals using a photoconvertible near-infrared cell membrane label. PLoS One 8, e69257
10. Jung KO, Kim TJ, Yu JH et al. (2020) Whole-body tracking of single cells via positron emission tomography. Nat Biomed Eng 4, 835-844
11. Herschman HR (2004). Noninvasive imaging of reporter gene expression in living subjects. Adv Cancer Res 92, 29-80
12. Shaner NC, Steinbach PA and Tsien RY (2005) A guide to choosing fluorescent proteins. Nat Methods 2, 905-909
13. Pan K, Deng H, Hu S et al. (2020) Real-time surveillance of surgical margins via ICG-based near-infrared fluorescence imaging in patients with OSCC. World J Surg Oncol 18, 96
14. Chiu CH, Chao YK, Liu YH et al. (2016) Clinical use of near-infrared fluorescence imaging with indocyanine green in thoracic surgery: a literature review. J Thorac Dis 8, S744-S748
15. Kosaka N, Ogawa M, Choyke PL and Kobayashi H (2009) Clinical implications of near-infrared fluorescence imaging in cancer. Future Oncol 5, 1501-1511
16. Hoffman RM (2015) Application of GFP imaging in cancer. Lab Invest 95, 432-452
17. Yang M, Baranov E, Jiang P et al. (2000) Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. Proc Natl Acad Sci USA 97, 1206-1211
18. Bouvet M, Wang J, Nardin SR et al. (2002) Real-time optical imaging of primary tumor growth and multiple metastatic events in a pancreatic cancer orthotopic model. Cancer Res 62, 1534-1540
19. Sueitsugu A, Osawa Y, Nagaki M et al. (2010) Simultaneous color-coded imaging to distinguish cancer "stem-like" and non-stem cells in the same tumor. J Cell Biochem 111, 1035-1341
20. Sueitsugu A, Jiang P, Moriwaki H, Saji S, Bouvet M and Hoffman RM (2013) Imaging nuclear - cytoplasm dynamics of cancer cells in the intravascular niche of live mice. Anticancer Res 33, 4229-4236
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37. Gilad AA, Winnard Jr PT, van Zijl PC and Bulte JW (2007) Developing MR reporter genes: promises and pitfalls. NMR Biomed 20, 275-290
38. Vande Velde G, Rangarajan JR, Toelen J et al (2011) Evaluation of the specificity and sensitivity of ferritin as an MRI reporter gene in the mouse brain using lentiviral and adeno-associated viral vectors. Gene Ther 18, 594-605
39. Kim HS, Woo J, Lee JH et al (2015) In vivo tracking of dendritic cell using MRI reporter gene, Ferritin. PLoS One 10, e0125291
40. Gilad AA, McMahon MT, Walczak P et al (2007) Artificial reporter gene providing MRI contrast based on protein exchange. Nat Biotechnol 25, 217-219
41. Massoud TF and Gambhir SS (2003) Molecular imaging in living subjects: seeing fundamental biological processes in a new light. Genes Dev 17, 545-580
42. Bull E, Madani SY, Sheth R, Seifalian A, Green M and Burch JJ (2007) Stem cell tracking using iron oxide nanoparticles. Int J Nanomedicine 2, 1641
43. Fan K, Lu C, Shu G et al (2021) Sialic acid-engineered mesoporous polydopamine dual loaded with ferritin gene and SPION for achieving endogenous and exogenous synergistic T2-weighted magnetic resonance imaging of HCC. J Nanobiotechnol 19, 1-17
44. Omami G, Tamimi D, Branstetter BF et al (2014) Basic principles and applications of 18F-FDG-PET/CT in oral and maxillofacial imaging: a pictorial essay. Imaging Sci Dent 44, 325-332
45. Keu VK, Witney TH, Yaghoubi S et al (2017) Reporter gene imaging of targeted T cell immunotherapy in recurrent glioma. Sci Transl Med 9, eaag2196
46. Maclaren DC, Gambhir SS, Satyamurthy N et al (1999) Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. Gene Ther 6, 785-791
47. Chung IK (2002) Sodium iodide symporter: its role in nuclear medicine. J Nucl Med 43, 1188-1200
48. Ronne P, Hellberg S, Kiuel M et al (2016) Comparison of Somatostatin receptor 2-targeting PET tracers in the detection of mouse atherosclerotic plaques. Mol Imaging Biol 18, 99-108
49. Furutaka T, Lohith TG, Takamatsu S, Mori T, Tanaka T and Fujibayashi Y (2006) Potential of the FES-hERL PET reporter gene system—basic evaluation for gene therapy monitoring. Nucl Med Biol 33, 145-151
50. Altmann A, Kissel M, Zitzmann S et al (2003) Increased MIBG uptake after transfer of the human nonepinephrine transporter gene in rat hepatoma. J Nucl Med 44, 973-980
51. Moroz MA, Sereganova I, Zannonicola P et al (2007) Imaging hNET reporter gene expression with 124I-MIBG. J Nucl Med 48, 827-836
52. Berberat P, Friess H, Kashiwagi M, Beger HG and Büchler MW (1999) Diagnosis and staging of pancreatic cancer by positron emission tomography. World J Surg 23, 882-887
53. Stockhofe K, Postema JM, Schierenstein H and Ross TL (2014) Radiolabeling of nanoparticles and polymers for PET imaging. Pharmaceuticals 7, 392-418
54. Neves AA, Xie B, Fawcett S et al (2017) Rapid imaging of tumor cell death in vivo using the C2A domain of Synaptophysin-I. J Nucl Med 58, 881-887
55. Schier AF (2020) Single-cell biology: beyond the sum of its parts. Nat Methods 17, 17-20
56. Kichimaru T, Iwano S, Kiyama M et al (2016) A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. Nat Commun 7, 1-8
57. Iwano S, Sugiyama M, Hama H et al (2016) Single-cell bioluminescence imaging of deep tissue in freely moving animals. Science 359, 935-939
58. Shapiro EM, Skrtic S, Sharer K, Hill JM, Dunbar CE and Koretsky AP (2004) MRI detection of single particles for cellular imaging. Proc Natl Acad Sci U S A 101, 10901-10906
59. Shapiro EM, Sharer K, Skrtic S and Koretsky AP (2006) In vivo detection of single cells by MRI. Magn Reson Med 55, 242-249
60. Shapiro EM, Skrtic S and Koretsky AP (2005) Sizing it up: Cellular MRI using micron-sized iron oxide particles. Magn Reson Med 53, 329-338
61. Heyn C, Ronald JA, Ramadan SS et al (2006) In vivo MRI of cancer cell fate at the single-cell level in a mouse model of breast cancer metastasis to the brain. Magn Reson Med 56, 1001-1010
62. Masedunskas A, Millberg O, Porat-Shliom I et al (2012) Intravital microscopy: a practical guide on imaging intra-cellular structures in live animals. Bioarchitecture 2, 143-157
63. Thuber GM, Yang KS, Reiner T et al (2013) Single-cell and subcellular pharmacokinetic imaging allows insight into drug action in vivo. Nat Commun 4, 1-10
64. Hoover EE and Squires JA (2013) Advances in multiphoton microscopy technology. Nat Photonics 7, 93-101
65. Morimoto A, Kikuta J and Ishii M (2020) Intravital multiphoton microscopy as a novel tool in the field of immunopharmacology. Pharmacol Ther 206, 107429
66. Liang Y and Walczak P (2021) Long term intravital single cell tracking under multiphoton microscopy. J Neurosci Methods 349, 103783-349
67. Lopez MJ, Seyedi-Razavi Y, Yamaguchi T et al (2020) Multiphoton intravital microscopy of mandibular draining lymph nodes: a mouse model to study corneal immune responses. Front Immunol 11, 39
68. Carney B, Kossatz S and Reiner T (2017) Molecular Imaging of PARP. J Nucl Med 58, 1025-1030
69. Xu M and Wang LV (2006) Photoacoustic imaging in biomedicine. Rev Sci Instrum 77, 041101
70. Xia J, Yao J and Wang LV (2014) Photoacoustic tomography: principles and advances. Electromagnetic waves 147, 1-22
71. Wang L, Maslov K and Wang LV (2013) Single-cell label-free photoacoustic flowoxigraphy in vivo. Proc Natl Acad Sci U S A 110, 5759-5764
72. Parker D, Broadbent C, Fowles P, Hawkesworth M and McNeil P (1993) Positron emission particle tracking - a technique for studying low within engineering equipment. Nucl. Instrum. Methods Phys Res A 326, 592-607
73. Lee K, Kim TJ and Pratz G (2015) Single-cell tracking with PET using a novel trajectory reconstruction algorithm. IEEE Trans Med Imaging 34, 994-1003
74. Ouyang Y, Kim TJ and Pratx G (2016) Evaluation of a BGO-based PET system for single-cell tracking performance by simulation and phantom studies. Mol Imaging 15, 1-8