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Invited Mini Review

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 Tomography (PET), Single Photon 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. [BMB Reports 2022; 55(6): 267-274]

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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). In

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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 *gaussia* 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

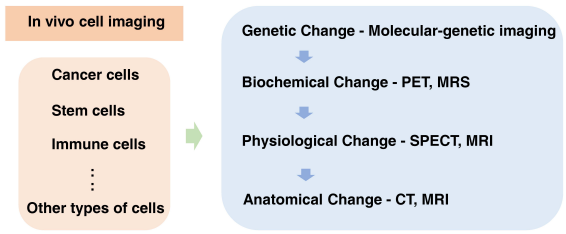

Fig. 1. Molecular imaging modalities for *in vivo* cell imaging.

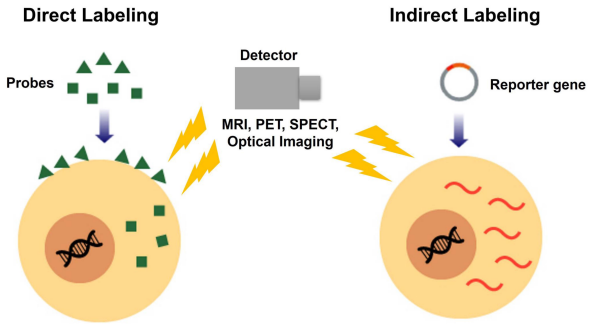

Fig. 2. Labeling methods for *in vivo* cell imaging.

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

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 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 Receptos (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 EgadMe (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).

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). Dopamine 2 receptor (D_2R) is used as a nuclear medicine reporter gene to combine the Dopamine 2 receptor ligand such as ^{18}F-fluoroethylspiperone (46). Sodium iodide symporter (NIS) is the cell membrane protein used to uptake radionuclides such as ^{125}I and ^{131}I (47). Somatostatin receptor (SSTR) reporter gene is a membrane receptors which uses PET tracers such as ^{68}Ga-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) is used for imaging probes such as ^{11}C-ephedrine (50, 51). For *in vivo* cell imaging, ^{18}F-fluorodeoxyglucose (FDG) is widely used for glucose metabolism, showing different uptake between cancer cells and normal cells (52). Since radioisotopes such as ^{18}F and ^{11}C has a short-half life, radioistopes such as ^{89}Zr and ^{64}Cu could be studied for long-term imaging (53). For SPECT imaging, this study monitored cell death with radiolabeled 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

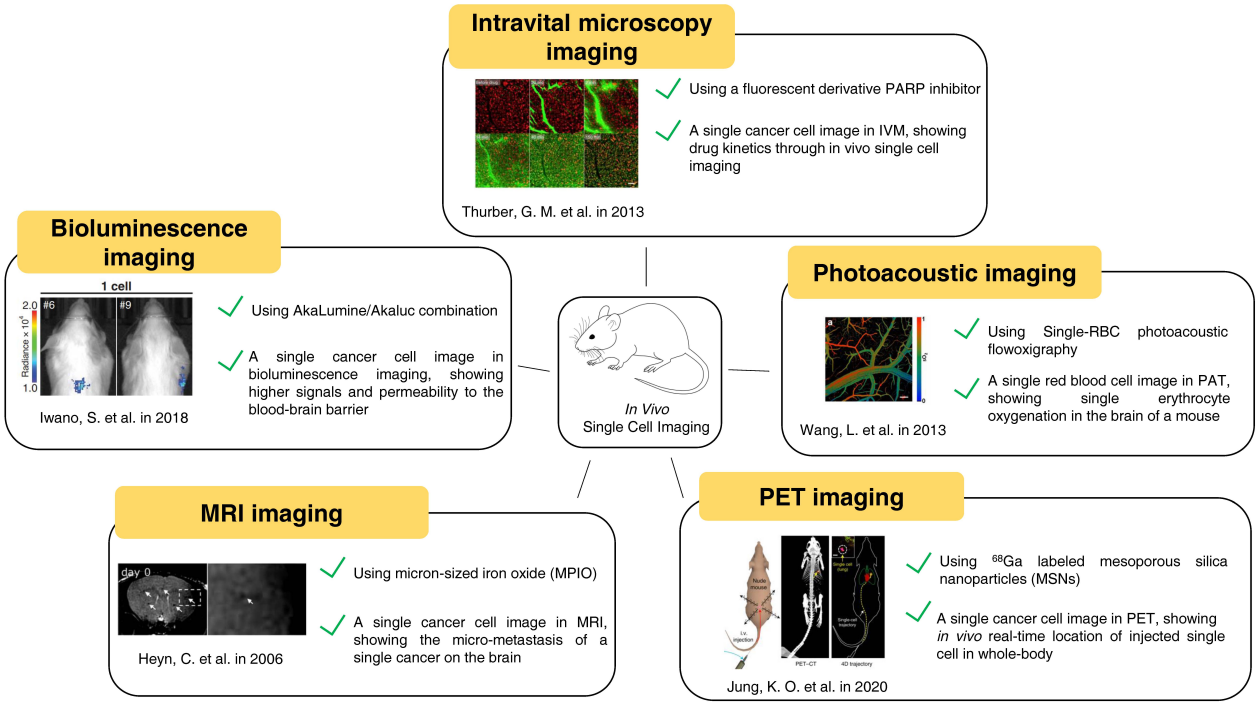

Fig. 3. Studies for *in vivo* single cell imaging and tracking.

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, the 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 intravital microscopy (MP-IVM) is a method that enables dynamic monitoring of cells under various physiological 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 ^{68}Ga 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.

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