CHAPTER ONE

Introduction

1.1 General introduction of Molecular imaging

Molecular imaging can be broadly defined as the science to non-invasively visualize and quantitatively analyze the function of *in vivo* biological processes at the cellular and molecular level [1,2]. The technique of molecular imaging has enabled real-time monitoring of expression of specific genes and the localization of probes or markers. It non-invasively provides the visualization, characterization and eventually quantification of cellular and molecular processes, under physiological or pathological conditions, in a living organism. Especially over the last two decades, the technique of molecular imaging has become increasingly important and currently plays a crucial role in the areas of fundamental and clinical research, including regenerative medicine, drug development, image guided therapy, cancer and stem cell therapies [3-6].

Molecular imaging makes use of various imaging agents and modalities and in recent years, the resolution and image quality of existing imaging systems have improved continuously by choosing a suitable molecular imaging agent for its matching imaging modality. The various existing imaging technologies differ in several aspects, like spatial resolution, depth penetration, energy expended for image generation, availability of injectable/biocompatible molecular probes and the respective detection threshold of probes for a given technology [7].

1.1.1 Imaging modalities

Current molecular imaging modalities mainly include optical imaging, nuclear imaging, ultrasound, MRI and computed tomography (CT). These modalities vary broadly in sensitivity and spatial resolution. Figure 1 shows an overview of the most common molecular imaging modalities used in pre-clinical settings. In general, the two most sensitive imaging modalities are optical imaging and nuclear imaging. Optical imaging mainly consists of bioluminescence imaging (BLI) and fluorescence imaging (FLI), whereas nuclear imaging includes single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Amongst the relatively low sensitivity but high spatial resolution modalities, ultrasound is a modality with a broad clinical availability. Another common modality that is found in hospitals and clinical centers worldwide is MRI. Compared to optical imaging and nuclear imaging, MRI requires larger quantities of the contrast agent, normally at micro-molar range. Besides optical imaging, nuclear imaging and ultrasound, other imaging techniques, e.g. CT and MRI, have been used more for diagnostic imaging as they predominantly provide anatomical pictures. Each imaging modality has its advantages and disadvantages and a number of reviews have summarized the complexity and diversity of current molecular imaging modalities [8-11]. The ideal

situation is to be able to obtain sophisticated imaging at the molecular level with a high sensitivity and high spatial resolution. A final unmet need is to integrate the different imaging modalities so that complementary information can be obtained from each modality.

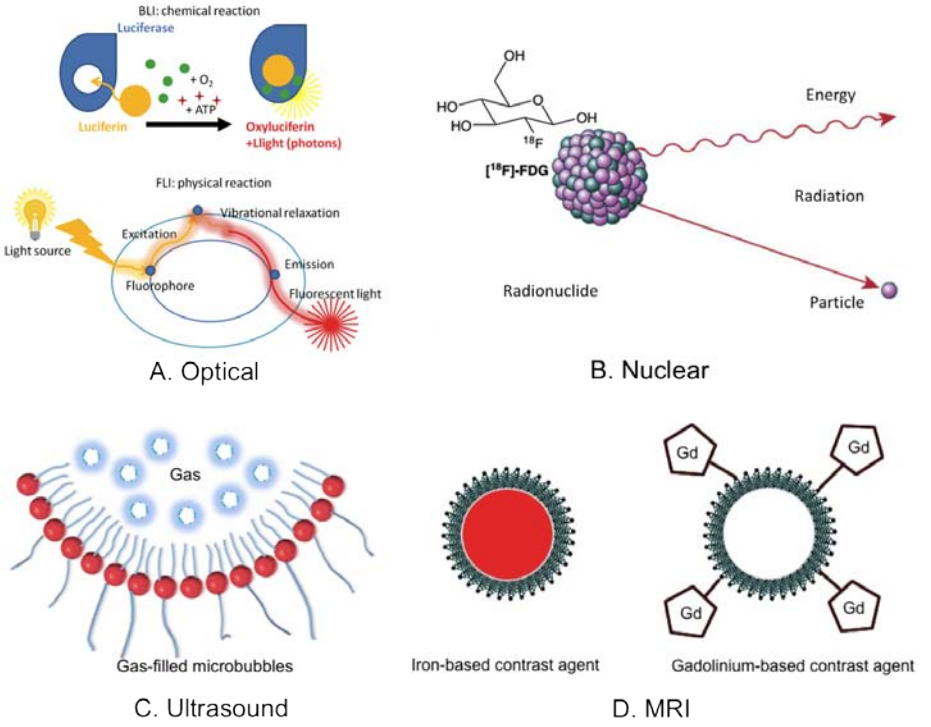

Figure 1. Overview of four common molecular imaging modalities. A. Optical imaging: for BLI, substrate (e.g. luciferin) is required to react with enzymes (e.g. luciferase) to enable native light emission; or for FLI, probes with fluorophores (e.g. fluorescein) consist of small molecules that may be quenched with fluorescence potential and only liberated after specific enzymatic cleavage or upon molecular target binding. B. Nuclear imaging (SPECT/PET): SPECT uses gamma rays to provide 3D information as cross-sectional slices and PET detects positron emission from radionuclides, commonly 18-Fluorine. Small molecules, including drugs, can be adapted to incorporate suitable radionuclides enabling both localization and quantification of molecular expression. C. Ultrasound: active microbubbles made from albumin or lipids are relatively in large structures (5 to 10 µm diameter). They are typically bound to activated endothelium. D. MRI: Depends on the type of delivery payloads, either ultra-small superparamagnetic-based molecular imaging agent (e.g. iron oxides) or paramagnetic-based molecular imaging agent (e.g. gadolinium chelates) can be employed. Iron oxide particles (size range ~10 nm to 5 µm) are commonly equipped within polymer shells. Gadolinium contrast requires interaction with local water molecules, so that the surface of the carrier is often decorated by the gadolinium chelates [12].

For all types of imaging modalities, the signal-to-background ratio (SBR) is always a parameter needs to be addressed. SBR is a measure used to compare the level of a desired signal to the level of background noise. SBR is defined as

the ratio of signal power to the noise power and a ratio higher than 1 indicates more signal than noise.

Most of molecular imaging modalities can obtain the desired signals without any enhancement or modification. However, sometimes for improving the SBR, the imageable signal needs to be enhanced to a level strong enough for visualization. The methods vary from improving target concentration by pretargeting, specific chemical activation, conformational changes, multivalency, or biological trapping to obtain maximized specificity [1,13]. For example, Barbet J. *et al* proved that target concentration in tumor tissues can be enhanced while their uptake in normal tissues is reduced by pretargeting labeled bivalent hapten with bispecific antibodies [14]. Whereas Weissleder R. *et al* made use of protease-activated near-infrared fluorescent probes for imaging, resulting in high SBR upon proteolytic activation of the fluorophores [15].

In this thesis, we will mainly focus on optical imaging and its pre-clinical applications, both at the microscopic and macroscopic level, because it has the potential to significantly enhance our understanding of basic cancer and cell death mechanisms. Compared to nuclear imaging, ultrasound, MRI and CT which are already in function in the clinic, optical imaging provide wider functional contrast than current clinical imaging techniques. This is done either by intrinsic monitoring of physiological changes, e.g. bioluminescence, and light absorption, or by external contrast such as the use of fluorescence probes. Importantly, optical imaging is generally non-invasive and high-throughput, the equipment needed for measurements is of low cost. Eventually, we hope optical imaging can be implemented as the next clinical modality in conjunction with the other already established clinical imaging techniques.

1.1.2 Molecular imaging agents

Next to a molecular imaging modality, a specific molecular imaging agent, which is related to the biological process of interest, is the key requirement for molecular imaging. The term molecular imaging agent can be broadly defined as a probe that is used to visualize and characterize biological processes in living organisms, where the probe can be detectable by a certain molecular imaging modality described above. Both endogenous and exogenous molecules can be used as molecular imaging agents [16]. Commonly an imaging agent is a high-affinity ligand conjugated to signal molecules, which can be detected by an appropriate imaging modality. This ligand recognizes a specific molecular target, like certain enzymes, adhesion molecules and their receptors, growth factors and cytokine receptors and reporter genes [1,17-19]. Examples of different reporter systems are illustrated in Figure 2.

After the selection of a molecular target, a high-affinity ligand possessing the

specificity for the selected target needs to be chosen. This means the ligand should have high specificity to reach the intended target at a sufficient concentration within a short amount of time, so that it can be detected *in vivo* before degradation or excretion occurs. Such ligand can be a peptide, an engineered monoclonal antibody or antibody fragment, a recombinant protein, an aptamer, an oligosaccharide or another small molecule [1,19]. The ligand can recognize the target specifically through various means, e.g. receptor or antibody recognition, enzymatic activation and/or cellular trapping [20].) Many antibodies have been used against numerous targets including epidermal growth factor receptor, $\alpha_v\beta_3$ integrin, glucose transporter and matrix metalloproteinases (MMPs). The production of antibodies, however, is relatively complex and expensive [21-24]. For this, small cell-penetrating peptides (CPPs) have been considered to be good alternatives. CPPs are able to penetrate cell membranes efficiently and labeled CPPs can also be specifically activated by certain proteases, like MMPs and Cathepsins [25-27]. Furthermore, dual-modality probes can be made by linking fluorescent CPPs to nanoparticles for *in vivo* fluorescence and magnetic resonance imaging (MRI) [28]. Other issues like biodistrubution, excretion rate, toxicity and non-specific binding should be considered when choosing an appropriate molecular imaging agent [1,9].

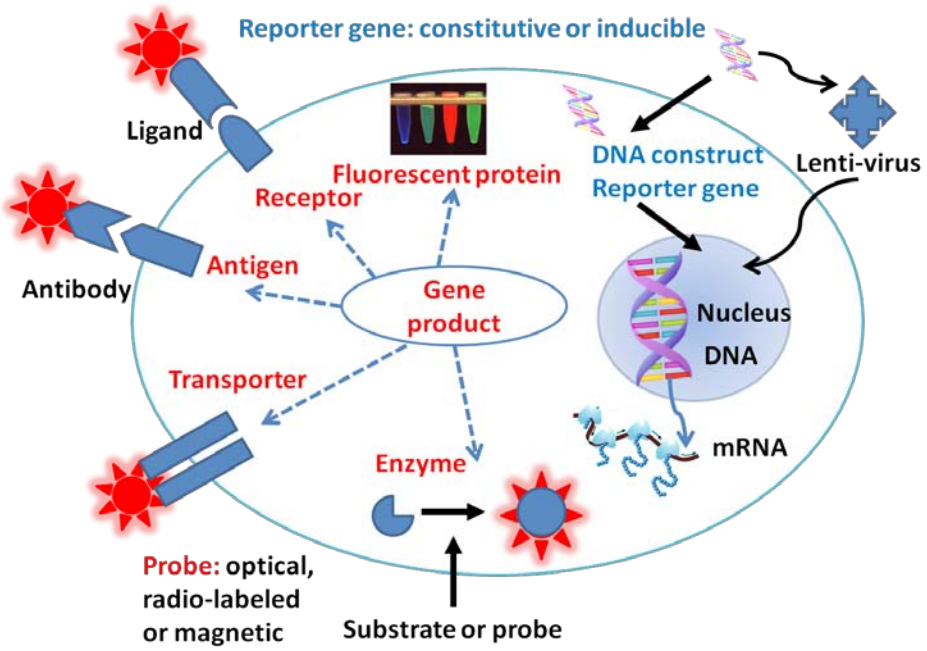

Figure 2. Schematic representation illustrating some selected reporter systems. Gene reporter constructs can be either driven by a constitutive active promoter resulting in a continuous production of the gene reporter product. It can also be driven by an inducible promoter that will be activated only when the specific promoter is turned on. If required, the promoter can also be tissue specific resulting in the production of the reporter gene product only in that specific tissue. For the delivery of a reporter construct, the reporter gene construct

has to be delivered to a cell (or tissue). This can be accomplished by classical transfection methods or using a viral vehicle to infect the cells. When adenoviral vectors are used the reporter gene will not be integrated into the genome of the cell and therefore will only be temporarily expressed. When classical transfection, retroviral or lentiviral constructs are used, the reporter gene will be stably integrated into the genome of the cell and the reporter will be passed on to every daughter cell after division. There are different types of gene reporters. The gene product of the reporter constructs can be an antigen, transporter, receptor, enzyme or fluorescent protein. For molecular imaging, an antibody tagged with a probe can be used to bind certain antigen; a ligand tagged with a probe can be used to recognize a specific receptor; a transported molecule tagged with a probe can be used for a transporter uptake; an substrate can be used for an enzyme reaction which leads to the generation of a signal (e.g. bioluminescence) or fluorescent proteins that generate light by themselves when excited with external light source.

1.2 Optical imaging

Optical imaging uses light to non-invasively interrogate cellular and molecular function in living organisms and ultimately obtains information from tissue composition and biomolecular processes. Images are generated by using photons ranging from ultraviolet to near-infrared [16,29].

Among different molecular imaging modalities, optical imaging is specifically appealing for *in vivo* non-invasive imaging, both at macroscopic (e.g. whole body small animal imaging) and microscopic (e.g. multi-photon microscopy and confocal microscopy) level, because of its high sensitivity and versatility [30]. There are a number of approaches used for optical imaging (e.g. BLI, FLI, fluorescence tomography, optical coherence tomography and photoacoustic microscopy). The various methods use bioluminescence, fluorescence, light scattering and/or absorption as the source of imaging contrast [29,31-33]. For example, bioluminescence is visible light generated by a living organism via a chemical reaction. So for BLI, a specific enzyme and its corresponding substrate are required. Fluorescence, however, comes from molecules that react to light in a physical process. In this process, a fluorescent object (e.g. a fluorophore) absorbs light of certain wavelengths and then emits it during vibrational relaxation. In most cases, some energy is lost as heat so that the emitted light has less energy and a longer wavelength than the light absorbed. So for FLI, an external light source for excitation is needed [6,34]. Nowadays, besides the conventional fluorescence reflectance imaging approach, developments in optical imaging are currently under way to extend FLI to three-dimensional imaging with volumetric information by fluorescence-mediated molecular tomography (FMT) and multispectral optoacoustic tomography (MSOT) [35,36].

1.2.1 Bioluminescence imaging

The high sensitivity of BLI makes it to be an excellent experimental tool for fundamental research in small animals like mice. With the negligible background, BLI is particularly useful for studying diverse biological processes, including 1) longitudinally tracking luciferase-labeled cancer cells, immune cells, and other cell

types in rodent models; 2) Monitoring gene expression changes associated with chemical stress, tumor hypoxia and heat-shock response; 3) Assessing protein stability and function to elucidate complex disease mechanisms; 4) Imaging therapeutic delivery to enhance the specificity and efficacy of targeted gene-based therapies and accelerate drug discovery [37-41].

However, BLI measurement requires a chemical reaction between luciferase and luciferin for light production, which luciferase binds its cognate luciferin and catalyzes the oxidation of the small molecule. So it cannot be employed in human or animals lacking expression of bioluminescent reporters, e.g. in syngeneic mouse models, spontaneous tumor models or chemical-induced cancer models [42-44]. Moreover, images acquired from BLI measurement mostly use algorithms to assign the positions of the light sources more precisely but still lack of 3D information [45]. These drawbacks of BLI limit its clinical translation.

To cover the drawbacks of BLI, FLI is considered to be a good alternative. Especially because of to the development of injectable NIR fluorescent probes with specific targeting or activating abilities, FLI in the NIR region will allow application in the clinic, e.g.for image-guided surgery, early disease diagnosis and therapy monitoring [3,46,47].

1.3 Near-infrared fluorescence imaging

Due to the fact that the absorption coefficient of tissue is considerably lower in the NIR region (700-900 nm), the light can penetrate deeper (several centimeters) [48]. Minimal light absorption by hemoglobin and water in the NIR spectrum enables advanced FLI systems to sensitively detect NIR fluorophores *in vivo* with high resolution [49].

The widely used NIR fluorophores, including cyanine dyes like Cy3, Cy5.5, Cy7, Alexa Fluor dyes like Alexa Fluor 750 and IRDye Infrared Dyes like IRDye 800CW have generated a great interest as they show favorable SBR. Currently, the NIR fluorescent probes can be broadly divided into two types: either using the NIR fluorophore solely as non-specific probes or the fluorophore coupled to a ligand serving as specific probes. Firstly, isotopes and fluorophores can be used interchangeably as non-specific imaging agents. For example, Evans blue has been commonly used in cell viability assays because of its penetratable to non-viable cells [50]. Indocyanine green is a cyanine dye which is used in the clinic, e.g. for the non-invasive monitor of lymph node mapping [51]. However, these imaging agents are associated with relatively high background signal due to the non-specific binding nature. To achieve a better SBR, many more NIR fluorescent probes are specifically targeted to recognize certain features of cells or activation-sensitive towards certain enzyme reactions [52-54]. Different tumor-specific targeting probes can specifically bind to different membrane targets like

glucose transporters (GLUTs) or epidermal growth factor receptors (EGFRs) [55,56]. For activatable probes, many are designed to be selectively activated by enzymes such as matrix metalloproteinases (MMPs) or cathepsins, which are highly expressed by, e.g. cancer cells, so that they can be used for cancer imaging [57,58]. Specifically, by using fluorescence resonance energy transfer (FRET) probes, more precise information about the functional specificity of certain process can be generated [59]. The probe is designed with linkers between pairs of fluorochromes in close proximity, typically <10nm. A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through non-radiative dipole-dipole coupling. However, when the fluorescence is quenched due to the close proximity of fluorochromes, the capacity for excitation will be restricted. Upon the enzymatic target-induced cleavage of the linkers, the strong increase in distance between donor and acceptor chromophores will result in detectable fluorescence.

1.3.1 Near-infrared fluorescence imaging of cancer

FLI is a rapidly developing discipline and among various research areas, much attention has been drawn to the development of disease-oriented molecular imaging, in particular the diagnosis of cancer at an early stage. The detection of changes at the molecular level, which occur before typical symptoms can appear, will greatly contribute to the treatment of many diseases, especially cancer [6]. Early detection and non-invasively monitor of treatment are essential for increasing the survival rate; promising imaging agents in combination with advanced imaging modalities have significantly contributed to this field in the last few years [10,49,60].

There are many hallmarks of cancer that can serve as appropriate imaging targets. Except the above mentioned (GLUTs, cathepsins, EGFRs, MMPs), other already in-use cancer hallmarks for FLI includes probes detecting $\alpha_v\beta_3$ integrin, Her2 receptors, vascular endothelial growth factor receptor (VEGFR) [61,62]. These hallmarks are mostly cellular transporters, proteases, growth factors and extracellular matrix receptors, which are strongly associated with the development and progression of different cancer types [63,64].

Another research area that greatly benefits from optical imaging is visualizing cell death. The first description about the association between cell death and cancer was raised in 1972 when Kerr *et al* described massive programmed cell death (“apoptosis”, a Greek word describing falling leaves) following hormone withdrawal in hormone-dependent tumors where the cells were populating rapidly [65]. Many recent studies have implicated the importance of imaging cell death during tumor growth and invasion because apoptosis and necrosis are two hallmarks of most and perhaps all types of cancer. Moreover, when tumor tissues are undergoing aggressive progression, necrotic core is always found

during the tumor development [66,67]. Finally, optical imaging of cell death can be employed as a biomarker, e.g. for anti-tumor strategies to monitor therapeutic efficacy or evaluate drug toxicity [68-70].

1.3.2 Near-infrared fluorescence imaging of cell death

Cell death has been classified into multiple types according to morphological, biochemical or functional differences. But it mainly consists of two distinctive forms, apoptosis and necrosis. Apoptosis appears to be an active process during the regulation of tissue size and the maintenance of a homeostatic status. On the contrary, necrosis is a degenerative phenomenon that always follows irreversible injury [71]. Morphologically, apoptotic cells exhibits condensation of the nuclear chromatin and cytoplasm, fragmentation of the nucleus, blebbing of the intact plasma membrane and budding of the whole cell to produce membrane-bounded bodies (known as apoptotic bodies) in which organelles are intact initially. These bodies are disposed of by adjacent cells without inflammation. Dying cells were originally catalogued as necrotic in a negative fashion. But recent studies reveal that necrotic cells exhibit some distinctive morphological features, including an increasingly transparent cytoplasm, swelling of organelles, minor ultra-structural modifications of the nucleus, increased cell volume and eventually disruption of the plasma membrane. However, their nuclei remain intact and can aggregate and accumulate in necrotic tissues. Biochemically, apoptosis has typical inter-nucleosome cleavage of DNA, which is distinguished from the random DNA degradation observed in necrosis [72,73]. Increased evidence indicated there are other types of cell death, e.g. autophagy, senescence and mitotic catastrophe [74-76].

In the human body, eventually all cells will undergo cell death. Cell death is not only involved in many biological processes, e.g. to maintain homeostasis but also in various pathological conditions when cell death is unbalanced [77]. An elevated level of cell death is a general phenomenon in many diseases, e.g. traumatic brain injury, reperfusion injury, carotid artery injury, cerebral stroke, acute myocardial infarction, ischemia, rheumatoid arthritis, myelodysplastic syndromes and cancer [66,78-81]. Thus, imaging of cell death plays a pivotal role both physiologically and pathologically. Currently, many biomedical researchers are aiming to facilitate early diagnosis of cell death related diseases or therapies by accurately identify and monitor cell death *in vitro* and *in vivo*.

In order to facilitate accurate and non-invasive detection of cell death both *in vitro* and *in vivo*, current efforts in the NIR fluorescence imaging field are focused on developing targeted or activatable cell death fluorescent probes [82]. Table 1 lists a number of fluorescent probes described in recent studies about detection of apoptosis and necrosis.

Type of detecting agent	Type of targeted biomarker	Type of cell death	Ref.
Peptide DEVD	caspases	apoptosis	83-87
Peptide CQRPPR	histones	apoptosis	88,89
Peptide Duramycin	phosphatidylethanolamine	apoptosis & necrosis	90
Small molecular GSAO	Heat shock protein-90	apoptosis & necrosis	91,92
Small molecular Hoechst-IR	extracellular DNA	necrosis	93
Small molecular DDC	membrane depolarization and phospholipid scrambling	apoptosis	94
Small molecular ML-10	anionic phospholipid surfaces	apoptosis	95
Small molecular ZnDPA	phosphatidylserine	apoptosis & necrosis	96,97
Protein Annexin V	phosphatidylserine	apoptosis & necrosis	98-102
Protein C2A domain of Synaptotagmin-I	phosphatidylserine	apoptosis & necrosis	103
Protein Lactadherin	phosphatidylserine	apoptosis & necrosis	104,105
Antibody 3B9 mAb	La autoantigen	apoptosis & necrosis	106

Table 1. Summary of fluorescent probes for detecting apoptosis and necrosis.

As shown, the development of molecular imaging has generated a rich amount of NIR fluorescent probes, which has shed light on various cell death-related research fields. Most of these cell death fluorescent probes are either reacting with intracellular biomarkers, like cytosolic proteins, caspase enzymes, exposed DNA and mitochondrial membrane electronic potential, or targeting extracellular biomarkers, like plasma membrane phospholipids. The biomarkers of apoptosis and necrosis have arouse intensive research interests in monitoring therapeutic effects which are mediated with cell death, either in a pro- or anti-apoptotic manner. For example, caspases can be either activated or inhibited by small-molecule drugs. Important protein-to-protein interactions can be interfered with or mimicked by peptides or organic compounds. Moreover, unwanted proteins, like pro-survival factors in cancer cells, can be specifically down regulated by antisense or other strategies [107-109]. Because of the importance to accurately detect and characterize cell death in different stages of diseases, also because of the need to use cell death as a biomarker for anti-tumor strategies, there is still an urgent demand for the refined optical imaging of cell

death in diseases like cancer. Ultimately, new cell death imaging agents which can characterize the progression of apoptosis and necrosis with high sensitivity and high spatial resolution needs to be developed. Such imaging agent can eventually help to diagnose patients that are likely to develop certain disease where cell death is involved at an early stage and finally assess response to the therapy.

1.4 Outline of this thesis

The pre-clinical molecular imaging of detecting cancer and cell death is rapidly expanding at the research frontiers of current life sciences. The aim of the work included in this thesis was to explore the diverse application possibility of using NIR fluorescent probes with specific properties to visualize and characterize cancer and cell death.

In **Chapter 2**, we imaged breast tumors and their metastases using combinations of four NIR fluorescent probes that possess different optical imaging properties. In **Chapter 3** and **Chapter 4**, we studied two different NIR fluorescent probes, PSVue and a heat shock protein-90 alkylator (NIR fluorescent conjugate of GSAO), which can be used to non-invasively imaging cell death with different optical modules in a mouse model of traumatic brain injury. **Chapter 5** describes the NIR fluorescently tagged GSAO as a biomarker for monitoring breast cancer cell death after chemotherapy. Next, **Chapter 6** provides a general discussion about the advantages and the challenging that the state-of-art optical imaging is facing and shares some future prospective. This thesis ends with a summary that outlined the major findings of studies described in different chapters and explored the clinical implications **(Chapter 7)**.

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