CHAPTER SIX

General Discussion and Future Scope

6.1 General Discussion

Optical imaging, both at microscopic and macroscopic level, is developing rapidly and spanning research in all current life sciences. It has been not only employed to study specific promoters, protein-protein interaction and gene transfer at molecular level, but also to follow the fate of GFP, RFP and/or luciferase expressing cells and detect NIR fluorescent probes at whole body scale [1-4].

At microscopic level, optical imaging benefits from the ongoing development of *in vivo* microscopic technologies. Optical Imaging by confocal and multi-photon microscopy can provide images with the highest sensitivity and spatial resolution. Although the limited light penetration depth through biological tissue restrains its diagnostic and therapeutic applications in humans, current development of new tunable lasers in the mid- and far-infrared range will substantially increase the tissue penetration depth. Optical imaging has in this respect the potential to deliver high-resolution, histology-type reconstruction of tumor and tumor microenvironment in the living organism [2,5-8]. Compared to other molecular modalities, optical imaging and optical reporter systems are very cost-effective and time-efficient, so that they are particularly well suited for *in vitro* assays, e.g. to validate different reporter systems.

At macroscopic level, the development of specific injectable NIR fluorescent probes enables the non-invasive and real-time whole body imaging of animal models, offering the potential to translate optical imaging into clinical applications [9]. Particularly, in cancer research, future applications should allow quantitative measurements of tumor progression and micro-metastasis and treatment response [10]. Due to its high sensitivity, optical imaging is extremely useful for early detection of micro-metastases and minimal residual disease states in animal models [11,12].

However, in term of cancer research, the major challenge of non-invasive optical imaging of cancer is to enhance tumor-to-background ratio (TBR). As described in this thesis, various injectable optical imaging agents, which are specific for detecting hallmarks of cancer, have been developed. These imaging agents in general provide a better TBR compared to the non-specific agents like indocyanine green (ICG), although ICG is the only FDA-approved cyanine dye as an optical imaging agent for medical diagnostics [13,14]. Even though there are already a variety of injectable optical imaging agents becoming available, the current state-of-art *in vivo* optical imaging is still a preclinical research tool. This is mainly because of limitations in penetration depth and light scattering, which consequently lowers the resolution of the image. More efforts should be addressed to make optical imaging agents that yield high local concentrations in deep tumor tissues but have minimal light scattering, minimum non-specific tissue extravasation, internalization by macrophages and rapid renal or hepatic removal. Considering these factors, a broad range of chemical or biological amplification strategies are emerging: 1) Using multivalency to label a single molecule with multiple fluorophores; 2) Using the quenching effect by paring fluorochromes with

quenchers or via distance-dependent interaction between excited states of dyes (FRET); 3) Using covalent reaction to bind targets (activity-based binding); 4) Using photolysis (uncaging) by releasing caged compounds; 5) Using biological processes like enzyme activation, cellular trapping or pre-targeting molecular agents first by a multivalent agent and then by a fluorescent conjugate [15-19].

Over the last decades, cell death has been considered to be a natural barrier to cancer development. Triggered by anticancer therapy or by various types of physiological stress during tumorigenesis, cancer cells are forced to follow apoptosis and/or necrosis [13,14,20]. However, others elucidated that cancer cells can resist therapy by evolving diverse strategies to circumvent or dampen apoptosis. Compelling functional studies supporting this include: apoptosis could be eliminated by abolishing TP53 tumor suppressor function, or by elevating the expression level of anti-apoptotic regulators (e.g. Bcl-2), or alternatively, by downregulating pro-apoptotic factors (e.g. Bax and Bak) [18,21,22]. Moreover, cell proliferative and apoptotic pathways are often coupled, as many promoters of cell proliferation have been found to also possess pro-apoptotic activity [23]. Thus, the resistance of cell death has been widely accepted as one of the major hallmarks of cancer.

Perhaps more importantly, recent study revealed that another major type of cell death, necrosis, has pro-inflammatory and tumor-promoting potential [24]. As compared to other types of cell death (e.g. apoptosis and autophagy), necrosis can release pro-inflammatory signals into the tumor surrounding tissue and recruit immune inflammatory cells to promote tumor angiogenesis, cancer cell proliferation and invasiveness [21,25,26]. In certain circumstances, cancer cells can undergo programmed necrosis (necroptosis) to release bioactive regulatory factors to stimulate neighboring viable cancer cells to proliferate so that incipient neoplasias can be sustained and facilitated [21,27]. By tolerating certain amount of necrosis, e.g. spontaneously forming a necrotic core during tumor development, aggressive cancer cells can benefit from attracting tumor-promoting inflammatory cells. These cells can then foster growth-stimulating factors to the surviving cells and eventually promote neoplastic progression [25,28].

Besides the complicated relation between tumor progression and cell death, a rich amount of cell death imaging agents has been developed to imaging tumor cell death, in order to follow the efficacy of anti-tumor therapies. As described earlier in the thesis introduction, most of these cell death imaging agents 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. In this thesis, we mainly analyzed two representative cell death optical imaging agent, PSS-794 (targeting the extracellular biomarker phosphatidylserine) and GSAO-AF750 (targeting the intracellular biomarker HSP-90 in cells which have lost membrane integrity). At the end of this chapter, a list has been made to summarize the pros and cons of current available tumor cell death imaging agent (Table 1).

6.2 Future Scope

In order to address important needs in clinical translation, new high resolution and deep penetration optical (or multimodal) imaging technologies are required. Simultaneous and combined readouts need to be acquired in order to couple relevant functional information to anatomical and spatial information. For this, optical imaging and SPECT/PET will be thrust together with MRI and/or CT. The advantage of multimodal imaging modality will enable to monitor a wide range of functional parameters especially in relation to molecular events with high sensitivity, great temporal and spatial resolution as well as deep tissue penetration. But the limitation of conventional optical imaging is that the analysis has only been based on two-dimensional planar images and that spatial resolution is relatively poor. To address this problem, optical imaging is now extended to three-dimensional (3D) imaging with volumetric information, e.g. by fluorescence-mediated molecular tomography (FMT) and multispectral optoacoustic tomography (MSOT) [29,30]. These new 3D optical imaging modalities will allow visualization of deeper inside tumor tissues compared to conventional optical imaging [31]. Current preclinical instruments limit optical imaging depth to 2-3 cm and resolutions of 1-2 mm. This would explain why optical *in vivo* imaging has remained mostly in the preclinical research arena. MSOT, for example, could be the promising next generation for cancer imaging, representing a paradigm shift for optical imaging, with resolutions in the 20-200 µm range and with penetration depths of several centimeters [30,32]. Moreover, multimodality imaging, in which optical technologies are combined with clinical technologies, will be an important and promising non-invasive approach to acquire structural and anatomical information [33-35]. The ongoing efforts will greatly facilitate the translation of multimodal molecular imaging into the clinic. The first new clinical application of optical imaging will be in image-guided surgery to visualize tumor tissue or to find sentinel lymph node. This will be done either during open surgery where tumor tissue is near the surface or during minimal invasive laparoscopic or endoscopic surgery. For this purpose, new sensitive NIRF camera systems have been developed [36].

Until now, only indocyanine green (ICG) has been used as a non-targeted probe, since it is the only FDA approved probe. The NIRF dye ICG has mostly been used to find sentinel lymph nodes but also to detect liver metastasis of colon cancer. In the later case, one takes the advantage that ICG, which strongly binds to serum albumin, leaks into the tumor tissue based on the EPR effect and retains as a ring around the tumor margin, mainly due to lack of proper clearance [37]. Such translation will be important for the clinic in providing sensitive, specific, and real-time intra-operative visualization of the molecular features of physiological and pathological processes [4,38-40].

All in all, compared to other imaging modalities, optical imaging offers superior sensitivity (e.g. single molecular level), great temporal resolution (e.g. picoseconds) as well as reasonable spatial resolution (1-2 millimeter)[19,41]. Thanks to these advantages, optical imaging can broaden its way for other clinical applications.

Cell death imaging agent	Mechanism of action	Animal Model	Treat-ment	Imaging Modality
PSS-794 Zinc(II)-dipicolylamine	Binds to multianionic phosphorylated biomolecules, including the binding of PS at the outer surfaces of anionic vesicles and cell membranes	Subcutaneous PAIII prostate adenocarcinoma in rats and EMT6 mammary tumours in mice	None	FLI
		Subcutaneous PAIII prostate adenocarcinoma in rats	Radiation	
GSAO 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide	Binds cytoplasmic Hsp90 following loss of plasma membrane integrity	Orthotopic human 4T1 breast tumour in mice	Cyclophosphamide	FLI
		Subcutaneous mouse Lewis lung carcinoma and human CT26 colorectal carcinoma in mice	None	SPECT
Fluorescent annexin V (Annexin V–Cy5.5)	Binds to extracellular-facing PS due to its externalization at the early stage of apoptosis	Lewis lung Carcinoma (LLC) in chemosensitive and chemoresistant mice	Cyclophosphamide	FLI
Radiolabeled annexin-V (^{99m}Tc-Annexin V)		Human breast cancer MDA-MB435 cells in athymic mice	Anti-CD95, quercetin, Siamois 1 and Siamois 2	SPECT
SPIO-C2A C2A domain of Synaptotagmin I	Binds to negatively charged phospholipids including PS, which redistributed from inner to the outer leaflet of plasma membrane during cell death	Subcutaneous EL4 lymphoma in mice	combination of cyclophosphamide and etoposide	MRI
^{99m}Tc-C2A C2A domain of Synaptotagmin I		Subcutaneous human H460 non-small cell lung cancer in mice	Paclitaxel	SPECT
^{18}F-ICMT-11	Binds to activated caspases 3 and 7	Subcutaneous 38C13 lymphoma xenograft in mice	Cyclophosphamide	PET
^{111}In-3B9 (APOMAB)	Binds to the La ribonucleoprotein upon loss of plasma membrane integrity	Subcutaneous EL4 murine lymphoma in mice	Combination cyclophosphamide and etoposide	SPECT
^{111}In-DAB4 (APOMAB)		Subcutaneous EL4 murine lymphoma in mice		

Table 1. Summary of current tumor cell death imaging studies.

Pros	Cons	Ref
1. Small molecular (<2KDa) with the same targeting capability as Annexin V; 2. Lower renal uptake compared to that of Annexin V; 3. Formulated as a zinc complex improves water solubility.	1. Binding to the phosphatidylserine (PS) exposed on dying cells is zinc-dependent; 2. Accumulation at the target limited by the surface area of the plasma membrane and the abundance of externalised of PS; 3. Can exhibit cross reactivity for PS in activated platelets, macrophages, endothelial cells and aging erythrocytes; 4. Can also bind reversible PS in situations of physiological stress; 5. Also selectively target bacteria in heterogeneous biological media; 6. Cannot discriminate apoptotic and necrotic tissues.	42 43
1. High stability, easy synthesis and versatility with respect to reporter group; 2. Small size (0.5kDa) contributes to fast clearance and favourable biodistribution; 3. Abundance of cytoplasmic target lends to high signal to noise ratio.	1. High renal uptake precludes imaging of kidney and adjacent structures; 2. Is unsuitable for discrimination between apoptosis and necrosis.	Chapter5 44
1. Strong affinity to PS; 2. Ligand readily available on cell surface; 3. Radiolabeled annexin-V has already been studied in humans with acceptable radiation burden.	1. Large size (>35KDa) results in slow delivery to site of interest, slow clearance, non-specific uptake in non-target tissues and especially strong renal uptake; 2. Binding to the PS exposed on dying cells is calcium-dependent; 3. As above PSS-794 point 2,3 and 4.	45 46
1. Small size (<15KDa) so that good penetration of tumor tissue; 2. Quick clearance of unbound material so that good tissue contrast; 3. Ligand readily available on cell surface.	1. Low affinity to PS (dissociation constant between 90-300 nM. compared to 1-2 nM for Annexin V) due to the imaging agent is a heterogeneous mixture of labelled molecules; 2. High renal and hepatic uptake precluding imaging of abdominal region; 3. Binding to the PS exposed on dying cells is calcium-dependent.	47 48
Early detection of caspase-3/7 associated tumor apoptosis.	1. High hepatic uptake precludes imaging of abdominal region; 2. Possible cross-reactivity with cathepsins; 3. Not suitable to detect necrosis.	49
1. Detection of cell death specific for both tumour cells and other cells, which apoptosis was induced by DNA damaging agents; 2. Chemo-sensitive tissue such as gut and bone marrow do not complicate image.	Detection limited to apoptotic malignant cells in response to DNA-damaging treatment.	50 51

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