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REVIEW ARTICLE

Two-photon microscopy in pre-clinical and clinical cancer research

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Abstract The applications of two-photon microscopy (TPM) on pre-clinical and clinical study of human cancer and diseases are reviewed in this paper. First, the principle of two-photon excitation (TPE) is introduced. The resulting advantages of TPM for imaging studies of animal models and human samples are then elaborated. Subsequently, the applications of TPM on various aspects of tumor studies, including tumor angiogenesis, invasion and metastasis, tumor microenvironment and metabolism are introduced. Furthermore, studies of TPM on clinical human skin biopsy and the development of two-photon microendoscopy are reviewed. Finally, potential future directions are discussed.

Keywords two-photon microscopy (TPM), intravital imaging, pre-clinical tumor studies, cancer early detection, cancer diagnosis, medical imaging

1 Introduction

Two-photon microscopy (TPM) has received explosive interest in the past few decades in biomedical research. It has been widely applied on various biologic samples, from a single protein or DNA molecule to whole cells and to live tissues. Combined with novel fluorescent reporters [1,2], researchers can now characterize the motion of motor proteins [1,2], investigate the interaction between protein and DNA molecules [3,4], measure the cellular movements, secretion, enzyme production, gene expression and modulation of these processes from environmental stimulations [5,6], and distinguish cancer from healthy tissues [7].

Cancer research is one of the emerging areas that TPM has proved its utility. As one of the major causes of death worldwide, cancer accounts for around 7.9 million deaths in 2008 according to World Health Organization. Imaging techniques have been applied in the screening, diagnosis/staging, treatment guiding, assessment of cancer treatment outcome and prognosis. Since its introduction in 1990 [8], TPM has gained applications in basic, pre-clinical studies by enabling further insights into various aspects of cancer mechanisms, including the initiation, proliferation, metastasis, metabolism and angiogenesis of tumor and the effects of tumor microenvironment on the physiology and gene expression of cancer cells. Recently, it has also been widely used in translational studies of drug delivery and gene therapy methods for human diseases, and has been further adopted to develop clinical diagnosis and staging technology for various cancer types. Together, these progresses have resulted in discovery of new drugs, novel diagnosis and treatment methods of cancer in human patients [9], showing the great potential of TPM as an imaging technique to help cure cancer and improve life qualities of human beings.

The major advantage of TPM in cancer research is the ability to image cellular activities in live tissues with subcellular resolution. Most pre-clinical researches on genetic and molecular mechanisms, that are involved in cancer initiation, progression and metastasis or to determine outcomes of experimental therapeutics, are usually carried out in two-dimensional (2D) cell cultures, where cancer cells grow as monolayer. However, overall, the reductionist 2D approach does not mimic the native *in situ* environment of cancer or normal tissues, nor reflect the three-dimensional (3D) cell morphology and may distort cell-integrin interactions in the tumor microenvironment [10]. Moreover, cellular behavior is influenced by many factors including cytokine gradients, interactions with other cellular and extracellular components, anatomical compartmentalization, and forces of fluid flow. As such, cellular behavior can be very different *in vitro* compared to *in vivo* settings. For an important instance, the tumor microenvironment, which consists of cells, soluble factors, signaling molecules, extracellular matrix, and mechanical

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cues, can promote neoplastic transformation, support tumor growth and invasion, protect the tumor from host immunity and foster therapeutic resistance. Especially, it provides niches for dormant invasion, intravasation, and metastases to take place. These differences could provide important clues as to the molecular mechanisms of invasion in the primary tumor and reflect interactions between cells and the microenvironment of the tumor that have not yet been duplicated *in vitro* [9,11]. Overall, cancer studies carried out in 2D cultures affect protein expression, cell proliferation, differentiation, and metabolism of cancer cells [12–15], which may partially explain commonplace discrepancies between bench-top and clinical efficacy of new therapies [16]. Therefore, since cell based studies with TPM are beyond the paper's scope and has been elegantly describes elsewhere [17], to emphasis the clinical potential of TPM, this paper explains the principle mechanism of TPM and the underlined advantages for *in viv*o studies. This paper then reviews the current applications in pre-clinical imaging studies on animal models, and in clinical early detection and diagnosis of human disease.

2 Two-photon excitation and advantages of two-photon microscopy

Two-photon excitation (TPE) was first introduced by Maria Mayer in 1931 [17]. Normally, upon excitation of light source, the electron of the atoms in a fluorophore will absorb one photon and will be excited to an orbit with higher energy (excited state). After a short period of time, usually 0.5–20 ns, the electron at the excited state releases the energy by emitting one photon with less energy and return back to the original state (ground state). It is worth noting that, the energy of the emitted photon, in another word, the color of the fluorescence is only determined by the intrinsic characters of the fluorephore (i.e., the type of the fluorophore, the chemical structure, etc.). The time period that the electron remains in the excited state is thus termed as fluorescent life time, and the excitation mechanism is called one photon (1P) excitation. TPE is the excitation of a fluorophore by two photons but with lower energy (i.e., smaller frequency and longer wavelength) than required in 1P excitation (Fig. 1). While the emission via TPE is the same as via 1P excitation, the only requirement of the TPE is the two photons arrive at the fluorophore at the same time to generate a photon flux in the range of 10^{20}–10^{30} photon/($cm^2 \cdot S$). As a result, the TPE has not been demonstrated until 1990 by Denk et al. [8], following the introduction of subpicosecond pulse mode-locked lasers. In the TPE, each excitation photon usually carries half of the energy that is needed to excite the fluorophore, so the wavelength for the TPE is roughly double of the wavelength used for 1P excitation. Since the 1P excitation spectra of most fluorohores are 400–600 nm, the TPE usually uses laser at 700–1500 nm, the near infrared range. Whereas near infrared light can not only penetrate through deeper tissue but also induce less photon damage, because most tissues do not have the same significant endogenous absorption in this wavelength range compared to 1P excitation. In addition, as the TPE requires two photons to be absorbed by the fluorophore at the same time, the probability of the simultaneous absorption and the following excitation is much lower than in 1P excitation. Therefore, much denser laser power is needed in TPE. TPM is therefore created with discriminative excitation and reduce background [8] compared with 1P excitation based imaging methods, such as confocal imaging. Briefly, in TPM imaging, the laser is focused to a tiny spot, diffraction limited in size, and the power is finely adjusted that only those fluorophores inside this tiny focal volume can be sufficiently excited while all other molecules outside this excitation spot will not. This is another key advantage of TPM in comparison to confocal microscopy, which may require additional optical elements (e.g., a pin hole) to reject the out of focus emission. Under optimal settings, TPM can image biologic tissue up to 100 μm in depth [18].

A broad range of image reporters is currently available, which provides maximal flexibility for two-photon microscopic studies. Firstly, various biological molecular and tissue structures have demonstrated their strong intrinsic fluorescence under TPEs: bio-molecules such as β-nicotinamide adenine dinucleotidehydrogen (NADH), and flavin adenine dinucleotide (FAD) are reported to associate with cellular metabolism level, thus are effective indicators for functional imaging [19,20]. Some structural components in tissue, including collagen and elastin exhibit second harmonic generation characteristics thus are favorable tools for label-free structural two-photon imaging [6]. Secondly, exogenous fluorescent labelers, e. g., fluorescent proteins has tremendously accelerated the applications of TPM, fluorescent proteins of the entire visible and the near infrared spectra has made multi-color two photon imaging possible [21–24]. In addition, fluorescent proteins with longer emission such as mKate2 experience less absorption and scattering in tissues thus are more favorable for deeper TPM where images of visible fluorescent proteins are too dim [25,26]. Genetically engineered fluorescent reporters obtained by fusing fluorescent protein to cellular reporters can thus label almost any interested cellular events with high specificity and efficiency [27–29]. Lastly, chemical labelers, such as quantum dots and organic dyes are also used in time-lapse *in vivo* two-photon imaging due to their superior brightness and photostability [30–34]. With the above advantages, the recent introduction of TPM has made it possible to study cancer at a subcellular resolution in real time *in vivo*.

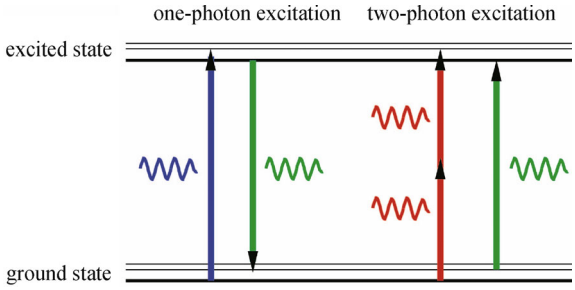

Fig. 1 Jablonski diagram illustrating one- and two-photon excited fluorescence. Two-photon excited fluorescence results from the simultaneous absorption of two photons, each of half the energy of that from one-photon absorption

3 Two-photon microscopy intravital imaging studies in pre-clinical cancer research

Microscopes were first used to perform *in vivo* imaging of tissue in living animals in the 19th century [35], as a technique referred to as intravital microscopy (IVM). By that time, most IVM studies could only examine the vasculature and the microcirculation, because the microscopes available at that time lacked of the resolution and contrast for the visualization of other tissues. The cancer cell metastasis by IVM was first demonstrated in a rabbit ear chamber in the 1950s [36]. When IVM imaging techniques were improved considerably and genetic tumor mouse model that expressed fluorescent proteins (FPs) became available, major breakthroughs in this field occurred in the 1990s. Since then, IVM has evolved into an important tool for investigating the processes underlying cancer and metastasis [37–39]. Recently, IVM based on TPM (IV-TPM) has been successfully used to image single cells in living organisms in their natural environment, advancing various studies in basic biologic research, including localized photoactivation of "caged" compounds such as calcium or the neurotransmitter glutamate [40–42], electrical activity in deep tissue [43–45], quantitative imaging of immune-cell motility and morphology, as well as embryo development [46,47]. In pre-clinical tumor studies, the angiogenesis, invasion and metastasis, microenvironment and metabolism are major aspects of tumor studies carried out by IV-TMP.

3.1 Tumor angiogenesis

IVM studies have characterized normal vascular networks, which consist of differentiated units such as arterioles, capillaries and venules, and form a well-organized architecture with dichotomous branching and hierarchic order. In contrast, tumor vessels are dilated, saccular, tortuous, and heterogeneous in their spatial distribution [48]. Jain and coworkers first demonstrated the potential of IV-TPM in studying gene expression and physiologic function in the deep internal regions of tumors, where the cell behaviors were not accessible with confocal microscopy techniques [49]. By applying the high spatial resolution and imaging depths of TPM, imaging host-generated vasculature in response to tumors by growth of xenograft tumors in transgenic mice that express enhanced green fluorescent protein (EGFP) under the control of the vascular endothelial growth factor (VEGF) promoter was performed (Fig. 2) [49]. They demonstrated the analyzing of the gene expression of VEGF inside 200 μm of the tumor, quantitatively resolving the tumor vascular architecture, giving an insight into the mechanisms of angiogenesis in tumors. Additionally, they were able to study the growth and localization of mutations in the tumor cells, such as those resistant to hypoxia-induced apoptosis. Quantitative measurements of red blood cell velocity and vascular permeability of individual tumor vessels could also be imaged in a similar method to those studies described earlier, to gain further mechanistic insight into tumor function. They also showed that it is possible to visualize the location of therapeutic drug delivery in the tumor architecture. Moreover, they showed that by using fluorescent semiconductor nanocrystals, also known as quantum dots, IV-TPM could allow high-resolution angiography-like imaging of tumor vasculature without significant extravasation often found with dextran-conjugated organic fluorophores [50]. They were also able to track multiple subpopulations of cells as they recruited to the tumor vasculature. These studies demonstrated the wide-ranging possibilities of utilizing TPM for studying tumor function, enabled by the high 3D spatial resolution and imaging depths.

3.2 Tumor invasion and metastasis

IV-TPM has also been applied to study cancer cell invasion from the primary tumor and metastasis to vasculature. Condeelis and colleagues used mammary carcinoma xenografts expressing green fluorescent protein (GFP) and cyan fluorescent protein (CFP) [51,52] to study cell motility and migration *in vivo* and identified fundamental differences in the mode of carcinoma cell migration when compared to the behaviors reported in studies of cell migration on planar 2D surfaces or *in vitro* invasion assays [51,53]. They showed that invading cells preferentially migrate along collagen fibers toward the vasculature with dynamic changes in shape and do not take on a fibroblastic, this demonstrated a novel interaction between mammary carcinoma cells and the stromal cells [51,54]; which is similar to phenotypes observed in carcinoma cells migrating within dense collagen networks *in vitro* [55]. Le Dévédec and coworkers used IV-TPM to track the movement of two distinct breast tumor cell populations, by tagging CFP to a gene known to relate to progressive metastasis and GFP to control tumor cells. Dual color imaging showed conditional expression of CFP among

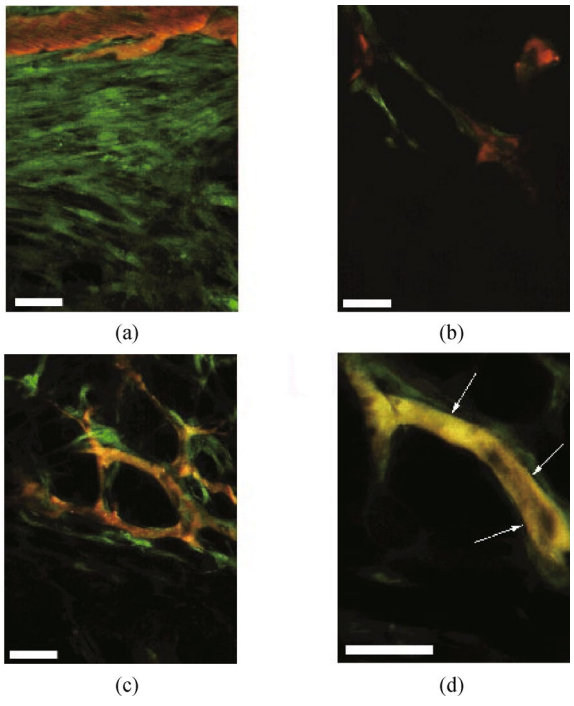

Fig. 2 Gene expression and vasculature imaging in transgenic mice model by IV-TPM. EGFP was expressed upon aviation of VEGF. (a) and (b) TPM imaging at different depth of tumor, (a) 35–50 μm from the tumor surface and (b) 200 μm inside tumor, angiogenic blood vessels was observed with EGFP expressed host cells; (c) colocalization of VEGF-expressing host cells with angiogenic vessels; (d) single layer of (c) at twice magnification. Scale bars, 50 μm. Reprint from Ref. [49] with permission from publisher

control cells, furthermore, stimulated CFP expression by doxycycline has no impact on cell motility compared to control cells (Fig. 3) [23]. This study proved the potential of IV-TPM to directly compare the metastasis activity of two cell populations in the very same tumor environment, and furthermore, to evaluate the effect of treatment methods that targets the cell motility and consequent intravasation and tumor cell dissemination.

3.3 Tumor microenvironment

Dynamic interactions among tumor cells, stromal cells, and the surrounding extra cellular matrix (ECM) are required for the tumor cells to exploit the functionality of stromal cells and generate a microenvironment favorable to malignancy. Boissonnas et al. have visualized the modification of tumor cells and the motility of cytotoxic T lymphocytes [47]. Wyckoff and coworker used IV-TPM to visualize the direct role of macrophages in the intravasation of tumor cells in mammary tumors by labeling them with GFP and observing their uptaking of fluorescent dextran delivered from blood vessels [56–58]. Fukumura and coworkers imaged the VEGF expression level, hypoxia, acidosis, the three important factors in the tumor microenvironment which determinate the tumor growth, metabolism, invasion and angiogenesis. By combining IV-TPM for VEGP-GFP, fluorescence ratio imaging microscopy and O_2-dependent phosphorescence quenching detection, they revealed independent regulation of hypoxia and acidosis to VEGF in live mousse brain tumors [49].

3.4 Tumor metabolism

Abnormal metabolism is an indicator of tumor progression. Many malignant cancer cells have been identified to exhibit excessive rates of glycolysis in the presence of oxygen [59]. Imaging cellular metabolism can be applied to classify tissues as normal pre-cancerous or invasive cancer. Furthermore, metabolism is particularly sensitive to upstream molecular interventions and therefore may be a powerful biomarker of early-drug response [20]. Imaging metabolic activity via the endogenous fluorescent cofactor NADH and FAD, a metabolic coenzyme is one of the most successful applications of IV-TPM to study tumor metabolism. TPM overcome the photobleaching of intrinsic fluorophores, photodamage to biologic samples, and significant light scattering and absorption in turbid cell and tissue environments from the 1P lasers in near-UV and visible wavelengths to excite NADH (350 nm) and FAD (450 nm), respectively. Goodman and coworkers imaged NADH to analyze the regulation of redox state on certain transcription regulatory elements, such as the repressor carboxyl-terminal binding protein (CtBP) [60]. They further found that CtBP played a significant role in controlling tumor cell metastasis, via hypoxia-induced NADH changes [61]. NADH imaging in excised pancreatic islets has also been used to compare the stimulatory effects of glucose and the mitochondrial substrate methyl succinate in metabolic and calcium activity as well as insulin secretion [62]. Skala and coworkers studied the NADH and FAD redox states in precancerous epithelia with IV-TPM and fluorescent lifetime microscopy (FLIM). They first combined the cellular redox ratio, NADH and FAD lifetime, and subcellular morphology imaging for *in vivo* 2P imaging of hamster cheek pouch tissues with dysplasia, the earliest form of pre-cancer lesion in epithelial cancer. They found significant increase in the protein-bound NADH in high-grade precancerous tissues compared with normal ones, and increased protein-bound FAD in low-grade, but decreased FAD in high-grade precancerous tissues (Fig. 4) [19]. Walsh et al. applied TPM and time-correlated single photon counting to measure the NADH and FAD in live breast cancer tumors, they found differences of basal metabolic levels between untransformed, malignant and cancer cells. Moreover, they detected the metabolic changes induced by the inhibition of HER2-an epidermal growth factor receptor, the overexpression of which has shown to be involved in the development and progression of various types of breast

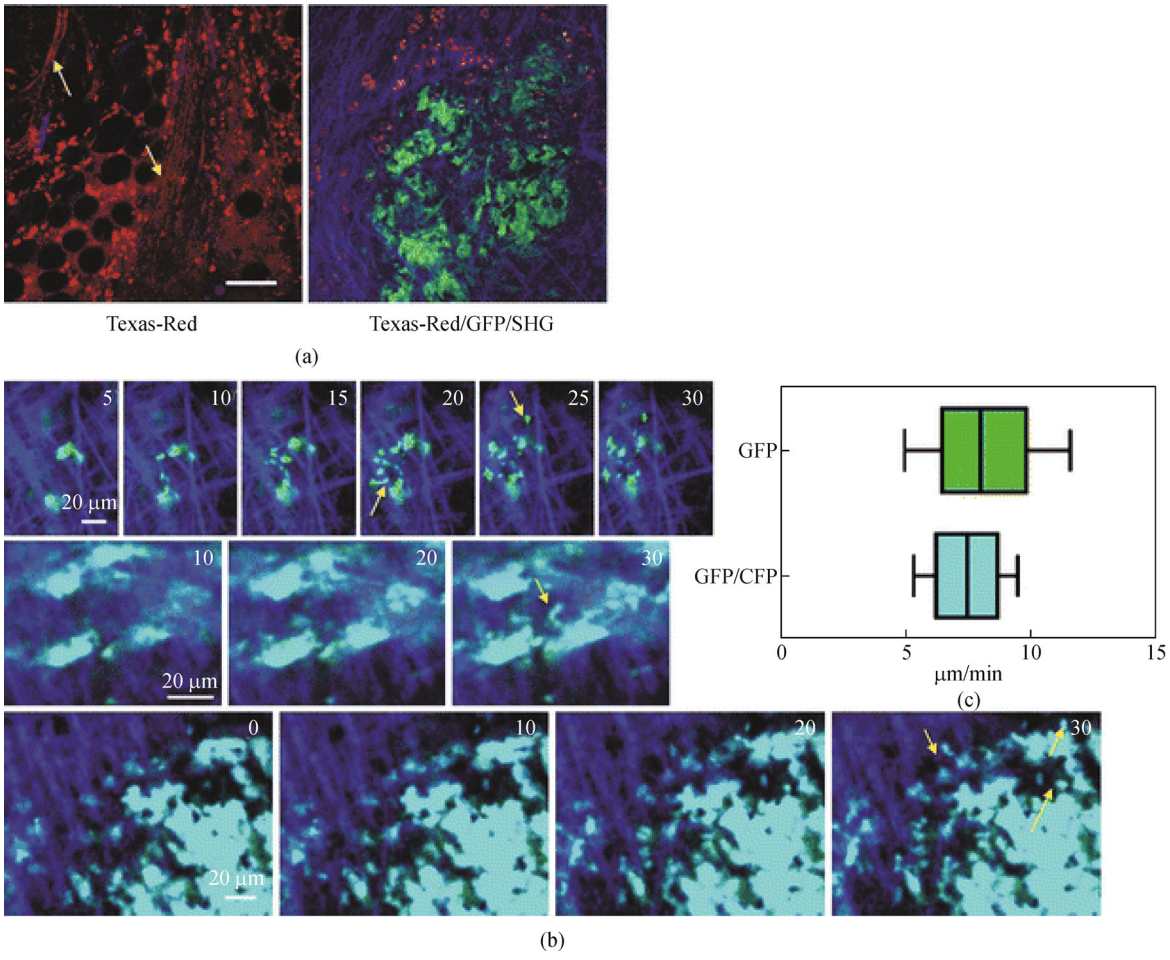

Fig. 3 Imaging motility of two cancer cell populations in mammary tumor with IV-TPM. (a) *In vivo* TPM imaging of tumor microenvironment. Macrophages (red), blood vessels (arrow), tumor cells (green and cyan), collagen (purple), labeled by Texas-Red, Dextran, GFP, intrinsic second harmonic generation (SHG) signal, respectively; (b) simultaneous imaging of motility of tumor cell of GFP or GFP/CFP expression induced by doxycycline; (c) cell motility with FP expression, induction of CFP expression did not affect cell motility. Scale bars, 100 μm. Reprint from Ref. [23] with permission from publisher

cancer by its antibody in human breast cancer xenografts in mice. The metabolic changes in tumor by inhibition were observed by IV-TPM in 48 hours, much earlier than that was observed by fluorodeoxyglucose-PET metabolism imaging [20]. Overall, these positive results indicate that IV-TPM metabolism imaging would have great potential to unravel the early stages of cancer development and to rapidly assess cellular-level metabolic response to molecular expression and drug action, which would greatly accelerate drug development studies.

4 Clinical applications of two-photon microscopy

The ultimate goal of basic and pre-clinical studies is to translate the insights from laboratory into bedside to develop novel treatment methods and instruments for human patients. Imaging has played an indispensable role in clinical cancer treatments for early detection screening and staging cancerous cells or lesions. Many pre-clinical investigations ultimately lead to studies of *ex vivo* or *in vivo* human samples, and to clinical drug or therapy trials. Optical microscopic techniques based on one photon excitation have been successfully applied in human *in vivo* skin and endoscopic imaging, as well as commercialization [63–66]. Generally, TPM has been demonstrated and applied in translational and clinical cancer studies in two main areas: 1) *in vivo* TPM imaging of human skin, and 2) *in vivo* TPM endoscopy of deeper tissue areas [6].

4.1 *In vivo* two-photon microscopy imaging of human skin

TPM analysis of human skin biopsy has been extensively applied to look for indicators of cancerous tissues from normal tissues. Dermatological imaging studies have demonstrated the ability of TPM to distinguish human skin biopsies with skin cancers [67–70]. Dimitrow and

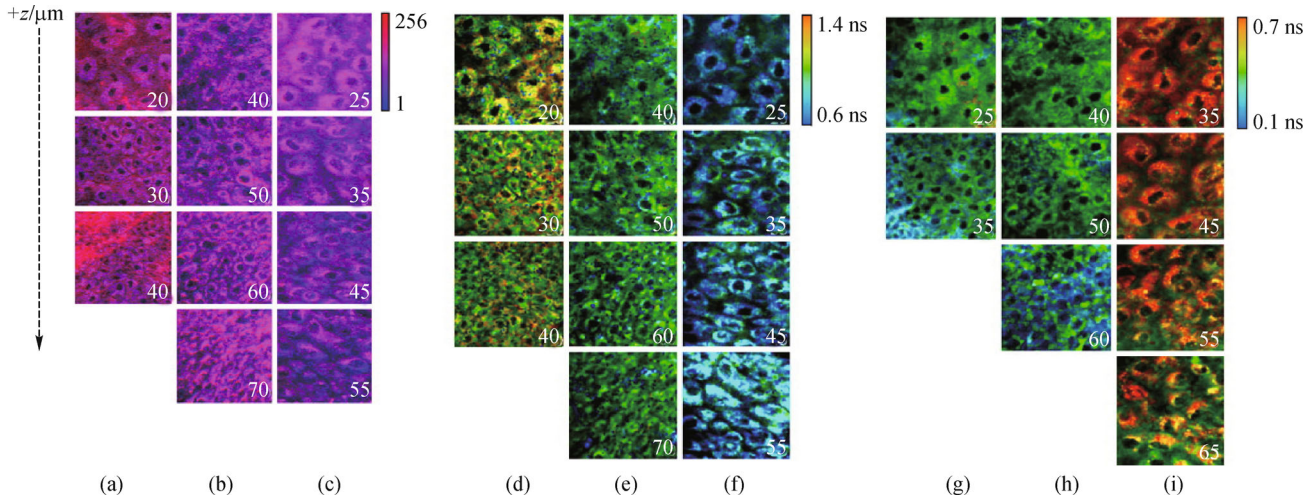

Fig. 4 *In vivo* imaging of metabolism level of precancerous tissues by IV-TPM combined with FLIM at different depths. (a)–(c) Redox ratio, fluorescence intensity of FAD/NADH; (d)–(f) mean NADH lifetime; (g)–(i) mean FAD lifetime by FLIM imaging. High-grade precancer tissues (c), (f) and (i) were associated with high redox ratio, low NADH level and high FAD level compared to normal (a), (d) and (g) and low-grade precancer tissues (b), (e), and (h). Image size 100 μm × 100 μm. Reprint from Ref. [19] with permission from publisher

coworkers [70] screened melanocytic skin lesions from 83 human patients for the pigmented skin melanoma with TPM. Four imaging features, such as architectural disarray of epidermis, poorly defined keratinocyte cell borders, presence of dendritic cells and presence of pleomorphic cells, are identified from large intercellular distance and ascending melanocytes (Fig. 5) as indicator for diagnosis of malignant melanoma with 85% and 97% accuracy for *in vivo* and *ex vivo* examination, respectively. They further demonstrated the potential of TPM-FLIM to differentiate cancerous skin tissues from healthy human skins [69]. Warren et al. have demonstrated further application of TPM in human melanoma diagnosis by combining TPM with pump-probe imaging [71,72], a method that can detect chemical variety of melanin sensitively. Along with the structural information provided by TPM, they were able to distinguish eumelanin from pheomelanin [71], and identify melanomas in pigmented lesions from nonmalignant nevi by the eumelanin content in both animal model and human tissues [72,73]. These studies positively indicate the great potential of TPM in accurate *in vivo* human skin disease detection.

4.2 *In vivo* two-photon microendoscopy

Besides the application on *in vivo* dermatological imaging, TPM has also received wide attention for clinical cancer detection in the development of technologies for miniaturized and flexible two-photon microendoscopy (TPME). Currently, TPME approaches deep human tissues by two main ways: intracorporeal and intracavitary [6], the former uses needle-like lenses to penetrate nearer the body surface, the later uses these or similar lenses attached to a flexible fiberoptic probe, thus potentially enabling deeper intracavity imaging. Although ideal TPME might eliminate the need for more invasive or surgical biopsy-based approaches to cancer detection, it may eventually further lead to target microsurgical or ablation approaches [74]. Major technical obstacles exist for TPME because of the difficulty of integrating of ultra-fast pulse laser source that is required for TPE, mainly the degradation of ultrashort excitation pulses that occur within optical fiber as a result of the combined effects of group-velocity dispersion and self-phase modulation [63,75,76]. Therefore, there have been relatively few human TPME studies, and fewer still pertaining specifically to cancer. Major effort on the clinical application of TPM has been mainly focused on the developing and improving TPME technologies [63,64]. Recently, the advance of photonic crystal fibers and pre-chirped multicore [77,78] have advanced the technology in this regard, and miniaturization of imaging lenses such as gradient index (GRIN) lenses and scanning units based on microelectrmechanical systems (MEMS) have further facilitated development fiber-based TPME systems toward clinical use [79–81].

Up to now, human *in vivo* demonstrations of TPME have thus far used rigid needle-like endoscopic GRIN lenses to image the skin. In a recent study, Llewellyn et al. used a GRIN lens based endoscope of 350 μm to image the sarcomeres in human muscle with minimized invasion. The TPME observed *in vivo* sarcomere length changes according to body gesture with millisecond-scale time resolution [82]. The technical improvements, initial demonstration in human skin endoscopic imaging, as

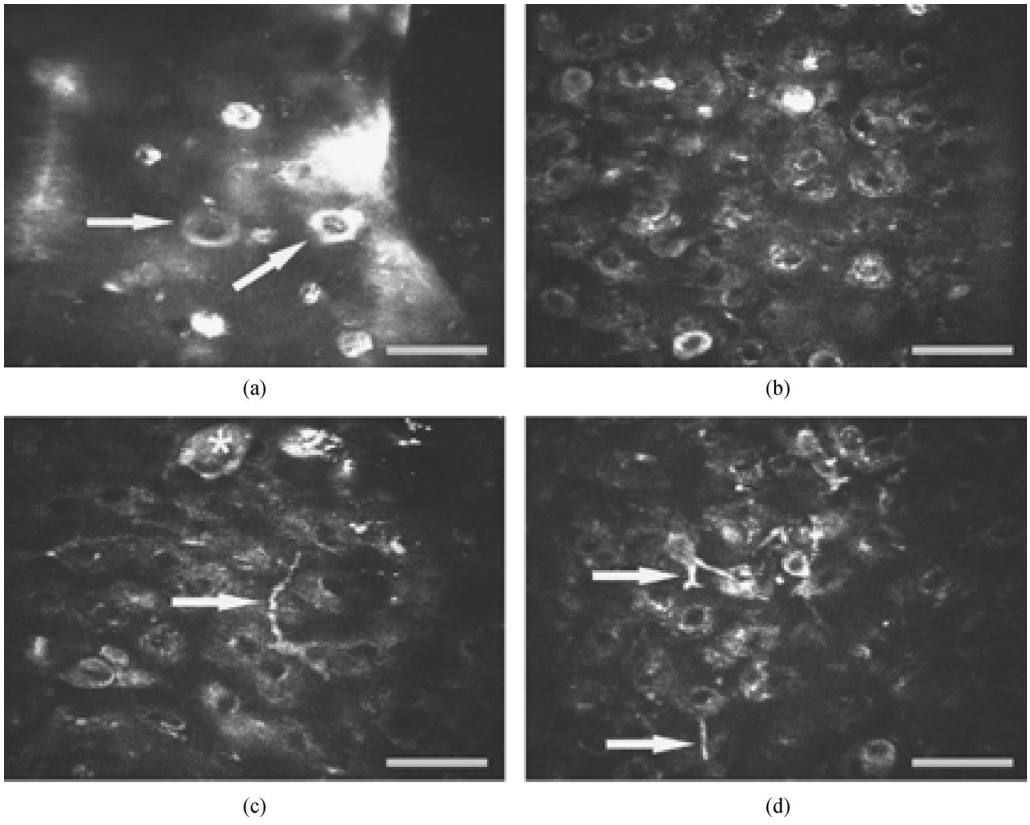

Fig. 5 TPM images of malignant melanoma in human skin sample at upper epidermal (a), granular (b), spinous (c) and (d) layers. Four factors, epidermis disarray (a, highly fluorescent melanocytes, white arrows), poorly defined keratinocyte cell borders (b, c, d), pleomorphic and dendritic cells (c, d, asterisk, and arrows, respectively) were presented in human melanoma skin lesions. Scale bar, 40 μm. Reprint from Ref. [70] with permission from publisher

well as the wide application of fiber based confocal microendoscopy in clinical procedure [83] indicate that, with further development of prototype fiber-based TPME devices and refined procedures of human studies, intracorporeal and intracavitary demonstrations of TPME specifically for clinical human cancer diagnosis will be promising in short future.

5 Conclusions

Early diagnosis of cancer and development of near-real-time monitoring of tumor response to therapy, as well as knowledge of tumor initiation, progression and invasion are needed to improve the outcome and the quality of life for cancer patients. Over the past decades, the application of TPM has received wide attention in both pre-clinical and clinical human cancer studies. Other applications of TPM are emerging in areas including eye imaging, cardiovascular imaging, brain imaging and neuron imaging. More valuable medical information for early cancer screening, guided biopsies and monitoring therapies could be obtained by multimodal imaging of TPM combined with powerful diagnostic methods such as positron emission tomography (PET), computed tomography (CT), magnetic resonance imaging (MRI) [84,85], and ultrasound imaging [86,87]. Such setup would allow the combination of structural information traditionally obtained with established CT, MRI and ultrasound techniques with functional and molecular information provided by optical imaging [88].

Overall, TPM has the potential to become a powerful and practical tool for a wide array of applications from tumor studies in animal models, to noninvasive early detection, image-guided biopsies and intraoperative procedures, and therapeutic monitoring of cancer.

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