Recent advances and applications of nonlinear optical microscopy: perspectives in cancer imaging

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Abstract

Background: Biomedical imaging is one of the important investigation processes for human diseases, especially in cancer diagnosis. Along with conventional light microscopic techniques, there are some high performance imaging systems are being used for cancer diagnosis, such as phase contrast microscopy, Optical coherence tomography, magnetic resonance imaging. But still these imaging modalities suffer from several limitations and medical facilities, research institutions started to use more specific and advanced microscopic techniques like, multi-photon fluorescence microscopy, Coherent anti-Stokes Raman scattering microscopy, second harmonic generation microscopy, third harmonic generation microscopy, etc. which are referred as non-linear optical microscopy.

Methods: Literature searches from PubMed and google scholar related to recent development of non-linear optical microscopies and their applications on cancer diagnosis were chosen in this review.

Results: After reviewing 93 related article, it was found that non-linear optical microscopies are playing a key role in the precise, rapid diagnosis, staging of different types of cancer.

Conclusions: Non-linear optical microscopic modalities are not only limited to image the cancerous tissues, but also have the potential for commercialized non-invasive, real time endoscopic and laparoscopic diagnosis of cancer. If so, the physician could use these modalities for early diagnosis, surgery, and treatment. Thus way non-linear optical microscopic techniques could pioneer in cancer imaging area.

Keywords: Non-linear optical microscopy, cancer imaging, multi-photon, label-free imaging

Background

Cancers are abnormal cell growth, where the abnormal cells have characteristics to spread another parts of the body. Currently, blood tests, computed tomography (CT) scans, magnetic resonance imaging (MRI), endoscopy and pathological investigation of tissue biopsy are used for cancer diagnosis. However, these conventional modalities have limitations. MRI, CT imaging techniques can not distinguish between benign and malignant carcinoma efficiently [1]. Also these techniques suffer from limited soft tissue resolution, low penetration depth, high scattering in tissue, inherent speckle pattern, less sensitivity, high cost issues [2]. One major drawback of this techniques is, use of ionizing radiation, which is toxic to the normal tissues, like as cell killing, DNA damaging, mutation [3]. Non-linear microscopic techniques could be the solution to overcome these. Physicians and researchers started to use these advanced techniques to overcome the limitations in the previous techniques. In the recent years, several non-linear microscopic and endoscopic modalities have been adapted for regular biopsy and non-invasive investigations for cancer diagnosis. Many researchers are contributing to improve these modalities and bringing them to be used widely in the medical imaging field, especially in the cancer diagnosis field. This review presents some of these recent (mostly since 2000) advances and applications of nonlinear microscopic techniques used in cancer diagnosis non-invasively at molecular level with high sensitivity and safety.

Methods

Literature searches from PubMed and google scholar were done. A total of 93 related to several non-linear optical microscopies and their applications on cancer diagnosis were included. The literature with the English language and publication date from 2000 to 2017 were chosen. Main

search keywords were ‘non-linear optical microscopy, cancer, cancer imaging, cancer diagnosis, Two-photon-excited fluorescence (TPEF) microscopy, multiphoton fluorescence (MPF) microscopy, second harmonic generation (SHG) microscopy, third harmonic generation (THG) microscopy, Coherent anti-Stokes Raman scattering (CARS) microscopy’. These TPEF, SHG, THG and CARS microscopy are the best-known nonlinear microscopy technique.

Concept of nonlinearity in microscopy

Figure 1: Linear vs Non-Linear Interaction

Non-linear optical microscopy (NLOM) is advantageous for investigating several endogenous and exogenous substances and structures in tissues also in cells for 3D imaging. They are capable of imaging specific substance in the complex live tissue samples.

NLOM uses nonlinear interactions (figure 1, Jablonski diagram) between light and matter to generate images in contrast with conventional microscopy which typically uses linear interactions, (absorption, refraction, scattering, fluorescence). In TPEF microscopy, number of virtual state is two (Figure 1) and similarly in three-photon modality, number of virtual state is three. As the

energy of a photon is inversely proportional to its wavelength, the two absorbed photons must have a wavelength about twice that required for one-photon excitation.

On the other hand, SGH microscopy is nonlinear optical process, where two photons consist of same frequency interacting with a nonlinear substance are effectively "conjugated" to generate new photons with double the energy, and hence twice the frequency (ω) and half the wavelength of the initial photons [4, 5]. In the same way, in THG light with optical frequency (ω), wavelength (λ) is converted into light with optical frequency 3ω .

CARS is a nonlinear four-wave mixing procedure, which is used to enhance the weak (spontaneous) Raman signal. In the CARS process, a pump laser beam (at frequency ω_{Pump}) and a Stokes laser beam (at frequency ω_{Stokes}) interact, producing an anti-Stokes signal at the optical frequency $\omega_{\text{CARS}} = 2 \omega_{\text{Pump}} - \omega_{\text{Stokes}}$. When the frequency difference between the pump and the Stokes beams matches the frequency of a vibrational mode, the molecular oscillators are coherently driven (figure 3). The result is an enhanced anti-Stokes (shorter-wavelength) Raman signal that is the base for the increased vibrational contrast in CARS microscopy [6].

NLOM basically uses laser of wavelength 700nm-1000nm, which has an advantage for high penetration depth. Increased signal to noise ratio, compatible design, less photo-bleaching [7] characteristics made them very effective in biomedical imaging.

Classification of nonlinear microscopy

Based on coherence property, NLOM is classified into two categories. First one is incoherent and second one is coherent. The incoherent modalities create random phase signal and their power is proportional to radiation producing molecules concentration, such as nonlinear versions of fluorescence microscopes. Nonlinear versions of fluorescence microscopes are based on the

continuous absorption of two or more photons. Examples of incoherent NLOM are TPEF microscopy, MPF microscopy.

On the other hand, second category of NLOM produce signals whose phase is fully prescribed by a variety of factors, including the excitation light phase and the geometric distribution of the radiating molecules. Coherent signal power is proportional to radiating molecules concentration squared. Nonlinear versions of coherent microscopy are based on the simultaneous scattering of two or more photons. Examples are SHG, THG and CARS microscopy. This review describes recent NLOM advances and applications in the field of cancer imaging.

Application of incoherent nonlinear microscopy

Multi-photon excitation fluorescence (MPEF)

In MPEF microscopy, photons are being absorbed simultaneously. These include two-photon excited fluorescence and three-photon excited fluorescence microscopy. Here every photon corresponds to the half of the energy. This two-photon absorption concept first described 1931 by Göppert-Mayer [8]. After that, in 1961 Wolfgang Kaiser practically first time observed this two-photon absorption [9]. Finally, this two-photon excited microscopy was combined with laser scanner and got patent in 1991 by Denk and Strickler [10].

Wang et al. (2002) [11] demonstrated TPEF microscopy in mammary carcinoma xenografts expressing green fluorescent protein (GFP) and cyan fluorescent protein (CFP) in the breast cancer animal model. They revealed extracellular matrix, cell motility, and chemotaxis behavior differences in mammary carcinoma. MPEF microscopy was also used for oral malignancy. Wilder-Smith et al. (2004) [12] demonstrated a non-invasive squamous cell carcinoma imaging for oral malignancy diagnosis in a hamster model. Their method was able to image of collagen matrix and fibers, cellular infiltrates, blood vessels, and microtumors. The same group [12] demonstrated

premalignancy and oral malignancy imaging through multiphoton microscopy which was combined with OCT, optical Doppler tomography (ODT). By using autofluorescence phenomenon, multiphoton autofluorescence microscopy (MPAM) applied for hamster oral mucosal neoplasia [13]. This group was successfully demonstrated quantitative aspects of oral cancer in a hamster model. Also recently in 2016, there was a development of multiphoton microscopy (MPM) for oral epithelial dysplasia based on MPAM by the same group [13]. This method was non-invasive and very important, because this dysplasia could progress to cancer [14]. Sipkins et al. (2005) [15] imaged a metastatic tumor spread in the bone marrow through MPM. Sahai et al. (2005) [16] reported a simultaneous in-vivo GFP, CFP as well as collagen imaging by MPM in mammary carcinoma. Lin et al. (2006) [17] used multiphoton fluorescence (MF) to distinguish basal cell carcinoma from the normal dermal stroma. This technique could replace the Mohs' surgery. Kirkpatrick et al. (2007) [18] evaluated ovarian cancer by TPEF. They used multiphoton fluorescence lifetime imaging protein-bound and free nicotinamide adenine dinucleotide in normal and precancerous epithelia. Role of macrophage during mammary tumor intravasation was first described through MPM by Wyckoff et al., (2007) [19]. Kedrin et al. (2008) [20] described an intravital two-photon excitation microscopic method imaging of metastatic behavior through a mammary imaging window.

Dermatological imaging demonstrated the efficiency of two-photon excited fluorescence microscopy to differentiate normal human skin from skin cancers. Dimitrow et al. (2009) ^[21] demonstrated this multiphoton based tomographic dermatological imaging to distinguish cancerous skin from normal skin. De Giorgi et al. (2009) [22] used combined two-photon excitation fluorescence microscopy, fluorescence lifetime imaging microscopy, multispectral MPM for cutaneous tumors imaging. This method was effective to distinguish between healthy

skin from basal cell carcinoma and malignant melanoma. Williams et al. (2010) [23] demonstrated MPM to ovarian Cancer by laparoscopic approach. Heuke et al. (2013) [24] described a similar multimodal imaging system for skin cancer, which composed of CARS, SHG and TPEF imaging. Zhuo et al. (2011) [25] described a label-free colonic cancer monitoring modality of TPEF microscopy. Xiong et al. (2011) [26] described TPEF imaging of human basal cell carcinoma and squamous cell carcinoma. Leupold et al. (2011) [27] used TPEF microscopy for optical detection of malignant transformation in melanocytes. Piletic et al. (2010) [28] demonstrated use of two-photon microscopy (TPM) for human melanoma diagnosis by combining it with pump-probe imaging in. Later in 2014, a nonlinear spectral imaging modality was introduced by Thomas et al. [29] for murine cutaneous squamous cell carcinoma model. This method was combination of both TPEF and SHG. Adur et al. (2011) [30] used TPEF microscopy for human serous ovarian tumor diagnosis. Cicchi et al. (2013) [31] used MPM for morphological and functional imaging of adenomatous polyp and adenocarcinoma. Yan et al. (2010) [32] demonstrated MPM to diagnose gastric cancer. The two-photon imaging system also used for bladder carcinoma diagnosis [33, 34]. Cicchi et al. (2010) [35] developed a combined two-photon and SGH imaging system for bladder carcinoma. Tewari et al. (2011) [36] used MPM for human prostate and periprostatic cancer imaging. Also Durand et al. (2015) [37] demonstrated a real-time MPM for periprostatic nerve tracking. This method does not require biopsy. Meyer et al. (2012) [38] used TPEF microscopic imaging for advanced carcinoma of the hypopharynx, larynx, and left tonsil. Chen et al. (2013) [39] demonstrated lung cancer imaging by multiphoton imaging modality. This is considered as real-time non-invasive ‘optical biopsy’.

Currently, breast cancer is one of most prevalent cancer in women and ranks second as a cause of cancer death in women. Multiphoton imaging could lead early diagnosis of this fatal cancer type.

Wu et al. (2013) [40] applied a label-free breast cancer imaging method. In the same year, Patsialou et al. (2013) [41] described an intravital multiphoton imaging of human breast tumor. Xu et al. (2013) [42] used Two-Photon Excited Auto Fluorescence (TPEAF) microscopy for quantitative label-free lung cancer imaging.

MPM could also play a tremendous role in ovarian cancer diagnosis as it is also fatal and widespread types of cancer. More than 100000 death incidence happens per year worldwide according to a report of 2011 [43]. Watson et al. (2013) [44] used MPM for ovarian tumorigenesis imaging in model mice. The same group also used MPM for endogenous contrast imaging in ovarian cancer of mouse model [45]. Chen et al. (2013) [46] used TPEF microscopy for cancerous esophagus tissue imaging. Makino et al. (2012) [47] developed multiphoton tomographic imaging modality for colon cancer. They used their multiple photon microscopy based endoscope prototype. Also recently Xu et al. (2017) [48] demonstrated further about MPM based esophageal cancer detection, which strongly suggest the potential of MPM for development of a multiphoton endoscope system for in vivo imaging in future. Zhuo et al. (2012) [49] demonstrated label-free two-photon microscopy of goblet cells for colon cancer. Yan et al. (2013) [50] developed low rectal cancer imaging modality in real time through MPM, which led to the feasibility of using MPM to make real-time multiphoton colonoscopy. Also recently Xu et al. (2017) [51] applied MPM for pancreatic ductal adenocarcinoma detection. It showed that MPM could provide in vivo real-time information of pancreatic neck resection margins (RM) status during surgery. Galli et al. (2013) [52] used two-photon excited fluorescence microscopy for kidney tumor imaging. Recently in 2016, there was a real-time multiphoton imaging method for gastric cancer demonstrated by Yan et al. (2016) [53]. This method suggests laparoscopic imaging of T4 gastric cancer which consists of serosal invasion. In association with nanoparticles, TPEF microscopy was also used

recently in vitro imaging of breast cancer cell line [54]. Recently a combined MPM was demonstrated by Xu et al. (2017) [55]. They combined two-photon excited fluorescence (TPEF), SHG microscopy for label-free human esophageal squamous cell carcinoma detection. Also recently Bower et al. (2017) [56] made MPM and applied in rat mammary tumor imaging. In the future, it is expected that MPM can be used in clinical research in the future with the development of a new MPM endoscope system.

Application of coherent nonlinear microscopy

Second-harmonic generation (SGH) microscopy

SGH microscope obtain contrasts from differences in a specimen's capacity to generate second-harmonic light from the incident light. It is different from the conventional optical microscopes; they obtain contrast by obtaining differences in optical density, path length, or refractive index of the specimen. SHG is a coherent process in which two photons are upconverted to exactly twice the frequency (half wavelength) of the excitation laser.

Figure 2: SGH generation

SHG does not associated with the excitation of molecules such other techniques such as fluorescence microscopy. Therefore, the molecules should not undergo the effects of phototoxicity or photobleaching. Label-free 3D imaging is possible by Second-harmonic generation microscopy. SHG was first discovered in crystals in 1961 by Franken et al. [57]. The first biological SHG imaging was demonstrated using rat tail tendon with a resolution of $\sim 50 \mu\text{m}$ [58]. Later Brown et al. (2003) [59] described SHG microscopy for dynamic imaging of collagen in melanoma. Lin et al. (2006) [17] used SHG to distinguish basal cell carcinoma from normal dermal stroma.

Kirkpatrick et al. (2007) [18] evaluated ovarian cancer by SHG microscopy. Kedrin et al. (2008) [20] described an intravital SHG imaging method for cancer metastatic characteristic imaging through a mammary imaging window. Provenzano et al. (2008) [60] demonstrated SGH based computational analysis of endogenous and exogenous fluorophores in breast cancer. Nadiarnykh et al. (2010) [61] described extracellular matrix's abnormalities in ovarian cancer through SHG. In the same year, Williams et al. [23] demonstrated a laparoscopic SHG module for epithelial ovarian cancer imaging. Ajeti, et al. (2011) [62] demonstrated Structural changes in mixed Col I/Col V collagen in human breast cancer. Adur et al. (2011) [30] showed SHG microscopy for human serous ovarian tumor imaging. In 2011, Chen X [63] showed a quantitative SHG imaging of cancerous thyroid tissue based on collagen change. Xiong et al. (2011) [26] described SHG imaging of human basal cell carcinoma and squamous cell carcinoma. In 2012, Adur et al. (2012) [64] showed SHG for human ovarian cancer imaging. Xu et al. (2013) [42] used SHG microscopy for quantitative label-free lung cancer imaging. Tilbury and Campagnola (2015) [65] demonstrated SGH Microscopy for Ovarian and Breast Cancer Imaging. Watson et al. (2012) [66] described a SHG module for ovarian carcinoma analysis in mice model. Ambekar et al. (2012) [67] demonstrated a method to quantify collagen structural change in breast cancer by SHG. Eliceiri et al. (2014) [68] demonstrated an automated method for aligned collagen determination in human breast cancer. Meyer et al. (2012) [38] used SHG microscopic imaging for advanced carcinoma of the hypopharynx, larynx, and left tonsil. Burke et al. (2012) [69] demonstrated matrix alterations during breast tumor progression by using SGH imaging modality. Zheng et al. (2011) [70] described a label-free SHG method for distinguishing of normal and fibroadenomal breast tissue. Wen et al. (2014) [71] described a texture analysis method for SHG generated image of ovarian cancer. Bredfeldt et al. (2014) [72] developed an automated tracking algorithm called fiber

extraction (FIRE) method of SHG image of breast cancer. Tilbury et al. (2014) [65] analyzed the collagen isoform Col I and Col III of ovarian cancer model by SHG. Chen et al. (2014) [46] used SHG for cancerous esophagus imaging. Han et al. (2008) [73] demonstrated SHG in tumor collagen and showed differences between reactive stroma and healthy stroma. Galli et al. (2013) [52] used SHG microscopy for kidney tumor imaging. Pal et al. (2015) [13] applied SGH for hamster oral mucosal neoplasia. Also recently in 2016, there was a development of SGH for non-invasive oral epithelial dysplasia imaging based on SHG by the same group [74]. Recently Zeitoune, et al. (2017) [75] demonstrated semiautomatic collagen fiber quantification based SGH imaging of epithelial ovarian cancer. Also recently in 2017, Bower et al. [56] developed SHG and applied in rat mammary tumor imaging.

Current progress in SHG microscopy, it is clear that there are a variety of possible future applications of SHG in the study of tumor progression.

Third-harmonic generation (THG) microscopy

THG is a third order NLO process and unlike SHG, is not limited to non-centrosymmetric structures. It is just frequency tripling as compare to SHG. Tsang (1995) [76] showed this THG for the first time and based on it, Barad et al. (1997) [77] developed a THG microscope for the first time. THG requires three photons to generate one at a tripled frequency. Tai et al. (2007) [78] used silver nanoparticles as THG contrast agent, and imaged cancer cell at molecular level in vitro. Adur et al. (2011) [30] successfully applied THG microscopy for human serous ovarian tumor imaging. They had successfully identified transformation of epithelium surface of human ovarian cancer through THG microscopy. Also this study suggests further in vivo studies to quantitatively analyze endogenous optical biomarkers of the ovary. Weigelin, Bakker, and Friedl (2012) [79] used third harmonic generation microscopy for collective melanoma cell invasion analysis. This

study showed potentials of THG in molecular classification of invasion modes and routes in melanoma cells. Harpel et al. (2016) [80] developed a label-free imaging of microbubbles done by THG. This study suggests that contact-free THG images of microbubbles attached to tumor cells could enable unseen cancer to be imaged during surgery. This study demonstrates the potential of THG for endoscopic module development for point-of-care diagnostics as well as early detection of cancer. TGH based human brain tumors was done by Kuzmin et al. (2016) [81]. This study THG has the potential to discriminate among the different cancer cell types. Recently THG microscopy was used to distinguish breast cancer cell subtypes (Gavgiotaki et al., 2017) [82].

Coherent anti-Stokes Raman scattering (CARS) microscopy

Maker and Terhune (1965) [83] mentioned the idea about CARS for the first time. Later Begley, Harvey, and Byer (1974) invented CARS microscopy [84]. CARS microscopy is a dye-free method which images structures by displaying the characteristic intrinsic vibrational contrast of their molecules. CARS probes chemical bond vibration levels, therefore, provides some degree of molecular specificity, particularly for small molecules.

Figure 3: CARS generation

In a sense, CARS contrast can be thought of like a cross between SHG and fluorescence. The main benefit of this method is that the sample remains almost unaffected. Légaré et al. (2006) [85] demonstrated CARS endoscopy for cancer imaging. Le, Huff, and Cheng (2009) [86] used CARS

for cancer cell behavior study in excess lipid environments in vivo and in vitro. Gao et al. (2011) [87] described label-free lung cancer diagnosis using CARS. Hammoudi et al. (2011) [88] demonstrated an automated nuclear segmentation based CARS imaging method by using artificial neural networks. Meyer et al. (2012) [38] used CARS microscopic imaging modality for advanced carcinoma of the hypopharynx, larynx, and left tonsil diagnosis. Xu et al. (2013) [42] used CARS microscopy for quantitative label-free lung cancer imaging. Galli et al. (2013) [52] used CARS microscopy for kidney tumor imaging. Chen et al. (2015) [63] designed an endo-microscopic probe, which is capable of identifying the location of the tumor in vivo during surgery. Legesse et al. (2015) [89] demonstrated a texture analysis based CARS microscopy for skin cancer diagnosis. Recently Shadfan et al. (2017) [90] developed CARS based endomicroscope for oral cancer diagnosis. In association with nanoparticles, CARS microscopy was also used recently in vitro imaging of breast cancer cell line [54]. Recently Weng et al. (2017) [91] combined a deep learning method and CARS for automated differential diagnosis of lung cancer.

Discussion

Van den Abbeele et al. (2016) [92] suggested a precision medicine based clinical model for cancer imaging which was combined with PET, CT and MRI. These techniques were efficient but still possess resolution limit in molecular level imaging. Better knowledge of cancer progression and metastasis at molecular level might be helpful for detecting cancer in its early phase and treating thereby. Precision medicine and large cohorts of cancer imaging data acquired from NLOMs could be combined and by machine learning methods, such as deep learning, artificial neural network, support vector machine and physicians could improve personalized diagnosis at molecular level imaging, radiation treatments with safety and efficiency. Though recently there is some contribution in the oncology field, but not many research groups are trying to implement these

non-linear imaging methods by automated methods. For instance, Coffey, Giedt and Weissleder (2013) [93] developed multiphoton based automated intravital imaging system. But this method was limited to specific cancer clonal cell line. This study suggest that, that kind of algorithm could be implemented for primary cancer also. Other representative work on this field are- Wen et al. (2014) [71] study on texture analysis based deep learning method of ovarian cancer through the SGH and CARS microscopy, Bredfeldt et al. (2014) [72] study on an automated algorithm based identification of breast cancer image acquired by second harmonic generation microscopy, Weng et al. (2017) [91] study on combined deep learning method and CARS for automated differential diagnosis of lung cancer. In future, these advanced non-linear imaging modalities could be integrated with various automated algorithms and predict concisely and rapidly about the presence, stage, predict the precision medicine for individual cancer patients.

Conclusions

Here we tried to describe prominently and leading microscopic cancer imaging modalities which are built through non-linear phenomenon. Now, these advanced techniques are being used for practical clinical cancer imaging. In future non-linear optical microscopic modalities could have the potential for commercialized non-invasive endoscopic and laparoscopic diagnosis of cancer. If so, the physician could use these modalities for early diagnosis, surgery, and treatment. Thus way non-linear optical microscopic techniques could pioneer in cancer imaging area.

List of abbreviations

NLOM (non-linear optical microscopy), TPEF (Two-photon-excited fluorescence), MPF(multiphoton fluorescence), SHG (second harmonic generation, THG (third harmonic generation), CARS (Coherent anti-Stokes Raman scattering).

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Competing interests

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