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Photoacoustic Imaging for Cancer Detection and Staging

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Abstract

Cancer is one of the leading causes of death in the world. Diagnosing a cancer at its early stages of development can decrease the mortality rate significantly and reduce healthcare costs. Over the past two decades, photoacoustic imaging has seen steady growth and has demonstrated notable capabilities to detect cancerous cells and stage cancer. Furthermore, photoacoustic imaging combined with ultrasound imaging and augmented with molecular targeted contrast agents is capable of imaging cancer at the cellular and molecular level, thus opening diverse opportunities to improve diagnosis of tumors, detect circulating tumor cells and identify metastatic lymph nodes. In this paper we introduce the principles of photoacoustic imaging, and review recent developments in photoacoustic imaging as an emerging imaging modality for cancer diagnosis and staging.

Keywords

Photoacoustic imaging; ultrasound; cancer; diagnosis; staging; exogenous contrast agents; spectroscopy

ROLE OF IMAGING IN CANCER DIAGNOSIS AND THERAPY

Cancer is one of the most challenging health problems throughout the world. It is the leading cause of death in developed countries and the second leading cause of death in developing countries [1, 2]. For example cancer caused 25% of deaths in the United States in 2011 [3]. Various diagnostic tools have been developed to assist physicians in the detection, differentiation, and staging of cancer at its curable stages, thus increasing the survival rates and reducing the healthcare cost [4–6]. Moreover, medical imaging paves the way for developing novel, non- or less-invasive, and highly targeted therapeutic procedures with less adverse side effects through accurately monitoring and assessing the outcome of treatments. During past decades, considerable research has been focused on development of new medical imaging techniques capable of characterizing anatomical, structural, functional and metabolic characteristics of cancerous tissues [4, 5]. Recent advances in molecular and cellular biology indicated that understanding the cellular and molecular pathways of cancer

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CONFLICT OF INTEREST

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PATIENT'S CONSENT

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can lead to diagnosis of the pathology at its early stages of development and affect the cancer treatment significantly [7]. In particular, the development of imaging contrast agents with specific targeting moieties expands the scope of several existing medical imaging modalities from their conventional anatomical and structural imaging to molecular and cellular imaging [7–10]. An optimal imaging modality to identify cancer at its early stages of development must have notable features such as being non-invasive (or minimally invasive), capable of high temporal and spatial resolution and able to provide information about cellular and molecular events with high sensitivity and specificity. Moreover, the ideal imaging modality must have minimal complexity and the potential of being widely available in clinical practice.

Currently, major imaging modalities capable of providing cellular and molecular information of cancer are optical imaging [11], magnetic resonance imaging (MRI) [12], and radionuclide imaging such as positron emission tomography (PET) [13] and single photon emission computed tomography (SPECT) [14]. These modalities have shown great capabilities and have been extensively studied in cancer diagnosis. However, they suffer from certain limitations such as system complexity and temporal resolution in MRI, limited imaging depth in optical imaging and limited spatial and temporal resolution in PET and SPECT. Therefore, there is still demand for the development of new robust imaging modalities, capable of addressing these limitations and reducing system complexity and implementation cost.

Photoacoustic (PA) imaging has shown tremendous potential in cellular and molecular-specific imaging of cancer. The key advantages which make PA imaging a suitable diagnostic modality for clinical applications are its ability to provide molecular information at clinically relevant depths with a high resolution and in real-time [15–23]. Moreover, it can be easily combined with ultrasound (US) imaging as both imaging modalities have shared hardware components and a common signal detection regimen. Therefore, through the combination of US and PA imaging, it is possible to obtain information on anatomical, functional and molecular content of diseased tissues [24–27].

PHOTOACOUSTIC IMAGING: PRINCIPLES

PA imaging is based on the principles of photoacoustic effect that were first explored by Alexander Graham Bell in 1880 [28]. Photoacoustic techniques were initially studied in non-biological fields such as physics and chemistry [29, 30]. Since Theodore Bowen introduced PA imaging technique as a biomedical imaging modality in 1981 [31], this technology has been developing quite rapidly by several early adopters of photoacoustic imaging [32–34].

Fundamentally, the photoacoustic technique measures the conversion of electromagnetic energy into acoustic pressure waves [31]. In biomedical PA imaging, the tissue is irradiated with a nanosecond pulsed laser, resulting in the generation of an ultrasound wave due to optical absorption and rapid thermal (or thermoelastic) expansion of tissue Fig. (1) [35]. The initial pressure, p_0 , generated by an optical absorber, is described as

$$p_0 = T\mu_a F$$

where F is the laser fluence at the absorber, μ_a is the optical absorption coefficient, and T is the Grüneisen parameter of the tissue [36]. By detecting the pressure waves using an ultrasound transducer, an image can be formed with the primary contrast related to the optical absorption of tissue. This unique mechanism through which a PA image is generated provides distinct advantages compared to other *in vivo* imaging modalities. First, the

contrast mechanism in PA imaging is based on the differences in optical absorption properties of the tissue components. PA imaging is suited for imaging structures with high optical coefficient such as blood vessels [37–39]. Second, PA imaging can be achieved using longer wavelengths in near-infrared (NIR) region, where tissue absorption is at a minimum. Light can penetrate up to several centimeters into biological tissues at wavelengths in the near-infrared range while remaining under the safe laser exposure limits for human skin [40, 41]. The photoacoustic technique enables imaging deeper into tissues than optical imaging methods that utilize ballistic or quasiballistic photons (e.g., optical coherence tomography or OCT) because photoacoustics does not rely on detection of photons – instead, weakly scattering acoustic waves are detected in response to laser irradiation. Third, a PA imaging system can be easily combined with a US system because both systems can share the same detector and electronics. The primary contrast in US imaging is derived from the mechanical properties of the tissue, which mostly describes anatomical information. Therefore, combined PA and US system can provide both anatomical and functional information [27, 42].

In PA, imaging depth is limited by light penetration and acoustic attenuation. In practice, the incident laser light and acoustic wave generated by the light beam will be attenuated in the tissue. However, the penetration depth is primarily limited by the optical scattering – a dominant component of the optical attenuation in the tissue. Specifically, the optical penetration depth is determined by the effective extinction coefficient $\mu_{\text{eff}} = (3\mu_a(\mu_a + \mu_s))^{1/2}$ obtained from diffusion theory, where μ_a and μ_s are the absorption coefficient and reduced scattering coefficient of the tissue, respectively [43, 44]. At depths greater than 1 mm, the light diffuses and decays exponentially with the exponential constant equal to μ_{eff} . The penetration depth is defined as the distance at which the intensity of the light inside the tissues falls to $1/e$ (~ 37%) of its intensity at the surface. The penetration depth, $1/\mu_{\text{eff}}$, is strongly wavelength-dependent and it may reach up to several centimeters in NIR region [40, 41].

Spatial resolution of PA imaging at depth beyond quasi-ballistic penetration of photons is determined by characteristics of an ultrasound transducer. The lateral resolution is dependent on numerical aperture (NA) and the center frequency of the transducer. The axial resolution is inversely proportional to the frequency bandwidth of the transducer [27, 45]. Therefore, a transducer with large NA, higher center frequency and higher bandwidth provides the best spatial resolution. In practice, sub-millimeter spatial resolution is achievable at depth up to several centimeters, and sub-100 μm is possible for penetration depth of several millimeters. Higher lateral resolution (~ 5 μm) in PA imaging can be achieved by using fine optical focusing within less than 1 mm imaging depth where the focused light is in the ballistic regime. In this case, the lateral resolution is determined by optical diffraction limit, however, the axial resolution is still limited by frequency bandwidth and acoustic attenuation [46]. Overall, spatial resolution of photoacoustic imaging decreases as imaging depth increases.

PA IMAGING USING ENDOGENOUS CONTRAST

Endogenous Contrast Agents in PA Imaging

Biological tissues contain several kinds of endogenous chromophores that can generate PA signal. The main sources of endogenous contrast in PA imaging are hemoglobin, melanin, and lipids. Depending on the wavelength, these endogenous contrast agents may have strong absorption coefficients in comparison with other tissue constituents. Photoacoustic imaging has been used in various applications where endogenous chromophores are present, such as in the visualization of blood vasculature structure and melanoma [38, 39, 46–50]. The absorption coefficients of these chromophores are strongly wavelength dependent.

Therefore, if PA imaging is performed at several wavelengths, referred to as spectroscopic PA imaging, it is possible to measure distributions of the chromophores [48, 50, 51]. The use of endogenous chromophores in biomedical imaging has two key advantages. First, the endogenous contrast agents are inherently biologically safe. Although exogenous contrast agents may provide strong contrast and ability to be targeted to specific molecules, one of the major obstacles inhibiting the clinical use of exogenous contrast agents is safety, such as toxicity and retention in the body. Second, physiological and metabolic changes that differentiate pathological tissue from normal tissue can be monitored because endogenous contrast agents provide the physiological changes, such as oxygen saturation and vascular blood volume in the body [34, 50, 52].

PA Imaging for Monitoring Angiogenesis and Hypoxia Using Oxygen Saturation (SO₂)

Solid tumors contain areas of low oxygen concentration referred to as hypoxic regions. Hypoxia is regarded as an important event leading to tumor progression and angiogenesis (the growth of new blood vessels in the body) [53]. For the growth of a tumor, large amounts of oxygen and fresh nutrients need to be continuously supplied. In order for the tumor to develop, new vascularization is required to provide sufficient amount of the oxygen and nutrients. Therefore, malignancy of the tumor can be determined based on the microvasculature structure and the oxygen saturation (SO₂) [54].

PA imaging has been used to image the microvasculature structure and angiogenesis because hemoglobin is strong absorber in NIR region [38, 39, 46–49]. Fig. (2a) shows a three dimensional PA image (at wavelength of 600 nm) of a subcutaneous tumor (LS174T colon carcinoma) 8 days after the inoculation [47]. The tumor with the associated vascular structure was clearly visualized as shown in Fig. (2a). Fig. (2b) represents x-y and x-z maximum intensity projection of the vasculature of a different tumor (SW1222) acquired 12 days after inoculation. In Fig. (2b), the tumor was imaged at 758 nm to achieve a deeper penetration depth (at least 9 mm) because of a larger size of the tumor [47].

Furthermore, spectroscopic PA imaging allows us to calculate the oxygen saturation, SO₂, of the blood [48] assuming that two forms of hemoglobin – oxyhemoglobin (HbO₂) and deoxyhemoglobin (Hb), are the major optical absorbers in a body. To calculate the oxygen saturation in a body, the wavelength dependent absorption coefficients (μ_a [cm⁻¹]) at two wavelengths can be calculated by the following equation:

$$\begin{aligned}\mu_a(\lambda_1) &= C_{HbO_2} \varepsilon_{HbO_2}(\lambda_1) + C_{Hb} \varepsilon_{Hb}(\lambda_1) \\ \mu_a(\lambda_2) &= C_{HbO_2} \varepsilon_{HbO_2}(\lambda_2) + C_{Hb} \varepsilon_{Hb}(\lambda_2)\end{aligned}$$

where C_{HbO_2} and C_{Hb} are the molar concentrations [M] of HbO₂ and Hb, and ε_{HbO_2} and ε_{Hb} are the molar absorption coefficients [cm⁻¹M⁻¹] of HbO₂ and Hb, which are generally well known.

The SO₂ level of hemoglobin can be described as:

$$SO_2 = \frac{C_{HbO_2}}{C_{HbO_2} + C_{Hb}}$$

To improve the accuracy of SO₂ level estimation, more than two wavelengths can be used. In addition, depth and wavelength dependent fluence compensation of PA signals can further improve the accuracy. Fig. (2c) represents *in vivo* PA functional imaging of oxygen saturation in a mouse brain with a glioblastoma xenograft [50]. The red arrow indicates the

hypoxic region where the brain tumor is located. As shown in Fig. (2d), this result clearly demonstrates that the SO_2 level in the tumor vasculature is lower than the surrounding normal tissue. In this study, the laser light penetrated through the skin and the skull to the mouse brain because of their relatively small thickness (~0.5 mm) [55]. However, PA imaging of a large animal or human brain through intact skull still remains challenging due to scattering and absorption by the skin and skull as well as the phase and amplitude distortions of ultrasonic waves by the skull [56, 57]. To increase light transmittance through the skull, the utilization of a photon recycler was recently reported for PA imaging of the human brain [56].

PA Imaging for Melanoma Detection

Melanoma is the most dangerous form of the skin cancer that arises from melanocytes. Melanocytes are specialized pigmented cells which produce melanin. If melanoma is diagnosed early, it can be removed by surgical procedure, and ~80% of cases are cured in this manner [58]. Therefore, early detection of melanoma is very important for its complete treatment. PA imaging with melanin has been demonstrated to obtain images of subcutaneous melanomas and their surrounding vascular structure *in vivo* [48, 51, 59]. Dual-wavelength PA imaging can be used to differentiate melanomas from tissue based on the optical absorption difference between hemoglobin and melanin [48]. The vascular structure surrounding the melanoma was acquired at 584 nm because both melanin and hemoglobin exhibit high absorption. At 764 nm, melanin provides higher photoacoustic contrast in comparison with hemoglobin. By combining the images collected at 584 nm and at 764 nm, melanoma can be differentiated from surrounding vasculature.

Melanoma is an extremely dangerous cancer with high metastatic potential. The metastatic cancer cells can easily migrate through the membrane and extracellular matrix and spread from one site to another via the lymphatic or blood systems [60]. Therefore, curing metastatic malignant melanoma remains a challenge and has a relatively poor prognosis, with a median survival rate of only 6 months [61]. To provide timely treatment strategies, it is very important to detect metastatic malignant melanoma. The most common methods for detecting metastasis are assaying lymph nodes or bone marrow when diagnosis and surgery are performed. However, these methods are invasive, time consuming and inaccurate [60]. *Ex vivo* blood assay of detection of circulating tumor cells (CTCs) could provide an alternative approach for evaluating metastasis, however, the sensitivity is limited by the small blood volume and the small fraction of cancer cells within the blood samples [62]. The sensitivity can be improved by analyzing a significantly larger volume of blood. The ability of PA technique to differentiate melanoma from the blood could be used to detect metastatic melanoma cells in the blood circulation [8, 63–65]. Photoacoustic flow cytometry was introduced to perform the blood screening [8, 64]. In one of its *in vivo* demonstrations, the laser light was irradiated into a mouse ear blood vessel through a minimally invasive optical fiber and PA signals were collected at two wavelengths (865 and 630 nm) to enable spectroscopic separation of melanoma from blood. In addition, in order to decrease false-negativity of PA flow cytometry, the improved signal-to-noise ratio can be achieved in various ways, such as using exogenous contrast agents [66], enhancing melanin synthesis using drugs [64] or other melanin activators [67], and using melanin-containing pathogens [68, 69]. In another study, label-free melanoma cells in flowing bovine blood were imaged *in vitro*, using a fast scanning PA microscope Figs. (3c–f) [65].

PHOTOACOUSTIC IMAGING USING EXOGENOUS CONTRAST AGENTS

Exogenous contrast agents have been introduced to expand the application of PA imaging in several ways [17, 18, 70, 71]. First, since the PA signal depends on the optical absorption of the photoabsorber, utilizing exogenous contrast agents with large optical absorption at a

desired wavelength can increase the PA signal [19, 72, 73]. This effect can subsequently allow imaging at greater depths or using lower optical fluence. Moreover, by tuning the optical absorption of these exogenous contrast agents to fall in the “tissue optical window” where tissue components have minimal absorption, it is possible to increase the imaging contrast and depth [74–77]. Second, by utilizing small enough contrast agents (such as nanoparticles) that can extravasate from the vasculature, get close to and ultimately tag specific cells or molecules, PA imaging can be utilized to visualize events at the cellular and molecular levels [78–86]. Moreover, having prior knowledge of the optical absorption of such contrast, opens a path to spectroscopic PA imaging, where the PA signals at several different wavelengths are utilized to suppress the signal from background tissue and thus reveal the presence of the contrast agents with a higher contrast [52, 79, 87–91]. Depending on the required imaging depth, desired light source to be used, and delivery mechanism of nanostructures, the choice of exogenous contrast agents may change.

Exogenous Contrast Agents for PA Imaging

Currently, the major contrast agents for PA imaging are metallic nanostructures [23, 66, 71, 72, 76, 79, 92–103], dyes (or fluorophores) [104–108], and carbon nanotubes [21, 83, 99, 109–112]. Recently, new PA contrast agents such as per-fluorocarbon droplets loaded with metallic nanostructures [113–115] were introduced to further enhance PA imaging abilities. Fig. (4) summarizes the optical absorption of tissue components Fig. (4a) and the peak of optical absorption for several types of exogenous contrast agents used in PA imaging Fig. (4b). Detailed reviews of exogenous contrast agents for PA imaging are available [17, 18, 70].

Metallic Nanostructures—Metallic nanostructures made of noble metals such as gold or silver can be synthesized with tunable optical absorption and can significantly enhance the contrast in PA imaging [17, 70]. The mechanism of optical absorption in these nanostructures is based upon surface plasmon resonance (SPR) that can result in a significantly higher optical absorption compared to other optical absorbers such as dyes [116]. Furthermore, the absorption spectrum of these SPR nanostructures can be tuned through controlling their sizes and geometries [117–119]. Various different SPR nanostructures have been utilized for PA imaging and are reported in literature, including: gold nanospheres [78, 79, 92, 120], gold nanorods [76, 77, 89, 90, 93, 94, 121], gold nanoshells [72, 92, 122], gold nanocages [75, 80, 98], gold nanoclusters [123], gold nanostars [124], gold nanoroses [97], gold nanowantons [71], and silver nanoplates [125] with different ranges in size, optical absorption spectra and PA imaging applications. These plasmonic nanostructures are synthesized with sizes varying from a few nanometers to a few hundred nanometers and their delivery or uptake depends on their size, geometry, and surface charge and functionalization [126].

Dyes—Dyes (or fluorophores) have been widely used for other medical imaging applications such as fluorescence imaging [127], Förster resonance energy transfer imaging (FRET) imaging [128], two photon microscopy [129] and Raman spectroscopy [130]. Recently, near infrared (NIR) absorbing dyes such as Alexa fluor 750 [87], indocyanine green (ICG) [41, 131, 132], and IRI dye 800CW [107] have been utilized as contrast agents in PA imaging. These dyes possess high absorption coefficients and low quantum yield, making them efficient contrast agents for PA imaging [87]. Most dyes are small organic molecules consisting of 20 to 100 atoms, thus making them bio-compatible and capable of being cleared from the body through renal clearance [133].

Single-walled Carbon Nanotubes (SWNTs)—Single-walled carbon nanotubes (SWNTs) are essentially folded single layers of graphite, exhibiting strong optical

absorption over a broad spectrum [134]. The optical absorption of SWNTs is broad and covers the tissues optical window, thus making them a suitable contrast agent for PA imaging. Moreover, SWNTs can be synthesized as small as 1 nm diameter and with various aspect ratios to increase both absorption cross section and effective surface area for bio-conjugation applications [135]. Besides their applications as photoacoustic contrast agents, their wide absorption spectrum allows them to be used in thermoacoustic imaging where the energy source is not restricted to optical sources [109]. The rapid blood clearance of functionalized SWNTs through the renal excretion route without adverse side effects, opens the door for their utilization in *in vivo* applications [136]. The evidence of SWNTs safety in small animal models has been previously demonstrated in the literature [110, 137]

Multimodal Hybrid Contrast Agents—Hybrid contrast agents are developed to act as a contrast agent in two or more imaging modalities. These hybrid contrast agents can provide further detailed molecular information with an enhanced contrast, or can be used to cross-validate the imaging results. Some contrast agents can provide contrast for multiple imaging modalities. For example, gold nanoparticles can provide contrast in PA, CT, and OCT. Besides, there are several types of hybrid contrast agents developed by combination of PA imaging contrast agents with other non-optical imaging modalities, such as magnetic resonance imaging (MRI) [71], magneto-motive ultrasound (MMUS) imaging [138–142], and computed tomography (CT) imaging [143]. Moreover, synthesis of contrast agents for more than two imaging modalities such as PA/MRI/Raman imaging [144] and MRI/CT/PA imaging [145] is reported in literature.

As PA imaging is complementary with an US imaging, design and development of multimodal agents capable of enhancing contrast in both US and PA imaging has received attention by researchers over the past few years. India ink or ICG loaded micro- and nano-bubbles are examples of such contrast agents [146]. Furthermore, US/PA contrast agents were developed by encapsulating plasmonic nanostructures of different types into nano-sized droplets made out of materials with low boiling points, such as perfluorocarbon [113, 114]. Upon depositing the energy into light absorbing plasmonic nanostructures, the generated localized heat causes vaporization of the carrier droplets resulting in a strong instantaneous PA signal followed by weaker PA signal due to the conventional mechanism of PA signal generation (thermoelastic expansion). Simultaneously, the light induced localized generation of a bubble enhances the local acoustic reflectivity, thus acting as an US contrast agent [113]. Passive accumulation of 200 nm perfluorocarbon droplets loaded with gold nanorods in a mouse pancreas and a strong PA signal due to vaporization of nanodroplets, followed by conventional PA signals due to the thermoelastic expansion of gold nanorods, was demonstrated [113]. The PA signal amplification due to vaporization and generation of nano and micro-bubbles can potentially play an important role in clinical applications of PA imaging if the sensitivity is a major limitation. The utility of these bubble-based PA contrast agents is studied and reported in several research studies [147]. The nonlinear bubble-related amplification of PA signals is reported in the literature [21, 148].

Molecular Targeting of PA Exogenous Contrast Agents—Besides generating a significant contrast in PA images, PA contrast agents can provide opportunities to perform pathology specific imaging through cellular and molecular targeting of specific targets in cancerous tissues. Over expression of specific receptors or antigens in cancer cells results in increased, selective receptor-mediated uptake of nanostructures with specific targeting moieties, opening the door for cellular and molecular imaging of cancer.

To perform nanostructures-assisted cellular and molecular PA imaging, the surfaces of the nanostructures are functionalized by ligands and antibodies with high affinity, avidity and

specificity for specific overexpressed cell receptors [149, 150]. Numerous research studies have been focused on design and development of molecular-targeted PA contrast agents. For example gold nanospheres, functionalized with antibodies are shown to selectively label cancer cells with over-expressed epithelial growth factor receptor (EGFR) [78, 79, 84]. Other studies demonstrated PA imaging augmented with molecular specific contrast agents to detect CTCs [22, 82], prostatic cancer [76], breast cancer [107], and pancreatic cancer [125]. SWNTs were also shown to possess a great capacity for molecular targeting and detection via PA imaging [151]. Dyes are also shown to have the capability of cellular and molecular targeting [152, 153]. Interestingly, some molecular-targeted PA contrast agents can be simultaneously utilized to perform localized photothermal therapy and even localized drug-delivery [154–156].

Applications of Exogenous Contrast Enhanced PA Imaging in Cancer Diagnosis

Contrast-enhanced PA molecular imaging (i.e. PA imaging using molecular targeted exogenous contrast agents) can provide anatomical, functional and molecular information of diseased tissues at clinically relevant depths, with high spatial resolution and obvious contrast. Several research groups have shown the abilities of PA imaging augmented with molecular-targeted contrast agents to detect cancer at the cellular level [76, 79, 82, 84, 157]. Contrast enhanced PA imaging has shown great promise in its ability to detect primary tumors [83, 103], tumor vasculature [158], CTCs [22, 82, 159, 160], and micro-metastasis in sentinel lymph nodes (SNL) [106, 161]. Table 1 summarizes some reported research studies related to exogenous contrast agent-assisted PA in detection of tumors, CTCs, mapping lymph nodes, and identifying lymphatic metastasis.

Primary Tumor Detection—Capabilities of contrast-enhanced PA imaging to detect various types of tumors are demonstrated in several *in vivo* animal studies. In the majority of the reported studies, plasmonic or multimodal nanostructures were accumulated in tumors either through enhanced permeability and retention (EPR) effect or were functionalized to accumulate through receptor-mediation. Then PA imaging was utilized to visualize the presence of the nanostructures and thus indicate the presence of the tumor.

Metallic nanoparticles are the main group of nanostructures utilized in PA imaging of tumors. For example, it was demonstrated that gold nanocages can be utilized to detect B16 melanoma at a wavelength of 778 nm [80]. The utilization of gold nanospheres accumulation and subsequent PA imaging at 532 nm was demonstrated in an additional study to detect human breast cancer tumor [120]. Spectroscopic PA imaging is demonstrated to be capable of visualizing the presence of exogenous contrast agents *in vivo* through obtaining PA signals at multiple wavelengths and finding a map of different optical absorbers. For example, the ability of spectroscopic PA imaging to monitor the passive accumulation of silica-coated gold nanorods in a xenografted tumor is demonstrated [91]. Using the known optical absorption properties of nanorods, Hb and HbO₂, spectroscopic PA imaging can clearly localize and differentiate these three components in a tumor. In another study, anti-EGFR conjugated silver nanoplates were utilized to image the pancreatic tumors in a mouse model Fig. (5) [125]. Functionalizing nanostructures to target different biological entities of interest can enable PA to image multiple targets by using nanostructures with distinct optical absorption spectra to target each of them. For example, the feasibility of using gold nanorods with different absorption spectra and multiplex targeting moieties to image multiple types of cancer is demonstrated both *ex vivo* [90, 162] and in a mouse model [94].

Besides metallic particles, hybrid multimodal nanostructures were shown to be capable of providing imaging contrast for PA and other imaging modalities. It is shown that triple-

modality hybrid nanoparticles (contrast agents for MRI, Raman spectroscopy and PA imaging), can extravasate through the leaky blood-brain barrier into the extravascular space, be internalized by cancer cells within an orthotopic glioblastoma brain xenograft tumor, and subsequently be detected by PA imaging [144]. The results from all three imaging modalities confirm the accumulation of hybrid nanostructures within the brain tumor, suggesting the feasibility of tumor detection following passive accumulation of nanoparticles.

Dyes have also been shown to be suitable contrast agents for labeling tumors in PA imaging. Spectroscopic PA imaging of IRDye800 dye targeted to $\alpha_v\beta_3$ integrin which is over-expressed in human glioblastoma tumors, was shown to be capable of identifying brain tumors in a mouse model [52]. In another study, the utilization of IRDye800CW targeted to neutropilin-1 receptor (NPR-1) enabled spectroscopic PA imaging to detect breast cancer xenograft tumor in nude mice [107].

Additionally, SWNTs were recently used as PA contrast agents in tumor detection. Labeling of human glioblastoma tumors in inoculated mice was achieved using antibody functionalized SWNTs to target $\alpha_v\beta_3$ integrins [111]. SWNTs targeted to $\alpha_v\beta_3$ integrin overexpressed in tumor neovasculature were shown to be capable of identifying human glioblastoma-astrocytoma xenograft tumor in a mouse [83].

Detecting Circulating Tumor Cells (CTCs)—Metastatic spread from the primary tumor often leads to death in patients with cancer. Highly sensitive detection of circulating tumor cells (CTCs) would greatly enhance overall patient survival, if treated. Although the utility of PA to detect melanoma cells due to intrinsic contrast of their melanin content has been demonstrated [8, 63–65], other types of cancer cells, such as breast and prostate, do not contain melanin pigments, and therefore exogenous contrast agents can significantly improve the ability of PA imaging in detecting such CTCs [159, 160]. The abilities of PA imaging using contrast agents to detect and monitor CTCs have been reported in several studies. The feasibility of using targeted gold nanoparticles to label breast cancer cells in human blood was demonstrated [159]. Gold nanoparticles have also shown capability for being used in detecting circulating prostate cancer cells [160]. In a more recent study, it was shown that by using both gold-coated SWNTs and magnetic nanoparticles, circulating breast cancer cells can be magnetically trapped by a static magnetic field and then PA imaging can detect their accumulation [86]. A different research group demonstrated the same concept by using hybrid magneto-plasmonic nanoparticles in a set of *ex vivo* studies [163, 164].

Mapping Lymph Nodes and Imaging Cancer Cell Metastasis Through

Lymphatic Systems—In most developed cancers, malignant tumor cells are metastasized through the lymphatic system. Reliable detection of metastatic cancer cells in the sentinel lymph node can play an important role in diagnosis, staging and treatment of cancer. Several studies have demonstrated the capability of using PA to map lymph nodes and also to identify the metastasis of cancer cells through the lymphatic system. For example, gold nanorods injected into a mouse forepaw accumulated in the sentinel lymph nodes (SLNs) and were subsequently imaged by PA [89]. PA signal enhancement due to the injection of ICG in a rat model is demonstrated in another study. The PA results were confirmed by performing fluorescent imaging of the ICG dye Fig. (7) [165]. Gold-coated SWNTs were also shown to be capable of identifying the SLN in a selected area of the mesenteric region of a mouse [99]. A real-time PA lymphatic mapping and quantification of disseminated tumor cells (DTCs) in prenodal lymphatics is also demonstrated [166]. In another recent study, PA augmented by gold nanocages was shown to be capable of SLN mapping in a rat model [98].

Beyond the previously described studies which showed the ability of PA imaging to map the lymph nodes, other researchers have investigated the utility of PA to detect micrometastasis of cancer cells within the lymphatic system [167–172]. A research group demonstrated that the principles of PA flow cytometry can be extended to enable *in vivo* assessment of migrating cancer cells within lymphatics [171, 172]. In their studies, the utility of gold nanorods, nanoshells, and carbon nanotubes as cellular markers was demonstrated [171]. It must be noted that detecting migrating cells in the lymphatic system can be performed without utilizing contrast agents to label cancerous cells. In other words, intrinsic absorption of some types of cancer cells (such as melanoma) can be used to identify the presence of cancer cells within the SLN and to monitor the cancer cells' migration. For example, the ability of PA to identify accumulation of melanoma cells in lymph nodes through lymphatic channels is demonstrated. It is shown that the sensitivity of PA is sufficient to identify as few as 500 melanoma cells in lymph nodes [168]. In a recent study, PA computed tomography was demonstrated to be capable of detecting melanoma metastases in human dissected SLNs [167]. Together, these studies suggest PA as a promising intraoperative imaging technique in identifying micrometastasis of cancer cells.

CHALLENGES FOR PA IMAGING OF CANCER

Laser Safety

During PA imaging, skin is irradiated with the laser light. For safety reasons, laser radiation should be kept below the maximum permissible exposure (MPE), which is the level of laser radiation to which a human can be exposed without hazardous effects or biological changes [173]. MPE for human skin to a pulsed laser defined by the American National Standards Institute (ANSI) is 20–100 mJ/cm² at the wavelengths ranging from 400 to 1500 nm [173]. Higher laser radiation provides higher signal-to-noise ratio (SNR) in PA imaging. However, using a fluence greater than the MPE limits leads to a temperature rise in the irradiated tissue, with the potential for causing pain and burning the patient's skin.

Safety of PA Exogenous Contrast Agents

PA imaging with exogenous contrast agents is rapidly emerging field because of the advantages described earlier. However, the long-term toxicity and accumulation of nanoparticles remains a concern [174]. These toxicity and accumulation risks are highly variable based on differences between sizes, geometries and surface characteristics of nanoparticles. For the nanoparticles to move into the clinic, it is important that more toxicity studies are conducted for each type of nanoparticle. Therefore, the safety of these metallic nanoparticles has become a focus of many research groups [175, 176]. Most of these studies are aimed to study the long term safety issues in living subjects as the nanostructures larger than a certain size (~5–6 nm) cannot be cleared through renal clearance and will accumulate in biologically important organs such as the liver and spleen. On the other hand, small nanoparticles are rapidly cleared from the body and therefore do not provide sufficient time for them to label their molecular targets [177]. Biodegradable clusters of nanoparticles were recently introduced as a promising tool to address the clearance time and long term side effects issues of metallic nanoparticles [123, 178, 179]. These nontoxic biodegradable plasmonic nanoclusters consists of ultra-small (< 5nm) individual gold particles stabilized by a weakly adsorbed biodegradable polymer. The small spacing between primary gold nanoparticles within the clusters results in strong NIR absorbance. The nanoclusters, are stable at pH 7. However, after cellular uptake, they degrade into primary gold nanoparticles at around pH 5 (the environment inside of the endosomes) within a few days. Once the polymer is biodegraded, the ultra-small primary nanoparticles can be excreted from the body [123, 179]. Interestingly it was shown that clustering the plasmonic nanoparticles can also enhance PA signal [148, 180–182].

Temporal Resolution

Temporal resolution of current existing PA imaging systems is one of the most important limiting issues. PA system utilized with optical parametric oscillator (OPO) lasers are mostly operating at low frame rates due to the low pulse repetition rates (PRF) (often less than 50 Hz) of the laser sources. Recently, efforts have been made to increase the temporal resolution of PA imaging with the goal of developing real-time PA imaging systems. PA imaging system, implemented on a clinical ultrasound machine equipped with a 128 element array transducer and a Nd:YAG laser with a 1000 Hz PRF was demonstrated to have capability of providing combined ultrasound and PA images of a human hand with a frame rate of 8 frame/sec [183]. Although this single wavelength is not satisfactory for several advanced PA techniques such as spectroscopic PA imaging, at 1064 nm, the tissue background signal decreases significantly and thus enable imaging contrast agents such as silver nanoplates for molecular PA imaging [74]. Similarly, other researchers investigated PA flow cytometry with a Yb-doped fiber laser with a high PRF (0.5 MHz) at 1064 nm which can reduce false-negative results caused by undetected cells or particles between laser pulses [184]. In another study, researchers developed a beamforming technique capable of generating one PA image per irradiation and thus by utilizing a Q-switched alexandrite laser with PRF of 7.5 Hz, they could achieve 7.5 Fr/sec [25]. Utilization of a high PRF Nd:YLF diode-pumped Q-switched laser and a 30 MHz linear array transducer to achieve 50 frame/sec PA imaging is also demonstrated [185].

Cost and Portability of Pulsed Lasers

The major cost associated with a PA imaging system is due to the need for expensive Q-switched lasers. The use of low-cost compact laser diodes with wide emission wavelength availability instead of conventional Q-switched pulsed systems opens the possibility of creating portable imaging instrumentation suitable for clinical applications. However, there are certain difficulties related to the use of CW lasers for PA imaging of tissue chromophores. For example, the relatively low optical power delivered to the targeted tissue samples results in generation of extremely weak acoustic waves. This is translated to a low signal-to-noise ratio (SNR) of the detected signals. To further increase the SNR, some research studies propose to use coded excitation for pulsed PA imaging.

PHOTOACOUSTIC IMAGING IN CLINICAL APPLICATIONS

With all the promise of PA imaging in cancer diagnosis, clinical applications of PA imaging in human subjects have been reported in a limited number of studies. Imaging breast cancer could be one of the potential clinical applications of PA imaging due to the flexibility of placing light delivery and ultrasound detection on breast tissue. In one of the first demonstrations of *in vivo* PA imaging of human subjects, 2-D PA images of breast were obtained from patients diagnosed with cancer prior to surgical mastectomy [186, 187]. In another study, the utility of a customized PA system, consisting of a hemispherical array of piezoelectric transducers, to reconstruct 3-D vascular images of human breast tissue was demonstrated [188]. A PA mammoscope was developed and used to demonstrate promising results in detecting breast tumors [189–192]. Evaluation of clinical feasibility of the PA mammoscope was reported for 10 patients with malignancies and two patients with breast cysts. PA imaging was capable of identifying malignancies with a higher contrast compared to x-ray and mammography Fig. (8). Moreover, PA images were shown to be capable of identifying malignancies independent from breast density which can potentially give PA a significant advantage over conventional breast screening techniques [189].

Contrast agents can play an important role in further utility of PA imaging in clinical applications. Various types of dyes have already been approved for specific clinical use.

However, most metallic PA contrast agents, made out of heavy metals, elicit concerns with regard to their long-term safety [17]. Further development of safe, clinically useful PA contrast agents can open up new horizons for the clinical utility of PA as a tool for cancer diagnosis.

It must be noted that besides PA imaging of cancer in human subjects, other studies demonstrate application of PA imaging in determining depth of port wine stain [193], monitoring blood hemoglobin concentration [194], monitoring of cerebral venous oxygenation [195], and imaging human peripheral joints for inflammatory arthritis diagnosis [196].

CONCLUSIONS

Photoacoustic imaging has shown promising capabilities to become a suitable clinical imaging modality with some notable advantages, including high resolution, clinically relevant penetration depth, non-ionizing signal generation, availability of non-radioactive imaging contrast agents, the potential for real time image acquisition, and the simple integration with widely used clinical ultrasound systems. Its superior abilities to detect deeply situated tumors and their vasculature, micrometastasis in lymphatics and other organs, and circulating tumor cells can greatly contribute to the better understanding of molecular pathways associated with various cancers. Furthermore, photoacoustic imaging can enable physicians to detect cancer at its early stages of development, thus increasing survival rates and reducing health care costs associated with cancer treatment procedures.

Photoacoustic imaging can be combined with other ultrasound-based and optical molecular imaging modalities to provide better contrast and further molecular information. One of the key advantages of photoacoustic imaging is that it can be easily implemented on clinical ultrasound machines to expand the scope of conventional ultrasound imaging by enabling cellular and molecular imaging. Having basic similarities with ultrasound imaging, photoacoustics inherits several notable advantages of ultrasound imaging such as non-ionizing signal generation, real-time imaging, and of the capacity to image deep lying tissue structures.

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Fig. 1.
A diagram shows the main components of a PA imaging system and the mechanism of PA signal generation.

Fig. 2.

Functional PA imaging of tumors using endogenous contrast agents. **(a)** Three dimensional PA images of LS174T tumor after 8 days of inoculation. The image was obtained at 600 nm with a scan step size of 70 μ m. **(b)** x-y and x-z maximum intensity projection of a larger SW1222 tumor after 12 days of inoculation. The images were acquired at 758 nm and the imaging depth is at least 9 mm (reprinted with permission from Laufer *et al.* [47]). **(c)** SO₂ image of a nude mouse brain with a glioblastoma xenograft. Hypoxia in the brain tumor was monitored by PA imaging. The red arrow indicates the location of the tumor, which shows lower SO₂ than the surrounding vasculature. **(d)** A quantitative comparison of normal and tumor vasculatures SO₂ in three mice. The results clearly show that SO₂ level in the tumor is lower than normal vasculatures (reprinted with permission from Li *et al.* [50]).

Fig. 3.

(a–b) *In vivo* imaging of a subcutaneously inoculated B16-melanoma in an immunocompromised nude mouse: (a) Combined two maximum amplitude projection images at 764 nm and 584 nm depict a melanoma and surrounding vasculature. The melanoma is pseudo-colored brown at 764 nm and blood vessels are pseudo-colored red at 584 nm. (b) Three dimensional PA image of the melanoma generated from the data at 764 nm. The overlaid image in Fig. (3a) was redrawn at the bottom (reprinted with permission from Zhang *et al.* [48]). (c–f) PA detection of label-free melanoma cells in flowing bovine blood *in vitro*. (c) B-scan PA image showing two melanoma cells clearly at $x = 588$ and $720 \mu\text{m}$. (d) M-mode PA image tracking three melanoma cells acquired through the center of the glass microtube. (e) PA amplitude profile from the melanoma cell at $x = 588 \mu\text{m}$ shown in (c) and its Gaussian fit. (f) PA amplitude profile from the cell around $t = 19 \text{ ms}$ in (d) and its Gaussian fit. Dashed lines in (c) and (d) mark the boundary of the glass tube (reprinted with permission from Wang *et al.* [65]).

Fig. 4.

(a) Optical absorption of endogenous photo absorbers in tissue. The optical window is shown with a yellow strip. (b) The range where peak absorption of major PA imaging exogenous contrast agents falls in. The peak absorptions depend on several parameters such as size, geometry, optical cross-section, etc. SWNTs are shown to have a high optical absorption over a wide range of spectrum including optical window spectrum.

Fig. 5.

(a) TEM image of silver nanoplates. The scale bar is 100 nm. (b) Extinction spectra of silver nanoplates. 2-D cross sections of an orthotopic pancreatic tumor in a nude mouse model. US image (c) shows the anatomical features, while the USPA image (d) shows molecular accumulation of a-EGFR conjugated nanoplates (yellow), oxygenated blood (red), and deoxygenated blood (blue). Image dimensions are 14.5 mm by 11.8 mm, (reprinted with permission from K. A Homan *et al.* [103]).

Fig. 6. (a) Schematic of *in vivo* magnetic trapping and multi-spectral PA detection of breast CTCs labeled with golden carbon nanotubes (GNTs). The laser beam is delivered either close to the external magnet or through a hole in the magnet using a fiber-based delivery system. (b) PA spectra of 70 μm veins in the mouse ear (open circles). Absorption spectra of magnetic nanoparticles (MNPs) and GNTs (dashed red and green curves) are normalized to PA signals from CTCs labeled with MNPs (filled red circle) and GNTs (filled green circle) (reprinted with permission from Galanzha *et al.* [22]).

Fig. 7.

Spectroscopic PA image of a rat auxiliary region (a) before injection of ICG. Blood vessels (BV) are seen. (b) Photoacoustic image acquired 0.2 hour after injection. Lymphatic vessels, as well as an SLN, are seen. (c) Graph shows results of comparison of photoacoustic signals within the SLN and lymphatic vessels (LV) versus time before and after the injection in a given rat. The photoacoustic signals were normalized by the photoacoustic signals of adjacent blood vessels. (d) Graph shows results of statistical comparison of photoacoustic signal enhancement in the background ($n = 5$), SLN ($n = 5$), and lymphatic vessels ($n = 4$) at 0.2 and 1 hour after injection. The P values for the SLN and lymphatic vessels, respectively, were less than .002 and .001 at 0.2 hour after injection and less than .001 and .13 at 1 hour

after injection. (e) Three-dimensional photoacoustic image processed from the 0.7-hour post injection. a.u. = Arbitrary units (AU), error bars in d = standard deviation, error bars in e = standard error of the mean, (reprinted with permission from Kim *et al.* [165]).

Fig. 8.

Diagnostic images of a 31 mm infiltrating ductal carcinoma in the right breast of a 64 year old woman. **a)** The cranio-caudal mam-mogram of the right breast shows a large region with atypical and suspicious microcalcifications (white square). **b)** The ultrasound image shows a large inhomogeneous lobed mass (white square) with microcalcifications, which is somewhat suggestive for a benign fibroadenoma. Close to this large lesion, there is a second comparable, but smaller lesion (not visible in this image). **c)** The T1 weighted contrast enhanced MRI shows two lesions in the lateral quadrant of the right breast. The biggest lesion (white square) is visible in this image and measures 34 mm, the second, smaller lesion (14 mm) is positioned caudal to this lesion and is not visualized here. **d)** Photoacoustic imaging also shows two abnormalities, separated less than 10 mm. **e)** The upper abnormality (5 mm depth) has a contrast of 4.7 and a maximum diameter of 26 mm and can be seen in this transversal cross-section (slice thickness 0.24 mm). **f)** The smaller, lower (13 mm depth) abnormality had a contrast of 5.3 and a maximum diameter of 14 mm, (reprinted with permission from Heijblom *et al.* [189]).

Table 1

Summary of nano-sized contrast agents assisted PA in detecting tumors, CTCs, mapping lymph nodes and identifying lymphatic metastasis

Goal of the study	Molecular probe	Imaging Wave-length (nm)	Target or Conjugation	Ref.
Tumor detection				
Spectroscopic PA molecular and Hypoxia Imaging of brain tumors	IRDye800	764, 784, 804, 824	α_3 integrin	[50]
PA imaging of LNCaP prostate cancer cells labeled with gold nanorods and implanted in a nude mice	Gold nanorods	725 nm	Human epidermal growth factor (HER2)/neu antigen	[76]
PA Imaging of B16 melanoma in mice	Gold Nanocages	778 nm	-melanocyte-stimulating hormone	[80]
PA imaging of targeted SWNTs to detect glioblastoma-astrocytoma tumor xenograft	SWNTs	690 nm	α_3 integrin	[83]
Spectroscopic 3-D PA imaging to monitor the accumulation of silica coated gold nanorods in human endolethial tumor	Gold nanorods	740, 760, 780, 800, 820, 840 nm	None	[91]
Spectroscopic PA imaging to detect pancreatic tumor in a nude mouse	Silver Nanoplates	740 to 940 nm	EGFR	[103]
PA imaging of breast tumor xenograft in nude mice	IRDye800CW	710 to 890 nm	neutropilin-1 receptor(NPR-1)	[107]
PA imaging of targeted SWNTs to detect human glioblastoma tumors in mice	SWNT	750 nm	α_3 integrin	[111]
PA detection of human breast cancer (BT474) tumor xenograft in mice	Gold nanospheres	532 nm	None	[120]
PA imaging of orthotopic glioblastoma mice brain tumor	Hybrid multimodal nanostructure	532 nm	None	[144]
PA imaging of gold nanoshell accumulated in a colon carcinoma tumor	Gold nanoshells	800 nm	None	[158]
CTC detection				
Imaging CTCs labeled both magnetically and optically and are captured using a	Magnetic nanoparticles (MNPs) and gold-coated SWNTs	905 nm	plasminogen activator receptors for MNPS None for Gold-coated SWNTs	[86]

Goal of the study	Molecular probe	Imaging Wave-length (nm)	Target or Conjugation	Ref.
static magnetic field in a mouse with breast cancer xenograft tumor				
Photoacoustic flowmetry of prostate cancer cells	Gold nanoparticles	530 nm	epigallocatechin gallate	[160]
Lymph node mapping and micrometastasis detection				
Mapping SLN using spectroscopic PA imaging of gold nanorods	Gold nanorods	757, 807, 820 nm	None	[89]
SLN mapping in a rat model using gold nanocages	Gold Nanocages	755 nm	None	[98]
PA imaging augmented with functionalized gold-coated SWNTs to trace and map lymphatic endothelial cells (LECs) in mouse model.	Gold-coated SWNTs	850 nm	lymphatic endothelial hyaluronan receptor-1 (LYVE-1)	[99]
Spectroscopic PA Imaging lymph nodes and lymphatic vessels in a rat using ICG	ICG	618 and 668 nm	None	[165]
PA imaging of cancer cells metastasis from primary tumor in mouse ear to SLN using golden SWNTs	Magnetic nanoparticles (MNPs) and Gold-coated SWNTs	639 and 850 nm	Folate	[166]
Utilizing PA flow cytometry to image lymphatics and lymphocytes <i>in vivo</i>	Gold nanorods, Gold nanoshells, CNTs	639, 850, 865 nm	None	[171]