

Review article

Photoacoustic clinical imaging

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A B S T R A C T

Photoacoustic is an emerging biomedical imaging modality, which allows imaging optical absorbers in the tissue by acoustic detectors (light in - sound out). Such a technique has an immense potential for clinical translation since it allows high resolution, sufficient imaging depth, with diverse endogenous and exogenous contrast, and is free from ionizing radiation. In recent years, tremendous developments in both the instrumentation and imaging agents have been achieved. These opened avenues for clinical imaging of various sites allowed applications such as brain functional imaging, breast cancer screening, diagnosis of psoriasis and skin lesions, biopsy and surgery guidance, the guidance of tumor therapies at the reproductive and urological systems, as well as imaging tumor metastases at the sentinel lymph nodes. Here we survey the various clinical and pre-clinical literature and discuss the potential applications and hurdles that still need to be overcome.

1. Introduction

In recent years, molecular imaging techniques have rapidly been translated and integrated into mainstream clinical practice and standard of care [2]. Such techniques improve understanding, characterization, and monitoring of pathologies, thus enabling earlier detection, more accurate diagnosis, and improved disease management. While magnetic resonance imaging (MRI) and radionuclide imaging methods have offered fertile ground for developing a huge variety of novel contrast agents and mechanisms, the relatively high costs and logistical restrictions of such modalities (such as magnetically shielded rooms or radiation safety equipment) limit their cost-effectiveness [4–6]. By comparison, the increased affordability and portability of ultrasound instruments, as well as the advent of contrast-based methods such as elastography and microbubble-based ultrasound molecular imaging, have made clinical ultrasound an invaluable tool for clinical imaging, both anatomy, and molecular content [7]. Photoacoustic imaging (PAI), which is also referred to as optoacoustic imaging, is an emerging technique that has immense potential for augmenting ultrasound with rich optical contrast and can serve as a portable and (relatively) low-cost standalone modality for regional imaging of blood vessels and other optical contrast agents [9,10]. The core strengths of PAI are its potential for high spatial/temporal resolution, clinically relevant imaging depth [11], ability to image both endogenous [12] and exogenous [13] chromophores, and the absence of ionizing radiation. Common

endogenous chromophores include water (both free and bound), oxy-hemoglobin (HbO₂), deoxyhemoglobin (Hb) melanin and lipids. Exogenous agents are mostly small molecule dyes such as Indocyanine green (ICG), Methylene Blue Dye (MBD), nanoparticles or even reporter gene agents. Unlike current microbubble-based ultrasound contrast agents, PAI can image small molecules that can readily extravasate, target cell membrane molecules, or even enter cells of interest to target intracellular molecules, and thus provides the clinician with potentially valuable molecular data [14–16].

The photoacoustic effect was first described by Alexander Graham Bell [17] over a century ago as a conversion of optical energy to audible pressure waves. However, little progress was made until the invention of the laser allowed for a much-improved signal generation. Subsequently, the use of photoacoustics grew in popularity, first in the field of gas spectroscopy and later for biomedical applications [18]. Over the past 20 years, PAI has rapidly evolved from the concept phase [19] to a tomographic modality for small animal imaging [14] to clinical devices [20].

The effect itself is based on pressure transients generated by absorption of pulsed or modulated light [9]. Such acoustic transients experience far less scattering and absorption than visible or near infrared (NIR) light as they propagate through the tissue. Measuring those transients by an acoustic transducer at tissue boundaries allows faithful reconstruction of the absorption sites. Owing to its inherent scalability, PAI can be performed over a wide range of depths and resolutions

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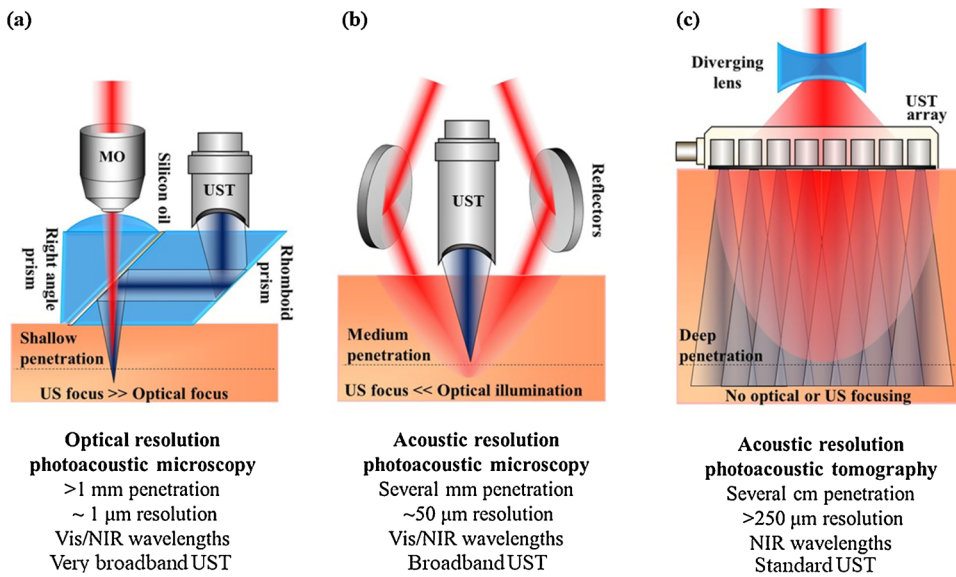


Fig. 1. various configurations for PAI.

(a) Optical resolution photoacoustic microscopy. Here the optical excitation is focused and co-aligned with the acoustic transducer. While many configurations exist to maintain alignment, a simple but effective one was devised by Wang et al. [1]. Here two prisms are joined together with a thin layer of silicone oil between them. This layer transmits light interruptedly but reflects the acoustic waves and thus acts as an optical/acoustic splitter. A rhomboid prism is required for the acoustic side to ascertain that a longitudinal wave (rather than shear) reaches the transducer. Penetration depth is very shallow in order to maintain the optical focusing. The optical focal region (mm scale) is much smaller than the acoustic one and thus determines the (lateral) resolution which can be sub-cellular. (b) Acoustic resolution photoacoustic microscopy. Here the light is delivered around the transducer to create uniform illumination. The unfocused light allows for greater penetration.

Both the lateral and axial resolutions are determined by the acoustic focal region. See for example Kim et al. [3] (c) Acoustic resolution photoacoustic tomography/macroscopy. Here neither the light nor the sound are focused which allows several cm of imaging depth. Imaging is based on multiple transducers which views each point in the region of interest (ROI) from a different angle and thus able to resolve it. See for example Witte et al [8].

US- Ultrasound, MO - Microscope objective, UST - Ultrasound transducer

(Fig. 1) [11]. Acoustic-resolution photoacoustic tomography (PAT) can achieve sub-millimeter resolution at depths up to several centimeters [21], while optical-resolution photoacoustic microscopy (PAM) can achieve even sub-micron resolution but is restricted to a few hundred microns of depth penetration [22] with a typical depth-to-resolution ratio of about 200. The field of view (FOV) usually also scales with depth as well as with the scanning time.

In recent years, advanced light sources technologies allow for mobility, tunability, compactness, and affordability [10]. Novel algorithms allow for real-time, high-resolution and accurate reconstruction of the initial pressure distribution even under non-ideal conditions [23]. Such non-ideal conditions often include a limited viewing angle, in which the sample is not fully enclosed with acoustic detectors. Other common conditions are finite sized and limited bandwidth detectors. The use of multiple wavelengths can, in principle, allow the concentrations of distinct chromophores to be simultaneously quantified, thus providing molecular-specific contrast [24]. Accurate spectral separation has been a long-standing challenge in PAI, mostly due to the effect of spectral coloring [25].

Here we review the recent advances in the clinical translation of PAI. This paper builds on our previous review from 2014 [26]. The goals of this review are to show the current progress in PAI toward clinical imaging, point out the remaining hurdles and finally discuss possible future directions to make PAI a truly clinically relevant modality. Thus, we have emphasized on published scientific literature which both reports of human imaging in that have been achieved in clinical settings and those that are close to being achieved. For the reader's convenience, this review is organized according to the anatomic location from top to bottom of the human body. Afterward, we will briefly discuss the outstanding challenges of PAI clinical translation as well as prospects.

2. Brain imaging

Clinical brain imaging is one of the more challenging applications for photoacoustic imaging, due to strong optical scattering by the skull and brain tissue, which severely limits the optical fluence. Furthermore, the acoustic signal became distorted and attenuated on transit from the brain back through the skull and scalp [27]. Consequently, at present, PAI for brain imaging is still in a preclinical stage, as experiments on

rodents are less challenging due to their thin skull.

Multiple studies have performed photoacoustic brain imaging in small animals *in vivo* using PAT. One focus has been on structural and functional brain imaging. Using the intrinsic optical absorbance of hemoglobin as an endogenous contrast agent, Wang et al. [28] imaged blood vessels in the healthy rat brain as well as in lesions and showed functional imaging of cerebral hemodynamic changes in response to whisker stimulation and response to hyperoxia and hypoxia. The intrinsic contrast of blood vessels to the background parenchyma ranged from 1.7 to 7.9, and the resolution achieved was 20 μ m, close to the detection limit. Since the system had a single detection element that was scanned around the head, the measurement time was ~16 min. A faster system, with a 512-element full-ring ultrasonic transducer array, was used by Nasirivanaki et al. [29] to record the resting-state functional connectivity (RSFC) in the mouse brain, which are the correlations between spontaneous brain activity in various parts of the cortex. Research has shown the RSFC is altered in many brain disorders, such as Alzheimer's disease and schizophrenia and therefore measurements of RSFC could constitute an appealing technique for the study of brain diseases in mouse models. RSFC was measured in live mice under either hyperoxic or hypoxic conditions, as well as in dead mice. The intrinsic contrast relied on the absorbance of hemoglobin as well, and the imaging was done with a 0.01–0.02 mm resolution. Strong intra-hemispheric and bilateral inter-hemispheric correlations in eight main functional regions and several sub-regions were recorded. Through three periods of hyperoxia to hypoxia modulation, weaker correlations during hypoxia were consistently observed. Laufer et al. imaged the vasculature in a mouse brain with a Fabry-Pérot polymer film sensing interferometer that is transparent to the optical illumination, and therefore allows to image noninvasively in reflection mode [30]. A second focused laser beam that is raster scanned over the surface of the sensor allows the readout of the 2D distribution of the acoustic transients which are incident upon the sensor. The time-varying acoustic waveform acquired at each point of the sensor allows for reconstruction of a 3D image. The system allows imaging over a 50 mm² area, with a lateral resolution of 50–100 μ m, and a vertical resolution of 50 μ m with a measurement time of 1 s. Using scanned broadband illumination, images based on hemoglobin absorbance contrast were acquired down to a depth of ~3 mm. PAT was also shown to accurately localize epileptic seizures in rats *in vivo* by monitoring changes in blood flow [31]. The

system used had a single illumination wavelength and a single scanned transducer element for acoustic wave detection and acquired an image in 5 min. Findings were verified with EEG, the gold standard in seizure measuring technique. Zhang et al. mapped the micro-vasculature network of the mouse brain, imaged spontaneous neuronal activity in deep tissue, and studied neuronal activity during epileptic seizures [32]. This was accomplished using a high-frequency linear transducer array (centered at 21 MHz), which was rotated around the mouse head to achieve whole-brain imaging based on hemoglobin absorbance, with a high spatial resolution for this large imaging window (75 μ m lateral, 500 μ m vertical).

Using an alternative approach with indocyanine green (ICG) as a contrast agent, PAT was utilized to perform angiography in rats [33]. The ICG was conjugated with polyethylene glycol (PEG) to prolong the circulation in blood. The contrast of the blood vessels to the background was enhanced from 1.8:1 to 2.8:1 in the presence of ICG-PEG, with an estimated concentration of $\sim 10 \mu$ M in the blood. The imaging system had a single scanned ultrasonic transducer and was used to perform imaging with 50 μ m resolution, with an image acquisition time of 23.5 min.

Brain tumor imaging is another promising clinical application of PAI. Since PAI can rely on endogenous contrast or small molecule contrast agents such as ICG, it circumvents the difficulties associated with the delivery of larger imaging agents across the blood-brain barrier. In one of the first demonstrations of brain tumor imaging *in vivo*, Ku et al. successfully identified regions of tumorigenesis in the rat brain by examining the distortion in vascular architecture and associated vascular changes based on the endogenous contrast of hemoglobin [34]. The PAT system used had three single element transducers, a 3.5 MHz, a 10 MHz, and a 20 MHz, whose corresponding spatial resolutions were approximately 210 μ m, 60 μ m and 30 μ m respectively and were scanned around the animal head. Several years later, Burton et al. performed multispectral optoacoustic tomography (MSOT) of glioblastoma tumors in mice, and monitored both hemodynamics and ICG uptake using tunable illumination in the 680–980 nm range and spectral unmixing [35] (see Fig. 2). The detection was through a 64-element curved transducer array, with an estimated in plane spatial resolution of 150 μ m and 800 μ m in the elevational direction. A version of this system is also available commercially and marketed by iThera Medical. Imaging was performed on the animals twice, 16- and 34-days following implantation of U87 glioblastoma cells into the striatum. At the 16-day mark, the animal was imaged following a carbon-dioxide challenge, producing the image shown in Fig. 2C. At the 34-day mark, the animal was imaged with multispectral illumination, producing the spectrally-unmixed Hb image in Fig. 2A. Next, ICG encapsulated into liposomes was injected into the animal and spectral-unmixing resolved the ICG image in Fig. 2E. All results were verified in cryosections with photographs (Fig. 2D) or fluorescence images (Fig. 2F and H) showing agreement with between the ICG and the PAT signals. Kircher, and coworkers have developed a triple-modality MRI-PAI-Raman imaging gold/silica nanoparticle for both preoperative and intraoperative brain tumor imaging, with PAI being used intraoperatively to localize deep tumors in mice [36]. The nanoparticle was shown to diffuse to the extravascular space through the disrupted blood-brain barrier in the tumor region without the need for a targeting agent, due to enhanced permeability and retention (EPR) effect. The photoacoustic signals were shown to increase by 75% after intravenous injection of the nanoparticles, while the MRI signal increased more than six-fold, and the Raman signal at least an order of magnitude. The nanoparticles were used to delineate the tumor margins with high precision, as validated with histology *ex vivo*. Fan et al. developed organic nanoparticles, based on Perylene-diimide [37], which were shown to increase the PA signal *in vivo* by $\sim 80\%$. The nanoparticles were used to detect a tumor at a depth of 4 mm in mice *in vivo*. The imaging was performed with a commercial LAZR system (LZ250 linear array transducer with 21 MHz center frequency) and tunable illumination in the range of 680–970 nm.

In order to increase the light penetration through the sculp and skull, Guo et al. developed conjugated polymer nanoparticles with strong absorption in the second near IR region [38]. The particles were surface functionalized with an active targeting ligand cyclo (c-RGD) for selective targeting of integrin on tumor cell surfaces. Using laser illumination at 1064 nm, these particles were used to detect a brain tumor through the sculp and skull at a depth of 3 mm with a signal-to-background as high as 90. The same particles were then used for photothermal therapy and were shown to effectively inhibit tumor growth and prolong survival rates compared to the controls.

Although no photoacoustic imaging has been performed on human brains to date, a few studies have addressed its feasibility. Imaging performed on both new-born rats, and a human neonatal skull phantom has shown the potential feasibility of PAT for imaging the brain of human neonates with thinner skulls [39–41]. Nie et al. performed PAT of an excised canine brain enclosed inside an adult human skull *ex vivo* [42], promoting the application of PAT to adult human brain imaging. To compensate for the scattering loss in the thick skull, they implemented a photon recycler that reflected back-scattered light to the skull. Using this system, PAT was performed with a spatial resolution of ~ 1 mm, and eight blood vessels were identified in the canine brain that was also visible in a photograph.

The development of contrast agents with stronger optical absorption, novel illumination methods, and new strategies to deliver light into the brain, for example through the openings in the skull such as the nasal passage, could pave the way to clinical PAI. Applications in humans could include intraoperative brain imaging (e.g., image-guided brain tumor resection), for example, where the depth of light penetration and the acoustic aberrations due to the skull are not an issue, thus favoring PAI use over other imaging modalities.

3. Thyroid imaging

Thyroid cancer is over diagnosed and over treated. Ultrasonography, in combination with fine needle aspiration cytology (FNAC) followed by histology, is the primary diagnostic tool for thyroid cancer. Although remarkably sensitive, this method does not discriminate between aggressive lesions and subclinical cancers that will not present any problems during the patient's lifetime. Patients with inconclusive FNAC results undergo radical thyroidectomy [43] of the thyroid of which 70–80% prove to be unnecessary as the post-surgical biopsies confirm no malignancy [44–46]. Other clinical imaging techniques [47–49] such as scintigraphy, MRI, CT, and PET/CT also lack the specificity to distinguish between benign and malignant follicular nodules or to identify patients with malignancies that would benefit from surgical treatment. Such imaging limitations lead to a high number of nodule biopsies as well as overdiagnosis and overtreatment. Since the thyroid gland is superficial (2–3 cm deep) and allows sufficient light penetration, PAI is an attractive modality to augment US/FNAC for molecular imaging of thyroid nodules.

Kang et al. have utilized a 5–14 MHz linear array transducer to study thyroid microcalcifications on resected human thyroid nodules [50]. PAI was compared with radiographs that were obtained immediately after surgery, ultrasonography images, visual inspection and histologic analyses of the resected tissue. They tested 36 samples from eight patients and compared specimens with and without calcifications. Their statistical analyses revealed that while ultrasonography images and radiographs showed statistically significant changes in their intensity values, PAI did not. The inability of PAI to identify calcifications, might be attributed to the fact that a single wavelength was used, and the intense blood signals could not be separated entirely from the microcalcifications. Dogra [51] et al. have combined a 2D photoacoustic detector array, a tunable laser system, and a fixed-focus acoustic lens for testing the ability of PAI to differentiate malignant tissue, benign nodules, and healthy human thyroid tissue, on resected human thyroid tissue. Multispectral PAI was performed on 50 *ex vivo* thyroid

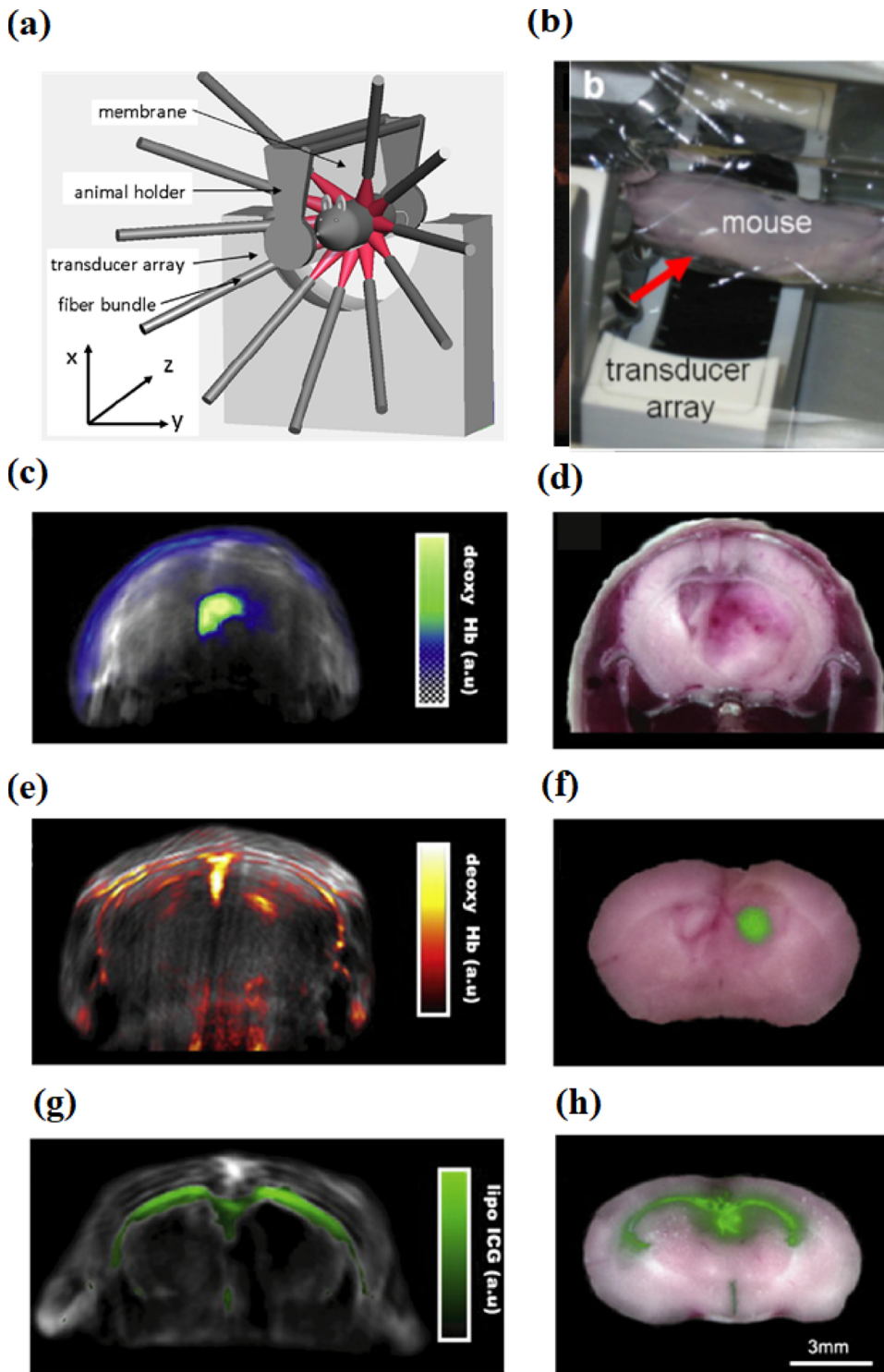


Fig. 2. Multispectral photoacoustic imaging of glioblastoma and liposomal ICG distribution in brain.

(a) Schematic representation of the MSOT system. A curved array of wideband and cylindrically focused ultrasound transducers enables parallel data acquisition. Optical fibers are used to homogeneously illuminate the object. A special animal holder with a transparent plastic membrane is used for animal positioning. (b) A picture of the mouse during a scan, showing the position of the mouse and illumination with respect to the array of focused wideband ultrasound transducers. (c) The spectrally unmixed Hb pseudo color overlay on an 800 nm single wavelength MSOT image from an animal 34 day following implantation with U87 glioblastoma cells (d) The corresponding *ex vivo* cryosection. (e) A Hb image 16 day following implantation following a 10% carbon dioxide challenge. (f) The corresponding *ex vivo* fluorescence image showing tumor size and location. (g) A 900 nm single wavelength MSOT image (grayscale) with an overlay (green) of the spectrally-resolved liposome-ICG signal, from a mouse injected intraventricularly with ICG encapsulated into liposomes. (h) The equivalent cryoslice with an overlay of the fluorescence from the injected particles.

Panels A, B were adapted from: Adrian Taruttis, Eva Herzog, Daniel Razansky, and Vasilis Ntziachristos, "Real-time imaging of cardiovascular dynamics and circulating gold nanorods with multispectral optoacoustic tomography," *Opt. Express* 18, 19592–19602 (2010); <https://doi.org/10.1364/OE.18.019592>

Panels C-H were from NeuroImage, Vol 65, Neal C. Burton, Manishkumar Patel, Stefan Morscher, Wouter H.P. Driessen, Jing Claussen, Nicolas Beziere, Thomas Jetzfellner, Adrian Taruttis, Daniel Razansky, Bohumil Bednar, Vasilis Ntziachristos, Multispectral Opto-acoustic Tomography (MSOT) of the Brain and Glioblastoma Characterization, p.522–528, Copyright (2013), with permission from Elsevier.

specimens, and chromophore images that represented optical absorption of Hb, HbO₂, lipid, and water were reconstructed. Classification of malignant tissue, benign nodules, and normal regions was performed by a pathologist in a blinded manner and compared to the PA images. Results showed a statistically significant difference in the estimated Hb levels between malignant and benign nodules, in the estimated Hb, lipid and water levels between malignant and normal tissue and the estimated Hb levels between benign nodules and healthy tissue. The sensitivity and specificity were found to be 69.2%, 96.9% respectively while positive and negative predictive values were 81.8%, and 93.9%, which demonstrates the potential of PAI for classification of thyroid

nodules. Yang et al. used a clinical photoacoustic/ultrasound device for clinical imaging of human thyroid *in vivo* [52]. The information attained with the color Doppler ultrasound were compared with photoacoustic imaging (1064 nm) and showed that PAI was able to detect more blood vessels than the color Doppler. Levi and coworkers devised a markedly different approach. They developed a clinically applicable photoacoustic imaging agent [53] that can add significant diagnostic value by providing information on the activity of the matrix metalloproteinase-9 and 13 (MMP-9/13) associated with both invasive papillary and follicular thyroid carcinoma [54,55]. The probe was based on a peptide cleavable by MMPs, which was conjugated with two different

chromophores (BHQ3 and Alexa750) and had high PA signals from both chromophores at different wavelengths (675 and 740 nm, respectively) prior to cleavage. However, after cleavage by MMPs, the negatively charged fragment that was conjugated to BHQ3 was washed away and the second fragment, containing a cell penetrating sequence (and conjugated to Alexa 750), was retained inside nearby cells due to its positive charge. Thus, prior to cleaving the PA signal was strong only for the lower wavelength. A differential measurement at the two wavelengths allowed assessing the MMP activity. Optimization of the probe was followed by PA imaging of subcutaneous thyroid cancer xenografts in nude mice [128]. They used two commercial PAI small animal imaging systems: Visual Sonics Vevo LAZR and Endra Life Sciences Nexus 128 commercial preclinical system. Both MMP-9 and MMP-13 were shown to activate the probe. After intratumoral and intravenous injections, both fluorescence imaging and PAI confirmed an increase in the signal from Alexa 750. Moreover, both imaging modalities showed significantly higher signals in tumors of mice injected with the activatable agent than in tumors injected with the control, uncleavable agent. Thus, such an agent can be used during surgical procedures for non-invasive detection of MMPs activities. Finally, Dima et al. have interrogated the application and imaging features obtained by non-invasive and handheld PAI of the thyroid of two healthy human volunteers at a single wavelength (800 nm) [56]. They developed a compact handheld version of the MSOT system described earlier using a detector adapted to the dimensions and geometry of the human neck. The device and relevant anatomy are described in Fig. 3A, B. To assess whether vascular structures could be better resolved using PAI, ultrasound Doppler imaging was also employed and compared to PAI images. The results depicted in Fig. 3C, D showed that large and medium-sized vessels, detected using Doppler ultrasound, were captured by the PAI system at a better resolution (a few-fold higher). In addition, PAI revealed finer vascular networks, which were unconfirmed by the Doppler ultrasound. The authors speculated that this is due to a combination of applied pressure on the neck (that caused a partial blockage) and their chosen Doppler parameters that render the blood flow too weak to be detected.

To conclude, the Thyroid is a superficial organ, accessible by PAI

with a few preliminary clinical systems already demonstrated. Moreover, molecular contrast is very much needed to augment ultrasound imaging and biopsy guidance. Thus, it stands to reason that as the right contrast mechanism is found (either endogenous or in the form of exogenous contrast agent) this application can advance quickly to clinical use and showcase the potential of PAI.

4. Breast imaging

Breast imaging is a promising area for PAI. Breast cancer is the most common cancer in women and a leading cause of cancer-related death worldwide. Current screening methods include X-Ray mammography (XRM) and ultrasound imaging. XRM suffers from a low positive predictive value, exposure to ionizing radiation, and reduced sensitivity in women with dense breast tissue as well as causing extreme discomfort [57]. Ultrasonography results are strongly dependent on the examiner's interpretation and suffer from a high false-positive rate. MRI of the breast has high sensitivity, but low specificity and high cost [58,59]. As a result, there is a strong need for improved breast cancer imaging techniques that can reduce false-positive rates and improve sensitivity [60–62].

PAI is particularly well suited to improve diagnostic imaging of the breast. The tissue of interest is superficially located and mostly within the maximum achievable imaging depth of PAI. Healthy breast tissue has low optical absorption and ultrasound scattering, allowing for highly efficient PAI. In addition, angiogenesis is an important diagnostic and prognostic factor in breast cancer. Since abnormally increased vasculature and hemoglobin at tumor sites produces strong intrinsic photoacoustic contrast, PAI is ideally suited for visualizing angiogenesis [63].

The use of PAI for breast imaging was first proposed in 1994 [19,64]. One of the first instruments used to image patients was the Twente Photoacoustic Mammoscope [65], wherein the patient lies prone with the breast suspended and lightly compressed between a glass plate and a planar US detector array. Using a 1064 nm laser, the PA Mammoscope has been used to image over 100 patients, and initial results demonstrate that it can identify malignancies with no difference

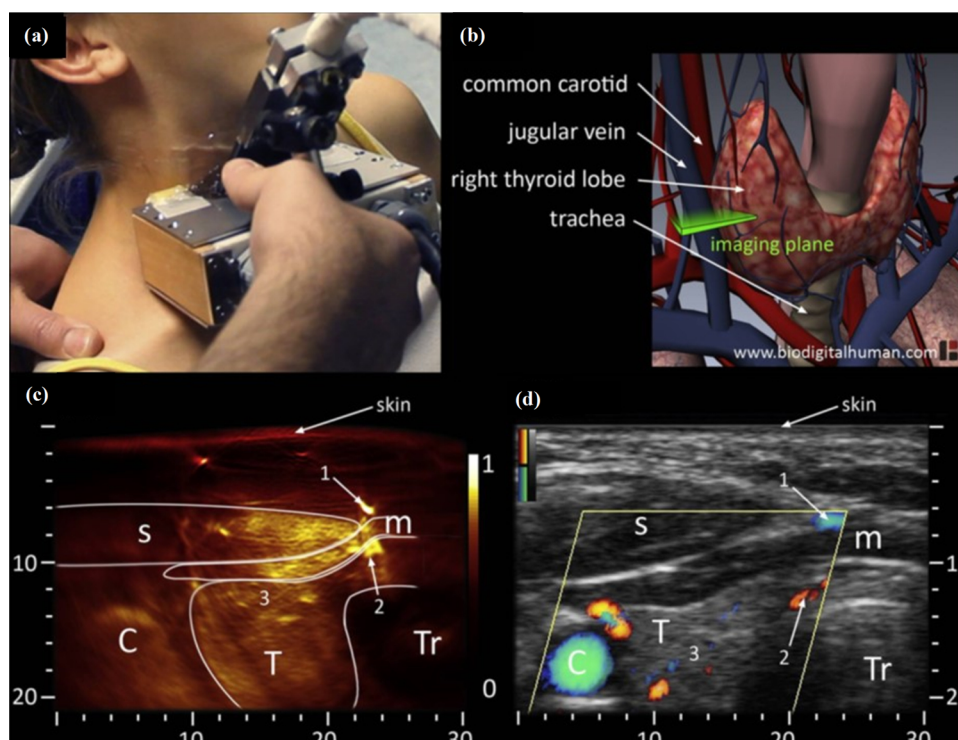


Fig. 3. photoacoustic and ultrasound Doppler thyroid imaging using a hand-held probe.

(a) The curved, hand-held photoacoustic device with wide optical illumination for *in vivo* thyroid imaging. (b) Anatomy of thyroid gland including cardio-vascular and respiratory system; the cross-sectional 2D imaging plane is highlighted in green. (c) PAI cross-section of the left thyroid lobe from a healthy volunteer. The image, leveled and normalized from 0 to 1, shows with high sensitivity vascular features of skin, muscles and within the thyroid lobe. (d) The corresponding ultrasound image in grayscale also depicts Directional Power Doppler signals superimposed in color (red/blue color-maps indicating opposite flow directions). C: Carotid, T: Thyroid, Tr: Trachea, s: sternocleidomastoid muscle, m: infrahyoid muscle; axes in mm.

From: Alexander Dima, Vasilis Ntziachristos, "In-vivo handheld optoacoustic tomography of the human thyroid," Photoacoustics Volume 4, Issue 2, Copyright June 2016, Pages 65–69; <https://doi.org/10.1016/j.pacs.2016.05.003>; <https://creativecommons.org/licenses/by/4.0/>

in the average PA contrast between patients with high- and low-density breast tissue [66,67]. Similarly, Ermilov et al. showed promising results, with the Laser-based Optoacoustic Imaging system (LOIS-64) [68]. For imaging, the patient lies prone with the breast suspended into a hemispherical cup with a 64-element detector. An initial study using a 755 nm laser and 27 patients showed a promising ability to detect malignant and benign lesions. A similar setup was used by Kruger et al. based on a Nexus 128 preclinical PAT system by Endra Life Sciences Inc. to demonstrate imaging feasibility; a single healthy volunteer was imaged with scan times and resolution of 12 s to 3.2 min for scans of 24 mm to 96 mm radius, respectively [69]. A second generation design of this device reduced the center frequency of the ultrasound transducer from 5 to 2 MHz in order to improve depth penetration (trading off spatial resolution) and introduced a spiral detector scan protocol that improved the field of view [70]. It was used to image 4 human volunteers and able to visualize the vasculature throughout a breast volume as large as 1335 mL. Kitai et al. developed a photoacoustic system wherein the patients again lie in a prone position with the breast suspended and compressed between two plates and imaged using four different wavelengths: 756 nm, 797 nm, 825 nm, and 1064 nm. A unique feature of this system is the dual-illumination through both plates. Analyzing images from 26 patients, lesions were correctly identified in 74% of the cases (20 of 27) [20]. Taking advantage of the multispectral images, the mean oxygen saturation (SO_2) was also calculated, and all detected lesions showed lower SO_2 levels as compared to the surrounding and contralateral breast tissues.

More recently, Toi *et al.* used a modified version of the PA mammoscope called PAM-03, (Fig. 4) which uses a hemispherical detector array with two laser wavelengths of 755 nm and 795 nm. In this study of 30 patients, their optimized system was able to resolve very fine vascular structures in the breast, even compared with MRI imaging of the same patient [71]. Additionally, the system can characterize cancer treatment-driven changes in vascular morphology and hemoglobin

saturation. This is described in Fig. 5. A recent study tested the capabilities of MSOT breast imaging on six patients using five wavelengths: 700 nm, 730 nm, 760 nm, 800 nm, and 850 nm. A handheld version of a commercial MOST system called MSOT Acuity Echo was used for this study. This system is marketed by iThera Medical and utilizes a tunable laser, allowing multiple illumination wavelengths in the 680–980 nm range. The study demonstrated reproducible data on tissue composition and physiological properties, potentially enabling differentiation of solid malignant tissue from healthy tissue [72]. Another handheld system utilizing two wavelengths (1064 nm and 757 nm) is the Imagio by Seno Medical Instruments, which integrates a linear ultrasound array. Recently, a large multicenter prospective clinical trial of over two thousand women was completed comparing Breast Imaging Reporting and Data System (BI-RADS) categories assigned using combined PAI and ultrasonography (Imagio) versus ultrasound alone. Using combined PAI and ultrasonography exceeded US specificity by 14.9% with similar sensitivity (US: 96% vs. 98.6%) [73]. Further details of PA breast imaging may be found in a separate article by A. Oraevsky et al. in this same journal issue.

Thus, PAI for breast imaging is quite advanced with a handful of clinical systems developed by multiple groups. As most of the technological barriers have been overcome, we can see a multitude of commercial companies aiming at developing a fully capable clinical system. While light penetration still poses some limitations (especially for larger breast tissue) The challenges now are shifting toward the clinical side - to prove the added clinical value and reduced costs / increased patient compliance over the current standard of care mammography.

5. Dermatologic imaging

Invasive biopsy combined with a histopathologic analysis is still the method of choice for accurately diagnosing many skin diseases since it is the most accessible and superficial organ in the human body.

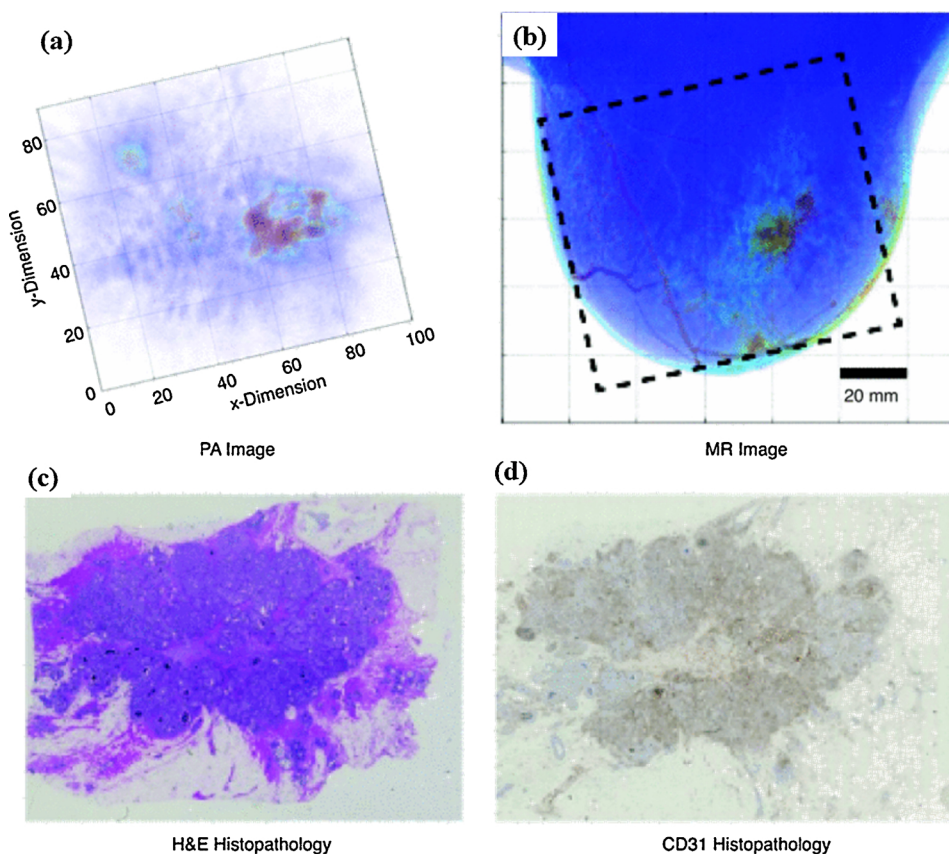


Fig. 4. An example of PA mass appearance seen in a 63-year-old patient (P55) with infiltrating ductal carcinoma (IDC).

The breast lesion was highly suspicious on XRM (not shown) by the presence of an irregularly shaped, unsharply delineated, 20-mm mass. (a) The average intensity projection (AIP) PA image is shown tilted due to the breast being tilted during the PA measurement to position the lesion favorably in front of the detector. In the PA image, the lesion is clearly visible as an irregular, high-contrast, 29-mm mass. The lesion colocalized perfectly with the lesion on XRM (not shown). The lesion also colocalized well with (b) the AIP MR image after tilting the PA image. The dashed box in the MR image indicates the area from which the PA image is acquired. The MR appearance is described as an irregularly shaped mass. (c) A histopathological assessment of the tissue specimen post-surgery revealed the presence of a 34-mm IDC, grade 2. (d) The CD31-stained tumor section shows the microvasculature spread over the entire lesion supporting the mass appearance observed in PA and MR images. It is intriguing that the patterns in 'a'–'d' appear roughly similar in appearance.

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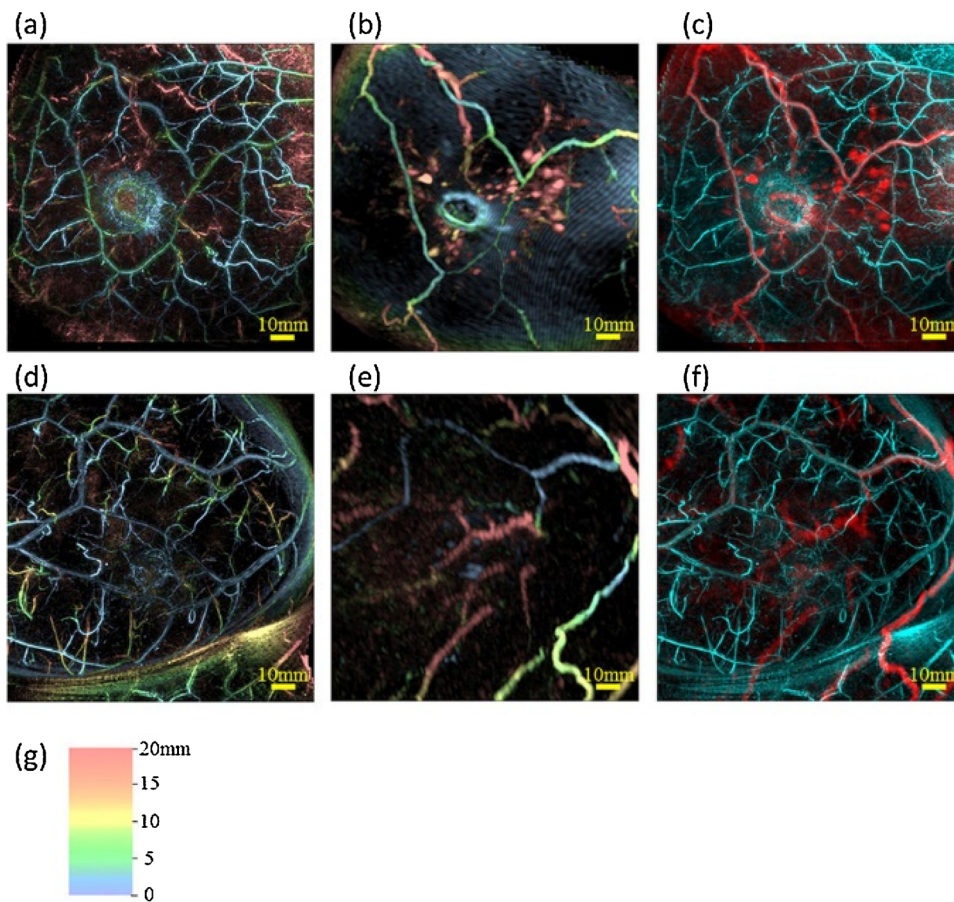


Fig. 5. Comparison of visibilities of blood vessels between PAI and MRI using maximum intensity projection (MIP) images on the healthy breast.

Case 1(a–c): (a) PA image, (b) MR image deformed to correspond to the PA image, and (c) fusion image of PA (cyan) and MR (red). Case 2 (d–f): (d) PA image, (e) MR image deformed to correspond to the PA image, and (f) fusion image of PA (cyan) and MR (red). All images are coronal views. We colored the signals according to the depth using the color chart shown in (g). Images were taken at 755 nm and 795 nm.

From: Toi, M. et al. Visualization of tumor-related blood vessels in human breast by photoacoustic imaging system with a hemispherical detector array. *Sci. Rep.* 7, 41970; doi: 10.1038/srep41970 (2017). <http://creativecommons.org/licenses/by/4.0/>

However, dermatological diseases are highly amenable to non-invasive optical diagnosis. Common dermatologic applications of PAI include the detection of skin cancers, burn depth estimation, diagnosis of psoriasis, and various cosmetic applications. Early detection of melanoma, an aggressive cutaneous cancer, is essential since it is difficult to diagnose yet is responsible for the vast majority of skin cancer deaths [74]. Existing imaging techniques for skin imaging in the clinic, such as optical coherence tomography (OCT) or high-frequency ultrasonography, are either fundamentally limited in their imaging depth (1–2 mm) or lack functional and molecular imaging capabilities [75].

PAI, on the other hand, is a promising modality for diagnosing skin disease due to its greater imaging depth and high resolution. Oh [76] and later Zhang [77], developed a dark-field functional acoustic-resolution photoacoustic microscope (PAM). The PAM achieves impressive performance with an imaging depth of up to 3 mm, an axial resolution of 15 μm , and a lateral resolution of 45 μm . The microscope was used to image subcutaneous melanoma in a xenograft mouse model, with tomographic views clearly showing the melanoma and its surrounding vascular supply. These imaging results were subsequently confirmed by histology. To achieve even a greater penetration depth compared to PAM while maintaining the high resolution, Zhang et al. have developed a photoacoustic tomography (as opposed to photoacoustic microscopy as explained in Fig. 1) combined with OCT [78]. The use of longer wavelength (1050 nm) allowed for improvement in penetration depth as well. They were able to achieve a penetration depth of 5 mm with respective OCT and PAT axial resolutions of 8 and 20 μm and the lateral resolutions of 18 and 50–100 μm . They also demonstrated three-dimensional *in vivo* imaging of the vasculature and the surrounding tissue micro-morphology in murine and healthy human volunteer's skin. Their study emphasized the complementary contrast and tissue information provided by each modality. The PAM system was also used for imaging angiogenesis and measuring the SO_2 of single

small blood vessels (on both the arterial as well as the venous sides) in rats, as well as total hemoglobin concentration in humans. Favazza et al. have imaged healthy human volunteers using a similar system [79] and showed functional images of the cutaneous microvasculature and a melanocytic nevus at depths of up to 2 mm. After excision of the nevus, multiple anatomical features that had been identified by PAI were confirmed by histologic examination. Staly et al. have used a similar system for tracking the growth of melanoma brain metastases noninvasively in a mouse model [80]. PAI was performed with an intact skull five days after tumor cell injection. The PA signal intensity from tumors was 15–30 dB greater than the background, which allowed tumor sizes to be tracked throughout the disease course. Importantly, PAI measurements of tumor size agreed with subsequent autopsy findings. Yao and colleagues have developed an optical-resolution photoacoustic microscope system for accurately measuring the metabolic rate of oxygen (MRO_2) in the superficial layers of skin in a nude mouse ear [81]. They used wavelengths of 584, 590, and 605 nm to measure SO_2 , total hemoglobin, and vessel diameter, as well as blood flow velocity and direction (using a broadband photoacoustic Doppler). They have shown a 300% increase in flow rate and a 40% increase in MRO_2 in a subcutaneous melanoma tumor model. Finally, Zhou et al. have developed a handheld version of the PAM system and successfully detected ~4 mm-thick melanomas in phantoms and *in vivo* in a mouse model with xenograft tumors [82]. Phantoms were made of gelatin and intralipid mixture with a high scattering coefficient to mimic real tissue. To mimic a melanoma, black ink and gelatin mixture, which had an absorption coefficient of 70 cm^{-1} at 650 nm, close to the real melanoma absorption coefficient, was used.

Such systems are highly translatable for clinical use in melanoma diagnosis, prognosis, and surgical planning. A different approach was taken by Zhang et al. [83], who used a Fabry-Pérot polymer film ultrasound sensor to image the superficial vasculature. Imaging the palm

of the hand in a single healthy volunteer as well as a xenograft mouse model with a subcutaneous colorectal tumor, the investigators achieved imaging depths of up to 5 mm with sub-100 μm spatial resolution. Burn injuries are complex traumatic events with various local and systemic effects [84]. They are characterized by an initial shock phase, which involves rapid vasodilation, leakage of fluids outside of the intravascular space and to the release of toxic metabolites as well as antigens and immunomodulatory agents. Later, the tissue response shifts to a hyperdynamic and hypermetabolic state in which increased oxygen consumption, carbon dioxide, and glucose production are experienced as the body strive to repair the tissue. Accurate estimation of the extent and depth of injury are the two most important parameters for the clinical evaluation of burns. Moreover, continuous, real-time monitoring of hemodynamics, especially of the peripheral circulation, is required for proper treatment and control of infections. PAI is ideally suited to this task, as it allows adequate imaging depth as well as intrinsic molecular contrast for imaging hemodynamics and assessing burn depth. Yamazaki et al. used a single element, ring-shaped PVDF/TRFE (polyvinylidene fluoride/trifluoroethylene) transducer to image superficial dermal burns (SDB, 0.07 to 0.12 mm deep), deep dermal burns (DDB, 0.12 to 2 mm deep), or deep burns (DB, depth over 2 mm) afflicted on the dorsal area in rats [85]. They tested various wavelengths and showed that a spectral range of 532–580 nm is particularly well-suited to assessing burn depth. Aizawa and co-workers have extensively investigated the behavior of the PA signal from burns over time and at different wavelengths [86]. Using the same PAI system and animal model, they showed that changes in the PA signal over time reflect *in vivo* changes from the shock phase to the hyperdynamic state. Hirao et al. have used antibacterial photodynamic therapy to control infections at the burn sites [87]. Again, the same PAI system and animal model were used, but in this case, the aim was to monitor photosensitizer distribution in burned skin. The therapeutic agents, hydrophilic Methylene Blue Dye (MBD) or hydrophobic porphyrin sodium solution, absorb strongly at NIR wavelengths, facilitating their high-contrast separation from endogenous tissue chromophores by subtracting a second PA image acquired at 532 nm. The distribution of PA signals was confirmed to coincide well with the distribution of photosensitizer, as measured by fluorescence in tissue biopsied following PA measurements. A comparison of the temporal behavior of the two agents showed that the hydrophobic agents clear from the burn faster and thus require more precise timing of the irradiation. Zhang et al. have used the PAM system described earlier to image burns in a porcine model. A region of skin was burned by cautery and excised, after which PAI was used to estimate burn depth and compared with histology which showed good correlation.

Finally, a dermatologic application of recent interest is the diagnosis and monitoring of psoriasis, a chronic inflammatory skin disease. Despite extensive research efforts, many aspects of psoriasis pathogenesis have yet to be elucidated. Moreover, the lack of biomarkers or objective methods to phenotype heterogeneous presentations of the disease create challenges for prognostication and individualized therapy. Aguirre [88] and co-workers have recently used a handheld raster-scan optoacoustic mesoscopy (RSOM) system that implements ultra-broadband (10–180 MHz) ultrasonic detection and can achieve precision assessment of label-free psoriasis biomarkers [89]. The system is described in Fig. 6a–b. It is capable of imaging epithelial thickness, mean vessel diameter, and inflammation markers. Their system achieved axial and lateral resolutions of 4.5 μm and 18.4 μm respectively with an imaging depth of 1.5 mm and a large FOV of 8 mm by 2 mm. Following the work of Omar [90], the PA signal was separated into two frequency bands. These are shown separately and combined in Fig. 6c–e. The smaller features (which produce lower PA signal), such as microvasculature, were mapped to the higher frequency range, while bulkier structures (such as large blood vessels) were mapped to the lower frequency range. Separating the reconstruction by frequency, followed by equalization of the intensities, allowed rendering of fine

spatial details together with lower-resolution structures. The authors thus demonstrated visualization of skin morphology and vascular patterns in the dermis and sub-dermis of psoriasis patients, as well as quantification and clinical scoring of inflammation and other biomarkers of psoriasis without the need for contrast agents. An example of these features is shown in Fig. 6g–i.

As the skin is potentially the most accessible organ for optical and acoustic imaging, it is clear that PAI can be used for clinical dermatological imaging. However, while the technological barriers are perhaps lower here, a single “killer-application” where PAI has a distinctive advantage over all other technologies was yet to be identified. The requirement of physical tissue contact, as opposed to other “purely optical” methods, poses some limitations. If such an application can be found, we predict that such technology can advance quickly into the clinic.

6. Intraoperative imaging

Proper patient selection prior to surgery and achievement of complete tumor clearance after surgical resection is crucial to providing optimal care for the individual patient. Patients with suspected locoregional or distant metastases should be identified during pre-operative planning and prior to potential surgery be treated with systemic neoadjuvant treatment. However, with current imaging modalities, the identification of very small lesions (< 5 mm) can be challenging or even impossible. As a result, patients may undergo surgery with little to no oncologic benefit, but with a high risk of an inferior quality of life. While a significant predictor of long-term survival in surgical patients is tumor-free resection margins, margin-positive resections are a frequent phenomenon, creating significant challenges for peri-operative decision-making. Tumor-specific molecular imaging can potentially provide crucial information to the surgeon in the above-described situations and enhance the surgeon's ability to visualize tumors intraoperatively. Together with tumor-specific fluorescence imaging, PAI can likely improve clinical decision-making during surgery and potentially decrease the rate of margin-positive resections.

Intraoperatively, PAI offers several benefits that could lead to improved surgical treatments and outcomes. The strengths of PAI include its clinically relevant depth of penetration compared to other intraoperative molecular imaging techniques, such as fluorescence imaging [91], and its potential to provide imaging in real-time. Thus, PAI could help delineate tumor boundaries and tumor infiltration in real-time in order to define resection margins and can help assess whether the residual tumor has been left behind after resection. In addition, PAI could play an essential role in the intraoperative assessment of metastases and lymph node status, as demonstrated in a recent study on pancreatic cancer patients utilizing a targeted fluorescent /PAI probe [92]. Moreover, PAI could be used in the future to diminish iatrogenic injury during surgery by improving visualization of nerves, ureters, and vessels.

Photoacoustic imaging is ideal for surgical navigation and biopsy guidance. Several groups have performed phantom studies to assess the feasibility of using acoustic-resolution photoacoustic imaging to accurately locate and identify features of interest, such as blood vessels, nerves, and tendons. These phantom studies mimicked endonasal endoscopic neurosurgeries near the internal carotid arteries [93], administration of local nerve blocks without causing intraneural damage [94], and teleoperated surgeries using the da Vinci robot [95]. In these approaches, an optical fiber (attached or adjacent to the surgical tool) was used to deliver light into the region of interest while a standard ultrasound probe was placed distally. Both ultrasound and photoacoustic images were reconstructed with either conventional delay-and-sum beamforming or the short lag spatial coherence (SLSC) technique.

Some preclinical studies have also utilized PAI for surgical guidance. For example, one group utilized a PA probe consisting of optical fibers integrated on top of a medical ultrasound transducer [96], or a

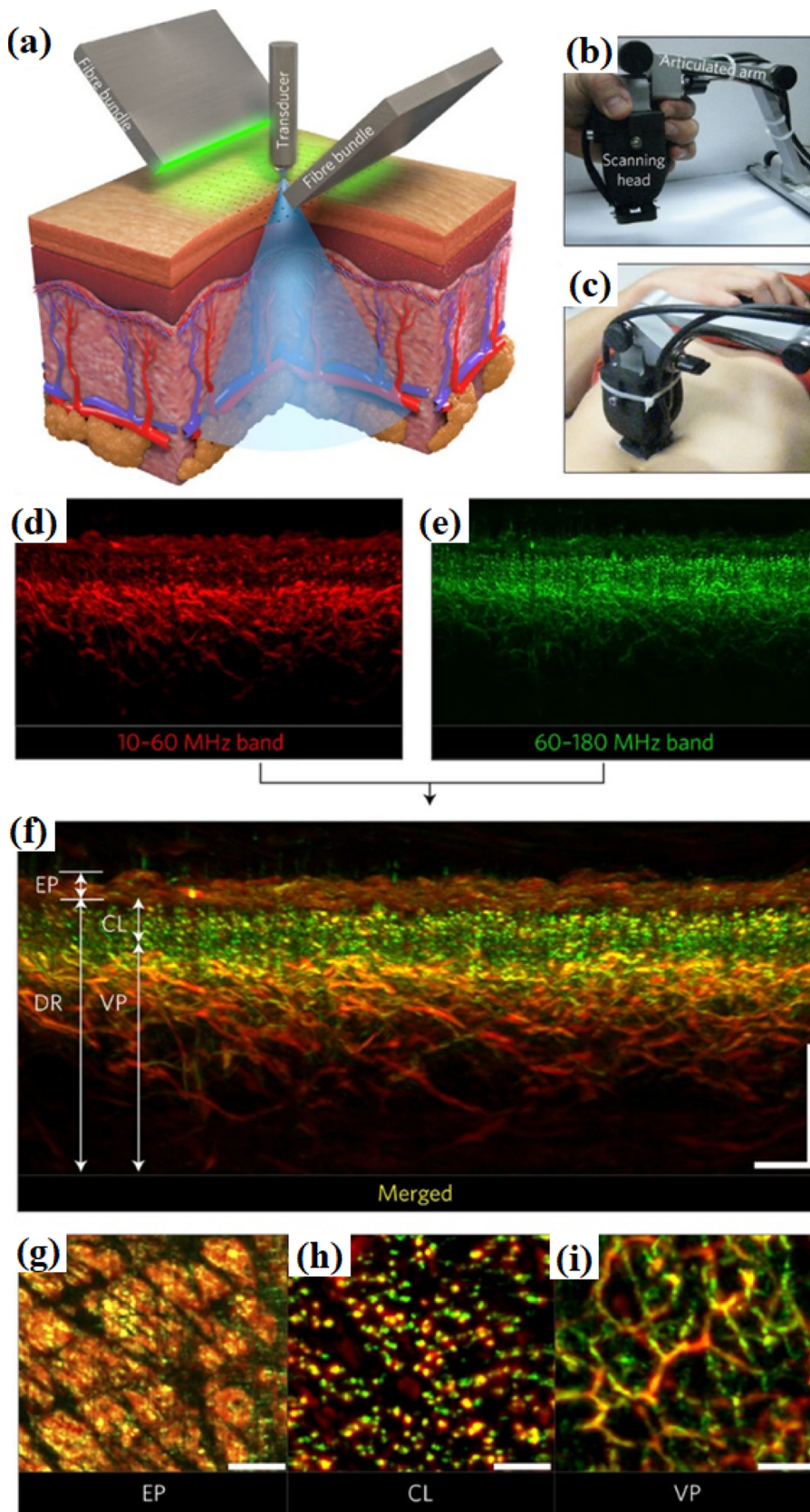


Fig. 6. PA Imaging of the psoriasis biomarkers in the skin.

(a), Schematic of the operation of UB-RSOM. The transducer is raster-scanned parallel to the skin surface, acquiring acoustic signals (A lines) that are generated by laser illumination from two fixed fiber bundles. The focal point of the transducer is kept above the skin surface. (b), Photograph of the scanning head and the articulated arm. (c), Photograph of the scanning head in position, ready for data acquisition. (d,e), The collected optoacoustic signals are filtered in two frequency bands and reconstructed into two images shown in red and green, representing low and high spatial frequencies, respectively. (f), UB-RSOM image of healthy skin that combines low and high spatial frequencies in (D,E) in a single color-coded image. Different biological structures appear in red or green depending on their spatial frequency. The epidermis (EP) appeared mostly in red, and could be clearly distinguished from the dermis (DR). In the dermal vasculature, the capillary loops (CL) could be distinguished from the epidermis and from the vascular plexus (VP) in the deep dermis. The larger vessels appeared mainly in red, whereas the smaller vessels appeared mainly in green. (g–i), Maximum intensity projections in the coronal direction of the epidermis, showing the indentations of the skin (g), the capillary loop layer (h) and the dermis (i). All scale bars, 500 μm .

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device combining ultrasound, PA, and fluorescence imaging [97]. These devices were used to assess the viability of different tissues and to image cancer in the axillary lymph nodes. The ability of these devices to accurately guide surgery and biopsies to relevant disease foci and to

monitor the local delivery of photoacoustic agents has been demonstrated in animal models. Another study demonstrated the use of a virtual intraoperative surgical photoacoustic microscope [98] for guided needle insertion and retraction. While the device achieves

superior resolution, the small field-of-view and penetration depth could limit its clinical use.

Like other imaging modalities, PAI provides limited molecular information in the absence of targeted contrast agents. However, tumor delineation requires a dedicated contrast agent. Fluorophores are often dually used as photoacoustic agents, of which biocompatible, highly-absorbing NIR dyes are preferred. While only a handful of dyes are currently approved for clinical use, many have been tested preclinically and are expected to receive clinical approval over the next few years. One example is Trastuzumab® labeled with black hole quencher 3 or fluorescein, which has been tested by both photoacoustic and fluorescence imaging to evaluate HER2 overexpression in breast cancer diagnosis, assess margins after tumor resection, and guide surgery [99]. Another example is a gastrin-releasing peptide receptor-targeted photoacoustic agent for prostate cancer imaging [100]. *In vivo* results on mouse models with both agents have shown high resolution and penetration depth, and the ability to provide tomographic views of cancerous lesions, demonstrating the advantages of PA over fluorescence. Finally, Tummers et al. have recently addressed the operative management of pancreatic ductal adenocarcinoma (PDAC) by using EGFR-specific cetuximab-IRDye800 as a targeted dual fluorescence and PA agent [101]. The authors have conducted a first-in-human multi-modality guided surgery on a small cohort of patients undergoing surgical resection for pancreatic cancer. They have successfully shown *ex-vivo* (i.e., after resection) that fluorescence and PA signals were 4-fold higher in the tumors compared to surrounding normal tissues during surgery (examples shown in Fig. 7). Moreover, no adverse events were seen after intravenous injection of cetuximab-IRDye800, thus demonstrating the safety and feasibility of this agent in multimodality molecular imaging of primary PDAC and its metastases.

In the authors' opinion surgical navigation might be the single most fertile and relevant application for PAI as A) There is no need for substantial imaging depth. B) Many other conventional methods of clinical molecular imaging such as MRI or PET are many infeasible for such a task, and thus molecular contrast is much lacking. C) Most of these procedures are currently being performed under ultrasound guidance. Augmenting ultrasound with PAI can provide the surgeon with molecular contrast with minimal interference to the current clinical workflow. D) PAI collects data from both exogenous and endogenous chromophores. Thus, it cannot only guide the surgeon toward a target to be resected but also away from crucial blood vessels and nerves to be protected.

7. Imaging of the lymphatic system

Sentinel lymph nodes (SLNs) are regional lymph nodes that are most likely to drain metastasizing cancer cells [102]. Thus, selective biopsy of relevant SLNs in the regional basin is an effective way to assess the spread of breast cancer/melanoma and has become the standard of care [103]. Unfortunately, Sentinel lymph node biopsy (SLNB), while far less invasive than lymph node dissection, still requires the use of radioactive tracers as well as surgery. Thus, a major challenge in the field is to assess clinically non-palpable SLNs using a non-invasive method. Thus, several groups have suggested guiding SLNB with PAI, which can potentially overcome the need for any surgical procedure.

Song and coworkers have suggested a non-invasive photoacoustic SLN identification system using MBD injection in a rat model [106]. Using the PAM system described earlier, they were able to successfully image SLNs following intradermal MBD injection with high optical contrast (> 40 dB) and resolution ($500\ \mu\text{m}$) *in vivo* at greater than 2 cm depth. They have also developed NIR gold nanocages as a new class of PA imaging agents for SLN mapping [107]. Compared to MBD, gold nanocages can easily be bio-conjugated with antibodies that target specific receptors, potentially obviating invasive axillary staging procedures in addition to providing noninvasive SLN mapping. Their gold nanocages have a peak optical absorption at 735 nm, enabling imaging

deep within the body. Moreover, the particles drain rapidly into lymphatic channels and tend to concentrate within the lymph nodes, thus generating a strong PA signal. The authors were thus able to achieve a clear PA image of the SLNs of rats using the PAM system at a depth of up to 3.3 cm. Similarly, Kim et al. have developed gold-plated carbon nanotubes (GNTs) for multimodal PAI and photothermal high-contrast molecular agents [104]. These particles have a 100-fold higher absorption than traditional single-wall carbon nanotubes, and preliminary cultured cell viability tests show that GNTs have minimal toxicity. Antibody-conjugated GNTs were used to map the lymphatic endothelial receptor in healthy nude mice. In a different study, Song et al. have demonstrated the feasibility of high-speed 3D PA for imaging the uptake and clearance dynamics of Evans blue dye in SLNs [105]. Their system was capable of generating 2D imaging cross-sections at 50 frames per second, or a full 3D scan every 5 s with 200 mm axial resolution. Chulhong et al. used ICG for noninvasive *in vivo* mapping of SLNs and lymphatic vessels in healthy rats [1] by both volumetric spectroscopic PAI (with the PAM system) and planar fluorescence imaging. A cooled charge-coupled device (CCD) camera equipped with a bandpass fluorescence filter was used to capture fluorescence images before and after intradermal injection of 1 mM ICG over more than 3 h. Both 618- and 668-nm optical wavelengths were used for spectroscopic PA imaging. The authors showed that ICG was cleared from the lymph nodes at a much slower rate compared to clearance from the lymphatic vessels. They also demonstrated that the two methods are complementary: while fluorescence imaging has a good temporal resolution, it lacks spatial resolution, whereas PAI is slow but has a high spatial resolution. The combination of the two modalities together with FDA-approved ICG has the potential to assist in mapping SLNs for axillary staging and in evaluating tumor metastases in patients. To facilitate clinical translation, the same group developed handheld PAI probes to guide SLN needle biopsies [106]. They used a linear ultrasound array (L8-4, Philips Healthcare) with a bifurcated multimode fiber bundle to guide the laser into the tissue. The end of the fiber bundle was split into two parts, which were mounted on both sides of the transducer, enabling handheld scanning. Again, the SLN was imaged before and after ICG injection; however, to test the clinical feasibility of the technique, the imaging depth was intentionally increased by placing a 2-cm thick slab of chicken breast tissue on top of the rat. Images acquired at 10 min post-injection showed that ICG accumulation enhanced the PA signal in the SLN by 9.5 ± 2.7 fold ($\pm$ standard deviation). The needle progression was also detected by PA and with 7-fold higher contrast without ICG, suggesting that PAI can be used for SLN biopsy guidance. In a different study, the same system was also tested on phantoms and rats [107]. In phantoms, MBD (~ 30 mM) were detected at a depth of 5.2 cm with an SNR of 7.5. In rats, structures with bound MBD were visible at a depth of up to 4.2 cm. Following the MBD injection, the SLN was visible (contrast-to-background ratio of 18.2) as was the injection needle. The same system was modified by Erpelding [108] to provide access to raw photoacoustic data (i.e., the recorded voltage traces per channel) while retaining the imaging capabilities of the commercial ultrasound scanner. While standard ultrasound systems beamform the data to generate B-mode ultrasound images (which is not ideal for PA reconstruction), access to the raw data allowed the researchers to apply dedicated PA reconstruction algorithms, boosting performance. Imaging the SLNs of rats, again with a slab of chicken breast placed on top, and injecting with MBD contrast, they achieved an average contrast-to-background ratio of 76. More recently, the same system with dual wavelength excitation was demonstrated on newly diagnosed stage I-III breast cancer patients with negative axillae [109] as was determined by ultrasound. MBD (5 mL, 2 mg/mL) was injected subcutaneously into the same breast quadrant as the primary tumor and PAI was performed 5 min post-injection. The process and device are described in Fig. 8a-e. PAI of the SLN and a needle are shown in Fig. 8f. Spectral subtraction of PAI at excitation wavelengths of 650 nm (Fig. 8g) and 1064 nm (Fig. 8h) allowed rejection of the blood vessel,

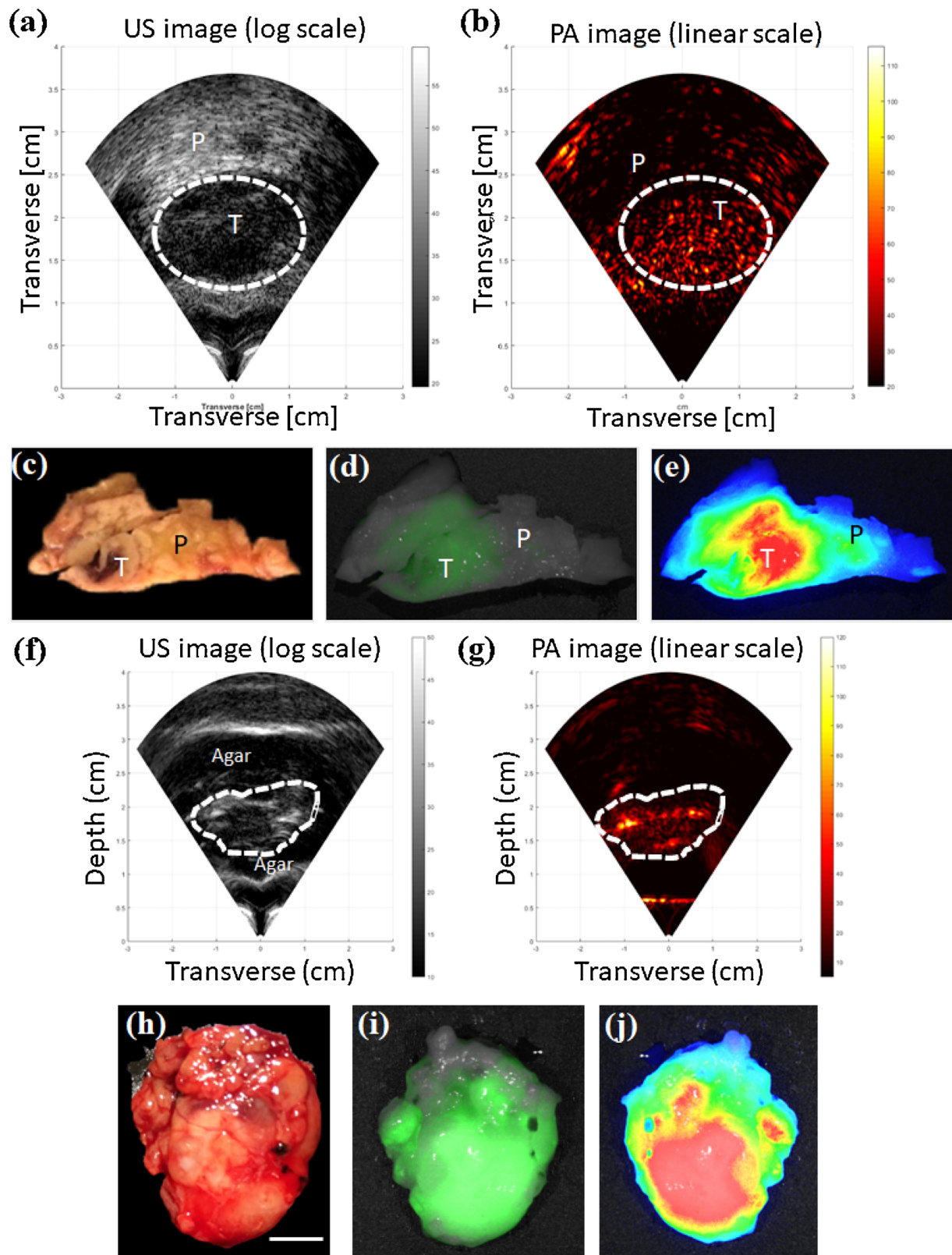


Fig. 7. Photoacoustic imaging with corresponding fluorescence images of resected pancreatic cancer.

(A) Bright field of primary pancreatic tumor in breadloaf section, (B) fluorescence overlay (C) heat-map fluorescent. (D) Corresponding B-mode ultrasound image of hypoechoic tumor, surrounded by white dotted line, (E) and corresponding photoacoustic image. (F). B-mode ultrasound (left) and Photoacoustic images (right) of tumor-positive lymph node surrounded by white dotted line. (G) Corresponding bright field (H), fluorescence overlay, (I), and heat-map fluorescent images of tumor-positive lymph node. Corresponding Scale bar represents 1 cm. T = tumor, P = normal pancreatic tissue.

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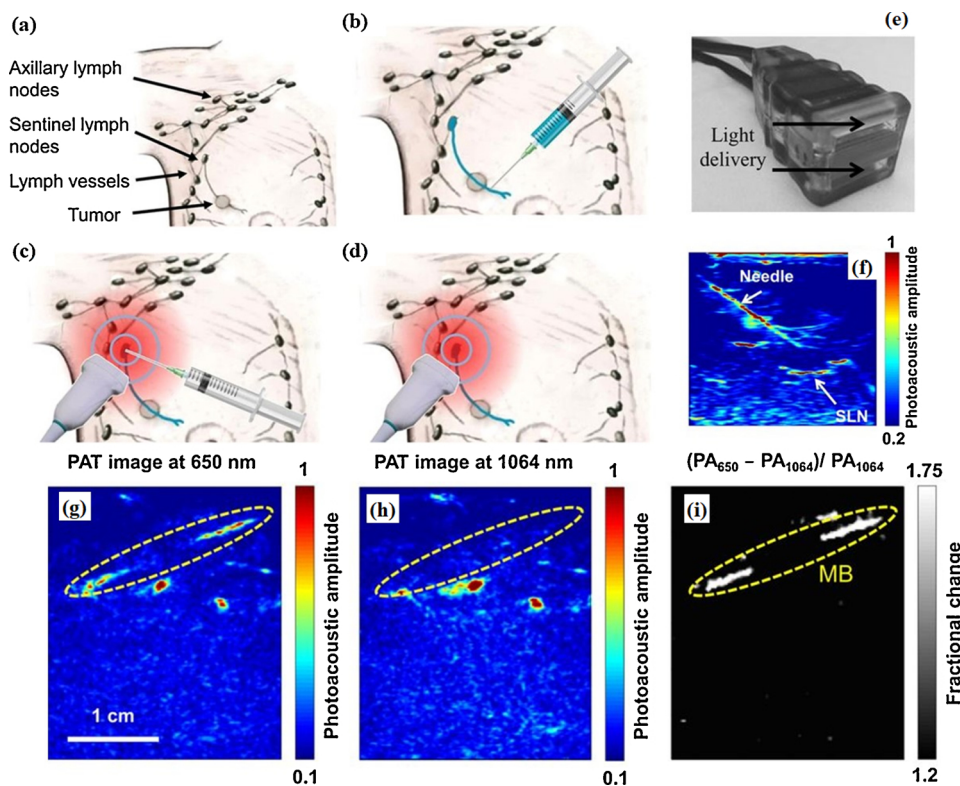


Fig. 8. Photoacoustic-US imaging approach for minimally invasive SLN staging.

(a) Anatomy showing the relevant structures. (b) Periareolar injection of methylene blue dye, which is collected by lymphatic vessels and drained to the sentinel lymph node. (c) PAT-US imaging pinpoints the SLN. (d) PAT-US imaging guides a FNAB of the SLN with high contrast. (e) The proposed probe features a flat surface for light delivery, and the fiber bundles are integrated into the ultrasonic transducer housing for ergonomic handling. (f) *In vivo* PAT image of the SLN and needle. (g) PAT image acquired at 650 nm showing both methylene blue dye in lymphatic vessels and hemoglobin in blood vessels. (h) PAT image acquired at 1064 nm showing primarily hemoglobin in blood vessels while suppressing the signals from methylene blue dye. (i) Image displaying the fractional change (650 nm signal relative to 1064 nm signal) convincingly highlights the locations of methylene blue dye. PA₆₅₀ and PA₁₀₆₄: photoacoustic amplitudes measured at 650 nm and 1064 nm, respectively.

From: Garcia-Urbe, A. et al. Dual-Modality Photoacoustic and Ultrasound Imaging System for Noninvasive Sentinel Lymph Node Detection in Patients with Breast Cancer. *Sci. Rep.* 5, Article number: 15748 (2015); <https://doi.org/10.1038/srep15748>. <http://creativecommons.org/licenses/by/4.0/>

and thus more robust detection of the MBD labeled SLN as shown in Fig. 8i. Finally, Akers et al. have developed a method to validate SLN images acquired by PAI [110]. They injected MBD radiolabeled with [125]I in a rat model for multimodal SLN mapping by PAI and single-photon emission computed tomography (SPECT). Their dual optical/nuclear contrast agent can be used to confirm the location of SLNs detected by noninvasive PAI in deep tissue, enabling pre-clinical and clinical validation of PAI studies with the potential to facilitate clinical translation of PAT.

Beyond applications for *in vivo* imaging of SLNs, there are some opportunities for PAI in the *ex vivo* assessment of SLNs. While histopathological and immunohistochemical assessment of excised SLNs is an accurate predictor of disease in the nodal basin, the turnaround time for results can take days to a week. If metastases are detected, the patient must then return for radical lymphadenectomy. However, if PAI could provide a fast and accurate assessment of resected lymph nodes, the decision whether to perform further lymphadenectomy can be made in the same surgical session, avoiding the need for patient recall and additional patient discomfort, morbidity, and healthcare costs. Grootendorst et al. used a tomographic PA setup with top illumination to study resected SLNs from six patients [111]. Results correlated well with histopathology and showed that multiple areas containing melanoma cells could be identified in the PA images based on their higher PA response. In all three malignant nodes, an increased PA response in the images could be correlated to the presence of melanoma cells verified by histopathology.

Luke and co-workers developed a molecularly activated plasmonic nanosensor for ultrasound-guided spectroscopic photoacoustic imaging [112]. The nanosensor imaging agent was engineered to target the epidermal growth factor receptor. Upon interaction with the target, the agent's optical absorption spectrum is shifted to the red and NIR regions, allowing spectral separation from the unbound agent and background tissue. The authors used a commercial small animal imaging PAI system - the Visual Sonics Vevo LAZR with a 40 MHz transducer and multiple NIR wavelengths. Using a murine xenograft model of oral squamous cell carcinoma that was given sufficient time to metastasize,

they were able to achieve centimeter-depth imaging of lymph micro-metastases in the lymph nodes as small as 50 μm with high sensitivity and specificity. Recently, Stoffels et al. initiated a study to detect melanin in the SLNs of patients with metastatic melanoma using the MSOT system described earlier [113]. First, in a melanin-embedded agarose phantom, the authors could detect four melanoma cells in a volume of 0.02 μl . Then, in 506 SLNs excised from 214 melanoma patients, MSOT achieved a sensitivity and specificity of 100% and 62.3%, respectively, and a false negative and false positive rate of 0% and 37.7%, respectively. Finally, the authors conducted an *in vivo* study of 20 patients using MSOT with ICG injections to detect melanin in 41 SLNs. The ICG was first to use for detecting the SLNs with depths up to 50 mm, and then the melanin spectral signature was used to determine if the SLN was metastatic or not. Here, the sensitivity and specificity were 100% and 48.6%, respectively, and the false negative and false positive rates were 0% and 51.4%, respectively.

Thus, PAI holds a great promise for both *in vivo* imaging and *ex vivo* analysis of SLNs in cancer patients. Again, we emphasize the relevance of surgical guidance. We expect that as commercial clinical devices will become more and more available and affordable, PAI that enjoys greater accuracy and greater safety may replace the nuclear imaging methods which are currently used. However, it must show a low-enough false positive rate to be a favorable alternative to the standard of care.

8. Gynecological and urologic imaging

Both the male and female reproductive and urological systems present attractive clinical opportunities for PAI since the illumination, and acoustic detection can be positioned in relative proximity to the target organ using an endocavity device. Of specific interest is prostate imaging. In the standard screening approach for prostate cancer, an abnormal digital rectal exam or elevated prostate-specific antigen level in the blood is followed by a transrectal ultrasound-guided prostate biopsy wherein needles are used to core random areas of the prostate [114]. This approach leads to over detection of indolent tumors that

have little clinical significance and under-detection of aggressive cancers. Some clinicians have recently adopted an MRI guided ultrasound biopsy approach (UroNav). First, patients undergo an MRI scan which is later loaded into the ultrasound machine to create an MRI overlay during the scan. Such an approach, when performed successfully, seems to improve the positive predictive values of transrectal ultrasound-guided prostate biopsy. However, the co-registration of those two modalities taken at different time points is challenging and not always possible.

To that end, several groups have developed promising pre-clinical systems. Kothapalli et al. have developed a Capacitive Micromachined Ultrasound Transducers (CMUTs) capable of deep tissue photoacoustic imaging [115,116]. They have shown on a chicken breast phantom, the capabilities of the CMUT array to detect and image structure up to 5.3 cm deep thus make those suitable for prostate and breast cancer imaging.

Other notable studies have focused on *ex vivo* canine prostate tissue models. Wang [117] et al. have tested a broad illumination scheme to test PA signals *ex vivo* in pork tissue-covered canine prostates. Yaseen et al. [118] have developed a system based on a fiber-coupled, Q-switched laser combined with a commercial endocavity transducer, while Or-aevsky and co-workers [119] have tested a convex wideband PVDF transducer and developed a maximum angular amplitude probability algorithm to negate the photoacoustic reconstruction artifacts. These studies have demonstrated the ability to visualize small-vascularized tumors, which were not visible in ultrasound images. An interesting study by Kumon [120] et al. analyzed the PA signals from a murine model of subcutaneous prostate cancer and was able to differentiate cancerous from healthy tissue based on spectral signatures. Dogra et al. [121] have used the 2D array system described earlier (Section 3- thyroid imaging) to study surgically excised prostates from 30 patients undergoing prostatectomy for biopsy-confirmed prostate cancer. Their results showed a statistically significant increase in mean Hb content and a significant decrease in lipid content in malignant prostate cancer tissues, compared to healthy prostate tissue. Bell [122] et al. have developed an integrated transrectal probe for translational ultrasound/photoacoustic imaging. These probes can be used *in vivo* to image the prostate during surgery or to guide the administration of therapy. Recently, Horiguchi et al. [123] developed the first combined ultrasonic/photoacoustic device for clinical use and tested it in a pilot study on seven patients to improve real-time visualization of the neurovascular bundle during radical prostatectomy. Preliminary phantom results were used for characterizing the device. Patient results have shown the ability of PAI to differentiate microvascular complexes and adjacent nerves from surrounding tissue intraoperatively, thus improve surgical outcomes. Later, a similar device was used to investigate the PAI of microvasculature in prostate cancer patients [124]. They compared the PAI intensity in each region before radical prostatectomy to the total vascular area (TVA), and total vascular length (TVL) assessed by CD34-immunostaining after resection. Their findings showed a high correlation between the TVA, TVL and the PAI intensity. Thus, they concluded that the intensity of PAI signals might reflect the microvasculature in normal prostatic tissues and index tumors and that PAI could be a novel modality for imaging prostate cancer angiogenesis. Finally, Kothapalli et al. recently demonstrated a dual-modality transrectal ultrasound and photoacoustic imaging system that combines the features of anatomical transrectal ultrasound with functional and molecular specific PAI to improve prostate cancer detection [125]. Their study included developing a CMUT based dual ultrasound and photoacoustic device (Fig. 9a), validation experiments in tissue-mimicking phantoms and *ex vivo* human prostates, pre-clinical *in vivo* experiments on mouse models of prostate cancer, and *in vivo* pilot clinical translational studies on prostate cancer patients (N = 20) without and with the intravenous injection of ICG. These clinical results demonstrated that the addition of photoacoustic imaging technology provides complementary vascular contrast (due to hemoglobin absorption) and enhancement of

intraprostatic photoacoustic contrast from ICG, including from the tumor region.

Other works have focused on PAI for placement of brachytherapy seeds that deliver localized radiotherapy. Accurate positioning of these metal implants is crucial to effective treatment; however, due to their small size, they are often missed in the ultrasound image. PAI, which relies on optical contrast rather than acoustic reflection, can image these seeds with high accuracy. Su and colleagues [126] used a standard linear array ultrasound imaging transducer coupled with optical fibers to experimentally evaluate PAI for visualization of 21 G and 30 G needles in excised porcine tissue. They showed that while the ultrasound signals decayed as the seed's angle became more perpendicular to the probe, the PA signal did not. Similarly, Harrison et al. [127] have tested the effects of various wavelengths using brachytherapy seeds in a chicken breast phantom. They found that PAI at 1064 nm allowed for better identification of the seeds at a depth of up to 5 cm. Kuo et al. [128] investigated the possibility of dose planning using a commercial ultrasound transducer in an *ex vivo* canine prostate with side illumination. Bell et al. [129,130] investigated the use of the SLSC method for imaging brachytherapy seeds. Because the seeds are small (especially in the transverse dimension), the resultant acoustic waves are coherent, and this technique thus affords a better signal-to-background ratio compared to delay and sum. Subsequently, the same group used transurethral illumination [58] for better visualization of the seeds. More recently, Singh et al. [131] have used US images to cancel out artifacts in PA images.

Another area of interest is the urinary bladder. Urethrocystoscopy is often not sensitive enough to discriminate between carcinoma *in situ* and inflammation. To that end, optical techniques may be utilized for added molecular data that can assist the clinicians. Several works have utilized PAI for this task because it can provide rich optical contrast. Kamaya et al.¹³² combined B-mode ultrasound imaging and PAI on different bladder phantoms and showed that PAI can complement diagnostic information obtained by cystoscopy and urine cytologic analysis, and could potentially obviate the need for biopsy in some tumors before definitive treatment. Xie et al. [133] have developed a miniaturized PAI microscope for imaging the microvasculature of *ex vivo* canine bladders. Their device holds promise for differentiating malignant tumors from healthy tissues, by assessing morphometric characteristics of neoangiogenic microvasculature in tumors. Nguyen et al. [134] have developed a dual system for combined imaging and focal treatment of bladder tumors. High-intensity focused ultrasound (HIFU) was combined with a 532 nm laser light to enhance therapeutic effects while a second, tunable laser was used for PAI. They tested 45 porcine urinary bladder tissues *ex vivo* and observed a significant increase in the PA image brightness (~9-fold) at the treated areas after ablation, suggesting that PAI can be used to guide the ablation process. Scheepbouwer et al. [132] developed a multi-modality system for animal imaging combining ultrasound, PAI, and bioluminescence imaging. While the BLI signal was very sensitive to tumor volume, the complementary anatomical data collected by ultrasound and the functional data on SO₂ from multispectral PAI afforded better monitoring of bladder tumor progression. Sivasubramanian and colleagues have developed a combined hand-held clinical ultrasound and photoacoustic probe [135]. They used it to image anatomical features and injected gold nanorods and black ink a healthy rat model for imaging reverse flow of urine from the bladder to the kidney as well as clearance of the nanoparticles from the body. An additional chicken breast layer on top of the rat demonstrated the device capabilities of 2 cm imaging. The system allows imaging over a 50 square mm area with ~30 dB of SNR. In addition, they demonstrated the capabilities of the device to image vesicoureteral reflux. Vesicoureteral reflux, a common urinary tract disease, is characterized by the abnormal flow of urine backward from the bladder into the kidneys. Currently, this is imaged with the help of X-Ray cystography, during micturition vs. normal state. Sivasubramanian and co-workers have demonstrated PAI of such an abnormal

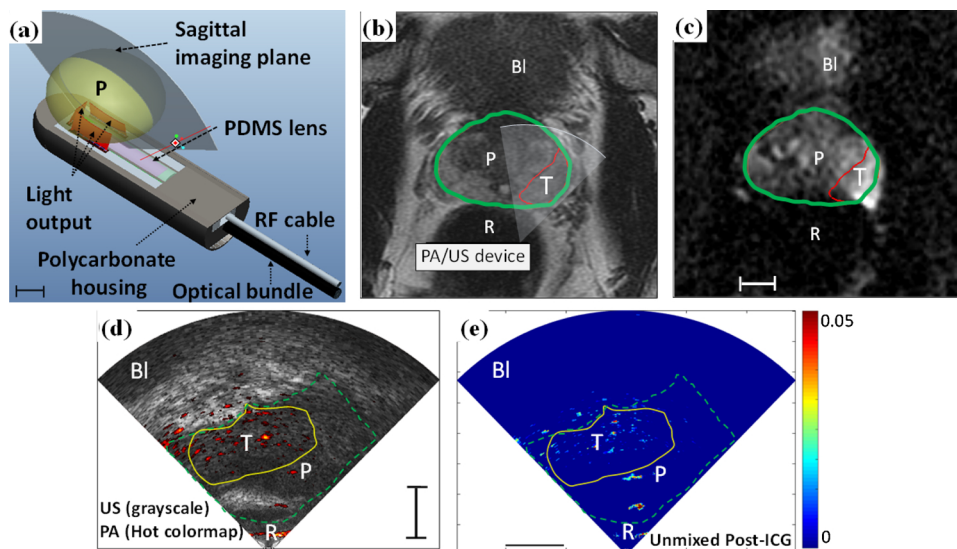


Fig. 9. Dual transrectal photoacoustic and ultrasonic imaging of prostate cancer.

(a) Schematic representation of the home built TRUSPA device imaging the human prostate. The device uses a linear capacitive micro-machined ultrasound array integrated with fiber optic bundles and encapsulated with polydimethylsiloxane (PDMS) in the lens shape. The RF cable connects the CMUT array to the external hardware. (b) T1-weighted MRI overlaid of a 53-year-old male patient was diagnosed with prostate cancer in the left base of the prostate. Bladder (Bl), rectum (R), and prostate (P) in green contour and Tumor region (T) in red contour in (b,c) and yellow contour in (d,e) are marked. (c) Diffusion-weighted-MRI showing the malignancy (red contour) in the left peripheral zone of the prostate. (d) Co-registered ultrasound (US) on gray-scale and photoacoustic (PA) image on red color scale with intravenous ICG injection (25 mg; 10 ml at 2.5 mg/ml). Image was acquired 6-minute

post-ICG injection. Green contour shows prostatic region and yellow contour surrounds the tumor region in the left base of the prostate. (e) Spectrally unmixed ICG image, obtained from multi-wavelength PA data of the post-ICG injection, shows ICG accumulation in the tumor region. Scale bars are 1 cm in length.

state with phantom imaging. Additionally, functional PA imaging of clearance of nanoparticles from circulation was also demonstrated by monitoring the accumulation of gold nanorods in the bladder.

Koo et al. used a PA microscope combined with ICG-enhanced single-walled carbon nanotubes for *in vivo* imaging of tumors in the bladder and sentinel lymph nodes of rats [136]. The enhanced contrast allowed them to achieve sub-nanomolar sensitivity and sub-mm spatial resolution. Kim et al. [137] have developed a photoacoustic cystography system for non-invasive *in vivo* imaging of the rat bladder. MBD contrast agent was used to highlight the bladder and enabled deep tissue. Kim et al. [137] have developed a photoacoustic cystography system for non-invasive *in vivo* imaging of the rat bladder. MBD contrast agent was used to highlight the bladder and enabled deep tissue imaging.

Current screening techniques for cervical cancers (Pap smear test or Human Papilloma Virus test) suffer from a high false negative rate. For ovarian cancer, the common Transvaginal Ultrasound (TVUS) or blood test for CA-125 protein have shown to have a very high false positive rate, and thus no screening test is usually performed. Molecular imaging of those cancer types has also become a clinical application of interest for PAI. PAI can substantially increase the positive predictive value of TVUS. Kumavor et al. have designed and built a combined ultrasound/PAI transducer based on a commercial transvaginal ultrasound transducer with circumferential fibers for illumination, housed in a protective casing [138]. Monte Carlo simulations were used to optimize the illumination patterns. The device was used to image phantoms as well as human ovaries obtained during oophorectomy, demonstrating that PAI can image deep-seated blood vessels, up to 15 mm below the surface. Peng and colleagues studied the differences in vascularity between healthy and cancerous tissues at various stages [139]. From each patient, they embedded healthy cervical tissue and a section of a cervical lesion in a cylindrical phantom and observed statistically significant differences in PA signal amplitude between healthy and cancerous tissue, with PA amplitude strongly correlating with cancer stage. It was shown that PAI could detect precancerous cervical intraepithelial neoplasia at a depth of 5 mm. Aguirre co-workers have reported the using combined PAI/ultrasound imaging of 33 *ex vivo* human ovaries from 24 patients [140]. They used a 1.75-dimensional ultrasound array (i.e., a two-dimensional array with an uneven number of elements in each axis) with 1280 elements to detect the acoustic signal from sample ovaries inside a bath filled with saline solution. Quantification of light absorption in ovaries from postmenopausal

women showed that malignant ovaries absorbed significantly more light than healthy ones, with both sensitivity and specificity of 83%. Alqasemi and co-workers have developed a recognition algorithm from co-registered ultrasound and PA images to assist with ovarian cancer diagnosis [141]. They used 400 images that were acquired by Aguirre [140] in a previous study as a training set and used a set of optimized filters to extract 15 tumor-specific features. An additional nine features were extracted based on observation (i.e., the appearance of the PA image in cancer vs. healthy tissue). These features were used to train a Support Vector Machine (SVM) algorithm and other algorithms, which were then used to classify 95 new images from 37 ovaries (20 patients). The SVM algorithm performed the best, achieving 76.9% sensitivity and 95.1% specificity (positive and negative predictive values were 71.4% and 96.3%, respectively) on a per-image basis, suggesting that analysis of co-registered ultrasound/PAI images holds promise for early detection of ovarian cancer. Yang et al. have developed a triple modality endoscope capable of PAI, ultrasound imaging, and OCT [142]. The incorporation of OCT enables high-resolution imaging of tissue scattering properties, albeit at very limited penetration depths. The device capabilities were demonstrated in *ex vivo* porcine and human ovaries and compared to H&E-stained histological section. PAI showed multiple small vessels near the surface (~1 mm deep), which agreed well with findings from both the OCT image and histology.

Bohndiek et al. used PAI for functional measurement of ovarian tumor response to the antiangiogenic drug, Trebananib [143]. PAI was performed on orthotopic xenograft mouse models ($n = 9$) before treatment and after each of three doses and compared to a control group of untreated, tumor-bearing mice. Imaging was performed *in vivo* using a commercial system - Nexus 128 by Endra which was described earlier. Results showed that using Trebananib significantly reduced the total hemoglobin and increased Hb, indicating normalization of the residual tumor vessels. H&E stains were used to confirm those findings.

Finally, Jokerst et al. also developed gold nanorods for ovarian cancer detection using PAI [144]. These nanorods can also be used to guide resection using Raman imaging. The investigators injected gold nanorods into subcutaneous xenograft tumors in mice at different concentrations and showed that PAI could detect nanorod concentrations as low as 0.4 nM within tumors. Moreover, the PA signals correlated well with the concentration of gold in the tumor and thus can be used for staging, assuming the concentrations of gold within the tumor increase with size.

Although a considerable body of research is aimed at urologic and

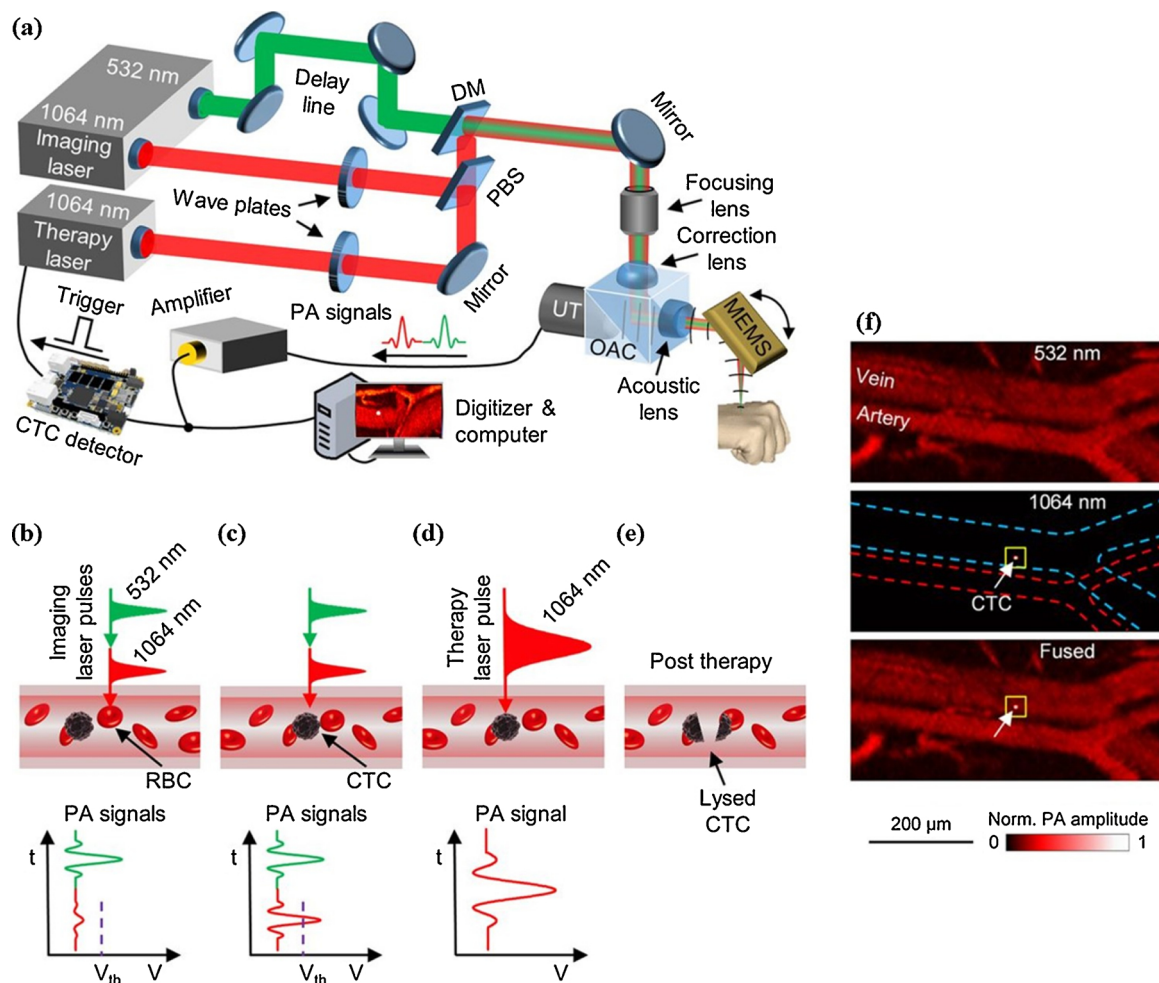


Fig. 10. Photoacoustic imaging of blood constituents.

(a) Schematic of selected components of the experimental system. DM, dichroic mirror; MEMS, micro-electro-mechanical-system scanning mirror; OAC, optical-acoustic combiner; PBS, polarizing beamsplitter; UT, ultrasonic transducer. The 1064 nm and 532 nm imaging lasers are employed to image CTCs and vasculature, respectively. (b–e) Scheme of real-time detection and laser killing of CTCs. The CTC detector compares the earlier 1064 nm laser-induced CTC-specific PA signal against an optimized threshold level (purple dashed line in 'b' and 'c') above the Hb signal, and thus can reliably distinguish CTCs and trigger the therapy laser 'd'. Within ~10 μ s, the therapy laser is fired and focused to the detected CTC location to photomechanically kill the CTC 'e'. (f) 532 nm laser-induced (Top), 1064 nm laser-induced (Middle), and fused (Bottom) flow cytography images. In the 1064 nm laser-induced image, the white arrow and yellow square indicate the detected CTC; the red and blue dashed lines delineate the artery and vein boundaries, respectively.

From: He, Y. et al. *in vivo* label-free photoacoustic flow cytography and on-the-spot laser killing of single circulating melanoma cells. *Scientific Reports* 6, Article number: 39616 (2016); <https://doi.org/10.1038/srep39616>. <http://creativecommons.org/licenses/by/4.0/>

gynecological applications, it seems that the technical difficulties for clinical translation here are higher as deep imaging is required and only limited space is available. It might be the case that more sophisticated and innovative ways to effectively deliver light to the target organs are needed. Transurethral illumination was proposed in the past but combined with a transrectal imaging device might cause great discomfort for the patient. It will be interesting to see how this field further evolves over the next few years.

9. Photoacoustic sensing of circulating cells and substances

Hemoglobin is one of the most prominent endogenous contrast agents in the NIR. As PAI is non-invasive and non-ionizing, there is a growing interest in continuous PA imaging and sensing of blood constituents. Of particular importance is the sensing of circulating tumor cells and tumor DNA. As most Melanomas are melanotic, circulating Melanoma tumor cell has a high melanin content that can be detected by PA. Weight et al. have developed a PA flow cell, in which Melanoma cells are flowing through due to a peristaltic pump and a 450 nm laser pulses are used for side illumination and PA excitation of the flowing

cells [145]. A thin PVDF membrane on top of the flow cell was used to pick off the acoustic transients. Both black polystyrene latex microspheres, as well as human malignant melanoma cells (SK-MEL-1) in saline, were used to demonstrate the capacities of such a system for detecting as low as ten cells within the flow cell volume. Later on, the same group, have replaced their thin film PVDF with an optical detection scheme that obviates the need for piezoelectric films [146]. This eliminates noise that was the result of light hitting the detector directly. Instead, perturbations in the index of refraction of water immersing the sample induced the photoacoustic pressure causes changes in probe beam reflection that can be then measured by a photodetector. This improved setup allowed increased sensitivity, so even a single melanoma cell was visible well above noise levels. Zharov and co-workers have developed a dual photothermal and photoacoustic microscope for real-time detection of circulating cells, nanoparticles, and contrast agents *in vivo* [147]. They showed that during imaging of the blood vessels in a mouse ear and after labeling the cells with ICG or gold nanorods, the threshold sensitivity is estimated as one cancer cell in the background of ten million normal blood cells. Later, Galanzha et al. have developed *in vivo* multispectral and multiparametric,

photoacoustic flow cytometry in the lymph node [148]. They utilized the lymph node's natural valves to hydrodynamically focus the flowing cells into a single file and thus were able to increase selectivity and sensitivity of their system greatly. The downside of such a method is the long measurement time due to the very weak flow in the lymph. They used endogenous absorption as intrinsic cell-specific markers, or gold nanorods, nanoshells, and carbon nanotubes as multicolor probes. They demonstrated label-free detection of metastatic melanoma cells in the lymph and achieved a detection limit of one melanoma cell in the background of million white blood cells. Later, the same group used a high pulse repetition rate laser operating at 820 nm and 1064 nm for preclinical studies for label-free PA detection of low-pigmented mouse and human CTCs [124]. The high-speed system reduces the rate of false-negative errors for fast moving CTCs in large blood vessels, and thus allows sampling of a considerable blood volume in a more realistic time frame. Their results showed that low-pigmented C8161 human melanoma cells produced a very high false negativity rate of about 60–80% compared to 12% for strongly pigmented B16F10 mouse melanoma cells. To overcome this limitation, the authors utilized functionalized magnetic nanoparticles to assist with *in vivo* enrichment and PA detection of melanoma cells. A magnetic field was applied to trap or slow down targeted cells within the laser beam and increased the signal strength (i.e., the number of cells detected per unit time) by order of magnitude. Finally, He et al. have developed a fast scanning PA flow cytography system for high-speed imaging at both 532 nm and 1064 nm with a single-cell resolution that was coupled with real-time selective CTC destruction by nanosecond-pulsed NIR laser-induced photothermalolysis [149]. The system is described in Fig. 10A. The use of both wavelengths allowed easy separation of melanin-rich blood continues (circulating Melanoma cells) from other hemoglobin-rich cells (red blood cells). This is described in Fig. 10B–E. The high scanning speed and extremely detailed imaging allow for the tracking of a single cell in both superficial arteries and veins *in vivo* as shown in Fig. 10F. An additional high-power laser was hardware-triggered for killing the CTC on the spot in a thermally confined manner without causing collateral damage. A pseudo-therapy feasibility study including both *in vivo* and *in vitro* experiments demonstrated the performance and the potential clinical value of this method, which can facilitate early treatment of metastasis by clearing circulating tumor cells from the vasculature.

While PAI of the different blood constituents is technologically achievable, the question remains whether it can produce clinically significant measurements. CTC's, while an essential topic for research, are also quite rare their exact biological role and significance are still unknown. It will be challenging to estimate the accuracy and impact of such a system. However, if bedside or even wearable systems can be developed, this will open the door to many exciting applications for constantly monitoring different blood analytes.

10. Challenges in clinical photoacoustic imaging

Over the last three decades, photoacoustic methods have shown great potential for imaging blood vessels in small animals (full body) and humans (superficially) [10]. Multispectral PAI has mostly been used for estimating blood oxygenation levels. However, deep tissue imaging beyond a few mm) or imaging of targets other than blood vessels remains a challenge, which limits the translation of PAI to the clinic for a wide range of applications.

One key factor that limits the clinical usefulness of PAI is the penetration depth [150]. PAI can image structures up to several centimeters deep inside the tissue. Such penetration depth significantly exceeds any optical imaging technique that relies on unscattered ballistic photons for image reconstruction such as confocal microscopy [151]. It also achieves much better spatial resolution (at comparable depths) compared to optical imaging techniques which rely on diffusely scattered photons such as diffused optical tomography [152]. However,

reliable and clinically useful imaging at depths exceeding 2–3 cm *in vivo* remains a major challenge [153]. This is in part due to the fact that, unlike B-mode ultrasonography, PAI is essentially a quantitative / semi-quantitative technique in nature. In other words, one expects the PA image intensity to be positively correlated with the local optical absorption. While this assumption might hold at shallow imaging depth, it does not hold as the imaging depth increases since:

- 1 The optical fluence is attenuated and spatially spread in a complex fashion and by order of magnitude or more for every cm of tissue [154]. Even if one assumes ideal reconstruction, unless a fluence compensation scheme is applied, the photoacoustic image is not an image of optical absorption but an image of the product of the local light fluence and the optical absorption. This means that a region in the image might appear “dark” due to poor light penetration and not due to decreased light absorption and vice versa [25]. This effect can be partially mitigated by applying optical models to predict and compensate for the light attenuation [155,156]. However, as the imaging depth becomes greater, it becomes considerably harder to precisely correct for this effect. This problem is exacerbated when multiple wavelengths are used since the fluence at each wavelength is perturbed differently. Fig. 11A–D demonstrates this effect on a tissue phantom with embedded absorbers.
- 2 For many clinical applications, it is not feasible to surround the ROI with detectors from all sides. Thus, many times one is restricted to a limited view problem - i.e., one is attempting to reconstruct the initial pressure based on a partial dataset [157]. It was shown in the literature that limited view causes errors and artifacts, which are exponentially increasing with the decrease in the viewing angle [157] (i.e., as the depth from a finite detection aperture become greater). Moreover, even under full-view conditions, the signal to noise ratio (SNR) is significantly degrading with depth due to optical and acoustic attenuation and dispersion. Thus, the reconstruction does not accurately represent the local fluence and absorption as the imaging depth increases [23].
- 3 The acoustic properties of the tissue such as speed of sound and the Grüneisen coefficient (which describes the efficiency of conversion of optical energy to acoustic pressure) are not constant throughout the different tissue types [158] by a few tens of percents. For example, both of those properties are dependent on the temperature which can change with depth [10]. As the imaging depth becomes progressively greater and the ROI contains more tissue structures at different depths beneath the skin surface, the assumption of constant acoustic properties across the ROI becomes progressively less accurate and thus leads to non-quantitative imaging.

These reasons might explain the discrepancy in imaging depth, which is reported for phantoms (6 or more cm), compared to reports regarding *in vivo* clinical imaging (2–3 cm at most).

The second major issue which limits the clinical use of PA is the imaging contrast. Tissues naturally contain multiple endogenous chromophores with different, yet overlapping, absorption spectra, such as water, HbO₂, and Hb and other pigments (shown in Fig. 11e) [159]. PAI can image those chromophores or even attempt to separate them using multispectral PA. Since various abnormal states cause changes in tissue composition, such as vascular fat deposition in atherosclerotic plaques [160] or angiogenesis in cancer development [161], PAI has the potential to visualize and quantify these changes in comparison with the healthy surrounding tissue. However, visualization of blood vessels and tissue oxygenation alone might not have sufficient clinical value for many diseases [162]. Changes in vasculature might not be dramatic enough for early detection or might not be specific enough to separate from benign or malignant tumors. Moreover, as mentioned earlier, since accurate quantification poses a significant challenge at depths, the accuracy of the PAI estimation of such parameters is still unclear [163]. Another technical difficulty is the need for a “wet” contact environment

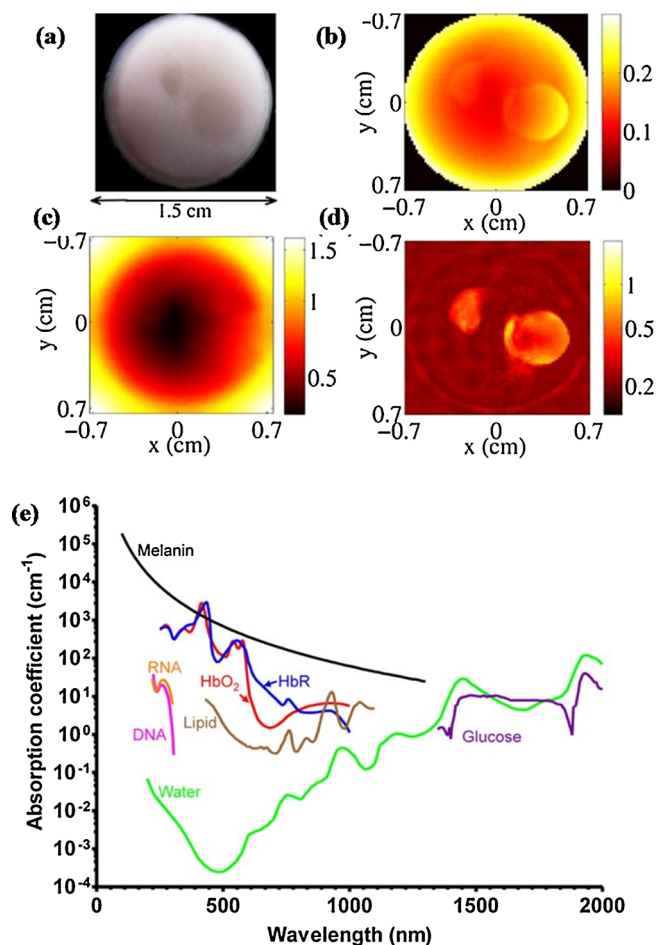


Fig. 11. Opportunities and challenges of PAI.

(a) Photo of a physical PA phantom contains a round and a semi-triangular insertions with similar absorption that were embedded inside. (b) Reconstructed PAI of the phantom. Only the boundary of the round insertion is visible in the image. (c) Calculated fluence map (d) Calculated absorption coefficient after fluence compensation. Notice the differences between 'B' and 'D'. (e) Absorption spectra of major endogenous contrast agents in biological tissue. Oxy-hemoglobin, red line (150 g/L in blood); Deoxy-hemoglobin, blue line (150 g/L in blood); Lipid, brown line (20% by volume in tissue); Water, green line (80% by volume in tissue); DNA, magenta line (1 g/L in cell nuclei); RNA, orange line (1 g/L in cell nuclei); Melanin, black line (14.3 g/L in medium human skin); Glucose, purple line (720 mg/L in blood).

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Panel E from Yao, J. et al. Photoacoustic microscopy. Laser Photonics Rev. 7, No.5, 758–778 (2013). DOI 10.1002/lpor.201200060. Published by John Wiley and Sons. © 2012 by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

for proper acoustic coupling between the tissue and the transducer. In recent studies, either dry coupling technique [164] or a non-contact schemes [165,166] were suggested, but those usually suffer from lower sensitivity and higher complexity which reduces their attractiveness. Some of the abovementioned limitations can be overcome, or at least reduced, by the usages of targeted exogenous molecular agents [26]. Such agents can potentially enjoy high absorption and unique spectra which will make it easy to separate them from the background. More importantly, they can convey important clinical data such as the presence of a particular biomarker or the level of activity of a specific

enzyme, which is much more valuable for clinical decision-making [15]. Unfortunately, although a considerable volume of pre-clinical studies has reported such agents, to date, no such targeted agent is FDA approved. However, fluorescent optical imaging agents have started to be FDA approved, and those can be used for PAI even if not optimal [15]. Due to depth limitation, unclear accuracy and dependence on the operator expertise, as well as the low availability of clinical PAI systems, there are only a handful of attempts for translating those agents into the clinic. It might be the case that one or more key imaging agents may drive PAI hardware dissemination into the clinic and improvements in clinical hardware may help drive more translation of the imaging agents.

11. Conclusions and future outlook

This paper reviews a wide breadth of the use of PAI for clinical imaging. Some clinical applications are not discussed here but have been covered comprehensively in recent review papers. These include PAI of the eye [167,168], intravascular [169] and cardiovascular PAI [170], bone and joint PAI [171] and PAI of the gastrointestinal tract [172]. Most of the work is still in its preclinical stage. However, efforts are being made for clinical translation of various applications. These, together with the increasing availability of clinical PAI instruments are expected to increase significantly the usage of PAI in clinical practice over the next decade.

There are still many outstanding challenges for incorporation of PAI in standard-of-care medical use. The first challenge is reproducibility and standardization of the photoacoustic images. Currently, there are multiple groups developing equipment [9], reconstruction algorithms [173], image analysis methods [174] as well as experimental protocols [175], imaging agents [176] and phantom fabrication [177]. The imaging results and consequent interpretations of the underlying disease state are highly dependent on all of those. Standardization and highly reproducible images are imperative prior to any clinical use. As most clinical systems are hand-held devices, the operator's training and capabilities also seem to play a significant factor and should be further researched.

It is worth noting that the clinical application(s) of any future PAI system is entirely dependent on its ability to produce real-time (or quasi-real-time) imaging. Unlike full-body imaging techniques, which can be processed offline post image acquisition and are less operator dependent, regional imaging techniques with handheld devices are highly dependent on real-time visual feedback for the operator to identify the right anatomical structures and imaging planes/volume. As the physical limitations (e.g., the speed of sound in tissue, the number of available parallel acoustic detectors, the number and rate of optical pulses needed to produce an image, etc.) indeed allow for real-time or quasi-real-time data acquisition, the imaging rate usually depends on the reconstruction algorithm to accurately and rapidly process the vast amount of RF data. There are several types of algorithms which were proposed over the years and were reviewed recently [173]. Delay and sum algorithms were shown to quantify the PA signal albeit their computational simplicity inaccurately. Thus, most of the current clinical devices are based on either a variation of the back-projection method [178] or its Fourier counterpart - the k-space algorithm [179]. That algorithm demonstrated their ability to provide real-time reconstruction in two and three dimensions [180] especially when implemented on Graphics Processing Units (GPUs) for parallel processing. Another emerging technique is the Interpolated Model Matrix Method [181] that is much more intensive computationally but allows a full description of the measurement system and can yield more accurate photoacoustic images. Very recently, such a technique was implemented at 20 frames per second imaging rate [182] which opens avenues to a more accurate PA reconstruction in real time.

Fluence compensation remains a significant challenge as it prevents the interpretation of PA images as absorption maps as well as reliable spectral decomposition. As of yet, there is no general and reliable solution, but it seems that the incorporation of additional data (in the form of multiple illuminations or imaging angles, switchable molecular imaging agents, direct fluence detection on the surface or *a-priori* assumptions regarding the spatial properties of fluence and absorption) is vital. We believe that further research can lead to robust and general strategies for reducing the effects of the unknown fluence on PAI.

Suitable optical sources for deep tissue clinical imaging are still required. Currently, most PA systems reported in the literature are based on pulsed lasers that often allow wavelength tunability, such as Q-Switched optical parametric oscillators, Ti:Sapphire lasers, and dye lasers. These sources emit short pulses at high peak power, which generate the PA signal most efficiently. Those have shown to be useful in the lab but are bulky and costly systems, often requiring specialized high-power supplies and cooling units which make their clinical translation challenging. In addition, there is often very limited control over their excitation parameters, such as the pulse duration and pulse repetition frequency. Pulse to pulse variation and jitter are also present and may degrade performance.

Therefore, to make the PA system more attractive for usage in the clinic, other types of optical excitation sources should be considered. One type of potential sources are laser diodes (LDs), which are low-cost, have a very compact size and simple for operation and maintenance. The main disadvantage of LDs, however, is their limited peak power. While tunable laser systems may reach peak power in the order of Mega-Watts, the common continuous-wave LDs are limited to about 1 W in the visible and NIR range. Pulsed operation of some laser diodes may allow a peak power of about 100 W. The inferior peak power can be partially compensated by controlling the LDs excitation parameters. Continuous-wave LDs allow flexible control over the excitation parameters through direct modulation of the current, and with the proper driver, such LDs can emit arbitrary excitation patterns at bandwidths up to hundreds of MHz. In addition, while the PRF of typical pulsed tunable lasers is often limited to a few tens of Hz, the PRF of CW or pulsed LDs can reach a few MHz, thus allowing signal averaging over many acquisitions in order to improve SNR. Further research is required for better utilization of LDs for clinical use. To improve light delivery at greater depths, several groups have explored “minimally invasive” interstitial illumination schemes [131,183] in which a fiber optic is inserted into the tissue. Such approach can be used for certain clinical applications such as guided-surgery or biopsy [184] (which already require invasive intervention), urological imaging (with transurethral [185], transrectal [123] or transcervical [186] illumination) or imaging of the GI tract [172]. However, for general diagnostic applications, interstitial illumination schemes may be hard to implement due to patients’ compliance.

Other improvements necessary for realizable reconstruction lie on the detector side. It was shown that a crucial parameter required for high-quality PAI is the element directivity [187]. This is especially true for linear or convex arrays. Capacitive Micro-machined Ultrasound Transducers (CMUTs) have shown superior directivity patterns at the expense of higher cost and complexity, as well as lower sensitivity [188]. In the future, it would be of interest to design composite piezoelectric transducer arrays (common in modern medical ultrasound transducer) specifically for PAI applications. Such arrays may enjoy both high sensitivity and wide directivity at costs that are comparable to current medical transducers.

One possible route for lowering the barriers of translating PAI to the clinic is by upgrading the existing ultrasound systems with light delivery augmentations. This can potentially bring down the cost of PAI/hybrid systems and shorten the operator’s training [107,135]. However, there are a few major pitfalls to be considered. Unlike ultrasound, PA

signals are relatively weak and omnidirectional. Thus, reliable PA reconstruction requires large detector elements and a wide viewing angle of the entire region of interest. In contrast, ultrasonography usually requires small, tightly pitched elements for effectively steering the beam and rarely requires a tomographic view. In addition, dedicated PAI reconstruction algorithms need to be implemented. Some transducers are pre-processing the raw RF waveforms at the hardware level using a dedicated Application Specific Integrated Circuits (ASICs) [189], which usually do not allow implementation of PAI-specific algorithms. Thus, upgrading existing ultrasound systems with light delivery might be as challenging as designing a dedicated dual-mode US-PAI system.

Another major obstacle to clinical implementation of PAI is the lack of commercially available photoacoustic imaging systems for intraoperative use. Hardware development meant for clinical use must be married with the specific needs and applications relevant for clinicians. Those are entirely different from the current pre-clinical machines that are aimed at small animal imaging in laboratory settings. However, as systems become available, an adaptation of this novel technique will likely be rapid since surgeons already routinely perform ultrasound imaging intraoperatively.

In addition, the stage is now set for developing and translating injectable molecular imaging agents. Those will have to overcome any potential toxicity and regulatory barriers first, but once approved will provide enhanced PA signal as well as invaluable and clinically relevant data. We expect that increased use of PAI for clinical imaging will allow the development of the relevant standards and the creation of PA-specific databases. The potential of clinical PAI can only be fully realized after the successful clinical translation of such agents. Thus, we conclude that albeit the impressive progress in recent years, there is still a substantial amount of technical and scientific work needed in order to obtain an appropriate imaging modality for useful routine clinical implementation. At this point, it seems that it is time for academia to collaborate with industrial partners as well as clinicians to push what is currently mostly lab work towards a real clinician-friendly diagnostic modality.

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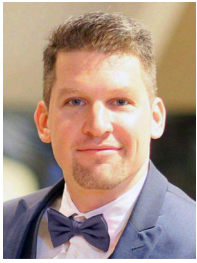
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