Review

Light In and Sound Out: Emerging Translational Strategies for Photoacoustic Imaging

S. Zackrisson1,2,3,4,5, S.M.W.Y. van de Ven1,2,3,4, and S.S. Gambhir1,2,3,4

Abstract

Photoacoustic imaging (PAI) has the potential for real-time molecular imaging at high resolution and deep inside the tissue, using nonionizing radiation and not necessarily depending on exogenous imaging agents, making this technique very promising for a range of clinical applications. The fact that PAI systems can be made portable and compatible with existing imaging technologies favors clinical translation even more. The breadth of clinical applications in which photoacoustics could play a valuable role include: noninvasive imaging of the breast, sentinel lymph nodes, skin, thyroid, eye, prostate (transrectal), and ovaries (transvaginal); minimally invasive endoscopic imaging of gastrointestinal tract, bladder, and circulating tumor cells (in vivo flow cytometry); and intraoperative imaging for assessment of tumor margins and (lymph node) metastases. In this review, we describe the basics of PAI and its recent advances in biomedical research, followed by a discussion of strategies for clinical translation of the technique. Cancer Res; 74(4); 1–26. ©2014 AACR.

Introduction

Photoacoustic imaging (PAI), also referred to as optoacoustic imaging, is an emerging new imaging technique with significant promise for biomedical applications. The key strength of PAI is its ability to collect functional and molecular information from most tissues. One of the major advantages of this technique over other imaging modalities is that PAI has the ability to provide tissue information without using an exogenous molecular imaging agent, by making use of endogenous contrast alone. Additional advantages over other imaging modalities are that PAI can provide images: (i) in real-time; (ii) at clinically relevant depths; (iii) with relatively high spatial resolution; (iv) and without the use of ionizing radiation. PAI can be performed either by (i) relying on intrinsic tissue contrast alone (e.g. mapping endogenous chromophores such as melanin, hemoglobin, and lipids) or by (ii) using exogenous molecular imaging agents that can either target specific molecular processes (targeted agents) or extravasate due to leaky vasculature and the enhanced permeability and retention (EPR) effects found in tumor tissue (nontargeted agents).

In this review, we will first describe the basics of PAI, followed by a comprehensive overview of its clinical applications. We will then discuss strategies for future translation to clinical practice.

Principles of Photoacoustic Imaging

PAI relies on the photoacoustic effect, first described by Bell (1). In short, a pulsed nanosecond-long red-shifted laser beam is used to illuminate the tissue(s) of interest causing photons to propagate diffusely inside. The absorption of these photons leads to a slight localized heating of the tissue causing thermoelastic expansion. This transient thermoelastic tissue expansion generates pressure waves (ultrasound), which can then be detected by broadband ultrasonic transducers and converted into images. These basic principles are illustrated in a potential clinical application (see Fig. 1). The shared detector platform (the detector array) facilitates a natural integration of PAI and ultrasound imaging creating a hybrid imaging technique that combines functional (PAI) and structural (ultrasound) information, which is favorable for clinical translation (Fig. 1). In addition, the fact that PAI is similar to ultrasound in technology and handling—it can also itself be real-time, portable, relatively cheap, and safe (nonionizing radiation)—makes it even more adoptable/integrable for clinicians.

The combined use of light and sound in PAI has an important advantage over other imaging techniques, such as optical imaging, ultrasound, MRI, computed tomography (CT), positron emission tomography (PET), and single-photon emission computed tomography (SPECT), namely the unique scalability of its spatial resolution and depth penetration across both optical and ultrasonic dimensions. In optical imaging, scattering of light hinders penetration depth, whereas in PAI the detection of acoustic signal, which has much lower tissue scattering, allows for superior depth penetration. Spatial resolution can be very high (microscopic level) at higher ultrasound...
frequencies, but with lower depth penetration, as a trade-off, due to the increasing attenuation of ultrasound with frequency. Photoacoustic microscopy (PAM) generates lateral resolutions down to 45 μm at a depth of 3 mm when using ultrasound frequencies of 50 MHz (2–5), and this can even improve to 200 nm (subcellular level) in optical-resolution PAM, but only at imaging depths of up to 1 mm (6). Macroscopic PAI systems allow for deeper tissue imaging by using lower ultrasound frequencies of 4 to 8 MHz and typically generate resolutions of 0.5 to 5 mm at reported imaging depths of approximately 3 to 7 cm (3, 6–10). A summary of the largest depths reached with macroscopic PAI systems is presented in Table 1. The unique scalability of spatial resolution and imaging depth may afford a wide breadth of imaging ranging from the microscopic to the macroscopic scale, something that is very challenging to do using most other imaging modalities. For example, comparing optical microscopy images with the CT images is difficult because the image contrast is obtained in entirely different ways (optical absorption vs. X-rays). Thus, PAI could open up inimitable opportunities to study complex biologic systems and pathways in the same organism from the subcellular level to the organ level, using the same optical absorption contrast. Some PAI systems can already image both at the macroscopic and microscopic level (11), although so far most systems have been designed to image specifically at one or the other of these levels. Although spatial resolution of PAI may be superior over other imaging techniques, such as PET and SPECT (12), we have to keep in mind that these modalities do not suffer from depth limitations (as attenuation of γ rays can be corrected for), which makes them suitable for whole-body imaging. Most likely PAI will be used in adjunct to clinically established imaging techniques, or in combination with them in a multimodality setting. More information on these multimodality imaging approaches in which anatomic and molecular information are combined, can be found under the organ-specific sections of this review.

Another key advantage of PAI, mentioned earlier, is that the optical component of the technique allows for molecular imaging even without the use of an imaging agent, by using multiple wavelengths (multispectral imaging of endogenous contrast). Different tissue components have unique optical scattering and absorption properties for each wavelength. In Fig. 2, the absorption spectra for the main light-absorbing tissue components (oxy- and deoxyhemoglobin, lipid, water, and melanin) are shown. As seen from this figure, total light absorbance in these tissue components is lowest in the...
wavelength range of 600 to 1,000 nm, referred to as the near-IR (NIR) or “optical window.” Because the absorbance is lowest in the NIR range, light of these wavelengths is used to obtain sufficient tissue-penetration depth (a few centimeters). Information on tissue composition, that is, relative concentrations of (de)oxyhemoglobin, water, lipid, or an injected imaging agent, can be calculated by combining photoacoustic data acquired at multiple wavelengths. Numerous relevant clinical parameters can be assessed this way, for example hypoxia, perfusion, and neoangiogenesis.

Many different PAI platforms for clinical use already exist or are under development including handheld probes and photoacoustic CT as exemplified under the organ-specific sections that follow. In short, PAI systems can be categorized as linear array systems (13–16) or tomographic systems (9, 17). Tomographic systems detect photoacoustic waves emanating from the “entire” perimeter of the subject of interest using either a single element or an array transducer. In linear array systems, an ultrasound array is used to detect photoacoustic waves from “limited angles” around the object. The limited field of view results in an image quality inferior to that of tomographic systems. However, linear array systems are more portable and can be integrated into already existing clinical ultrasound systems, enabling the

### Table 1. Summary of the largest imaging depths reported for macroscopic PAI in experimental and clinical studies

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<th>Subjects</th>
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<td>Prostate pseudo lesions with fresh canine blood</td>
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<td>Wang and colleagues (112)</td>
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<td>~20</td>
<td>LOIS-P (laser optoacoustic imaging system for the prostate)</td>
<td>Dog prostate in vivo</td>
<td>Blood rich-induced prostate lesions</td>
<td>—</td>
<td>Yaseen and colleagues (113)</td>
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<tr>
<td>~32</td>
<td>Photoacoustic mammoscope prototype</td>
<td>Phantom</td>
<td>Breast lesion-mimicking objects</td>
<td>Ecoline 700 black water color dye</td>
<td>Manohar and colleagues (95)</td>
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<tr>
<td>~50</td>
<td>Hand-held photoacoustic/ultrasound probe</td>
<td>Phantoms/chicken breast and rats</td>
<td>SLNs</td>
<td>Methylene blue</td>
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<td>~52</td>
<td>PAT</td>
<td>Phantoms/chicken breast</td>
<td>Lesion-mimicking objects</td>
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<tr>
<td>~52</td>
<td>Hand-held photoacoustic/ultrasound probe</td>
<td>Phantoms/chicken breast and rats</td>
<td>SLNs</td>
<td>Methylene blue</td>
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<td>Optoacoustic tomography</td>
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<td>~70</td>
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<td>~28*</td>
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<td>Patients (n = 26)</td>
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<tr>
<td>~60</td>
<td>Photoacoustic mammoscope</td>
<td>Patients (n = 13)</td>
<td>Breast cancer</td>
<td>—</td>
<td>Manohar and colleagues (8)</td>
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\*Depth reported for one case only.
visualization of organs less accessible with tomographic systems (e.g., via endoscopes or catheters).

During the last few decades, major progress has been made in source and detector development, allowing for instance for faster (real-time) imaging at more wavelengths and greater depth penetration (18). Light propagation models and image reconstruction algorithms have also improved significantly (18–23), which is critical to image quantitation. Obtaining truly quantitative images is only possible when correcting for the amount of light delivered to an actual tissue depth, and this remains challenging because light attenuation depends on several factors, not only the imaging depth but also the tissue type in which the light propagates.

The abovementioned progress in technology plus the inherent advantages of PAI are paving the way for clinical translation of the technique. A summary of potential clinical applications of PAI in different organ systems is illustrated in Fig. 3. More detailed descriptions of the principles of photoacoustics can be found in book chapters (24, 25) and other articles covering this topic (17, 26–28).

Imaging Contrast

**Endogenous contrast**

PAI takes advantage of the fact that the body tissues contain a variety of endogenous chromophores with different absorption spectra, such as melanin, water, fat, HbO2 (oxyhemoglobin) and Hb (deoxyhemoglobin), as shown in Fig. 2. Many diseases cause changes in tissue composition, such as neovascularization in cancer development (29) and vascular fat deposition in atherosclerotic plaques (30). PAI has the potential to visualize and quantify these changes in comparison with the normal surrounding tissue. PAI based on endogenous contrast alone has been studied for various diseases (e.g., atherosclerosis, breast cancer, and prostate cancer; refs. 30–32). However, not all absorption spectra for different tissues are known. Multiwavelength PAI makes it possible to image several tissue components in the same image, although the need for a tunable pulsed laser source increases the costs of the system significantly. Because some tissue components have very similar absorption spectra within the NIR range, there is sometimes a need for exogenous molecular imaging agents to enhance the contrast. Moreover, exogenous imaging agents can potentially facilitate deeper tissue imaging.

**Exogenous imaging agents**

Exogenous imaging agents can greatly enhance the PAI contrast. One could use nontargeted imaging agents that mostly extravasate from the blood stream due to increased vascular permeability and the EPR effect in tumorous tissue, or targeted imaging agents that target a specific molecular process, that is, a certain receptor, protein, or enzyme. The advantage of targeted imaging agents is that they can provide specific information on molecular or cellular processes in the tissue. There is a great variety of imaging agents that can be used for PAI (see Table 2 and Fig. 4). The selection of a particular imaging agent will depend upon the application for which it will be used and one should thoroughly consider various characteristics, such as the size, shape, toxicity, stability, surface chemistry, and targeting moieties of the imaging agent. Besides a few imaging dyes that have been approved for human use [indocyanine green (ICG), Evans blue, and methylene blue], the rest of the imaging agents are not yet approved,
and have only been evaluated in phantoms or animal models. So far, not much information is available on the mass range that is needed for any of the potential imaging agents to perform their respective PAI studies. This is an important issue and the toxicity of these imaging agents will have to be determined relative to the mass needed to be injected to perform a given study. It is likely that higher masses will be necessary for PAI studies compared with very sensitive techniques such as PET and SPECT. Mass ranges will have to be assessed for each specific agent and its applications.

Small-molecule dyes

There is a range of small-molecule dyes that absorb in the NIR window and can thus be used for PAI. Many of these dyes are fluorescent and are commonly used in optical fluorescence imaging. The difference with PAI is that for this technique only the absorption part of the spectrum is used and not the fluorescent emission part, making it more efficient to have a low quantum yield (i.e., a low ratio of photons emitted to photons absorbed, as more of the absorbed energy can be converted into photoacoustic signal). The dyes can be used as agents that are not targeted, but can also be functionalized to enable molecular targeting. For example, a dye can be directly conjugated to a targeting antibody such as trastuzumab to image HER2 expression (33) or to a caspase-binder to image apoptosis (34). Furthermore, an activatable probe has been designed by our laboratory especially for PAI, using activatable cell-penetrating peptide as its peptide platform with a NIR chromophore (quencher or fluorophore) on either side of this platform to enable dual-wavelength imaging. The intact probe is photoacoustically silent through subtraction of the images obtained at the two wavelengths, but after activation by matrix metalloproteinases (MMP), specifically MMP-2, the cleaved probe shows a change in photoacoustic signal because now only the cell-penetrating peptide portion of the probe (carrying one of the chromophores) accumulates in the cells and the rest of the probe (carrying the other chromophore) diffuses away (35). This platform is generalizable and can be tailored to the target proteases of interest, but its potential was recently shown in mice in vivo for PAI of thyroid carcinoma (36). In addition, Razansky and colleagues showed that the commercially available activatable fluorescent probe MMPSense 680 can be used for molecular imaging with PAI systems as well (37). MMPSense 680 also undergoes changes in light absorption after activation by MMPs. These changes can then be spectrally resolved over the inactivated probe and the background by acquiring the photoacoustic signal over a range of wavelengths, compared with the two-wavelength imaging used for the previously described activatable photoacoustic probe.

The advantages of the small-molecule dyes (~1 nm in size) are their biocompatibility and their fast and complete clearance from the body due to their small size. This makes them
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<td>Alexa Fluor750</td>
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<td>Gold nanocages</td>
<td>Silver cubes coated with a layer of gold</td>
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<td>Pearl necklaces</td>
<td>Gold nanorods decorated with iron oxide spheres</td>
<td>90 nm</td>
<td>785</td>
<td>In vitro</td>
<td>Dual mode cancer imaging with PAI and MRI</td>
<td>Wang and colleagues (80)</td>
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<td>Iron oxide cores with gold spikes</td>
<td>120 nm</td>
<td>800–900</td>
<td>Rat model</td>
<td>Imaging lymphatic vessels and nodes</td>
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<td>Nanoroses</td>
<td>Clusters of iron oxide cores with thin knobby gold coating</td>
<td>20–80 nm</td>
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<td>Cancer imaging, EGFR targeting; atherosclerosis imaging</td>
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<td>Wonton-shaped cobalt cores coated with layer of gold</td>
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<td>Phantoms and mouse model</td>
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<td>30–40 nm</td>
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<td>Magneto-plasmonic nanoparticles</td>
<td>Liposomes encapsulating gold nanorods and iron oxide spheres</td>
<td>100–200 nm</td>
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<td>Phantoms</td>
<td>Dual mode PAI and MRI</td>
<td>Qu and colleagues (82)</td>
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Abbreviation: EGFR, EGF receptor.

*aFDA approved.*
valid candidates for clinical use. As mentioned earlier, a few biocompatible dyes have already been approved for clinical use (ICG, Evans blue, and methylene blue). These dyes have been studied in various animal models for PAI (3, 38–43), but not yet in clinical trials. The downside of their small size is that it is less likely for the imaging agents to be delivered to or retained at the target site during their short circulation time. In addition, photostability is an important issue for these dyes as they suffer from photobleaching (loss of optical absorption) after exposure to prolonged laser irradiation. Circulation time and photostability can be improved by encapsulating more dye molecules into a bigger nanoparticle, which was shown by Kim and colleagues who developed ICG-embedded nanoparticles based on PEBBLE (photonic explorers for biomedical use by biologically localized embedding) technology (44). In PEBBLE, high dye concentrations are encapsulated into biocompatible nanoparticles, for example, an ormosil matrix was used by Kim and colleagues for nanoparticle synthesis and each particle was loaded with >20,000 ICG molecules. Gupta and colleagues also encapsulated many ICG molecules into bigger nanoparticles, but they made a construct composed of a protein shell, purified from the plant-infecting brome mosaic virus (BMV), to encapsulate the ICG (45).

Nanoparticles

Nanoparticles are larger in size than small molecules. Their size varies between a few to several hundred nm. On the basis of their mechanism, the nanoparticles can be divided into two categories: plasmonic and nonplasmonic nanoparticles. The plasmonic nanoparticles are made of a noble metal (gold or silver) and use the surface plasmon resonance effect (SPR). This effect takes place when the electromagnetic field of incoming light interacts with the conduction electrons on the surface of the nanoparticle, resulting in mutual oscillation of these electrons at a resonance frequency relative to the lattice of positive ions. At this resonance frequency, the incoming light is absorbed by the nanoparticle, with an optical absorption that is five orders of magnitude greater than the absorption for dyes (on a per particle basis; ref. 25).

The plasmonic nanoparticles exist in numerous sizes and shapes (see Table 2). The most commonly used kinds in PAI are nanoshells, nanorods, and nanocages, usually made of gold (46–57). Besides these regular shapes, some more complex shapes have been designed, such as nanostars, nanoroses, nanowontons, and nanoclusters (52–56, 58–62). The advantage of these plasmonic nanoparticles is that their optical properties (absorption and scattering) are highly tunable over the NIR spectrum by changing their size or shape, which makes them very attractive for biomedical applications. An additional important advantage is that the surface characteristics of these nanoparticles are easy to chemically modify, which is important for in vivo use (e.g., to decrease cytotoxicity or to increase circulation time and stability) and for molecular targeting. Functional groups such as polyethylene glycol (PEG) or integrins can be attached to the surface in a straightforward way. The disadvantages of plasmonic nanoparticles are that they can deform after extended exposure to laser irradiation and that their long-term safety is a concern, especially with the larger particles that do not fully clear the body as they are taken up by the reticuloendothelial system (RES). The deformation of nanoparticles affects the photoacoustic signal and results in inconsistent imaging results over time. A thin layer of silica coating can increase stability of the nanoparticles and allow them to maintain their original shape for a longer time (63). An extra advantage of the silica coating is that these hybrid nanoparticles can act as photoacoustic nanoamplifiers due to more efficient heat dissipation to the tissue (64). Zhang and colleagues have demonstrated a new type of nanoparticle for PAI. In addition to using conjugates of fluorescent nanodiamonds and gold nanoparticles to enhance their otherwise low photoacoustic signal (65), they have shown the feasibility of using nanodiamonds damaged by helium ion irradiation (66). These radiation-damaged nanoparticles gave a 70-fold higher photoacoustic signal as compared with gold nanoparticles of similar dimensions, caused by increased optical absorption due to vacancies in the diamond crystal lattice.

The nonplasmonic nanoparticles are strong light absorbers that do not rely on the SPR effect. Single-walled carbon nanotubes (SWNT) are the most widely used example of nonplasmonic nanoparticles. They absorb light over a broad spectrum, including the NIR range. Their broad absorption spectrum even allows for "thermoacoustic imaging" using lower energy electromagnetic waves (microwaves instead of NIR light; ref. 67). Like the plasmonic nanoparticles, the
nonplasmonic nanoparticles can also be modified to improve their functionality. For example, several dyes can be attached to the SWNTs to increase light absorption and targeting agents can be conjugated to the SWNTs for molecular imaging purposes (Fig. 4; refs. 68–70). In addition, the SWNTs can be coated with a thin layer of gold, which enhances the photoacoustic signal as with this layering the SPR effect is used (71). Another type of nonplasmonic nanoparticle is a structure in which several dye molecules are grouped together, with stronger light absorption and longer circulation time than for the free dye. For example, the ICG-embedded PEBBLEs by Kim and colleagues are ormosil spheres containing many ICG dye molecules, and it was shown that HER2-antibodies could be attached to the surface of these nanoparticles for cancer targeting (44). Quantum dots are semiconductor nanoparticles that are strongly fluorescent with sizes ranging from 2 to 10 nm. Shashkov and colleagues showed in their studies that these quantum dots can also be used as PAI agents (72). Copper sulphide nanoparticles, approximately 11 nm in diameter, also have these semiconductor characteristics and produce photoacoustic signal at longer wavelengths (1,064 nm) as shown by Ku and colleagues (73).

Limitations of SWNTs for human clinical applications comprise mainly the (long-term) toxicity concerns (74). Although the first systematic toxicity evaluation of functionalized SWNTs following intravenous injection in living mice did not show signs of acute or chronic toxicity, more evidence is needed to determine the safety of these SWNTs for in vivo medical applications (75). The nanoparticles encapsulating dyes have the problem of photobleaching, however, their stability can increase up to 500% as compared with the free dye (76). In addition, the surface of the encapsulated dyes can potentially be easier to chemically modify than just the free dye by itself. Quantum dots are highly photostable, but their main drawback for biomedical applications is that their major components, cadmium and selenium, are toxic to cells and organisms (77).

Depending on their characteristics, the nanoparticles can also be loaded with drugs and used as delivery vehicles to allow for simultaneous delivery of therapeutic and imaging agents in vivo (78, 79), or their intrinsic properties can be used for photothermal ablation therapy (80). This provides great opportunities for image-guided therapy/theranostics (combining therapy and diagnostics).

**Multimodality imaging agents**

Because imaging is becoming increasingly important in the diagnosis and treatment of disease, imaging agents that can be tracked by multiple imaging modalities can be very useful in overcoming the limitations of a single imaging modality in the different steps of the diagnosis/treatment process (e.g., using different modalities for whole-body diagnostic imaging before surgery vs. real-time intraoperative imaging). Multimodality imaging agents that can enhance contrast for PAI in combination with other imaging modalities are under development. Nanoparticles that are both plasmonic and superparamagnetic can be used for combined photoacoustic and MRI; examples are nanostars, nanoroses, pearl necklaces, nanowontons, iron oxide and gold-coupled core–shell nanoparticles, and liposomes containing iron oxide nanospheres and gold nanorods (58–62, 80–82). Kim and colleagues used multifunctional microparticles and microspheres for concurrent photoacoustic and ultrasound imaging in phantoms (83). Wilson and colleagues developed photoacoustic nanodroplets, consisting of a droplet of liquid-phase shift perfluorocarbon with a bovine serum albumin shell in which optically absorbing nanoparticles have been suspended (84). These nanodroplets act as dual-imaging agents for both photoacoustic and ultrasound imaging through optically triggered vaporization. Gold nanorods have been labeled by 125I to allow for dual-mode SPECT and PAI after intra-articular injection in rat tails (85). For a triple-modality approach of MRI, PAI, and Raman imaging, our research group recently developed “MPR” nanoparticles by modifying gold-based Surface Enhanced Raman Scattering (SERS) nanoparticles with gadolinium (86). This novel molecular imaging agent was then used to accurately help delineate the margins of brain tumors in living mice (Fig. 5). Recently, Grootendorst and colleagues proposed to use a commercially available MRI agent (Endorem; superparamagnetic iron oxide nanoparticles), as a photoacoustic probe for intraoperative imaging of lymph nodes and demonstrated the feasibility ex vivo in rats (87).

**Genetically encoded probes**

Genetically encoded fluorescent proteins have been widely used for more than a decade in preclinical optical imaging studies. The use of PAI for reporter gene imaging was first demonstrated by Li and colleagues in 2008 (88). They used the lacZ gene, which encodes the enzyme β-galactosidase. After injection of a colorless analog of lactose, “X-gal,” cleavage of this substrate by β-galactosidase eventually leads to the formation of a blue product, which can then be detected by PAI due to its high optical absorption. In later studies, they showed that they could reach imaging depths of approximately 5 cm with this technique, with spatial resolutions of approximately 1 mm, which is a major improvement over traditional optical (bioluminescence or fluorescence) reporter gene imaging, reaching depths of up to approximately 1 cm (89). Filonov and colleagues showed that a bacteriophytochrome-based NIR fluorescent protein named iRFP could also serve as a probe for PAI (90). It can produce stronger photoacoustic signal than blood in the NIR window, does not require injection of exogenous substrates, and can be delivered into the body by standard genetic manipulations of cells. Ha and colleagues then reported the potential of ferritin as a reporter gene suitable for PAI in vitro (91). Recently, Qin and colleagues demonstrated in vitro and in vivo in mice that the tyrosinase gene as a single reporter gene could be used for triple-modality imaging by PAI, MRI, and PET, through catalyzing the synthesis of melanin (92). These genetically encoded proteins have vast potential for whole-body animal imaging, however, the use of genetically encoded probes remains challenging for human applications due to the requirement of introduction of a reporter gene. Nevertheless, for many small animal model applications or cell therapy applications in humans, reporter gene strategies provide a potentially powerful approach.
Clinical Applications

Breast imaging

The conventional techniques for breast imaging include digital mammography, ultrasonography, scintimammography, and MRI. A combination of these methods usually yields relatively high sensitivity and specificity for cancer detection. Each of the techniques has some drawbacks. Mammography is associated with ionizing radiation and has decreased...
sensitivity for cancer detection in dense breasts. The results of ultrasound greatly depend on the instrument performance and examiner’s skill. MRI requires more time and cost and requires the use of imaging agents, to get dynamic information about neovascularization. New imaging technologies are needed that can aid in early detection of breast cancer and provide comprehensive information of the tumor properties potentially important for treatment decision and especially in assessment of response to therapy. Increased use of preoperative systemic therapy and differentiation of therapy in general, depending on the tumor properties, require more functional/molecular tumor information that PAI may be able to provide.

PAI is now being investigated in initial patient studies in breast imaging. Five studies published so far have involved patients (8, 9, 31, 93, 94). Two of the studies are continuing clinical attempts with The Twente Photoacoustic Mammoscope (PAM), developed at the University of Twente (Enschede, the Netherlands). It consists of a bed on which the patient lies in the prone position with her breast pendant through an aperture. In the scanning compartment of the system, the breast is gently compressed between a glass plate and a flat ultrasound detector matrix, with acoustic coupling gel applied to a depth of 40 mm without the use of a molecular imaging agent (see Supplementary Data; http://www.optosonics.com/breast-images.html). So far, no patients with breast cancer have been imaged with this system, but neoplastic breast lesions could potentially be visualized on the basis of their hemoglobin content.

Kitai and colleagues recently published an article in which 27 tumors in 26 subjects were visualized using a prototype photoacoustic mammography system (93). The patients were examined lying in the prone position with the breasts mildly compressed in a craniocaudal direction between holding plates. Pulsed laser beams irradiated the breast from both sides, and photoacoustic signals were detected on the caudal side by a transducer array. The measurable area was 30 mm × 46 mm for one scan, which took 45 seconds. The lesions
included 21 invasive breast cancers (IBC), five ductal carcinoma in situ (DCIS), and one phyllodes tumor. Nine of 21 patients with IBC had received primary systemic therapy (PST). Eight of 12 IBC without PST were visible.

Detection was possible in all 5 cases with DCIS, whereas it was not in the 1 case with phyllodes tumor. Seven of nine IBCs with PST were assigned as visible despite decreased tumor size after PST. The mean value of hemoglobin saturation in the blood vessels of visible lesions was 78.6% (they reported values of >92% for normal subcutaneous blood vessels) and the average total hemoglobin content in these lesions was 207 μmol/L. These encouraging results show the potential to evaluate and quantify functional information, which may be useful in diagnosing and assessing treatment response in breast cancer.

Other systems with potential applications for breast cancer imaging have been developed, but not yet clinically tested. The optoacoustic plus ultrasound system (OPUS), is a handheld combination of a wavelength-tunable pulsed laser and a commercial ultrasound scanner (97). By using suitable image reconstruction algorithms and quantification procedures, a linear relation between local optical absorption and photoacoustic signal was found for absorbers at different depths and optical matrix properties.

The Wang laboratory has developed an integrated PAT and thermoacoustic tomography (TAT) system that combines nonionizing radiofrequency waves and NIR laser light to obtain additional information from breast tissue, such as water/ion concentration, blood volume, and oxygenation level of hemoglobin (98). As opposed to breast compression from the sides, used in X-ray mammography, this PAT/TAT scanner uses front compression to give the breast a cylindrical shape and obtain a full 3D dataset. The front compression may possibly allow for imaging the regions close to the chest wall. Also, dry coupling is used instead of the usual coupling gel in conventional ultrasound imaging. Good quality PAT and TAT images on tissue mimicking phantoms have so far been achieved (98, 99).

So far, out of all of the potential clinical applications, translation of PAI has reached the furthest in breast imaging. Several groups are working on improved clinical systems with better depth penetration and higher spatial resolution (100, 101), which will help move photoacoustic breast imaging forward. Sentinel lymph node detection in breast cancer. The presence of axillary lymph node metastases is the most important factor in staging of early breast cancer and during the last decade, sentinel lymph node biopsy (SLNB) has taken a leading role in the staging procedure. Although SLNB could also be useful in other type of cancers, for example, melanoma or breast tumor site with the combination of a radiolabeled sulfur colloid and a blue dye. The clinical procedure varies, but can involve presurgical scintigraphic imaging, and pre/peri-operative use of a gamma probe or Geiger counter to locate the SLN. After visual confirmation, the SLN is surgically resected, and the definitive presence of cancer is established with histologic methods. SLNB can help avoiding morbidities associated with complete axillary nodal dissection, which was the prior standard. So far, radiocolloids and blue dyes are generally considered complementary in SLNB and should both be used routinely. However, the procedure could be done without involving radioactivity if the signal from the blue dye alone could be detected in real-time pre/perioperatively with PAI and in combination with ultrasound for anatomic information. Although to a lesser extent, morbidity has also been reported after SLNB (103, 104). Possibly, a PAI-guided approach could avoid surgical intervention of the sentinel node and instead guide a fine-needle aspiration. If PAI allows for noninvasive identification of the SLN, the combination of fine-needle aspiration biopsy and real-time reverse transcription PCR to confirm lymph node status (malignant/benign) might be a minimally invasive alternative to SLNB.

Using PAI, Song and colleagues accurately mapped SLNs noninvasively in living mice and rats upon injection of Evans blue, a blue dye currently used in clinical SLNB (41). They were also able to mimic PAI of “deeper” SLNs by layering additional biologic tissues on top of the rats (>3 cm) and injecting either methylene blue (40), ICG (38), or gold nanocages (105). ICG-stained SLN photoacoustic imaging is shown in Fig. 7. In addition, Kim and colleagues have reported in vivo photoacoustic and ultrasound mapping of ICG- or methylene blue–stained SLNs in rats using a clinical ultrasound array system, and they also demonstrated guidance of SLN fine-needle aspiration biopsies using this technique (7, 106, 107). SNL detection by PAI and ICG-enhanced SWNTs was evaluated in rats by Koo and colleagues (70). Akers and colleagues recently reported the use of SPECT/CT to validate PAI in SLN detection (108). Although SLN imaging is a PAI application under intensive research in animal models in vivo, its feasibility remains to be shown in humans.

Urologic imaging

Prostate imaging. Standard screening and diagnostic methods for prostate cancer such as blood screening for prostate-specific antigen (PSA) and other biomarkers, digital rectal examination (DRE), and transrectal ultrasound (TRUS)– guided prostate biopsy—have limited ability (sensitivity and specificity) in the early diagnosis of prostate cancer (109, 110). Furthermore, the available techniques cannot fully differentiate between aggressive and more latent prostate cancers with the consequence that there is no significant impact on survival. This leads to overdiagnosis and overtreatment (110). A combined transrectal probe with ultrasound and PAI for prostate imaging to combine anatomic, functional, and molecular imaging is a potentially useful approach to move prostate imaging and diagnosis forward. To date, there are no reports on PAI for prostate imaging in humans, yet several groups have conducted promising studies in vivo. In canine prostate, induced blood lesions have successfully...
been imaged (Fig. 8; refs. 111–113); in a rat model, prostate adenocarcinoma tumors have been visualized (32) and in a mouse window chamber model, prostate tumor progression has been imaged with 3D photoacoustic and pulse echo imaging, both without the use of a molecular imaging agent (114) and with the use of gold nanorods to enhance image contrast (115). ICG embedded PEBBLEs conjugated with HER-2 antibody showed high contrast and high efficiency for binding to prostate cancer cells in initial in vitro characterization studies (44).

Among the treatment options available for prostate cancer, the use of brachytherapy seeds, metal implants that deliver localized radiotherapy, is an important clinical approach (116, 117). Because visualization of the small seeds can be difficult, the TRUS-derived position of the needles delivering the seeds to the tissues is often relied upon to infer seed placement. Correct seed placement is important for dose planning management. Because metals have an optical absorption that is orders of magnitude greater than that of soft tissue, PAI may be very valuable as an adjunct to TRUS in seed placement imaging. Su and colleagues experimentally evaluated PAI in combination with TRUS for brachytherapy seed visualization in excised bovine prostate tissue. They found that PAI improved imaging contrast from 2.7 to 27.9 dB over TRUS alone (for seeds in the long-axis orientation) at imaging depths ranging from 4 to 13 mm (118). Harrison and Zemp investigated PAI for brachytherapy seed visualization in a tissue phantom, comparing the received intensity to endogenous contrast. They found that PAI at 1,064 nm could identify brachytherapy seeds uniquely at laser penetration depths of 5 cm in biologic tissue at the ANSI limit for human exposure with a contrast-to-noise ratio of 26.5 dB (119). Kuo and colleagues recently showed imaging of brachytherapy seeds in an ex vivo dog prostate phantom (120).

In summary, to move the clinical translation of photoacoustic prostate imaging forward, there is a need for the development of a transrectal PAI/ultrasound device with sufficient depth penetration and spatial resolution. Also, better characterization of biologic prostate tumor signatures (e.g., cell surface receptors, degree of vascularization) is important to identify potential targets for a more specific diagnosis of prostate cancer by PAI, with or without the use of extrinsic molecular imaging agents.

**Bladder imaging.** Because urethrocystoscopy cannot determine the extent of invasive bladder cancer and is not always able to discriminate between flat carcinoma in situ and inflammation, PAI may be able to give additional diagnostic and/or prognostic information in these cases. In an initial study by Xie and colleagues, the bladder microvasculature was successfully visualized ex vivo using microscopic PAI on canine and human bladder specimens (121).

Figure 7. Photoacoustic images of SLN in a rat in vivo after ICG injection. A, image at 668 nm 0.2 hour after ICG injection. B, image at 618 nm 2.2 hours after injection. C, image at 668 nm 2.8 hours after injection. D, graph shows comparison of spectroscopic photoacoustic (PA) signals within the SLN region over a period of time. E, graph shows comparison of spectroscopic photoacoustic signals within blood vessels (BV) over a period of time. LV, lymphatic vessel. Numbers in colored bar at top of D and E equal wavelength in nanometers. Reprinted with permission from The Radiological Society of North America, Kim and colleagues (38). Copyright 2010 RSNA.
A different application is evaluation of vesicoureteral reflux (VUR). VUR is abnormal leakage of urine from the bladder to the upper urinary tract, which may be a source of infections and eventually renal scarring, especially in children. Current imaging methods to diagnose and monitor VUR are X-ray or radionuclide based and the possibility to use a combination of ultrasound and PAI instead, and hence avoid ionizing radiation in children, would be a step forward. Kim and colleagues explored noninvasive photoacoustic bladder imaging in vivo by mapping rat bladders filled with methylene blue (122), whereas Koo and colleagues visualized the rat bladders using ICG-enhanced SWNTs (70). These methods may in the future be useful for VUR monitoring in children.

Photoacoustic bladder imaging is certainly in its infancy and many technical issues need to be resolved before clinical translation is feasible. In addition, further investigations of exogenous molecular imaging agents, especially about safety and suitability for this application, are warranted before exploration of the technique in humans.

Dermatologic imaging

Melanoma is the most aggressive form of cutaneous cancer, and the most difficult to diagnose and stage (123). Detection typically relies on visual evaluation of the morphology of a lesion, followed by biopsy and histopathologic assessment of suspicious lesions. To improve melanoma detection, various noninvasive imaging techniques, including PAI, are under investigation. Most melanomas contain melanin, which is a highly light-absorbing pigment. Melanin provides great contrast for PAI in the “optical window” (~600–1,000 nm; see Fig. 2) without having to inject an imaging agent. PAM is most frequently explored for melanoma detection and staging, using various imaging systems such as dark-field PAM, with depth penetrations of up to 3 mm (17). The first proof-of-principle of this technique in vivo was shown by Zhang and colleagues, imaging angiogenesis, melanoma, and SO2 of single vessels in animals, and total hemoglobin concentration in humans (2). This group illustrated that dual-wavelength imaging can separate the contributions from two different pigments, in this case hemoglobin and melanin, based on their absorption spectra. In further studies, they measured the metabolic rate of oxygen in melanoma using PAM (124). In addition, they demonstrated that targeted melanoma imaging could enhance the photoacoustic contrast by approximately 300%, using biocjugated gold nanocages targeted to the α-melanocyte–stimulating hormone receptors overexpressed on melanomas (Fig. 9; ref. 53). Favazza and colleagues investigated PAM in vivo in volunteers by imaging a melanocytic nevus and assessing the vascular and structural differences between palm and forearm skin (125). Recently, photoacoustic detection of melanoma metastases based on their melanin content proved to be possible in resected human lymph nodes (126).
summary, PAM can noninvasively (without the use of an imaging agent) obtain functional information on tissue that may be helpful in the diagnosis and staging of melanoma. Biopsy is the standard method for histology and diagnosis of melanoma, although it relies on biopsying a representative area of the often heterogeneous tumor. With the use of PAM, it might be possible to "map" the lesion and guide what area to biopsy, potentially increasing the diagnostic accuracy and avoiding unnecessary biopsies. One of the challenges is that a subgroup of melanomas does not contain melanin, and thus require a different PAI approach, possibly involving the injection of a molecular imaging agent. Another critical point in the development of more accurate diagnostic tools is the need for a high-resolution technique to decide whether the cancer cells have invaded through the basement membrane (123), enabling them to spread to distant tissues. Further in vivo studies are thus required to determine whether PAM can meet these clinical needs.

In addition to assessing malignant melanoma, PAI may also be valuable in the evaluation of other skin conditions, such as burn wounds. Because clinical characterization of burn wounds can be challenging, the assessment of tissue viability by imaging intact dermal vasculature may help in determining an appropriate treatment plan. So far, PAM has been explored for burn wound assessment in rat and pig models with promising results (127–130). Human studies have not been performed yet, but this seems to be within reach in the near future.

Gynecologic imaging

Ovarian cancer has the highest mortality of all gynecologic cancers due to the late onset of symptoms in combination with a lack of effective methods for early detection (58). At present, there is no existing evidence that any screening test, including CA-125, ultrasound, or pelvic examination, reduces mortality from ovarian cancer (131). The currently used imaging techniques, for instance ultrasound, have poor sensitivity and specificity for the detection of early-stage cancers (132). Because PAI can be easily integrated in a clinical ultrasound system, such a dual system could potentially enhance the diagnostic accuracy of ovarian cancer detection. Aguirre and colleagues developed a noninvasive transvaginal coregistered 3D ultrasound and PAI-system, and evaluated the optical properties of normal porcine ovarian tissue with this system in vivo (133). The results showed strong light absorption from highly vascularized corpora lutea and low absorption from follicles, demonstrating that PAI is capable of detecting vascularized structures in the ovaries, otherwise not visible with ultrasound. These findings were further evaluated in 33 ex vivo human ovaries (134). Quantification of the light absorption levels in the ovary indicated that, in the postmenopausal group, malignant ovaries showed significantly higher light absorption than normal ones ($P = 0.0237$) corresponding to a sensitivity of 83% and a specificity of 83%. The same group presented an integrated optical coherence tomography (OCT), ultrasound, and PAI prototype endoscopy system, with a diameter suitable for insertion through a standard endoscopic laparoscopic port during minimally invasive surgery (5 mm; ref. 135). Porcine and human ovarian tissues were imaged successfully ex vivo, showing the potential of the hybrid device over each modality alone in ovarian tissue characterization. Kumavor and colleagues have recently developed a coregistered pulse-echo/photoacoustic transvaginal probe and in initial experiments human ovaries ex vivo were imaged (136). The mentioned combined techniques for ovarian imaging show promise for clinical translation, but results remain to be validated in vivo and eventually tested for diagnostic accuracy compared with existing imaging techniques. PAI could potentially also play a role in other gynecologic diseases, such as cervical and endometrial cancer, but so far these areas have not been explored.

Hematologic imaging

Circulating tumor cells (CTC) can often be detected in the blood of patients with early or advanced cancer by antibody-based assays or molecular methods. CTC detection is an area of intense research, studying patients with different types of cancer, including breast, colorectal, prostate, and skin cancer. In several studies, CTC detection in early and/or metastatic disease has been shown to correlate with unfavorable clinical outcome (137). There are several different technologies for CTC detection in vivo, but the two main approaches are the use of monoclonal antibodies directed against histogenic proteins (e.g., cytokeratins and epithelial cell adhesion molecule) and PCR-based molecular assays amplifying tissue-specific transcripts (138). The major drawback for both of these techniques is their limited sensitivity due to the analysis of only small blood volumes. To overcome this problem, flow cytometry techniques could be used to assess larger blood volumes in vivo. The fluorescent labels commonly used in flow cytometry have certain limitations, such as their potential toxicity and immune response to the fluorescent tags. Photoacoustic flow cytometry has the advantage that label-free detection of cells with high absorption/scattering characteristics, such as the CTCs from melanomas for instance, is possible. Malignant melanomas are known to metastasize at early stages and the presence of CTCs seems to be a prognostic marker (139, 140). The intrinsic photoacoustic contrast, coming from the melanin in the cells, makes photoacoustic flow cytometry an ideal candidate for the label-free detection of CTCs from melanoma, as has been shown by several research groups in vitro (141–145) as well as in vivo (146–148). Nedosekin and colleagues recently reported on in vivo ultrafast photoacoustic flow cytometry, showing the detection of circulating human melanoma cells in large blood vessels in mice by using the intrinsic photoacoustic signal of melanin, and by targeting the tumor cells with magnetic nanoparticles (148). McCormack and colleagues used targeted gold nanoparticles to increase the signal from melanoma cells in a stationary in vitro system (144). The same group was able to use targeted gold nanoparticles in a breast cancer cell line and detect the nonpigmented CTCs with their system. Although they did not specifically show the detection of individual cells due to cell clumping, they claim that their system may be capable of this in clinical samples (149). Label-free or targeted photoacoustic CTC detection methods may be translated into human studies in the near future. Possible clinical applications to be evaluated include blood screening...
Figure 9. In vivo noninvasive photoacoustic (PA) time-course coronal maximum amplitude projection (MAP) images of B16 melanomas using targeted [Nle4, D-Phe7]-α-melanocyte-stimulating hormone gold nanocages [Nle4, D-Phe7]-α-MSH-AuNCs and nontargeted PEG gold nanocages (PEG-AuNCs). A and E, photographs of nude mice transplanted with B16 melanomas before injection of [Nle4, D-Phe7]-α-MSH-AuNCs (A) and PEG-AuNCs (E). Time-course photoacoustic images of the B16 melanomas after intravenous injection with 100 μL of 10 nmol/L [Nle4, D-Phe7]-α-MSH-AuNCs (B–D) and PEG-AuNCs (F–H) via tail vein. The background vasculature images were obtained at 570 nm (ultrasonic frequency = 50 MHz), and the melanoma images were obtained at 778 nm (ultrasonic frequency = 10 MHz). Red, blood vessels; yellow, the increase in photoacoustic amplitude. Reprinted with permission from Kim and colleagues (53). Copyright 2010 American Chemical Society.
for early CTCs before the development of metastases to predict patient prognosis; testing for disease recurrence and individualized treatment monitoring through CTC counting; evaluation of surgical margins for CTCs; and even the inhibition/prevention of the development of metastases through therapeutic elimination of CTCs (150).

In addition to tumor cell detection in the circulation, PAI may also be very useful for visualization of the vessel wall itself, as several reports on atherosclerotic plaque characterization illustrate (37, 151, 152). Catheters that integrate intravascular ultrasound (IVUS) and intravascular photoacoustic (IVPA) imaging have been designed to approach tissues of interest through the blood vessels (153–159). In the future, these types of catheters could be used during intravascular procedures in a cardiovascular disease setting (e.g., coronary plaque characterization and stent placement), but possibly also in an oncologic setting (e.g., guidance of intravascular drug delivery directly to the tumor site).

**Ophthalmic imaging**

Ultrasound, OCT, and fluorescent angiography play an important role in diagnostic imaging of the eye. Because PAI depicts optical absorption, which is independent of the tissue characteristics imaged by OCT or ultrasound, this technique can provide additional physiologic information in ocular imaging. The eye is a very suitable application for optical imaging due to its accessibility and optical properties. The major light absorbing molecules in the eye are melanin and hemoglobin. Melanin is present in the uvea (iris, choroid, ciliary body) and in pigmented tumors, whereas hemoglobin is present in the abundant microvasculature of the uveal tract, in superficial microvessels of the eye (retina, conjunctiva) and in pathologic neovascularization (including tumors). The physiologic information provided by PAI, for example, changes in microcirculation, hypoxia, and nevi formation, could prove highly valuable in the diagnosis of ocular disease. Not only for the detection and characterization of cancer of the eye (e.g., intraocular melanoma, lymphoma, retinoblastoma, and metastases), but also for other blinding diseases such as diabetic retinopathy, age-related macular degeneration, and glaucoma. To date, several efforts have been made to develop PAI methods suitable for ophthalmologic applications, although so far only tested in experimental settings. Our laboratories have developed a photoacoustic ocular imaging device and demonstrated its utility in imaging in living rabbits (the deeper layers of the eye including the retina, choroid, and optic nerve; Fig. 10 (160). Hu and colleagues have shown label-free photoacoustic angiography of the eye in mice (161). This is an alternative to the commonly used fluorescence angiography that requires injection of an imaging agent (fluorescein or ICG), which may have side effects. Song and colleagues have built several PAI platforms for ophthalmic imaging in rodents, including hybrid systems that combine photoacoustic ophthalmoscopy with other techniques such as OCT and fluorescence imaging (162–165). Nevertheless, there is a need for further optimization and evaluation of ocular PAI systems before they can be tested in humans and evaluated against existing diagnostic techniques.

**Bone and joint imaging**

Peripheral joint imaging seems to be a highly accessible and promising area for PAI, as demonstrated in several pilot studies of human finger joints (166–169). In combination with conventional US, dual-modality imaging can potentially provide both morphologic and physiologic information on joints, such as blood flow, blood volume, hemoglobin oxygenation, and vascular density. It would be an advantage to be able to show that PAI early on can visualize the physiologic changes in the articular tissue appearing before the late morphologic changes. This would help to diagnose and treat joint disease at an earlier stage before it has become more manifested. In addition, by visualizing these physiologic features, PAI may be able to play a role in the diagnosis of primary bone cancer or bone metastases. A first, study by Hu and colleagues demonstrated in vivo PAI of osteosarcoma in a rat leg, monitoring tumor development and showing apparent changes in imaging characteristics over the course of 2 weeks after cell injection (170). Although these initial results are encouraging, more research is needed to verify findings in human studies and to overcome certain critical challenges of PAI, such as the limited depth penetration in bone (even more limited than in soft tissue).

**Neonatal brain imaging**

Ultrasound imaging is an established pediatric brain imaging modality that is used before the fontanelles are closed. After the closure of the fontanelles, the image quality degrades significantly because the skull severely attenuates and scatters ultrasonic waves. Transcranial ultrasonic brain imaging of adults is also limited by the inhomogeneous aberrating effect of the skull bone. Yang and Wang applied PAT to image the brain cortex of a monkey through the intact scalp and skull cx vivo. The reconstructed PAT image showed the major blood vessels on the monkey brain cortex (171). Because the human skull scatters light strongly, the same group added a photon recycler to the PAT system, to reflect back-scattered light back to the skull, which in turn increased light transmittance through the skull (172). Recently Sussman and colleagues used PAT in a neonatal rat model to demonstrate in vivo alterations in cerebral hemodynamics in a noninvasive and near real-time fashion (173). These results suggest that PAT may have the potential for human brain cortex imaging in infants or even adults although further technical development is needed before proceeding to humans.

**Gastrointestinal imaging**

Endoscopic imaging is the main diagnostic tool in gastrointestinal diseases. Mostly, endoscopic imaging relies on wide-field white-light optical methods, which are limited by what the human eye can see and therefore lack sensitivity to subsurface processes and physiologic changes. Attempts to overcome these limitations include techniques such as autofluorescence imaging, narrow-band imaging, and OCT (174, 175). Photoacoustic endoscopic imaging may have the ability to visualize physiologic processes and to resolve molecular imaging agents that target specific molecular changes in tissue. Subsurface imaging is very well possible with this technique because of the favorable penetration depth (up to several centimeters). The
combination of deeper tissue penetration, high resolution, and real-time imaging makes PAI a valuable add-on to endoscopy. It may allow for the detection of very small lesions (e.g., micrometastases) or otherwise invisible "flat" lesions, probably even more so with the use of targeted molecular imaging agents. Intraoperatively, PAI could help in determining the extent of the tumor before surgical removal, and the completeness of the excision could be checked immediately. Also, this technique could assist in guiding the anastomosis procedures.

Only a few studies have been published in this application area. Recently, Yang and colleagues presented their endoscopy device that can acquire photoacoustic and ultrasonic data simultaneously. This device was tested in vivo in rabbit esophagi and in rat colons. With this technique, the esophagus and surrounding organs could be visualized, including the adjacent vasculature that was only visible in photoacoustic mode. Also, the vasculature in the colon wall could be seen, and after injection of Evans blue dye, the adjacent lymph structures became visible. Moreover, differences in oxygen saturation values could be indicated between the aorta and caudal vena cava in the rabbits (176). Yuan and colleagues and Sheaff and colleagues both describe a preclinical photoacoustic
endoscopic techniques that they are developing (177, 178). The endoscope built by Yuan and colleagues was able to discriminate cancerous human colorectal tissue from normal human colorectal tissue \textit{ex vivo}, with contrast ratios of approximately 2 (177). No clinical studies have been performed yet.

**Thyroid imaging**

Follicular thyroid carcinomas cannot be distinguished from adenomas based on their clinical, imaging, or cytologic assessment, but always require surgical biopsy for diagnosis. Because the majority of nodules (3/4) are benign, the need for new noninvasive detection methods to avoid unnecessary surgeries is clear, and PAI may be a valid candidate. To date, there are no studies published on photoacoustic thyroid imaging, but this application is currently under exploration by our research group (36). We developed an enzyme-activatable photoacoustic probe that was previously described in this review (35). The presence and activity of two members of the MMP family, MMP-2 and MMP-9, suggested to differentiate between malignant and benign lesions, were studied in a thyroid tumor mouse model. \textit{In vivo} PAI results showed significantly higher signal in tumors injected with the enzyme-activatable probe than in tumors injected with the control (noncleavable) probe, indicating the probe's potential to distinguish between benign adenomas and MMP-rich follicular carcinomas. We believe that with the combination of high spatial resolution and signal specificity, molecular PAI holds great promise as a noninvasive method for early diagnosis of follicular thyroid carcinomas.

**Intraoperative imaging**

Intraoperative is another area where PAI has great potential, particularly because it can help the surgeon to see immediately beyond the resection surface. A portable PAI could be brought into the operating room and provide real-time images similar to intraoperative ultrasound. However, the optical contrast provides additional information over ultrasound images and could for instance help assess oxygen saturation and perfusion status of an organ (viability), useful in, for example, bowel resections, organ transplants, and amputations. Surgical resection also plays a critical role in the treatment of many cancers. It is of ultimate importance to reassess complete resection of the tumor mass to diminish the risk for local recurrence and improve survival rates. CT, MRI, or PET-CT imaging (depending on tumor type) can give information on tumor extent before surgery, but during the operative procedure, the surgeon currently relies on visual appearance and palpation. Several intraoperative imaging methods have been evaluated, such as ultrasound and intraoperative MRI, but they have been found either too time consuming or lacking sufficient spatial resolution. PAI could potentially provide a solution to this problem. The exploration of PAI in the intraoperative setting is still in its infancy. Xi and colleagues report a microelectromechanical system–based intraoperative PAT (iPAT) technique, and demonstrate its ability to accurately map tumors in 3D and to inspect the completeness of tumor resection during surgery in a tumor-bearing mouse model (179). They propose potential use of the iPAT technique in breast cancer surgery although they point out the need for development of a handheld probe with increased depth resolution before clinical translation of the technique. Furthermore, to deal with the strong effect of blood (from intraoperative bleeding) on PAI absorption, they suggest the use of targeted molecular imaging agents to provide enough contrast. In a recent review, Su and colleagues discuss potential future intraoperative imaging methods for malignant gliomas, a common type of brain tumor. Among other techniques, they point out that PAI in combination with optical imaging agents could be promising (180). This application has been explored by our laboratory, with the development of a triple-modality MRI–PAI –Raman imaging nanoparticle that can accurately help delineate the margins of brain tumors in living mice both preoperatively and intraoperatively (see also Fig. 5; ref. 86).

Besides tumor delineation and assessment of its margins, PAI could also play a role in the assessment of metastases, in particular the lymph node status. Previously, we discussed the SLN procedure for breast cancer. The lymph node status for other types of cancer could also be explored by PAI during tumor resection, for instance, in the case of an abdominal tumor, the surgeon would be able to make quick decisions on the resection of suspicious (mesenteric) lymph nodes during laparotomy or laparoscopy. For this situation, most likely an imaging agent would have to be injected to provide enough sensitivity and specificity. Grootendorst and colleagues have shown in healthy rats that superparamagnetic iron oxide nanoparticles (Endorem) can be used as a PAI agent for lymph node visualization (87). More recently, they have shown promising results using the same nanoparticles in an \textit{ex vivo} intraoperative setting in a metastatic prostate cancer model in rats (181).

**Combination of diagnostics and therapy**

PAI (combined with other modalities) could also be used in a clinical setting to guide treatment and to combine diagnostic information with therapy ("theranostics"). PAI has recently been proposed to guide and/or monitor therapy based on high-intensity focused ultrasound (HIFU; ref. 182). Prost and colleagues show a lesion obtained \textit{in vitro} in chicken breast by focusing HIFU based on the photoacoustic signals emitted by an optical absorber placed in the tissue (183). Gold nanoparticle–mediated hyperthermia shows particular promise in animal studies and is currently being investigated in early clinical studies (184). The visualization and tracking of temporarily or permanently implanted metal devices, such as biopsy needles and needles used for localized drug delivery, have been studied with PAI (185). The results suggest that PAI, combined with ultrasound imaging, is capable of real-time, high-contrast, and high-spatial resolution visualization of metal implants within anatomic landmarks of the background tissue.

**Outlook**

PAI holds the promise of becoming a valuable tool in multiple clinical areas in which there are unmet needs. This technique has several important strengths over the currently

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available imaging techniques; in particular, its ability to obtain functional and molecular information from tissue, even without the use of a molecular imaging agent, along with its low cost, portability, lack of ionizing radiation, and feasibility of real-time imaging at high resolution and clinically relevant depths. PAI can be used not only in a noninvasive way, but also in an intraoperative or endoscopic imaging setting. The combination of PAI with existing imaging techniques (multimodal imaging) or delivery of therapeutics will likely add even more to its potential.

Besides these major advantages, there are still important challenges to overcome. Clinicians will need to get familiar with the PAI technique and become aware of its capabilities and limitations. The most obvious limitation for clinical applications of PAI is its penetration depth, dependent on the limited penetration of light in tissue, which will likely not reach beyond several cm (~7 cm; Table 1) in the NIR wavelength range. Because spatial resolution is associated with penetration depth in PAI, the detection of smaller lesions (a few mm) located deeper in the tissue will need to be investigated. As for the propagation of ultrasound waves in tissue, the same limitations apply as in ultrasound imaging, that is, limited penetration through bone and air.

Although the PAI technology has already reached a level that can be used in clinical studies, there is still much room for improvement. Fast and tunable systems that can provide multispectral images in real-time should be developed, and PAI systems need to become stable and user-friendly with regard to data acquisition and analysis; in particular, progress in quantitative data analysis is essential. The development of miniaturized probes is needed for endoscopic, intravascular, and intravascular PAI. For intravascular clinical use, the photoacoustic probe should be scaled down to <1 mm in diameter (157).

Image contrast and sensitivity of detection represent another area of needed improvement for PAI. Because one of the main advantages of PAI is that endogenous chromophores can be imaged without the injection of an exogenous imaging agent, this intrinsic contrast will most likely be the primary focus of initial clinical studies. Therefore, we will need to learn more about the distribution of intrinsic features, such as neoangiogenesis and hypoxia/oxygen saturation, within and between the various cancer types and benign lesions. Tumors that are less vascularized could be a caveat, but overall this intrinsic contrast may provide us with substantial functional information on tissue. To enhance photoacoustic image contrast, exogenous molecular imaging agents are under development and will need to be approved for use in humans. Several key concerns about these molecular imaging agents remain. These include (i) the ability to produce sufficient PAI signal with a relatively low dose of injected imaging agent. It may be the case that for some agents, insufficient amounts can be delivered to the target site(s) because of the limits in the total mass that can be injected while still being nontoxic to the subject. (ii) Regulatory issues that have held up the translation of optical imaging agents will likely slow down the clinical translation of PAI agents as well. (iii) The ability to show the added value of using an imaging agent in addition to intrinsic PAI signal will have to be carefully studied. Nevertheless, once relevant molecular imaging agents become available for clinical PAI, this technique may play an even more significant role in a variety of diseases with respect to their detection, staging, treatment planning, and monitoring (see also Fig. 3).

In conclusion, the recent surge in interest in PAI has provided us with a wealth of experience and knowledge from preclinical studies. Promising results and technologic advances lay the foundation for clinical translation of this technique. Although challenges remain, few of them are beyond reach. Further developments in laser sources and detectors, image processing algorithms, and molecular imaging agents, will open up opportunities for PAI in disease detection, treatment surveillance, and targeted therapy in a range of pathologies. Its ability for high-resolution molecular imaging in real-time, its nonionizing properties, and its compatibility with existing imaging systems (ultrasound in particular), will help strengthen PAI’s position in the fast-growing imaging arena in the near future.

Disclosure of Potential Conflicts of Interest

S.S. Gambhir has ownership interest (including patents) in Endra Inc. and is a consultant/advisory board member of Visualsonics Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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