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Recent progress in photoacoustic molecular imaging

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By acoustically detecting the optical absorption contrast, photoacoustic (PA) tomography (PAT) has broken the penetration limits of traditional high-resolution optical imaging. Through spectroscopic analysis of the target's optical absorption, PAT can identify a wealth of endogenous and exogenous molecules and thus is inherently capable of molecular imaging with high sensitivity. PAT's molecular sensitivity is uniquely accompanied by non-ionizing radiation, high spatial resolution, and deep penetration in biological tissues, which other optical imaging modalities cannot achieve yet. In this concise review, we summarize the most recent technological advancements in PA molecular imaging and highlight the novel molecular probes specifically made for PAT in deep tissues. We conclude with a brief discussion of the opportunities for future advancements.

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Introduction

Photoacoustic tomography (PAT, also referred to as optoacoustic or thermoacoustic tomography) is based on the photoacoustic effect [1], in which ultrasonic waves generated by optical excitation are detected to map the original optical energy deposition [2–6]. PAT naturally utilizes rich optical absorption contrast and weak acoustic scattering inherent in biological tissue, lending it a clear advantage over traditional high-resolution optical imaging in retrieving anatomical, functional, molecular, metabolic, and histologic information at large depths.

One of the strengths of PAT is its inherent molecular sensitivity. Unlike fluorescent imaging which relies on the fluorescent molecule's radiative relaxation, PAT depends on the molecule's thermoelastic expansion through nonradiative relaxation. PAT has a 100% relative sensitivity to small optical absorption variations, meaning that a given percentage change in the optical absorption coefficient yields the same percentage change in the PA signal amplitude [7]. All molecules have unique optical absorption features that can serve as their 'fingerprints' for PA identification. The spatial distribution and optical properties of molecules are often closely related to their microenvironment (e.g. hypoxia in tumors), allowing PAT to probe physiological functions and pathological conditions. Moreover, because acoustic scattering in tissue is much weaker than optical scattering, PAT can harness scattered photons to explore molecular information with high spatial resolution at depths far beyond the optical diffusion limit (~1 mm).

PA molecular imaging has advanced rapidly since the first reports on PAT of the tumor microenvironment in the 2000s [8–10,11•,12]. Almost every technical aspect in PA molecular imaging has progressed, including the detection sensitivity and penetration depth of the imaging system [5,13–19,20••,21••,22•], the quantification accuracy of the signal unmixing [3,13,23–25], and the design and application of molecular probes in deep tissues [10,26–35,36••,37–39]. In this concise review, we focus on the major advancements in PA molecular imaging reported in the last several years (2014–2017) including novel imaging systems, signal unmixing methods, and molecular probes. We also overview the opportunities that may lead to future advances. Readers are referred to recent review articles to gain a more comprehensive knowledge of the principles of PAT [40,41], the molecular contrast agents [34,37,42,26,43], and the biomedical applications [6,16,44–46].

Basic principles of PAT

A typical PAT system includes a short-pulsed laser for efficient wideband PA signal generation, an ultrasonic transducer (or transducer array) for signal detection, a signal amplification and digitization system, and a computer for image formation. PAT has been implemented with two major image formation methods [2]. The first method, direct image formation, is based on mechanical scanning of a focused or unfocused excitation light beam and a focused single-element ultrasonic transducer. The second method, reconstruction image formation, is based on wide-field light illumination and parallel acoustic detection by a multi-element ultrasonic transducer array.

Direct image formation is commonly used in photoacoustic microscopy (PAM), whereas reconstruction image formation is the basis for photoacoustic computed tomography (PACT). Compared to PAM, PACT typically has a higher imaging speed and greater penetration depth but lower spatial resolutions [41]. Depending on the image formation method, PAT may require mechanical or electronic scanning to form two-dimensional (2D) and three-dimensional (3D) images.

PAT complements other imaging methods in contrast mechanism, spatial–temporal resolution, and penetration depth, and has found broad applications in the biomedical research, especially in functional brain mapping [47], cancer diagnosis and staging [44,48], tissue engineering and regenerative medicine [49], developmental biology [50], and molecular cell biology [51], as comprehensively reviewed elsewhere [6,52,53]. In particular, PAT has been widely used for various cancer studies [44], including fundamental research of cancerogenesis [54], cancer detection and staging [55], and navigation and evaluation in cancer treatment [56]. Using either endogenous contrast (e.g. melanin in melanoma cells) or exogenous contrast (e.g. targeted nanoparticles or organic dyes), PAT has become increasingly popular in providing accurate and early diagnosis of cancers [44].

Advances in PAT implementations

Continuous developments in laser technology, ultrasonic detection, digitization electronic systems, and parallel computation have driven technical breakthroughs in PAT technologies. Notably, inspired by PAT's rapid development and its increasingly important role in biomedical research, more and more manufacturers are developing commercial products specifically designed for PAT, including high-energy, high-speed pulsed lasers (e.g. pulsed laser diode illuminator, Quantel-Laser, Inc.), ultra-wideband ultrasonic transducers (e.g. 225 MHz bandwidth transducer, Olympus, Inc.), and high-speed, multi-channel data acquisition systems (e.g. 128 channel DAQ, Ultrasonix, Inc.). Industrial support in the development of PAT technology is critical for accelerating its commercialization and clinical translation.

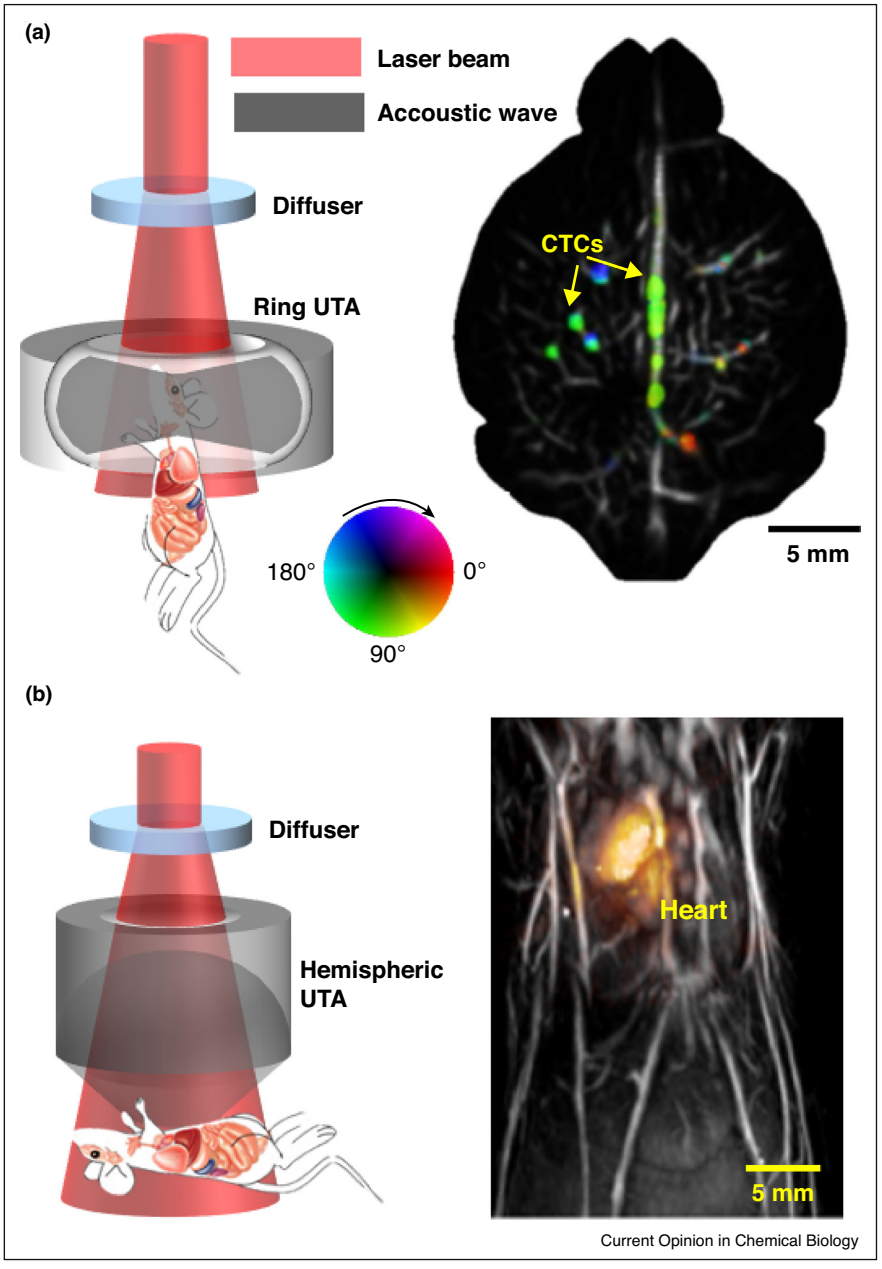
Here, we highlight several recent technological breakthroughs in PAT. First, Real-time, whole-body small animal imaging has been achieved due to high-speed laser sources and data acquisition systems [20••,21••]. We reported a panoramic PACT system with a 125 μm in-plane resolution, 50 Hz 2D frame rate, and 48 mm penetration depth, which is capable of capturing circulating tumor cells in mouse brains (Figure 1a) [20••]. Fehm *et al.* developed a 3D PACT system to capture the dynamics of an entire heart beat with a 100 Hz 3D frame rate within a 1.5 cm^3 volume (Figure 1b) [21••]. These real-time, whole-body PAT systems are extremely powerful when tracking exogenously labeled drug molecules in pharmacokinetic studies on small animal models, thereby enabling biomedical researchers to test new drugs and monitor longitudinal therapy in the future. Second, The spatial resolutions of PAT have been pushing the existing limits through the use of high-frequency wideband ultrasound detection [22•,57•]. Agurirre *et al.* recently reported an ultra-broadband PAM system for human skin imaging with a spatial resolution of 7 μm, enabled by an ultrasonic detection band of 10–180 MHz [22•]. Guggenheim *et al.* developed a PAM system using an ultrasensitive plano-concave microresonator with an ultrasound detection band of 0–40 MHz and a large acceptance angle of 75 degrees [57•]. By matching the ultrasonic detection band with the detectable PA signal spectrum, which is primarily limited by the depth of the target, ultra-wideband ultrasonic detection has enabled multi-scale PA molecular imaging with the highest possible resolutions at different depths.

Advances in signal unmixing methods

Traditionally, spectroscopic imaging is used in PAT to extract the weak signals of molecular probes from the strong background signals of blood, by taking measurements at multiple optical wavelengths [3]. However, this method performs optimally only in superficial tissue because it requires knowledge of the local optical fluence (J/m^2), which is difficult to estimate in deep tissue [13,25]. Novel methods based on two different strategies have been developed to improve the signal unmixing accuracy in PAT [3,16,18,23,25]. The first strategy focuses on optical fluence compensation with tissue property modeling [18,23,24,58–71]. Instead of assuming homogenous optical properties, these advanced model-based methods typically treat the wavelength-dependent local optical fluence as another unknown parameter and then iteratively solve for the concentrations of molecular probes as an inverse problem. For example, Tzoumas *et al.* have recently reported an eigenspectrum-based method that has shown improved accuracy in quantifying deep-tissue blood oxygenation. This method models the local optical fluence as an affine function of only three reference base spectra (Figure 2a,b) [72••]. While this strategy has the potential to recover the concentrations of weakly-absorbing molecular probes, a large number of optical wavelengths (or reference fluence spectra) are needed, which slows down image acquisition. Moreover, the inverse problem is typically ill-posed and computationally intensive.

The second strategy seeks to recover the signal contribution from the molecular probes by exploring the temporal changes of the detected signals, assuming that the changes are confined only to the local molecular probes of interest [27,73–81,82••]. The temporal signal changes can be induced externally (e.g. light illumination) [36••,73] or internally (e.g. chemical cleavage) [74,75,82••]. For

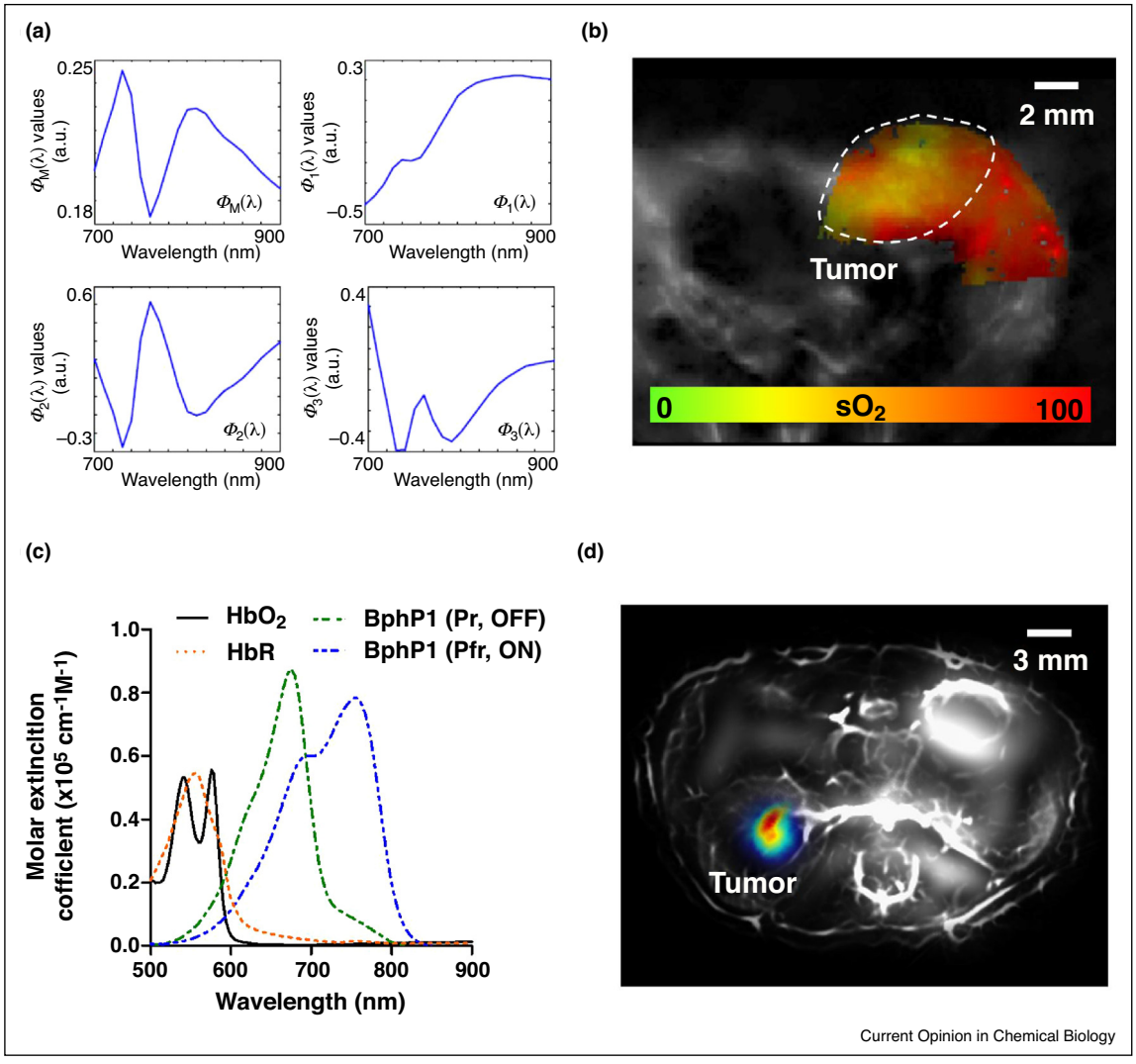
Figure 1



Advances in real-time, whole-body small animal PAT. **(a)** A ring-shaped ultrasonic transducer array (UTA) based panoramic PACT system with a 50 Hz 2D frame rate and 48 mm penetration depth, which is capable of capturing circulating tumor cells (CTCs) in mouse brains [20••]. The colors represent the flow direction of CTCs. Flow speed is radially encoded in the color disk by hue saturation (a greater radius indicates a faster flow speed). **(b)** A hemispherical-shaped UTA based PACT system with a 100 Hz 3D frame rate in a 1.5 cm^3 volume, which is capable of capturing the mouse heart beating [21••].

example, several groups (including the authors') have explored the reversible photoswitching capability of several fluorescent (Dronpa, rsTagRFP) and non-fluorescent (BphP1, AGP1) proteins [36••,73,83]. By turning the molecular probe's optical absorption on or off at a certain wavelength, this temporal modulation can effectively eliminate the constant background signals without needing to know the local optical fluence, thus dramatically enhance the image reconstruction robustness and detection sensitivity (Figure 2c,d). However, the applicability of this strategy is limited to special types of molecular probes whose optical properties can be physically or chemically modulated, such as activatable nanoparticles or photoswitchable proteins [27,36••].

Figure 2



Advances in PAT signal unmixing. **(a)** An eigenspectrum-based signal unmixing method assumes the local optical fluence in deep tissue can be modeled as an affine function of three reference base spectra (Φ_1, Φ_2, and Φ_3) [72••]. **(b)** Eigenspectrum-based blood oxygenation mapping of the breast tumor in a mouse, showing the hypoxia tumor core. **(c)** A photoswitching-based signal unmixing method explores the two absorbing spectra of non-fluorescent protein BphP1. The constant background signals from hemoglobin can be suppressed through differential imaging [36••]. **(d)** Photoswitching-based differential image of the BphP1-expressing tumor in a mouse kidney.

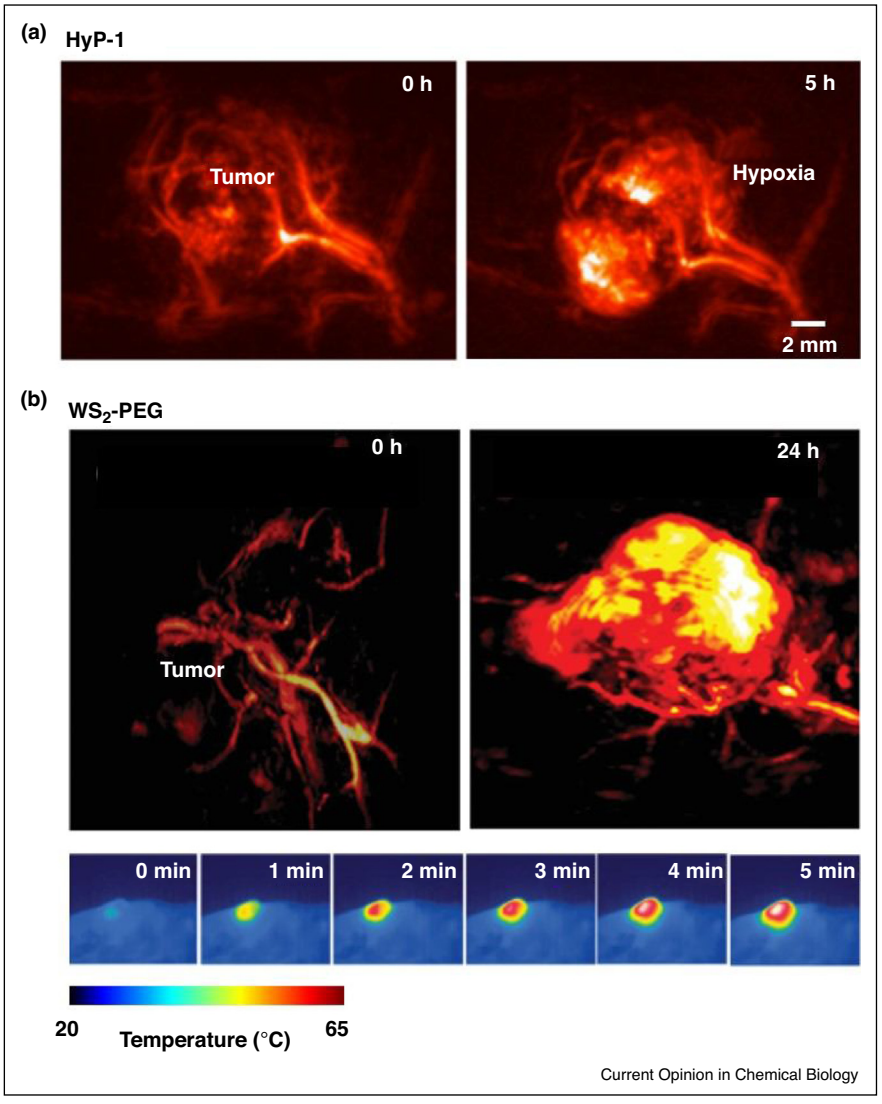
Advances in molecular probes made for PAT

PAT does not rely on fluorescence emission of molecules, giving it the ability to image nearly all molecules, fluorescent or not. Taking advantage of wavelength-tunable optical parametric oscillator (OPO) lasers and Ti:Sapphire lasers, PAT has been implemented to explore numerous molecular probes with primary absorption wavelengths ranging from the ultraviolet to the near-infrared (NIR) region [26]. The ideal molecular probe for PAT should have the following attributes: be specific to the biological process of interest; exhibit maximal absorption in the NIR window for deep *in vivo* imaging; have zero or low fluorescent quantum yield (not strict); be nontoxic to the cells; and be resistant to photobleaching. As PAT draws increasing attention from the biomedical community at large, more and more molecular probes are being developed specifically for PAT by researchers in synthetic chemistry, protein engineering, and nanotechnology. The availability of commercial PAT systems has also accelerated the adoption of these molecular probes in fundamental research areas, including cancer biology [8,31,33,35,39,44,84,85], neuroscience [86,87•,88,89], and regenerative medicine [90–93].

So far, three major strategies have been individually or concurrently implemented in developing PAT-specific

molecular probes. First, Aiming to maximize the penetration depth of PAT, the first strategy focuses on developing contrast agents that have strong optical absorption in the NIR wavelength range with low fluorescent quantum yield [16,85,94–99,100••]. Taking advantage of melanin's strong absorption in the NIR range, Jathoul *et al.* developed a tyrosinase reporter gene system that introduced the key enzyme in melanin synthesis into non-melanogenic cells [100••]. Although melanin's relatively featureless absorption spectrum could make it hard to distinguish from intrinsic signals from hemoglobin, *in vivo* PAT of tyrosinase-expressing cells has shown high sensitivity [99,100••]. Zhou *et al.* recently developed a phosphorus phthalocyanine (P-Pc) dye that has an absorption spectrum peaking around 1000 nm [98••]. P-Pc takes maximum advantage of its large molar extinction coefficient ($1.1 \times 10^5\ cm^{-1}\ M^{-1}$ at 1064 nm) and the strong 1064 nm light from the Nd:YAG lasers, and thus has enabled deep tumor imaging *in vivo*. Second, Aiming to suppress the background signals from blood and improve the detection sensitivity, the second strategy focuses on developing contrast agents that can change their optical absorption in response to external or internal modulations [27,34,44,74–81,101]. Knox *et al.* reported an NIR agent for PA imaging of tissue hypoxia, which features an N-oxide-based trigger that can undergo facile

Figure 3



Advances in PAT molecular probes. **(a)** PACT images showing that HyP-1, an NIR hypoxia-response dye, changed its absorption peak from 670 nm to 760 nm five hours after exposure to the hypoxic environment of a breast tumor in a mouse [82••]. **(b)** PACT images of a mouse breast tumor before and 24 hours after i.v. injection of WS_2-PEG nanosheets, showing the accumulation of WS_2-PEG in the tumor region [102]. The bottom-row images show that, when exposed to 808 nm light, the photothermal effect of WS_2-PEG increased the local tumor temperature by 40 °C within 5 min.

bioreduction in the absence of oxygen and shifts the optical absorption peak from 670 nm to 760 nm (Figure 3a) [82••]. By taking ratiometric measurement, the hypoxic tissue environment (e.g. tumor and ischemia) can be imaged. Third, Aiming to improve the theranostic efficiency in personalized medicine, the third strategy focuses on developing contrast agents that have simultaneous functionalities of imaging and therapy (e.g. photothermal, photodynamic, drug delivery) [77,81,102–105]. Cheng *et al.* demonstrated PEGylated nanosheets for dual-modal CT/PAT guided photothermal therapy of tumors [102]. The strong NIR absorption of the nanosheets provides excellent signals for PAT of the tumor structure, and efficient heating for ablating the tumor cells (Figure 3b).

Conclusion and discussions

Enabled by the advances in system implementations, signal unmixing methods, and molecular probes, PA molecular imaging has become increasingly popular in fundamental research and precision medicine. While this concise review can only cover a small portion of the exciting developments in PA molecular imaging, it has demonstrated the strong potential of this promising technology to continue growing and developing. It is also clear that the development of PAT has become a multidisciplinary effort from laser technology, ultrasound detection, high-speed electronics, mathematics, parallel computation, synthetic chemistry, protein engineering, and nanotechnology. The rapid growth of PAT technologies and their broad applications in biomedical research have, in turn, triggered new opportunities for each discipline.

With a series of long-standing engineering challenges overcome, we believe that PA molecular imaging will see even faster growth in the coming years. In particular, we anticipate four key breakthroughs. First, PA molecular imaging at depths around the optical dissipation limit (~10 cm) will be possible by developing molecular probes that can strongly absorb light in the NIR optical window, while other intrinsic tissue components present the least optical attenuations [106–108]. For example, the effective attenuation coefficient spectrum of human breast tissue has a minimum near 730 nm. Moreover, when the optical scattering effect is compensated for by using wavefront engineering technologies [109,110], PA molecular imaging may approach a sufficient penetration beyond 10 cm. Second, Single-molecule detection by PAT is highly promising using novel ultrasonic detectors with high piezoelectric efficiency (for piezoelectric ultrasound receivers) or high Q-factors (for optical ultrasound receivers) [57•,111,112]. Third, Quantitative PA molecular imaging with high accuracy at depths will be enabled by new imaging methods and mathematical models that can better map the optical properties of the tissue [72••]. Fourth, Finally, PA molecular imaging of neural activities in the deep brain will be achieved by using novel genetically encodable indicators of action potentials or surrogates (e.g. voltage-sensitive or calcium-sensitive proteins) with strong absorption in the NIR spectral region [113].

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