Photoacoustic microscopy: principles and biomedical applications

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Abstract

Photoacoustic microscopy (PAM) has become an increasingly popular technology for biomedical applications, providing anatomical, functional, and molecular information. In this concise review, we first introduce the basic principles and typical system designs of PAM, including optical-resolution PAM and acoustic-resolution PAM. The major imaging characteristics of PAM, i.e. spatial resolutions, penetration depth, and scanning approach are discussed in detail. Then, we introduce the major biomedical applications of PAM, including anatomical imaging across scales from cellular level to organismal level, label-free functional imaging using endogenous biomolecules, and molecular imaging using exogenous contrast agents. Lastly, we discuss the technical and engineering challenges of PAM in the translation to potential clinical impacts.

Keywords Photoacoustic microscopy · Structural imaging · Functional imaging · Molecular imaging · Endogenous biomolecules · Exogenous contrast agents

1 Introduction

Photoacoustic signals are acoustic waves excited by pulsed or amplitude-modulated optical radiations. The amplitude and phase of the generated photoacoustic signals not only depend on the light sources but also the optical, thermal and mechanical properties of the excited objects. While photoacoustic effect has been a known physical phenomenon for over a century [1], it is only in the past three decades that photoacoustic tomography (PAT) has gained momentum, due to the development of modern laser technologies, inverse reconstruction algorithms and sensitive ultrasonic detectors. PAT is now one of the fastest growing technologies for biomedical imaging [2–5]. Compared to other biomedical imaging technologies, such as magnetic resonance tomography [6], ultrasonic imaging [7], and X-ray computed tomography [8, 9], PAT provides complementary merits by combining the strengths of optical imaging and ultrasonic imaging [2]. The implementation of a typical PAT system always combines optical excitation and acoustical detection [3], leading to

the following three features. First, PAT relies on optical absorption, hence it can provide high-contrast spectral properties of biological tissues, including the physiological functions [10] and the molecular compositions [11]. Second, PAT detects the local acoustic pressure induced by pulsed-laser excitation, hence it can provide relatively large penetration depth, as acoustic pressure waves has two to three orders of magnitude less attenuation than light in tissues [12]. Third, PAT system is highly scalable by combining different light delivery and acoustics detection methods [4], hence it can perform multi-scale imaging with a flexible spatial resolution and penetration depth.

According to the configurations of optical excitation and acoustic detection methods, the implementations of PAT system can be classified into three groups, including photoacoustic computed tomography (PACT), optical-resolution photoacoustic microscopy (OR-PAM), and acoustic-resolution photoacoustic microscopy (AR-PAM). PACT employs wide-field optical illumination and parallel acoustic detection, providing relatively low spatial resolutions (hundreds of micrometers) and deep penetration depth (several centimeters); PAM employs either tightly/loosely focused optical illumination and focused acoustic detection, providing high spatial resolutions (from several micrometers to tens of micrometers) and relatively shallow penetration depth (from one to several millimeters). The complementary imaging characteristics of PACT and PAM

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systems make photoacoustic imaging a promising technology for a variety of biomedical applications [2]. In this concise review paper, we will focus on PAM. First, we will introduce the basic principles and system designs of PAM systems. Second, the major imaging parameters of PAM systems (including OR- and AR-PAM) will be discussed in detail. Third, the major biomedical imaging applications of PAM will be introduced. Lastly, technical and engineering challenges in PAM are presented.

2 Principle of PAM

For a PAM system, optical excitation and acoustic detection are the basic configurations. The PA signals, i.e. the acoustic waves, are generated by optical absorption of the target. If the optical excitation satisfies the stress and thermal confinements [5], the generated initial acoustic pressure amplitude can be expressed as

$$P(\vec{r}) = \Gamma \eta \mu_a(\vec{r}) F(\vec{r}), \quad (1)$$

where Γ is the Grüneisen parameter, η is the percentage of absorbed light energy that is converted into heat through non-radiative relaxation, μ_a is the absorption coefficient, and F is the local optical fluence. The amplitude of the initial pressure is linear to the optical absorption and the local optical fluence, providing PAM with high optical-absorption-based contrast mechanism.

In PAM, the implementations of focused optical excitation and acoustic detection are similar to confocal microscopy. The confocal configuration maximizes the detection sensitivity. Figure 1 shows the typical setups of OR- and AR-PAM. The difference between OR- and AR-PAM is the size of the focused laser spot on the target. OR-PAM employs tightly focused light as the excitation source, as shown in Fig. 1a and b, and its lateral resolution is determined by the focused light spot size, which is approximately given by $0.51\lambda_o/\text{NA}_o$. Here λ_o is the light

wavelength and NA_o is the numerical aperture (NA) of the optical objective. A larger optical NA can provide a better lateral resolution. For example, an NA of 0.1 provides a lateral resolution of $\sim 3 \mu\text{m}$ [13] and 1.23 provides $\sim 0.22 \mu\text{m}$ [14] at 532 nm. OR-PAM in transmission mode (Fig. 1a) is only applicable to thin tissue slices due to the limited penetration, while the reflection mode (Fig. 1b) is capable of imaging larger tissue volumes. AR-PAM employs weakly focused or collimated light beam, as shown in Fig. 1c. The optical beam spot of AR-PAM is larger than the acoustic focal spot. As a result, the lateral resolution of AR-PAM is acoustically determined by the acoustical focal spot size, which is given by $0.71\lambda_a/\text{NA}_a$. Here λ_a is the acoustic wavelength. NA_a is the NA of the focused ultrasonic transducer. The lateral resolution of AR-PAM is more than ten times worse than OR-PAM. The axial resolutions of OR- and AR-PAM are both determined by the bandwidth (Δf) of the ultrasonic transducer, which is given by $0.88c/\Delta f$ [15]. c is the speed of sound.

The optical spot size of PAM system also determines the optical fluence that can be safely delivered into the tissue. Due to the safety concern for in vivo study, the optical fluence is limited by the maximum permissible exposure (MPE) regulated by American National Standard for Safe Use of Lasers [17]. Compared to OR-PAM, more optical energy can be used for AR-PAM, due to the larger optical spot size. Generally, the pulse energy is less than $1 \mu\text{J}$ for OR-PAM and less than 1 mJ for AR-PAM [3]. Because AR-PAM can harness the PA signals generated by scattered photons, it can achieve larger penetration depth. Nevertheless, the PA signals also suffer from tissue attenuation before reaching the transducer. The acoustic penetration depth of AR-PAM system is inversely proportional to the central frequency of the transducer [18], as low-frequency acoustic waves have weaker scattering. For example, by using a 5-MHz transducer, the imaging depth of AR-PAM is $\sim 30 \text{ mm}$ [4].

Fig. 1 The schematics of typical PAM systems. **a** Transmission-mode OR-PAM. UT, ultrasonic transducer. **b** Reflection-mode OR-PAM. SOL, silicone oil layer. **c** Reflection-mode AR-PAM. Figures reproduced with permission from [16]

The working distance of a PAM system depends on the focal length of the objective or ultrasonic transducer. A relatively large working distance can be obtained by using low-NA optical objective and ultrasonic transducer, at the cost of lateral resolution. The field of view (FOV) of the PAM system is determined by scanning either the sample or the light/sound. Most of the reported conventional PAM systems use stepper motor stages, with a maximum scanning speed of ~ 5 cm/s. To provide high-quality images, the scanning step size for PAM should be at most half of the lateral resolution [19], which is on the order of several micrometers for OR-PAM and tens of micrometers for AR-PAM. Therefore, it is time consuming to scan a large FOV using conventional PAM systems. The imaging speed can be improved by using scanning mirrors with various driving mechanisms [20–30]. For example, a commercial Galvanometer mirror has been used to provide a B-scan rate of 60 Hz over an ~ 4 -mm scanning range [28]. Another OR-PAM system employed a water-immersible MEMS scanning mirror, providing a B-scan rate of 400 Hz over a 3-mm scanning range [27]. Recently, a 2-axis waterproof MEMS scanner was developed for handheld OR-PAM [30].

The imaging characteristics of PAM systems can be tuned optically or acoustically. A longer excitation wavelength provides a larger penetration depth in the near-infrared (NIR) window of 650–950 nm [31]. In addition to spatial resolution, different optical and acoustic NAs can also affect the imaging depth. A high optical/acoustic NA induces a short depth of focus (DOF), so depth scanning may be necessary for volumetric imaging if a high NA is used.

3 Biomedical applications

In the following sections, the major biomedical applications of PAM are presented, including (1) structural imaging across scales from single cells to organs, (2) label-free functional imaging, and (3) the molecular imaging with exogenous contrast agents.

3.1 Structural imaging

3.1.1 Single cell imaging

Single cell imaging requires at least a submicron/sub-wavelength spatial resolution [32], hence it is usually implemented by using OR-PAM systems. The submicron/subwavelength OR-PAM can be achieved by either using a high optical NA [14, 33–36] or a transducer with a ultra-high central frequency [37]. The former is more commonly used for single cell imaging [32]. Due to the

limited working distance and small penetration, OR-PAM systems with high-NA objectives, as shown in Fig. 1a, were usually implemented in the transmission mode [14, 33–36, 38, 39]. Zhang et al. reported the first sub-wavelength OR-PAM by employing a 1.23-NA objective [14], obtaining a lateral resolution of 0.22 μm . The single cell imaging capability was demonstrated on individual melanoma cells and red blood cells (RBCs) *ex vivo* (Fig. 2). Compared to the optical microscopy (OM) images, PAM images show a higher contrast between cells and the background. Later, the similar OR-PAM configuration was applied to image the mitochondria [35], MC3T3 osteoblast cells [36], and MCF7 cells [39].

The high-optical-NA OR-PAM, however, has a poor axial resolution, due to the limited ultrasonic detection bandwidth. To address this issue, Strohm et al. [37] reported another approach for resolving single cells by using ultrahigh frequency ultrasonic transducers. In this case the lateral resolution was also acoustically determined, as the laser spot size was larger than the acoustical spot size. The central frequencies of the employed ultrasonic transducers were up to 1200 MHz, while the NA of the objective was fixed at 0.25. The lateral and axial resolutions both reached at 1 μm . The images of a single melanoma cell obtained at different ultrasonic frequencies were shown in Fig. 3a, b. Alternatively, Shelton et al. employed an objective with an NA of 0.8 to image a single bovine RBC [34], taking advantage of multi-photon optical absorption. This non-linear multiphoton OR-PAM has provided a lateral resolution of ~ 0.4 μm and an optically-determined axial resolution of ~ 1.5 μm , as shown in Fig. 3c and d. Nevertheless, both the high-frequency OR-PAM and multi-photon PAM have reduced penetration depth, compared with traditional OR-PAM systems.

Several other methods, based on nonlinear effects or sophisticated signal detection, have improved the axial resolution of OR-PAM [38, 40–42]. Shelton et al. [40] used the nonlinear transient absorption to achieve an axial resolution of 6 μm ; Zhu et al. [42] developed a multi-view OR-PAM enhanced by a deconvolution method, providing an axial resolution of 4.7 μm ; Yao et al. [41] developed photo-imprint OR-PAM using the intensity-dependent photobleaching effect and obtained an axial resolution of 0.37 μm ; Dong et al. [38] employed an ultra-wideband micro-ring resonator ultrasonic detector to achieve an axial resolution of 2.12 μm .

3.1.2 Microvasculature imaging

The microvascular system is responsible for exchanging oxygen, nutrients and metabolism waste with the surrounding tissues. Imaging microvasculature can provide physiological and pathophysiological information for

Fig. 2 Ex vivo images of melanoma cells and RBCs obtained by OR-PAM with an optical NA of 1.23. **a** Melanoma cells on a glass slide. From left to right: PAM image, OM (0.5 NA) image, and a composite of the PAM image and the fluorescence OM image. The blue regions show the fluorescent stained nucleus. CN: cell nucleus. **b** PAM and OM (1.0 NA) images of RBCs. Figures adapted with permission from [14]. (Color figure online)

Fig. 3 Images of melanoma cells obtained by high-frequency OR-PAM and red blood cells obtained by multiphoton OR-PAM. **a** Top-view MAP images obtained by the 200-, 375- and 1200-MHz ultrasonic transducers. Scale bar, 30 μm . **b** A cross-sectional image around the cell nucleus as indicated by the arrow in (a). Scale bar, 15 μm . **c** Volumetric images of several RBCs on a glass slide within the volume of $50 \times 50 \times 20 \mu\text{m}^3$. **d** Close-up image of a single RBC marked with a yellow asterisk in (c). Figures reproduced with permissions from [34, 37]. (Color figure online)

diagnosing many diseases [43], such as uremia [44], diabetes [45] and rheumatoid arthritis [46]. PAM has played an important role in the imaging and characterization of the microvasculature [47]. The diameters of micro vessels generally fall in the range of 2–100 μm [48], hence both OR-PAM and AR-PAM are capable of imaging microvasculature. OR-PAM is usually employed for

superficial microvasculature imaging [49], while AR-PAM is usually employed for subcutaneous microvasculature imaging [50–54].

The mouse ear is a widely used model for OR-PAM, as it has abundant capillary network. The thickness of the mouse ear is around 0.2–0.3 mm [55]. Therefore, the capillaries in the ear skin can be imaged by OR-PAM without losing the lateral resolution [47], as shown in Fig. 4. The top-view MAP image was shown in Fig. 4a, and the corresponding volumetric image was shown in Fig. 4b. Small vessels can be clearly resolved with high imaging contrast. OR-PAM has also been used for imaging the mouse brain [27] and eyes [49]. AR-PAM is usually used for the deep-penetration imaging of microvasculature. Zhang et al. for the first time used AR-PAM to image the human hand in vivo, showing the microvascular network at

Fig. 4 A typical in vivo OR-PAM image of the microvasculature in a mouse ear. **a** Top-view MAP image. **b** Volumetric image. Figures adopted with permission from [47]

a depth of ~ 400 – 650 μm below the skin surface [54], as shown in Fig. 5. The largest vessel is 350 μm in diameter, and the smallest is within 50 μm . From the B-scan image in Fig. 5c, the three layers of skin surface, stratum corneum and vessels can be clearly identified.

3.1.3 Whole organ imaging

As most endogenous biomolecules are non-fluorescent and the organs are larger than 1 mm, it is generally a challenge for traditional optical microscopy to image the whole organs. PAM is a potential technology for addressing the above issues. Wong et al. [56] demonstrated the whole-organ imaging capability of a microtomy-assisted OR-PAM (ma-OR-PAM), using UV light as the optical excitation source. The microtomy was used for automatically shaving off the thin layer of tissue after being imaged. Compared to optical sectioning microscopy, the ma-OR-PAM can generate high-resolution three-dimension (3D) images of whole organs embedded in agarose, by simultaneous imaging DNA/RNA, hemoglobin and lipids, with a lateral resolution of 0.9 μm . Agarose was used for keeping the tissue fresh. A 3D ma-OR-PAM image of an unstained agarose-embedded mouse brain is shown in Fig. 6. The imaged volume of the mouse brain was

$9.5 \times 7.5 \times 11.0$ mm^3 . The volumetric image of the mouse brain is shown in Fig. 6a, obtained with a sectioning thickness of 0.2 mm. The lateral ventricles, hippocampus, and cerebellum can be observed in the 3D image. The high-resolution 3D image can also provide histology-like imaging of cell nuclei, blood vessels, axons, and other anatomical structures of the organs.

3.2 Label-free functional imaging

Since almost all endogenous biomolecules in biological tissues can absorb light at their corresponding wavelengths, they can potentially provide absorption contrast for PAM without labelling. The optical absorption spectra of major endogenous biomolecules [16] are shown in Fig. 7. The multispectral PAM can be implemented with multiple-wavelength excitation [57, 58] and the relative concentrations of the biomolecules can be quantified through a variety of signal inmixing methods. Label-free functional studies, such as monitoring the oxygen saturation of hemoglobin (sO_2) and oxygen metabolism, can be performed using PAM with intrinsic contrast agents. We will introduce two representative applications of PAM in functional studies.

3.2.1 Brain activity imaging

PAM is an effective tool to visualize the brain activities with high resolution and relatively deep penetration. OR-PAM has been widely used for brain imaging, in which the mouse scalp is typically removed but the skull is intact. Yao et al. developed a fast-speed OR-PAM using a water-immersible MEMS scanning mirror to monitor blood perfusion and oxygenation activities of a mouse brain in vivo [27], as shown in Fig. 8. It was observed that the PA amplitude in the contralateral somatosensory region and sagittal sinus region increased until the end of the electrical stimulations. The sO_2 variations responded to the stimulations were quantified using a single-wavelength pulse-width-based method. The changes of cerebral blood flow (CBF), oxygen extraction fraction (OEF) and cerebral metabolic rate of oxygen (CMRO_2) in the core responding region were also measured. AR-PAM has also been used for functional brain imaging, with improved penetration but compromised resolution [59–61]. For example, Liao et al. reported AR-PAM of rat brain hemodynamic changes during forepaw electrical stimulation [59], and Stein et al. used AR-PAM to monitor the blood oxygenation dynamics in hypoxic and hyperoxic challenges [61]. Compared to the single-wavelength pulse-width-based method, multiple-wavelength excitation method is more sensitive to the wavelength-dependent optical attenuation in tissue, but it doesn't require the extremely short laser pulse.

Fig. 5 A typical in vivo AR-PAM image of the microvasculature in a human palm. **a** Photograph of a human hand. Solid rectangle indicates the imaged area. **b** Top-view MAP image after removing the skin surface and the stratum corneum. **c** B-scan image along the dashed line in (b). **d** Top-view MAP images from different layers. Figures reproduced with permission from [54]

Fig. 6 3D ma-OR-PAM image of an unstained agarose-embedded mouse brain. **a** Volumetric image of a mouse brain with a sectioning thickness of 200 μm . **b–k** Ten representative coronal views as positioned by the dashed lines in (**a**). Figures adopted with permission from [56]

Nevertheless, both methods have found successful applications for functional brain imaging.

3.2.2 Blood flow imaging

Most diseases have adverse effect on the blood flow and thus disrupt the normal oxygen metabolic activities of cells. It is therefore clinically significant to measure the blood flow and oxygen metabolism for diagnosis and therapeutic purposes [62]. PAM is a promising technology for this task because it does not require additional exogenous contrast agents to label the blood or oxygen [13, 63–69]. For example, Yao et al. developed a metabolic OR-PAM system that can simultaneously measure sO_2 and blood flow in vivo [13], as shown in Fig. 9. In particular, the blood flow was measured based on the photoacoustic Doppler bandwidth broadening with bidirectional motor scanning. By combining the essential anatomical and functional parameters of the tissue, the metabolic rate of oxygen can be quantified in absolutely value, as first demonstrated in a hyperthermia model and a tumor model [13].

3.3 Molecular imaging with exogenous contrast agents

Although PAM is capable of high-resolution imaging with endogenous contrast, many diseases and physiological processes usually lack sufficient intrinsic PA contrasts [11, 70], such as tumor cell receptor expression and protease activity. Various exogenous PA contrast agents, from small-molecule dyes to gold, polymer and carbon nanostructures to liposome encapsulations [11, 63, 71–75], have been invented to enhance the specificity and sensitivity of PAM. The development of PA contrast agents can be of great importance to advance the molecular imaging applications of PAM, especially for detecting early-stage cancers and tracking immunotherapy mechanisms against diseases. We will only list two representative applications below. More comprehensive studies can be referred to the recent review articles [70, 76].

Fig. 7 Optical absorption spectra of major endogenous biomolecules in tissues. Figure adopted with permission from [16]

3.3.1 Molecular imaging of gene expression

Cai et al. [77] demonstrated the capability of AR-PAM for noninvasive and simultaneous imaging of a subcutaneous lacZ-expressing tumor and its surrounding microvasculature networks in vivo. The lacZ gene is a widely used reporter gene in biology and its expression can be studied by several chromogenic assays. In this study, Cai et al. employed the X-gal as the molecular probe which absorbed the light in the wavelength range between 605 and 655 nm.

Fig. 8 In vivo OR-PAM of the mouse brain responses to the electrical stimulations of the hindlimbs. **a** Fractional PA amplitude response to left hindlimb stimulation (LHS) and right hindlimb stimulation (RHS). LH, left hemisphere; RH, right hemisphere. **b** Depth-resolved response. **c** sO_2 changes in response to the stimulations. **d** The changes of cerebral blood flow (CBF), oxygen extraction fraction (OEF) and cerebral metabolic rate of oxygen (CMRO₂) in the core responding region. Figure adopted with permission from [27]

The lacZ-expressing tumor cells were subcutaneously injected into the mouse. The AR-PAM images of the malignant tumor and surrounding tissues acquired at 584 and 635 nm were shown in Fig. 10. The tumor only contributed to the PA signals at 635 nm. The fused MAP image of the two wavelengths shows the spatial distributions of the tumor and the surrounding microvasculature. With the dual-wavelength excitation scheme, the expression of the lacZ gene was successfully identified.

3.3.2 Molecular imaging of tumors targeted by bioconjugate gold nanocages

There have been numerous nanostructures developed as exogenous contrast agents for PAT [11, 70, 71]. Gold nanocages (AuNCs) have been demonstrated as one effective contrast agent for molecular PA imaging [78]. Kim et al. [72] have conducted a study to quantify the contrast enhancement of the active and passive targeting of AuNCs to B16 melanomas. The AuNCs was bioconjugated with [Nle4, D-Phe7]- α -melanocyte-stimulating hormone ([Nle4, D-Phe7]- α -MSH-AuNCs) for active targeting and poly (ethylene glycol) (PEG-AuNCs) for passive targeting. The MAP images of the B16 melanomas targeted by [Nle4, D-Phe7]- α -MSH-AuNCs and PEG-AuNCs are shown in Fig. 11, which were acquired by the dual-wavelength AR-PAM (570 nm for microvasculature and 778 nm for AuNCs). It was observed that [Nle4, D-Phe7]- α -MSH-AuNCs provided three-times higher contrast enhancement than PEG-AuNCs. Except for the gold nanocages, other nanostructures, such as the palladium nanosheets [11], semiconducting polymer nanoparticles [75], silver nanoplate [71] and carbon nanotube [79] were also demonstrated as effective PA contrast agents.

Fig. 9 In vivo OR-PAM images of the oxygen saturation of hemoglobin and blood flow speed measured on a mouse ear. **a** The total concentration of hemoglobin acquired at 584 nm. **b** The measured sO_2 in the area indicated by the dashed box in (**a**). **c** The measured flow speed in the area indicated by the dashed box in (**b**). **d** The profile of the flow speed along the dashed line indicated in (**c**). Figures reproduced with permission from [13]

4 Conclusion and Challenges

In conclusion, PAM is a powerful tool for anatomical, functional and molecular imaging for preclinical studies, with great potential for clinical applications. In this concise review, we have introduced the basic principles and the major imaging characteristics of PAM. The major biomedical imaging applications of PAM, from multiscale structure imaging to functional imaging using endogenous biomolecules to molecular imaging using exogenous contrast agents, have been presented with representative examples.

The strong momentum of PAM development is fueled by its increasingly popular applications in the life sciences and its commercialization. PAT has enabled biomedical studies that require high-resolution imaging at depths far beyond the optical diffusion limit and filled a void of high-resolution optical imaging in deep tissue. We strongly believe that PAM will find broad impact in fundamental research and clinical practice.

Fig. 10 In vivo AR-PAM images showing the lacZ-expressing tumor and the surrounding microvascular networks. **a** MAP image of the lacZ-expressing tumor acquired at 635 nm. **b** MAP image of the surrounding microvasculature acquired at 584 nm. **c** Fused MAP image of the two wavelengths. **d** Fused 3D image of the two wavelengths. Figures adopted with permission from [77]

Nevertheless, to tap the full potential of PAM, there are still several technical and engineering challenges in PAM that need to be addressed.

1. *Limited detection view* Most of AR-PAM with weakly focused light and OR-PAM systems using low-NA optical objectives suffer from the limited view problem [80, 81]. The PA signals from vertical structures aligned with the acoustic axis cannot be detected due to interference cancellation of coherent pressure waves. Limited view can be corrected by using either multi-view transducers or structure illumination [82], requiring complex system design. Recently, deep learning has been validated as a promising tool to correct the imaging artifacts in traditional optical imaging [83], which may also be a possible solution for PAM to correct the limited view.
2. *Imaging speed and field of view* Most of the reported PAM systems are only applicable to small-region imaging ($< 10 \times 10 \text{ mm}^2$), limited by small scanning range or the slow scanning speed. For scanning mirror-based OR-PAM systems, the field of view can be enlarged by increasing the rotation angles of scan mirrors, which, however, may suffer from the limited detection region of the ultrasonic transducer. For PAM systems using stepper scanning motor stages, the

Fig. 11 In vivo AR-PAM images of the melanomas targeted by [Nle4, D-Phe7]- α -MSH-AuNCs and PEG-AuNCs, respectively. **a** Photographs of the nude mice bearing melanoma. **b–d** MAP images of the melanomas and surrounding microvasculature after injection of [Nle4, D-Phe7]- α -MSH-AuNCs. **f–h** MAP images of the melanomas and surrounding microvasculature after injection of PEG-AuNCs. Figures adopted with permission from [72]

scanning speed is limited by the motor translation speed and the required step size. For samples with curvature surfaces, such as the brain, the imaging requires even more time by performing depth scanning. Nevertheless, a fast PAM with ultra-wide field of view is highly desired for preclinical or clinical researches.

3. **Miniaturization** For most small animal imaging studies based on table-top PAM, the animals are typically in

anesthesia to avoid any motion artifacts. However, the anesthetic status affects the normal physiological functions, such as the metabolism and neuron activities. Therefore, the imaging results of anesthetized animals may lack fidelity, compared with the results obtained with freely behaving animals. The current table-top PAM systems are not able to image the awake animals in motion. This issue can be addressed by miniaturizing PAM systems and mounting it onto the animal body or head, similar to miniaturized two-photon microscopy [84]. The miniaturization of PAM system can benefit tremendously for functional brain studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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