Published in final edited form as:

Opt Lett. 2010 December 1; 35(23): 4018–4020.

Simultaneous dual molecular contrasts provided by the absorbed photons in photoacoustic microscopy

Xiangyang Zhang¹, Minshan Jiang¹, Amani A. Fawzi¹, Xiang Li², K. Kirk Shung², Carmen A. Puliafito¹, Hao F. Zhang^{3,4}, and Shuliang Jiao^{1,5}

¹Department of Ophthalmology, Keck School of Medicine, University of Southern California, 1450 San Pablo Street, Los Angeles, California 90033, USA

²Department of Biomedical Engineering, University of Southern California, Los Angeles, California, USA

³Department of Electrical Engineering and Computer Science, University of Wisconsin–Milwaukee, 3200 North Cramer Street, Milwaukee, Wisconsin 53201, USA

Abstract

We investigated the feasibility of simultaneously imaging two distinctive molecular contrasts provided by the absorbed photons in biological tissues with a single light source. The molecular contrasts are based on two physical effects induced by the absorbed photons: photoacoustics (PA) and autofluorescence (AF). In an integrated multimodal imaging system, the PA and AF signals were detected by a high-sensitivity ultrasonic transducer and an avalanche photodetector, respectively. The system was tested by imaging ocular tissue samples, including the retinal pigment epithelium and the ciliary body. The acquired images provided information on the spatial distributions of melanin and lipofuscin in these samples.

Photoacoustic microscopy (PAM) [1,2] is an optical absorption-based microscopic imaging modality that detects laser-induced ultrasonic waves as a result of specific optical absorption. Traditionally, PAM detects the ultrasonic waves generated in a sample by that part of the absorbed photons whose energy is converted to heat. However, apart from generating heat, the absorbed photons may also undergo other physical processes, such as stimulating autofluorescence (AF), when fluorophores are present. Unfortunately, this effect of the absorbed photons is not utilized in conventional PAM, although the absorbed photons may provide important and complementary molecular contrasts of the biological tissues. On the other hand, existing fluorescence microscopy is unable to measure the part of the absorbed photons that are converted to heat.

The motivation for this study is to explore the dual molecular contrasts provided by the absorbed photons to simultaneously image both melanin optical absorption and lipofuscin AF in retinal pigment epithelium (RPE). The RPE is a monolayer of pigmented cells located between the choriocapillaris and light-sensitive outer segments of the photoreceptors. RPE dysfunction, which can result from a lifetime of oxidative stress, contributes to numerous retinal diseases, including age-related macular degeneration (AMD), a leading blinding disease in industrialized countries. A feature of the RPE cells that contributes to their stress susceptibility is the complement of their two major pigments, lipofuscin and melanin—

pigments that are believed to have opposite effects on stress susceptibility. Lipofuscin is an autofluorescent, photoreactive pigment that can exacerbate visible light stress. In contrast, melanin is a highly light absorbing pigment that protects against light stress by acting as an optical screen and an antioxidant. As a result, measuring the concentration and spatial distribution of melanin and lipofuscin provides important information in the aging retina, which is important for AMD research and clinical diagnosis [3,4], especially when the images of the two substances are obtained simultaneously and with automatic spatial registration.

PAM is an ideal candidate for imaging the RPE melanin because melanin strongly absorbs visible light. We have been successful in noninvasive imaging of retinal vessels and RPE pigment *in vivo* in rodents with a novel PAM configuration called photoacoustic ophthalmoscopy (PAOM) [5]. Although techniques now exist to image AF as a measure of RPE lipofuscin, which fluoresces in the wavelength range 500 to 800 nm when stimulated by a light 488 to 633 nm [6,7], to our knowledge, no single technique exists to image melanin and lipofuscin simultaneously.

Figure 1 shows a schematic of the experimental system. The illumination light source is a frequency-doubled *Q*-switched Nd:YAG laser (SPOT-10-100-532, Elforlight Ltd, UK: 532 nm; 10 μ J=pulse; 2 ns pulse duration; maximal pulse repetition rate: 30 kHz). The output laser light was first attenuated with a neutral density (ND) filter before being coupled into a single-mode optical fiber. The light reflected from the surface of the ND filter is detected by a photodiode (DET10A, Thorlabs Inc.), which provided a trigger signal for both AF and PAM signal acquisition. With this arrangement, the two imaging modalities were synchronized and the effect of laser jittering can be avoided. The output laser light from the fiber was collimated to a beam diameter of 10 mm, reflected by a dichroic mirror (DMLP567, Thorlabs, Inc.), scanned by a *X*-*Y* galvanometer, and then focused on the sample by an objective lens ($f = 50$ mm, Achromatic Lens, VIS-NIR, Edmund Optics Inc.). The galvanometer scanner was controlled by an analog-output board, whose sample clock was used to trigger the laser.

The back-traveling fluorescent photons emitted from the sample passed through the dichroic mirror and a longpass filter (FEL0550, cut on wavelength: 550 nm, Thorlabs, Inc.) then was focused on an avalanche photo-detector (APD110A, Thorlabs, Inc.). The AF signal was digitized and stored by a high-speed digitizer (CompuScope 22G8, Gage Applied Technologies) at a sampling rate of 2 GS/s.

The induced photoacoustic (PA) waves from the sample were detected by a custom-built needle ultrasonic transducer (30 MHz; bandwidth, 50%; active element diameter, 1 mm). The detected PA signals were first amplified by 80 dB and then digitized by another high-speed digitizer (CompuScope14200, Gage Applied Technologies) at a sampling rate of 200 MS/s.

The laser pulse energy was 40 nJ. As reported in our previous publications [8,9], the system has a lateral resolution of 3:2 μ m. The depth resolution is 23 μ m, which is determined by the bandwidth and center frequency of the ultrasonic transducer [8,9]. In the experiments, the laser was triggered at a pulse repetition rate of 24 kHz, the simultaneous acquisition of the PAM and AF images, each consisting of 256×256 scanning positions, took 2:7 s. During imaging, the samples were immersed in a water tank and the transducer was placed about 4 mm away from the sample.

In our previous *in vivo* retinal imaging experiments, one question remained unanswered: whether lipofuscin molecules had any observable contribution to the PA signals due to optical absorption [5]. Answering this question is important, because we need to establish

the specificity of PAM in imaging melanin in the RPE. To answer this question, we first imaged lipofuscin control slides (American MasterTech Scientific Inc., Lodi, California, USA). A strong AF signal was detected, while we did not observe any PA signal. The results showed that the contribution of lipofuscin to the PA imaging mode can be neglected (image not shown) at the 532 nm stimulating wavelength.

We then imaged an unfixed slide of an aged human RPE. The maximum-amplitude-projection (MAP) images of PAM and AF are shown in Figs. 2(a) and 2(b), respectively. To verify the experimental results, we also imaged the same sample with a commercial fluorescence microscope (Leica, DM LB2) and a bright-field microscope (Leica, DM LB2). The corresponding images are shown in Figs. 2(c) and 2(d), respectively. The PAM image in Fig. 2(a) showed two distinct bright lines separated by a region without a visible PA signal. In reference to Fig. 2(d), we can see that these two bright lines correspond to the RPE on the right and choroidal melanin to the left. The area between them represents drusen deposits, the hallmark lesion of AMD. In contrast to the PAM image, the AF image in Fig. 2(b) showed only a single bright line. In reference to Fig. 2(d), we can see that this line corresponds to the RPE. Figure 2(c) shows identical features as in Fig. 2(b), which confirms that the AF signal corresponds to the lipofuscin distribution within the RPE cells. By comparing the images in Fig. 2, we can see that the contribution of choroidal melanin to the AF signal is negligible.

From this experiment, we confirmed that (1) lipofuscin is only present in the RPE not in the choroid (at least in the samples tested here) and (2) the AF imaging mode of our system reliably detected lipofuscin AF in the RPE.

After verification of the capability of imaging dual molecular contrasts we moved on to image the RPE of a human eye cup acquired from the San Diego eye bank. The sample was fixed in 10% formalin, and the retina was gently peeled to reveal the underlying RPE layer. The acquired images (not shown here due to page limit) showed different distributions of melanin and lipofuscin in the sample. It can be seen that melanin and lipofuscin are not distributed homogeneously.

We also imaged the ciliary body in a pig eye. The eye was acquired from a local slaughter house and was fixed in 10% formalin. The eye ball was cut in half to expose the ciliary body. The PAM and AF images are shown in Fig. 3. The two images showed strikingly different but complementary features of the sample. We also acquired an image of the same sample with a commercial fluorescence microscope in Fig. 3(c). Although we did not image the same area, the fluorescent microscope image captured the same features as our AF image. We measured the fluorescence spectrum of the sample, which showed an emission range of 550–700 nm peaked at 680 nm (data not shown). These measurements confirmed that the AF image showed the distribution of a fluorophore (we call it lipofuscin-like fluorophore). Whether the fluorophore is lipofuscin or not needs to be verified with other histopathologic techniques. We pseudocolored the AF image (green) and overlaid it with the PAM image. The result is shown in Fig. 3(d), which showed that, in the sample, melanin accumulated in granular areas lined by lipofuscin-like fluorophore. To our knowledge, it is the first time that this feature of melanin and lipofuscin-like fluorophore distribution in the ciliary body was reported. Because the ciliary body pigmented epithelium is an extension of the RPE, it is anticipated that lipofuscin accumulates either in the pigmented epithelium or in the muscle fibers in the ciliary body.

In summary, we have investigated the dual molecular contrasts provided by the absorbed photons in biological tissues using an integrated PAM and AF microscopy. The imaging contrasts were verified by imaging controlled samples. When applied to imaging *ex vivo*

ocular samples, the technique revealed interesting features of melanin and lipofuscin distribution that were not seen before. We did not recognize individual RPE cells and, hence, were unable to differentiate the signal originating from melanin and lipofuscin within the RPE cells, possibly because of the limited system resolution. Our study laid the foundation for future quantitative imaging of the distributions of lipofuscin and melanin in RPE *in vivo*, which are important for the research and clinical diagnosis of AMD. The imaging speed, which is currently limited by the pulse repetition rate of the illuminating laser, makes the system potentially suitable for routine *in vivo* applications in ophthalmic research.

Acknowledgments

This work is supported in part by the National Institutes of Health (NIH) grant 7R21EB008800-02 and the Juvenile Diabetes Research Foundation Innovative Grant 5-2009-498. We acknowledge the contribution of J. Chen at USC, who provided unfixed human-donor RPE slides. We also thank Dr. Jeannie Burke from Medical College of Wisconsin for thoughtful discussions.

References

1. Zhang HF, Maslov K, Wang LV. Nat. Protoc. 2007; 2:797. [PubMed: 17446879]
2. Zhang HF, Maslov K, Stoica G, Wang LV. Nat. Biotechnol. 2006; 24:848. [PubMed: 16823374]
3. Weiter JJ, Delori FC, Wing GL, Fitch KA. Invest. Ophthalmol. Visual Sci. 1986; 27:145. [PubMed: 3943941]
4. Delori FC, Goger DG, Dorey CK. Invest. Ophthalmol. Visual Sci. 2001; 42:1855. [PubMed: 11431454]
5. Jiao S, Jiang M, Hu J, Fawzi A, Zhou Q, Kirk K, Carmen S, Puliafito A, Zhang HF. Opt. Express. 2010; 18:3967. [PubMed: 20389409]
6. Marmorstein AD, Marmorstein LY, Sakaguchi H, Hollyfield JG. Invest. Ophthalmol. Visual Sci. 2002; 43:2435. [PubMed: 12091448]
7. Gibbs D, Cideciyan AV, Jacobson SG, Williams DS. Invest. Ophthalmol. Visual Sci. 2009; 50:4386. [PubMed: 19324852]
8. Zhang HF, Wang J, Wei Q, Liu T, Jiao S, Puliafito CA. Opt. Express. 2010; 18:1278. [PubMed: 20173952]
9. Xie Z, Jiao S, Zhang HF, Puliafito CA. Opt. Lett. 2009; 34:1771. [PubMed: 19529698]

Fig. 1.
 (Color online) Schematic of the experimental system: laser, frequency-doubled Q -switched Nd:YAG laser; ND, neutral density filter; PD, photodiode; I, iris; SMF, single-mode optical fiber; L1-4, lenses; DM, dichroic mirror; LF, longpass filter; APD, avalanche photodiode; UT, ultrasonic transducer.

Fig. 2.
(Color online) Comparison of the PAM and AF images acquired simultaneously from a human RPE slides: (a) PAM image; (b) AF image; (c) from a commercial fluorescence microscope; (d) from bright-field microscopy. Bar: 100 μm.

Fig. 3.
 (Color online) PAM and AF images of the ciliary body of a pig eye: (a) PAM image; (b) AF image; (c) fluorescence microscopy photograph; (d) overlaid PAM and AF image where lipofuscin-like fluorophore is pseudocolored green. Bar: 100 μm .