High-resolution imaging in a deep turbid medium based on an ultrasound-switchable fluorescence technique

Baohong Yuan,^{1,2,a)} Seiichi Uchiyama,³ Yuan Liu,^{1,2} Kytai T. Nguyen,^{2,4} and George Alexandrakis^{2,4}

¹Ultrasound and Optical Imaging Laboratory, Department of Bioengineering, The University of Texas at Arlington, Arlington, Texas 76019, USA

²Joint Biomedical Engineering Program, The University of Texas at Arlington and The University of Texas Southwestern Medical Center at Dallas, Texas 75390, USA

³Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

⁴Department of Bioengineering, The University of Texas at Arlington, Arlington, Texas 76019, USA

(Received 18 March 2012; accepted 22 June 2012; published online 17 July 2012)

The spatial resolution of fluorescence imaging techniques in deep optically turbid media such as tissues is limited by photon diffusion. To break the diffusion limit and achieve high-resolution and deep-tissue fluorescence imaging, a fundamentally different method was demonstrated based on a concept of ultrasound-switchable fluorescence. The results showed that a small fluorescent tube with a diameter of $\sim 180\ \mu\text{m}$ at a depth of $\sim 20\ \text{mm}$ in an optical scattering medium ($\mu'_s \approx 3.2$ and $\mu_a \approx 0.026\ \text{cm}^{-1}$) can be clearly imaged with a size of $\sim 260\ \mu\text{m}$. The depth-to-resolution ratio is shown to be about one order of magnitude better than other deep-tissue fluorescence imaging techniques. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4737211>]

Fluorescence microscopy is an invaluable tool for many scientific fields.¹ Unfortunately, it is limited by two fundamental challenges:^{2,3} (1) the diffraction limit and (2) the diffusion limit. The diffraction limit leads to a finite spatial resolution. The diffusion limit results in a finite imaging depth in biological tissues due to strong light scattering ($<1\ \text{mm}$).^{3–6} To be able to image fluorophores in tissue with a depth significantly larger than an optical photon mean free path ($>1\ \text{mm}$), one has to take into account the effect of multiple, or diffuse, light scattering in the image formation process by use of appropriate photon propagation models. Examples of this approach are fluorescence laminar optical tomography (FLOT; resolution: $\sim 200\ \mu\text{m}$; imaging depth: 2–3 mm) (Ref. 7) and fluorescence diffuse optical tomography (FDOT; resolution: $\sim 3\text{--}5\ \text{mm}$; imaging depth: 30–50 mm).⁵ Unfortunately, in these techniques the improvement in imaging depth comes at the expense of the spatial resolution. The depth-to-resolution ratio is limited to ~ 10 for both FLOT and FDOT.

To break the diffusion limit and achieve deep-tissue and high-resolution fluorescence imaging, ultrasound-mediated fluorescence (UMF) was investigated recently.^{8–10} A highly focused ultrasound beam was employed to modulate fluorophores.^{8–11} The UMF signal can be detected and used to quantify the fluorophore information in the small ultrasound focal volume. Thus, a fluorescence image with ultrasound resolution can be achieved at a large depth.¹⁰ Further studies have shown that microbubbles or fluorophore-labeled quenchable microbubbles can be used to enhance ultrasound-modulated signal.^{9,12} Unfortunately, low signal-to-noise ratio (SNR) of UMF techniques significantly limits the further development.^{10,11,13} Recently developed photoacoustic tomography (PAT) has been reported to be

able to image fluorescent molecules with large depth-to-resolution ratio.^{14,15} However, the fluorescence measurement is indirect because PAT is based on the optical absorption property of fluorophores. Therefore, it cannot take advantages of the unique emission properties of fluorophores, such as fluorescence lifetimes and emission spectra.

To significantly improve SNR and further increase spatial resolution without sacrifice of imaging depth, an imaging technique, ultrasound-switchable fluorescence (USF),⁹ is demonstrated in this study. The idea is to use a short ultrasound pulse to turn on fluorophore emission (so the fluorophores become bright) only inside the ultrasound focal volume, while the fluorophores outside of the focal volume remain non-fluorescent (dark). Thus, ultrasound-induced fluorescence photons from the focal volume can be greatly increased and the unwanted fluorescence emission from the background can be dramatically suppressed compared to the UMF method. Because of the existence of a threshold to ultrasonically switch on a fluorophore, the spatial resolution of the USF technique eventually depends on the physical size of the volume in which fluorophores are switched on, which is generally smaller than the physical size of the ultrasound focus. Thus, the USF technique can potentially improve spatial resolution in deep tissue. The combination of the three techniques, (1) a USF technique, (2) a light-pulse-delay technique, and (3) a photon counting technique, is the key to achieve this goal.

A DBD-AA (fluorophore) labeled PNIPAM (thermo-sensitive polymer) has been found to exhibit an excellent switch-like relationship between the environmental temperature and the fluorescence intensity and lifetime (DBD-AA: N-{2-[(7-N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazol-4-yl](methyl)amino}ethyl-N-methylacrylamide; and PNIPAM: poly N-isopropylacrylamide).¹⁶ Although its peak excitation light is in the visible light region, as a proof-of-concept study, it provides a good opportunity to investigate the feasibility of the USF technique. It has been found that

^{a)}Author to whom correspondence should be addressed. Electronic mail: baohong@uta.edu.

the fluorescence lifetime of the DBD-AA labeled PNIPAM remains almost constant at ~ 4 ns when the environment temperature (T) is below 31°C . However, the lifetime quickly jumps to another constant of ~ 14 ns for temperatures above 32°C . Similar results are found for the fluorescence intensity, which is ~ 4 – 5 times stronger when $T > 32^\circ\text{C}$ than for $T < 31^\circ\text{C}$. Therefore, a temperature-change-mediated jump in emission from a low fluorescence intensity (off-state) to a high fluorescence intensity (on-state) can be defined at $T < 31^\circ\text{C}$ and $T > 32^\circ\text{C}$, respectively. A transition band is defined as $31 < T < 32^\circ\text{C}$. The 31.5°C can be considered as the temperature threshold to switch on the fluorophores. The underlying mechanism of temperature-induced change in fluorescence intensity for these polymers has been discussed previously.¹⁶ A high intensity focused ultrasound (HIFU) transducer can be used to externally and rapidly increase the tissue temperature above the threshold to switch on the fluorophores only in its focal volume (due to tissue absorption of acoustic energy). After ultrasound exposure and USF data acquisition, thermal energy is diffused and temperature recovers back to background temperature. Thus, fluorophores are switched off.

One of the challenges is that the fluorophores are not completely dark, even when temperature is below the “off” threshold. These are called non-100%-off fluorophores in this study. The non-100%-off fluorophores can generate significant unwanted background fluorescence due to the large volume of the background medium and should be eliminated. To avoid this type of noise, a light-pulse-delay technique is developed by taking advantage of the significant change in fluorescence lifetime between the off and on states.⁹ The basic idea is to use pico-second (ps) light pulses to illuminate the sample. The emitted fluorescence pulses are delayed (or expanded) mainly due to two mechanisms: (1) tissue light scattering and (2) finite fluorescence lifetime. The delay caused by tissue scattering is much less significant than that caused by finite fluorescence lifetime and can be ignored for the tissue depths considered in this work.⁹ Because of the significant increase in lifetime, fluorescence pulses emitted from the switched-on fluorophores are significantly delayed compared with those emitted from non-100%-off fluorophores. Consequently, this change in lifetime provides a very efficient way to temporally separate the background fluorescence from the switched-on fluorescence by only acquiring the late-arriving photons. Thus, background noise can be significantly avoided. Photon counting, which is so sensitive that one single photon can be detected, is adopted in this study due to the relatively weak signal. To avoid thermal diffusion during the heating period, the ultrasound exposure time τ_p should be much shorter than the thermal diffusion time constant τ_{th} of the sample. In this study, the ultrasound exposure time τ_p is limited to 100 ms, which is shorter than the estimated sample’s thermal diffusion constant of 315 ms (assuming the sample has a thermal diffusivity of $\sim 1.4 \times 10^{-3} \text{ cm}^2/\text{s}$ and the HIFU transducer has a lateral focal size of $420 \mu\text{m}$). In addition, the time constant of fluorescence switching between the two states is usually between 0.1 and 1 ms,^{17,18} which is much shorter than HIFU exposure time.

Fig. 1(a) shows the major components of the experimental system. A picosecond pulsed supercontinuum light source

FIG. 1. (a) A schematic diagram showing the measurement system. PDG: pulse-delay generator; FG: function generator; RF-Amp: radio frequency power amplifier; MNW: matching network; ps: pico-second; HIFU: high intensity focused ultrasound; PMT: photomultiplier tube. (b) The configuration of the phantom, laser and detector.

was adopted (SC-450, Fianium, Eugene, Oregon; pulse width: ~ 5 ps; with a broad illumination band). To narrow the excitation spectrum, a bandpass filter (FF01-475/35-25, Semrock, New York; central wavelength: 475 nm; bandwidth: 35 nm) was used as the excitation filter. A light guide (NT42-345, Edmund Optics, New Jersey) was used to collect emitted fluorescence photons. To more efficiently reject the excitation photons and possible autofluorescence from the sample, two long pass emission filters were used (BLP01-594R-25 and FF01-593/LP-25, Semrock, New York; pass-band > 610 nm and > 604 nm, respectively). A cooled photomultiplier tube (PMT) (H7422P-50, Hamamatsu, Japan) driven by a high voltage source (C8137-02, Hamamatsu, Japan) was used as a photon detector. The signal was further amplified by a broadband amplifier (SR445A, Stanford Research Systems, California) and acquired by a multi-channel oscilloscope (DPO 7254, Tektronix, Oregon). A pulse-delay generator (PDG) (DG645, Stanford Research Systems, California) sent a pulse to gate a function generator (FG) (AFG3252, Tektronix, Oregon). Thus, a gated sinusoidal wave signal with a central frequency of 2.5 MHz and a gate width of 100 ms was generated and was further amplified by a radio-frequency (RF) power amplifier (2100 L, E&I, New York). The amplified signal was input into an impedance matching network to drive the HIFU transducer (H-108, Sonic Concepts, Washington; central frequency: 2.5 MHz; active diameter: 60 mm; focal length: 50 mm). The HIFU transducer was focused on a silicone sample (VST-50, Factor II, Inc, Lakeside, AZ) that functioned as an acoustic absorber¹⁹ and was located at the HIFU focal zone. A small silicone tube (Instech Lab, BSIL-T031, PA) that was filled with the thermo-sensitive fluorescent polymer solution (10 mg/ml) and inserted into the silicone phantom along Y direction was used to simulate a small blood vessel. The original inner diameter of the tube was around $787 \mu\text{m}$. When it was tightly inserted into the silicone phantom, the inner diameter was significantly reduced to around $180 \mu\text{m}$.

FIG. 2. Lateral distribution of the HIFU-induced temperature increase.

due to the inward compression generated by the surrounding silicone material (the diameter was measured after the experiments using a micrometer by cutting the silicone phantom along the direction perpendicular to the tube). The sample, HIFU transducer, and light guide were submerged into a water tank that was filled with Intralipid solution ($\mu'_s \approx 3.2$ and $\mu_a \approx 0.026 \text{ cm}^{-1}$) as a tissue-like phantom. The distances among the laser, silicon phantom and detector have been shown in Fig. 1(b). Because the silicone phantom is semi-transparent, the depth of the tube in the scattering medium is 20 mm. The HIFU transducer is mounted on a 3-dimensional translation stage for both initial HIFU positioning and subsequent scanning. Right after the sample is exposed for 100 ms, another channel of the PDG generates a short pulse to trigger the oscilloscope for data acquisition (DAQ). A small thermocouple with a junction size of $75 \mu\text{m}$ (CHCO-003, Omega Engineering, Connecticut) is embedded into the silicone phantom to measure HIFU-induced temperature changes.

Fig. 2 displays the measured HIFU-induced temperature distribution along the lateral (X) direction in the silicone phantom. At each location, the temperature was measured 5 times and the average was shown in the figure (error $< 0.1^\circ\text{C}$). A sharp peak exists when the HIFU focus is overlapped with the thermocouple junction. The peak value

can reach about 8°C when the peak-to-peak voltage of the RF amplifier is 220 V. However, it is unclear what causes the occurrence of the side lobes. If considering the main lobe from $\sim 3^\circ\text{C}$ to the peak value of $\sim 8^\circ\text{C}$, the FWHM is about $600 \mu\text{m}$, which is wider than the lateral FWHM of the ultrasound profile ($\sim 420 \mu\text{m}$), and the full-width at three-quarter maximum (FWTM) is about $260 \mu\text{m}$.

Fig. 3 shows one example of the acquired background fluorescence photons (with the thermo-sensitive fluorescent solution filled in and excitation light turned on, but without HIFU applied). The solid line represents fluorescence photons and the dotted line shows the synchronized signal that was generated by the excitation light pulse via an internal fast photodiode. To display two lines in one figure for comparison, the data of the excitation light pulses were artificially shifted below the emission photon data. The oscillation artifact of the photon signal is mainly due to the instrument including PMT ringing effect (an intrinsic problem of some PMTs due to the internal photoelectron multiple reflection or scattering) and other parasitic capacitance (maybe from the PMT and/or cables). The time interval between any two adjacent excitation pulses is 50 ns, due to the 20 MHz laser repetition rate (see the double arrows marked as (a)). Therefore, the time interval between two adjacent primary fluorescence photons is 50 ns, too (see the double arrows marked as (b)). The numbers from (1) to (10) indicate the locations of the primary fluorescence photons (see the pulse right after each number). Each fluorescence pulse after the number represents a fluorescence photon, which may be mainly generated from the non-100%-off fluorescent polymers. Compared with the excitation pulses, the primary fluorescence photons are delayed about 17 ns (see the two arrows marked as (c)), which is mainly due to the insertion delays of the PMT, amplifier and cables. Because this delay is fixed, the primary fluorescence photons can be easily identified based on the location of the excitation pulses and this delay. After the primary photons are identified, those fluorescence photons between any two adjacent primary fluorescence photons can be analyzed. For example, a few very obvious secondary fluorescence photons are indicated in the figure by large gray arrows. The delay between the secondary photons and their primary photons are 18, 5, 7, and 17 ns, respectively (see the marks of (d), (e), (f), and (g), respectively).

FIG. 3. One example showing the background fluorescence photons. The tube was filled with fluorescent polymer solution and the HIFU was off.

FIG. 4. One example showing USF photons. The tube was filled with fluorescent polymer solution. The HIFU was turned on for 100 ms before the rising edge of the trigger signal and maintained off after the rising edge.

Fig. 4 shows an example of USF photons. The dotted-dashed line is the delayed pulse from the PDG for triggering data acquisition. This means that the 100-ms HIFU exposure is complete right before the rising edge of the trigger pulse. The primary photons are indicated by the numbers in parentheses from (1) to (10). Unlike Fig. 3, a few very late-arriving fluorescence photons have been observed, which are marked by the large gray arrows. For example, the first one is delayed by about 27 ns compared with its primary photon (see the two arrows marked as (d)). Other very late-arriving photons are detected at delay times of 30, 32, and 23 ns as indicated in (e), (f), and (g), respectively. Compared with the secondary photons in Fig. 3, these very late-arriving photons in Fig. 4 are clearly correlated with the HIFU heating event. Therefore, they are indicated as USF photons. In this study, the criterion for differentiating a USF photon from background fluorescence photons is that the delay time (relative to their primary photons) is ≥ 23 ns, which is based on the observation on a large amount of background fluorescence photons.

By laterally scanning the HIFU transducer, the USF photon distribution along the X direction can be acquired. Due to the statistical nature of the USF photons in the proposed method, to acquire a statistically meaningful histogram about the lateral distribution of the detected USF photons, a large amount of excitation events at each location are needed, which is difficult to attain using the current system because photons are counted manually. Therefore, the following criteria were adopted to stop data acquisition at each location when either one of them was satisfied: (1) if more than three USF photons are detected, the location is marked as one, which means that USF photons are detected; or (2) if less than three USF photons are detected within a total number of 300 excitation pulses, the location is marked as zero, which indicates that USF photons are not likely to be detected at that location. The results are shown in Fig. 5. For comparison, a part of the temperature data (shown in the central part of Fig. 2) is also plotted in the same figure. The left vertical axis represents temperature and the right vertical axis shows the (digitized) USF photons. Clearly, USF pho-

tons are observed in the peak area of the temperature main lobe. The size is $260 \mu\text{m}$, which happens to be equal to the FWTM of the temperature main lobe (see Fig. 2). No USF photons were observed outside of this range. To explain this result, three horizontal lines are displayed on the figure. From the bottom to top, the three lines represent 31, 31.5, and 32°C , respectively, after considering the background temperature of the phantom (25°C). It can be seen that the locations where USF photons are detected are above 32°C . On the contrary, the locations where USF photons are not observed are below 31.5°C (the temperature threshold of switching on the fluorophores). The size of the $260 \mu\text{m}$ is larger than the physical size of the tube ($\sim 180 \mu\text{m}$). This is mainly due to the fact that the size of the area where the temperature is above 32°C is not infinitely small. The size of the USF signal eventually depends on both the fluorescent target size and the size of the area where the fluorophores can be switched on. Nevertheless, the outcome of this study was that a small fluorescent tube with a diameter of $\sim 180 \mu\text{m}$ at a large depth of ~ 20 mm in an optically turbid medium was clearly imaged with a size of $\sim 260 \mu\text{m}$, which has not been previously achieved by other fluorescence techniques for deep tissue imaging.^{5,20,21}

FIG. 5. USF photons distribution along lateral (X) direction. The left and right vertical axes represent the temperature and the (digitized) USF photons (indicated by the two dotted arrows).

In conclusion, a fundamentally different fluorescence imaging method was developed based on a concept of USF. By combining the three techniques (USF, light-pulse-delay and photon counting methods), it is demonstrated that high-resolution fluorescence imaging in a deep tissue-like phantom is feasible. Compared with FLOT and FDOT, the depth-to-resolution is improved from ~ 10 to ~ 100 . However, a few key issues need to be solved in future studies to enable the translation of this method to the *in vivo* setting. Namely, a red or near-infrared thermo-sensitive fluorescent polymer with a threshold slightly above body temperature ($\sim 37^\circ\text{C}$) should be developed for *in vivo* applications.^{22,23} For 3D imaging, a laterally and axially uniform temperature distribution is highly desired. This may be achieved by using an ultrasound transducer with a relatively uniform focal size along both the lateral and axial directions or two crossed ultrasound beams. When the pulse energy is high enough, we expect that the fluorescence decay curve can be detected analytically (instead of discrete photons). Thus, the data acquisition speed can be dramatically improved. Compared with the technique of using a continuous wave laser, our technique showed significant improvement in the SNR and spatial resolution (see the supplementary material for detailed comparison and some experimental details).²⁴

This work was supported by funding from the NIH/NIBIB 7R15EB012312-02 (Yuan), the CPRIT RP120052 (Yuan), the Research Enhancement Program at University of Texas at Arlington (Yuan) and the new faculty startup program at University of Texas at Arlington (Yuan), and Japan Science and Technology Agency (Uchiyama). Also, thank Dr. Hanli Liu and Dr. Haiying Huang for sharing some equipment with us.

- ¹B. R. Masters and P. T. C. So, *Handbook of Biomedical Nonlinear Optical Microscopy* (Oxford University Press, New York, 2008).
- ²K. R. Chi, *Nat. Methods* **6**(1), 15 (2009).
- ³L. V. Wang, *Nature Photon.* **3**(9), 503 (2009).
- ⁴D. M. McDonald and P. L. Choyke, *Nat. Med.* **9**(6), 713 (2003).
- ⁵A. Corlu, R. Choe, T. Durduran, M. A. Rosen, M. Schweiger, S. R. Arridge, M. D. Schnall, and A. G. Yodh, *Opt. Express* **15**(11), 6696 (2007).
- ⁶B. Yuan and Q. Zhu, *Opt. Express* **14**(16), 7172 (2006).
- ⁷B. Yuan, S. A. Burgess, A. Iranmahboob, M. B. Bouchard, N. Lehrer, C. Bordier, and E. M. Hillman, *Rev. Sci. Instrum.* **80**(4), 043706 (2009).
- ⁸M. Kobayashi, T. Mizumoto, Y. Shibuya, M. Enomoto, and M. Takeda, *Appl. Phys. Lett.* **89**(18), 181102 (2006).
- ⁹B. Yuan, *J. Biomed. Opt.* **14**(2), 024043 (2009).
- ¹⁰B. Yuan, Y. Liu, P. M. Mehl, and J. Vignola, *Appl. Phys. Lett.* **95**(18), 181113 (2009).
- ¹¹B. Yuan and Y. Liu, *J. Biomed. Opt.* **15**(2), 021321 (2010).
- ¹²C. E. Schutt, M. J. Benchimol, M. J. Hsu, and S. C. Esener, *Proc. SPIE* **8165**, 81650B (2011).
- ¹³L. H. V. Wang, *Dis. Markers* **19**(2–3), 123 (2003).
- ¹⁴J. Yao and L. V. Wang, *Contrast Media Mol. Imaging* **6**(5), 332 (2011).
- ¹⁵D. Razansky, M. Distel, C. Vinegoni, R. Ma, N. Perrimon, R. W. Koster, and V. Ntziachristos, *Nature Photon.* **3**(7), 412 (2009).
- ¹⁶C. Gota, S. Uchiyama, T. Yoshihara, S. Tobita, and T. Ohwada, *J. Phys. Chem. B* **112**(10), 2829 (2008).
- ¹⁷X. Ye, Y. Lu, L. Shen, Y. Ding, S. Liu, G. Zhang, and C. Wu, *Macromolecules* **40**(14), 4750 (2007).
- ¹⁸N. Kikuchi, J. F. Ryder, C. M. Pooley, and J. M. Yeomans, *Phys. Rev. E* **71**(6), 061804 (2005).
- ¹⁹K. Zell, J. I. Sperl, M. W. Vogel, R. Niessner, and C. Haisch, *Phys. Med. Biol.* **52**(20), N475 (2007).
- ²⁰Y. Lin, L. Bolisay, M. Ghijsen, T. C. Kwong, and G. Gulsen, *Appl. Phys. Lett.* **100**(7), 073702 (2012).
- ²¹Y. Lin, T. C. Kwong, L. Bolisay, and G. Gulsen, *J. Biomed. Opt.* **17**(5), 056007 (2012).
- ²²S. Uchiyama, Y. Matsumura, A. Prasanna de Silva, and K. Iwai, *Anal. Chem.* **76**(6), 1793 (2004).
- ²³M. Rahimi, S. Kilaru, G. E. H. Sleiman, A. Saleh, D. Rudkevich, and K. Nguyen, *J. Biomed. Nanotechnol.* **4**(4), 482 (2008).
- ²⁴See supplementary material at <http://dx.doi.org/10.1063/1.4737211> for detailed comparison between CW methods and time-domain method used in this study, and some experimental details.