High resolution imaging beyond the acoustic diffraction limit in deep tissue via ultrasound-switchable NIR fluorescence

Yanbo Pei1,2*, Ming-Yuan Wei1,2*, Bingbing Cheng1,2, Yuan Liu1,2, Zhiwei Xie1,2†, Kytai Nguyen1,2 & Baohong Yuan1,2

1Department of Bioengineering, The University of Texas at Arlington, Arlington, TX 76019, USA, 2Joint Biomedical Engineering Program, The University of Texas at Arlington and The University of Texas Southwestern Medical Center at Dallas, TX 75390, USA.

Fluorescence imaging in deep tissue with high spatial resolution is highly desirable because it can provide details about tissue’s structural, functional, and molecular information. Unfortunately, current fluorescence imaging techniques are limited either in penetration depth (microscopy) or spatial resolution (diffuse light based imaging) as a result of strong light scattering in deep tissue. To overcome this limitation, we developed an ultrasound-switchable fluorescence (USF) imaging technique whereby ultrasound was used to switch on/off the emission of near infrared (NIR) fluorophores. We synthesized and characterized unique NIR USF contrast agents. The excellent switching properties of these agents, combined with the sensitive USF imaging system developed in this study, enabled us to image fluorescent targets in deep tissue with spatial resolution beyond the acoustic diffraction limit.

Fluorescence microscopy has been widely used in biological and medical studies because it can provide subcellular images with structural, functional, and molecular information1,2. In addition to cellular or subcellular information at the superficial tissue level, micro-information, such as microcirculation in sub-centimeter- or centimeter-deep tissue, is also important for studying both healthy and diseased tissues1–4. Unfortunately, microscopy is limited to imaging superficial tissues (submillimeter in depth) because tissue scatters light so strongly that the light cannot be optically focused in deep tissue1,2. Instead of focusing light, fluorescence diffuse optical tomography (FDOT) detects highly scattered light and thus can image centimeter-deep tissues but suffers from poor spatial resolution (millimeters)5–8. As a result, the micro-information is lost in FDOT. To reveal such essential information, high resolution fluorescence imaging in deep tissue is highly desirable.

To achieve this aim, several techniques recently have been proposed and demonstrated, such as multispectral optoacoustic tomography9, ultrasound-modulated fluorescence10,11 or luminescence12, ultrasound-induced temperature-controlled fluorescence13–15, and time-reversed ultrasonically encoded optical focusing (TRUE)16–20. These techniques take advantage of the large penetration depth of diffused light (meaning highly scattered light) and tightly focused ultrasound to acquire fluorescence images in deep tissues (>1 mm). Compared with FDOT, the resolution of these techniques depends on ultrasound frequency and is dramatically improved without sacrificing the imaging depth. Although significant progress has been made, the acoustic diffraction limit, a new barrier, essentially hinders further improvement in the resolution.

To break the acoustic diffraction limit, spatially or temporally applied multiple sound–light interactions based on TRUE have been adopted to focus the light into a region smaller than the focal size of the adopted ultrasound wave19,20. Thus, the spatial resolution can be improved beyond the acoustic diffraction limit. While TRUE-based techniques have shown promising results in static samples, significant challenges remain for imaging dynamic samples because the time reversal of light (one of the key techniques in TRUE-based methods) in deep living tissue is intrinsically vulnerable to dynamic processes, such as blood flow19,21.

Recently, we proposed a concept of ultrasound-switchable fluorescence that has the potential to break the acoustic diffraction limit based on its unique switching features15. Two major components are included in USF imaging: (1) USF contrast agents and (2) an ultrasound-controlled optical imaging system. First, USF contrast agents are used to label the tissue. Then, the labeled tissue is scanned by the system through an area of interest to image the distribution of the USF contrast agents. Unlike TRUE-based methods, USF does not rely on optical time...
reversal and therefore is not susceptible to tissue’s dynamic processes. The principle of USF imaging is based on the unique switching properties of the contrast agent whose fluorescence can be switched on and off via a focused ultrasound wave. To apply this technique for imaging subcentimeter- or centimeter-deep biological tissue beyond the acoustic diffraction limit, one major challenge is the need to develop near infrared (NIR) USF contrast agents that have outstanding switching properties, such as a large ON-to-OFF ratio in fluorescence intensity \( I_{\text{ON}}/I_{\text{OFF}} \), a sharp transition between OFF and ON states \( T_{\text{BW}} \), and an adjustable switching threshold \( T_{\text{th}} \). NIR light can efficiently minimize tissue absorption and therefore can penetrate centimeter-deep tissue. It excites minimal tissue autofluorescence and thus can avoid background noise. A large ratio of \( I_{\text{ON}}/I_{\text{OFF}} \) is essential for suppressing the background fluorescence noise generated by fluorophores in the OFF state (so-called non–100% off fluorescence) and enhancing the signal produced by ultrasonically switched-on fluorophores. Unfortunately, such an NIR USF contrast agent has not been developed to date. The challenge in the imaging system is how to efficiently differentiate the USF signals from the noise. In the current study, we developed a family of NIR USF contrast agents that have a large ratio of \( I_{\text{ON}}/I_{\text{OFF}} \) (~3–9), which has not been achieved by other contrast agents (~1.8). We also developed a sensitive USF imaging system. Combining the unique contrast agents and the imaging system, we demonstrated that the NIR USF technique can break the acoustic diffraction limit for high-resolution imaging in deep tissue.

**Results**

**NIR USF contrast agents.** We synthesized a family of USF contrast agents by encapsulating an environment-sensitive NIR dye of indocyanine green (ICG) into thermo-sensitive nanoparticles (NPs). Fig. 1A schematically shows the USF concept of the synthesized NPs. When the environment’s temperature is below a threshold (the lower critical solution temperature (LCST) of the NPs), the NPs are hydrophilic and absorb a dramatic amount of water. Therefore, their size is relatively large. ICG molecules fluoresce weakly in water-rich microenvironments because water provides a polar and nonviscous solvent microenvironment, which can increase the nonradiative decay rate of the excited fluorophores. When the temperature increases above the threshold (LCST), the NPs experience a phase transition from hydrophilic to hydrophobic. Thus, the water molecules are dramatically expelled from the NPs, and the NPs significantly shrink. Accordingly, the ICG molecules inside the NPs are exposed to a polymer-rich microenvironment, which has a relatively lower polarity and higher viscosity compared with the water-rich microenvironment. This type of microenvironment can suppress the nonradiative decay rate of the excited fluorophores, and therefore the fluorescence intensity from the ICG increases dramatically. This phase transition caused by the environment’s temperature crossing LCST is reversible. A high intensity focused ultrasound (HIFU) transducer can be used to control the temperature in its focal volume and then locally switch on/off the contrast agents.

The NPs are made of thermo-sensitive polymers of either poly (N-isopropylacrylamide) (PNIPAM) or its copolymer with acrylamide (AAm) or N-tert-butylacrylamide (TBAm). For the chemical structures, see Fig. S1 in Supplementary Information. Copolymerizing an appropriate amount of AAm or TBAm can increase or decrease the LCST (and therefore the switching threshold) of the copolymer compared with the pure PNIPAM polymer. ICG is a commonly used NIR dye that has a peak excitation at 780 nm and a peak emission at 830 nm. Our data show that ICG is more sensitive to the change of the solvent’s polarity than to that of the solvent’s viscosity (see Fig. S2). The size of the NPs was found to be between 70 and 150 nm via dynamic light scattering and transmission electron microscopy (see Fig. S3). The chemical structures of the dye can be found in Fig. S1, and the synthesis protocols are provided in the Methods.

Four USF contrast agents were synthesized, including (1) ICG-encapsulated P(NIPAM-TBAm 185:15) NPs, (2) ICG-encapsulated PNIPAM NPs, (3) ICG-encapsulated P(NIPAM-AAm 90:10) NPs, and (4) ICG-encapsulated P(NIPAM-AAm 86:14) NPs. The ratio in each NP refers to the molar ratio between the monomer of PNIPAM and the monomer of TBAm or AAm. The composition of these NPs was confirmed by Fourier transform infrared results (see Fig. S4).

The switching curve of each synthesized NP is shown in Fig. 1B, in which the fluorescence intensity is plotted as a function of the sample temperature. The sharp switching features clearly can be seen for all the four USF NPs with different switching thresholds (LCSTs: 28, 31, 37, and 41 °C, respectively; see the figure caption for details). The \( I_{\text{ON}}/I_{\text{OFF}} \) can reach 2.9, 3.3, 9.1, and 9.1, respectively, corresponding to the four LCSTs, which are 1.6–5.1 times higher than that of other contrast agents. This is mainly attributed to the extremely high temperature sensitivity of PNIPAM. To verify whether the NPs can be repeatedly used, Fig. 1C shows the data measured from one of the samples of ICG-encapsulated P(NIPAM-AAm 90:10) NPs at low (25 °C) and high (44 °C) temperatures. The fluorescence intensity can be repeatedly switched between the two temperatures. The results suggest that the ICG molecules are not likely to be released in a short period because of their relatively larger molecular weight (774.96 g mol\(^{-1}\)) compared with that of a water molecule (18 g mol\(^{-1}\)).

**Sample configuration.** A small silicone tube (with a mean inner diameter of 0.69 mm) was filled with the aqueous solution of the ICG-encapsulated PNIPAM NPs (LCST = 31 °C) and embedded into a piece of porcine muscle tissue to simulate a blood vessel as a target for USF imaging. Fig. 2A shows the configuration of the tissue sample, the tube, the excitation light, the fluorescence collection fiber, and the high intensity focused ultrasound (HIFU) transducer. The porcine tissue has a thickness of ~4 mm (z) and a width of 20 mm (x). The tube was inserted into the tissue along the y direction. The distance from the tube center to the top surface of the tissue is ~4 mm. A fiber bundle with a diameter of ~3 mm (Edmund Optics NT39-366, New Jersey) was used to deliver the excitation light from a laser to the bottom of the tissue to excite the HIFU switched-on fluorophores. The second fiber bundle (Edmund Optics NT42-345) was placed on the top of the tissue to collect USF photons. A 2.5 MHz HIFU transducer (H-108, Sonic Concepts, Washington; active diameter: 60 mm; focal length: 50 mm) was positioned at the bottom of the tissue and focused on the tube region. To efficiently transmit the acoustic energy into the tissue, the HIFU transducer, the bottom surface of the tissue sample, and the fiber bundle for delivering the excitation light were submerged into water. For imaging the tube two dimensionally, the HIFU transducer was scanned on the x-y plane.

**USF imaging system.** The setup of the USF imaging system is shown in Fig. 2B. The system mainly consists of four subsystems: (1) an optical system, (2) an ultrasonic system, (3) a temperature measurement system, and (4) an electronic control system. The optical system includes the delivery of the excitation light and the collection of the emission light. The excitation light is generated from an 808 nm laser and is delivered to the bottom of the sample tissue via the fiber bundle. Although the laser is operated in a continuous wave mode (continuously illuminates once turned on), the time delivering the light to the sample and the illumination duration are controlled using a fast mechanical shutter (UNIBITZ LS3T2, New York) that is triggered by a pulse delay generator (PDG). The collected fluorescence photons via the second fiber bundle are delivered to a set of emission filters and then received by a photomultiplier tube (PMT). The carefully designed combination of the four emission filters can maximally reject the excitation photons and pass the emission fluorescence photons. The signal after the PMT is further amplified and

---

**Fig. S4:** The chemical structures of the dye can be found in Fig. S1, and the synthesis protocols are provided in the Methods.

Four USF contrast agents were synthesized, including (1) ICG-encapsulated P(NIPAM-TBAm 185:15) NPs, (2) ICG-encapsulated PNIPAM NPs, (3) ICG-encapsulated P(NIPAM-AAm 90:10) NPs, and (4) ICG-encapsulated P(NIPAM-AAm 86:14) NPs. The ratio in each NP refers to the molar ratio between the monomer of PNIPAM and the monomer of TBAm or AAm. The composition of these NPs was confirmed by Fourier transform infrared results (see Fig. S4). The switching curve of each synthesized NP is shown in Fig. 1B, in which the fluorescence intensity is plotted as a function of the sample temperature. The sharp switching features clearly can be seen for all the four USF NPs with different switching thresholds (LCSTs: 31 °C). The ICG molecules are not likely to be released in a short period because of their relatively larger molecular weight (774.96 g mol\(^{-1}\)) compared with that of a water molecule (18 g mol\(^{-1}\)).
then acquired by an oscilloscope. The ultrasonic system consists of the HIFU transducer and the driving components, including an impedance matching network (NWM), a radio-frequency (RF) power amplifier, and a function generator (FG). The temperature at the HIFU focus is measured by a micron-sized thermocouple via an amplifier and the second oscilloscope. The entire system is controlled by the PDG, including the firing of the HIFU heating pulse, the firing of the excitation light pulse, and the data acquisition of the oscilloscope. The time sequence of these processes is plotted in Fig. 2C. In this study, the ultrasonic exposure time is 300 ms, determined by the width of the gating pulse from the PDG. During the ultrasonic exposure period, the tissue temperature at the HIFU focus rises. After the exposure, the temperature reduces as a result of thermal diffusion. The excitation light illuminates the tissue for 2 ms right before the end of the ultrasonic exposure. At the same time, the fluorescence signal is acquired by the oscilloscope, which is triggered by a pulse from the PDG. The HIFU transducer is scanned by a two-dimensional translation stage. The details about the system can be found in Methods.

High resolution USF images. Fig. 3A shows a USF image of the tube on the x-y plane. The two vertical dashed lines indicate the locations of the inner edges of the tube. The full-width-at-half-maximum (FWHM) and the full-width-at-one-eighth-of-the-maximum (FWEM) of the USF image profile along the x direction at each y location were calculated. The averaged FWHM and FWEM at different y locations were 0.48 ± 0.13 mm and 0.68 ± 0.19 mm, respectively. Although the FWHM (0.48 mm) is narrower than the inner diameter of the tube, the FWEM (0.69 mm) is very close to the tube’s inner diameter (0.69 mm). This is understandable because
the inner diameter is a parameter describing the full size of the tube. Therefore, the FWEM, instead of the FWHM, can be considered a parameter describing the full size of the USF image.

To compare the USF image with a pure ultrasound image, the same sample was scanned on the x-y plane using the same HIFU transducer via the commonly used pulse-and-echo method (see the details in Methods). At each x-y location, the reflected ultrasonic echo from the top inner boundary of the tube was recorded and used to generate the ultrasound image. The result is shown in Fig. 3B. Its averaged FWHM and FWEM are 0.76 ± 0.01 mm and 1.12 ± 0.02 mm, respectively, and both are larger than those of the USF image.

Fig. 3C and Fig. 3D shows the comparison among the profiles of USF, diffused fluorescence light, ultrasound, and temperature along the horizontal dashed line marked in Fig. 3A. The FWHM of the diffused fluorescence signal is 3.9 mm (see the black line with “x” in Fig. 3C and the details in Methods about how the signal was measured). It is significantly larger than the FWHM of the corresponding USF’s profile (0.48 mm; see the blue solid line) and the tube’s inner diameter (0.69 mm). This overestimation is commonly seen in FDOT, mainly because of the dramatic light scattering caused by the thick tissue. This result indicates that the USF has much higher resolution than does FDOT. The FWHMs of the temperature profile (0.66 mm; see the red line with squares in Fig. 3D) and the ultrasound profile (0.76 mm; see the black line in Fig. 3D) are also larger than that of the USF profile (0.54 mm; see the blue line with squares in Fig. 3D). These results indicate that the USF achieves the highest resolution among the techniques mentioned above.

Estimation of the FWHM of the point spread function. To estimate the FWHM of the point spread function of the USF imaging system, we adopted the following strategies. (1) A smaller tube (inner diameter: 0.31 mm) was also scanned. (2) Because further reducing the tube size was difficult and limited by the signal-to-noise, we mathematically de-convolved the tube profile data from the acquired USF profile data. The average FWHM of the estimated point spread functions of both the large and small tubes was 0.29 mm. For the ultrasound imaging system, if one assumes that...
the ultrasound speed in muscle is between 1,542 and 1,626 m/s, the FWHM of the diffraction-limited focal size (equivalent to the FWHM of the lateral point spread function) of the adopted HIFU transducer (frequency = 2.5 MHz and f-number = 0.83) is theoretically between 0.512 and 0.54 mm. Therefore, the estimated size of the point spread function of the USF system is smaller than that of the ultrasound system. The above result clearly indicates that USF can achieve a resolution beyond the acoustic diffraction limit.

Discussion

The synthesized USF contrast agents in this study have the following advantages. First, they are NIR contrast agents and therefore can be used for deep-tissue imaging. Second, their ON-to-OFF ratios (I_{ON}/I_{OFF}) are a few times higher than those of the existing contrast agents. Third, the switching threshold (T_{th}) can be well controlled and therefore can be potentially used for in vivo animal studies in the future where the T_{th} should be slightly higher than body temperature (37°C). Fourth, the transition between on and off states is sharp (generally ~3–5°C). Such a narrow transition bandwidth (T_{FWHM}) is necessary for efficiently switching on/off fluorescence and avoids potential tissue thermal damage in animal studies. Fifth, the excellent switching repeatability of the developed USF contrast agents allows acquisition of multiple images at the same location for either improving signal-to-noise ratio (SNR) via averaging or monitoring tissue dynamic processes.

Generally, the USF signal is weak and may be contaminated with noise. This problem becomes even more severe when high resolution is desired. This is because the high resolution requires the small focal size and therefore the small amount of USF contrast agents in the focal volume can be switched on. Thus, the amount of the detectable USF photons (the desired signal) dramatically decreases as the resolution increases, while the amount of the noise photons remains stable because noise is not correlated with the focal size and the resolution (see the next paragraph). Accordingly, to successfully image the small tube in deep tissue, it is necessary to optimize the USF imaging system and use the NIR USF contrast agent with a large I_{ON}/I_{OFF}.

The PMT-received photons potentially consist of four components: (1) excitation photons from the laser due to the leakage of the emission filters, (2) tissue autofluorescence photons within the pass band of the emission filters, (3) photons emitted from the non–100% off USF contrast agents, and (4) USF photons. The first three components are the major noise sources. They can be generated from the entire tissue sample and are not correlated with the ultrasound focus. Therefore, they can be called global noise. The last component is the desired signal, which is uniquely related to the ultrasound focus. It can be called local signal.

Because of the very short HIFU exposure time (see the detailed discussion in the Supplementary Information), the thermal damage can be completely ignored because of the high resolution. However, it is worth pointing out that MI = 2.5 is not required and lower MI should also work for USF imaging. Quantitative investigation between the USF signal-to-noise ratio and MI is necessary in future. In addition, this value can be reduced by adopting several strategies that are discussed in the Supplementary Information.

In conclusion, we synthesized a family of NIR USF contrast agents based on ICG-encapsulated PNIPAM NPs. These NPs have excellent switching properties based on the fluorescence-intensity ratio between on and off states, the narrow transition bandwidth, the adjustable switching threshold, and the switching repeatability. The developed USF imaging system was optimized to clearly differentiate the USF photons from the background noise. When combining the contrast agents and the imaging system, we demonstrated that the USF imaging technique could break the acoustic diffraction limit for high-resolution imaging in deep tissue.

Methods

Materials.

N-isopropylacrylamide (NIPAM), acrylamide (AAm), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), N,N,N’,N’-tetramethyl ethylene diamine (TEMED), N,N’-methylenebisacrylamide (BIS), N-tert-butylacrylamide (TBAm), sodium ascorbate, and ICG were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were used as purchased without further purification.

Synthesis protocols.

The ICG-encapsulated PNIPAM NPs were used as one example to describe the protocol, and others are similar. ICG was added directly into the reaction solution prior to the polymerization and then loaded into the NPs, likely as a result of the amphiphilic property of the ICG molecule itself. NIPAM (monomer, 0.6822 g), acrylamide (AAm), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), N,N,N’,N’-tetramethyl ethylene diamine (TEMED), N,N’-methylenebisacrylamide (BIS), N-tert-butylacrylamide (TBAm), sodium ascorbate, and ICG were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were used as purchased without further purification.

Scientific Reports | 4 : 4690 | DOI: 10.1038/srep04690
was used as the solvent for synthesis, 15 mol% of TBAm that can be dissolved in water.

Combination of optical and PECT/PET modalities. *Journal of Nuclear Medicine* 49, 169–172, doi:10.2967/jnumed.107.043331 (2008).

Wang, L. V. Multiscale photocoustic microscopic and computed tomography. *Nature Photonics* 3, 503–509, doi:10.1038/nphoton.2009.157 (2009).

Yuan, B. & Zhu, Q. Separately reconstructing the structural and functional parameters of a fluorescent inclusion embedded in a turbid medium. *Optics Express* 14, 7172–7187, doi:10.1364/OE.14.010717 (2006).

Razansky, D. et al. Multispectral opto-acoustic tomography of deep-seated fluorescent proteins in vivo. *Nature Photonics* 3, 412–417, doi:10.1038/nphoton.2009.98 (2009).

Yuan, B., Liu, Y., Mehl, P. & Vignola, J. Microbubble-enhanced ultrasound-modulated fluorescence in a turbid medium. *Applied Physics Letters* 95, 181113–181113, doi:10.1063/1.3262959 (2009).

Kobayashi, M., Mizumoto, T., Shibuya, Y., Enomoto, M. & Takeda, M. Fluorescence tomography in turbid media based on acousto-optic modulation encoding. *Applied Physics Letters* 98, 181102, doi:10.1063/1.2364600 (2006).

Huynh, N. T., Hayes-Bill, B. R., Zhang, F. & Morgan, S. P. Ultrasound modulated imaging of luminescence generated within a scattering medium. *J. Biomed. Opt.* 18, 011117, doi:10.1117/11.2020505 (2013).

Lin, Y., Bolisay, L., Ghaisan, M., Kwong, T. C. & Gulsen, G. Temperature-modulated fluorescence imaging in a turbid media. *Applied Physics Letters* 100, 73702–737024, doi:10.1063/1.3681378 (2012).

Lin, Y., Kwong, T. C., Bolisay, L. & Gulsen, G. Temperature-modulated fluorescence tomography based on both concentration and lifetime contrast. *J. Biomed. Opt.* 17, 056007–0560601, doi:10.1117/1.1056607 (2012).

Yi, W. K., Uchiyama, S., Liu, Y. K. N. T. & Alexandrakis, G. High-resolution imaging in a deep turbid medium based on an ultrasound-switchable fluorescence technique. *Applied Physics Letters* 101, 033703, doi:10.1063/1.4737211 (2012).

Xu, X. A., Liu, H. L. & Wang, L. V. Time-reversed ultrasonically encoded optical focusing in scattering media. *Nature Photonics* 5, 154–157, doi:10.1038/Nphoton.2010.306 (2011).

Wang, Y. M., Judkewitz, B., DiMarzio, C. A. & Yang, C. H. Deep tissue focal fluorescence imaging with digitally time-reversed ultrasound-encoded light. *Nat Commun* 3, 1083, doi:10.1038/Ncomms1925 (2012).

St. K., Fiolka, R. & Cai, M. Fluorescence imaging beyond the ballistic regime by ultrasound-encoded light focusing in a confined channel. *Nature Photonics* 6, 657–661, doi:10.1038/nphoton.2012.205 (2012).

Judkewitz, B., Wang, Y. M., Horstmeyer, R., Mathy, A. & Yang, C. Speckle-scale focusing in the diffusive regime with time reversal of variance-encoded light (TROVE). *Nature Photonics* 7, 300–305, doi:10.1038/nphoton.2013.31 (2013).

Si, K., Fiolka, R. & Cai, M. Breaking the spatial resolution barrier via iterative sound-light interaction in deep tissue imaging. *Scientific Reports* 2, doi:10.1038/srep00748 (2012).

Konecky, S. D. & Tromberg, B. J. IMAGING Focusing light in scattering media. *Nat Photonics* 5, 155–157, doi:10.1038/Nphoton.2011.200 (2011).

Wodicka, L. & Ritsch, C. Fluorescence imaging using liquid targets. *Nature Medicine* 9, 123–128, doi:10.1038/Nm0103-123 (2003).

Chen, Y. & Li, X. Near-Infrared Fluorescent Nanocapsules with Reversible Response to Thermal/pH Modulation for Optical Imaging. *Biomacromolecules* 12, 4367–4372, doi:10.1021/bm201350d (2011).

Gotz, C., Uchiyama, S., Yoshihara, T., Tobita, S. & Ohwada, T. Temperature-Dependent Fluorescence Lifetime of a Fluorescent Polymeric Thermometer, Poly(N-isopropylacrylamide), Labeled by Polarity and Hydrogen Bonding Sensitive 4-Sulfamoyl-7-aminobenzofurazan. *The Journal of Physical Chemistry B* 112, 2829–2836, doi:10.1021/jp070981g (2008).

Wang, D. P., Miyamoto, R., Shiratani, Y. & Hirai, T. BODIPY-Conjugated Thermoresponsive Copolymer Fluorescent Thermometer Based on Polymer Microrosivity. *Langmuir* 25, 13176–13182, doi:10.1021/la901860x (2009).

Yuan, B. H., Chen, N. G. & Zhu, Q. Emission and absorption properties of indocyanine green in Intralipid solution. *J. Biomed. Opt.* 9, 497–503, doi:10.1117/1.1569541 (2004).

Yuan, B. H., Pei, Y. B. & Kandukuri, J. Breaking the acoustic diffraction limit via nonlinear effect and thermal confinement for potential deep-tissue high-resolution imaging. *Applied Physics Letters* 102, doi:10.1063/1.4792736 (2013).
28. Cheng, B. et al. Development of Ultrasound-Switchable Fluorescence Imaging Contrast Agents Based on Thermosensitive Polymers and Nanoparticles. *IEEE Journal of Selected Topics in Quantum Electronics* **20**, 6801214, doi:10.1109/JSTQE.2013.2280997 (2014).

29. Yang, Q., Xu, X., Lai, P., Xu, D. & Wang, L. V. Time-reversed ultrasonically encoded optical focusing using two ultrasonic transducers for improved ultrasonic axial resolution. *J. Biomed. Opt.* **18**, 110502–110502, doi:10.1117/1.jbo.18.11.110502 (2013).

30. Rahimi, M. et al. Synthesis and Characterization of Thermo-Sensitive Nanoparticles for Drug Delivery Applications. *J Biomed Nanotechnol* **4**, 482–490, doi:10.1166/jbn.2008.014 (2008).

Acknowledgments
This work was supported in part by funding from the NIH/NIBIB 7R15EB012312-02 (Yuan), the CPRIT RP120052 (Yuan) and the NSF CBET-1253199 (Yuan). The authors are grateful to Drs. Hanli Liu and Yi Hong for sharing lab equipment and to Ms Jyothi U. Menon for TEM measurement.

Author Contributions
B.Y. designed the experiment. Y.P. implemented the imaging experiment and analyzed the image data. Z.X. and K.N. designed the synthesis protocol of the USF contrast agents. M.-Y.W. and B.C. synthesized the contrast agents and M.-Y.W., B.C. and Y.L. characterized the contrast agents. B.Y., M.-Y.W. and Y.P. prepared the manuscript. All authors reviewed the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pei, Y.B. et al. High resolution imaging beyond the acoustic diffraction limit in deep tissue via ultrasound-switchable NIR fluorescence. *Sci. Rep.* **4**, 4690; DOI:10.1038/srep04690 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. The images in this article are included in the article’s Creative Commons license, unless indicated otherwise in the image credit; if the image is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the image. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/