An in-house built temperature-controlled fluorescence spectrometer

An in-house built temperature-controlled fluorescence spectrometer system was developed to study the fluorescence intensity change of the USF contrast agents versus the solution temperature. A 3.5 mL quartz cuvette (Hellma, Germany) was filled with 3 mL sample and placed into a temperature-controlled sample compartment (qpod 2e, Quantum Northwest, Inc., USA; temperature precision: ±0.01 ℃; temperature accuracy: ±0.15 ℃ from –20 to +105 ℃). The solution temperature was measured by the qpod system via inserting a thermometer probe (WD-93824-00, Oakton, USA; temperature accuracy: 0.1 ℃ from 0 to 70 ℃) into the sample. The excitation light with a wavelength of 808 nm generated by a laser (MGL-II-808-2W, Dragon lasers, China) was passed through the open window on the cuvette holder and delivered to the sample via a fiber bundle. The emitted fluorescence from the sample was filtered by a longpass filter (BLP01-830R-25, Semrock Inc., USA) and collected by a modular USB spectrometer (USB2000+, Ocean Insight, USA) attached to the cuvette holder at a 90-degree angle from the excitation light beam. A MATLAB-based program was developed to read the solution temperature from the interface of the Q-Blue software (Quantum Northwest, Inc., USA) which controlled the cuvette holder. The spectrometer received the commands from the program to acquire the spectrum at the preset temperature points automatically during the heating of the sample.

ICG-liposome characterization

Three independently synthesized ICG-liposome samples were tested by the in-house built fluorescence spectrometer system with same setting parameters. The wavelength of the excitation light was 808 nm and the emitted fluorescence was filtered by a 830 nm-longpass filter. The preset solution temperature of the sample was increased from 35.0 to 45.0 ℃ with an increment of 0.1 ℃ (limited by the precision of the thermometer probe). The exposure time of the spectrometer was 100 ms. The fluorescence intensity at each temperature point was calculated by summing the acquired spectrum data from 830 to 1020 nm. The hydrodynamic size of the ICG-liposome was measured using a dynamic light scattering (DLS, NanoBrook 90PlusPALS, Brookhaven Instruments, USA) system at room temperature. The sample was diluted 100 times with PBS buffer before conducting the measurement to avoid aggregation.

Calculation of image's SNR

Along the y axis, each USF image had three lines and each line had 41 scan points. From each line, we could calculate a SNR value based on the following definition. The SNR of each USF image was defined as the mean of the three SNRs of the three lines. The background defined as the average of the 12 scan points at the two edges of each line (i.e., 8 points at each edge, two maximum values and two minimum values of the total 16 points were excluded) was subtracted first. To calculate the SNR of each line, the noise was defined as the standard deviation of the 12 scan points (used for calculating the background) and the signal was defined as the root-mean-square of the maximum six signal values from the 11^{th} to 31^{th} scan points (the silicone tube was shown in this range). The SNR was then calculated by the following equation:

$$SNR = 20\log_{10}\frac{Signal}{Noise}.$$

A brief discussion about the background photons in USF imaging

To investigate the stability of the EM gain under different trigger modes, a weak and stable light source is needed to illuminate the EMCCD camera. Tissue's autofluorescence is a reasonable light source for this purpose because it is weak under the 808 nm excitation and also stable in a short period time. A brief discussion about the background photons is given here. In USF imaging, it is common that some background photons can be detected, which are independent of ultrasound and usually consist of tissue autofluorescence, excitation light leakage from the laser, and/or non-100%-off fluorescence from the USF contrast agent. Usually, the excitation light leakage has been well minimized by using the multiple and high-quality emission filters, which should not be dominant in the background photons. When the silicone tube is injected with water only, the background photons should not have any fluorescence photons from the non-100%-off fluorophores. Thus, the major light source is from the tissue autofluorescence.

http://dx.doi.org/10.21037/qims-20-796

Background images (I_{BG}) and background fluorescence images (I_{BGF}) of the tissue samples

A background image (I_{BG}) is defined as the image acquired by the EMCCD camera when the silicone tube is filled with water (i.e., no USF contrast agents are injected and no ultrasound is exposed). The background image is usually formed by tissue's autofluorescence (I_{AF}) and also some minor excitation photons leaked through the emission filters from the laser due to the imperfect property of the emission filters (I_{EL}). In general, we have $I_{BG}=I_{AF}+I_{EL}$. When the silicone tube is filled with the USF contrast agent solution, one more component, i.e., the background fluorescence (I_{BGF}) from the non-100%-off contrast agents, is included in the acquired image (i.e., $I_{UCA}=I_{BGF}+I_{BG}=I_{BGF}+I_{AF}+I_{EL}$). Again, no ultrasound is applied when acquiring these images. Thus, by subtracting the image acquired when the tube is filled with water (I_{BG}) from the image acquired when the tube is filled with USF contrast agent (I_{UCA}), we can have the background fluorescence image (i.e., $I_{BGF}=I_{UCA}-I_{BG}$), which is generated only from the non-100%-off USF contrast agent because tissue's autofluorescence (I_{AF}) and the laser leakage (I_{EL}) have been subtracted.

When the EM gain is set to 1, *Figure S2A,B,C,D,E* show the white light photo, background image (i.e., I_{BG}, tissue's autofluorescence and laser leakage) and the background fluorescence image (i.e., I_{BGF}, fluorescence coming from the non-100%-off USF contrast agent) of the silicone tube embedded in chicken breast tissue with a thickness of 2.5, 3.5, 4.5, 5.0 and 5.5 cm, respectively. The average intensity (the spatial average of the whole 2D image) of these figures are quantitatively shown in *Figure S2F*. The average intensity of all the background images (I_{BG}) acquired with different tissue thickness is similar to each other (444, 573, 533, 545 and 564 counts corresponding to 2.5, 3.5, 4.5, 5.0 and 5.5 cm, respectively). This is because the background photons are from tissue's autofluorescence and laser leakage, which are usually independent of tissue's thickness when the thickness is large enough (such as ≥3.5 cm in this example). However, the average intensity of all the background fluorescence images (I_{BGF}) reduces dramatically with the increase of the tissue thickness (5113, 706, 182, 88 and 56 counts corresponding to 2.5, 3.5, 4.5, 5.0 and 5.5 cm, respectively). When tissue thickness >3.5 cm, the background fluorescence intensity (I_{BGF}) becomes smaller than the background intensity (I_{BG}). This result is understandable because the background fluorescence photons are mainly caused by the non-100%-off USF contrast agents in the tube. When increasing the depth of the tube in tissue samples, the fluorescence intensity will exponentially decay due to tissue's scattering and absorption and the attenuation of the excitation light in tissue. When depth is small enough, the background fluorescence intensity (I_{BGF}) may be higher than the background intensity (I_{BG}) depending on the fluorophore concentration, its emission efficiency and the excitation light intensity. This happens in this example when tissue thickness is 2.5 and 3.5 cm. With the increase of the depth, the background fluorescence intensity (I_{BGF}) reduces so quickly that it may be lower than the background intensity (I_{BG}). This happens in this example when tissue thickness is >3.5 cm.

2D-USF-signal images

A 2D-USF-signal image ($I_{2D-USF-sig}$) at a specific scan position of the ultrasound focus is defined as the subtracted image between the two images acquired from the EMCCD camera after (I_{US-On}) and before (I_{US-Off}) the ultrasound is applied. The following equation illuminates the relationship among these images: $I_{2D-USF-sig}=I_{US-On}-I_{US-Off}=(I_{2D-USF-sig}+I_{BG}+I_{BGF})-(I_{BG}+I_{BGF})$, where $I_{2D-USF-sig}$ represents ultrasound-induced fluorescence increase and is the real USF signal that we are detecting. I_{BG} and I_{BGF} are the background image and background fluorescence image, respectively, discussed in the previous section. By subtracting I_{US-Off} from I_{US-On}, both the I_{BG} and I_{BGF} can be removed and the real 2D-USF-signal image $I_{2D-USF-sig}$ can be found.

*Figure S3*A shows a typical example of a 2D-USF-signal image when the ultrasound focus is scanned on the silicone tube embedded in chicken breast tissue with three different thicknesses, 2.5, 3.5 and 4.5 cm, respectively. *Figure S3B* shows the one-dimensional (1D) profiles of the *Figure S3A* across the geometric center along the Y direction for the tissue thickness of 2.5 cm (the blue line) and 3.5 cm (the red line). The FWHM of the 1D profiles significantly increases from 21.32 to 34.84 mm when the tissue thickness rises from 2.5 to 3.5 cm. The circular shape of the 2D-USF-signal image is even not recognizable in the 4.5 cm-thick chicken breast due to the increased light scattering from the thicker tissue.

http://dx.doi.org/10.21037/qims-20-796

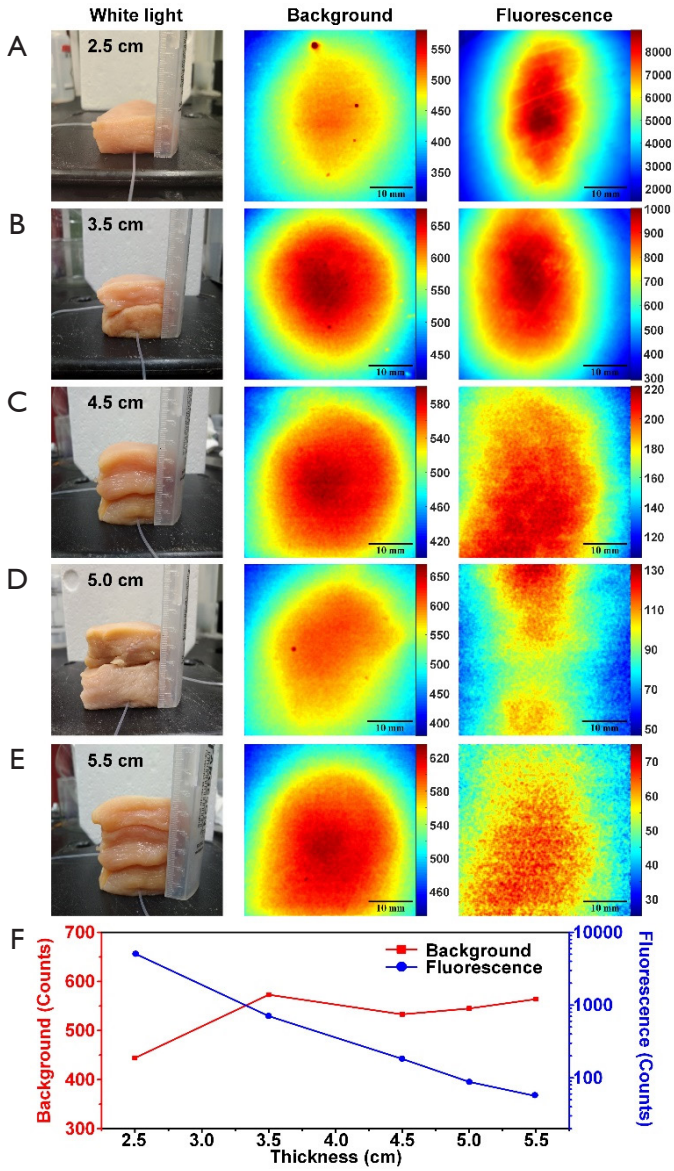

Figure S1 The white light photo, background image (I_{BG}) and background fluorescence image (I_{BGF}) of the silicone tube embedded in chicken breast tissue with a thickness of (A) 2.5 cm, (B) 3.5 cm, (C) 4.5 cm, (D) 5.0 cm and (E) 5.5 cm. (F) The average intensity of the background images (the red line with squares, and see the left y axis with a linear scale) and the average intensity of the background fluorescence images (the blue line with circles, and see the right y axis with a logarithm scale) at different thicknesses.

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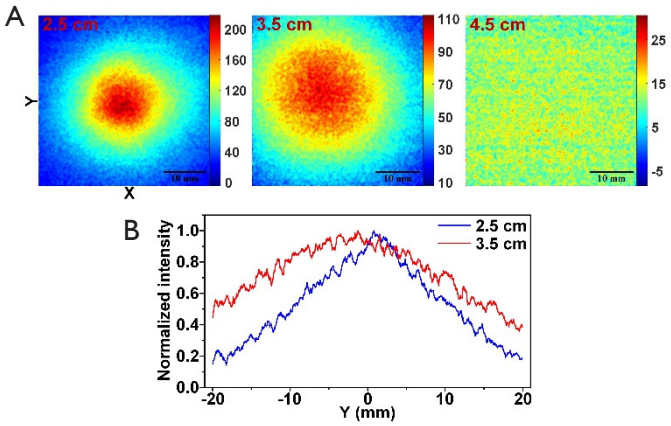

Figure S2 USF-signal images in different thick tissues. (A) Three 2D-USF-signal images when the ultrasound focus is scanned on the silicone tube embedded in chicken breast tissue with three different thicknesses, 2.5, 3.5 and 4.5 cm, respectively. (B) The 1D profiles of the 2D-USF-signal image across the geometric center along the Y direction with the tissue thickness of 2.5 cm (the blue line) and 3.5 cm (the red line). USF, ultrasound-switchable fluorescence.

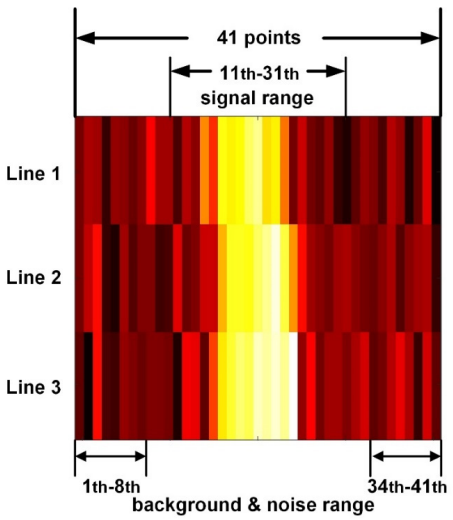

Figure S3 The schematic diagram of SNR calculation. SNR, signal-to-noise ratio.

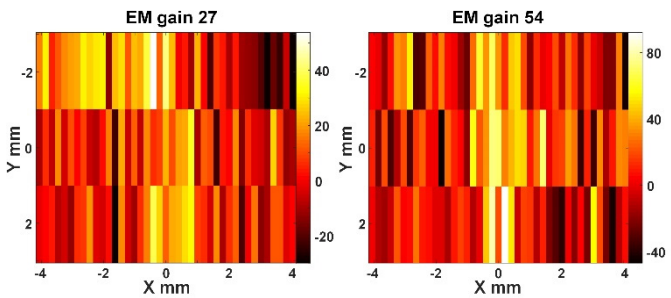

Figure S4 The USF images of the silicone tube filled with ICG-liposomes and embedded in 5.5 cm-thick chicken breast tissue at a gain of 27 (left) and 54 (right), respectively. USF, ultrasound-switchable fluorescence; ICG, indocyanine green.

http://dx.doi.org/10.21037/qims-20-796