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Title Nanoscale Ultrasound-switchable FRET Based Liposomes for Near-infrared Fluorescence Imaging in Optically Turbid Media

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Abstract: A new approach to fluorescence imaging in optically turbid media centered on the use of nanoscale ultrasound-switchable FRET based liposome contrast agents is reported. Liposomes containing lipophilic carbocyanine dyes as FRET pairs with emission wavelengths located in the near-infrared window were prepared. The efficacy of FRET and self-quenching for liposomes with a range of fluorophore concentrations was first calculated from measurement of the liposome emission spectra. Exposure of the liposomes to ultrasound resulted in changes in the detected fluorescent signal, the nature of which depending on the fluorophores used, detection wavelength and the fluorophore concentration. Line scanning of a tube containing the contrast agents with 1 mm inner diameter buried at a depth of 1 cm in a heavily scattering tissue phantom demonstrated an improvement in image spatial resolution by a factor of 6.3 as compared with images obtained in the absence of ultrasound. Improvements were also seen in image contrast with the highest obtained being 9% for a liposome system containing FRET pairs. Overall the results obtained provide evidence of the potential the nanoscale ultrasound-switchable FRET based liposomes studied here have for in vivo fluorescence imaging.

1 Introduction

The potential *in vivo* fluorescence imaging has to non-invasively provide direct visualization of physiological processes in native tissue has sparked many investigations identifying potential strategies for this to be achieved. The realization of this, however, is hampered by factors such as high optical scattering, absorption, and autofluorescence that collectively seriously limit the spatial resolution, penetration depth and signal to noise ratios that can be achieved in tissue imaging.

In recent years the use of fluorescent probes that are excited and emit in the near infra-red region has been investigated to improve the capabilities of *in vivo* fluorescence imaging. This is a useful approach as the absorption coefficient of near infra-red light is at least one order of magnitude lower than in the visible region^[1] and, due to the comparative increase in wavelength, light scattering and autofluorescence are also reduced.^[2] As a result light penetration depths in tissue can reach several centimeters^[3] and due to reduced autofluorescence unwanted background signals are decreased as well.

Despite the improvement in imaging capabilities that can be made using near-infrared probes *in vivo* fluorescence imaging still suffers from poor spatial resolution and low signal to noise ratios. To address both of these issues hybrid imaging approaches combining ultrasound (US) and optical imaging have been considered which involves the use of a focused US beam to modulate only the fluorophores within the US focus. In this way fluorescence imaging with US level spatial resolution can be achieved.^[4] One such hybrid approach is US modulated fluorescence tomography (USMFT)^[5-8] which produces fluorescent emission modulated at the US frequency from excited fluorescent probes within the US focal zone. Despite gains in spatial resolution this technique produces extremely low modulated light levels (modulation depth 10^{-6} to 10^{-4} ^[9]). The modulated fluorescent signal has been enhanced through the use of fluorophore labelled microbubbles that act as contrast agents which cyclically emit or quench

fluorescent emission via US induced oscillation of the microbubbles.^{[10][11]} To date microbubbles labelled with donor-quencher pairs have been the most commonly used approach.^[12]

Unfortunately, despite the improvements contrast agents offer, USMFT is still hampered by low signal to noise ratios.^[4] Recently an imaging technique based on the use of US-switchable fluorescence has been demonstrated to significantly improve SNR of fluorescent imaging in turbid media. This approach uses thermoresponsive probes that ‘turn on’ in response to local temperature changes induced by the application of high intensity US pulses. The emission effectively turns off when the temperature reduces below a threshold level.^[4,13] In comparison to USMFT greater On-to-Off ratios (defined as the ratio of the fluorescence intensity with US on to the intensity prior to sonication) can be attained as the on and off states are more distinct. However, more work is required to produce probes with temperature thresholds slightly above body temperature as compared to temperature changes of the order of 10 degrees Celsius reported to date.

Here a new approach to fluorescence imaging in optically turbid media that uses nanoscale US-switchable liposome fluorescent probes is reported. This approach is based on the effect of US on Fluorescence Resonance Energy Transfer (FRET) between an excited donor fluorophore and an acceptor fluorophore. In practice liposomes labelled with donor and acceptor fluorophores are exposed to US which effectively switches the distance dependent transfer of energy between fluorophores on and off. This FRET based method is advantageous as compared to techniques based on fluorescence self-quenching (SQ) alone as there is a large gap between the excitation maxima of the donor and the emission maxima of the acceptor which significantly reduces the background fluorescence induced by breakthrough of the excitation light.

This work describes the preparation and characterization of nanoscale liposomes containing lipids labelled with the carbocyanine dyes DiIC18(5) (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt) (“DiD”, Life Technologies Ltd, CA, USA) and DiIC18(7) (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide) (“DiR”, Life Technologies Ltd, CA, USA). Experimentally the emission spectra from liposomes labelled at a range of different concentrations and ratios of DiD to DiR in their equilibrium state are obtained to identify the effect the increase in concentration and hence proximity has on fluorescence emission and quenching. The results of these measurements are then used to investigate the efficiency of FRET and SQ for each liposome system studied to determine which mechanism and concentration has the potential for the greatest ‘on/off’ US induced switching of fluorescence. Liposomes are then exposed to US and the resulting changes in fluorescence emission are recorded. Finally, line scans of the fluorophore labelled liposomes buried deep inside a scattering phantom are made to assess the potential improvements in resolution and contrast the use of nanoscale US-switchable FRET based liposomes offers to *in vivo* optical imaging. The spectral region of DiD-DiR pair is largely red shifted to close or within the NIR window, with the excitation maxima at ~640 nm, emission maxima of DiD (donor) and DiR (acceptor) at ~670 nm and ~770 nm respectively and as such the findings of this work will have direct applicability to *in vivo* tissue imaging.

2. Results

2.1 Fluorescence Spectroscopy Measurements

DiD-DiR labelled liposomes with six groups of DiD concentrations (0.05 mol %, 0.1 mol %, 0.25 mol %, 0.5 mol %, 0.75 mol %, and 1 mol %) at four DiD to DiR ratios (1:0, 1:0.25, 1:1, and 0:1) were prepared by the manufacturing process described in the Experimental Section.

The fluorescence emission spectra of these liposomes are shown in Figure 1.

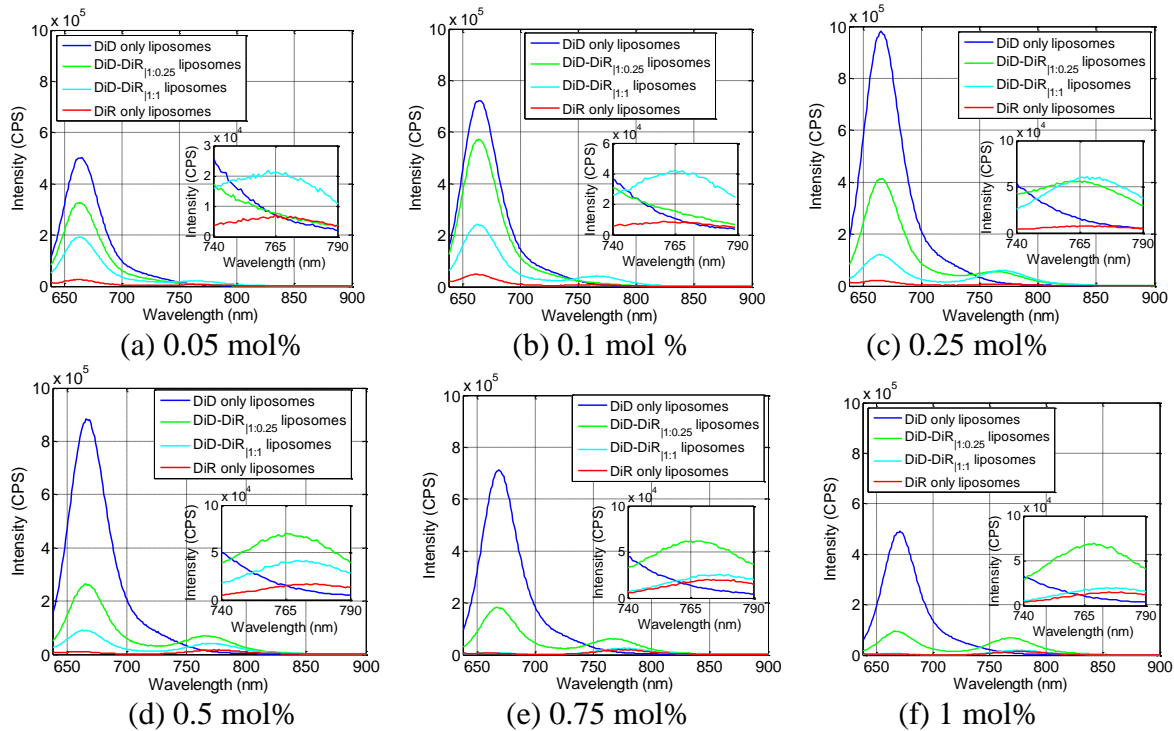


Figure 1. Fluorescence emission spectra of DiD only liposomes, DiD-DiR labelled liposomes with DiD to DiR ratio 1 to 1 (DiD-DiR_{1:1} liposomes), DiD-DiR labelled liposomes with DiD to DiR ratio 1 to 0.25 (DiD-DiR_{1:0.25} liposomes), and DiR only liposomes at six different concentrations of DiD: (a) 0.05 mol%, (b) 0.1 mol%, (c) 0.25 mol%, (d) 0.5 mol%, (e) 0.75 mol%, (f) 1 mol%. The insets show a magnified view of emission over the wavelength range between 740 nm and 790 nm. CPS: counts per second.

In the DiD only liposomes strong peaks are seen spanning the DiD emission wavelength

range (center wavelength: ~ 664 nm) for all DiD concentrations studied (Figures 1 (a) – (f)).

The spectra recorded from DiD-DiR labelled liposomes are characterized by lower emission over the DiD range as compared to the DiD only liposomes for all DiD-DiR ratios considered.

The decrease corresponding to the emission range of the FRET pair donor DiD (referred to as *donor quenching*) is due to energy transfer via the FRET process. At the same DiD

concentration the DiD emission from DiD-DiR labelled liposomes with DiD to DiR ratio 1:1

(DiD-DiR_{1:1}) is lower than that from liposomes with DiD to DiR ratio 1:0.25 (DiD-DiR_{1:0.25}),

suggesting higher donor quenching in liposomes with higher acceptor concentration.

Comparing DiD-DiR_{1:1} with DiR only liposomes, for DiD concentrations of 0.05 mol% to

0.5 mol% (see Figure 1 (a), (b), (c), and (d)), the DiR emission from DiD-DiR labelled

liposomes is higher than that from DiR only liposomes. This increased acceptor emission can

be attributed to FRET. DiR emission from the higher DiD labelling concentrations of 0.75 mol % and 1 mol % (see Figure 1(e) and (f)) are similar indicating SQ is high at these concentrations.

Through a comparison of the emission from DiD-DiR_{1:1} and DiD-DiR_{1:0.25} (Figure 1(a) and (b)), it can be seen that the acceptor emission from the former is higher than the latter and this is due to a higher amount of DiR for the DiD-DiR_{1:1} system. However, with increase in concentration it can be seen that the acceptor emission from the DiD-DiR_{1:0.25} system approaches that of DiD-DiR_{1:1} and even goes beyond the latter, as shown in Figure 1(c), (d), (e) and (f). This can be attributed to DiR SQ being stronger at the higher DiR concentration. It is interesting to observe that at 0.5 mol % (Figure 1(d)), 0.75 mol % (Figure 1(e)) and 1 mol % (Figure 1(f)), both DiD emission and DiR emission from DiD-DiR_{1:1} system are much lower than for the case of DiD-DiR_{1:0.25}.

2.2 Calculation of SQ and FRET Efficiencies

To further analyze the concentration dependent energy transfer in the fluorophore labelled liposomes, the detected emission over the DiD wavelength band (658 nm - 695 nm) and emission over the DiR wavelength band (755 nm - 816 nm) were extracted from the spectra shown in Figure 1. This was achieved by integrating the detected emission intensity over the wavelength ranges of DiD and DiR emission based on the transmission bands of the optical filters used in the acousto-fluorescence setup described in the Experimental Section.

The intensities obtained via integration as described above were normalized for concentration of DiD. Figure 2(a) shows the normalized results for DiD emission from the DiD only liposomes and the DiD-DiR labelled liposomes. The auxiliary line is an extension of the normalized intensity over the DiD wavelength range from DiD only liposomes with 0.05 mol% DiD concentration. The normalized intensities decrease with increase in DiD concentration. The intensity decrease from the DiD only liposomes can be attributed to the

existence of concentration dependent SQ. It is reasonable to assume that the further decrease from the normalized intensity from the DiD only liposomes as compared to that from the DiD-DiR labelled liposomes is due to FRET. The SQ efficiency of DiD and the DiD based FRET efficiency are calculated using Equation 1 and Equation 2 respectively and shown in Figure 2(b). As expected the efficiencies are monotonically increasing functions of the concentration as the fluorophores become more tightly packed, which provides an insight into the changes in packing US will induce.

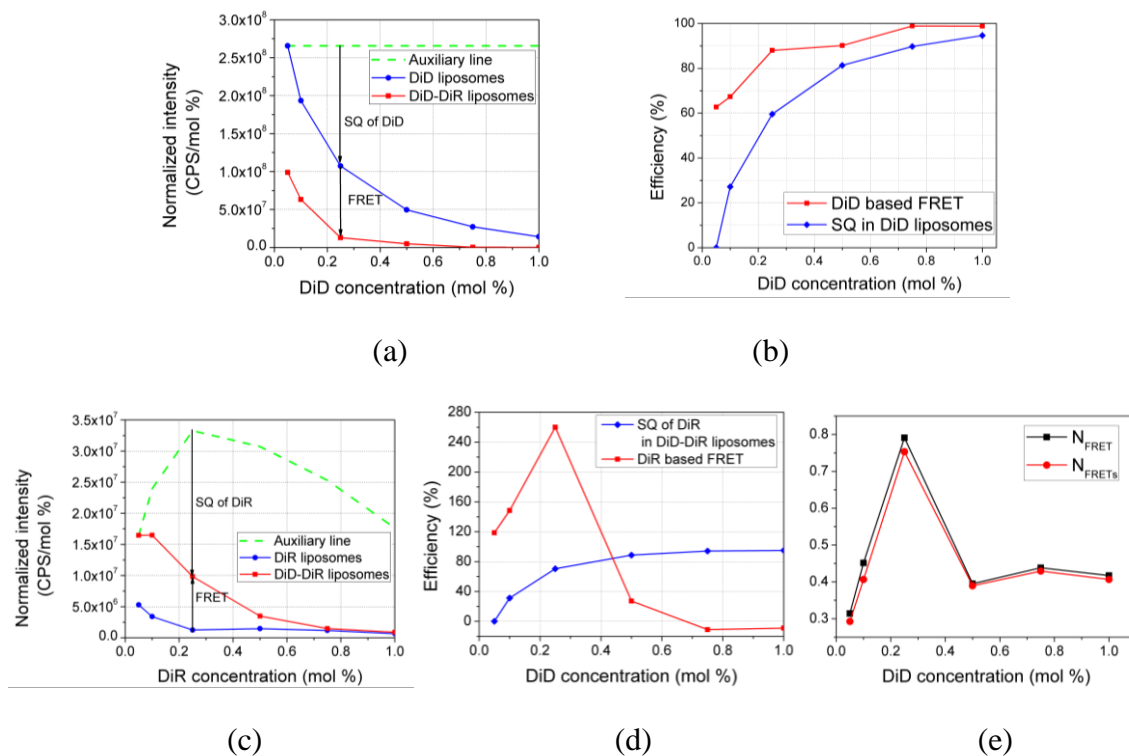


Figure 2. (a) Normalized intensity of DiD emission; (b) DiD based SQ and FRET efficiency; (c) normalized intensity of DiR emission; (d) DiR based SQ and FRET efficiency; (e) N_{FRET} and N_{FRETs} .

Similarly, the intensity of DiR emission from DiR only liposomes and DiD-DiR labelled liposomes normalized to DiR concentration with an excitation wavelength of 633 nm were calculated and shown in Figure 2(c). Without SQ of DiR and DiD the normalized DiR emission from DiD-DiR liposomes would be anticipated to increase with rise in concentration and the associated closer proximity of fluorophores increasing the likelihood of FRET. The

results shown in Figure 2(c), however, are contrary to this prediction. This observation can be attributed to SQ of DiR emission which directly reduces DiR emission combined with SQ of DiD emission indirectly reducing DiR emission via reduction in FRET. The auxiliary baseline was plotted via Equation 4 to calculate SQ of DiR emission. The DiR SQ efficiency and DiR based FRET efficiency are calculated using Equation 3 and Equation 5 and shown in Figure 2(d). The normalized FRET values N_{FRET} and N_{FRETs} , calculated using Equation 6 and Equation 13, are shown in Figure 2(e). For the concentration range considered N_{FRET} and N_{FRETs} display similar levels indicating when excited at 633 nm the emission from DiR over the DiD emission band is negligible. It can be seen that the rate of change of N_{FRET} with concentration follows a similar trend to the DiR based FRET efficiency.

2.3 Measurements of US Mediated Fluorescence

The emitted intensity from the fluorophore labelled liposomes when exposed to US with peak pressure of 1.2 MPa and 4 s duration is shown in Figure 3 (see Experimental Section for detailed experimental setup). The extent of emitted fluorescence can clearly be seen to vary with the application of US thus demonstrating the principle of these nanoscale liposomes as US switchable contrast agents. The fluorescence emitted increased for DiD emission from DiD-DiR labelled liposomes at DiD concentration of 0.5 mol % (Figure 3(e)); decreased for DiR emission from DiD-DiR labelled liposomes at DiD concentration of 0.1 mol % (Figure 3(b)); decreased for all DiD only liposomes (Figure 3(c) to (h)); increased following an initial small decrease for DiD emission from DiD-DiR labelled liposomes at DiD concentration of 0.1 mol % (Figure 3(a)) and DiR emission from DiD-DiR labelled liposomes at DiD concentration of 0.5 mol % (Figure 3(f)). These results demonstrate that the efficacy of US switching of fluorescence depends on the wavelength of emission (i.e. DiD or DiR emission), DiD labelling concentration (0.1 mol % or 0.5 mol %) and the combination of fluorophores used (i.e. DiD only or both DiD and DiR). These dependencies are summarized in Table 2.

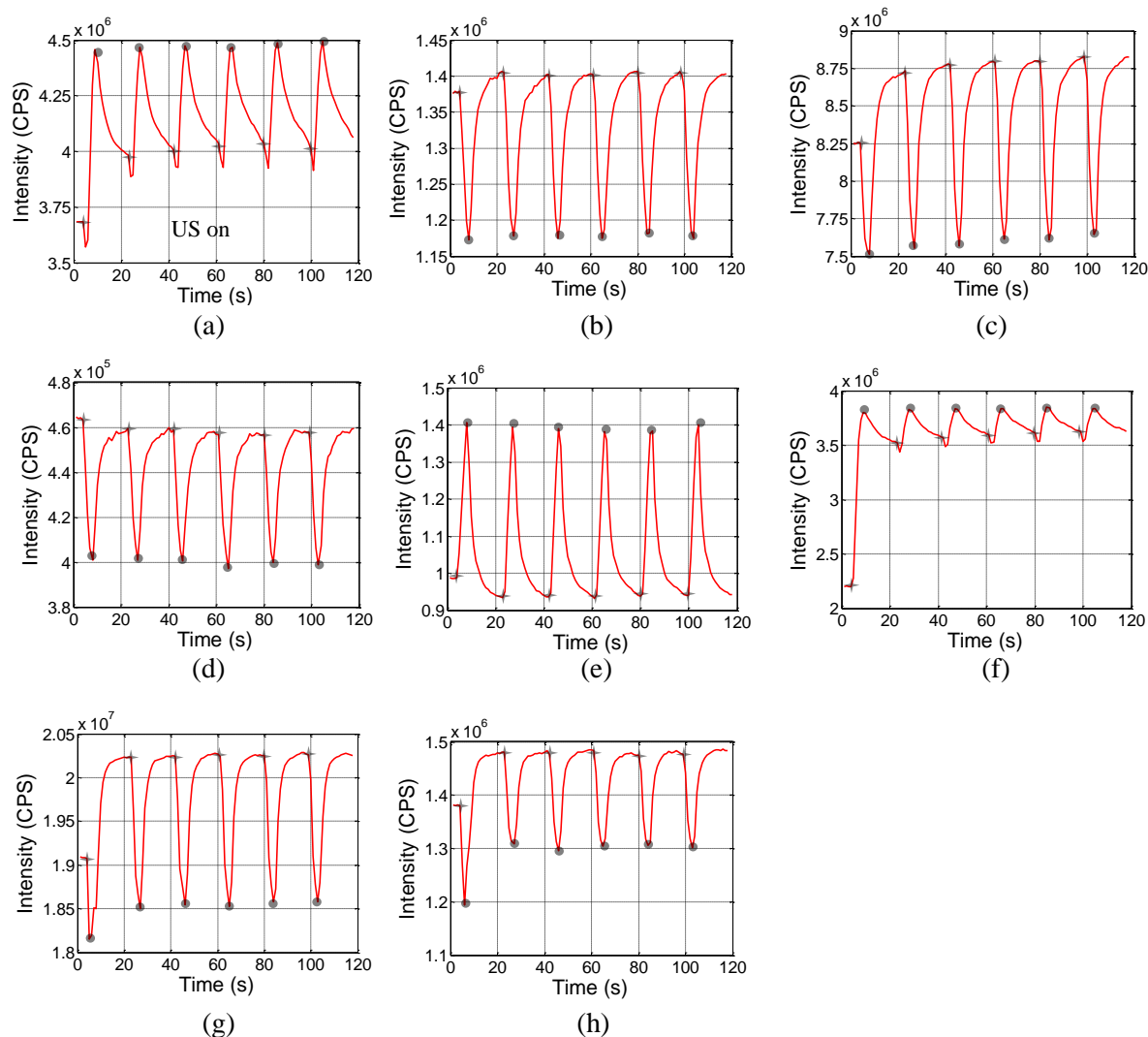


Figure 3. US mediated change in: (a) DiD emission from DiD-DiR labelled liposomes 0.1 mol %; (b) DiR emission from DiD-DiR labelled liposomes 0.1 mol %; (c) DiD emission from DiD only liposomes 0.1 mol %; (d) DiR emission from DiD only liposomes 0.1 mol %; (e) DiD emission from DiD-DiR labelled liposomes 0.5 mol %; (f) DiR emission from DiD-DiR labelled liposomes 0.5 mol %; (g) DiD emission from DiD only liposomes 0.5 mol %; (h) DiR emission from DiD only liposomes 0.5 mol %. \dagger : Turn US on; \bullet : Turn US off.

Table 2. US mediated change in fluorescence emission from liposomes (\uparrow : increase, \downarrow : decrease).

Sample	DiD-DiR liposomes DiD emission	DiD-DiR liposomes DiR emission	DiD liposomes DiD emission	DiD liposomes DiR emission
0.1 mol %	$\downarrow\uparrow$	\downarrow	\downarrow	\downarrow
0.5 mol %	\uparrow	$\downarrow\uparrow$	\downarrow	\downarrow

Control experiments with the solution degassed and with water soluble quantum dots (PL-QDN-700, PlasmaChem GmbH, Berlin, Germany) were also obtained, the results shown in Figure 4. For the degassed solution, diluted DiD-DiR labelled liposomes were put inside a

vacuum pump for 30 mins with a pressure difference with the atmosphere of 65 mm Hg. The intensities were normalized to their mean intensities. It can be seen that the changes of the intensity for the two control experiments are much smaller compared with the original solution without degass process.

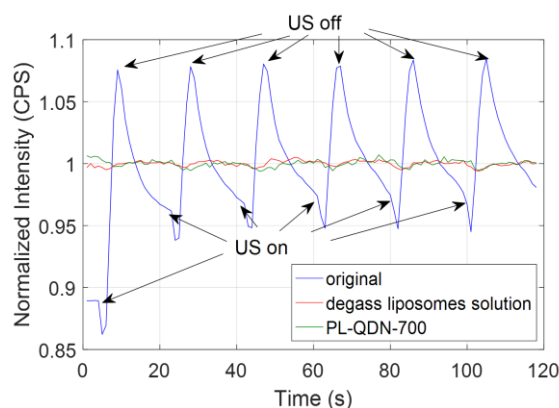


Figure 4. Comparison of the intensity change for original solution (DiD-DiR labelled liposomes with DiD labelling concentration of 0.1 mol %), degassed solution, and water soluble fluorophores (PL-QDN-700).

It also needs to be mentioned that one can argue the US mediated fluorescence is due to a temperature change induced by the US. To investigate this the temperature in the US focal zone was monitored using a calibrated temperature sensor, with the results shown in Figure S1. It was observed that the temperature indeed increased slightly with US on and decreased with US off. However, the maximum change during the whole US exposure period is only 0.24 °C. To investigate how sensitive the fluorophore labelled liposomes are to this change of temperature, the fluorescence emission spectra of the fluorophore labelled liposomes were measured at a range of temperature from 20 °C to 45 °C, and the integrated fluorescence intensity for DiD emission range and DiR emission range were plotted, results shown in Figure S2. It was observed that for all the fluorophore labelled liposomes, the intensity change for a temperature change of 0.24 °C is neglectable. Therefore the temperature effect of the US transducer should not be a mechanism to induce the intensity change.

2.4 Line Scanning Imaging in Scattering Phantom

Line scanning imaging was performed using 0.1 mol % DiD-DiR labelled liposomes, 0.1 mol % DiD only liposomes, 0.5 mol % DiD-DiR labelled liposomes, and 0.5 mol % DiD only liposomes. The fluorophore concentrations of 0.1 mol % and 0.5 mol % were chosen based on the calculation of FRET and SQ in Section 2.2. This choice was based on finding a balance between liposome systems with concentration of fluorophore not too high for FRET or SQ to be strong and a concentration that is sufficient to generate detectable levels of FRET and SQ. From Figure 2(a) and Figure 2(c) it can be seen that liposomes with DiD concentrations of 0.1 mol % and 0.5 mol % are suitable for detection at both DiD emission wavelengths and DiR emission wavelengths. The detailed method for line scanning imaging is present in the Experimental Section.

Figure 5 **Error! Reference source not found.** shows the obtained On-to-Off ratio of fluorescence following a scan of the US beam across a tube filled with either DiD-DiR labelled liposomes or DiD only liposomes. Image contrast is then calculated as the difference between the highest On-to-Off ratio and the lowest On-to-Off ratio. It can be seen that image contrast obtained from scanning DiD-DiR labelled liposomes is always higher than contrast in the case of DiD only liposomes. Assessment of the image spatial resolution obtained by using US switching of fluorescence shows it is greatly improved as compared to the resolution obtained without US for the four cases studied. Here the resolution is defined as the full width half maximum (FWHM) of the scanned image. The contrast and resolution improvements are summarized in Table 3. The highest contrast is 9.0%, obtained from DiD-DiR labelled liposomes at 0.5 mol % via detection of emission over the DiD wavelength range. Detection of DiR emission is preferable to DiD emission for deep tissue imaging due to its longer wavelength range. The contrast achieved in this case was 8.5% from the DiD-DiR labelled liposomes with 0.5 mol % DiR concentration. As the spatial resolution is highly

dependent on the size of the US focus there are no obvious differences in the resolution attained for the different liposome systems studied. The average resolution obtained is 1.87 mm which is an improvement by a factor of 6.3 as compared to images acquired without US.

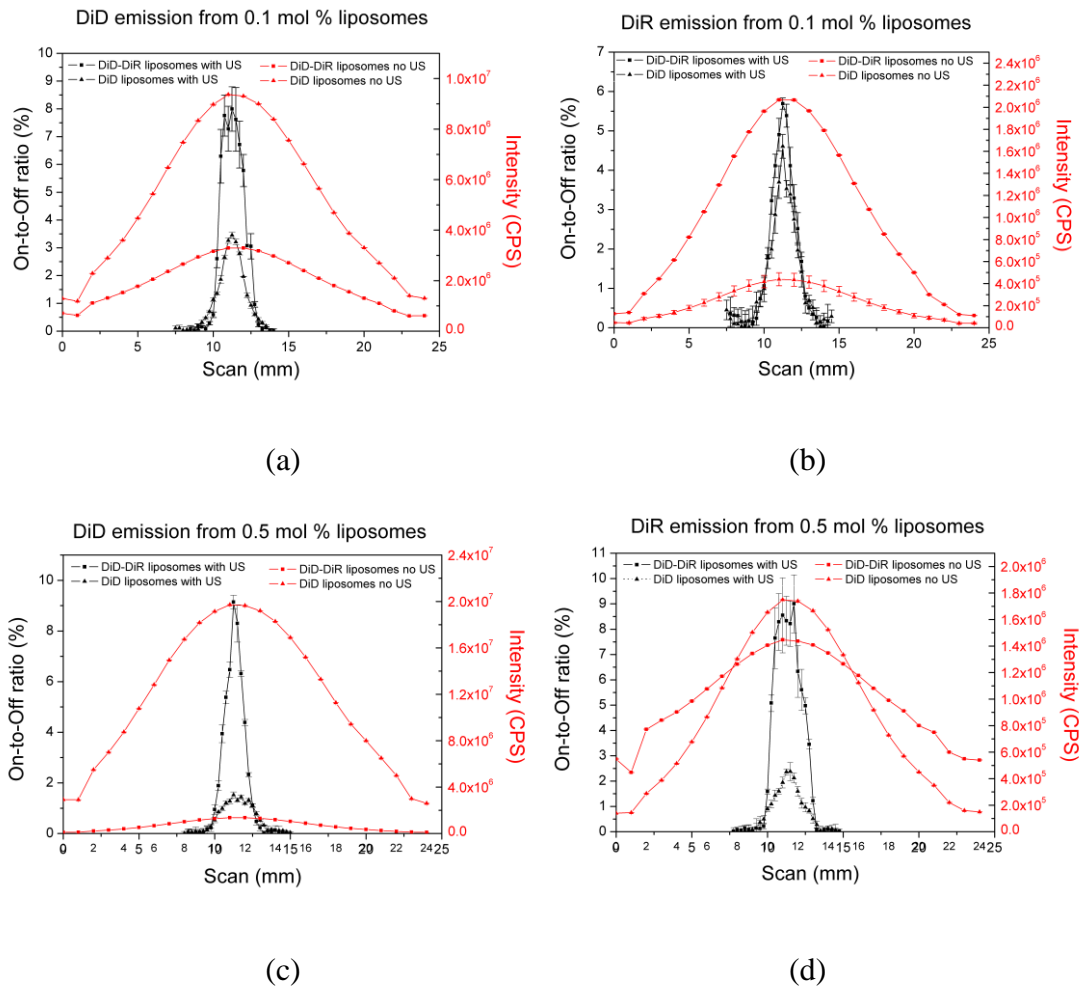


Figure 5. Scan of a tube filled with fluorophore labelled liposomes at the mid-plane of a scattering phantom. (a) DiD emission from liposomes with DiD concentration 0.1 mol %; (b) DiR emission from liposomes with DiD concentration 0.1 mol %; (c) DiD emission from liposomes with DiD concentration 0.5 mol %; (d) DiR emission from liposomes with DiD concentration 0.5 mol %.

Table 3. Contrast and spatial resolution of the line scan images in Figure 5. **Error! Reference source not found.**

Sample	Detection wavelength	Contrast	Spatial Resolution		
			With US (mm)	Without US (mm)	Improvement
0.1 mol % DiD-DiR liposomes	DiD	7.8%	1.79	11.81	6.6
0.1 mol % DiD liposomes	DiD	3.3%	1.63	11.61	7.1
0.1 mol % DiD-DiR liposomes	DiR	5.8%	1.70	10.67	6.3

0.1 mol % DiD liposomes	DiR	4.6%	1.55	10.87	7.0
0.5 mol % DiD-DiR liposomes	DiD	9.0%	1.37	10.85	7.9
0.5 mol % DiD liposomes	DiD	1.3%	2.59	13.34	5.2
0.5 mol % DiD-DiR liposomes	DiR	8.5%	2.38	11.84	5.0
0.5 mol % DiD liposomes	DiR	2.5%	1.93	11.12	5.8

Scanning images were also obtained with the DiD-DiR labelled liposomes static inside the tube, and compared with the situation when the solution has a flowing speed of 0.01 ml/min. The results were shown in Figure 6. It can be seen that the maximum On-to-Off ratio decreased for the first static scan compared with the case with the solution flowing, and it decreased further for the second static scan.

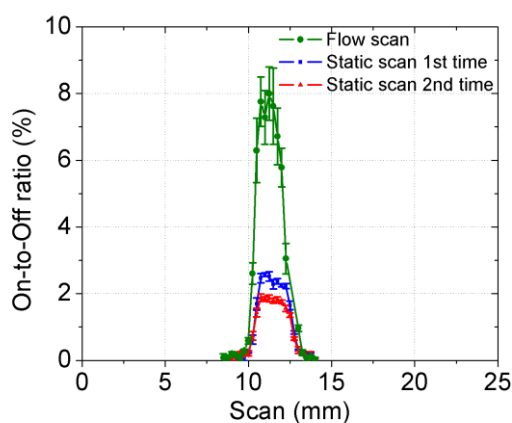


Figure 6. Comparison of the On-to-Off ratio with the DiD-DiR labelled liposomes (0.1 mol % DiD labelling concentration, detection at DiD emission wavelength) flowing at a speed of 0.01 ml/min and with the liposomes solution static. The tube was scanned two times consecutively with the solution static.

3 Discussion

The results of this work demonstrate that US can effectively switch fluorescence emission from nanoscale labelled liposomes on and off. This approach to fluorescence imaging in optically turbid material was shown to increase spatial resolution by up to a factor of 6.3 as compared to conventional fluorescence imaging in the absence of US. There are a number of underlying mechanisms that can be attributed to the US induced change in fluorescence

emission and hence observed improvement in spatial resolution. An understanding of these mechanisms can be gained from the experimental results.

For example, FRET and SQ are dependent on the intermolecular distances of fluorophores and they are also diffusion controlled process.^[14,15] Figure 3 demonstrates that US can either increase or decrease the fluorescence depending on fluorophore concentration. This may be due to a combination of two effects: (1) US increases the lateral diffusion of dye molecules and lipids; (2) US leads to volumetric changes of the liposomes. The comparison of the liposomes solution with and without degass process in Figure 4 suggests that the origin of these two effects can be due to US induced non-inertial cavitation.^[16] During non-inertial cavitation, gas bubbles pre-existing in the fluid can oscillate or move, which generates small cavities and creates free volume in the lipid bilayer. The increased free volume can facilitate quicker lateral diffusion of the lipids^[17]. Non-inertial cavitation also involves the nucleation of gas bubbles in the hydrophobic region of lipid bilayers,^[18] which supports the hypothesis that US leads to volumetric changes in liposomes. Rectified diffusion can also increase liposome size.^[16]

The two mechanisms need to be considered when investigating the role US plays in changing fluorescence emission as follows. First, the increased lateral mobility increases the likelihood that fluorophore molecules become close enough for non-radiative energy transfer, which acts to increase FRET and SQ. This effect is thus similar to an increase in fluorophore concentration. On the other hand, size expansion decreases the concentration of fluorophores located in the lipid bilayer which leads to a decrease in the intermolecular distance between fluorophores. Due to an inverse sixth power law dependence of energy transfer efficiency on distance, FRET and SQ are reduced and the fluorescence emission intensity over both DiD and DiR emission bands can be changed. The normalized intensity can therefore be changed by both the increased mobility and the size expansion. It needs to be mentioned that since

FRET is effective over a very short distance ranging from 1 nm to 10 nm, a slight change in molecule mobility or size can lead to a detectable fluorescence variation. Second, for detection at DiD emission wavelengths the effects of SQ and FRET are consistent i.e. decrease of either of the aforementioned effects leads to increased intensity while increase of either effect leads to decreased intensity. However, for detection at DiR emission wavelengths, the effects of FRET and SQ conflict with each other i.e. increase of FRET leads to increase of DiR emission intensity, while increase of SQ of DiR leads to a decrease of DiR emission intensity. In addition, increase of SQ of DiD leads to an indirect decrease of the DiR emission intensity via reduction of FRET. Third, relating the above effects described by (1) and (2) with the detected fluorescence variation shown in Figure 3, both effects occur at a time scale of milliseconds to seconds. Figure 3 also indicates that the increased lateral diffusion initially has the strongest contribution to the fluorescence emission which is followed by the increased dominance of the effects of size expansion. The origin of US mediated changes in emission intensity from the labelled liposomes, as seen in Figure 3, are summarized below.

DiD Emission from 0.1 mol % DiD-DiR Labelled Liposomes:

The fluorescence intensity over the DiD emission wavelength range from DiD-DiR labelled liposomes with 0.1 mol % DiD concentration (DiD to DiR ratio: 1:1) was observed to decrease slightly upon initial application of US, followed by a more significant increase after the second application, as seen in Figure 3(a). In the model proposed here, the initial signal decrease is attributed to increased lipid mobility, which makes FRET and SQ stronger. The evolving size expansion reduces FRET and SQ, which can subsequently lead to an increase in donor emission. The results suggest that volumetric expansion of liposomes is the dominant mechanism underlying the observed US switching of the fluorescence emission.

DiD Emission from 0.5 mol % DiD-DiR Labelled Liposomes:

The DiD emission intensity from 0.5 mol % DiD-DiR labelled liposomes was observed to increase with application of US (Figure 3(e)). This difference from the previous case of 0.1 mol % DiD-DiR labelled liposomes can be considered to be due to the comparative increase in FRET and SQ efficiency in the absence of US for the 0.5 mol% case, as seen in Figure 2(b). Since the FRET efficiency (90.2%) and SQ efficiency (81.3%) without US exposure have been very high already, the increase in mobility in the lipid bilayer US induces has little influence on the donor emission intensity. The size expansion, which reduces both FRET and SQ efficiency, is considered for this liposome system to have the greatest effect on the variation in fluorescence emission.

DiR Emission from 0.1 mol % DiD-DiR Labelled Liposomes:

The DiR emission intensity from 0.1 mol % DiD-DiR labelled liposomes was observed to decrease with application of US (Figure 3(b)). This can be explained by referring back to the curve with square markers in Figure 2c, which describes the relationship of the normalized intensity at DiR emission wavelength from DiD-DiR labelled liposomes with concentration of fluorophores. It can be seen that at 0.1 mol %, further increase of DiD concentration leads to a decrease of the DiR emitted intensity, while further decrease of DiD concentration has no influence on the DiR emitted intensity. This observation suggests that the increased mobility is the dominant factor to determine the variation of the intensity, and the effect of size expansion is comparatively negligible. With increase of concentration, FRET, SQ of DiD, and SQ of DiR increase. The fluorescence decrease seen in Figure 2(c) indicates that SQ of DiD and DiR play an important role in the overall US induced fluorescence variation. On the other hand, the increased mobility of dye molecules increases FRET, SQ of DiD, and SQ of DiR. The observed rate of change of DiR emission from 0.1 mol % DiD-DiR liposomes in Figure 3(b) also suggests that the increased SQ of DiD and DiR contributes strongly to the

fluorescence variation as compared to the increased FRET. This is in accordance with the observation in Figure 2(c).

DiR Emission from 0.5 mol % DiD-DiR Labelled Liposomes:

Figure 3(f) shows that DiR emission intensity from 0.5 mol % DiD-DiR labelled liposomes decreases slightly with application of US, followed by a more significant increase. In the model proposed here the initial signal decrease is due to an increased lateral mobility. However, referring back to the normalized intensity detected from DiD-DiR liposomes in Figure 2(c), it can be seen that at 0.5 mol %, the fluorescence will change as a result of either an increase or decrease in the fluorophore concentration. Therefore, the influence of size expansion needs to be considered in this case. Size expansion decreases SQ of DiD and SQ of DiR. In contrast to the previous case, size expansion increases FRET for 0.5 mol % DiD-DiR liposomes. This is because above 0.25 mol % FRET decreases with increase of concentration (Figure 2(d)), which potentially is due to the significant effect of SQ of both DiD and DiR at high concentrations. Because all of the three effects described lead to an increase in fluorescence emission over the DiR wavelength range, subsequent increase in signal intensity is observed.

Fluorescence Emission from DiD Only Liposomes:

It was observed from Figure 3(c), (d), (g), (h) that the fluorescence emission (both DiD and DiR emission) from DiD only liposomes (both at 0.1 mol % and 0.5 mol %) decreases when US is applied. This may be due to increased mobility which increases SQ of DiD that makes a greater contribution to the fluorescence intensity, suggesting both the concentrations 0.1 mol % and 0.5 mol % are relatively low for DiD only liposomes.

In summary, the US mediated variation of fluorescence emission is different for the eight liposomes systems studied. The increased lateral mobility of dye molecules and size

expansion are the two primary factors that can potentially lead to the fluorescence variation. In practice, the increased lateral mobility decreases the fluorescent signal while the size expansion increases the fluorescent signal. Both effects exist for detection of DiD emission from DiD-DiR labelled liposomes with 0.1 mol % DiD concentration and DiR emission from DiD-DiR labelled liposomes with 0.5 mol % DiD concentration, therefore US induced a bimodal variation in fluorescence emission. For detection of DiD emission from DiD-DiR labelled liposomes with 0.5 mol % DiD concentration, only the size expansion contributes to the fluorescence intensity due to high FRET and SQ efficiency, therefore only an increase in emission is observed. For detection of DiR emission from DiD-DiR labelled liposomes with 0.1 mol % DiR concentration, only the increased mobility is considered to contribute to the fluorescence intensity and correspondingly only a decrease in emission is observed. The DiD concentration of 0.1 mol % can therefore be regarded as the threshold for detection at DiD emission wavelengths from DiD-DiR labelled liposomes: below this concentration US leads to a decrease of the fluorescence intensity; above this concentration US leads to an increase of the fluorescence intensity; around this concentration a bimodal change is observed. Similarly, the DiR concentration of 0.1 mol % can be regarded as the threshold for detection at DiR emission wavelengths from DiD-DiR labelled liposomes. DiR has a higher threshold than DiD which is likely to be due to the opposite effect of FRET and SQ. Since there is only SQ in the DiD only liposomes, the threshold is higher than 0.5 mol % therefore no signal increase was observed.

4 Conclusion

In conclusion, the efficacy of nanoscale liposomes incorporated with donor-acceptor FRET pairs to improve image spatial resolution of optically turbid samples was investigated based on the principle of US switching of fluorescence emission. The emission wavelength used in this work is located in the NIR window which is well suited for non-invasive in vivo tissue

imaging. The liposomes systems studied were found to display high levels of FRET and SQ which changed in response to US. The specific changes in fluorescence emission via US were found to depend on the fluorophores used, detection wavelength and the fluorophore concentration. Line scanning of a representative tissue structure composed of a tube with 1 mm inner diameter buried at a depth of 1 cm in a heavily scattering tissue phantom was carried out. Results obtained demonstrated an improvement in image spatial resolution by a factor of 6.3 as compared with conventional fluorescence imaging. The highest image contrast obtained is 9%, which is a significant improvement over alternative techniques relying on direct modulation of the fluorescent signal using US. Further, unlike previously reported systems of US switchable fluorescent probes, the approach presented here is not dependent on changes in local temperature and as such can remove the possibility of detrimental thermal effects on samples associated with changes in sample temperature of the order of 10 degrees Celsius. The findings of this work highlight the enhancement achievable in US switching of fluorescence via the incorporation of FRET pairs in liposome contrast agents as can be seen from the much greater contrast achieved with the DiD-DiR labelled liposomes as compared to the case when DiD only liposomes were used. Moreover, DiD or DiR has also been used largely to track cells in live mice^[19] and has not been reported to cause serious cytotoxicity^[20] which supports in vivo application of the contrast agents. For effective in vivo imaging, factors such as stability, biodistribution and biocompatibility of the DiD-DiR labelled liposomes need to be investigated. Future work will also involve verification of the model proposed here to explain the mechanisms underlying the US switching of fluorescence.

5 Experimental Section

Preparation of DiD-DiR, DiD and DiR Labelled Liposomes: DiD-DiR, DiD and DiR labelled liposomes were produced at six DiD labelling concentrations (0.05 mol %, 0.1 mol %, 0.25

mol %, 0.5 mol %, 0.75 mol %, and 1 mol %) based on the freeze-thaw extrusion method.^[21] The labelling concentration is the molar percentage of the amount of DiD to total amount of all materials used (DiD, DiR and DPPC). These concentrations were chosen considering the dose dependency of liposomal stability. The suggested concentration to minimize the effects caused by integration of the carbocyanine dyes on liposomal membranes is in the range of 10 µg/ml dye to 2.5 mg/ml liposomal-lipid,^[22] corresponding to a molar percentage of 0.3 mol %. Experimentally the carbocyanine dyes were introduced at the time of liposome formation.^[23] The DiD only liposomes and DiR only liposomes were prepared as donor only controls and acceptor only controls, respectively.

DiD and DiR were reconstituted at 2 mg/ml in ethanol respectively to prepare a stock solution. The DiD or DiR solution was mixed with DPPC phospholipids (#850355, Avanti Polar Lipids Inc, AL, USA) and the solvent was evaporated with oxygen-free nitrogen gas (BOC Group plc, Manchester, UK). The dried lipids were resuspended in phosphate buffered saline (PBS) which was made with autoclaved water. The buffered lipids underwent 5 freeze-thaw cycles using liquid nitrogen (-196 °C) and water (45 °C) before extruding 10 times through 100 nm track-etched membranes (Whatman Plc, Bucks, UK) using a barrel extruder (Northern Lipids Inc, Burnaby, Canada) at 45 °C. The aforescribed PBS buffer was used for all subsequent dilutions. A total lipid concentration of 1.28 mM was used for all the measurements.

Measurements of Fluorescence Emission and Absorbance Spectra: The fluorescence emission spectra of DiD-DiR, DiD, and DiR labelled liposomes were measured using a spectrofluorometer (Fluoromax-4, Horiba Scientific, Kyoto, Japan) following excitation at a wavelength of 633 nm with a 2 nm slit width. The emission was collected from 638 nm to 900 nm wavelength, using a 2 nm slit width.

The absorbance spectra of DiD only liposomes and DiR only liposomes were measured using a spectrophotometer (Biochrom Libra S32 PC, Cambridge, UK). Wavelengths from 450 nm to 900 nm were scanned with a step of 1 nm and scan speed of 1856 nm/min. PBS was used as a reference with the spectra obtained acting as a temporary baseline for the other samples. The absorbance at 633 nm was extracted from the absorbance spectra for calculation of the DiR based FRET efficiency and can be found in Table S1 in Supporting Information.

Calculation of SQ and FRET Efficiencies: To investigate FRET and SQ quantitatively a normalized intensity was defined as the ratio of the intensity to the labelling concentration (unit: CPS/mol %). Since the same amount of lipid was used in all cases, the normalized intensity indicates the averaged fluorescence emission intensity from a unit fluorophore. The SQ efficiency of DiD in DiD only liposomes was calculated by the decreasing percentage of the normalized DiD emission intensity from DiD only liposomes at 0.05 mol % ($\bar{I}_{DiD|DiD0.05mol\%}$) with the normalized DiD emission intensity from DiD only liposomes at the other concentrations ($\bar{I}_{DiD|DiD}$):

$$SQ_{DiD} = (\bar{I}_{DiD|DiD0.05mol\%} - \bar{I}_{DiD|DiD}) / \bar{I}_{DiD|DiD0.05mol\%} \quad \text{Equation 1}$$

The donor based FRET efficiency^[24] was calculated from the decreasing percentage from $\bar{I}_{DiD|DiD}$ to the normalized DiD emission intensity from DiD-DiR labelled liposomes ($\bar{I}_{DiD|DiD-DiR}$), namely:

$$FRET_{DiD} = (\bar{I}_{DiD|DiD} - \bar{I}_{DiD|DiD-DiR} + \bar{I}_{DiD|DiR}) / \bar{I}_{DiD|DiD} \quad \text{Equation 2}$$

where $\bar{I}_{DiD|DiR}$ (normalized DiD emission intensity from DiR only liposomes) is used to correct detection of the emission intensity at the donor wavelength from the acceptor.

The SQ efficiency of DiR is calculated as follows:

$$SQ_{DiR} = (\bar{I}_{baseline} - \bar{I}_{DiR|DiD-DiR}) / \bar{I}_{baseline} \quad \text{Equation 3}$$

where $\bar{I}_{baseline}$ is an auxiliary line based on regarding DiD emission as excitation light to DiR and it is calculated as follows:

$$\bar{I}_{baseline} = \bar{I}_{DiR|DiD-DiR0.05mol\%} \cdot I_{DiD|DiD} / I_{DiD|DiD0.05mol\%} \quad \text{Equation 4}$$

where $\bar{I}_{DiR|DiD-DiR0.05mol\%}$ is the normalized DiR emission intensity from DiD-DiR labelled liposomes at 0.05 mol %, $I_{DiD|DiD}$ is the absolute DiD emission intensity from DiD only liposomes, $I_{DiD|DiD0.05mol\%}$ is the same quantity as $I_{DiD|DiD}$ but at a concentration of 0.05 mol %.

The DiR based FRET efficiency was calculated from the increased percentage from the normalized DiR emission intensity of DiR only liposomes ($\bar{I}_{DiR|DiR}$) to the normalized DiR emission intensity of DiD-DiR labelled liposomes ($\bar{I}_{DiR|DiD-DiR}$):^[25]

$$FRET_{DiR} = \frac{\varepsilon_{DiRat633nm} \bar{I}_{DiR|DiD-DiR} - \bar{I}_{DiR|DiR} \bar{I}_{DiR|DiD}}{\varepsilon_{DiDat633nm} \bar{I}_{DiR|DiR}} \quad \text{Equation 5}$$

where $\varepsilon_{DiRat633nm}$ and $\varepsilon_{DiDat633nm}$ are the extinction coefficients of DiR only liposomes and DiD only liposomes respectively at $\lambda = 633$ nm. Based on Lambert-Beer's Law for the same amount of samples and path length of the radiation beam used for recording the absorption spectrum, $\varepsilon_{DiRat633nm} / \varepsilon_{DiDat633nm} = A_{DiR} / A_{DiD}$, where A_{DiR} and A_{DiD} are the absorbance of DiR only liposomes and DiD only liposomes at $\lambda = 633$ nm respectively. Therefore $\varepsilon_{DiRat633nm} / \varepsilon_{DiDat633nm}$ can be obtained from absorbance measurements of A_{DiR} and A_{DiD} . $\bar{I}_{DiR|DiD}$ is for correction of the emission at the acceptor wavelength from the donor.

In addition to the FRET efficiencies, the normalized FRET value N_{FRET} was defined as a reliable and global calculation method for FRET quantification and it can be used to compare between different measurements.^[26] It is a normalized quantity only dependent on the FRET efficiency and the complex percentage P (twofold of the percentage of the DiD-DiR complex exhibiting FRET to total donors and acceptors). Here a complex means an interacting pair of donor and acceptor. With the DiD to DiR ratio of 1:1, it is reasonable to have the assumption

that $P = 1$ for all the samples studied. Therefore N_{FRET} is only dependent on the FRET efficiency. N_{FRET} is calculated as:^[26]

$$N_{FRET} = \frac{FRET1}{\sqrt{Af \cdot Df}} \quad \text{Equation 6}$$

where

$$FRET1 = \frac{Ff - (Fd/Dd)Df - A\bar{f}a[(Fa/Aa) - (Fd/Dd)(Da/Aa)]}{1 - (Da/Fa)(Fd/Dd)} \quad \text{Equation 7}$$

$$A\bar{f}a = \frac{Af - (Ad/Fd)/Ff}{1 - (Fa/Aa)(\frac{Ad}{Ff})} \quad \text{Equation 8}$$

Ff , Fd , Dd , Df , $A\bar{f}a$, Af , Fa , Aa , and Da are symbols expressing the fluorescence emission intensity from a specific sample (DiD only liposomes, DiR only liposomes, or DiD-DiR labelled liposomes) at a specific detection wavelength range (DiD emission band or DiR emission band) excited by either 633 nm or 710 nm. The symbols and their interpretation can be found in Table 1. Since Ad was measured to be zero, $A\bar{f}a$ can be simplified as:

$$A\bar{f}a = Af \quad \text{Equation 9}$$

Table 1. Symbols and their interpretation.

Symbols	Meaning
Ff	DiR emission intensity from DiD-DiR labeled liposomes excited at 633 nm
Fd	DiR emission intensity from DiD only liposomes excited at 633 nm
Dd	DiD emission intensity from DiD only liposomes excited at 633 nm
Df	DiD emission intensity from DiD-DiR labeled liposomes excited at 633 nm
Af	DiR emission intensity from DiD-DiR labeled liposomes excited at 710 nm
Fa	DiR emission intensity from DiR only liposomes excited at 633 nm
Aa	DiR emission intensity from DiR only liposomes excited at 633 nm
Da	DiD emission intensity from DiR only liposomes excited at 633 nm
Ad	DiR emission intensity from DiD only liposomes excited at 710 nm

Since the fluorescence intensity at the donor emission band from the acceptor is usually very small, in literature it is usually supposed that $Da=0$, $FRET1$ can thus be simplified as *net FRET*^[24] with a new symbol $nFRET$:

$$nFRET = Ff - Df \cdot a - Af \cdot b \quad \text{Equation 10}$$

where a and b are correction coefficients of the percentage of DiD and DiR bleed-through respectively and calculated from DiD only liposomes and DiR only liposomes respectively as follows:

$$a = \frac{Fd}{Dd} \quad \text{Equation 11}$$

$$b = \frac{Fa}{Aa} \quad \text{Equation 12}$$

N_{FRET} can therefore also be simplified as follows with a new symbol N_{FRETs} :

$$N_{FRETs} = \frac{nFRET}{\sqrt{Af \cdot Df}} \quad \text{Equation 13}$$

Measurements of US mediated fluorescence: The dynamics of the fluorophore labelled liposomes in the US field were studied using the setup shown in Figure 7. The solution was controlled by a syringe pump through a light transparent Fluorinated Ethylene Propylene (FEP) tube with 1.1 mm inner diameter and 0.20 mm wall thickness with a flowing speed of 0.01 ml/min. The water tank was filled with deionized water for coupling of US. The US transmission efficiency of the FEP tube was measured to be 67.8%. A signal generator (AFG3022, Tektronix, Beaverton, USA) and a radio frequency power amplifier (75A250A, Amplifier Research, Souderton, USA) were employed to drive a focused 2.25 MHz US transducer (A304-SU, Olympus, Massachusetts, USA). A HeNe laser (20 mW power) with 633 nm wavelength was used as the excitation light. The laser beam (diameter: 1 mm) was expanded 10 times by a beam expander to increase the volume of liposomes exposed to light. To reduce bleed-through of the excitation light, its direction was adjusted by two mirrors so that it is not directly detected. The tube, US focal zone, and the laser light were aligned to be confocal using a needle hydrophone. The emitted light was collected by a long working distance objective lens (M Plan Apo 20, NA 0.42, 20×, Mitutoyo, Japan). A Notch filter (NT63-347, Edmund Optics, York, UK) and a high performance emission filter were used to

achieve a high rejection of the excitation and room light. For the detection at the donor emission wavelength a bandpass filter with 676 nm center wavelength (CWL) and 37 nm bandpass (FF01-676/37-25, Semrock, New York, USA) was used. For the detection at the acceptor emission wavelength a bandpass filter with 785 nm CWL and 62 nm bandpass (#87-759, Edmund Optics, York, UK) was used. The fluorescence intensity was detected using a photon counting system (ET Enterprises electron tubes, Uxbridge, UK) which includes a photomultiplier (PMT, 9108A), amplifier-discriminator (AD6), counter (CT2), and power supply (PM20). The signal generator and photon counting system were controlled and synchronized using Labview. For the measurements of the US mediated fluorescence, the US was turned on at 4 s for 4 s with a peak pressure of 1.2 MPa, and then turned off for 15 s. This procedure was repeated six times to check the repeatability. During this procedure light intensity was recorded with a photon counting system at a sampling period of 100 ms. 10 continuous values (corresponding to a 1 s time bin) were averaged and the mean values plotted.

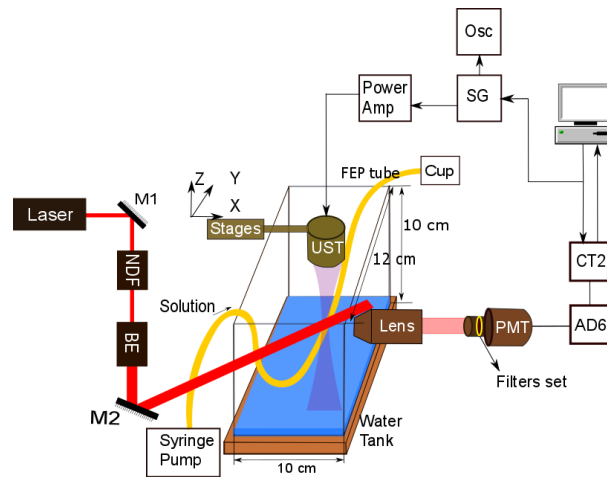


Figure 7. Setup for measurements of the US mediated fluorescence. M1: mirror 1; M2: mirror 2; NDF: neutral density filter; BE: beam expander; UST: US transducer; SG: signal generator; Osc: oscilloscope; PMT: photomultiplier tube; AD6: amplifier-discriminator; CT2: counter/timer module.

Line Scanning Imaging: The setup used for line scanning was similar to the setup shown in Figure 7. However, in the case of line scanning the FEP tube was buried in the center of a scattering phantom ($2 \times 7.7 \times 5$ cm, x - y - z) at a depth of 1 cm. The scattering phantom was made from agarose gel mixed with polystyrene microspheres (reduced scattering coefficient $\mu_s' \sim 1 \text{ mm}^{-1}$, anisotropy factor $g = 0.93$). No additional absorption is added so the absorption coefficient μ_a is comparable to that of water.^[28] The US transducer was mounted on a three dimensional translational stage (Standa, 8SMC1-USBhF, Vilnius, Lithuania). The translational stage, SG, and CT2 were synchronized using Labview. After the initialization of the translational stage, SG and CT2, the US was turned on for 4 s and off for 15 s repeating six times. At the same time the fluorescence intensity was detected and recorded by the photon counting system. After this the translational stage was moved 250 μm along the x direction to the next position and the US and photon counting system were operated again. This procedure was repeated until the translational stage reached the pre-set final position. In contrast to the setup shown in Figure 7, the laser light illuminated the scattering phantom perpendicularly. For the scans obtained without applying US the water tank was scanned along the z direction for 24 mm with a step of 250 μm . At each position 1000 values of the intensity were recorded with a time window of 100 ms. Mean and standard deviation of intensity measured for each position were calculated. Due to the cylindrical shape of the tube used, it can be regarded that the same region of the tube is imaged for scanning along the x direction with application of US and along the z direction without application of US.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Supporting Information

Title Nanoscale ultrasound-switchable FRET based liposomes for near-infrared fluorescence imaging in optically turbid media

*Qimei Zhang, Stephen P. Morgan, Melissa L. Mather**

Table S1. Absorbance at 633 nm of DiD only liposomes and DiR only liposomes.

Concentration (mol%)	$A_{\text{DiD}}^{\text{a}}$	$A_{\text{DiR}}^{\text{b}}$	$A_{\text{DiR}}/A_{\text{DiD}}$
0.05	0.35	0.36	1.03
0.1	0.50	0.27	0.54
0.25	0.70	0.35	0.50
0.5	1.23	0.50	0.41
0.75	1.52	0.60	0.39
1	1.67	0.63	0.38

^{a)} Absorbance of DiD only liposomes; ^{b)} Absorbance of DiR only liposomes.

To investigate the influence of temperature on the fluorescence emission intensity from the fluorophore labelled liposomes, the temperature in the US focal zone was monitored using a customized temperature sensor based on Fibre Bragg Grating (FBG).^[1] The use of the optical fibre based temperature sensor, rather than a thermocouple with metal sensing tip, was used here to avoid disturbance of the US focal zone. The FBG based temperature sensor, connected to an interrogator unit (Smart Scan Interrogator, Smart Fibres Ltd, Bracknell, UK), is calibrated based on a thermocouple (TC-08, Pico Technology, St Neots, UK) in a water bath from 20 °C to 45 °C. The US was operated the same as for measurement of the US mediated fluorescence - the US was turned on for 4 s with a peak pressure of 1.2 MPa, and then turned off for 15 s. The procedure was repeated six times. The monitored temperature is shown in Figure S1. It can be seen that the temperature indeed increased slightly with US on and decreased with US off. However, the maximum change during the whole US exposure period is only 0.24 °C. To investigate how sensitive the fluorophore labelled liposomes are to this temperature change, the fluorescence emission spectra of the fluorophore labelled liposomes were measured at a range of temperature from 20 °C to 45 °C, and the integrated fluorescence intensity for DiD emission wavelength range (658 nm - 695 nm) and DiR emission wavelength range (755 nm - 816 nm) were plotted, as shown in Figure S2. It can be seen for all the fluorophore labelled liposomes, the intensity change for a temperature change of 0.24 °C is neglectable. Therefore the temperature effect of the US transducer is not a mechanism for the observed intensity change.

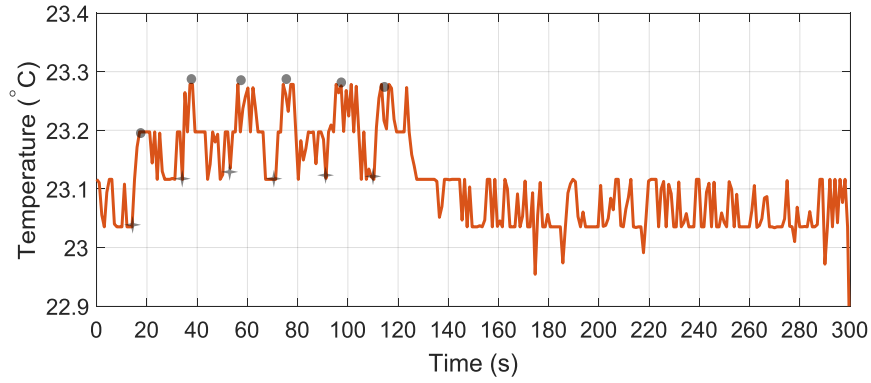


Figure S1. Monitored temperature in the US focal zone. \times : turn US on; \bullet : turn US off.

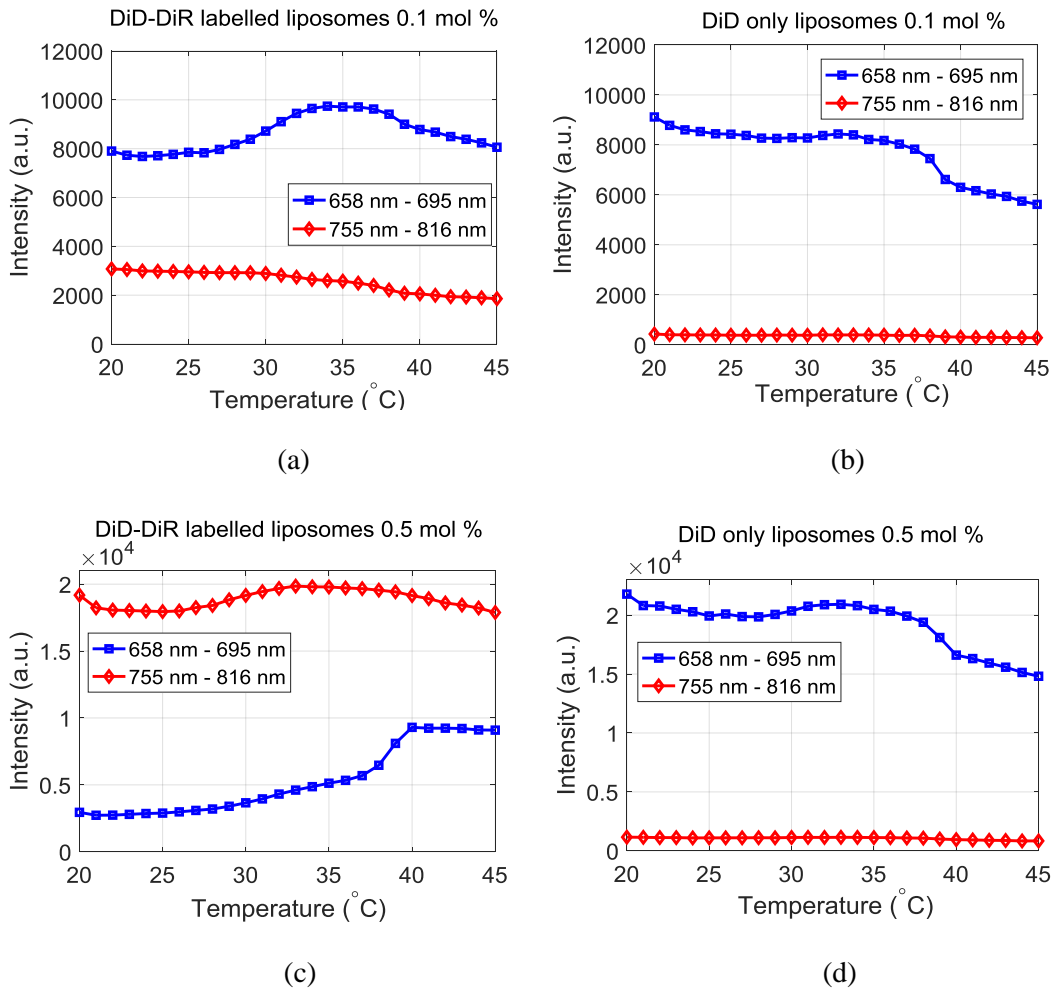


Figure S2. Fluorescence emission intensity change with temperature for (a) DiD-DiR labelled liposomes at 0.1 mol %; (b) DiD only liposomes at 0.1 mol %; (c) DiD-DiR labelled liposomes at 0.5 mol %; (d) DiD only liposomes at 0.5 mol %.

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