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Comparison of illumination geometry for lifetime-based measurements in whole-body preclinical imaging

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Abstract

Macroscopic fluorescence lifetime imaging (MFLI) has been proved to be an accurate tool to quantify FRET lifetime-based assessment of receptor-ligand engagement *in vitro* and *in vivo*. Herein, we report on the quantitative comparison of MFLI for whole-body preclinical studies in transmittance and reflectance geometries. The comparative study was conducted both for *in vitro* and *in vivo* conditions. FRET quantification performance in both geometries was similar in high fluorescence concentration samples. However, the reflectance geometry performed better at low fluorescence concentration. In addition, reflectance geometry could acquire sub-surface imaging of the main whole-body organs of small animals without being compromised by tissue attenuation.

Graphical abstract

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Keywords

fluorescence lifetime imaging; preclinical imaging; targeted delivery

1 | INTRODUCTION

Preclinical molecular imaging is central to the drug discovery process, allowing for fast screening of drug candidates and thus reducing the development cost of translating new drugs to the clinic. [1,2] Typically, a therapeutic compound is labelled, injected into a small animal model and then imaged at various time points to retrieve the whole-body bio-distribution as provided by intensity or brightness. The co-localization of an imaging reporter with the pathological site is then interpreted as a parameter for targeted delivery. However, intensity measurements do not provide the quantitative information required to assess efficient drug delivery. Indeed, they are limited in providing macroscopic local accumulation that can be both associated with passive and active delivery. For true *in vivo* drug efficacy assessment, the cellular delivery context is the critical information sought. Attaining such information directly by intensity measurements is not yet feasible with current preclinical imaging modalities. The technique of choice is then to use fluorescence optical microscopy on animal models that are surgically implanted with intravital chambers. However, these approaches are still restricted to small FOV with limited depth penetration that may not be representative of the whole tumour drug delivery process. Furthermore, intravital microscopy approaches are not well suited for longitudinal studies in fast-growing pathologies, may alter the biological process under investigation, and do not provide the pharmacokinetics context associated with the other animal organs. The recent development of a macroscopic fluorescence lifetime imaging (MFLI) platform has overcome the limitation of intensity-based imaging approaches by providing lifetime-sensitive measurements that can be leveraged to quantify target-receptor engagement non-invasively. Indeed, the integration of MFLI and Förster Resonance Energy Transfer (FRET) has enabled the assessment of cellular delivery based on a receptor-ligand interaction in a whole intact animal. [3]

FRET is a physicochemical phenomenon occurring when two different fluorophores, named donor and acceptor, which have an overlapping spectrum of donor emission and acceptor excitation, come into proximity under appropriate inter-molecular orientation. The main FRET application is to study nano-scale biochemical interactions, including association, activation, and conformational changes of proteins and oligonucleotides. [3–8] The occurrence of FRET leads to increased acceptor fluorescence intensity, reduced donor fluorescence intensity, and reduced donor fluorescence lifetime. Fluorescence microscopy is the main tool employed to visualize and quantitate FRET. Historically FRET has been sensed via intensity-based approaches, but the current FRET quantification standard relies on fluorescence lifetime microscopy (FLIM). FLIM has the main benefit of a robust and highly reproducible quantification of FRET with less experimentally cumbersome protocols. [9,10] However, translation of FLIM-FRET to macroscale FRET applications was limited until the development of Macroscopic fluorescence lifetime imaging (MFLI) platforms and red-shifting of the FRET fluorophore pair. [11,12]

We have developed and optimized an MFLI platform designed around a gated ICCD and structured light illumination. This platform, coupled with the computational tools required to perform optical tomography, [13–16] enables performing 3D fluorescence imaging of live intact animals. [17] For tomography, the structured light illumination is implemented in transmittance for optimal performances. [18] However, in many scenarios, the model of choice for *in vivo* studies includes the subcutaneous implantation of tumour xenografts. Such models are hence only located a few millimetres deep below the skin and can be readily imaged via photographic methods. Therefore, we have updated our imaging platform with a reflectance channel that can also illuminate the live animal with structured light. In this work, we compare the performances of both our trans-illumination and reflectance channels in imaging FRET. The near-infrared (NIR) spectral window was chosen because light absorption and autofluorescence of tissues are minimal at this range. [19] The investigation of NIR FRET quantification accuracy was conducted using *in vitro* and *in vivo* experiments. IgG-IgG coupling was used for *in vitro* validation, and the transferrin delivery system was used for *in vivo* validation. Overall, we report that both illumination channel configurations lead to accurate lifetime-derived FRET measurements but that the reflectance channel provides more robust results in photon-starved conditions and allows to monitor concurrently important organs such as the liver which are essential in pharmacokinetics studies.

2 | MATERIALS AND METHODS

2.1 | *In vitro* FRET samples preparation

Fluorescently-labelled IgG was purchased from Thermo Fisher Scientific. The donor was Alexa Fluor 700 conjugated to mouse IgG1 (isotype control, Cat. No. MG129), and the acceptor was Alexa Fluor 750 conjugated to anti-mouse goat IgG (H+L) secondary antibody (Cat. No. A-21037). The ratios of acceptor to the donor (A:D) were either 0:1 (non-FRETing condition) or 2:1 (FRET condition). The donor IgG concentration was 134.4 nM (20 µg/ml) for the highest concentration sample and was two-fold serially diluted to 67.2, 33.6, 16.8, 8.4 and 4.2 nM. The acceptor IgG concentration was adjusted proportionally to A:D ratios. The mixtures were prepared in a 96-well microplate. Phosphate-buffered saline (PBS) was added to normalize the total volume to 200 µl/well. The plate was incubated at 4°C overnight to allow the reaction to complete before the imaging session.

2.2 | *In vivo* cancer cells-Matrigel plug preparation

The fluorescent probes were prepared by conjugating Alexa Fluor 700 (AF700, donor) or Alexa Fluor 750 (AF750, acceptor) (ThermoFisher Scientific cat# A10010 and A10011 respectively) to iron-loaded transferrin (Sigma cat# T4132) per manufacturer's instruction. T47D human breast cancer cells were obtained from ATCC cell bank and were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% foetal calf serum (ATCC), 4 mM L-glutamine (Life Technologies), 10 mM HEPES (Sigma). They were then plated in 60 mm dishes and cultured until confluent. The cells were incubated for an hour at 37°C with AF700 and AF750 labelled transferrin at A:D ratios 0:1, 1:1, 2:1 or 3:1 (using constant donor amount: 40 µg) diluted in serum-free medium (phenol red-free DMEM, 5 mg/mL bovine serum albumin (Sigma), 4 mM L-glutamine, 20 mM HEPES

(Sigma) pH 7.4). The cells were washed with PBS, fixed with 4% paraformaldehyde, scraped, pelleted and then mixed with Matrigel at a volume ratio of 1:1. The cell-Matrigel mixtures were injected into either thoracic or inguinal mammary fat pads at approximately 2×10^6 cell/site. The gels were allowed to solidify for 60 minutes before the imaging. The mouse was placed on the imaging stage while being anesthetized with isoflurane and supplied with warm air. The anesthetized animal was monitored for respiratory rate and pain reflex. The images were acquired in transmission and reflectance configuration as per the study focus. The use of animals was approved by Institution Animal Care and Use Committee at Rensselaer Polytechnic Institute and Albany Medical College.

2.3 | *In vivo* tumour xenograft preparation

A female SCID nude mouse was injected with live 3×10^6 T47D cancer cells mixed with Matrigel at the right inguinal mammary fat pad. The tumour was allowed to grow until it reached 5 mm in diameter. The animal was intravenously injected with fluorescently labelled transferrin at A:D 2:1 (acceptor:donor 80:40 μ g). The imaging was performed at 6 hours post-injection, using the same animal imaging protocol mentioned above.

2.4 | Imaging platform

The time-gated fluorescence lifetime imaging was designed and implemented for pre-clinical applications. [11] The schematic of the imaging platform is displayed in Figure 1. The excitation source was a near-infrared pulsed laser (Mai-Tai HP, Spectra-Physics), tuneable from 690 – 1050 nm. The laser pulse width was 100 fs, and the repetition rate was 80 MHz. The excitation was tuned to 695 nm for Alexa Fluor 700. The laser was coupled to digital micromirror devices (DMDs), in which the projecting pattern was controlled by the computer. The transmission projection system was DLi 4410 (Digital Light Innovations), and the reflectance projection system was PG-D3010X (Sharp). The wide-field laser projected from the reflectance DMD was reflected onto the imaging stage by a dichroic mirror (FF700-Di01-25 $\times$ 36, Semrock). The detector was a time-gated intensified CCD (ICCD) (LaVision, Germany). Emission filters (FF01-720/13-25 and FF01-715/P-25, Semrock) were installed to the detector. Each time-gated data set consisted of a series of 160 12-bit images representing a fluorescence delay at 40-ps gate interval and 300-ps gate width. These temporal parameter settings were identical to the ones used in our previous studies. [3,11] The system settings, which include the laser power, the detector integration time and the illumination pattern intensity, were varied to optimize photon counts to acquire optimal time-resolved fluorescence decays. The laser output power was set between 300–600 mW (steps 100 mW), which was approximately 125–750 μ W/cm², given 30% DMD coupling efficiency and 10% active DMD illumination area. The detector exposure time was set between 100–800 ms (steps 100 ms), and the detector voltage gain (multichannel plate gain, MCP) was fixed at 550 V. The variation of the laser power and the detector exposure time created 32 system settings that were applied to both imaging geometries. Grey level illumination of the DMD, which was controlled by Microsoft PowerPoint, was applied to improve the dynamic range of detection of low concentration samples. [12,20] Decays with high photon counts and hence good SNR are essential to quantitative lifetime imaging. This is especially true for applications in which multiple lifetimes are considered simultaneously. Especially, FRET is a photon-starved application due to the donor quenching but requires bi-

exponential fitting. Moreover, FRET in the NIR is characterized by short lifetime, as low as <300ps for the quenched donor. Hence, NIR FRET is one of the most challenging *in vivo* lifetime imaging applications.

2.5 | Data Analysis

Fluorescence decays were analysed using the bi-exponential fitting model (Equation 1).

$$I(t) = IRF \otimes \left(A_1 \cdot e^{-\frac{t}{\tau_1}} + A_2 \cdot e^{-\frac{t}{\tau_2}} \right) \quad (1)$$

The short (τ_1) and long lifetimes (τ_2), which represented FRETing and non-FRETing donor lifetimes, were set to 240 ± 40 and 950 ± 50 ps, respectively. [21] IRF is the instrument response function, which was measured by using a diffuse white paper as a sample. A_1 was used as the FRET quantitative parameter, hereafter termed FRETing donor fraction (FD%). Any pixel having peak photon counts less than 500 was excluded from the analysis. The statistical difference of FRET quantification performance was calculated using the equivalence test at $p = 0.05$ and 95% confidence interval.

3 | RESULTS AND DISCUSSION

3.1 | In vitro validation

The quantification of the FRETing donor fraction (FD% as estimated by bi-exponential fitting) for the mixtures of fluorescently labelled primary and secondary antibodies prepared at A:D ratios 0:1 (non-FRET condition) and 2:1 (FRET condition) is provided in Figure 2. This data set summarizes the quantification over 32 imaging parameter conditions as data were acquired for the combination between four laser power output (300–600mW, steps 100mW) and eight detector exposure time (100–800ms, steps 100ms) settings, both under transmission and reflectance geometries. In all cases, the FD% was clearly distinct between the two A:D ratios. Using reflectance, the FD% quantification was stable regardless of the concentrations and imaging conditions. The variations between FD% from all concentrations obtained using reflectance were $\pm 4.9\%$ at A:D 0:1 and $\pm 3.7\%$ at A:D 2:1. In the case of high concentrations (>33 nM), both reflectance and transmittance provided consistent results. Conversely, the FD% quantification at low concentration deviated from high concentrations especially in the case of A:D 0:1. For trans-illumination geometry, the variations were respectively $\pm 15.2\%$ at A:D 0:1 and $\pm 6.7\%$ at A:D 2:1.

To provide a different view of this comprehensive data set, in Figure 3 we show the collated FD% for all imaging parameter conditions (laser power and exposure time) versus pixel intensity as defined by the maximum of the fluorescence decay curves. In the case of reflectance, the FD% is relatively stable over the dynamic range investigated herein as demonstrated by a linear regression with minimal slope. Conversely, transmission geometry demonstrated a more pronounced slope, especially for the A:D 0:1, indicating a higher FD% estimate at low intensities. The R-squared of the regression analysis between FD% and

fluorescence intensity was 0.0034 (A:D 0:1) and 0.0084 (A:D 2:1) in reflectance geometry and 0.1386 (A:D 0:1) and 0.0789 (A:D 2:1) in transmission geometry. To relate this difference between the two illumination channels' performance, we computed an acquisition factor that provides insight into system settings (acquisition factor = laser power $\times$ exposure time $\times$ grey level). A higher acquisition factor corresponds to higher overall system settings, whereas a lower acquisition factor corresponds to lower overall system settings. We summarize in Figure 4 the FD% quantification of the same data set versus this acquisition factor. Unlike transmission geometry, in case of reflectance, the FD% quantification is stable even if the acquisition parameters are increased towards the maximum power and integration times allowable in our system. On the other hand, the transmission channel demonstrates a pronounced increase in FD% estimation as the acquisition factor increases. We should note that high acquisition factors do not correlate systematically to high pixel intensities. Indeed, for low concentrations, even working at a high acquisition factor will lead to fluorescence decay curves with relatively low photon counts. Increasing acquisition factor will also result in higher excitation bleedthrough to the detector. This effect is more noticeable in transmission geometries when acquired from a blank well (Figure 5).

3.2 | In vivo validation

The *in vitro* study suggested an improved performance of reflectance configuration compared to transmission under highly dynamic fluorescence conditions. *In vivo* validation was then conducted to confirm the applicability of the reflectance geometry in live imaging settings for pre-clinical studies.

In the first *in vivo* experiment, cancer cells-containing Matrigel plugs were subcutaneously implanted into thoracic and inguinal mammary fat pads in live anesthetized animal (Figure 6). Each plug had cells loaded with transferrin AF700-AF750 at different A:D ratios (0:1, 1:1, 2:1 and 3:1 respectively). In this experiment, as the cells were labelled prior to implantation, the fluorescence biodistribution was located uniquely at the plugs. The performance of *in vivo* FRET quantification was comparable using both transmission and reflectance geometries (Figure 6A–D). The difference of FD% between the two configurations was $\pm 2\%$ (Figure 6E–F). This study confirmed that reflectance geometry could be an alternative imaging configuration for *in vivo* wide-field quantitative lifetime-based FRET imaging. An important consideration criterion for choosing an imaging geometry is the depth of the sample. Reflectance configuration is suitable for imaging of sub-surface or limited depth of tissues, while transmission configuration is suitable for tomography.

The next *in vivo* validation involved the biodistribution of the transferrin. A mouse carrying a breast cancer tumor xenograft was intravenously injected with an NIR labelled transferrin pair (Tf AF700-AF750 at A:D ratio 2:1). The images were acquired at 6 hours post-injection under transmission and reflectance geometries. After the injection, the fluorescence probes were mainly distributed to the tumour xenograft, the urinary bladder and the liver (Figure 7A–D). There was no difference of FD% in the tumour xenograft imaged on reflectance and transmission geometries (4.83 ± 4.18 vs. 4.73 ± 5.24 , $p = 0.774$). There was also no difference of FD% in the urinary bladder imaged on both imaging geometries (1.8 ± 2.84 vs. 1.73 ± 2.92 ,

$p = 0.798$). FD% in the tumour xenograft was statistically different from that in the urinary bladder (reflectance: $p = 9.53 \times 10^{-26}$, transmission: $p = 1.35 \times 10^{-14}$) (Figure 7E). Importantly, the liver was only visible when imaged under reflectance geometry but not under transmission geometry. FD% in the liver was significantly higher (53.87 ± 5.24).

4 | DISCUSSION

Macroscopic fluorescence lifetime imaging (MFLI) is a preclinical imaging platform that provides, beyond intensity-based bio-distribution studies, the capability to quantitate fluorescence lifetime over the whole animal body at fast acquisition speeds. Unlike other optical imaging techniques, MFLI is as sensitive as positron emission tomography (PET) imaging with the detection limit at the nano- to pico-molar level. [22] In addition, its ability to quantitate lifetime over the animal whole body offers a unique wealth of information. Especially, in the case of oncological studies, we have demonstrated that MFLI in conjunction with lifetime-based FRET quantification has enabled us to report, for the first time, on the quantitative imaging of receptor-target engagement and associated cellular drug delivery in intact live animals. As intensity-based studies are limited to reporting target accumulations of therapeutic compound, invasive methodologies have been required so far to confirm the cellular accumulation, including biopsy and histopathology. These invasive approaches are not well suited for longitudinal studies as well as monitoring drug delivery in its whole context as required for pharmacokinetics studies. Our MFLI-FRET platform for preclinical study was initially developed in transmission geometry. [11] This implementation was designed to enable 3D tomographic optical imaging of small animals. [17,18] Such approach is required for deep seated pathologies. However, due to the large variation in optical properties of live organs as well as changes in thickness probed in non-contact systems, acquiring useful fluorescence signals beyond the pathological site can be challenging. Even though using the near-infrared imaging window improves penetration, high attenuation associated with organs such as the liver, which is far greater than muscle or skin for instance, [23] can limit whole body biodistribution assessment. The penetration of the excitation light can be improved by increasing the excitation power and increasing camera gain settings. However, increasing these parameters also increases the chance of the excitation bleedthrough, which can negatively impact fluorescence quantification accuracy in both intensity and lifetime measurements. Conversely, reflectance geometry can prevent direct exposure of the detector to the excitation light. Hence, excitation bleedthrough can be largely mitigated, and the dynamic range of detection improved. Additionally, by leveraging the ability of DMDs in controlling locally the illumination intensity, one can improve efficiently the dynamic range of detection in reflectance geometry by applying the inhomogeneous pattern to the DMD. [20] Hence, the intensity of the excitation wide-field pattern can be locally reduced at high fluorescence intensity areas, while retained at low fluorescence intensity areas. The actual intensity can be recovered by correction with the laser power, the detector exposure time and DMD grey-level calibration. This illumination approach was validated to enhance the sensitivity of detection. [20] Though, due to the differences in illumination optics as well as tissue sampled, it is not obvious that both transmission and reflectance geometries yield similar lifetime-based quantification, especially in the challenging case of NIR FRET.

Our quantitative validation of *in vitro* FRET shows comparable results between the two geometries. IgG-IgG reaction was chosen as a model due to strong intermolecular binding, providing a distinct FRET reaction. The binding of donor IgG (Alexa Fluor 700) and acceptor IgG (Alexa Fluor 750) resulted in lifetime reduction of the donor, which was quantified by the bi-exponential fitting of fluorescence decay curves to retrieve relative short lifetime (FRET) component or FD%. There was no difference in FD% obtained from transmission and reflectance geometries at higher concentration range (> 33.6 nM). As expected, the difference was observed at lower concentration range due to the fewer photons collected. To acquire sufficient photon count (> 500 at peak) for bi-exponential fitting, the laser power was increased to enhance fluorescence intensity. With the laser power increased, transmission geometry has more chances of direct exposure of the detector to the excitation light than reflectance geometry because of the DMD and the detector alignment. The regression analysis showed a slightly positive relationship between illumination power and FD% in transmission geometry, while this relationship was minimal in reflectance geometry.

In vivo validation was demonstrated by the transferrin delivery system. The expression of transferrin receptor is highly elevated in many types of cancer, including breast cancer cells. Transferrin has been used as a drug delivery carrier for cancer treatment. [24–26] Binding of iron-bound transferrin (holo-transferrin) to the receptor, which is formed as homo-dimer at a distance less than 10 nm between the receptor monomers, allows FRET to occur. The cancer cells-Matrigel plug model allowed us to bypass the vascular delivery, by providing complete binding of transferrin and removing unbound transferrin. The difference of FRET quantification performance of transmission and reflectance geometries was within $\pm 2\%$ difference for all A:D ratios (0:1, 1:1, 2:1 and 3:1).

An important aspect of preclinical imaging is a study of whole-body drug distribution. Wide-field MFLI in transmission geometry can be impractical to visualize the whole body of a small animal due to limited tissue penetration of the light. Reflectance geometry can visualize the sub-surface of the whole body at a few millimetres depth, which is still sufficient to capture fluorescence lifetime information from many tissues, including the liver, urinary bladder, gastrointestinal tract, lungs, kidneys, superficial lymph nodes and, importantly, subcutaneous tumour xenografts. MFLI-FRET imaging of mice with intact breast tumour xenografts showed high accumulation of transferrin in the tumour xenograft, the liver, and the urinary bladder. FD% was increased in the tumour xenografts compared to the urinary bladder. This finding was expected due to the transferrin receptor-ligand engagement in T47D cancer cells. FD% in the xenograft was significantly higher than that in the urinary bladder, in which there was only the excretion of degraded probes. FD% in the T47D tumour xenograft was much lower than that in T47D Matrigel plugs for two main reasons. First, there was unbound transferrin probes accumulated at the tumour. The unbound probes relatively increased the component of non-FRETing donor. In addition, the photon counts at the tumour xenografts was much less compared to Matrigel plug because only a tiny fraction of intravenously injected transferrin ($\sim 5\%$) ends up in the tumour. There was a substantial fluorescence accumulation in the liver and high level of FD%. This was only visible under reflectance but not transmission geometry due to limited light penetration. The liver is a well-known primary organ for iron metabolism and profoundly expresses transferrin receptor. [27] Overall, reflectance geometry was more robust and provided higher

information content for whole body drug distribution study in small animals with subcutaneous tumour xenografts.

5 | CONCLUSION

Wide-field MFLI is available in both transmission and reflectance geometries for preclinical study. While both geometries provide similar lifetime-based FRET quantification, reflectance geometry provides more robust performance when subcutaneous tumour xenografts are investigated. Indeed, reflectance geometry enables a high dynamic range of detection and can achieve whole body visualization because it is less sensitive to excitation bleedthrough and does not have to penetrate through the entire depth of the tissues. Such configuration also is expected to facilitate the acquisition of dense spatial, spectral and temporal data sets for multiplexed studies, [28] which are more and more desired for guiding targeted drug development and assessing drug delivery *in vivo*.

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imaging session.

FIGURE 1.

Schematics of wide-field MFLI platform. The alignment of illumination devices of reflectance and transmission geometries are shown on the left panel. The characteristics of fluorescence decays and instrument response function (IRF) of both imaging geometries are displayed on the right panel. The fluorescence decays of FRETing donor (blue line) showed faster decay compared to non-FRETing donor (red line).

FIGURE 2.

Comparison of IgG-IgG FRET *in vitro* reaction. Top panels: Pixel distribution of FD% obtained from reflectance (A) and transmission (B) geometries. Each bar shows a relative distribution of FD% at a specific concentration collected from all imaging parameter conditions (for a total of 32 conditions). Bottom panels: Fluorescence intensity and FD% maps of the samples obtained under the highest system setting condition (C: reflectance, D: transmission).

FIGURE 3.

Independence of FD% from fluorescence intensity. Histograms show the relative frequency of FD% at a specific fluorescence intensity (after correction by system parameters) from IgG-IgG reactions under non-FRETing (A:D 0:1) and FRETing (A:D 2:1) conditions. The red lines are regression fittings.

FIGURE 4.

Reflectance imaging geometry was less susceptible to high system settings compared to transmission. Histograms show the relative frequency of FD% at a specific acquisition factor. The red lines are regression fittings. (Acquisition factor = laser power $\times$ detector exposure time $\times$ grey scale level).

FIGURE 5.

The effect of acquisition factor on the excitation bleedthrough. The figure shows increasing bleedthrough excitation on a blank sample under varied laser excitation powers and detector exposure times. Transmission geometry was more susceptible to high acquisition factor than reflectance geometry.

FIGURE 6.

Intensity and FD% maps on a mouse implanted with cancer cells-Matrigel plugs. A– B: intensity and FD% map under reflectance geometry; C–D: intensity and FD% map under transmission geometry. FRET quantification performance in cancer cells-Matrigel plugs *in vivo*. E: Scatter plots showing pixel distribution of FD% at varied A:D ratios (R: reflectance geometry; T: transmission geometry). The green diamonds inside the boxplots represent means. F: Equivalence tests between FD% of the same A:D ratio from transmission and reflectance geometries show ± 2 FD% different ($p < 0.05$, alpha 0.05).

FIGURE 7.

In vivo FRET quantification of T47D xenografted mouse. Fluorescence intensity and FD% maps showing major organs detected by reflectance (A–B) and transmission (C–D) geometries at 6 hours post-injection. The urinary bladder (UB) and the tumour xenograft (TM) were visible under both imaging geometries, but the liver (LV) was visible under reflectance geometry only. E: Scatter plot showing FD% pixel distribution from the urinary bladder and the tumour xenograft. There was no significant difference of FD% between reflectance (R) and transmission (T) geometries in the urinary bladder ($p = 0.774$) and the tumour xenograft ($p = 0.798$), but there was significant difference between the urinary

bladder and the tumour xenograft (reflectance: $p = 9.53 \times 10^{-26}$, transmission: $p = 1.35 \times 10^{-14}$). FD% of the liver, which could only be obtained from reflectance geometry, showed significantly higher FRET level.

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