Mesoscopic Fluorescence Tomography of a Photosensitizer (HPPH) 3D Biodistribution in Skin Cancer

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Rationale and Objectives: Photodynamic therapy (PDT) is a promising strategy for treating cancer. PDT involves three components: a photosensitizer (PS) drug, a specific wavelength of drug-activating light, and oxygen. A challenge in PDT is the unknown biodistribution of the PS in the target tissue. In this preliminary study, we report the development of a new approach to image in three dimensions the PS biodistribution in a noninvasive and fast manner.

Materials and Methods: A mesoscopic fluorescence tomography imaging platform was used to image noninvasively the biodistribution of 2-[1-hexyloxyethyl]-2 devinyl pyropheophorbide-a (HPPH) in preclinical skin cancer models. Seven tumors were imaged and optical reconstructions were compared to nonconcurrent ultrasound data.

Results: Successful imaging of the HPPH biodistribution was achieved on seven skin cancer tumors in preclinical models with a typical acquisition time of 1 minute. Two-dimensional fluorescence signals and estimated three-dimensional PS distributions were located within the lesions. However, HPPH distribution was highly heterogeneous with the tumors. Moreover, HPPH distribution volume and tumor volume as estimated by ultrasound did not match.

Conclusions: The results of this proof-of-concept study demonstrate the potential of MFMT to image rapidly the HPPH three-dimensional biodistribution in skin cancers. In addition, these preliminary data indicate that the PS biodistribution in skin cancer tumors is heterogeneous and does not match anatomical data. Mesoscopic fluorescence molecular tomography, by imaging fluorescence signals over large areas with high spatial sampling and at fast acquisition speeds, may be a new imaging modality of choice for planning and optimizing of PDT treatment.

Key Words: 3D biodistribution; photosensitizer; fluorescence tomography; ultrasound.

The most common cancer treatments include surgery, radiation therapy, chemotherapy, immunotherapy, and monoclonal antibody therapy. These therapies may be used either alone or in combination with other therapies. Although improvement of these treatment modalities to enhance their tumor selectivity is under way, development of novel alternative treatment approaches that may be safer, reduce functional tissue damage, and improve patients’ quality of life is a major focus area of research. Among all new modalities investigated, photodynamic therapy (PDT) emerges as a promising alternative treatment strategy (1). PDT is a noninvasive treatment using a combination of a photosensitizer (PS) drug, a specific wavelength of drug-activating light and oxygen. Light activation of a PS results in energy transfer cascades that ultimately yield cytotoxic reactive oxygen species.

PDT has undergone extensive investigations in cancer treatment and various PS drugs have been approved for clinical use in the United States and other countries (2,3). PDT is being used mainly to treat skin diseases and easily accessible malignant and premalignant lesions (4,5). PDT is a local therapy with the major advantage that the PS itself is minimally toxic in the absence of light; therefore, PS accumulation in nonspecific tissues confers minimal systemic toxicity. Furthermore, light activation is performed below the maximum permissible exposure limit and at wavelengths that do not harm the tissues. Hence, PDT can be applied repeatedly should a single treatment fail. The efficacy of PDT is largely dependent on the functional state of the tissue, the PS uptake, and light fluence delivered locally. If these parameters can be controlled/assessed relatively easily in superficial lesions, it is a significant challenge for pathologies that are thick or located a few millimeters deep. In such scenarios, the PS
biodistribution profile in tissue is not homogeneous, even in the case of local topical application. Second, light propagation in tissues is highly affected by the optical properties distribution in the sample. Hence, PDT efficiency can be hampered by intralesion PS heterogeneous biodistribution and light flux profiles.

The most accepted method to measure PS concentration and distribution in tissue has been by biopsy sampling of the tissue and chemical extraction of the compound for spectrophotometric assay quantification (6). However, this is an invasive, time-consuming, and expensive method that is prone to sampling errors. To provide a direct mean to image the PS biodistribution, there is a critical need to develop new imaging modalities that could provide the three-dimensional (3D) PS biodistribution at bedside for optimal therapy planning, and, potentially, during treatment to assess therapy efficacy (7–11).

One key property of the main PS drugs is their ability to fluoresce; therefore, fluorescent signals can be used to directly measure the biodistribution and concentration of the PS in vivo (12,13). However, two-dimensional (2D) surface measurements have been shown to be unreliable because of variations in tissue optical properties and heterogeneous drug depth distribution (14). Researchers have proposed either spatially resolved spectroscopic and/or tomographic methods to overcome these issues (15). In addition, some alternative approaches, such as small-fiber probes, have been developed to limit the effect of optical properties on the fluorescence signal. However, such probes still lead to local measurements prone to error sampling and could require minimally invasive protocol to obtain depth profile (16,17).

For noninvasive 3D imaging, classical fluorescence microscopic techniques have been used to image the PS biodistribution and/or assess therapy efficacy. For instance, confocal microscopy (CM) was used for imaging PpIX accumulation in the target tissue (18), whereas two-photon microscopy was used for posttreatment imaging (19) to assess cell death after radiation. Although these latter two imaging methods offer high-resolution performance and great sensitivity, they are restricted in their depth penetration (<1 mm) and field of view (<1 mm²), limiting their usefulness for fast imaging of deep pathologies. To probe bio-tissues beyond the traditional microscopic depth penetration, a novel method known as mesoscopic fluorescence molecular tomography (MFMT) has been developed.

MFMT is an optical tomography technique that can reconstruct absorption or fluorescence contrast over relatively large areas in a fast manner. MFMT is able to image highly scattering tissues that are a few millimeters thick (~3–5 mm) with a relatively high resolution (~100–200 μm). Similarly to diffuse optical tomography or fluorescence molecular tomography, MFMT uses several detectors to collect scattered photons at multiple distances away from the illumination spot, but at distances of a few hundred microns to interrogate tissues at the mesoscopic scale. MFMT uses discrete detectors analog to the pinholes in CM. Smaller or larger optode separations lead to shallower or deeper photon collection, enabling depth sensing. Combining these depth-sensitive measurements with an optical inverse formulation allows the retrieval of a 3D quantitative image of the fluorescence biodistribution. This unique combination of small-source detector separation, discrete detectors, and optical tomography offers unique capabilities for thick tissue imaging. A detailed explanation of the inverse problem and reconstruction schemes can be found in the Materials and Methods section.

MFMT works in epi-configuration and it is well suited for in vitro tomographic imaging (20) as well as for in vivo preclinical (21) and clinical imaging scenarios (22). Furthermore, it can be fused with other traditional optical imaging modalities such as Optical Coherence Tomography (21) to obtain molecular, functional, and structural information. One advantage of MFMT is its ability to image a large number of readily available fluorescent probes at fast acquisition speeds (23). Here, we apply MFMT to retrieve the distribution of 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), a PS that has been used in patients since 1999 within skin cancers in preclinical models. This is a first step toward establishing MFMT as a bedside imaging modality to assess noninvasively the PS biodistribution before treatment for best therapy planning, and potentially during therapy for light dose delivery optimization.

This article summarizes the imaging platform characteristics, image formation algorithms, imaging protocols, and present HPPH 3D biodistribution imaging on seven preclinical skin tumor models and on one sample exhibiting no lesions.

MATERIALS AND METHODS

Preclinical Model Protocol

All animal experiments followed protocols approved by the Institutional Animal Care and Use Committee of Roswell Park Cancer Institute. Transgenic K5-Gli mice were bred in Roswell Park Cancer Institute’s domestic colony. These mice have an activated Sonic hedgehog signaling pathway and overexpress the Gli2 downstream transcription factor, which drives proliferation and leads to the development of spontaneous multiple basal cell carcinoma (BCC). These spontaneous BCCs possess a histologically similar representation of nodular BCCs as that observed in human patients, which is the most common skin cancer in human with increasing incidence over the past decades (24). Three Gli mice (6–12 months old) from the breeding colony were selected for imaging after they spontaneously developed visible BCC tumors on their tails. All mice in this study were euthanized immediately before imaging. Although live imaging sessions under anesthesia (isoflurane) would have been preferable, working with freshly euthanized subjects allowed avoiding any movement during the imaging session for seamless nonconcurrent registration between MFMT and ultrasound (US) imaging, but still working under similar conditions as in live studies. Of the three mice
imaged, mouse A had three lesions imaged, mouse B had four lesions imaged, and mouse C exhibited no lesions based on visual inspection. All mice were injected intraperitoneally with 0.47 μmol/kg (<0.3 mg/kg) of HPPH (Roswell Park Cancer Institute) and were kept under reduced light conditions (<60 lux) until being sacrificed. Mouses A and C were imaged 20 hours after injection, whereas mouse B was imaged 40 hours after injection. In clinical settings, HPPH treatment is commonly applied 24 hours and 48 hours after injection (25).

**Tumor Imaging**

Areas of the tail that exhibited lesions, based on visual inspection (Fig 1a), were selected and then imaged sequentially with MFMT and high-resolution US A-scan imaging. Imaging sessions were performed within 30 minutes. Both data sets were registered a posteriori using a commercial software. To mitigate for the curvature of the tail over the area imaged, a matching liquid solution was used during the imaging sessions (Fig 1a). A diluted intralipid solution (30% Fresenius Kabi, Chicago, IL) matching the optical properties of the mouse skin (26,27) was created and the tail immersed halfway to generate a planar boundary condition.

**Mesoscopic Fluorescence Imaging System**

MFMT is a noncontact laser-scanning imaging technique similar to a confocal microscope. However, MFMT achieves sensitivity to fluorescence by selectively measuring the scattered light emerging from the tissue at different distances (0–4 mm) from the laser beam’s focal point. Whereas wide-field camera-based imaging suffers from the overlapping contributions of light from different regions of the tissue, MFMT serially injects light and detects emerging light at multiple discrete positions on tissue surface. The wider the distance between the optode positions, the deeper the detected light has traveled. This results in a dataset that contains rich information about the depth-resolved properties of the tissue. Then 3D images of the depth-resolved absorption and fluorescence contrast can be reconstructed based on an optical inverse problem using mathematical models of light propagation through the sample. MFMT allows obtaining depth-resolved 3D quantitative images of molecular probes at depths of several millimeters (~3–5 mm) with high sensitivity and relative high resolution (~100–200 μm) (21,23,28,29).

Succinctly, our MFMT imaging system is based on raster scanning a focused laser beam over the sample (Fig 1b). The fluorescence light is then detected at seven radial positions away from the illumination spot in an epi-configuration (the minimum and maximum source-detector distances are respectively 800 μm and 3900 μm). The optical setup is similar to the one in Zhao et al. (20) and is operated at 658 nm (laser diode L658P040, Thorlabs Newton, NJ) to match HPPH (Roswell Park Cancer Institute, Buffalo, NY) excitation spectra. A high-pass emission filter (FF01-692/LP-25, Semrock, Rochester, NY) is used in the detection channel to collect HPPH emission light. To image a large area, the illumination beam is raster scanned over a 6 × 3 mm² surface on the selected area of the tail. The overall light dose applied during the MFMFT imaging sessions is <0.1 J/cm², which is significantly below the light dose applied for PDT treatment (50–200 J/cm²) (25). Both an emission signal and a matching background are sequentially acquired (both with the same emission filter). Data acquisition over the previously mentioned area took 1 minute at a ~7.6 fps rate. Following the postprocessing methodology described elsewhere (30), averaged background signals were subtracted from averaged emission signals to minimize the autofluorescence and the bleed-through effect a posteriori.
and to render the system more sensitive to deep-seated fluorescence signals.

**MFMT Reconstructions**

MFMT is a subsurface-sensing modality that requires solving an optical inverse problem to retrieve the biodistribution of the fluorescent probe in three dimensions. Because of the limited volume probed with this mesoscopic imaging modality, the classical diffusion equation forward model used in fluorescence molecular tomography cannot be used. Herein, we used a central processor unit–based Monte Carlo photon propagation model to compute the Jacobian for such a shallow interrogation volume. The forward simulations were carried with 10^4 photons and 100 μm^3 voxel size. The MFMT image reconstruction process can be expressed using the following equation:

\[ x' = (A^T A + \lambda D)^{-1} A^T b \]  
(1)

where \( b \) is obtained from the fluorescence measurement, \( x' \) is the reconstructed fluorescence distribution, \( \lambda D \) is a regularization depth-dependent parameter, and \( A \) is the whole Jacobian that is computed based by the Monte Carlo–forward model. Thanks to the symmetry of the imaging space, \( A \) is populated for all the positions of the raster-scanned beam, based on a single set of sensitivity matrices, which are computed for one optode set (one source and seven detectors). Optical properties, \( \mu'_s \) and \( \mu_a \) for Jacobian (A) matrix are chosen as 3 mm\(^{-1} \) and 0.002 mm\(^{-1} \) \((26,27)\), respectively. Both 3D reconstruction of absorption and/or fluorescence contrast can be performed using this model (31). A depth-dependent regularization technique is used to mitigate the ill-posedness of the reflectance geometry. \( D \) is a diagonal matrix whose elements are the square root of the corresponding diagonal elements of \( A^T A \). The scaling factor (\( \lambda \)) for this diagonal matrix is selected based on the L-curve analysis (32). The conjugate gradient method is applied to solve this linear system (function \( 
\text{cg}, \) MATLAB R2009b, MathWorks, Natick, MA). The iterative algorithm was stopped if 100 iterations or if a tolerance of 10^{-2} was reached. Overall, the Monte Carlo–forward model and optical reconstructions were computed in less than 4 minutes for the typical 106,400 data measurements (15,200 sources positions x seven detectors) on a personal computer (Intel Core i7-3820 central processor unit, 3.6 GHz, 64 GB).

**Ultrasound Imaging**

The preclinical US system used in this study is a one-dimensional A-scanner that operates at 50 MHz (Longport Episcan, Chadds Ford, PA). The US probe allows investigating tissue along a 14.9- and 5.39-mm depth with a resolution of ~30 μm. US scans were taken immediately after MFMT imaging without altering the position and the condition of the mice. The US probe was wrapped in a sealing membrane to operate the US transducer in water for minimal impedance mismatch. A-scans were taken along the two sides of the tail with 100-μm steps, leading to 30–60 slices acquired depending on the broadness of the tail. A-scan slices provided both the geometrical information about the tail boundaries and about the tumor location and structure.

**Co-registration of US and MFMT Data Sets**

The MFMT and US data sets were co-registered a posteriori. First, to validate our approach, phantom experiments were conducted. A 3% agar (Sigma Aldrich, St. Louis, MO) was mixed with 2% intralipid suspension (20% emulsion, Sigma Aldrich St. Louis, MO) in deionized water. Solution was kept at 80°C on a hot plate (Isotherm, Fisher Scientific, Waltham, MA) and stirred at 350 rpm (Magnetic Stirring Bars, Fisher Scientific) for 1 hour. We aimed to produce a homogeneous turbid matrix with optical properties similar to that used in the optical forward model for tail imaging. Before solidification of the agar, we placed a cylinder-shaped clear solid inclusion (PDMS, Sigma Aldrich St. Louis, MO), ~4 mm in diameter and ~4–6 mm in height, just beneath the surface of the matrix. This inclusion, placed inside the matrix, mimics a tail immersed in intralipid as used in the tumor imaging cases. The PDMS inclusion, shown in Figure 2a, allowed inserting a 1.5-mm-diameter capillary, filled with fluorophore (Alexa 660, Life Technologies, Carlsbad, CA). The phantom was imaged nonconcurrently with US and MFMT. The border of the scanning areas was marked via cutting the matrix with a scalpel (Fig 2b), leading to strong
contrasts in both MFMFT and US, similarly to tails immersed in intralipid. These cuts were used as 2D fiducial markers for registration between the two modalities. The 2D MFMT measurements from the first detector were matched with the corresponding 2D US projection. The US data were rescaled through linear scaling and resampled to match the MFMT images. Registration was performed by pixel-to-pixel matching. We applied this co-registration scenario to three different cases: fluorescence capillary within the PDMS inclusion, fluorescence inclusion next to the PDMS inclusion, and fluorescence inclusion at the top part of PDMS cylinder. A similar registration methodology was employed to co-register the MFMFT and US data for tumor imaging. The boundaries of the tail were clearly delineated on both imaging modalities and used as 2D fiducial markers for rigid registration.

Image Analysis

Estimations of the tumor volume and PS biodistribution volume were calculated from US and MFMT, respectively. Tumor volume was obtained via image segmentation of the 3D US data. Before creating a volume for tumors, A-scan images were segmented, and interspacing between slices was adjusted to the same scale with 2D images. Because of the high contrast, simple intensity threshold at 10% of maximum intensity was performed and enclosed volume was calculated by an embedded software function (function SurfaceAreaVolume, Amira 5.2, Visage Image Inc., San Diego, CA). PS biodistribution volume was estimated using same protocol, though with various threshold values (see the following section). An example of US data, segmentation, and overlay with MFMT data is provided in Figure 3. Because of the regularization term and diffuse nature of the optical reconstructions, multiple isovolume thresholds were used to compute the PS biodistribution volume (10%–40%).

RESULTS AND DISCUSSION

Phantom Study Results

MFMT raw data presented in Figure 2c depict fluorescence signals acquired by the first detector of the optical imaging platform for various positions of the capillary. By design, the fluorescence signal was constrained within the capillary and MFMT captured those localized signals effectively (Fig 4a–c). US images were segmented via software (Amira 5.2, Visage
Imaging Inc., San Diego, CA) and fused with MFMT data as described in the Co-registration of US and MFMT Data Sets section. In all three cases investigated here, successful nonconcurrent registration was obtained between MFMT data and US data as shown in Figure 4. MFMT and US data were positioned at their respective correct locations as expected from the phantom design.

**Tumor Imaging Results**

MFMT data acquired on all seven tumors and a tail without lesions are provided in Figure 5. The measurements are provided for the first detector (800 μm offset) (Fig 5a) and the second detector (1320 μm offset) (Fig 5b). In the case of no tumor, the fluorescence signal was weak. However, no strong contrast or localized signal was observed. On the contrary, in all tumors, strong fluorescence signals were acquired and the fluorescence signals were always confined within the area of the lesion. No remnant fluorescent signals were observed outside the lesion regions. However, the fluorescence signals captured by the first detector (which is close to epi-configuration) were highly heterogeneous over the tumor area. Strong focal signals were captured that were confined to small area of the tumor. Conversely, the fluorescence signals acquired from the second detector exhibited a greater spatial distribution. For this set of data, the fluorescence signals were acquired over the majority of the lesions spatial extent. These results are expected because when the detector offset increases, the fluorescence signal acquired originates from deeper tissue in the tail. The discrepancy between shallow fluorescence signals and deeper fluorescence signals, as seen in all cases here, exemplifies the heterogeneous depth distribution of the PS. As a note, the 2D fluorescence map for the offset detectors can display signals outside the tail tissue. As the illumination beam is raster scanned, the offset detector can be located outside the tail tissue and still collect fluorescence signal emitted within the tail, thanks to the matching liquid. This location blurring is corrected when performing optical reconstructions as the photon propagation is modeled accurately.

![Figure 5. Background subtracted raw data for the no tumor tail and seven different tumors. (a) First-detector raw data; (b) second-detector raw data. Both detectors deliver high-contrast and highly localized signal. Images show a 3 x 6 mm² scanning area.](image-url)
Overall, these raw data demonstrate the potential of our imaging platform to sense HPPH fluorescence signals over a large area and with enough sensitivity to provide depth dependent measurements. Even after 40 hours, the captured fluorescence signals are still strong for all seven detectors in this BCC model. Moreover, these data also support the need to monitor the PS biodistribution in 3D before and during PDT treatment with dense spatial sampling. Our MFMT system is based on a resonant galvanometer mirror for ultrafast scanning. Such implementation allows collecting dense spatial measurements on the imaged surface. In this specific study, measurements were collected each 100 μm (laser beam positions) over the full area. Such dense spatial sampling allows revealing the highly heterogeneous distribution of PS in the tumor both in the illumination plane and in depth. Such information is not attainable using fiber based systems that are limited in their measurements channels and hence, prone to error sampling. To analyze the PS biodistribution further, MFMT reconstructions were performed and co-registration with US was achieved.

**MFMT Reconstructions and US Co-registration**

An example of 3D co-registration between MFMT and US for tumor 7 is provided in Figure 6. The co-registration allows visualizing the tumor volume and PS biodistribution as estimated by the methods described previously. The visualization is expected to provide the overall structure of the lesions via US and local drug delivery via fluorescence 3D imaging. The same process was applied to all tumors imaged and the 2D projections of the co-registration are provided in Figure 7, whereas the transverse projections are provided in Figure 8. The corresponding estimated volumes are reported in Table 1. In all cases investigated, the PS biodistribution was found to be located within the tumor volume as estimated by US. However, as described previously, the PS biodistribution was highly heterogeneous. In the majority of the cases, the PS biodistribution was limited to a subarea of the lesions. Only in the case of tumor 1, which was a very small tumor (0.22 mm³), was the PS biodistribution volume bigger than the tumor volume for isovolume threshold equal and larger than 20%. Also, the PS distribution had complex depth profiles. These results indicate, as expected, that there is no one-to-one match between tumor volume as obtained via structural imaging and PS biodistribution. The PS biodistribution is unique to each tumor and is highly heterogeneous in all dimensions. Even, in some cases, such as tumor 2, the PS seems to accumulate in the peripheral tissues.

**DISCUSSION**

This preliminary study aimed at investigating the potential of MFMT in retrieving the 3D biodistribution of a PS before treatment. One key aspect of the investigation was to establish that MFMT had enough sensitivity to acquire fluorescence signals emitted by the PS over a large surface, with high spatial sampling and at a fast acquisition speed. Our results demonstrate that our MFMT platform is able to acquire such data sets within a minute and with a 100-μm spatial sampling rate. Moreover, tomographic reconstructions were performed using these data sets to visualize the PS biodistribution in 3D. The computation time required for computing the forward model and solve the inverse problem was <4 minutes on a personal computer. The overall imaging session was achieved in 5 minutes, which is compatible with clinical practice. Indeed, according to the American Cancer Society, a PDT treatment takes 45–60 minutes, which indicates that our current system can be integrated into a PDT patient’s workflow without significant interference. We foresee that with the combination of more sensitive detectors to reduce the data
integration time and optimized reconstruction algorithms, that MFMT imaging procedure could be reduced to a few seconds. Then, MFMT could be also a promising imaging modality candidate to monitor PDT efficacy noninvasively during treatment.

Additionally, the data acquired on the seven tumors imaged in this study demonstrated a highly heterogeneous PS biodistribution. The overall PS volume did not match one to one with US data. This was expected because the contrast function difference between US and MFMT inherently yield ...
planning and optimization(33). Imaged, they cannot be used as prior information for PDT as obtained by US could be useful in defining the area to be mapping the PS uptake. Moreover, if anatomical data such techniques that have a high spatial sampling are required for our results address these questions and suggest that imaging different information. Diffusion, localization and compartmentalization may affect the heterogeneous distribution of HPPH between blood vessels and tumor interstitial space. Our results address these questions and suggest that imaging techniques that have a high spatial sampling are required for mapping the PS uptake. Moreover, if anatomical data such as obtained by US could be useful in defining the area to be imaged, they cannot be used as prior information for PDT planning and optimization (33).

Another point of importance is that we focused on the relative distribution of the PS agent, not on the quantification of the PS concentration. Quantifying fluorophore concentration absolutely is challenging because it is greatly influenced by the optical properties of the tissue. Here, we used a classical linearized tomographic formulation that employs the average optical properties of the tissue to compute the optical forward model. A more accurate approach would be to map optical properties at both excitation and emission wavelengths and provide the optical properties as a priori information for the fluorescence reconstruction. Such an approach has been described by Rohrbach et al. (34) and Saager et al. (35), who used spatial frequency domain imaging to estimate the optical properties of tissue over large areas, which in turn allowed extracting absolute fluorescence concentration in 2D. Still, it is a challenging task to be able to extend such work in 3D, although Konecky et al. (36) demonstrated that spatial frequency domain imaging data sets can be also used for tomographic reconstruction of optical properties. This 3D map of the optical properties could then be used as a priori information to compute accurately the optical forward model for absolute reconstructions of the PS concentrations.

**CONCLUSIONS**

This work reports the initial evaluation of using MFMT for retrieving the 3D distribution of PS in skin cancer before PDT treatment. To this end, HPPH was injected intraperitoneally into the mice expressing spontaneous skin cancers. Tumors located in the tail of the animal were imaged using MFMT and US. In all cases investigated here, all lesions exhibited strong fluorescence that allowed for mesoscopic optical reconstructions. Overall, the acquisition time of fluorescence signals over a 6 × 3 mm² area was completed within 1 minute, whereas the optical reconstructions were performed within 4 minutes on a personal computer. This preliminary investigative study highlights the potential of MFMT to retrieve the 3D PS biodistribution within a few minutes. Future study will focus on imaging the 3D PS biodistribution changes over treatment and optimize the reconstruction algorithms for allowing near real-time feedback during therapy.

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**REFERENCES**


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ISO, isosurface value for MFMT reconstruction.