Fluorescence background subtraction technique for hybrid FMT-XCT imaging of a mouse model of early stage lung cancer

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Abstract

The ability to visualize early stage lung cancer is important in the study of biomarkers and targeting agents that could lead to earlier diagnosis. The recent development of hybrid free-space 360° Fluorescence Molecular Tomography (FMT) and X-ray Computed Tomography (XCT) imaging yields a superior optical imaging modality for three-dimensional small animal fluorescence imaging over stand-alone optical systems. Imaging accuracy was improved by using XCT information in the fluorescence reconstruction method. Despite this progress, the detection sensitivity of targeted fluorescence agents remains limited by non-specific background accumulation of the fluorochrome employed, which complicates early detection of murine cancers. Therefore we examined whether X-ray CT information and bulk fluorescence detection could be combined to increase detection sensitivity. Correspondingly we researched the performance of a data-driven fluorescence background estimator employed for subtraction of background fluorescence from acquisition data. Using mice containing known fluorochromes *ex vivo* we demonstrate the reduction of background signals from reconstructed images and sensitivity improvements. Finally, by applying the method to *in vivo* data from K-ras transgenic mice developing lung cancer, we found small tumors at an early stage compared to reconstructions performed using raw data. We conclude on the benefits of employing fluorescence subtraction in hybrid FMT-XCT for early detection studies.

Keywords: fluorescence tomography; image reconstruction; multimodality; targeted fluorescence agent.

1. Introduction

Fluorescence Molecular Tomography (FMT) is a non-invasive optical imaging method aiming at monitoring fluorescence bio-distribution in small-animals *in vivo* [1,2]. In FMT, typically a fluorescent agent is injected into a mouse, and focused light is subsequently scanned across the animal surface, after which excitation and fluorescence photons that have propagated through the tissue are collected. The acquired data is combined in a mathematical inversion scheme to produce quantitative images of fluorescence bio-distribution. Over the past years, the technique has evolved from limited-angle trans-illumination geometries to CCD camera based 360-degree trans-illumination systems, enabling FMT to deliver molecular contrast with millimeter resolution [3]. A significant challenge in optical imaging is the highly diffusive nature of photons propagating in tissue [4, 5]. Furthermore, while FMT delivers molecular contrast, the modality does not provide anatomical information. To address these challenges, recent technical and performance advances have focused on the combination of FMT with modalities that offer anatomical information including MRI and X-ray CT [6-9]. The use of anatomical data can improve optical tomography by building geometrically accurate forward photon propagation models in the tissue imaged [10-12]. Second, the anatomical information has been employed to reduce the uncertainty of the ill-posed FMT inverse problem by using anatomical information as image priors [10,13]. In post-processing the FMT data, the anatomical information is used to provide the anatomical context for the reconstructed fluorescence signals. The improved imaging capabilities have been recently demonstrated in complex areas of the mouse, such as the thorax area for example imaging bone disease or lung cancer in animal models [13]. Detection of lung tumors with fluorescence tomography has also been reported using limited-projection FMT [14] and early-photon FMT [15]. An important next step towards improved image quality is to include the newly available anatomical information in the pre-processing of the FMT data before reconstruction of the fluorescence signal, by using it to apply a background fluorescence correction, which will be described in this paper.

Background fluorescence can be largely attributed to non-specific distribution or binding of the fluorescence agents employed for imaging, which are typically administered intravenously. After circulation through the body, the fluorescent agent typically accumulates at the target site, for example a tumor, resulting in a larger concentration of probe at the specific site. However, presence of the agent throughout the body, even at low concentrations,

suffices to generate signals that can be detected and contribute to reducing the contrast in the image or even masking small target lesions. Background fluorescence contributions may also be due to the auto-fluorescence of tissue and fluorochromes present in the mouse diet, but these contributions are typically minor in the near-infrared, unless the skin or the gastro-intestinal system are imaged [16-18].

Reduction of background fluorescence in the tissue is especially important when the aim is to visualize small targets with a low fluorescence intensity. This situation occurs particularly when imaging lung tumor development at an early stage. Early stage imaging can allow identification of biomarkers involved in disease development and progression. However, the imaging of small and non-bright tumors that characterize early stage lung cancer can be obstructed by the presence of background fluorescence in the tissue.

Methods for reducing the influence of background fluorescence on the reconstruction result have been investigated previously [18-24]. One approach constrained the inversion step of the reconstruction of the fluorescence distribution in the presence of background fluorescence. The maximum level of background fluorescence was calculated by taking the ratio of the measurements at the detectors divided by the corresponding weight matrix integrated over the complete volume. This maximum level was used to set a lower bound for the reconstructed fluorescence concentration [19]. Several multi-spectral approaches for reducing the effects of background fluorecence have been investigated [20,22,23]. Deliolanis et al. [20] performed additional multispectral measurements using shorter wavelengths, which were substracted from the measurements obtained by exctitation at the peak excitation wavelength of the probe. Tichauer et al. [23] recently developed a method that involves subtraction of the signal of a second, untargeted fluorescent tracer with similar characteristics as the targeted tracer. Pre-processing of the measured data has also been considered, before using them as input for the inversion [18, 21]. Gao et al. [18] plotted the normalized born ratio as a function of the 2D radial distance from the source point projected on the CCD camera plane. A cubic polynomial was fitted to the lower bound of the plot and subsequently this polynomial was subtracted from the normalized born ratios. Soubret et al [21] proposed a subtraction method for a slab geometry, under the assumption that the background fluorescence and the tissue/object imaged were both homogeneous. This method observed a linear relationship between the tissue background fluorescence and the normalized Born values measured, as a function of the distance between the source and the detector involved in each measurement. A proportionality

constant was then calculated and applied as a data pre-processing step to demonstrate imaging improvements. Although the assumption of a slab geometry is appropriate when using an imaging chamber, it remains an approximation. The latest developments of FMT systems are generally free-space implementations, for which the assumption of a slab geometry is less appropriate. Furthermore, it has been shown recently that also in systems where the mouse is imaged in a cassette, reconstruction of the fluorescence distribution is significantly improved by including accurate anatomical information [25]. The same is expected when incorporating anatomical information for performing a background fluorescence subtraction.

In this paper we interrogate whether the fluorescence subtraction scheme developed in Ref. [21] for slab geometry can be applied to 360-degree acquisition geometry, as it relates to imaging with a hybrid FMT-XCT system. A major advantage of hybrid FMT-XCT is that both modalities are implemented in the same system, which minimizes the time difference between acquisition of FMT and XCT data and eliminates the need to move the animal in between scans, resulting in a very accurate match of data from both modalities which was not previously available. We describe the steps of implementing the background fluorescence subtraction method for 360-degree geometries and employing XCT information. Importantly, we implemented it in a data-driven way. We demonstrate our findings on imaging performance and quantification using *ex vivo* mouse measurements, as well as *in vivo* studies employing transgenic mice developing spontaneous lung tumors [26].

2. Methods

Expression for normalized background fluorescence

The normalized Born approximation is a data conditioning method employed in fluorescence tomography to minimize the sensitivity of the reconstruction on experimental uncertainties, in particular the unknown gain and attenuation factors involved in tomographic measurements (gain of each detector employed, strength of each source etc.). A further benefit of this normalization is the reduced sensitivity of the reconstruction to spatial variations in absorption properties within tissues, whereby scattering variations remain challenging [12]. According to the normalized Born method, the emission measurements at the wavelength of the fluorochrome $U^{fl}\left(\vec{r}_d,\vec{r}_s\right)$ are normalized by the measured transmittance $U^{exc}\left(\vec{r}_d,\vec{r}_s\right)$ at the excitation wavelength of the laser:

$$\left[U^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right)\right]_{\mathrm{measured}} = \frac{U^{\mathrm{fl}}\left(\vec{r}_d,\vec{r}_s\right)}{U^{\mathrm{exc}}\left(\vec{r}_d,\vec{r}_s\right)}, \qquad\qquad (1)$$

where $\left[U^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right)\right]_{\mathrm{measured}}$ represents the normalized measurements corresponding to a source at $\vec{r}_s$ and a virtual detector at $\vec{r}_d$. For the case of a homogeneous medium with a constant fluorescence level throughout the medium, Soubret and Ntziachristos [21] derived a linear relationship between the Born ratio and the distance from source to detector, given by:

$$U_{\mathrm{target}}^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right) = \left[U^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right)\right]_{\mathrm{measured}} - a\left\|\vec{r}_d - \vec{r}_s\right\|, \qquad\qquad (2)$$

where $\left\|\vec{r}_d - \vec{r}_s\right\|$ is the distance from a source at $\vec{r}_s$ to a detector at $\vec{r}_d$, a is a constant proportional to the constant fluorescence level in the medium and $U_{\mathrm{target}}^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right)$ is the corrected background fluorescence. The parameters that need to be estimated to obtain $U_{\mathrm{target}}^{\mathrm{nB}}\left(\vec{r}_d,\vec{r}_s\right)$ are the proportionality constant a and the distance from source to detector $\left\|\vec{r}_d - \vec{r}_s\right\|$.

Calculation of distances

In this work we explored whether the relationship in Eq. 2 is applicable to measurements collected over 360° as available by hybrid FMT-XCT systems. XCT anatomical information was employed to calculate the distance between source and detector FMT pairs, as they are established in a non-contact manner on the mouse surface.

For each CCD viewing angle, the points on the mouse surface seen by the camera were extracted from the cross-section of the XCT derived surface and lines perpendicular to the CCD detection plane, forming the 3D surface of the imaged medium at the side of the CCD plane, S_{CCD}. In the same way, all points on the mouse surface as seen by a virtual camera at the source plane were extracted, forming the 3D surface of the medium at the side of the source plane S_{source} (Figure 1a).

The extracted surface S_{CCD} was interpolated on a grid of 512*512 points corresponding to the pixels in the CCD camera image, resulting in a 512*512 image. The 3D Euclidian distance from the source point $\vec{r}_s$ projected at the source surface S_{source} to all points on the interpolated surface image at the detector side S_{CCD} was calculated. This resulted in a 512*512 image of

distances, IM_{BG} (Figure 1b), representing the term $\left\| \vec{r}_d - \vec{r}_s \right\|$, for each pixel in a corresponding acquisition image IM_{acq} (Figure 1e) obtained by measurement with the hybrid FMT-XCT system.

Data driven proportionality estimation

The next step was to derive a method that automatically calculates a proportionality factor a as per Eq. 2, for each mouse studied. To perform this calculation, all measurements in an acquired data set obtained from a mouse scan were considered. In a typical FMT-XCT imaging protocol data from 18 angular projections are acquired. For each projection a total of 18 sources were scanned resulting in a total data volume of 324 images per wavelength. Then, a region of interest was automatically selected on a per-image basis as the area that was covered by the scanned sources. This area was employed for the calculation of the proportionality factor. This calculation assumes that there is an underlying fluorescence background distribution that is present in all measurements (scaled with distance). In case the medium would contain background fluorescence only, i.e. $U_{\text{target}}^{\text{nB}}\left(\vec{r}_d, \vec{r}_s \right)$ is zero in Equation 2, an estimate of the proportionality constant a calculated for one source detector pair $\hat{a}(\vec{r}_d, \vec{r}_s)$ would be given by:

$$\hat{a}(\vec{r}_d, \vec{r}_s) = \frac{\left[U^{\text{nB}}\left(\vec{r}_d, \vec{r}_s \right) \right]_{\text{measured}}}{\left\| \vec{r}_d - \vec{r}_s \right\|} . \tag{3}$$

where $\hat{a}(\vec{r}_d, \vec{r}_s)$ is a constant proportional to the background fluorescence level, $\left\| \vec{r}_d - \vec{r}_s \right\|$ is the distance from a source at $\vec{r}_s$ to a detector at $\vec{r}_d$, and $\left[U^{\text{nB}}\left(\vec{r}_d, \vec{r}_s \right) \right]_{\text{measured}}$ represents the normalized measurements corresponding to a source at $\vec{r}_s$ and a detector at $\vec{r}_d$. To estimate one proportionality factor a on a per mouse basis we assumed that the mice scanned contained only background fluorescence. Generally, this assumption is approximately true if the fluorescent target tissue volume for an injected fluorochrome is smaller than the total mouse volume visualized, which is typically the case when imaging tumors or specific organ parts. Using Eq. 3, the $\hat{a}(\vec{r}_d, \vec{r}_s)$ for each source detector pair was calculated and the distribution of the $\hat{a}(\vec{r}_d, \vec{r}_s)$ values for all source-detector pairs $(\sim 10^7)$ was plotted. With the assumption then that the fluorescent source is small compared to the total mouse volume scanned, the most

frequent value observed (the mode), was used as the estimate of the proportionality constant a on a per mouse basis:

$$a = \text{bin}(\max(\text{hist}(\hat{a}(\vec{r}_d, \vec{r}_s)))) ,\qquad\qquad (4)$$

thus, providing a robust and fully data driven method to determine a. In Figure 1c the distribution of the ratio of the pixels in the region of interest of IM_{BG} (Figure 1b) divided by IM_{acq} (Figure 1e), a can be found at the peak value, in this case a is approximately 0.2. The last step in the background correction was the subtraction of the background images IM_{BG} from the acquisition data IM_{acq} using the proportionality factor a and Eq. 2:

$$IM_{corrected} = IM_{acq} - a * IM_{BG}\qquad\qquad (5)$$

The corrected acquisition images $IM_{corrected}$ are used as input for the reconstruction of the fluorescence distribution, i.e. the operation is performed on the acquisition images directly, before source-detector pairs are extracted from the images for reconstruction.

Hybrid FMT-XCT imaging and reconstruction

The system that was used for hybrid FMT-XCT imaging was described in detail in [6]. It was a hybrid combination of a gantry based micro-CT system (eXplore Locus, GE HealthCare, Ontario, Canada) and free-space FMT instrumentation in trans-illumination setup. The rotating gantry enabled FMT and XCT acquisition over the full 360° range, resulting in a hybrid FMT-XCT data set.

The 3D fluorescence distribution was reconstructed from the acquisition data using a "double prior" hybrid reconstruction method, described in detail in [13]. This method combines a Finite Element Method (FEM) based optical forward model that employs XCT information to allocate organ-specific optical properties with a self-regularized inversion scheme also based on XCT priors. For the background correction, the background fluorescence was subtracted from the data and then used as an input for the reconstruction as in Ref. [13].

Animal studies

Ex vivo animal studies were performed to evaluate the performance of the method using controlled tissue measurements. For this purpose an euthanized nude mouse was employed and a 1 mm diameter translucent tube was inserted in the esophagus until it reached the upper stomach. The translucent tube was filled with three mixtures, containing intralipid, India ink,

water and 670/710 nm excitation/emission fluorochrome at three concentrations 1) control (0), 2) 1.4 mM and 3) at 11 uM.

In vivo animal studies were also performed to investigate the performance of the method in living tissues. We were particularly interested in studying small tumors, early in their development and interrogated whether detection improvements could be afforded using fluorescence subtraction. For this reason we employed a K-ras mouse model bearing mutated human K-ras[G12D] allele, which develops lung cancer with high incidence upon spontaneous recombination events [26]. We created double transgenic K-ras+/- and BL6 Tyr-/- mice, derived from mouse lines purchased from The Jackson Laboratory, Maine, USA and crossed in our laboratory. This double transgenic mouse line is white and develops lung tumors similar to a parent KRas+/- line. 2 nmol of a probe targeting αvβ3 (IntegriSense 680, excitation: 680nm, emission: 710nm, Perkin Elmer) was injected 24 h prior to imaging. We imaged 4 week old mice. All animal experiments were approved by the government of Bavaria and conform rules and regulations of Helmholtz Zentrum München.

Validation methods

Animals imaged *in vivo* by FMT-XCT were euthanized by an intra-peritoneal Ketamin and Xylazine injection, were embedded in Optimal Cutting Temperature (O.C.T.) compound mixed with black India ink and frozen to -80° C. For validation of the *in vivo* imaging results, the mice were placed in a cryotome (CM 1950, Leica Microsystems GmbH, Wetzlar, Germany) and imaged using multi-spectral-cryo-slicing imaging using a white light source and a sensitive CCD camera (PCO AG, Donaupark, Kelheim, Germany) as described in Ref. [27]. Fluorescence images at the emission wavelength of the probes and white light color images were collected at several cross-sectional positions in the thorax area and employed for comparison with the FMT-XCT images.

3 Results

Background fluorescence calculation and subtraction

Fig. 1 illustrates the steps involved in the calculation of the background fluorescence subtraction.

Fig.1a shows the first step in the calculation of the 3D surface for each projection angle, as described above in methods, at the side of the source S_{source} and the side of the CCD camera S_{CCD}. The calculated distances between the source position $\vec{r}_s$ projected to the source surface S_{source} and all points in the interpolated surface at the side of the CCD camera S_{CCD} constitute the estimate of the normalized background fluorescence IM_{BG} (Fig. 1b). A distribution (Fig. 1c) of the calculated background fluorescence image pixels in IM_{BG} divided by the pixels of the acquisition image IM_{acq} (Fig. 1e) is calculated as described in methods, showing a peak. We take the peak to correspond to the proportionality factor a, because we assume there is a constant level of background fluorescence throughout the medium, and the peak in the histogram corresponds to the most frequently observed value of $\hat{a}(\vec{r}_d, \vec{r}_s)$ for which $\left[U^{nB}\left(\vec{r}_d, \vec{r}_s\right) \right]_{measured} = \hat{a}(\vec{r}_d, \vec{r}_s) * \left\| \vec{r}_d - \vec{r}_s \right\|$. We assume that values higher and lower than this value are due to the fluorescence targets of interest that are also present inside the mouse, or because of inhomogeneities in the optical properties of the tissue.

The second row displays a white light image of the mouse for one projection angle (Fig. 1d), an original normalized acquisition image for one source IM_{acq} (Fig. 1e) and the corresponding image after fluorescence background subtraction $IM_{corrected}$ (Fig. 1f). It can be understood from the expression derived for the background fluorescence level, which includes the distance from source to detector surface, that areas where the mouse diameter is larger will result in higher levels of background fluorescence in the acquisition image. The image of the calculated background fluorescence level (Fig. 1b) reflects this, larger values are calculated for the abdomen, and smaller values are calculated for the sides of the mouse. The same is true for the normalized acquitision image (Fig. 1e). In the background corrected image (Fig. 1f) the signal from the abdomen is reduced and the liver shape is more clearly visible. In the lung area the level of normalized signal is reduced in general in the corrected image, and small irregularities due to fluorescent targets inside the mouse come to the surface that were not distinguishable in the image before correction.

Effect on quantification
We evaluated the effect of background fluorescence subtraction on the quantitative properties of FMT-XCT using an *ex vivo* study of a mouse with a tube inserted in the esophagus (Fig. 2). In this case the background fluorescence arises due to auto-fluorescence of the tissue only. The tube was filled with a mixture of intralipid, India ink, water and 1) high concentration

fluorochrome protein (Fig. 2a-d), 2) low concentration fluorochrome (Fig. 2e-h) and 3) no fluorochrome (control) (Fig. 2i-l). Because the same mouse was used for all experiments and the mouse was not moved during tube insertions, the background fluorescence images (Fig. 2m) only needed to be calculated once. The background corrected acquisition images for the two measurements involving a tube filled with a fluorescent mixture (Fig. 2 b,f) show a clear indication of the tube, whereas the background corrected acquisition image of the control measurement is almost clear of fluorescence signal (Fig. 2j). The background subtraction has a positive effect on the reconstructed images (Fig. 2c, g, k, d, h, l); before background subtraction, visible artifacts appear in the same place for each of the three cases, which is expected as exactly the same mouse is imaged in exactly the same position, while after background subtraction, the quantification of the values reconstructed for the tubes is not affected (Fig. 2 o), but the amount of artifacts due to the background fluorescence is reduced (arrows in Fig. 2c, g, k, d, h, l). The color bar displays signals of low intensity as dark red, and signals of high intensity as bright yellow; additionally we use a linear transparency map that displays signals of low intensity more transparent than signals of high intensity. This way of visualizing the data clearly shows that the number of high intensity artifacts, which are not affected by the transparency map, and could be misinterpreted as target signal, is reduced by using the background subtraction scheme. We quantified the signal in the tube by taking the maximum value. The quantification of the signal in the tube remains the same before and after applying the background subtraction method, demonstrating that the correction method results in reduction of undesired background fluorescence only, and that signal from fluorescent targets present in the mouse is not subtracted.

In vivo study of K-ras lung tumor model

In young 4 week old K-ras mice, tumor targets were around 0.8 mm, which is close to the maximum resolution that is expected to be achievable with FMT. Not only are the targets in young K-ras mice relatively small, the targets are not easily distinguished from the background fluorescence level either. Fig. 3a-b show cryoslicing results for a 4 week old K-ras mouse. The slices are taken from the thorax area of the mouse, displaying the heart, lungs and two tumors are indicated with arrows. Figure 3a shows a white light image, while figure 3b shows the fluorescence signal in orange overlayed on the white light image reduced to black and white. Background fluorescence is most clearly visible in the tissue surrounding the lung area. Transversal and sagittal slices of the corresponding FMT-XCT reconstructions with and without the background correction applied to the data are displayed in Fig. 3c-f. There is

a clear reduction in artifacts between the reconstructions based on uncorrected and corrected data for the 4 week old K-ras mouse. The uncorrected reconstruction displays several clear additional fluorescent signals not corresponding to the two small tumors in the lung area, demonstrating the typical effect of background fluorescence not being reconstructed as a homogenous background fluorescence level but as localized artifacts [18]. The resulting corrected reconstruction corresponds well with the validation image (Fig. 3b); it shows two small tumors in the lung in the transversal slice. The coronal slice additionally displays a signal corresponding to the liver area of the mouse. Before applying the background fluorescence correction, it is difficult to distinguish the two small tumors among the reconstructed artifacts, while after applying the background fluorescence correction the two small tumors can be clearly distinguished from signals of different origin. Also the correspondence of the liver signal with the shape of the liver as seen in the XCT image is improved.

4. Discussion

In this work we studied the recovery of small objects with hybrid FMT-XCT in the presence of background fluorescence. We exploited the hybrid capabilities of the FMT-XCT system by using the XCT volume for the data-driven calculation of images of the estimated background fluorescence, which we used for pre-processing of the FMT data before reconstruction of the fluorescence bio-distribution.

For the estimation of the background fluorescence we employed a linear relationship between the 3D distance from source to detector and the level of background fluorescence, which we assumed to be constant throughout the mouse body. We used the calculated constant together with the XCT volume to estimate normalized images IM_{BG} that would be acquired in case a homogeneous background fluorescence level would be present, which can be subtracted directly from the Born normalized acquisition images IM_{acq} (emission/excitation image) Furthermore, we defined a data-driven estimation method for the calculation of the proportionality constant $a,$ based on the ratio between calculated background fluorescence image and acquired normalized acquisition images for all pixels in a region of interest in the images defined by the area covered by the source positions scanned for the acquisition angle. By performing the background correction directly on the acquisition images, the resolution of the correction is much higher than when it would be done on the source-detector points used

in the weight matrix calculation, which is often a reduced representation due to memory restrictions.

Application of the implemented data-driven correction for background fluorescence resulted in improved estimates of the fluorescence bio- distribution *ex vivo* and *in vivo*, characterized by reduction in artifacts attributed to the presence of background fluorescence.

Results of the *ex vivo* mouse study showed that the background correction decreases the amount of artifacts in the reconstruction, while importantly it does not compromise quantification ability; the reconstructed value for the tube stayed the same before and after background correction. We showed that even in mice without an injected fluorescent probe, a low level of background fluorescence can be present and results in undesired artifacts in the reconstruction. Before background correction, these artifacts could be incorrectly interpreted as signal.

In general more varieties of targeted probes (probes that are fluorescent upon injection) exist than varieties of activatable probes (probes that become fluorescent when reaching the site of disease), consequently targeted probes are being used for most applications. The use of targeted probes inherently results in a distribution of a low level of background fluorescence throughout the mouse. The constant level of background fluorescence can result in artifacts which can be misinterpreted as signal.

The ability to distinguish small targets from artifacts arising due to background fluorescence is particularly important when imaging early stage disease progression when targets are smaller and less bright than in later stages. The *in vivo* study of early stage lung cancer displayed that it is possible to reconstruct small targets expressing a low signal compared to the background fluorescence level more accurately after using the correction method. When quantifying the signal strength of fluorescent targets for comparison of disease development in different mice, often relative measures are used that relate the signal in the area of interest to a reference area inside the same mouse, for example the muscle tissue. Calculation of such a relative measure would be complicated in case the surrounding tissue contains significant artifacts, such as the artifacts visible in the reconstruction of the *in vivo* study before correction. Reduction of artifacts due to background fluorescence is therefore not only important for interpretation of the signal in the area of interest, but also for quantification of the targets relative to other areas of the mouse.

The studies described in this paper show that the algorithm can be used for correcting for a background fluorescence level arising mainly from tissue autofluorescence, which was the

case in the *ex vivo* study, as well as when the majority of background fluorescence is due to a targeted probe used, as was the case in the *in vivo* study. The assumption is that the background fluorescence level is homogeneous, whether it is autofluorescence from the tissue or fluorescence from the probe used will not influence the applicability of the algorithm.

The method and work presented here can be expanded in several directions. In the calculation of the background fluorescence correction, we assumed a homogeneous object with homogeneous optical properties and homogeneous background fluorescence, which allowed us to use a simple calculation for the estimation of the background fluorescence. However, the background fluorescence level can be heterogeneous as well. When imaging the abdomen for example, large amounts of background fluorescence can be expected from e.g. the liver or the lower intestines, depending on the clearance properties of the probe used, and the diet of the mouse. Using the implementation described above, fluorescence originating from distinct structures such as the liver or kidneys will not be removed. This was also demonstrated by the liver signal that is still visible in the reconstruction of the *in vivo* study before and after correction.

A method for subtracting heterogeneous background fluorescence using a second untargeted tracer was recently successfully developed [23]. However, such an approach may not always be feasible, as this requires additional laser and filter sets at the second wavelength, an untargeted tracer with the same characteristics may not be available, furthermore it comes with additional cost and additional burden for the animal. The method described in this paper could be extended for cases in which the background fluorescence is inhomogeneous using for example the anatomical segmentation of the XCT data for the development of a heterogeneous or layered homogeneous model. After segmentation of the XCT data in anatomical regions based on contrast provided by the use of a CT contrast agent, the expected background fluorescence from these organs could be calculated and subtracted from the acquisition data before reconstruction by forward modeling their contribution. This can enable imaging of targets that would otherwise be hidden by potentially high background fluorescence levels.

In conclusion, the pre-processing of hybrid FMT-XCT data using the described background fluorescence subtraction method increases the imaging capabilities of hybrid FMT-XCT, leading to a wider range of possibilities for future directions of study.

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Figure 1. Steps in the calculation of the background fluorescence corrected data set. (a) Example of calculated surfaces at the side of source S_{source} and camera S_{CCD} for one gantry angle (205°), units in cm corresponding to distance from the indicated plane. (b) Calculated background fluorescence image, IM_{BG} for one source based on the 3D distances from source point on source surface to camera surface. (c) Histogram of background fluorescence / acquisition image per pixel in a region of interest on the mouse surface, estimated value of a is indicated by the dotted line. (d) white light image of the mouse (ventral side). (e) normalized acquisition data, IM_{acq} (f) background corrected data, $IM_{corrected}$.

Figure 2. Effect on quantification. Background correction applied to mouse with tube in esophagus, filled with a mixture containing (a-d) fluorochrome in high concentration, conc. 2 (e-h) fluorochrome in low concentration, conc. 1 (i-l) no fluorochrome. (a,e,i) normalized acquisition image for one representative source and projection angle (IM_{acq}). (b,f,j) background corrected normalized acquisition data (c-k) coronal slice of 3D FMT-XCT reconstruction intersecting the tube using uncorrected acquisition data (d-l) reconstruction using corrected acquisition data (m) calculated background fluorescence image (IM_{BG}) (n) white light image (p) graph of max. reconstructed values for control and low concentration fluorescence, normalized to high concentration fluorescence. (arrows indicate artifacts).

Figure 3. Hybrid FMT-XCT reconstruction and validation results for 4 week old K-ras. (a) RGB cryoslice image. (b) Validation cryoslice showing planar fluorescence in orange. (c,e) Normalized transversal slices and (d,f) sagittal slices of FMT-XCT reconstruction based on (c,d) uncorrected data and (e,f) corrected data. (Arrows indicate tumors, Sp=Spine, Lu=Lung, He=Heart, Ab=Abdomen, colormap displayed in (c) applies to all images, transparency map indicated by checkered pattern)