Imaging workflow and calibration for CT-guided time-domain fluorescence tomography

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Abstract: In this study, several key optimization steps are outlined for a non-contact, time-correlated single photon counting small animal optical tomography system, using simultaneous collection of both fluorescence and transmittance data. The system is presented for time-domain image reconstruction in vivo, illustrating the sensitivity from single photon counting and the calibration steps needed to accurately process the data. In particular, laser time- and amplitude-referencing, detector and filter calibrations, and collection of a suitable instrument response function are all presented in the context of time-domain fluorescence tomography and a fully automated workflow is described. Preliminary phantom time-domain reconstructed images demonstrate the fidelity of the workflow for fluorescence tomography based on signal from multiple time gates.

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OCIS codes: (170.0110) Imaging systems; (170.3010) Image reconstruction techniques; (170.3660) Light propagation in tissues

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1. Introduction

Small animal fluorescence molecular imaging provides a low-cost, ionizing radiation-free alternative to more conventional nuclear medicine imaging modalities, enabling high-throughput preclinical studies for drug discovery and development [1]. However, substantial absorption and scattering of visible and near-visible light by biological tissue limits the size of specimens that can be imaged [2]. The optimization of FMI for whole-body three-dimensional imaging in small animals is contingent upon developments in three distinct categories: fluorescent probe design, light propagation modeling through tissue, and imaging system design [3]. Developments in fluorescent probe design have resulted in fluorophores that absorb and emit light in the near-infrared range of the electromagnetic spectrum (600-1000 nm), a range where biological tissue absorption is at a minimum, allowing maximal light penetration through tissue [4]. Numerous imaging systems and light propagation models have been investigated to capitalize on the deeper penetrating light of these new fluorophores to accomplish whole body 3D imaging in small animals [5]. Perhaps the two most promising recent advances in fluorescence molecular tomography have been the development of hybrid FMI-anatomical imaging systems [6–15], which can provide anatomical priors to enhance image reconstructions, and systems that employ time-domain data acquisition [16–29], which various researchers have used to either improve the accuracy of image reconstructions or map fluorescence lifetime [16,20,21,25,27,30], which in turn can be used to interrogate the tissue microenvironment.

To probe the limits of fluorescence molecular imaging, these advances have been combined in a state-of-the-art fluorescence tomography (FT) system, employing the ultimate sensitivity of single photon counting and information-rich time-domain light detection in an easy-to-use non-contact conformation [31]. Highlights of this system include the simultaneous collection of transmitted excitation and emission light, automatic exposure control, laser referencing, and co-registration with a small animal computed tomography (microCT) system. Simultaneous fluorescence and transmittance collection provides a means of carrying out Born normalization (fluorescence/transmittance), a method of simplifying fluorescence imaging quantification while minimizing for errors from inaccuracies in light propagation modeling [32], instantaneously, avoiding potential errors associated with subject motion or changes in physiology or fluorescence distribution over time. The automatic exposure control, which is a software optimization, ensures that the highest possible signal is collected for each source-detector projection without damaging the detectors and data with optimal signal-to-noise characteristics can be collected at every projection [33]. The laser referencing utilized by the system provides a means of adjusting collected data to drift and fluctuations in the laser and co-registration with a microCT provides anatomical information that can be used to assist optical image reconstructions [34].

Though the workflow of this system has been presented previously for continuous-wave type imaging [31], translation of this workflow to full time-domain fluorescence imaging, to realize the full potential of this state-of-the-art device, is non-trivial and warrants considerable discussion. In this study, system calibration, laser referencing to pulse drift, jitter, and dispersion, instrument response function measurement, Born normalization, avoiding data corruption by interference from a specialized imaging bed, and co-registering the coordinate systems of the FT and microCT systems are all presented in the context of time-correlated
single photon counting (TCSPC)-detected data. Furthermore, an inversion approach is presented for utilization of the full time-domain fluorescence data set and the effectiveness of the entire workflow is validated with tissue-like phantom imaging using signals associated with weakly diffused photons.

2. System design

An in depth description of the FT system investigated in this study has been presented previously [31]. Briefly, the system is built around a picosecond-pulsed 80-MHz multimode laser diode (PicoQuant Photonics North America Inc. Westfield, MA) and 11 photomultiplier tubes (H7422P-50 PMTs; Hamamatsu Photonics K. K., Hamamatsu City, Japan), each of which are attached to time-correlated single photon counting instrumentation (Becker & Hickl GmbH, Berlin, Germany). Currently the system is set up to switch between 2 lasers, a 635 nm laser and a 755 nm laser. Excitation light from the chosen laser is coupled into a 50-μm multimode optical fiber, which is then passed through an in-line automated variable attenuator (OZ Optics, Ottawa, ON), for accurate control of the source power. The light is then split into 2 channels with a 96%/4% beamsplitter (OZ Optics, Ottawa, Canada), with the 4% of light directed to a reference PMT, and the other 96% of the light being carried to the outer radius of a 20 cm-diameter imaging gantry. The excitation light is then focused in free space through a lens with a focal distance of 97 mm toward the center of the gantry. Five detection channels oppose the source on the gantry with an angular separation of 22.5° (evenly spaced over 90°), each of which couples transmitted light into a 400-μm optical fiber using a lens with a working distance of 97 mm. In each detection channel, the collected light is then separated into a further 2 channels using 96/4 beamsplitters. The light in all channels is then collimated and passed through a choice of filters arranged on a motorized slider before being illuminated onto one of the ten remaining PMTs. In fluorescence imaging mode, the 96% fiber of each detection channel is passed through a long-pass filter (650 nm LP for the 635 nm laser; 780 LP for the 755 nm laser manufactured by Chroma Technology Corp, Bellows Falls, VT), while the 4% fiber of each detection channel is passed through a neutral density filter. The

![Fig. 1. Frame from a video (Media 1) of the system that portrays the motorized linear stage and motorized rotational imaging gantry in action. The five detection fibers (capped in green) can be seen at the top of the screen at the beginning of the video with the single source fiber at the bottom (capped in red). The specimen attached to the phantom bed is a mouse-shaped phantom from Caliper Life Sciences Inc.](image-url)
TCSPC instrumentation coupled to each PMT is used to measure the dispersion and attenuation of the laser pulse through the imaging medium: referred to hence as the temporal pulse spread function (TPSF). The excitation and detection fibers in the system are arranged such that the fully automated imaging gantry can be rotated over 360° (with a minimum resolution of 1°) to obtain TPSFs from as many projections about an object as desired.

The imaging bed of the FT system is attached to a translational stage (Fig. 1) allowing multiple slices of an object to be imaged. Furthermore, it was designed to be compatible with a co-registered microCT unit (xPlore Locus, GE Healthcare, London, ON) to provide anatomical priors to assist fluorescence image reconstructions. Figure 2 illustrates the schematic of the bed that was design to be adaptable to both mouse and rat imaging. The bed cantilevers off of a jack that provides flexibility in the height of bed chosen. This is important because the focal points of excitation and detection in the FT system are very narrow so the subject to be imaged has to be placed in the middle of the imaging gantry with some precision. At the imaging side, the bed is equipped with a small animal anesthesia mask with a bite-bar for stabilizing the head of rodents and delivering anesthetic gas during imaging. To support rodents, the bed was manufactured with 23 circular holes on its face, into which 5/64”
diameter fiberglass rods can be inserted in various configurations to accommodate different sizes and weights of rodents (Fig. 2b). This support infrastructure was chosen to minimize the number of source-detector projections that would be attenuated by the bed during imaging. Fiberglass was chosen as a supporting rod material because it has significantly higher x-ray attenuation than soft tissue and therefore can be easily located and segmented out of microCT images. The method for removal of data based on potential bed attenuation of specific source-detector projections is discussed further in Section 3.

3. Imaging workflow

Figure 3 depicts an overview of the basic workflow carried out to create time-domain fluorescence image reconstructions for rodents using the FT and microCT systems. Much of the workflow is similar to the previous CW FT system, but the salient features are described briefly here. Prior to each scan, the subject (generally either a mouse or a rat) is anesthetized and placed onto the FT/CT bed and positioned onto the bite-bar in the bed’s gas anesthesia hook-up (Fig. 2). Motion of the subject is further restricted with tape to avoid disrupting the subject position when transiting the bed between systems. The bed is then placed into the FT system and the height of the bed is adjusted, as well as the lateral position of the subject (by

Fig. 3. A simplified workflow depicting the full experimental procedure for carrying out time-domain fluorescence tomography in small animals.
moving the positions of the support rods), to center the subject in the FT imaging gantry. This is achieved by rotating the imaging gantry around the subject with the laser on, ensuring that the excitation point of the laser is always roughly normal to the circumference of the subject. Once the position of the subject on the bed has been optimized, the bed is removed from the FT system and placed in the microCT to collect a 3D anatomical image of the subject. At this stage, the CT image is used to select coronal slices of interest that can be used to guide the linear stage position in the FT system, achieved through a co-registration of the coordinate systems of the microCT and FT (the specifics of how the co-registration was carried out are discussed in Section 3.3). The bed is then moved back into the FT system and time-domain fluorescence and excitation light is collected for a customizable number of source-detector projections about each slice of interest as determined from the microCT image.

Once collected, TPSFs (fluorescence, transmitted excitation, and reference) from each source-detector projection or time-point are background-subtracted to remove any non-time-correlated signal in the detection. Then, two calibration procedures are applied to the time-domain optical data. First, the data is corrected for differences in detector sensitivity, time delay, and pulse dispersion, as well as any filtering or differences in filter performance in each channel (Section 3.1). Second, the data is corrected for temporal changes in the laser pulse during data collection, such as drift and jitter in the intensity, timing, or dispersion. These attributes are tracked by the reference PMT (Section 3.2). The final data calibration step is to create a Born normalized data set by taking a ratio of the fluorescence to the transmittance for each source-detector projection to mitigate any inconsistencies between the data and the model used for the image reconstruction [35]. The Born ratio data is then multiplied by a forward model of transmittance to create a normalized fluorescence data set that is inherently calibrated to the arbitrary source strength of the model [32].

After FT data calibration is completed, the anatomical microCT image stack is re-incorporated into the workflow in two ways. One, it is used to localize the position of the fiberglass bed supports within the coordinate system of the FT unit in order to remove FT data from any source-detector projection that could have been attenuated by the bed (Section 3.3). Second, it is used to create a finite-element mesh of the subject, which is translated into the FT system coordinates and incorporated into a fluorescence image reconstruction algorithm as a priori anatomical information to localize the position of each source and detector relative to the subject geometry and to constrain the inverse problem (Section 3.6).

Many different image reconstruction approaches can be employed for time-domain fluorescence data, two of which are investigated in this study; however, no matter the approach, if the full benefits of the time-domain data are to be leveraged, a final pre-reconstruction step that is required is the integration of the instrument response function into the forward model used in the reconstruction algorithm. The time required for this full protocol is highly variable depending on the size of the subject, the area to be imaged, etc. A typical mouse brain scan may require about 30 minutes from start to finish: about 5 minutes for the CT scan, 15 minutes for the fluorescence/transmittance scan, with a final 10 minutes of data processing and image reconstruction.

The following subsections provide in-depth descriptions of the salient workflow procedures discussed above preceding an experimental demonstration of the utility of the workflow in phantoms in Sections 4 and 5.

3.1. System calibration and instrument response function measurement

Full calibration of a time-domain optical imaging system requires accounting for detector channel differences with respect to not only signal intensity and filter efficiency, as with continuous-wave systems, but also with respect to pulse delay and temporal dispersion of signal. The intrinsic sensitivity, pulse dispersion, and time delay properties of each detector were determined with a single calibration experiment that can be easily repeated before system use.
The calibration experiment is depicted in Fig. 4a and entails placing a line diffuser (Thorlabs, Newton, NJ) in the center of the imaging gantry to send equal portions of light to all detection channels. The diffuser is engineered to diffuse a collimated laser beam with angular uniformity into a fan beam spanning 100° with a width of 4°. With the detector channels spanning 90° of the imaging gantry, the excitation beam is diffused evenly into each detection channel, with negligible pulse dispersion or delay. Then, the long-pass filters in all “fluorescence” detection channels are replaced with neutral density filters. This way both the “fluorescence” channels and the “transmittance” channels are set to detect transmitted excitation light, and detector-by-detector sensitivity differences can be accounted for (see below) because every detector should have been exposed to an identical signal in terms of intensity, pulse delay, and pulse dispersion. Therefore, any observed differences can be corrected for by applying calibration factors for intensity, pulse delay, and pulse dispersion to equalize all detectors to an arbitrary detector (for the purposes of this study, the detector that measured the highest fluence was used as the reference point)—see Fig. 4b and Fig. 4c.

Fig. 4. System calibration and instrument response function collection. (a) A cartoon layout of the fluorescence tomography imaging system depicting the arrangement used to calibrate the system and measure the instrument response functions. Specifically, a line diffuser is placed at the center of the imaging gantry to disperse the excitation beam evenly into each detection channel. (b) Raw temporal pulse spread functions collected at each detector. Each color denotes a different detection PMT. (c) The same curves as shown in (b) after applying amplitude and time shift calibrations to the detection channels.
Not only can the line diffusor experiment be used to calibrate inherent intensity and time-delay differences between the different detectors, it can also be used as the instrument response function (IRFs) for each detector. By calibrating all detectors, the TPSFs from line diffusor experiment will be roughly equivalent and can be used to model laser pulse dispersion by convolving with forward model time-domain Jacobians for image reconstruction purposes. A depiction of the IRFs for all detectors is presented in Fig. 4c (the average FWHM of the IRF is 650 ps). One caveat to this is that these IRFs are calculated for signal that has traversed the full diameter of the imaging gantry. When a specimen is placed in the middle of the gantry for imaging, the mean free path of the light outside of the specimen will be shorter than in the IRF experiment by a factor roughly equivalent to the diameter of the specimen. Therefore the calculated IRFs are shifted earlier in time by a factor of \((d_1 + d_2)/c\), where \(d_1\) is the distance between the focal point of the source on the surface of the specimen and the center of the gantry, \(d_2\) is the distance from the middle of the gantry to the projection point of a given detector on the surface of the specimen, and \(c\) is the speed of light in air (the typical time-shift for a mouse is approximately 100 ps). Furthermore, it should be noted that this IRF calculation is specific to the laser and PMT parameters used, so this must be kept the same between calibration and experiment. This calibration is much less time-consuming and involved than a previously described TCSPC approach [36]. The total time required for this simplified calibration and IRF calculation is about 2 min, including setup, making it possible to run the calibration before and after every experiment for improved system characterization of the system on an experiment-to-experiment basis.

Even though this first calibration experiment provides all necessary time-domain calibrations in theory, it relies on the assumption that the engineered diffuser is aligned properly and provides an angularly uniform dispersion of the source. If Born normalization is used for image reconstruction, a more robust approach of calibrating intensity can be employed. Essentially, any type of a diffusing medium, homogeneous or not, can be placed into the center of the FT imaging gantry and both fluorescence and transmittance detection channels can be set to detect excitation light (Fig. 4a). In this arrangement, each pair of detectors in each detection channel is expected to measure transmitted signal that is identical regardless of the shape or inhomogeneity of the diffuser used, since each pair receives light from the same collection optics. By this mean, calibration factors for each fluorescence-to-transmittance ratio measurement in each detection channel can be determined.

In general, if the same detection filter-sets are used to calibrate the system as to collect data, then it is not necessary to account for discrepancies in individual filters because they would be accounted for in the system calibration. However, since the presented calibration experiment requires both fluorescence and transmittance detection channels to detect excitation light, and different long-pass filter sets have to be used for detecting fluorescence using the two laser wavelength options, the inter-filter differences have to be taken into account for both calibration experiments and for data acquisition so that the calibration of signal is independent of filter differences. For the experiments presented below, the inter-filter differences were determined by placing a phantom fluorescent at both laser wavelengths (635 nm and 755 nm) was positioned at the center of the gantry and photon fluence was measured in the central detector while all filters were sequentially placed in the light path to determine inter-filter differences in efficiency.

Another key aspect to collecting robust time-domain data is being able to account for system drift over time. The laser referencing used to account for this is discussed in the following Section.

3.2. Laser reference

By monitoring the laser pulse in the FT system with a designated reference PMT, it is possible to correct for drift and jitter — in terms of intensity, pulse delay, and pulse dispersion — throughout data acquisition to improve the fidelity of the results. A major advantage to this is
that the user does not have to wait for the system to warm up to a quasi-stable level prior to imaging, being able to automatically correct for variability in the system in real time. Another advantage of this setup is that by incorporating the in-line motorized attenuator (used to adjust laser power during scanning to maximize signal-to-noise characteristics without going over detection limits [33]) upstream of the reference PMT, the laser reference signal can be used to calibrate tomography data without having to assume that the attenuation predicted by the motorized attenuator is accurate.

To demonstrate the utility of the laser reference, excitation TPSFs were collected through a line diffusor (see Section 3.1) as well as with the reference detector at 1s intervals for up to 20 minutes immediately after turning the FT system on in 2 separate experiments. In the first experiment, the laser attenuator was adjusted sequentially through 15, 10, 5, and 0 dBs at intervals of approximately 100 s to mimic an automatic exposure control experiment of an irregularly shaped specimen (Fig. 5a displays the intensity of one PMT detector channel and the reference detector over time). In the second experiment, TPSFs were collected in the same manner, but without adjusting the laser attenuator, and then one of the detectors was shut down and re-started at 600 s (Fig. 5c displays the mean-time of this detector and the reference detector over time). The results of the first experiment demonstrated that normalizing TPSFs by the signal intensity of the reference detector is a viable option for correcting for intensity

![Fig. 5. Laser referencing is illustrated here, where changes in the detected signal intensity (a) & (b) and in mean time (c) & (d) for one detection channel (blue curves) and for the laser reference channel (red curves). In (b) the normalized intensity relative to the signal in (a) to either the assumed change in laser attenuation (blue curve) or to the intensity in the laser ref. channel (red curve). (d) The mean time in the detection channel (c, blue-line) after correction by using the drift observed in the laser reference (c red-line).]
drift and changes in the laser attenuator during an experiment; whereas, simply adjusting the
signal by the assumed change in the laser attenuation turned out to be unreliable (Fig. 5b). The
results of the second experiment demonstrated that there could be considerable drift in the
mean time of the laser pulse during warm-up: upwards of 200 ps within the first 10 min (Fig.
5c). However, the magnitude of the shift was equivalent in all imaging detectors and in the
reference detector, suggesting one, that the mean time measured by the reference detector can
be used to correct for mean time drift in the imaging detectors (Fig. 5d), and two, that, at least
in this system, the major source of system drift during warm-up results from drift in the laser.
Further corroborating the second point: no appreciable change in detected intensity or mean
time was observed when one of the imaging detectors was shut off and turned back on at the
600 s (Fig. 5c). After correction, the mean time drift rate in the system was $-3.5 \pm 20.1$ ps/h.
These findings suggest that the reference PMT can be used for real-time data calibration in the
same manner as the combined TPSF/IRF approach presented by Ntziachristos et al. [37] but
without having to share the total photon count of the TPSF with the IRF in each detector, thus
providing a larger dynamical range.

3.3. Co-registration between microCT and FT system coordinates

Specifics of the spatial coordinate registration between the microCT and the FT instruments
have been described in detail previously [38]. More concisely, a rigid-body translation is used
to transform the Cartesian coordinate system of the microCT images to a polar coordinate
system of the FT system to co-register the two. The choice of coordinate system is arbitrary;
however, the cylindrical symmetry of the FT system makes generation and source-
detector placement easier in polar coordinates. The translation is repeatable and does not
require fiducial markers, assuming that the local coordinate system of each instrument does
not alter over time.

The translation is performed in two steps. The first is a two-dimensional translation in the
plane of the FT imaging gantry and the second is a one-dimensional linear translation in the
out-of-plane or stage axis. A simplified approach of co-registration was employed in this
study. A 2-mm diameter fiberglass rod, fixed to the imaging bed, was positioned into the
center of the imaging gantry. The positioning was verified by focusing the laser onto the rod
and rotating the gantry 360° to ensure that the focal point of the excitation beam was focused
onto the center of the rod at every projection angle. Once the position of the rod was verified,
the bed was transferred to the microCT system and imaged. Then the center of mass of the rod
in a coronal slice of the CT image was used to demarcate the location of the center of imaging
gantry in the FT system within the imaging plane. Furthermore the z-axis (stage axis) location
of the tip of the rod was noted in both imaging system and used to co-register the out-of-plane
dimension between the two systems.

With the microCT and FT system coordinates co-registered, it is possible to use the
anatomical information provided by the microCT to assist FT image reconstruction. In this
study, the mesh creation package provided by NIRFAST was employed [39]. The microCT
image was used to determine the outer surface of the imaged specimen and to localize the
position of the bed supports to remove FT source-projection data points that had the potential
to be interrupted by the supports. FT images were then reconstructed assuming homogeneous
optical properties within the specimen using Born normalization to account for data-model
mismatches [40].

The fiberglass rods used for supports are easy to locate in the microCT images because
they have relatively high x-ray attenuation compared to biological tissue, but not too high to
cause imaging artifacts. Likewise, the outline of the specimen can be located in the microCT
images since biological tissue has significantly higher x-ray attenuation than air. Based on
these properties, microCT images can be thresholded to create a mask for the rods and a mask
for the specimen (e.g., top of Fig. 6c). These two masks are then translated into the polar
coordinates of the FT system and the specimen mask is separated into a user-defined number of finite elements to create the FEM mesh [41]. All source and detector positions employed during the FT scan are then projected to the surface of the specimen mesh along a line connecting the location of the detection or source optics on the gantry and the center of the gantry. Any source or detector projection that subtends the rod mask is removed from the data set to avoid data corruption from the bed supports.

Figure 6a presents a microCT image of a mouse phantom (Caliper Life Sciences, Hopkinton, MA)—see Fig. 2c—with 2 cylindrical inclusions that were filled with a 100 nM solution of the AlexaFluor 647 fluorescent dye (Invitrogen, Carlsbad, CA) mixed with 1% Intralipid® in water. After the phantom was imaged in the microCT it was imaged in the time-domain FT system on top of the rods. In order to test out the mesh creation and rod removal algorithm, a fluorescence image was reconstructed using a pulse-integration approach, i.e., by summing the collected time-domain data (TPSFs) at each source-detector position over all

![Image](image_url)

(a) (b) (c) (d)

Fig. 6. The finite-element model mesh was created as illustrated here, accounting for rod disturbance in the optical measurements. (a) A cross-sectional CT image of the optical mouse phantom shown in Fig. 2. The bright circles correspond to the cross-section of the fiberglass rods that hold the phantom in place and the dark circles are cylindrical holes in the phantom made to accommodate the addition of up to two fluorescence inclusions. (b) A mask (top) and finite element mesh with source-detector locations (bottom) of (a) if the rods are not accounted for. The blue lines are the boundaries of each finite element while the red circles represent the location of the sources and detectors projected on the surface of the specimen. (c) The three layered mask (top) and finite element mesh with source-detector locations (bottom) of (a) when the rods and all sources and detectors interfering with the rods are removed. A reconstruction of the mouse phantom with both inclusions filled with 100 nM of AlexaFluor 647 dye is presented (d).
time bins. The fluorescence image reconstruction is presented in Fig. 6d, displaying a relatively accurate representation of the location of the two fluorescent inclusions.

4. Time-domain fluorescence tomography

The use of such signals as time-gates (local data type) or mean photon time of arrival (global data type) requires the TPSFs in a tomography data set to be calibrated. A crucial step in this calibration consists in insuring, as is done here, that the time-domain curves are time-referenced with respect to one another. In this section it is demonstrated that the approaches presented in Section 3 have led to the development of a CT-guided fluorescence tomography system allowing time-domain reconstructions to be achieved using such local data types as TPSF time gates.

4.1. Image reconstruction technique

A finite-element (FEM) time-domain approach, introduced previously [42,43], was applied to reconstruct fluorescence images using two approaches: a pulse-integration approach and a seven-gate time-domain approach. The inputs to the image reconstruction in both cases were the object mesh, created from the microCT image as described in Section 3.3, and a forward model matrix of time-domain data for fluorescence and transmittance collected at each unique source-detector pair. The fluorescence and transmittance data were calibrated and corrected (for possible system drift) based on the methods discussed in Sections 3.1 and 3.2. In order to calibrate the fluorescence data to the arbitrary source strength modeled in the FEM forward model, as well as to correct for discrepancies in source to specimen coupling or optical property anomalies in the specimen, the time-domain fluorescence data was multiplied on a source-detector-by-source-detector basis by the ratio of the experimentally measured transmittance at that source-detector (summed over all time points) and the FEM time-domain simulated transmittance (also summed over all time points) at that source-detector [35]. This model-calibrated fluorescence time-domain data set, which incorporates the benefits of the Born ratio while retaining quantitative reconstruction accuracy [8,31], was then used for iterative image reconstruction based on minimizing the objective function $\chi$ in the expression,

$$\chi = \min_{\mu} \left[ y - F(\mu) \right],$$  \hspace{1cm} (1)

where $y$ is the measured (and calibrated) fluorescence and $F(\mu)$ is the FEM-modeled diffused fluorescence for a mesh with a set of optical parameters $\mu$. The optical properties include the reduced scattering ($\mu'_s$) and the tissue absorption, which is a sum of the contribution from intrinsic absorbers ($\mu_a$) and the fluorescent molecules ($\mu_{af}$). The minimization is made into an iterative process by the update equation,

$$\hat{\delta\mu}_f = J^T \left( JJ^T + \lambda I \right)^{-1} \delta\Phi,$$  \hspace{1cm} (2)

where $\delta\mu_f$ is the update parameter, $\delta\Phi$ is the difference between the modeled and measured data at each iteration, and $\lambda$ is the regularisation parameter. The Jacobian, $J$, is the matrix that relates a small change in the measured fluorescence boundary data, to a small change in $\mu_{af}$. Time-referencing of the signal is achieved through a time-dependent convolution of each line of the Jacobian with the measured IRFs for each specific detector (Section 3.1). Equation (1) and Eq. (2) can be generalized for multiple time-gates reconstruction by the following generalization:
where $t_2-t_1$ up to $t_n-t_{n-1}$ denote the first to the $n^{th}$ time-gates. In the present study, Eq. (1) was solved by either summing up the model and data over all time-gates (pulse-integration) or by using seven 400 ps-wide time gates about the peak of the fluorescence TPSFs.

4.2. Image reconstruction with multiple time-gates

The fidelity of the workflow for acquiring time-domain fluorescence data with the FT system was investigated by comparing multi-time-gate to pulse-integration image reconstructions.

![Image](image_url)

Fig. 7. Reconstructions of experimental data are shown using a pulse-integration reconstruction (a), and a seven-gate time-domain reconstruction (b), overlaid onto a CT image of the phantom. The comparison between the fluorescence cross-sections of both reconstructions (a vertical cross-section through (a) and (b)) is presented in (c). The green line corresponds to the pulse-integrated reconstruction, the blue line to the multi-time-gate reconstruction, and the black line to the expected fluorescence profile. Zero on the x-axis denotes the center of mass of the phantom. The fluorescence is normalized to the peak fluorescence in each cross-section.
applied to experimental data collected from a cylindrical epoxy phantom of length 10 cm and diameter 3 cm. The homogeneous optical properties of the phantom were characterized at the excitation and emission wavelengths (absorption and reduced scatter at excitation was 0.18 and 10.7 cm^{-1}, respectively, and 0.16 and 10.1 cm^{-1} at emission), and used as prior knowledge to compute the Jacobians. The phantom was designed with two 3 mm-diameter cylindrical holes running 7 cm of the length of the phantom, 10.5 mm radial to the phantom’s center. These holes were filled with a 100 nM solution of IRdye-800CW (LI-COR Biosciences, Lincoln, NE) mixed with 1% Intralipid in water. The phantom was imaged in the FT system at an excitation wavelength of 755 nm with fluorescence emission collected at wavelengths longer than 780 nm. Fluorescence and transmittance time-domain data were collected at 80 source-detector positions axially about the phantom (a 10 min scan), applying all approaches described in Section 3. Specifically, laser referencing (Section 3.2) was used to correct any drift or jitter in the system as a function of time during data acquisition and IRFs were collected and the system was calibrated as described in Section 3.1. Figure 7a and 7b display the fluorescence image reconstructions of this experimental data for both described methods, the pulse-integration and the multi-time-gate, respectively. Both approaches were able to locate the fluorescence inclusions; however, the quality of localization and the quantitative accuracy of the multiple time-gate approach were considerably improved compared to the pulse-integration approach (Fig. 7c). The total reconstructed signal within the inclusion for the pulse-integration and time-gate reconstructions were 19 and 29%, respectively, and the average location error of the two reconstructed centroids of the reconstructed fluorescence were 2.16 and 1.17 mm. This supports the contention that the time-domain data collection workflow is highly robust, since the signal in some of the individual time-gates represents less than 1% of the total collected signal, and is therefore expected to be highly sensitive to any inaccuracies in data calibration, specifically time-referencing. Furthermore, a theoretical analysis suggested that the quality of the multi-time-gate reconstruction would be significantly compromised if the time-referencing was off by more than 50 ps in just one of the detection channels (results not shown).

5. Discussion and conclusions

Time-domain optical imaging is a rapidly growing field of research for small animal fluorescence tomography applications, offering a rich data set with enticing implications for the improvement of fluorescence image reconstructions and the mapping of fluorescence lifetime. A number of groups have investigated the theoretical side and the hardware side [16–29] of time-domain fluorescence tomography. In this paper, all aspects involved with acquiring a robust time-domain data set that can be used to reconstruct images for any, or many, time-gate(s) is presented in the context of a state-of-the-art time-correlated single-photon counting, non-contact fluorescence tomography system that provides simultaneous acquisition of transmitted excitation and emission light and employs a laser reference channel. This system has been introduced previously [31], where it was characterized for continuous-wave-type data acquisition and image reconstruction. However, a number of key obstacles had to be overcome before the system could be reliably used to produce robust time-domain fluorescence and transmittance data that could be used with more sophisticated image reconstruction algorithms. These obstacles, and their solutions, are outlined in the present paper and include a 2-minute calibration protocol to characterize pulse dispersion, delay, and amplitude, as well as the IRF, in all detection channels; the employment of laser referencing for laser drift and jitter corrections that can also be applied to all detectors for on-the-fly calibrations; a description of an automated method to avoid data corruption by interference from a specialized imaging bed; and an automated, fiducial-free means of co-registering the coordinate systems of the FT and microCT systems.

To validate the time-domain data collection workflow, an experiment was conducted using a cylindrical phantom with two fluorescence inclusions. The image reconstruction results (Fig.
demonstrate that it is possible to achieve improved localization and quantification by reconstructing on multiple time-gates as opposed to simply integrating the time-domain signal. This type of result is only possible because of the accuracy of the system calibration, laser referencing, and IRF measurement, which is needed to account for time-shifts, data drifts, and pulse dispersion in the data for each detection channel, independently. The results presented here demonstrate that CT-guided fluorescence tomography can be achieved and that this can be done using the full information content provided by TCSPC-based time-domain data acquisition.

Acknowledgments

This work has been funded by NCI grants RO1 CA120368 (KMT, RWH, FEG, QZ, HD, BWP) and K25 CA138578 (FL). The authors wish to thank Niculae Mincu and Guobin Ma at Advanced Research and Technologies Inc. for insightful discussions on time-domain system optimizations during the completion of this work. We would also like to thank our colleagues Scott C. Davis and Michael Jermyn for useful comments on forward model scaling and image reconstruction. Finally, we would like to acknowledge the contribution of Tennile Sunday for photography and videography presented in this work.