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Toward whole-body optical imaging of rats using single-photon counting fluorescence tomography

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Abstract

We used single-photon counting (SPC) detection for diffuse fluorescence tomography to image nanomolar (nM) concentrations of reporter dyes through a rat. Detailed phantom data are presented to show that every centimeter increase in tissue thickness leads to 1 order of magnitude decrease in the minimum fluorophore concentration detectable for a given detector sensitivity. Specifically, here, detection of Alexa Fluor 647 dyes is shown to be achievable for concentrations as low as 1 nM (<200 fM) through more than 5 cm in tissue phantoms, which indicates that this is feasible in larger rodent models. Because it is possible to detect sub-nM fluorescent inclusions with SPC technology in rats, it follows that it is possible to localize subpicomolar fluorophore concentrations in mice, putting the concentration sensitivity limits on the same order as nuclear medicine methods.

Advances in diffuse fluorescence tomography (FT) instrumentation are continually improving the limits of fluorescence sensitivity and subject size that can be imaged [1]. To date, the majority of FT systems have employed CCD cameras for photon detection, owing to the large number of optical projections they provide; however, the development of single-photon counting (SPC) technologies with ultralow noise characteristics has the potential to further improve the lower limit of FT system sensitivity. In this study, the lowest sensitivity of an *in vivo* SPC-based FT system was investigated, and the results suggest that it is possible to image nanomolar (nM) concentrations of fluorescence dyes in rats. This conclusion is supported by the results of three experiments. First, the absolute fluorescence detection sensitivity is evaluated in terms of tissue thickness and dye concentration. Second, the ability of the system to reconstruct low-concentration fluorescence inclusions in a rat-size tissue-simulating phantom is demonstrated. Finally, a rat cranium is imaged as a proof of concept that rat models of disease can be imaged using FT.

The noncontact tomography instrument used for this study is shown schematically in Fig. 1 [2]. During an imaging session, the focused beam of a laser diode illuminates the surface of the animal. Excitation of fluorescent molecules is achieved with one of two lasers with center wavelengths at $\lambda = 635$ or 755 nm. Five photon detection channels are arranged in a fan-beam transmission geometry, allowing focused time-correlated single-photon counting (TCSPC) detection using photomultiplier tubes (PMTs). The total angular dispersion of the detectors is limited to 90° , minimizing photon fluence variance between channels. Each

detection channel is separated into two PMTs to measure transmitted fluorescence and excitation light simultaneously. The simultaneous collection of both transmission and fluorescence data facilitates use of the Born normalization technique [3]. Fluorescence is detected by using long-pass interference filters with a cutoff at $\lambda = 650$ or 780 nm, depending on the laser used. The laser diodes can be operated either in pulsed or cw mode. The pulsed mode can allow time-resolved imaging to be achieved, while operation in the cw mode provides significant signal-to-noise (S/N) benefits without affecting the detection sensitivity (in this study, photons counts are summed up over all arrival times in both modes). Noncontact whole-body interrogation of a specimen is achieved through motorized lateral motion of a custom-built mouse cradle along the central axis of the optical system. For each position of the cradle along this axis, automated rotation of the gantry allows up to 360 laser projections to be acquired, i.e., as much as 1800 fluorescence measurements per 2D slice. The system has an imaging bed designed to fit both the optical system and a micro-computed-tomography (CT) system (eXplore Locus SP, GE Healthcare, London, Ontario) to provide CT-derived anatomical priors that are used to constrain FT reconstructions.

The sensitivity of the system was first evaluated by investigating the lowest concentration of fluorophores that can be detected through different thicknesses of scattering media. A wedge-shaped tissue-simulating phantom (Fig. 2) made of a polyurethane-TiO₂ matrix with absorption and reduced scattering properties of $\mu_a = 0.012 \text{ mm}^{-1}$ and $\mu'_s = 1.23 \text{ mm}^{-1}$ ($\lambda^x = 635 \text{ nm}$) and having a 4 mm diameter hole located 7 mm from the base of the phantom was used. The inclusion was filled with solutions of Alexa Fluor 647 dye (Invitrogen Life Sciences, Carlsbad, Calif.) in 1% Intralipid per unit volume in water, and optical data were acquired in cw mode for tissue thicknesses and AF647 concentrations ranging from 2.9 to 6.7 cm, and 0.07 to 70 nM, respectively.

Figure 3 shows the signal-to-background ratio (SBR) in channel 3 (Fig. 1) as a function of thickness, for different dye concentrations. The SBR was evaluated using the following equation:

$$\text{SBR} = \frac{I_F - I_F^{\text{bg}}}{\text{D.N.}}, \quad (1)$$

where I_F is the detected fluorescence signal; I_F^{bg} is the background fluorescence measured in the absence of dye (Intralipid and water only); and D.N. is the dark noise of the instrument, measured with the laser turned off under the same experimental conditions. As seen in Fig. 3, for each concentration, a 1 cm increase in thickness lead to approximately a 1 order of magnitude decrease in SBR. Moreover, fluorescence detection of concentrations lower than 0.07 nM could be achieved for thicknesses as large as 4 cm, while 0.7 nM could be detected up to 5 cm. The noncentral position of the inclusion leads to an overestimation of the sensitivity. If the inclusion was in the center of the phantom, simulations showed the minimum observable concentration is 1.4 times greater at the 2.9 cm thickness and 2.6 times greater at the 6.7 cm thickness. Moreover, simulations showed that increasing the absorption by a factor of 2 reduces the sensitivity by a factor of 10.

The ability to provide optical datasets with sufficient S/N levels for fluorescence image reconstruction was then evaluated in a rat-sized tissue-simulating phantom. A 5 cm diameter cylindrical phantom was made using the same bulk matrix as the wedge phantom. A 6 mm diameter cylindrical inclusion, 12 mm from the edge, was filled with concentrations of AF647 ranging from 0.7 to 700 nM. Simulations were used to show that the increased depth of the inclusion compared to the wedge phantom is countered by its increased size. Fluorescence and transmitted excitation light were collected for 32 equally spaced laser

projections around the phantom, and images were reconstructed from the Born-normalized signal with NIRFAST software [4]. The finite-element mesh was created from the boundaries of the object using the coregistered CT image. Figures 4(a) and 4(b) show the FT images associated with dye concentrations of 70 and 0.7 nM, respectively. The low level of S/N in the 0.7 nM dataset explains why the quality of the image was inferior to that obtained for 70 nM. Figure 5 displays the average values of the Born-normalized fluorescence as a function of dye concentration when the system was operated in cw versus pulsed mode. It demonstrates that the system response is linear until it reaches the noise floor, which is around 7 nM in pulsed mode and less than 1 nM in cw mode, owing to increased signal levels and improved S/N ratios provided by cw operation. Although it is not shown here, the reconstructed fluorescence values were also linear in terms of actual concentration values.

In the final experiment, a 5 mm diameter tube filled with a 10 nM solution of IRDye 800 (LI-COR Biosciences, Lincoln, Nebr.) was inserted through a cadaverous rat, and the rat was imaged using the 755 nm laser diode operated in cw mode. A near-IR dye was used to minimize the signal contribution associated with tissue autofluorescence. Figure 6 demonstrates the resulting FT-CT image where the center of mass of the fluorescent distribution was located within the boundaries of the tube, as seen in the CT image.

The results presented in this Letter demonstrate for the first time, to our knowledge, that FT can be used to image fluorophore distributions in specimens with thicknesses significantly larger than mice at sensitivity thresholds consistent with the requirements for imaging rat models of disease. This is shown to be achievable for concentrations that are significantly lower than what has been observed in the past with mouse-size phantoms. In fact, the results presented here show that concentrations lower than 1 nM can be imaged in a phantom with a thickness of 5 cm. Diffusion modeling for light transport has been used to show that this corresponds to an amount of sampled fluorescent molecules of approximately 200 femtomoles. This enhanced sensitivity of the FT system can be partly explained by a robust and reproducible data calibration methodology; however, the intrinsic photon detection gains result from the use of TCSPC with PMTs. Specifically, this detection technology provides extremely low levels of dark noise and is essentially free of readout noise. Moreover, because signals are acquired on a point-by-point basis, the system provides an almost unlimited dynamic range because of the automatic exposure control method [5]. Readout noise, dark noise, and limited dynamic range are all factors that can severely limit the sensitivity of CCD-based FT systems.

An important result from this study is that every increase of 1 cm of tissue thickness leads to an order of magnitude decrease in SBR. Based on this, Fig. 3 suggests that 1 and even 0.1 fM could be detected in mice, based upon their size. To our knowledge, this is the first instance indicating that FT could be used to detect such low levels of fluorescent dyes in mouse models of disease.

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Fig. 1.
(Color online) Noncontact SPC FT system.

Fig. 2.
(Color online) Rear and side views of the tissue-simulating wedge phantom designed to emulate different interrogated thicknesses.

Fig. 3.
(Color online) Fluorescence SBR as a function of thickness for four different AF647 concentrations. The line of detectability is at an SBR of 1.

Fig. 4.
(Color online) FT images where an inclusion was filled with an AF647 solution of (a) 70 and (b) 0.7 nM.

Fig. 5.
(Color online) Born-normalized fluorescence as a function of dye concentration.

Fig. 6.
(Color online) FT-CT image of a rat cranium acquired postmortem where a 5 mm diameter fluorescent tube has been inserted.