Time-resolved scanning system for double reflectance and transmittance fluorescence imaging of small animals.

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Abstract: We developed a time-resolved scanning system for fluorescence molecular imaging in diffusive media, such as biological tissues. In the present work the system is described and characterized in terms of linearity against optical parameters of the sample and against homogeneously diffuse fluorescent dye. Finally, preliminary measurements performed on phantom are presented, pointing out the ability of our system to produce projective images of the fluorophore distribution into the sample with a 200 fmol sensitivity and to decouple fluorescent amplitude from depth by means of fluorescent transmittance imaging.

OCIS codes: 170.6920 Time-resolved imaging; 170.7050 Turbid media; 170.6280 Spectroscopy, fluorescence and luminescence; 170.3880 Medical and biological imaging

1. INTRODUCTION

In the last years, optical imaging is emerging as greatly promising technique both for diagnostic purposes in the clinical area\(^1\),\(^2\) and as a powerful research aid in biomedical investigations. Optical molecular imaging, especially, is one of the most attractive, given its many advantages over classic techniques, such as MRI and nuclear imaging.\(^1\),\(^2\) Commonly optical molecular imaging is performed by tomographic reconstruction based on continuous wave\(^3\) or frequency domain systems.\(^4\),\(^5\) More recently also time-resolved techniques were studied and applied.\(^6\)–\(^8\) In this framework, we developed a novel system for optical molecular imaging of small animals based on a double transmittance and reflectance setup. The system works in the time-domain and is capable of producing projective reconstructions of the fluorophore distribution in the sample.

2. MATERIALS AND METHODS

2.1 Experimental setup

A schematic of the experimental setup used for data recording is depicted in figure 1. The system is symmetric with respect to the sample axis: for brevity, only one part will be described, even though it must be noticed that both sides can record at the same time, thus giving the possibility to gather a reflectance and a transmittance measurement simultaneously. Two pulsed laser diodes (S1, S2) (PDL, Picoquant GmbH, Germany) emitting at respectively the excitation and emission wavelengths of the selected fluorophore (\(\lambda_1\), \(\lambda_2\) hereafter) are coupled to delivery fibers (50/125 µm multi-mode fused silica fiber) through a \(2 \times 2\) optical switch that allows injection wavelength and position selection. The two sources are coupled to a free-beam optical stage that delivers the input pulse to the sample and collects the output pulse guiding it to the photomultiplier tubes (PMT, Becker & Hickl, Berlin, Germany). A filter wheel is placed just before the PMT to allow the selection of the recorded signal wavelength. Three different combinations of injection/collection wavelengths are possible, namely \(\lambda_1 - \lambda_1\), \(\lambda_2 - \lambda_2\), \(\lambda_1 - \lambda_2\), to obtain information respectively on the optical properties of the sample at the excitation wavelength and position selection. The two sources are coupled to a time correlated single photon counting board (TCSPC, Becker & Hickl, Berlin, Germany). A photodiode is also coupled to the optical path, providing a straightforward means of monitoring laser power fluctuations. The PMT electronic signals are driven through an electronic router (Becker & Hickl, Berlin, Germany) to a time correlated single photon counting board (TCSPC, Becker & Hickl, Berlin, Germany). Moreover, a precision translation stage is used to hold the sample, so that a multi-point scan is easily achievable for imaging.

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Figure 1: System setup configuration. The system is symmetrical to the sample, allowing simultaneous double recording of reflectance and transmittance measurements. Translation stage holds sample/inclusions to allow scanning.

2.2 System characterization

Three different kinds of measurements on phantom were performed to assess the system performances: first we realized a set of punctual measurements injecting and collecting at the same wavelength ($\lambda_1 - \lambda_1$ first, $\lambda_2 - \lambda_2$ then) to evaluate the system linearity with respect to the optical parameters of the bulk solution; then, a second set of punctual measurements was accomplished, injecting at the excitation wavelength and collecting at the emission wavelength of the fluorophore ($\lambda_1 - \lambda_2$), to verify the system linearity against different concentrations of the fluorophore in the bulk solution; finally we realized scanning measurements of the phantom with a fluorescent point inclusion, to estimate the capability of the system to distinguish point fluorescence from non fluorescent background.

For all the measurements, the sample used was a liquid phantom contained into an 18mm thick tank, prepared with a solution of Intralipid (Fresenius Kabi Italia S.p.A., Italy) and blue ink (Rotring, Germany) to simulate the scattering and the absorbing properties of living tissues in the 600 – 750 nm wavelength range. Nile Blue was chosen as a fluorophore, since its absorption (peak at 633nm) and emission (peak at 670nm) wavelength ranges are very close to that of other more usual to the biotechnological practice dyes, like Cy5 and Alexa Fluor. As a consequence, the two laser diodes were chosen at 633nm for $\lambda_1$ and 671nm for $\lambda_2$; they are driven at 80MHz repetition rate, with a pulse duration of 200ps.

The linearity of the system against the optical properties of the sample was tested, measuring in transmittance geometry a set of twelve phantoms with different values of $\mu_a$ and $\mu'_s$; for each of them, the recorded time-resolved curves were fitted to the analytical solution of the diffusion equation for a slab geometry.

The other important aspect of the characterization of the system is the linearity of the recorded signal against the fluorophore concentration: it was verified taking the time-integral of the curves collected measuring a set of 8 phantoms with different concentrations of Nile Blue dispersed into the bulk solution, whose nominal optical parameters were selected according to literature as 10 cm$^{-1}$ for the reduced scattering coefficient and 0.3 cm$^{-1}$ for the absorption coefficient (see and references therein).
Finally, the phantom with a punctual fluorescent inclusion and no bulk fluorophore was imaged by means of a 61 mm × 61 mm at 1 mm steps scan, to demonstrate the ability of our system to identify fluorescence embedded in a turbid medium. The inclusion was made with a transparent plastic cone of 3 mm diameter, 14 mm height and 20 µl volume, held by a stiff steel wire 0.4 mm thick. Measurements were repeated at fixed bulk optical properties for different inclusion concentrations and positions to verify signal dependence on these parameters.

3. RESULTS

The system has shown a good linearity in the estimation of bulk optical properties, even though an overestimation is present as it can be seen in figures 2 and 3. This is probably due to the fact that, since we chose the thickness of our sample to mime that of a mouse, it results quite small and does not perfectly lie in the application range of the diffusion theory. Figure 4 shows that also the diffuse fluorescence signal has a good linearity in both geometries. As we expected, reflectance signals are strongly dependent in intensity and in temporal shape and delay not only on inclusion concentration, but also on its depth inside the medium. On the contrary, transmittance signals are slightly dependent on inclusion depth, naturally candidating themselves for projection imaging. Moreover, reflectance signals are much more affected by noise than transmittance ones. In fact collecting exactly from the same point of injection intensely excites bulk fluorescence, which masks the signal of interest and limits the maximum penetration depth to no more than 8 − 9 mm.
Figure 4: Bulk fluorescence linearity.

In figure 5.a and 5.b two projection images of the transmitted fluorescent late gate intensity of an inclusion located 3 mm deep from the injection side of the tank and respectively $10^{-5} M$ and $10^{-8} M$ concentrated are reported as an example, while figure 5.c shows the reflectance image of a $10^{-5} M$ concentrated inclusion in the same position. From this last image it is evident how the signal intensity is much higher than that of transmittance ones, even though the contrast is lower, because of the bulk fluorescence shadowing effect. Moreover we report also transmittance image contrast as a function of dye concentration inside the inclusion: as it can be seen in figure 6, contrast is approximately 1 for a concentration of $10^{-8} M$, equivalent to an amount of fluorophore of 200 fmol, which we take as the smallest detectable concentration.

From the comparison of the inclusion physical size with its imaged spatial profile, we could determine that spatial resolution of the system can be estimated in about 3 mm, even if it is related to the optical properties of the bulk solution, in particular to the scattering coefficient. Since our measurements are time-resolved, it was also possible to estimate fluorescence lifetime by means of a linear interpolation of the curves tails in log scale, which for our fluorophore gave as a result $\tau = 1.3 \text{ ns}$ (cfr. fig. 7). Figure 8, reports the estimated fluorescence amplitude obtained by simultaneously fitting both side transmittances for each scan point to the time-resolved diffusion model for transmittance using concentration and position of the inclusion as free parameters. This procedure was aimed to obtain a map of the fluorophore distribution as independent as possible of the inclusion depth. The data we obtained show that the flattest behavior (i.e. the most independent) is anyhow the experimental transillumination signal when the inclusion is closer to the collection point.
4. CONCLUSIONS

We showed a novel system based on the time-correlated single photon counting technique for molecular imaging with the ability to detect fluorescent inclusions submerged into a tissue-like diffusive medium by fluorescence intensity and lifetime, also capable of measuring sample optical properties at both excitation and fluorescence wavelengths. We characterized the system and assessed its working ranges and showed some preliminary results obtained from phantom measurements. We are currently working for improving data interpretation model and developing the instrumentation for in-vivo measurements of nude mice.

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