Tomographic fluorescence lifetime multiplexing in the spatial frequency domain

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Abstract

The ability to simultaneously recover multiple fluorophores within biological tissue (multiplexing) can have important applications for tracking parallel disease processes in vivo. Here we present a novel method for rapid and quantitative multiplexing within a scattering medium, such as biological tissue, based on fluorescence lifetime contrast. This method employs a tomographic inversion of the asymptotic (late) portion of time-resolved spatial frequency (SF) domain measurements. Using Monte Carlo simulations and phantom experiments, we show that the SF-asymptotic time domain (SF-ATD) approach provides a several-fold improvement in relative quantitation and localization accuracy over conventional SF-time domain inversion. We also show that the SF-ATD approach can exploit selective filtering of high spatial frequencies to dramatically improve reconstruction accuracy for fluorophores with subnanosecond lifetimes, which is typical of most near-infrared fluorophores. These results suggest that the SF-ATD approach will serve as a powerful new tool for whole-body lifetime multiplexing.

Keywords

(170.3010) Image reconstruction techniques; (170.3880) Medical and biological imaging; (170.6920) Time-resolved imaging; (170.3650) Lifetime-based sensing; (170.6960) Tomography

Structured illumination techniques have been exploited for rapid and quantitative optical property mapping in diffuse optical tomography [1] and for improved spatial resolution, contrast, and dynamic range performance in fluorescence tomography [2–5]. We have previously demonstrated using point-scanning tomography that time domain (TD) fluorescence imaging using the asymptotic (or late-arriving) photons provides zero cross talk and optimal relative quantitation for tomographic imaging of multiple fluorophores in turbid media (multiplexing) [6]. In this Letter, we present a novel formalism that extends the asymptotic TD (ATD) approach to spatial frequency (SF) domain measurements, thereby combining the inherent benefits of wide-field measurements, such as rapid whole-body imaging and high resolution, with quantitative multiplexing capabilities using TD measurements. We validate SF-ATD using Monte Carlo (MC) simulations with MCX, a GPU-accelerated high-speed MC computing platform [7] that can accept arbitrary spatial

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See Supplement 1 for supporting content.
patterns as input. We show that SF-ATD provides a dramatic improvement in localization and relative quantitation accuracy compared to conventional direct inversion of SF-TD fluorescence data. We also show that by allowing selective filtering of high spatial frequency data, SF-ATD can quantitatively recover shorter lifetimes than possible using point-scanning methods. We validate the feasibility and advantages of SF-ATD over SF-DTD using experimental measurements with tissue-mimicking phantoms.

Consider a turbid medium obeying the radiative transport equation with absorption \( \mu^x_{a,m} \) and scattering \( \mu^s_{a,m} \) at the excitation (\( \lambda_x \)) and emission (\( \lambda_m \)) wavelengths, and with \( N \) embedded fluorophores, each having a lifetime \( \tau_n = 1/\Gamma_n \) and yield distribution \( \eta_n(r) \) (product of the quantum yield and extinction coefficient). It is straightforward to show (Supplement 1) that when \( \tau_n > \tau_a \), where \( \tau_a = (\mu^x_{a,m}(r))^{-1} \), the TD fluorescence signal in the asymptotic region, defined as \( t > \tau_a \), is factorized into functions of spatial frequency and time as

\[
U_F(k_s, k_d, t) \rightarrow \tau_a \sum_{n=1}^{N} a_n(k_s, k_d) e^{-\Gamma_n t}, \tag{1}
\]

where \( k_s \) and \( k_d \) are the source and detector spatial frequencies (SF) on the boundary of the medium, respectively, and \( a_n \) are time-independent decay amplitudes, which are related to corresponding yield distributions

\[
a_n(k_s, k_d) = \int d^3r \overline{W}_n(k_s, k_d, r) \eta_n(r). \tag{2}
\]

where \( \overline{W}_n = G^x_n(k_s, r)G^m_n(r, k_d) \) is a continuous wave (CW) fluorescence weight matrix evaluated as the product of transport Green’s functions, \( G^x_n, G^m_n \), computed at a reduced absorption \( \mu^x_{a,m}(r) - \Gamma_n \nu \). The reduced absorption is crucial for the accurate relative quantitation of multiple lifetime components. Equations (1)–(2) compactly and rigorously describe asymptotic TD fluorescence and are valid for arbitrary complex-shaped heterogeneous transport media, provided \( \tau_n > \tau_a \) (\( \approx 0.45 \) ns for \( \mu^x_{a,m} = 0.1/cm \)). We will show that the use of high spatial frequencies extends the applicability of Eq. (1) to even shorter lifetimes by effectively lowering \( \tau_a \).

Equation (2) separates the TD fluorescence from a turbid medium containing multiple fluorophores into independent forward problems for each lifetime. To see the advantage of this separation, consider the inverse problem of recovering \( \eta_n \) from TD measurements \( U_F \). Discretizing the problem into \( V \) medium voxels, \( L \) time points, and \( M \) pairs of sources and detector SFs at the medium boundaries, the full TD forward problem and its asymptotic limit [Eq. (1)] can be expressed as a matrix equation:
\[ y = W \eta^{t > \tau_a} = A \bar{W} \eta. \quad (3) \]

Here, \( y \) is a \((ML \times 1)\) data vector, \( W \) is the full \((ML \times NV)\) TD weight matrix, \( \eta = [\eta_1, \eta_2, \ldots, \eta_N]^T \) is a \((NV \times 1)\) vector of unknown fluorescence yields for both lifetimes, \( A = [\exp(-t/\tau_1) \otimes I, \exp(-t/\tau_2) \otimes I, \ldots, \exp(-t/\tau_N) \otimes I] \) is a \((ML \times MN)\), over-determined “basis” matrix containing Kronecker products of exponential decay terms and the \((M \times M)\) identity matrix, \( I \), and \( \bar{W} = \text{diag}(W_1, W_2, \ldots, W_N) \) is a \((NM \times NV)\) block diagonal matrix containing reduced absorption CW weight matrices for each lifetime. The inversion of Eq. (3) to recover \( \eta \) can now proceed along two alternate ways. In the first way, called the SF-asymptotic TD (SF-ATD) method, we first invert the well-conditioned matrix \( A \) using its Moore–Penrose pseudo inverse \( A^\dagger \), followed by a Tikhonov inversion of the matrix \( \bar{W} \) [6]. The inverse problem takes the form

\[ \eta_{\text{SF-ATD}} = \bar{W}^T (\bar{W} \bar{W}^T + \lambda I)^{-1} a, \quad (4) \]

where \( a = A^\dagger y = [a_1, \ldots, a_N]^T \) is a \((MN \times 1)\) vector of decay amplitudes for the \( N \) lifetimes and \( M \) pairs of source–detector spatial frequencies. The conventional approach for spatial frequency domain time-resolved fluorescence tomography [3], which we call the spatial frequency-direct TD (SF-DTD) approach, does not exploit the two-step nature of the inverse problem but rather performs a direct inversion of the full TD weight matrix \( W \). It can be shown that in the asymptotic limit, we can still exploit the factorization, \( W = A \bar{W} \), implicit in Eq. (3), to arrive at the following expression for the SF-DTD inverse problem [6]:

\[ \eta_{\text{SF-DTD}} = \bar{W}^T (\bar{W} \bar{W}^T + \lambda C_a)^{-1} a, \quad (5) \]

where \( C_a = (A^T A)^{-1} \), \( C_\eta = \sigma^2 \eta \) is the data covariance matrix, and \( \lambda = (\sigma_y/\sigma_\eta)^2 \), with \( \sigma_y \) and \( \sigma_\eta \) as the variances in the measurement and yield. Note that the size of the matrix to be inverted in Eq. (5) is \( NM \times NM \) (for \( \bar{W} \bar{W}^T \)), which is much smaller than the matrix for full TD inversion \((LM \times LM \text{ for } W W^T)\), since typically \( N \ll L \). Thus, Eq. (5) offers a dramatic reduction in the computation of SF-DTD, which can otherwise be prohibitive for more than a few gates. The key distinction between SF-ATD [Eq. (4)] and SF-DTD [Eq. (5)] is that the inverse problem in Eq. (4) is block diagonal, implying zero cross talk between multiple lifetimes, whereas Eq. (5) is not block diagonal. We will see that this difference results in significant performance difference between the two methods.

We first demonstrate the advantages of SF-ATD [Eq. (4)] over SF-DTD [Eq. (5)] using Monte Carlo (MC) simulations, performed using “MCX,” a GPU-based accelerated MC computing platform [7]. We consider a slab medium \((6 \text{ cm} \times 4 \text{ cm} \times 1.9 \text{ cm}, 1 \text{ mm}^3 \text{ voxels})\) with absorption \( \mu_a^x = \mu_a^m = 0.1 \text{ cm}^{-1} \), scattering \( \mu_s^x = \mu_s^m = 10 \text{ cm}^{-1} \), anisotropy of 0.01, and refractive index of 1.37. Two \( 1 \text{ mm}^3 \) fluorescent inclusions of equal concentration are placed
at a height of \( z = 13 \) mm, with varying separations of 10 mm, 2 mm, and 0 mm. We first consider a case where the inclusions have lifetimes of 0.8 ns and 1.2 ns, which are longer than the intrinsic absorption timescale \((\nu_{\text{\textmu}}a)^{-1} \approx 0.45 \) ns. The sources were 2D sinusoidal patterns of the form \( S_{\phi} = 0.5 \left[ 1 + \cos(2 \pi (k_x x + k_y y) + \phi) \right] \), where the three phases \( \phi_n = \frac{2\pi n}{3} (n = 0, 1, 2) \) are used to eliminate the D.C. background [1,8]. \( k_x \) and \( k_y \) ranged from 0 to 0.5/cm in six steps, resulting in 36 sources at \( z = 0 \). The detectors consisted of the same 36 patterns at \( z = 1.9 \) cm. The source and detector MC TD Green’s functions \( G_{n,m} \) were computed for \( 2 \times 10^9 \) photons, and subsequently, the CW weight functions \( W_1 \) and \( W_2 \) were calculated for the 36 source and detector patterns and 64 time points between 0 and 6.3 ns (resulting in \( 1296 \times 64 \) measurements). Separately, the decay amplitudes for the two lifetimes, \( a_1 \) and \( a_2 \) were recovered from the decay portion of the TD signal for all M measurements, using a linear bi-exponential fit with the known lifetimes. The linear fit employed 12 gates ranging from 1.9 ns to 6.3 ns (use of more gates did not significantly improve the quality of the fits). The \( a_n \)s were finally used in Eqs. (4) and (5) to recover the yield distributions for the two lifetime components using the SF-ATD and SF-DTD methods. The regularization \( \lambda \) was chosen to provide the least reconstruction error.

Figures 1(a)–1(c) show 2D slices of the SF-DTD reconstructed yields, \( \eta_1 \) (0.8 ns) and \( \eta_2 \) (1.2 ns) as the red and green components of a single RGB image, normalized to the maximum of both yields. Figures 1(d)–1(f) show the corresponding line profile along the X-direction, through the Y–Z location of the maximum of each yield distribution. Similarly, Figs. 1(g)–1(i) show slices of the SF-ATD reconstructions and Figs. 1(j)–1(l) the corresponding line profiles. It is clear that SF-DTD results in significantly higher cross talk, which can be seen for the 10 mm case [Fig. 1(d)] as a shoulder in the line profiles at the incorrect location. The SF-DTD results in a \( \eta_1 : \eta_2 \) ratio of 0.6, or a 40\% error in relative quantitation, and a \( \sim 1 \) mm error in localization for the 2 mm case. The SF-ATD results in <2\% error in recovering \( \eta_1 : \eta_2 \) and accurate localization for all three separations. The significantly better performance of SF-ATD can be attributed to the block diagonal form of the SF-ATD inverse problem in Eq. (4), which implies zero cross talk between \( \eta_1 \) and \( \eta_2 \). The optimal regularization (least error) for SF-ATD was higher than that for SF-DTD, resulting in broader distributions (low precision) with highly accurate centroids and yield values (high accuracy) compared to SF-DTD. We note that 2 mm separation is shorter than previously used separations for fluorescence tomography. Also note that the simulations do not consider possible model errors, such as incorrect estimation of optical properties and medium geometry, which affect SF-DTD more than SF-ATD, as will be seen with experimental data.

While SF-ATD thus provides superior performance for lifetimes longer than the absorption timescale, \( \tau_{\text{\textmu}} \), a more interesting scenario is when lifetimes are comparable to, or even shorter than, \( \tau_{\text{\textmu}} \). In this case, the late time evolution of the TD fluorescence is dominated by intrinsic photon scattering timescales and no longer reflects the true lifetime of the fluorophore. Thus, the benefits of the asymptotic factorization [Eq. (3)] can no longer be exploited for quantitative multiplexing when using all SFs (or equivalently, point illumination and detection). However, the use of high SFs can alleviate the influence of scattering and allow detection of short lifetimes in turbid media. This can be understood
based on an increase in the “effective” absorption of the medium with increasing SF as $\mu_a + k^2 D$, where $k$ is the spatial frequency and $D$ is the diffusion coefficient [8]. The increased $\mu_a$ implies that the highly scattered, late-arriving photons are attenuated more, thereby decreasing $\tau_a$ and resulting in a faster approach toward the asymptotic limit and also lowering the limit for the shortest lifetimes that can be detected in the asymptotic portion. This phenomenon is illustrated in Fig. 2, which shows the error in lifetime recovered from single exponential fits to the asymptotic TD signal for a single inclusion with a lifetime of either 0.3 ns [Fig. 2(a)] or 0.5 ns [Fig. 2(b)] for the same geometry as in Fig. 1. It is clear that for low frequencies, the error in lifetime is significant for the 0.3 ns case but decreases toward higher SF. At very high SFs, the lifetime error increases again due to a decreased signal-to-noise ratio (SNR) with increasing SF, which is in turn due to the medium acting as a low pass filter.

Figure 2 suggests that the use of nonzero SFs up to a threshold high SF (to avoid the low SNR of high SF data) can allow us to exploit SF-ATD for quantitative multiplexed tomography of short lifetimes in turbid media. To show this, we repeated the simulations of Fig. 1 for the 2 mm separation case, with the inclusions having lifetimes of 0.3 ns (shorter than $\tau_a$) and 0.5 ns, and with 11 SFs between 0 and 0.5 cm$^{-1}$. Figures 3(a) and 3(c) show SF-ATD reconstruction slices and the corresponding line profiles using the entire SF spectrum (which is Fourier equivalent to point excitation across the entire surface). While the localization of both lifetimes is recovered accurately, there is a more than 40% error in relative quantitation. However, the SF-ATD reconstruction using a high SF data set ($k > 0.1/cm$) shows a dramatic improvement in quantitation, with an error $<2\%$ [Figs. 3(b) and 3(d)]. The faster approach of the high SF-TD data toward the asymptotic portion allowed the use of 13 time gates in the reconstructions, with the inclusion of an additional time gate at 1.4 ns, close to the peak of the TD signal. Besides lifetimes shorter than $\tau_a$, SF-ATD with high SF data improves quantitation, even for lifetimes comparable to or slightly longer than $\tau_a$ (Supplement 1). SF-ATD therefore enables quantitative multiplexing using a wider range of near-infrared fluorophores, which typically have short (subnanosecond) lifetimes.

We evaluated the feasibility of SF-ATD using experiments with a tissue phantom. Two parallel tubes (0.965 mm outer diameter, 0.58 mm inner diameter) separated by 4 mm were embedded at a height of 12 mm in a circular petri dish (8.8 cm diameter) filled with an Intralipid+nigrosin mixture ($\mu_a = 0.3$ cm$^{-1}$ and $\mu_s' = 10$ cm$^{-1}$) to a height of 1.7 cm [Fig. 4(a)]. The tubes contained IRdye800CW (Licor biosciences, $\tau_1 = 0.45$ ns) and 3, 3’ diethylthiatricarbocyanine iodide ($\tau_2 = 0.65$ ns). The sample was excited at 745 nm (Ti:sapphire, Spectra Physics MaiTai, 150 fs, 80 MHz) and the transmitted TD fluorescence was detected with a $\lambda > 800$ nm filter (Chroma) attached to a intensified CCD camera (LaVision Picostar HRI, 400 ps gatewidth, 500 ms exposure, 600 V gain). To generate spatial patterns, we modified a Pico projector (Aaxa P300) with a digital micromirror device (DMD) to accept collimated fiber input from the Ti:sapphire laser. Sinusoidal patterns at 19 frequencies ranging from $k_x = 0$ to 0.51 cm$^{-1}$ along the axis perpendicular to the tubes ($x$) were projected under the dish. Figure 4(a) shows the lifetime maps of the tubes without Intralipid for $k_x = 0$. Figure 4(b) shows examples of the input patterns (integrated over all time gates) for zero (top) and the highest SF (0.51 cm$^{-1}$) used, measured directly with a

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white paper placed on the imaging plate. These patterns were used directly as the input source patterns in MCX (rather than relying on perfect sinusoids), thereby implicitly accounting for experimental factors, such as variation in the response across the DMD, laser illumination, and the ICCD sensitivity. For simplicity of analysis, we used point detectors, assigned as 26 pixels (2 × 2 binning) on the CCD images with a 2 mm spacing. The decay amplitudes, \( a_1 \) and \( a_2 \), for the 0.45 ns and 0.65 ns components were recovered for each measurement from linear bi-exponential fits [Fig. 4(c)] to the asymptotic portion of the TD data using 9 time gates.

The SF-DTD [Fig. 4(d)] and SF-ATD [Fig. 4(e)] yield reconstructions \( \eta_1 \) and \( \eta_2 \) for the 0.45 ns (red) and 0.65 ns (green) were performed using a high SF (\( k > 0.05 \text{ cm}^{-1} \)) data set. Figures 4(f) and 4(g) show the corresponding 1D line profiles through the location of the peak value of the respective yields, along with the true yields normalized to the maximum of \( \eta_1 \) and \( \eta_2 \) as vertical red (0.45 ns) and green (0.65 ns) lines at the true location of the tubes. The true ratio of the fluorescence yields was \( \eta_1 : \eta_2 = 1.36 \), as estimated directly from the CW fluorescence of the dye-filled tubes without Intralipid [as in Fig. 4(a)]. While SF-ATD provided 3.5% error in relative quantitation and perfect lateral localization, the depth localization of \( \eta_2 \) had an error of 1 mm. SF-DTD resulted in 44% quantitation error and was unable to delineate the lifetimes as spatially separate. The poorer localization of SF-DTD with experimental data compared to the simulations could possibly be attributed to model errors not accounted for in our reconstructions, including errors in optical properties and positioning, incorrect noise model, or poorer SNR of the experimental data. The SF-ATD is, however, more robust to these unknown parameters and provides accurate relative quantitation and spatial localization under the same conditions.

We have presented a novel approach for tomographic multiplexing using time-resolved SF domain measurements that provide high accuracy for quantitative tomography of multiple fluorophores simultaneously present in a turbid medium. We have also shown that SF filtering allows quantitative tomographic imaging of shorter lifetimes than possible with point measurements. This result has high importance for whole-body molecular imaging given that the lifetimes of most near-infrared fluorophores are in the subnanosecond range [9]. Further studies will focus on in vivo applications and on optimizing experimental parameters, including time gates and spatial frequencies, to further improve the imaging performance of SF-ATD.

**Supplementary Material**

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Fig. 1.
Comparison of (a)–(f) SF-DTD with (g)–(l) SF-ATD reconstructions of two fluorescent inclusions (indicated by “x”) with lifetimes of 0.8 ns and 1.2 ns and equal fluorescence yields, embedded at a height of 13 mm in a 1.9 cm thick turbid slab, with separations of 10 mm (left), 2 mm (center), and 0 mm (right). The 2D slices of the (a)–(c) SF-DTD and (g)–(i) SF-ATD yield reconstructions $\eta_1$ and $\eta_2$ for 0.8 ns and 1.2 ns lifetimes are shown as red and green components of a single RGB image, normalized to the maximum of both yields (yellow thus indicates equal yield for overlapping inclusions, i.e., for the 0 mm case). (d)–(f) Normalized line profiles across the centroid of SF-DTD and (j–l) SF-ATD reconstructed $\eta_1$ (red) and $\eta_2$ (green) along the X-direction, with true inclusion locations (vertical gray lines).
Fig. 2.
Dependence of fluorescence lifetimes on source and detector spatial frequencies, simulated for a 1.9 cm thick slab with an inclusion of lifetime 0.3 ns (left) or 0.5 ns (right) placed at a height of 1.3 cm. The images show the error, $|\tau_{\text{fit}} - \tau_{\text{actual}}|/\tau_{\text{actual}} \times 100$, of the lifetimes recovered from a single exponential fit to the asymptotic data.
Fig. 3.
Tomographic multiplexing of lifetimes shorter than the absorption timescale $\nu_\mu^{-1}$ using high spatial frequency data. The simulation conditions were as in Fig. 1, with 2 mm separated inclusions of lifetimes 0.3 ns and 0.5 ns and equal yield. (a), (c) SF-ATD reconstructions, $\eta_1$ and $\eta_2$ for the 0.3 ns (red) and 0.5 ns (green) using all spatial frequencies from 0 to 0.5 cm$^{-1}$. (b), (d) SF-ATD reconstructions using $k > 0.1$ cm$^{-1}$. (c)–(d) Normalized line profiles of $\eta_1$ (red) and $\eta_2$ (green) through the location of their individual maxima.
Fig. 4.
Experimental demonstration of the SF-ATD approach. (a) Dish phantom with two tubes filled with IRdye800CW (0.45 ns, red) and DTTC (0.65 ns, green), separated by 4 mm, shown with the illumination area and the lifetime map of the tubes. (b) Spatial patterns for the lowest ($k_x = 0$) and highest ($k_x = 0.51 \text{ cm}^{-1}$) frequency used. (c) Representative TD fluorescence data (dots) for $k_x = 0$ and a detector (CCD pixel) above the tubes, and linear bi-exponential fit (black) using the known lifetimes of 0.45 ns and 0.65 ns. The individual decay components at 0.45 ns (red) and 0.65 ns (green) intersect $t = 0$ (dotted line) at the
value of the decay amplitudes $a_1$ and $a_2$ used in tomography [Eqs. (4)–(5)]. $t = 0$ is determined from the peak of the instrument response function (IRF; dashed line shows IRF for $k_x = 0$ at a single detection pixel), measured with a white paper in place of the dish. $X$–$Z$ slices of (d) SF-DTD and (e) SF-ATD reconstructions of the 0.45 ns (red) and 0.65 ns (green) components shown as a RGB image. (f), (g) Line profiles along X for the reconstructions shown in (d) and (e) for the 0.45 ns (red line) and 0.65 ns (green line) components, shown with the true yields of the tubes normalized to the maximum yield (green and red dashed lines).