Imaging Metabolism with Light: Quantifying Local Fluorescence Lifetime Perturbation in Tissue-like Turbid Media

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Abstract. Fluorescence lifetime imaging creates a map of spatial differences in local lifetime. These lifetime differences can be correlated with local metabolic levels which makes this a valuable biomedical imaging modality. We examine the ability to localize a fluorescence signal from deeply embedded sites in highly scattering, turbid, tissue-like media. We show how a specific point-spread-like function may be used to quantify an isolated lifetime perturbation between two sites in tissue. This new minimally invasive method offers the opportunity to infer near-real-time estimates of metabolic activity.

1 Introduction

Optical techniques are among the most valuable diagnostic tools available in medicine. Microscopy-based histology, for example, is the definitive diagnostic tool for cancer. Optical contrast agents, such as the stains used in histology, improve the ability to distinguish tissue types. Fluorescence contrast agents also have proven useful in biomedical research; for example, in examining cellular functions. Fluorophores may be linked to selectively binding molecules and inserted into tissue. Then, localization of those fluorophores indicates the presence of specific receptor molecules. Alternatively, fluorescence lifetime may be used as specific contrast. Fluorescence lifetime techniques have an advantage over intensity-based fluorescence techniques where pharmacokinetics are very non-uniform; differences in intensity must be weighed against non-uniform distribution and washout of the fluorophore. In contrast, lifetime techniques rely upon a change in the fluorophore behavior, not the concentration, and thus will provide good results as long as a sufficient signal exists.

The characteristic fluorescence lifetime is the mean time that a specific type of fluorophore spends in an excited state before emitting a photon. Intensity at the fluorophore emission wavelength falls off exponentially, $I_f = I_0 \exp(-t/\tau)$,

where I_0 is the initial intensity after the fluorophores receive a brief pulse of light at the exciting wavelength, t is the time after the pulse and τ is the lifetime. Thus, lifetime may be estimated by fitting time-resolved measurements of intensity to the exponential model.

The lifetime may be dependent on environmental factors such as pH, temperature, or the presence of specific molecules such as oxygen [1]. When the relationship between lifetime and a specific environmental factor is known, the local environment may be quantified by measuring the lifetime of fluorophores at the site. Thus, fluorescence lifetime methods may be used as remote probes; for example, metabolic processes indicated by changes in pH. This technique has been applied in transparent media, such as cells under a microscope. Extending this technique to probe deep into tissue is made difficult by multiple scattering of light. After traveling one millimeter in tissue, photons enter a diffusion-like state in which there is large dispersion in the path lengths of photons traveling between two points. This path length dispersion introduces variability in times-of-arrival of photons at a detector, making it impossible to measure lifetime by an inverse exponential-based method.

For fluorescence lifetime imaging in tissue, the inverse method must reconstruct the optical parameters of the tissue, as well as the fluorescence properties at each site of interest in the tissue. The minimum required number of intensity measurements on the surface of the tissue is determined by the number of unknown parameters. Several researchers [2, 3, 4, 5, 6] have developed frequency-domain methods for extracting a lifetime map that are based on the diffusion approximation to transport theory. For time-resolved measurements, those methods require large data memory space and complicated numerical methods to solve. We have taken a different approach and used a random-walk-based method to arrive at analytical solutions for fluorescence intensity that suggest a much simpler method for quantifying fluorescence lifetime at sites in highly turbid media [7].

Our method uses a unique linearizing function both to quantify lifetime and to spatially localize the source of particular lifetime differences in tissue-like media. In this paper, we will show how this function is able to extract lifetime from a specific site in the tissue using just two sets of intensity measurements. We will look only at the performance obtained from single measurements of contrast. We believe that the additional information present in multiple contrast measurements may be used to obtain better localization and lifetime quantification and still have lower data memory requirements than approaches based on the diffusion approximation. Those enhancements will be necessary before this technique may be used to obtain useful information about metabolic activity from tissue sites deep in the body.

2 Results

To quantify lifetime in highly scattering tissue, one must have a model that describes photon transit properties. Subtracting the characteristics of photon transit will leave only those aspects related to fluorescence; for example, lifetime,

concentration, and quantum efficiency. We begin by examining the case of a single fluorophore site in the tissue and then extend the problem to account for many fluorophore sites.

For a single isolated fluorophore as shown in Fig. 1, the probability of a detected fluorescent photon is the product of the probabilities of three events: a source photon reaches the fluorophore site; the source photon has a fluorescent encounter that results in the emission of a photon; the emitted photon reaches the detector. For time-resolved measurements, there is a duration associated with each of those three probabilities. The product of the three probabilities yields a time-of-flight plot that provides the probability of photon arrival at a detector for each point in time.

Fig. 1. For a single fluorescent lifetime contrast measurement, two isolated fluorophore sites are required (fluorophore isolated to dashed box regions), contrast is obtained from two measurements (at locations r and r') where the fluorophore site at depth d is centered between a source and detector separated by ρ and contrast is a function of difference in lifetime, $\tau_d - \tau'_d$, between the two sites

The problem may be separated into a transport component and a fluorescent component. The transport part includes only the first and third events as described above. The probability of a photon starting at the source, passing through the fluorophore site and arriving at the detector may be expressed in the frequency domain as the product of two Green's functions [8, 9], one describing the probability of motion from the source to the fluorophore site, $\hat{G}_1$, and the other the motion from the fluorophore site to the detector, $\hat{G}_2$. In the time-domain, we will define the inverse Laplace transform of the product as W_t :

$$W_t \equiv L^{-1}\{\hat{G}_1\hat{G}_2\} \quad (1)$$

where W_t may be shown to be analogous to the point-spread-function (PSF) of an imaging system [10, 11]. W_t is dependent on the source-detector separation, fluorophore site, and separate absorption coefficients, μ_{ai} for the source wavelength and μ_{ae} for the fluorescence emission wavelength, as well as the respective scattering coefficients μ'_{si} and μ'_{se} .

There are three probabilities in step two that describe fluorescence at the fluorophore site. First is the probability that a photon at the fluorophore site will interact with the fluorophore. This probability, μ_{af}/μ'_{si} , is a function of fluorophore concentration, μ_{af} , also known as the fluorescence absorption coefficient.

Next is the probability of a fluorescent emission given the interaction, Φ , which is the quantum efficiency of the fluorophore. The third component quantifies the mean delay while the fluorophore is in the excited state which is the fluorescence lifetime. The lifetime of fluorophores that emit in the near-infrared, where tissue has low absorption and a lifetime probe may be used to quantify deep tissue properties, is typically 10 to 1000 ps. Making the assumption that fluorescence lifetime is small compared to typical transit times, which is appropriate for deep sites [7], we may write the probability of a fluorescent photon from an isolated fluorophore located at s , reaching a detector located at r given a source at r_0 as a function of time, t :

$$\gamma(t, r, s, r_0) = \frac{\mu_{af}}{\mu'_{si}} \Phi \left[W_t - \tau c \mu'_{si} \frac{dW_t}{dt} \right] \quad (2)$$

where W_t is dependent on the spatial variables, r, s, r_0 and c is the speed of light in tissue. Thus, the effect of lifetime is proportional to the time derivative of the PSF. For 100 ps lifetime fluorophores, source-detector separations should be approximately 10 mm or greater to ensure that transit times remain short compared to lifetime.

The intent of lifetime methods is to use time-resolved measurements of intensity on the tissue surface to infer lifetime differences occurring within the tissue. To extend Eq. 2 to the case where the fluorophores are uniformly distributed throughout the tissue, fluorescence-based absorption, μ_{af} , must be added to the μ_{ai} absorption term used in W_t to account for increased absorption during photon transit through the tissue. With this change, the intensity from a collection of fluorophores may be expressed as the sum of contributions from each fluorophore site, s , in the tissue. If measurements are made at two or more locations with the same source-detector distance, ρ , we may define contrast as the difference in intensity between two detector locations, r and r' as shown in Fig. 1, as

$$C(t, r, r') = \sum_s \gamma(t, r, s, r_0) - \sum_{s'} \gamma(t, r', s', r'_0) \quad (3)$$

where contrast is also a function of time. We will examine in detail the case where the optical and fluorescent characteristics of the tissue at two locations are identical except for two sites, s_d and s'_d , each located at depth d below a point midway between their respective sources and detectors. The lifetimes at those sites are different: τ_d and τ'_d respectively. Given only the intensity measurements from the two detector positions, we would like to quantify both the lifetime perturbation, $\tau_d - \tau'_d$ and the fluorophore location. From Eqs. 2 and 3, we may now write

$$C(t, r, r') = \mu_{af} \Phi (\tau_d - \tau'_d) c \frac{dW_t}{dt} \quad (4)$$

If contrast is plotted as a function of dW_t/dt , the result will be linear with a slope of $\mu_{af} \Phi (\tau_d - \tau'_d) c$. Since W_t is a function of position, including depth, one may try numerous positions until the result is linear, yielding the actual position. In practice, this requires prior knowledge of tissue scattering and absorption coefficients. These values may be estimated using diffuse reflectance

measurements at the source and emission wavelengths concurrently with the fluorescence contrast measurements. Given typical tissue optical properties [12] where $\mu'_{si} = \mu'_{se} = 1/\text{mm}$ and $\tilde{\mu}_a = 0.005/\text{mm}$, Fig. 2 shows how the function yields various curves for estimated positions other than the correct one corresponding to 10 mm depth. Lateral offsets, as well as offsets in depth, will produce non-linear plots. Since small lateral offsets in detector position may be used to determine the lateral position of a fluorophore site and are required to image, we will consider the more difficult problem of estimating the site's depth.

Fig. 2. Contrast plotted as a function of dW_t/dt for estimated depths of 5, 10, 13, 15, 20, and 25 mm. At the actual fluorophore depth of 10 mm, the plot is linear and passes through the origin

Using linear regression, the coefficient of regression, R^2 , may be used as a measure of linearity. An R^2 value near 1.0 will indicate a good estimate of fluorophore depth. For a number of source-detector separations, R^2 is plotted as a function of estimated depth for a fluorophore at actual depths of 10, 15 and 20 mm in Fig. 3. The R^2 values for all source-detector separations peak at the actual fluorophore depth. In addition, there are secondary peaks at depths both greater and smaller than the actual fluorophore depth. These secondary peaks, however, correspond to negative differences in lifetime; the sign indicates whether the lifetime at the site is shorter, or longer than the reference site. Under the assumption of uniform fluorophore concentration (which was used to obtain Eq. 4), however, the line should pass through the origin. At the secondary peaks, the y intercept is much larger than at the primary peak as may be seen in Fig. 2. For example, the line with a positive slope at an estimated depth of 25 mm has a larger y intercept than the line corresponding to the correct depth of 15 mm (which passes through the origin as expected). Thus, the actual fluorophore depth is unambiguous despite the presence of secondary local peaks in R^2 .

Since intensity measurements may be obtained using a variety of source-detector separations, we would like to know the optimum separation given a fluorophore site at a particular depth. It may be seen in Fig. 3 that some source-

Fig. 3. Linear regression R^2 as a function of estimated fluorophore depth for source-detector separations of 12, 18, 24 and 30 mm and actual fluorophore depths of 10, 15 and 20 mm. Peak R^2 values occur when the estimated depth is the fluorophore's actual depth. An optimum source-detector separation exists for each fluorophore depth which yields a sharp and narrow peak in R^2 when the estimated and actual fluorophore depth are in agreement

detector separation curves peak more sharply at the actual fluorophore depth than others. For a fluorophore at 15 mm depth, a source detector separation of 24 mm has a very sharp peak at the fluorophore depth of 15 mm. This implies that the PSF is able to filter very selectively for the lifetime of a fluorophore at 15 mm depth which may be useful given noisy data. By contrast, the much broader 30 mm source-detector separation curve implies that the PSF is less selective for the 15 mm deep fluorophore site. As may be seen in Fig. 3, for fluorophores at greater depths, the most selective source-detector distance is smaller and for shallower depths, the optimum source-detector distance is larger.

3 Conclusion

We have presented a method for obtaining localized fluorescence lifetime differences from sites deep within highly scattering, tissue-like media. Both scattering and fluorescence lifetime create a spread in photon times-of-arrival at a detector. This spread precludes the use of simple measures of fluorescence lifetime. Instead, one must first model the spread that results from photon transit to and from the fluorophore site. The analytical method we have used is based on random walk theory and starts by showing the effect on photon times-of-arrival from a single isolated fluorophore. For media with fluorophores distributed throughout, the effect of each site would be added after first adjusting absorption in the tissue to account for fluorescence-based absorption. This method quantifies differences in lifetime at two sites from intensity measurements made near each site. The time-dependent difference in the two intensity measurements is the contrast.

To localize the site within the tissue, we use the time derivative of a specific PSF-like function. Values of this PSF are calculated for each point in the tissue which requires knowledge of the tissue scattering and absorption properties at the source and emission wavelengths. In practice, those values may be obtained from diffuse reflectance measurements at the two wavelengths. Contrast, when plotted versus the time-derivative of the PSF, will be linear for the case of a difference in lifetime at a single site. The value of the slope is a function of the local fluorescence concentration and the difference in lifetime at two sites in the same position relative to the two intensity measurements making up the contrast measurement.

In the presence of multiple fluorophores, each with small differences in lifetime, performance of the method may be shown to degrade. The differences in lifetime at sites other than the one of interest may be considered noise and make small contributions to contrast. Due to the excellent spatial selectivity of the PSF, this noise results in small errors in localization and lifetime quantification; less than 13 percent error with lifetime noise $\sigma = 10$ ps as will be presented in a paper currently being prepared for publication. The ability of the PSF to resolve a lifetime difference at a particular depth is a function of source-detector separation. For each fluorophore depth, there is an optimal source-detector separation.

These results were obtained from single measures of contrast. To construct a lifetime map of the tissue, many measurements will be required. The non-

linearity of the PSF indicates that there may be additional information to be obtained by using multiple measurements, such as the measurements at adjacent points on the tissue surface and measurements at multiple source-detector separations, that may improve the quantification of both location and fluorescence lifetime. Localization of the lifetime perturbation is already impressive and further improvement will make this method a potentially valuable tool for utilizing fluorescence lifetime as a probe of metabolic status in living tissue.

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