Instrumentation and Methods for Time-resolved Diffuse Fluorescence Imaging

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Niksa Valim

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Abstract

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Niksa Valim

Mark Niedre

Diffuse optical tomography (DOT) and fluorescence mediated tomography (FMT) are emerging as valuable tools for non-invasive visualization of native tissue chromophores and fluorescent markers for clinical and preclinical applications. These have been used in a wide range of applications from breast, brain, and joint imaging in humans, to molecular imaging with targeted fluorescent probes and fluorescent proteins in small animals.

Despite this progress, imaging resolution – resulting from the intrinsically high degree of photon scatter in biological tissue – is a significant limitation in DOT and FMT. Time-resolved measurement of early-arriving photons from a pulsed laser has been shown to be a powerful approach to improve tomographic resolution. However, the quantitative improvement obtainable using this approach was previously poorly understood, for example as a function of target properties and instrument parameters. To study this, we first experimentally measured the time-dependant reduction in photon scatter through biological tissue compared to continuous-intensity photons over a range of arrival times, optical properties, pathlengths, and instrument geometries. This improvement was compared against a number of theoretical models of photon propagation, including the diffusion approximation to the Boltzmann Transport Equation and time-resolved
Monte Carlo models. We then studied the impact of instrumentation on early-arriving photon measurements and showed that this had a significant effect on instrument performance compared to theoretical models. Specifically the effects of i) instrument temporal impulse response function, ii) detector sensitivity and iii) detected signal levels were studied. Based on this work, an improved early-photon tomography prototype was developed and validated in our lab.

Complimentary to this work, we studied time-resolved and multispectral analysis of FMT data sets with multiple fluorescent targets. In general, the narrow (~200 nm) “diagnostic window” in biological tissue in the red and NIR wavelength range, combined with the broad emission spectra of organic fluorophores limits the number concurrent fluorescent targets in an imaging system to one or two. We developed a novel instrument that allows simultaneous collection of spectral and temporal data sets. Chemometric analysis of joint temporal and spectral data sets allowed us to separate, quantify, and image up to five fluorochromes concurrently with 93% accuracy. This significantly outperformed more conventional methods, where only spectral or temporal information was used independently.
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## Contents

Chapter 1 .......................................................................................................................... 1
Introduction ......................................................................................................................... 1
  1.1 Introduction .................................................................................................................. 1
  1.2 Limitations of Diffuse Optical Tomography .............................................................. 6
  1.3 Light Propagation in Biological Tissue ......................................................................... 9
  1.4 Fluorescence .............................................................................................................. 13
  1.5 Contribution of This Thesis ....................................................................................... 15

Chapter 2 .......................................................................................................................... 17
Experimental measurement of time-dependant photon scatters for diffuse optical tomography . 17
  2.1 Introduction .................................................................................................................. 17
  2.2 Methods and Materials ............................................................................................. 18
    2.2.1 Instrumentation .................................................................................................... 18
    2.2.2 Optical Phantom and Data Collection .................................................................. 20
    2.2.3 Data Analysis ...................................................................................................... 21
    2.2.4 Analytical Calculation of PDSFs ....................................................................... 22
    2.2.5 Monte Carlo Simulation ...................................................................................... 23
  2.3 Results ........................................................................................................................ 23
    2.3.1 Analysis of PDSF at Different Time Gates ......................................................... 23
    2.3.2 Effect of Chamber Path Length ......................................................................... 25
    2.3.3 Effect of Media Optical Properties .................................................................... 28
    2.3.4 Fluorescence PDSF ............................................................................................ 30
    2.3.5 Instrument Noise Performance at Different Time Gates ...................................... 31
    2.3.6 Theoretical Modeling ......................................................................................... 33
  2.4 Discussion and Conclusions ....................................................................................... 35

Chapter 3 .......................................................................................................................... 38
Instrumentation Consideration for Measurement of Early Arriving Photons in Diffuse Optical Tomography ........................................................................................................... 38
  3.1 Introduction .................................................................................................................. 38
3.2 Methods and Materials ........................................................................................................... 39
  3.2.1 Instrumentation .................................................................................................................. 39
  3.2.2 Data Collection and Analysis ............................................................................................ 40
  3.2.3 Effect of Source and Detector Geometry on Instrument PDSF ............................................. 41
  3.2.4 Effect of Detector Sensitivity on Instrument PDSF ............................................................. 42
  3.2.5 Effect of light intensity on instrument PDSF ...................................................................... 43
3.3 Results ..................................................................................................................................... 43
  3.3.1 Effect of Source and Detector Geometry on Instrument PDSF ............................................. 43
  3.3.2 Effect of detector sensitivity on instrument PDSF ............................................................... 46
  3.3.3 Effect of Illumination Intensity on Instrument PDSF .......................................................... 47
3.4 Discussion and Conclusions .................................................................................................... 48
Chapter 4 ...................................................................................................................................... 50
The Effect of Temporal Impulse Response on Experimental Reduction of Photon Scatter in
Time-resolved Diffuse Optical Tomography .................................................................................. 50
  4.1 Introduction ............................................................................................................................. 50
  4.2 Methods and Materials .......................................................................................................... 52
    4.2.1 Instrumentation .................................................................................................................. 52
    4.2.2 Modulation of the instrument TIRF .................................................................................. 54
    4.2.3 Experimental Measurement of the instrument PDSF .......................................................... 55
    4.2.4 Time-resolved Monte Carlo Simulations .......................................................................... 56
    4.2.5 Measuring PDSFs with a fast Avalanche Photo Detector .................................................. 57
  4.3 Results ................................................................................................................................... 59
    4.3.1 Varying System Temporal Impulse Response Function with Multimode Optical Fibers .......................................................... 59
    4.2.3 Measurement of Instrument Photon Density Sensitivity Functions ..................................... 62
    4.3.3 MC analysis of Time-resolved Instrument PDSFs ............................................................... 67
    4.3.4 PDSFs measurement at early time points with APD .......................................................... 69
  4.4 Discussion and Conclusions .................................................................................................... 74
Chapter 5 ...................................................................................................................................... 78
Time-resolved Multispectral Fluorescence Imaging ...................................................................... 78
  5.1 Introduction ............................................................................................................................. 78
List of Figures

Figure 1.1 (a) Reflectance mode where re-emitted light is collected on the same side as the illumination light. (b) Trans-illumination shines light through the volume of object and transmitted light is collected on the opposite side.[14] ................................................................. 2

Figure 1.2 (a) Continuous wave optical tomography image of cerebral ischemia in a rat. (a-f) The images show coronal cross sections of changes in oxy-, deoxy- and total hemoglobin through the rat brain midway as labeled in figure (g). (g) 12 detectors were placed on the top of rat head[2]. 3

Figure 1.3 (a) Illustration of the tumor location. (b) the gadolinium enhanced sagittal MR image slice that positioned tumor around y = 5 cm position in the DOT configuration. (c) Fluorescence trans-illumination image obtained from patient. (d) total hemoglobin concentration, blood oxygen saturation, reduced scattering coefficient, and fluorescence image slices at y = 5 cm are displayed (e) with their values along a horizontal line passing through the center of tumor [18]. 5

Figure 1.4 Light propagation through a diffusive media at different time points.......................... 8

Figure 1.5 The optical window that shows relative absorbance of biological component at red and NIR wavelength. ................................................................. 10

Figure 1.6 Jablonski diagram illustrating fluorescence process. The fluorescent molecule absorbs the excitation photon and moves to a higher level of energy S1’. Part of the energy is dissipated by a non-radiative transfer to the energy state S1. The electron relaxes to the ground state by emission of longer wavelength fluorescent photon. ................................................................. 14

Figure 2.1 Schematic of the system used to measure time-dependant PDSFs. ......................... 18

Figure 2.2 The measured temporal impulse response function of the system. The system TIRF FWHM was 163 ps ................................................................. 19

Figure 2.3 (a) Measured time-resolved intensity for a pulse transmitted through a 2-cm wide chamber filled with a homogenous solution of 1% Intralipid and 50 ppm ink. The curve was analyzed at each rod position to give the instrument PDSF at different time points (see text for
details). Example PPDSFs at (b) the 50% point on the rising edge of the transmitted time-
resolved curve, (c) the peak of the curve, (d) and the 50% point on the falling edge of the time-
resolved curve. (e) The FWHM of the PDSF as a function of measurement time. Reduction of
PDSF FWHM of more than 50% was observed for early photons versus quasi-CW photons..... 25

**Figure 2.4** Effect of chamber path length on (a) the absolute PDSF FWHM at different time gates
and (b) the relative FWHM of the PDSF time gate at 5% and 50% points on the rising edge of
the full-time curve compared to the quasi-CW PDSF FWHM. In all cases 1% Intralipid and 50
ppm ink were used. ........................................................................................................... 27

**Figure 2.5** Effect of (a) changing the Intralipid concentration (ink concentration was 50 ppm in
all cases) and (b) changing the ink concentration (Intralipid concentration was 1% in all cases)
on the relative PDSF FWHM at different time points. .................................................................. 29

**Figure 2.6** Measured PDSF FWHM as a function of the quasi-CW width for a 2-mm-diam tube
filled with Alexafluor-680. .................................................................................................... 31

**Figure 2.7** SNR at different time gates for 0.2- and 1.0-sec acquisition times. In both cases, the
photon counting rate was 1×106 photons/sec. ........................................................................... 32

**Figure 2.8** Theoretical system PDSF FWHMs for the rising and quasi-CW portion of the curves
calculated using (a) time-resolved Monte Carlo simulations and (b) the time-resolved diffusion
approximation to the BTE ......................................................................................................... 34

**Figure 3.1** (a) Schematic of instrument used to measure system PDSFs, (b) example measured
time-resolved intensity for a pulse transmitted through a 2-cm chamber length which was filled
with a liquid phantom with optical properties of μs’=17 cm⁻¹ and μa=0.15 cm⁻¹ .................. 42

**Figure 3.2** Example measured instrument PDSFs for 0 cm (a-c) and 2 cm (d-e) source and
detector offset distances, at the (a, d) 50% point on the rising edge of the time-resolved curve,
for (b, e) un-gated, quasi-CW photons, and for the (c, f) 50% point on the falling edge of the
time-resolved curve ................................................................................................................. 44
Figure 3.3 The absolute (a) and relative (b) FWHM of the instrument PDSF is summarized as a function of source and detector offset distance. ................................................................. 45

Figure 3.4 (a) The relative FWHM of the PDSF for photons arriving at the 10 % and 50 % points on the time-resolved curve for varying detector sensitivity (gain). (b) The relative FWHM of the PDSF for photons arriving at the 10 % and 50 % points on the time-resolved curve for varying detected signal levels (photon count rates). ................................................................................. 46

Figure 4.1 Schematic of instrument used to measure system PDSFs. Two multimode optical fibers of varying lengths were coupled at the system input and output to alter the instrument temporal impulse response function. .............................................................................................................. 53

Figure 4.2 Schematic of the experimental system used for evaluation of SPAD detector in measurement of EPs. Abbreviations: lens 1,2 (L1, L2), filter (F), inverter and attenuator (I/A). 58

Figure 4.3 (a) Measured system TIRFs for 1, 5, 10, and 20 m source and detector fiber lengths. The effect of fiber length from 1 m to 20 m on system TIRF FWHM for varying (b) SFL, (c) DFL, and (d) SFL and DFL. .......................................................................................................................... 61

Figure 4.4 (a) Example measured diffusely transmitted light through the liquid phantom for 1, 5, 10, and 20 m source and detector fiber lengths. Example measured photon density sensitivity function for 1 m source and detector fiber lengths (b) at the 50% point on the rising edge of transmitted time-resolved curve, (c) quasi-CW photons, and (d) and at the 50% point on the falling edge.............................................................................................................................................. 63

Figure 4.5 The FWHM of the middle plane of PDSF at early time points (fraction of the peak point) for varying (a) SFL, (b) DFL, and (c) SFL and DFL. (d) The FWHM of the middle plane of PDSF vs. the measured rise time for varying SFL and DFL....................................................................................... 65

Figure 4.6 a) Convolution of the output of time-resolved MC simulations with experimentally measured instrument TIRFs at different points on the rising edge of the transmitted curve. The effect of TIRF on broadening of the instrument PDSF at the middle plane of the imaging...
chamber are shown for MC calculations, and experimental measurements at the (b) 10% and (c) 50% of maximum on the rising edge of the time-resolved curve.

**Figure 4.7** (a) Measured instrument TIRF. The FWHM was 59 ps, (b) Measured TR curve through diffusive liquid phantom. The SPAD diffusion tail is evident (arrow, blue curve) compared to the TR curve measured with a PMT through the same media.

**Figure 4.8** (Colored online) (a) Measured TR curve through liquid phantom. (b) Measured PDSF profile in the center of the imaging chamber at 5 time points corresponding to the red arrows in (a), illustrating increasing photon scatter with time. (c) PDSF FWHM versus time. Error bars represent the standard deviation from 3 trials. (d) The relative PDSF FWHM (normalized to quasi-CW) as a function of time on the rising edge of the TR curve (normalized to fraction of peak) measured with the SPAD and obtained with TR-MC simulations. For comparison, the same curve measured with a PMT (TIRF FWHM = 163 ps) is shown.

**Figure 4.9** (Colored online) (a) Cylindrical cast resin phantom with two absorbing inclusions. (b) TR measurements were made as the phantom was rotated through 360°. (c) Normalized measured intensity as a function of rotation angle for 4 selected time points along with quasi-CW data.

**Figure 5.1** Schematic of the system used in time-resolved multiplexed imaging with solid phantom.

**Figure 5.2** The measured fluorophore RL used in time-resolved multiplexed imaging.

**Figure 5.3** (colored line) Calculated fluorophore RL (blue line) compared with measured fluorophore RL (red line).

**Figure 5.4** Mean concentration error of different fluorophore combinations based on i) spectral and temporal data, ii) purely spectral data, and iii) purely temporal data for simulated multiplexed data.

**Figure 5.5** Effect of signal to noise ratio on the mean concentration error of joint spectral and temporal data, pure spectral data, and pure temporal data, with simulated data. The equivalent
mean concentration error of the measured multiplexed data with phantom is shown in the figure.

Figure 5.6 An example of measured multiplexed imaging with tissue mimicking solid phantom.

Figure 5.7 Mean concentration error of different fluorophores combinations based on i) spectral and temporal data, ii) spectral data, and iii) temporal data for phantom measurements.

Figure 5.8 An example of measured multiplexed imaging in mice using joint spectral and temporal information.
To my beloved family and mentors.
Chapter 1

Introduction

1.1 Introduction to Diffuse Optical Tomography and Fluorescence Mediated Tomography

Diffuse optical tomography (DOT) and fluorescence mediated tomography (FMT) are emerging as valuable tools for non-invasive visualization of native tissue chromophores and fluorescent markers in humans and in laboratory animals [1-3]. These have been used in a wide range of biomedical applications from clinical-scale breast, brain, and joint imaging [4-6], to pre-clinical small animal molecular imaging with targeted fluorescent probes and fluorescent proteins [7-12]. DOT and FMT instrumentation require measurement of photons that propagate through bulk biological tissue in the diffusive regime either in transmittance or reflectance (epi-illumination) modes [2, 13]. Figure 1.1 (a) and (b) show the two common imaging geometric orientations, i.e. reflection and transmission modes. In reflectance mode, the source and detector pair are placed in one side of diffusive media, i.e. the light shine in through the diffusive medium and re-emitted light collects on the same side. In trans-illumination technique, diffusive media is surrounded by source and detector; the propagated photons exit the volume of the object and collects by the detector on the opposite side.
Figure 1.1 (a) Reflectance mode where re-emitted light is collected on the same side as the illumination light. (b) Trans-illumination shines light through the volume of object and transmitted light is collected on the opposite side.[14]

Images are obtained by relating measurements to the unknown quantity of interest (e.g. tissue optical properties or fluorescence concentrations) with physical models of light propagation through diffusive tissue, and then solution of the subsequent inverse problem to obtain tomographic image [15].

An example of DOT in small animal imaging is shown in figure 1.2. These figures show the application of optical imaging for cerebral ischemia study in a rat [2, 16, 17]. The main goal of this study was to study the concentration of oxyhemoglobin [HbO2], deoxyhemoglobin [Hb], and hemoglobin concentration [THb] = [HbO2] + [Hb]. In this work, absorption coefficient ($\mu_a$) is assumed as a linear combination of [HbO2] and [Hb]:

$$\mu_a^\lambda = \varepsilon_{Hb}^\lambda[Hb] + \varepsilon_{HbO}^\lambda[HbO]$$

Therefore, the concentration of [Hb] and [HbO2] was measured by reconstruction of $\mu_a$ at two wavelengths.
The imaging instrument operated in continuous wave mode with two different laser diodes operating at two different wavelengths (760 and 830 nm) [2, 16, 17].

**Figure 1.2(a)** Continuous wave optical tomography image of cerebral ischemia in a rat. (a-f) The images show coronal cross sections of changes in oxy-, deoxy- and total hemoglobin through the rat brain midway as labeled in figure (g). (g) 12 detectors were placed on the top of rat head[2].

An example clinical fluorescence optical imaging application is shown in figure 1.3 (a-e) [18]. Fig. 1.3a indicates a palpable mass in a breast tissue of female patient. The optical imaging system consisted of four sinusoidal intensity modulated laser diodes (70 MHz) operating at 650, 690, 786 and 830 nm. Optical measurements were made with a CCD camera.

These were performed in three stages: i) the intrinsic optical and physiological properties of the breast tissue were obtained from a full scan using four wavelengths and 45 sources positions that directed to the sample via fiber optics, ii) fluorescence imaging of ICG (0.125 mg/kg) using just
one excitation wavelength at 786 nm, and iii) the spectral filters were removed, and a baseline measurement were conducted at four wavelengths.

The location of the mass was around 8 o’clock (y = 5 cm) and the dimension of the mass was measured to be around 2.5 cm with ultrasound, mammogram, MRI, and PET. Figure 1.3b shows an MRI sagittal slice of the mass, which is the lighter region on the image. Figure 1.3c shows an example diffuse fluorescence trans-illumination image. The view of the camera was from foot to head, and the right and left side of the image correspond to the lateral and medial sides. Figure 1.3d shows a selected slice (y=5 cm) from the full 3D diffuse optical tomography reconstructed image. This figure shows the important optical parameters in a tissue, i.e. hemoglobin concentration, blood oxygen saturation, diffuse scattering coefficient, and fluorescent concentration. [18].
Figure 1.3(a) Illustration of the tumor location. (b) the gadolinium enhanced sagittal MR image slice that positioned tumor around $y = 5$ cm position in the DOT configuration. (c) Fluorescence trans-illumination image obtained from patient. (d) total hemoglobin concentration, blood oxygen saturation, reduced scattering coefficient, and fluorescence image slices at $y = 5$ cm are displayed (e) with their values along a horizontal line passing through the center of tumor [18].
1.2 Limitations of DOT and FMT

In a DOT or FMT imaging system, the tissue is illuminated with one or several source array(s) and the diffused light exiting the tissue is measured with several detectors positioned around the tissue in either reflectance or trans-illumination geometry.

The goal is to reconstruct the optical properties of the biological tissue (in DOT) or the concentration of the target fluorophore (in FMT). Image reconstruction in a DOT or FMT imaging system consists of three steps. First, a “forward model” of diffuse photon propagation between a source and detector position is developed (detailed information on forward modeling is provided in section 1.3). Second, a system of linear equations is developed that relates the measurements to the unknown quantity of interest (optical properties of fluorescence concentration) via the forward models. Images are then reconstructed by solving the resulting inverse problem,

\[ \mu = [W]^{-1}[U_{SD}] \]

where \( \mu \) is the reconstructed optical properties (absorption coefficient or scattering coefficient), \( W \) is the forward models, and \( U_{SD} \) is the measured data between source and detector pairs.

Due to the high degree of light scatter in biological tissue, the inverse problem in DOT and FMT is generally ill-posed and ill-conditioned. As such, imaging resolution is poor; estimates vary in the literature, but generally this is understood to be about 10–20% of the imaging length [19]. Generally speaking, the image resolution in DOT and FMT is determined by three factors: i) the physical properties of turbid media, ii) the instrumentation, and iii) the image reconstruction algorithm. Although it has been shown that the image resolution in DOT and FMT can be improved using optimal instrumentations and optimal instrumentation algorithms, imaging
resolution is generally understood to be one of the most significant limitations of FMT and DOT.

[20-22]

Two emerging approaches to improve DOT and FMT imaging resolution are, i) multi-modality imaging, and ii) time-resolved imaging. In multimodality imaging, anatomical information is obtained with a complimentary high-resolution imaging system, such as magnetic resonance imaging (MRI), X-ray computed tomography (CT), and positron emission tomography (PET) [23-25]. The higher resolution anatomical information is incorporated into the optical image reconstruction routine, either through reduction of parameters or by guiding regularization of the inverse problem [26]. This converts highly ill-posed inverse problem to a less ill-posed one. Although a highly promising approach, multimodality imaging relies on expensive instrumentation (relative to DOT and FMT instrumentation) or in the case of X-ray CT potentially hazardous ionizing radiation. Therefore, it is important to design high-resolution imaging system that uses just optical technology.

Second, time-resolved (TR) detection of ‘early-arriving’ or ‘weakly diffuse’ photons has also been shown to improve imaging resolution in DOT and FMT. As shown in figure 1.4, photons that arrive at a detector “early” from a fast (~femto second) pulsed laser travel in less scattered paths between the source and detector pair.
Figure 1.4 Light propagation through a diffusive media at different time points.

As such, the width of the imaging photon density sensitivity function (PDSF; alternatively referred to as the “weight function”, “photon measurement density function”, Jacobian, or “banana function”) is correspondingly reduced. This results in a narrower forward model, a better conditioned image reconstruction problem, and improved imaging resolution. Early work by Feld et al. [27] demonstrated this in localizing fluorescent inclusions with a fiber-coupled streak camera in bulk diffusive media, and the approach was studied by other groups in trans-illumination imaging geometries [28-30]. In recent years, there has been renewed interest in this concept – in our group and others - with the development of new classes of fast time-gated and time-resolved detectors [31-40]. Moreover, although the “early photon” effect been demonstrated previously, significant quantitative details with respect to measurement of early photons was absent in the literature of time-resolved optical imaging. The motivation of this PhD thesis was to better understand photon propagation in biological diffuse media in the
early time regime, with respect to physical and instrumentation considerations, as well as modeling with TR and diffusion theory.

### 1.3 Light Propagation in Biological Tissue

Modeling of light propagation in biological issue is critical for developing “forward models” or “weight functions” between source and detector pairs, and likewise for image formation in DOT and FMT. To characterize the physical behavior of light at red and near infrared wavelengths in biological tissue, optical properties are defined. There are four components that contribute in physical interaction of photons with tissue: the index of refraction($n$), absorption coefficient ($\mu_a$), scattering coefficient ($\mu_s$), and anisotropy of scattering($g$).

- **Index of refraction ($n$)** or refractive index is a dimensionless number that describes how light propagate through the medium.
  \[ n = \frac{c}{v} \]
  , where $c$ and $v$ are the speed of light in vacuum and medium respectively.

- **Absorption coefficient ($\mu_a$)** quantifies the propensity of photon absorption per unit length. Optical absorption in biological tissue originates primarily from hemoglobin, melanin and water.

- **Scattering coefficient ($\mu_s$)** quantifies the propensity of photon scatter per unit length.

- **Anisotropy of scattering ($g$)** or “mean cosine of the scattering angle” quantifies the net directionality of photon scatter in a material.
  \[ g = < \cos \theta > \]
\[ g = 1 \] indicates pure forward scatter, \[ g = -1 \] indicates pure back-scatter and \[ g = 0 \] indicates isotropic scatter.

- The reduced scattering coefficient is defined as
  \[ \mu'_s = \mu_s (1 - g) \]

Therefore, there are two major interactions for each photon that undergo through a tissue, i) absorption and ii) scattering. The extinction coefficient is defined as

\[ \mu_t = \mu_s + \mu_a \]

In biological tissue, photons primarily interact by scattering with tissue molecules (\( \mu_s \gg \mu_a \)), and the mean free path for photon scattering (\(1/\mu'_s\)) is much smaller than the mean absorption length (\(1/\mu_a\)).

![Figure 1.5](image)

**Figure 1.5** The optical window that shows relative absorbance of biological component at red and NIR wavelength.

Figure 1.5 shows the important molecules in biological tissue that contribute to absorption. The “optical window” or “diagnostic window” represents a wavelength range where biological components have their lowest relative absorption and scattering properties and is in the red and near infrared region.
When light enters highly scattered medium, the photons undergo a random walk. Two important models of light transport through diffuse media are described as follows:

1) Diffusion Approximation to the Boltzmann Transport Equation

Photon transport in tissue can be analytically modeled by diffusion approximation to the Radiative Transfer Equation (RTE) or Boltzmann Transport Equation (BTE). The RTE equation is written as [19]

$$\frac{\partial L(\vec{r}, \hat{s}, t)}{\partial t} = -\hat{s} \cdot \nabla L(\vec{r}, \hat{s}, t) - \mu_t L(\vec{r}, \hat{s}, t) + \mu_s \int_{4\pi} L(\vec{r}, \hat{s}', t) P(\hat{s}', \hat{s}) d\Omega' + S(\vec{r}, \hat{s}, t) \quad (1.1)$$

where $L/c$ represents the propagation energy (expected number of photons) per unit volume per unit solid angle, $\vec{r}$ denotes position, $\hat{s}$ denotes unit direction vector, and $t$ denotes time.

In spherical coordinates,

$$\hat{s} = (\sin\theta\cos\phi, \sin\theta\sin\phi, \cos\theta) \quad (1.2)$$

where $\theta$ and $\phi$ denote the polar and azimuthal angles respectively, and $\mu_t$ represents the extinction coefficient which is the summation of absorption or scattering events.

$P(\hat{s}', \hat{s})$ is called the “phase function” and physically $P(\hat{s}', \hat{s}) d\Omega'$ is the probability of light scatter from $\hat{s}'$ into $\hat{s}$ in $d\Omega'$, and $S(\vec{r}, \hat{s}, t)$ represents the source of energy.

Since the RTE has six independent variables $(x, y, z, \theta, \phi, t)$, mathematically it is difficult to solve for all but the simplest situations. The diffusion approximation is a simplification of the BTE which is frequently used to model photon propagation used in biological tissue since it is significantly easier to solve analytically. The diffusion equation can be written as:

$$\frac{\partial \Phi(r, t)}{c \partial t} + \mu_a \Phi(r, t) - D \nabla^2 \Phi(\vec{r}, t) = S(\vec{r}, t) \quad (1.3)$$

Where $\Phi(r, t)$ is fluence; $c$ is the speed of light; and $D$ is the diffusion coefficient.
It should be noted that diffusion equation approximates the RTE only if three assumptions are held:

1) The media has much stronger scattering property compared with absorption property \((\mu_s \gg \mu_a)\)

2) Photon propagation is considered far away (at least ten times of transport mean free path) from the source or boundaries.

3) The time for substantial fluence density change is much longer than the time to transverse one transport mean free path. \((t \gg 0)\)

In biological tissue the mean scattering path is much smaller than the mean absorption path and the above mentioned conditions are valid and the diffusion approximation is generally valid for modeling light propagation in biological tissue. Time-independent (continuous wave), time-dependant, and frequency-domain solutions of the diffusion approximation have been described in the literature. However – and of particular interest for this thesis – due to the above assumptions the diffusion approximation is not a valid approach for modeling early-arriving photon propagation. The time-resolved diffusion approximation to the BTE and experimentally disagreement with photon propagation measurements at early times is discussed in more detail in chapter 2. We also showed that time-resolved Monte Carlo simulations (described in the next section) are significantly more accurate in modeling propagation of early photons.

2) Monte Carlo Simulations

Alternatively, photon transport in biological tissue can be modeled by Monte Carlo (MC) simulation. Briefly, in an MC simulation, a large number of photons are initiated at the surface of
the sample. The random photon walk – including step-size, absorption, scatter, direction, specular reflections at boundaries – are modeled stochastically. Each individual photon (or photon packet) is tracked through the media until it gets absorbed or exits the media, and quantities of interest are tracked. By running a sufficiently large number of photons in the simulation, group behavior is accurately modeled. Comparing with the diffusion approximation, MC simulations can be performed for media with any optical properties, i.e. there is no need to have $\mu_s \gg \mu_a$ assumption in the media. Arbitrary geometries with heterogeneous optical properties can be modeled. Moreover, there is no need to trace photons far away from the boundaries and accurate results are obtained near source and detector. Most importantly for this work, photon behavior at early time points is accurate relative to the BTE. Therefore, time-resolved MC is significantly more accurate for computation of weight functions in early-photon FMT and DOT. The main disadvantage of MC over the diffusion approximation is that it is computationally expensive, and for complex simulations may require long computation times.

1.4 Fluorescence

The fluorescence process and its corresponding Jablonski energy diagram are shown in figure 1.6. The sample (fluorophore) under study is illuminated at a wavelength tuned to the excitation spectra of the molecule. The excitation photon elevates an electron from ground state ($S_0$) to an excited state energy ($S_1'$). The exited electron remains in excited state for a finite time, and then rapidly relaxes and reaches relaxed excited state energy ($S_1$) via non-radiative transfer [41]. As electrons in energy level ($S_1$) decay to ground state they emit photons with lower energy level relative to the excitation energy level. Therefore, fluorescence process is a red-shifted (lower
energy), i.e. the emission wavelength is longer than the irradiated wavelength. The mean time that a fluorophore remains in the excited state after excitation is called fluorescence life time ($\tau$ between 0.1-1ns).

**Figure 1.6** Jablonski diagram illustrating fluorescence process. The fluorescent molecule absorbs the excitation photon and moves to a higher level of energy $S1'$. Part of the energy is dissipated by a non-radiative transfer to the energy state $S1$. The electron relaxes to the ground state by emission of longer wavelength fluorescent photon.

The use of fluorescence in studying cellular and molecular events such as gene expression, protein function, and protein-protein interaction has been established using optical imaging systems. Fluorescence labeling of proteins allows detection of multiple targets in the same sample, or the study of multiple molecular functions in a single cell or biological organ [42-44]. High resolution microscopy imaging techniques have been developed and have enabled studying of molecular events in cell cultures in vivo. Since absorption of light is not a significant effect at microscopic scales and these imaging systems can use the whole visible spectrum of light and several fluorescence targets can be selected and imaged in this range with no or little spectral overlap.

However, in order to perform multiplexed fluorescence imaging in a deep biological tissues (i.e. whole animals or in humans) the choice of fluorophores is limited to those with spectral emission
in the red and NIR wavelengths, i.e. inside the diagnostic window in biological tissue, fig 1.5. In such a case, the spectral properties of multiple fluorophores (e.g. more than two) are highly overlapped and the separation of fluorophore components in a multiplexed image is challenging. Solving this problem with joint temporal and spectral data sets is the second major focus of this thesis (Chapter 5).

1.5 Contribution of This Thesis

The organization of this thesis is outlined briefly as follows. In chapter 2, the quantitative reduction on the instrument photon density sensitivity function (PDSF) and the tomographic image reconstruction problem using early arriving photons versus quasi-CW photons was studied in detail. The sensitivity of this effect with respect to physics of the system, including time-gate following the laser pulse, optical path-length, and optical properties was studied. This work was published in Journal of Biomedical Optics in 2010 [34]. Experimentally we observed a “leveling out” of the effect at very early times. However, time-resolved Monte Carlo predicted greater degree of improvement using very early time points.

The discrepancy of experimental measurement versus theoretical simulation was then studied in detail in chapter 3. The effect of a series instrumentation (hardware) parameters on the experimentally measured time-dependent PDSF including, source and detector geometry, detector sensitivity, and illumination intensity was quantified. This work was published in the Proceedings of the SPIE in 2012 [45].

Following this, in chapter 4, the impact of the instrument temporal impulse response function (TIRF) or “instrument speed” on PDSF width and imaging resolution was studied in detail.
These results have significant implications, i) in correct calculation of imaging PDSFs for time-resolved DOT and FMT, and ii) in design of time-resolved DOT and FMT systems that utilize the early-photon effect. This work was published in Journal of Physics, Medicine and Biology in 2013 [46]. With respect to the importance of TIRF in the reduction of imaging PDSFs, a new time-resolved imaging system was designed with fast Avalanche Photo Diode (APD). This improved imaging system had a significantly faster TIRF compared our previous imaging system. We demonstrated that the reduction in the width of the PDSF was correspondingly greater than our previous work, and approached the theoretical limit predicted by Monte Carlo simulations. This work was co-performed and co-authored with Ying Mu and was published in Optics Letters in 2013[47].

In chapter 5, time-resolved multispectral detection and imaging of multiple fluorophores in bulk diffusive media was studied. We showed that it was possible to use joint temporal and spectral information of fluorophore emission in a multiplexed imaging to accurately separate fluorophore components. Time-resolved multiplexed fluorescence imaging with up to five fluorophores were tested first in a tissue mimicking phantom and then implemented in mouse. A paper on this material (to be submitted to the Journal of Biomedical Optics) is currently in preparation.
Chapter 2

Experimental measurement of time-dependant photon scatters for diffuse optical tomography

2.1 Introduction

Time-resolved measurement of early-arriving photons through diffusive media has been shown to effectively reduce the high degree of light scatter in biological tissue. However, the experimentally achievable reduction in photon scatter and the impact of time gated detection on instrument noise performance is not well understood. In this work, we measured time-dependent photon density sensitivity functions (PDSF) between a pulsed laser source and a photomultiplier tube operating in time-correlated single photon counting mode. Our data show that with our system, measurement of early-arriving photons reduced the full width half maximum of the PDSF on average by about 40-60% versus quasi-continuous wave photons over a range of experimental conditions similar to those encountered in small animal tomography, corresponding to a 64-84% reduction in PDSF volume. Factoring in noise considerations, the optimal operating point of our instrument was determined to be about the 10% point on the rising edge of the transmitted intensity curve. Time-dependant Monte Carlo simulations and the time-resolved diffusion approximation were used to model photon propagation and were evaluated for agreement with experimental data.
2.2 Methods and Materials

2.2.1 Instrumentation

The system used for time-resolved measurement of instrument PDSFs is shown in figure 2.1. A customized pulsed supercontinuum fiber laser with an 80 MHz repetition rate was used as the light source (Koheras Super K Power, NKT Photonics, Birkerod, Denmark). The laser was modified by the manufacturer so that it had a shortened fiber compared to standard models which reduced the pulse-width to approximately 300 ps.

![Figure 2.1](image-url)

*Figure 2.1* Schematic of the system used to measure time-dependant PDSFs.
Figure 2. The measured temporal impulse response function of the system. The system TIRF FWHM was 163 ps

The output of the broadband laser was passed through a visible-NIR splitter device (SpectraK Split; NKT Photonics) to isolate the 550 to 850 nm section of the pulse and then was further filtered with a 670 nm band-pass interference filter (30 nm FWHM; Chroma Technology, Bellows Falls, VT). It is a property of supercontinuum pulse generation that different temporal positions in the broadband output pulse correspond to different emission wavelengths; therefore wavelength selection with the interference filter reduced the pulse-width to about 30 ps (i.e. reduced by a factor of the ratio of the filter band-pass divided by the full pulse spectral width).

The 670 nm light was focused to a 1 mm spot size at the surface of the sample chamber with a 300 mm focal length achromatic plano-convex lens (Edmund Optics, Barrington, NJ). The width of the custom made chamber could be adjusted between 1.5 and 6 cm and the chamber was filled with liquid media approximating the optical properties of biological tissue at red and NIR
wavelengths (see Section 2.2.2). A 1mm pinhole was placed at the exit plane of the chamber, co-
axial with the input position of the laser. Light from the chamber was collimated with a plano-
convex lens (f = 50 mm; Edmund) and passed through a second 670 nm interference filter.

Transmitted light was measured with a 16-channel photomultiplier tube assembly with a large 16
× 16 mm active cathode area (PML-16-C, Becker and Hickl, Berlin, Germany) connected to a
time-correlated single photon counting card (SPC-130, Becker and Hickl). The temporal
resolution (i.e. time bin width) of the TCSPC card was set to 7 ps in all cases. The PMT
assembly was pre-selected by the manufacturer to have an impulse response time of about 150
ps, suitable for photon counting. For the current experiments, the signals from each channel were
summed after correction for the minor time-skew between channels (about ±75 ps per channel)
to yield a single measurement.

In future experiments we intend to treat the 16 channels independently, for example, by adding
spectroscopic capabilities to our system. The measured instrument temporal impulse response
function (TIRF) is shown in figure 2.2. The full width half maximum of the TIRF was 163 ps,
which agrees well the performance specifications of our laser source (~30 ps) and multichannel
PMT (~150ps).

2.2.2 Optical Phantom and Data Collection

Homogeneous liquid optical phantoms were prepared from a mixture of 20% intralipid stock
solution (Baxter Healthcare Corporation, Deerfield Illinois) and ink (Higgins Ink, Sanford
Corporation, Bellwood, Illinois) which modified the scattering and absorption coefficients of the
solution, respectively. Optical properties were varied over a range that is relevant for red and
NIR imaging in mice [40, 48]; specifically the final intralipid concentration was varied from 1% to 5% (corresponding to approximately $\mu_s' = 17 \text{ to } 85 \text{ cm}^{-1}$ at 670 nm) and the final ink concentration was varied from 50 ppm to 150 ppm (corresponding to approximately $\mu_a = 0.15$ to 0.45 cm$^{-1}$ at 670 nm) [40]. Measurements were repeated at least 4 times for each condition. A 1 mm diameter absorbing rod was translated through the medium (x-step size = 0.25 mm z-step size = 0.5 mm) using computer-controlled stepper motor stages (XSlide, Velmex Inc., Bloomfield, NY) and the transmitted full-time curve was recorded at each point. This allowed us to measure the relative effect of the absorber at each position for each time gate. Acquisition times were 2 s per position and the laser power was adjusted between 0.5 and 10 mW at the sample to give comparable photon count rates for each condition.

To measure the fluorescence PDSF, the absorbing rod was replaced with a 2 mm diameter by 1 cm long tube filled with intralipid (1% intralipid with 50 ppm ink) and 10μM Alexafluor-680 dye (Invitrogen Corporation, Carlsbad, CA) attached to the end of a fine rod. The fluorescent tube was positioned so that the middle was at the same vertical position as the laser. In this case, the filter in front of the PMT was replaced with a 730 nm filter (30 nm bandpass; Chroma) to detect the fluorescent light.

2.2.3 Data Analysis

Full time curves were first acquired for each experimental condition without the absorbing rod in place. In each case, curves were analyzed to find time points corresponding to 0.5%, 1%, 5%, 10% and 50% on the rising and falling edges of the measured time-resolved curves as well as the peak and quasi-CW (integrated) intensities. The rationale here was that this allowed comparison of data for different optical properties and chamber pathlengths (as opposed to absolute time
The length of the full time curves vary significantly between conditions. Signals for each rod position and each time point within a narrow (7 ps) time window were normalized to those from the control signals (i.e. without an absorbing rod in place) and then subtracted from unity. These data were then plotted as a function of rod position to yield the experimental PDSF. Data was then analyzed to determine the FWHM in the middle plane of the PDSF for each condition and time gate.

2.2.4 Analytical Calculation of PDSFs

Analytical calculation of time-dependent PDSFs has been comprehensively described previously in the literature. The photon field at a time $t$ between a source and detector pair following a laser pulse can be written as the weight function[27]:

$$ W(r_s, r_d, t) = \int_0^t U_o(r_s, r_d, \tau) G(r_d, r, t - \tau) d\tau d^3r \quad (2.1) $$

where, $\tau$ is an integrating factor for time, $r$ is the position inside the diffuse media, $r_s$ and $r_d$ are the source and detector positions of each source–detector pair, respectively. The Green’s function $G$ and $U_o$ are normally calculated using an analytical approximation to the BTE. In this work we used the diffusion approximation to the BTE, since it is commonly used in modeling light propagation in diffusive media, for example in small animal tomographic imaging. Importantly, diffusion theory uses the P-1 approximation to the spherical harmonic expansion of the photon radiance and also assumes that all sources emit isotropically [49]. Using the diffusion approximation, it can be shown that the time-resolved photon fluence rate following an infinitely short laser pulse in an infinite medium is [50]

$$ N(r, t) = \frac{1}{(4\pi Dct)^{1/2}} \exp(-r^2 / 4Dct) \exp(-\mu_a t) \quad (2.2) $$
Where \( t \) is the time following the pulse, \( r \) is the position in the media, \( D \) is the diffusion coefficient where \( D = \{3[\mu_a + \mu_s']\}^{-1} \), \( \mu_s' \) is the reduced scattering coefficient, \( \mu_a \) is the absorption coefficient and \( c \) is the speed of light in the media.

### 2.2.5 Monte Carlo Simulation

We developed a set of C-code to calculate time-resolved PDSFs based on the Monte Carlo code publically available on the Oregon Medical Laser Center website [51]. Semi infinite slab geometries of varying thickness (\( l \)), scatter (\( \mu_s \)) and absorption (\( \mu_a \)) were used to model our experimental conditions. In all cases the anisotropy factor \( g \) for intralipid was taken to be 0.85. Photons were launched into the media from a pencil beam source, and the scattered photon path and exit time was logged for all photons that exited the media at a 1 mm by 1 mm “detector” position co-axial from the entry point. In each case, 1 billion photons were tracked, which required about 72 hours of computation on a 3.2 GHz Dual-Core PC running Linux. Paths of photon propagation for the rising edge of the transmitted intensity curve were then analyzed to yield the PDSFs.

### 2.3 Results

#### 2.3.1 Analysis of PDSF at Different Time Gates

The basic analysis performed in these experiments is shown in figure 2.3. As described above, full-time transmitted intensity curves were analyzed to yield time points corresponding to 0.5%, 1%, 5%, 10% and 50% of the peak intensity on the rising and falling edges of the curve. A typical transmitted full time curve for a 2 cm chamber pathlength with 1% intralipid and 50 ppm ink (corresponding to \( \mu_s' = 17 \text{ cm}^{-1} \) and \( \mu_a = 0.15 \text{ cm}^{-1} \)) is shown in figure 2.3(a). These data
were analyzed as described above to yield the PDSF at each time point. Example measured PDSFs at the 50% rise, peak, and 50% decay points along the curve are shown in figure 2.3(b-d), respectively. As expected, the plots became broader with time due to the photon scatter following the laser pulse.

As a metric of the extent of this photon scatter, the full-width half maximum (FWHM) of the PDSF at the middle plane (i.e. where photon scatter was maximal) of the sample chamber was measured. The FWHM of the PDSF as a function of time gate is shown in figure 2.3(e). In this case, the FWHM of PDSFs for time gates earlier than the 10% rise point of the TPSF was 48% ± 8% of the quasi-CW width. Since this represents the reduction in the PDSF radius, the corresponding reduction in the three-dimensional PDSF volume was therefore approximately 77%. The quasi-CW FHWM measurement here corresponds to the first moment of the time-resolved curve in figure 2.3(a) (as opposed to the peak), so that the peak of the curve is actually slightly “early” compared to the quasi-CW measurement. Photons arriving later than the peak of the TPSF had a larger PDSF FWHM compared to quasi-CW photons. The absolute photon arrival times were difficult to measure but the 0.5% rise point was approximately 180 ps after the laser pulse entered the front surface sample chamber.
Figure 2.3(a) Measured time-resolved intensity for a pulse transmitted through a 2-cm wide chamber filled with a homogenous solution of 1% Intralipid and 50 ppm ink. The curve was analyzed at each rod position to give the instrument PDSF at different time points (see text for details). Example PPDSFs at (b) the 50% point on the rising edge of the transmitted time-resolved curve, (c) the peak of the curve, (d) and the 50% point on the falling edge of the time-resolved curve. (e) The FWHM of the PDSF as a function of measurement time. Reduction of PDSF FWHM of more than 50% was observed for early photons versus quasi-CW photons.

2.3.2 Effect of Chamber Path Length

To test the effect of the pathlength on the width of the PDSF, the width of the sample chamber was varied from 1.5 cm to 6 cm. As above, the chamber was filled with liquid solution containing 1% intralipid and 50 ppm ink (corresponding to $\mu_s' = 17$ cm$^{-1}$ and $\mu_a = 0.15$ cm$^{-1}$). The absolute FWHM of the PDSF in mm is shown in figure 2.4(a), along with the relative PDSF
FWHM (fig. 2.4(b)) for the 5% and 50% rise points of the transmitted curve. As expected, increasing the chamber pathlength in both cases resulted in a wider PDSF since more scatter occurs through the longer pathlength. However, the relative width of the PDSF did not vary significantly, particularly for chamber pathlengths between 1.5 and 4 cm. Specifically, the PDSF FWHM was 42-48% and 50-58% of the quasi-CW PDSF for the 5% and 50% points on the rise portion of the curve respectively for 1.5 to 4 cm chamber widths. Chamber pathlengths longer than 4 cm resulted in slightly broader relative PDSF FWHMs at early time gates.
Figure 2.4 Effect of chamber path length on (a) the absolute PDSF FWHM at different time gates and (b) the relative FWHM of the PDSF time gate at 5% and 50% points on the rising edge of the full-time curve compared to the quasi-CW PDSF FWHM. In all cases 1% Intralipid and 50 ppm ink were used.
2.3.3 Effect of Media Optical Properties

To understand the influence of phantom optical properties on the FWHM of the measured PDSF, we varied $\mu_s'$ and $\mu_a$ over a range meant to mimic optical properties found in biological tissues at red and NIR wavelengths. Figure 2.5(a) shows the effect on the relative FWHM of the measured PDSFs for 1%, 3% and 5% intralipid ($\mu_s' = 17, 34$ and $81$ cm$^{-1}$, respectively) with 50 ppm ink added ($\mu_a = 0.15$ cm$^{-1}$) in all cases. When scattering was increased, the absolute width of the FWHM decreased, but the relative width of the FWHM for early-arriving photons compared to quasi-CW photons increased slightly from $48 \pm 8\%$ to $60 \pm 2\%$. The decrease in the absolute PDSF FWHM with increasing scatter results from a corresponding reduction in the diffusion coefficient $D$ and a more-rapid radial fall-off of the fluence rate from the source as in equation 2.2. Similarly figure 2.5(b) shows the absolute width of the FWHM decreased when the absorption coefficient was increased, whereas the relative width increased from about $48 \pm 8\%$ to $56 \pm 4\%$ of the quasi-CW PDSF. Therefore, larger optical properties resulted in slightly less reduction in photon scatter when measuring early photons.
Figure 2.5 Effect of (a) changing the Intralipid concentration (ink concentration was 50 ppm in all cases) and (b) changing the ink concentration (Intralipid concentration was 1% in all cases) on the relative PDSF FWHM at different time points.
2.3.4 Fluorescence PDSF

The absorbing rod was then replaced with a 2 mm diameter tube filled with 10 μM Alexafluor-680 dye in intralipid. For these experiments the chamber was filled with a solution of 1% intralipid with 50 ppm ink (μs′ = 17 cm⁻¹ and μa = 0.15 cm⁻¹). The measured FWHM of the PDSF as a function of the quasi-CW fluorescent signal is shown in figure 2.6. Although not indicated here, the length of the measured fluorescence curve was significantly longer than the excitation curve due to convolution with the 1.2 ns fluorescence lifetime. In this case the PDSF FWHM at early gates was 52% ± 6% of that for quasi-CW photons. The reduction was therefore slightly less than was observed in the case of the absorbing rod. It is known that the scattering coefficient for intralipid (as well as biological tissue) decreases with increasing wavelength so that the contribution of the scatter at the emitted wavelength (730 nm) may have slightly increased the width of the PDSF versus the excitation light (670 nm). Nevertheless, the reduction in the measured fluorescent PDSF at early gates was observed to be similar to that for the absorption PDSF.
2.3.5 Instrument Noise Performance at Different Time Gates

Generally, it was observed that the uncertainty in the PDSF measurements was larger at early time gates due to increased noise at early time points. To quantify this, we performed 100 measurements with either 0.2s or 1s total acquisition time without an absorbing rod in place and determined the mean (I) and standard deviation (σ) of the intensity at each of the time points shown in figure 2.7. For these measurements, the photon count rate was $1 \times 10^6$ counts / s. Here the chamber length was set to 2 cm, 1% intralipid and 50 ppm ink ($\mu_s' = 17$ cm$^{-1}$, $\mu_a = 0.15$ cm$^{-1}$). The resulting signal to noise ratio – defined as $SNR = 20 \log(I/\sigma)$ – is shown in figure 2.7.
These data indicate that the SNR for photons arriving before the 5% rise point of the time-resolved curve is less than 20dB, resulting in the larger measurement uncertainties. Therefore, while our other data show that measurement of ‘earlier’ photons than about the 5-10% rise point marginally reduced the measured width of the PDSF, the system SNR performance was significantly worse. It should also be noted that the measurement noise at very early time gates was about 2 times larger than would be expected from photon counting (Poisson) noise. Analysis of our data shows that there appeared to be a small (5 -10 ps) low frequency wobble of the full time curve during measurements. This had negligible effect at later time gates, but at very early times contributed to the reduced SNR.

![SNR at different time gates for 0.2- and 1.0-sec acquisition times. In both cases, the photon counting rate was 1×10^6 photons/sec.](image)

Figure 2.7SNR at different time gates for 0.2- and 1.0-sec acquisition times. In both cases, the photon counting rate was 1×10^6 photons/sec.
2.3.6 Theoretical Modeling

Time-resolved Monte Carlo simulations were performed to model the experimental measurements performed above. Figure 2.8(a) shows the results of a simulation (parameters: 2 cm chamber length, \(\mu_s = 112.5 \text{ cm}^{-1}\), \(g = 0.85\), \(\mu_a = 0.15 \text{ cm}^{-1}\)) compared to the experimentally measured data (intralipid = 1%, ink = 50 ppm). The results show that the simulation results agreed well with the experimental data for the rising edge and quasi-CW data except at very early time gates (<5% of the peak) where the simulation predicted a larger reduction in PDSF FWHM than was experimentally observed. As we discuss below, this is most likely due to the non-ideal (i.e. finite width) temporal impulse response function of the system. Further, the simulations show that average number of photon scattering events for photons arriving in the 5% rising time point was about 1/3 of the average number of all photons, i.e. ~600 scatters and 2000 scatters, respectively. This is close to the decrease in instrument PDSF predicted from the simulation.

Finally, we used the time-resolved diffusion approximation to the Boltzmann Transport Equation to calculate our instrument PDSF at early and quasi-CW time gates. The PDSF FWHM is shown in figure 2.8(b) along with the experimental data for the same conditions. From these data it can be seen that the TRDA underestimates the reduction in the PDSF FWHM observed by measuring early photons by about 20% at the 5 and 10% rise points. The implications of the theoretical modeling are discussed below.
Figure 2.8: Theoretical system PDSF FWHMs for the rising and quasi-CW portion of the curves calculated using (a) time-resolved Monte Carlo simulations and (b) the time-resolved diffusion approximation to the BTE.
2.4 Discussion and Conclusions

In all cases investigated the use of early-photons significantly reduced the amount of photon scatter and the corresponding instrument PDSF. Our data shows that on average, about 40-60% reduction in the FWHM of the PDSF was achievable by measuring early compared to quasi-CW photons over the range of optical properties and pathlengths investigated herein. This implies the volume of imaging PDSFs can be reduced by a factor of approximately 64-84% with a corresponding improvement in imaging resolution. The improvement is most likely highly dependent on the temporal response function of the measurement system; in this case, our system TIRF FWHM was 163 ps. The quantitative effect of altering instrument components and thus the system TIRF – for example, by replacing the filtered supercontinuum source with a faster pulsed laser – is not well understood and is the subject of ongoing study. Further, as we have demonstrated the reduction in photon scatter by measuring early photons comes at a cost of reduced noise performance and instrument sensitivity.

The optical properties and pathlengths used here were chosen to be relevant for red and NIR light propagation in biological tissue. As noted in the main text, while increasing the pathlength, decreasing $\mu_s'$ and $\mu_a$ all increased the absolute width of the PDSF for all time gates, the fractional reduction in FWHM was observed to be relatively consistent. This implies that measurement of early photons will give similar tomographic imaging improvement in different organs and tissue types in small animals [40, 48]. Further, the observed reduction in scatter at larger physical scales implies that the use early photons may indeed be valuable for tomographic imaging of larger laboratory animals or even at clinical scales.
Detection of “earlier” photons than about the 5-10% rise point of the time-resolved curve did not result in significantly narrower PDSFs, whereas the noise performance of the instrument was significantly worse. Therefore, we conclude that the 5-10% rise point is approximately the optimal operating point for early-photon measurements for our system. As above, this operating point is most likely highly instrument dependent, since source pulse width, detector temporal impulse response, detection sensitivity and noise properties are different for each configuration.

With respect to modeling photon propagation, the diffusion approximation was found to agree well with our measured data for quasi-CW measurements but to underestimate the observed reduction in PDSF FWHM observed at early time gates (i.e. <50% of the rise portion of the measured curve). While the discrepancy was small – about 20% overestimation at the 5 and 10% rise points – it was consistently observed versus our experimental data. This disagreement has been noted qualitatively previously [27, 33] and is most likely due to the underlying P-1 approximation as well as the assumption that sources emit isotropically. Therefore, we conclude that the TRDA will lead to data model disagreements if used in the calculation of early-photon forward models for the tomographic image reconstruction problem. Other models such as time-resolved P-3 or SP-N models or the full Boltzmann Transport Equation [52] were not evaluated here but may yield better data-model agreement over the complete time curve.

In contrast, time-resolved Monte Carlo simulations agreed well with our experimental data with the exception of very early time gates (earlier than the 5% rise point). Simulations predict that a greater fractional reduction in the PDSF FWHM – by a factor of 4 or better - should be achievable, but we were not able to observe this with our setup. This is most likely due to the finite (non-ideal) TIRF of our system (FWHM of 163 ps) effectively ‘smearing’ the reduction in
scattering at very early times. However, alternative experimental configurations with a shorter TIRF may yield greater experimental improvement versus what was measured here. Again, the use of earlier time gates would most likely yield decreased measurement SNR, so that noise resolution tradeoffs would be an important consideration in the design such an instrument.
Chapter 3

Instrumentation Consideration for Measurement of Early Arriving Photons in Diffuse Optical Tomography

3.1 Introduction

In chapter 2, we showed that measurement of early-arriving photons resulted in the reduction of the instrument photon density sensitivity function (PDSF) width by a factor of 2 to 2.5 over a wide range of relevant small-animal imaging conditions using a pico-second pulsed laser and time-resolved photon counting combination. However, we also showed that this experimental improvement was less than predicted from time-resolved Monte Carlo simulations. Specifically, a reduction by a factor of 4 or better was predicted, but this could not be achieved with our system. To better understand this, in this chapter we have experimentally tested the effect of a series instrumentation (hardware) parameters on the experimentally measured time-dependant PDSFs including, i) source and detector geometry, ii) detector sensitivity, iii) laser illumination intensity. The effect of instrument temporal response function is investigated with more details in chapter 4. Our ongoing research indicates that all of these parameters affected the relative PDSF width by as much as 10-25%, particularly at early time points. The results of this work are significant because they show in a number of cases that significant disagreement between experimental PDSFs and theoretical models exist as a result of minor changes in experimental configuration. We also anticipate that these results will be useful in the design of future time-resolved DOT and DFT imaging systems.
3.2 Methods and Materials

3.2.1 Instrumentation

The basic system used for these experiments is shown in figure 3.1. A fast, pulsed Super-continuum fiber laser with an 80 MHz repetition rate was used as the light source (KoherasSuperK Power, NKT Photonics, Birkerod, Denmark). The output of the laser was passed through a 670 nm bandpass interference filter (30 nm FWHM; Chroma Technology, Bellows Falls, Vermont). After filtering, the pulse width of the laser was approximately 30 ps. The light was focused onto the surface of a custom made sample chamber which was set to 2 cm in length and filled with liquid phantom solutions. For this work, intralipid solutions were prepared so that the optical properties were equivalent to typical literature values for biological tissue at red and near-infrared wavelengths [40, 53]; specifically, the reduced scattering coefficient ($\mu'_s$) was 17 cm$^{-1}$ and the absorption coefficient ($\mu_a$) was 0.15 cm$^{-1}$. A 1 mm pinhole was placed at the exit plane of the chamber. Initially this was placed co-axially with the input position of the laser (i.e. $d = 0$ cm in fig 3.1(a)), but as we discuss this was varied for subsequent experiments. Transmitted light was collected with a plano-convex lens ($f = 50$ mm; Edmund) and a second, 670 nm bandpass filter (Chroma) and then measured with a 16–channel photomultiplier tube (PMT) array (PML-16-C, Becker and Hickl, Berlin, Germany). The gain across the multi-anode PMT was controlled with a detector control card (DCC; Becker and Hickl) that was operated at 90% of maximum unless otherwise specified below. The output of the PMT array was amplified with internal preamplifiers and passed into a time-correlated single photon counting card (SPC-130, Becker and Hickl) with a 7 ps temporal resolution. The overall temporal instrument response function of the system (FWHM) was explicitly measured to be 154
ps, which agreed well the performance specifications of our laser source (~ 30ps) and multichannel PMT (~ 150 ps).

3.2.2 Data Collection and Analysis

Time-resolved photon density sensitivity functions (PDSFs) were measured with this system as described in sections 2.2.2 and 2.2.3. A black 1 mm diameter absorbing rod was translated through the medium using computer-controlled stepper motor stages (XSlide, Velmex Inc., Bloomfield, New York) and the full-time transmitted intensity curve was recorded at each point. The rod was translated through 121 steps with a step size of 0.25 mm in the lateral directions (for a total of 3 cm), and through 39 steps with a step size of 0.5 mm in the depth direction. As we have described previously, full-time curves were analyzed for each experimental condition to find time points corresponding to 1, 5, 10 and 50%-of maximum on the rising and falling edges of the measured curves as well at the quasi-CW (time-integrated) intensity. The rationale of this approach – as opposed to using absolute time windows following the laser pulse - was that this allowed direct comparison of results over a range of experimental conditions (such as pathlengths and optical properties) with significantly different time-resolved transmitted intensity curves. PDSFs were then determined using these measurements as follows:

\[ PDSF(x, z, t) = \left[ \frac{I_{max}(t) - I(x, z, t)}{I_{max}(t)} \right] \quad (3.1) \]

, where \( I_{max}(t) \) was the transmitted intensity at time \( t \) when no absorbing rod in place (i.e. the un-attenuated signal), and \( I(x, z, t) \) was the transmitted intensity at position \( x, z \) at time \( t \). This allowed us to investigate the effect of a number of instrumentation parameters on the shape of the instrument PDSF. As a quantitative metric of this width, we considered the full width at half
maximum (FWHM) of the measured PDSF in the middle plane of the sample chamber (i.e. where the effect of photon scatter was greatest) at each time point. These were compared to the PDSF FWHM for the time-integrated, quasi-continuous wave (CW) case.

### 3.2.3 Effect of Source and Detector Geometry on Instrument PDSF

Since DOT and FMT imaging systems typically utilize a range of illumination and collection geometries, we were interested in the effect of the source and detector geometry on the ability to measure early-arriving photons. As shown in figure 3.1 (a), for these experiments we utilized a planar transmission geometry where the detector arm was offset either 0, 1, 2 or 3 cm from the location of the source illumination position on the X-Y plane. PDSF measurements were then performed as described above. Acquisition times were 1 s at each rod position and the laser power was adjusted between 0.5 and 12.5 mW at the sample to give photon count rates of approximately $3 \times 10^6$ counts/s for each geometry (higher powers were required for wider source-detector separations). Measurements were repeated at least 3 times for each condition.
Figure 3.1(a) Schematic of instrument used to measure system PDSFs, (b) example measured time-resolved intensity for a pulse transmitted through a 2-cm chamber length which was filled with a liquid phantom with optical properties of $\mu'_s=17 \text{ cm}^{-1}$ and $\mu_a=0.15 \text{ cm}^{-1}$.

3.2.4 Effect of Detector Sensitivity on Instrument PDSF

To study the effect of detector sensitivity on the time-dependant instrument PDSFs, we modulated the gain of our multi-anode PMT. Here, we used the control software provided with the DCC card to modulate the gain of the multi-anode PMT from 75 to 90% of maximum. Because of the proprietary nature of the software it was not possible to directly translate the DCC gain percentage levels into voltages, but this approach nevertheless allowed us to study this effect and observe a general relationship between detector sensitivity and time-dependant PDSFs. Experiments were repeated at least 3 times for each experimental condition.
3.2.5. Effect of light intensity on instrument PDSF

We also considered the effect of the incident light intensity on the measured instrument PDSFs. For these experiments, we varied the intensity of the laser source at the sample chamber surface from 0.05 and 0.5 mW (0 cm offset) so that the photon count rate was increased from $3 \times 10^5$ and $3 \times 10^6$ counts/second, respectively. The acquisition time was adjusted from 1 to 10 s so that the total measured photon counts were consistent between experiments.

3.3 Results

3.3.1 Effect of Source and Detector Geometry on Instrument PDSF

To study the influence of source and detector displacement on the measured PDSF, the detector arm of our instrument was moved between 0 and 3 cm off-axis in 1 cm step sizes (fig 3.1(a)). Example measured PDSFs for 0 cm displacement for the 50% rising, quasi-CW, and 50% falling points on the time-resolved transmitted intensity curve (fig 3.1(b)) are shown in figures 3.2(a-c), respectively. Similarly, example data for 2 cm displacements are shown in figures 3.2(d-f). As we have demonstrated previously in chapter 2, the breadth of the instrument PDSF increased significantly with time due to increased photon scatter. At time later points than the center-of-mass of the time-resolved curve, the measured PDSF was wider than the CW case as anticipated.
Figure 3.2 Example measured instrument PDSFs for 0 cm (a-c) and 2 cm (d-e) source and detector offset distances, at the (a, d) 50 % point on the rising edge of the time-resolved curve, for (b, e) un-gated, quasi-CW photons, and for the (c, f) 50 % point on the falling edge of the time-resolved curve.

As shown in figure 3.3 (a-b), when the detector was moved off-axis from the source, the absolute PDSF width increased significantly. For example, for the 50%-of-peak point on the rising edge of the curve, the PDSF FWHM increased from 10 ± 0.5 to 24 ± 2 mm when the detector offset changed from 0 to 3 cm. For the 10%-of-peak point on the rising edge of the curve, the FWHM similarly increased from 6 ± 0.5 to 17 ± 1 mm. However, the relative PDSF (as a percentage of the CW case) also increased from 56 ± 4 % to 68 ± 5 % for the 50%-of peak point, and from 48 ± 3 % to 56 ± 6 % for the 10% of peak point. These data are in contrast to our previous data in chapter 2, which showed that increasing the chamber width from 2 to 4 cm for no lateral offset.
(i.e. $d = 0$ cm) resulted in no significant change in the relative PDSF. In other words, the increase in *relative* PDSF observed when moving the detector off-axis was not simply a result of the increased photon pathlength. We also tested the same geometries using time-resolved Monte Carlo simulations, and the computed results were in good agreement with our experimental data. Specifically, it was found that by increasing the source-detector offset from 0 cm to 3 cm simulations predicted an increase in relative PDSF FWHM from $50 \pm 5\%$ to $59 \pm 2\%$ for photons arriving at 10% rise point of transmitted intensity curve and from $56 \pm 6\%$ to $70 \pm 3\%$ for photons arriving at the 50% rise point of transmitted curve. Taken together, these data imply that greater reduction in the instrument PDSF widths is obtained when measuring early photons at small transmitted angles. Further quantification of this effect is the subject of ongoing work.

![Figure 3.3](image)

**Figure 3.3** The absolute (a) and relative (b) FWHM of the instrument PDSF is summarized as a function of source and detector offset distance.
3.3.2. Effect of detector sensitivity on instrument PDSF

To study the effect of detector sensitivity on the measured time-dependant instrument PDSF, we performed similar measurements while increasing the applied gain of the PMT. These results are summarized in figure 3.4 (a). As shown, increasing the gain on the multi-anode PMT from 75% to 90% (with a corresponding increase in photon count rate from $1 \times 10^5$ to $3 \times 10^6$ counts/s) resulted in a significant decrease in the PDSF FWHM from $78 \pm 1\%$ to $49 \pm 3\%$ ($p < 0.05$) for photons arriving at the 10% rise point on the transmitted curve and from $86 \pm 3\%$ to $57 \pm 3\%$ ($p < 0.05$) for photons arriving at the 50% rise point on the transmitted curve.

![Figure 3.4(a) The relative FWHM of the PDSF for photons arriving at the 10% and 50% points on the time-resolved curve for varying detector sensitivity (gain). (b) The relative FWHM of the PDSF for photons arriving at the 10% and 50% points on the time-resolved curve for varying detected signal levels (photon count rates).](image)

We note that by increasing the PMT gain, several secondary effects occur in addition to increased sensitivity; the shape of the instrument temporal impulse response function, the photon count rate change, and timing of the measured time-resolved signal through diffusive media (in particular the time corresponding to the peak of the transmitted curve) are all generally
understood to change by altering the applied gain in a photon counting system [54]. As such, attributing the change in relative PDSF to a specific effect is the subject of ongoing work. For example, to consider the possibility that this reduction in PDSF was actually due to the increased photon count rate (as opposed to the detector sensitivity), we repeated these experiments while adjusting the photon counting threshold from -58 to -100 mV while changing the detector gain as above so that the detected photon count rate was constant at $3 \times 10^6$ counts/s. Similar results were obtained; specifically, the relative PDSF FWHM decreased from $78 \pm 2\%$ to $48 \pm 4\%$ ($p < 0.05$) for photons arriving at the 10\% rise point on the transmitted curve and from $86 \pm 2\%$ to $58 \pm 2\%$ ($p < 0.05$) for photons arriving at 50\% rise point on the transmitted intensity curve. Therefore, the specific effect (or combination of specific effects) responsible for the change in relative PDSF width is still unknown and is the subject of ongoing work. Regardless of the underlying effect, substantial variability in experimental performance in measuring early-arriving photons was clearly observed simply by altering the applied PMT gain in our setup.

3.3.3 Effect of Illumination Intensity on Instrument PDSF

To study the influence of the incident light intensity on the FWHM of the measured PDSF, we varied the source laser power from 0.05 to 0.5 mW so that the detected photon count rate increased from approximately $3 \times 10^5$ to $3 \times 10^6$ counts/s and the acquisition time was varied from 1 to 10 s so that the total detected photon counts were consistent between experiments. As shown in figure 3.4(b), increasing the laser power and the photon count rate by a factor of 10 resulted in a decrease of the relative PDSF width at early time gates compared to quasi-CW photons. Specifically, a reduction from $62 \pm 2\%$ to $49 \pm 3\%$ for photons arriving at the 10\% rise point on the transmitted intensity curve ($p = 0.001$) and from $71 \pm 2\%$ to $57 \pm 3\%$ for photons
arriving at the 50\% rise point on the transmitted intensity curve \( (p = 0.006) \) was observed. As above, altering the incident light intensity may have affected a number of secondary parameters such as instrument temporal response due to the longer acquisition time (i.e. due low frequency ‘wobble’ of the laser trigger timing). At 3 \times 10^6 \text{ counts/s}, modest “pulse-pileup” also may have occurred, thereby possibly biasing the measured curve toward early photons. Therefore, the exact cause of the PDSF width reduction in altering the applied gain is unclear also an ongoing area of work.

### 3.4 Discussion and Conclusions

In recent years, there has been significant renewed interest in the fields of DOT and DFT in the detection of early-arriving photons to improve imaging resolution. As indicated in chapter 2, a significant reduction in the relative instrument PDSF width (by at least a factor of 2) could be realized over a wide range of conditions by measuring early photons using our particular pulsed laser and detector combination. It was also determined that this reduction was about 50\% less than predicted by time-resolved Monte Carlo simulation analysis at very early times. As such, the experiments described in this work were intended to provide insight into some reasons for the experimental and theoretical disagreement. To summarize, our results indicate that all of: i) detector sensitivity, and ii) detected light intensity had significant (10\%–25\%) impact on the width of the measured instrument PDSFs compared to Monte Carlo theory. Altering source-detector offset also reduced the “early photon effect”, but as we have noted this \textit{was} predicted by Monte Carlo simulations. As noted above, these results are significant because narrower instrument PDSFs lead directly to improved tomographic imaging resolution for time-resolved DOT and DFT systems[32].
We have attempted to design our experiments so as to measure the effect of these instrumentation parameters on the measured PDSFs as directly as possible but as we have noted isolating the particular effect (or combination of effects) responsible is not straightforward and is the subject of ongoing work. For example, increasing detector sensitivity would be most easily achieved simply by replacing the PMT with a more “red-sensitive” version, but this would also alter the detector temporal response and noise characteristics. Increasing the applied gain while maintaining the same photon counting threshold also resulted in an effective increase in detector sensitivity, but also altered the photon count rate and instrument temporal response.

However, a number of general conclusions can be drawn from this work. The first direct implication is that specific components and configuration parameters of a time-resolved DOT and DFT imaging system can lead to significant deviation of the width of the imaging PDSF compared to theoretical models. In particular, this was observed in studies with applied gain, and incident laser intensity. Therefore, in performing the time-dependant inverse image reconstruction problem, care must be taken to ensure that the “correct” sensitivity functions (Jacobians) are used - particularly at early time gates - to ensure data - model agreement.

Secondly, the intention is that these results may guide the design of better optimized DOT and DFT instruments for measurement of early-arriving photons. Overall, our results imply that measurement of early photons is best achieved with a time-resolved instrument with high-sensitivity detectors positioned at small offset angles from a rapid, high-intensity input light source. We also note that implementation of these results in the design of a DOT instrument would entail practical trade-offs. In addition to further studying the effects discussed herein, the effect of instrument temporal response function will be discussed in more detail in chapter 4.
Chapter 4
The Effect of Temporal Impulse Response on Experimental Reduction of Photon Scatter in Time-resolved Diffuse Optical Tomography

4.1 Introduction

In chapter 2, we experimentally measured time-dependent PDSFs for a particular pulsed light source (super-continuum laser) and detector (fast photomultiplier tube) combination and showed that with this configuration, early photons yielded reduction in PDSF width by about a factor of 2 over a wide range of experimental conditions [34]. This improvement was observed at the 10%-of-peak point on the rising edge of the transmitted intensity curve. However, at even earlier times a “leveling out” of the early photon effect was observed (i.e. no further reduction in PDSF width could be experimentally measured) despite the fact that time-resolved Monte Carlo simulations predicted that a reduction by a factor of 3-4 or better could be obtained. At the time, we hypothesized that this effect was chiefly due to the finite (non-ideal) temporal response time of our system, which was 163 ps. Although we did not confirm this explicitly, it is generally understood that “faster is better” in the time-resolved tomography literature. For example, other authors have shown that non-ideal instrument TIRFs can yield incorrect estimation of tissue optical properties from full time curves [55, 56], or can introduce of imaging artifacts when performing time-resolved imaging with mean, variance and “skew” time-resolved data-types [57].

However, to our knowledge the quantitative effect of instrument TIRF on measurements in the early-photon regime has never been studied in detail. Of particular interest is the degree of
experimental resolution improvement obtainable relative to the theoretical maximum. A survey of the literature published in time-resolved DOT and FMT shows that previously reported time-resolved systems have (had) intrinsic system TIRFs in the range of approximately 80-600 ps. Multi-channel plate (MCP) based systems have reported TIRFs in the range of 80-200 ps [28, 58], photomultiplier tube (PMT) based systems in the range of 160-600 ps [7, 59, 60], intensified charge-coupled device camera (ICCD) based systems in the range of 200-400 ps [40, 61, 62], streak camera based systems in the range of 150-250 ps [27]. Fast avalanche photodiode (APD) systems have recently been used in time-resolved tomography with stated detector response time of about 40 ps [63], although the overall system TIRF was not stated. Interferometric (i.e. transmission geometry optical coherence tomography) approaches have also been used to measure time-resolved curves through diffusive media, but to date these have been limited to relatively small (~1-5 mm) imaging volumes [64, 65].

In this chapter we quantitatively studied the impact of instrument TIRF on imaging resolution by systematically modulating our instrument TIRF in approximately the range of reported literature values (specifically, between 163 and 716 ps) using a fiber dispersion method similar to previous authors [56, 66] and measured the impact of this on experimentally measured PDSFs. As we demonstrate, all instrument TIRFs that we tested resulted in significant disagreement (up to 40%) in PDSF width compared to time-resolved MC theory. Experimental disagreement was most pronounced at very early time points (for example, at the 10%-of-peak point on the rising edge of the curve or earlier), and varied by about 0.3 mm per 100 ps of instrument TIRF in this regime. We found that modulation of the system TIRF by elongating the response on either the source or detector side had comparable effects, implying that use of fast sources or detectors will yield approximately the same improvement in imaging resolution in a time-resolved tomography
system. This was also confirmed by temporal convolution of system TIRFs with the output of time-resolved Monte Carlo (MC) simulations. To our knowledge, this effect has never been explicitly studied previously. These results have significant implications, i) in correct calculation of imaging PDSFs for time-resolved DOT and FMT, and ii) in design of time-resolved DOT and FMT systems that utilize the early-photon effect.

4.2 Methods and Materials

4.2.1 Instrumentation

The basic system used for these experiments is shown in figure 4.1 and is similar to the system we described previously in chapter 2. A fast, pulsed Super-continuum fiber laser with an 80 MHz repetition rate was used as the light source (Koheras Super-K Power, NKT Photonics, Birkerod, Denmark). The output of the laser was passed through a 670 nm bandpass interference filter (30 nm FWHM; Chroma Technology, Bellows Falls, Vermont) and after filtering, the laser pulsewidth was approximately 30 ps. Light was coupled into a 0.37 NA, 600 μm core step-index multimode optical fiber (BFL37-600; Thorlabs) of varying length by illuminating a diffusing glass plate (220-Grit ground glass diffuser, Edmund Optics, Barrington, NJ) placed directly in front of the fiber end (see section 4.2.2). Light exiting the fiber was focused to a 1 mm diameter spot on the surface of a custom made 15 cm × 15 cm × 2 cm glass sample chamber with a pair of plano-convex lenses (Edmund Optics). Neutral density filters were placed in the beam path so that the illumination intensity at the chamber surface was 1.25 mW. The sample chamber was filled with liquid phantoms prepared from a stock solution of 10% Intralipid (Baxter Healthcare Corporation, Deerfield, Illinois) and India Ink. These were diluted so that the final liquid optical properties at 670 nm were approximately as follows: reduced scattering coefficient $\mu_s' = 10$ cm$^{-1}$.
and absorption coefficient $\mu_s = 0.15 \text{ cm}^{-1}$. These values were chosen since they are approximately equivalent to typical literature values for biological tissue at red and near-infrared wavelengths [40, 53, 67].

**Figure 4.1** Schematic of instrument used to measure system PDSFs. Two multimode optical fibers of varying lengths were coupled at the system input and output to alter the instrument temporal impulse response function.

Light transmitted through the 2 cm wide chamber was collected with a second multimode optical fiber that was placed co-axially with the position of the input beam. Light exiting the detector optical fiber was collimated using a SMA-coupled achromatic plano-convex lens package (F260 SMA-B; Thorlabs) and then passed through a second 670-nm bandpass filter (Chroma). The transmitted light was measured with a 16–channel photomultiplier tube (PMT) array (PML-16-C, Becker and Hickl, Berlin, Germany). The gain on the multi-anode PMT was controlled with a detector control card (DCC; Becker and Hickl) that was operated at 90% of maximum. The output of the PMT array was amplified with internal preamplifiers and coupled into a time-correlated single photon counting card (SPC-130, Becker and Hickl) configured so that it had a 16.3 ps temporal resolution. Although the PMT array had 16 individual anodes, it was determined that the detected light illuminated approximately the middle 6 detection channels. Therefore, the time-correlated photon count signals from only the middle 6 channels were summed after correction of the minor inter-channel time skew (<25 ps in this case). The overall
system TIRF FWHM was measured to be 154 ps, which generally agreed well with performance specification of our light source (~30 ps) and multichannel PMT (~150 ps).

4.2.2 Modulation of the instrument TIRF

We used a fiber dispersion method similar to that described by [66] and [56] to modulate the temporal impulse response function (TIRF) of our system. As shown in figure 4.1, this was achieved by varying the length of the multimode optical fibers on the source and detector sides. The intermodal dispersion properties of the fibers temporally broadened transmitted light pulses with the amount of dispersion directly proportional to fiber length. To ensure as complete modal filling as possible (and therefore to maximize this effect) we placed a diffusing glass plate in front of the source fiber. The overall TIRF of the instrument was then altered by using either, i) a source fiber length (SFL) of 1, 5, 10 or 20 m, with a constant detector fiber length (DFL) of 1 m, ii) a DFL either 1, 5, 10 or 20 m, with a constant SFL of 1 m, and, iii) SFL and DFL of equal lengths between 1 m and 20 m. The rationale here was that we were interested in separating the effects of either a slower source or detector on the measured instrument PDSF at early time points. Instrument TIRFs for each combination were determined by measuring the temporal transmitted light through non-scattering water filled chamber. For these measurements, the source intensity was attenuated by placing neutral density (ND) filters in front of source fiber to avoid saturation of the PMT array. A thin scattering layer (i.e. a piece of white paper) was placed in front of the detector fiber to ensure complete mode filling, although this was removed for subsequent experiments (section 4.2.3) where intralipid solution performed a similar function. As we demonstrate, this fiber dispersion approach allowed broadening of the overall instrument TIRF FWHM from 163 ps to 716 ps.
4.2.3 Experimental Measurement of the instrument PDSF

To measure the photon density sensitivity functions we used a similar method to the previous work in chapter 2 and translated a 10 cm × 10 cm absorbing sheet with a centered 2 mm × 50 mm vertical aperture stepwise through the intralipid media (in chapter 2, we used an absorbing rod, but the aperture method was empirically found to reduce inter-experimental variability between measurements). The absorbing sheet was made from anodized foil (BKF12, Thorlabs) and was sufficiently large and opaque to absorb photons not passing through the aperture. The slit was translated through the liquid phantom using computer-controlled stepper motor stages (XSlide, Velmex Inc., Bloomfield, New York). A complete set of measurements were performed by translation of the slit through 121 steps with a step size of 0.25 mm in the lateral direction (for a total of 3 cm), and through 39 steps with a step size of 0.5 mm in the depth direction. The transmitted light was measured with the PMT array at each slit position. The maximum detected photon count rate was ~10^6 counts/s when the slit was positioned directly in front of the laser. By moving the slit to an off axis position, the count rate dropped to approximately 10^4 counts/s. We verified that no significant temporal shift of the transmitted photon intensity curve was measured over this range of photon count rates for otherwise identical conditions. We also showed numerically that the measured PDSF with a slit aperture had the identical FWHM as a point aperture at the center plane (data not shown for brevity) but had the benefit of increasing the photon count rate and improving the measurement SNR.

To compute the experimental PDSF, the full time curve through the liquid phantom media was first measured without the slit in place for each fiber combination. This temporal transmitted curve was analyzed to find the timing of the peak point and the 5, 10, 25 and 50%-of-peak time
points on the early-time range (i.e. rising edge) of the detected signal. The total photon counts at each slit position were summed for each 16.3 ps time window and the PDSF was then computed as follows:

\[
PDSF(x, z, t) = \frac{I(x, z, t)}{I_{\text{max}}(t)}
\]

Where \(I_{\text{max}}(t)\) was the maximum transmitted intensity at a given time \(t\) and \(I(x, z, t)\) was the transmitted intensity at position \(x, z\) at time \(t\). As a metric of the overall breadth of the instrument PDSF, we considered the full-width at half maximum (FWHM) of the PDSF in the middle plane of the chamber (i.e. \(z = 1\) cm) where the extent of photon diffusion was greatest. For all experiments, the acquisition time was 1 s per slit position. Each experiment was repeated at least 3 times for each source and detector fiber length combination.

4.2.4 Time-resolved Monte Carlo Simulations

Theoretical PDSFs were computed with a time-resolved Monte Carlo simulation based on the publically available MCML code on the Oregon Medical Laser Center website [51]. Monte Carlo is a gold standard method for modeling time resolved photon propagation – particularly at early time gates where the diffusion approximation to the Boltzmann Transport Equation (BTE) has been shown to be inaccurate – and is considered a numerical implementation of the BTE. We compiled the paths of photons from a pulsed (infinitely short) pencil beam emerging from a 2 cm semi-infinite slab media with \(\mu_s = 66.7\) cm\(^{-1}\), \(\mu_a = 0.15\) cm\(^{-1}\), anisotropy coefficient \(g = 0.85\) at 1-mm by 1-mm square detector located directly opposite from the input position. A total of 1 billion photons were tracked and the simulation yielded a temporal resolution of 30 ps.
Since time-resolved MC simulations characterize photon propagation through the media for an infinitely short incident pulse, it was also possible to calculate the effect of finite instrument temporal impulse response functions on the measured signal using a point-by-point temporal convolution as follows:

\[ Measured(r, z, t) = \int_{-\infty}^{+\infty} MC(r, z, t - \tau) TIRF(\tau) d\tau \quad (4.2) \]

, where \( MC(r, z, t) \) is the photon density at position \( r \) and \( z \) at time \( t \) obtained from the MC simulation respectively, and \( \tau \) is an integrating factor for time. Here \( TIRF(t) \) was first explicitly measured with our instrument for each of the SFL and DFL combinations as above.

### 4.2.5 Measuring PDSFs with a fast Avalanche Photo Detector

The instrument impulse response time is a function of several factors: source pulse width, detector response time and delays in optical fiber, lens and filters. The main important limitation of system TIRF is detector response time and transit time which usually is much larger than source pulse width or temporal delays in optical fiber, lens, and filters. Moreover, modifying the detector in a DOT system is much cost effective than changing the laser source. Therefore, it is more legitimate to minimize the instrument TIRF by using a fast detector and lowering the detector transit time. To evaluate the impact of a commercial fast detector, we substituted our 16-channel PMT with a fast Avalanche Photo Detector (id 100-50, id Quantique, Geneva, Switzerland). Although SPADs have been used in a small number of DOT and FMT systems previously - e.g. imaging systems [37, 68]- to our knowledge they have never been tested in measurement of EPs to improve resolution previously. In this context, SPADs have a number of potential advantages including a rapid response time in the range of 30-40 ps, high quantum
efficiency, relatively low cost, and high tolerance to damage from direct illumination versus, for example, photomultiplier tubes (PMTs) or multi-channel plates.

Figure 4.2 Schematic of the experimental system used for evaluation of SPAD detector in measurement of EPs. Abbreviations: lens 1,2 (L1, L2), filter (F), inverter and attenuator (I/A).

The system used for these experiments is shown in fig. 4.2 [47]. We used a femtosecond Titanium: Sapphire laser (MaiTai XF-1, Newport Corporation, Irvine CA) operating at 730 nm and fitted with a variable attenuator (VA), so that the incident power at the sample was set to 10 mW. Transmitted light from the sample was measured with the SPAD, which was fitted with a 2 mm aperture, a lens pair (f = 25 mm, Edmund Optics), and a 730 nm bandpass filter (Chroma Technology, Belmont Falls, VT). The SPAD produced a +2 V pulse for each measured photon, which was inverted and attenuated (26dB) before coupling into a time correlated single photon counting module (SPC-130; Boston Electronics, Brookline, MA). The measurement was synchronized to the laser pulse using an optical constant fraction discriminator (OCFD), and the entire instrument was controlled with a personal computer.

We first measured the overall instrument TIRF by placing a thin glass diffusing target in the path of the beam. We next replaced the diffusing target with a 2 x 20 x 20 cm³ plexi-glass imaging
chamber (2 cm depth) filled with a homogenous liquid phantom solution with intralipid (1%; Baxter healthcare) and India ink (50 ppm) added. Based on previously published characterization of similar phantoms at 730 nm, we estimated that the resulting optical properties in the media were $\mu'_s = 10 \text{ cm}^{-1}$, and $\mu_a = 0.1 \text{ cm}^{-1}$, which are close to reported values for biological tissue in this wavelength region [40].

We next measured the width of the PDSF through the diffusive media using a technique we have described in detail in section 4.2.3. We then replaced the chamber with the 25 mm diameter x 100 mm long resin cylindrical phantom (as above, with optical properties approximately equal to $\mu'_s = 10 \text{ cm}^{-1}$ and $\mu_a = 0.1 \text{ cm}^{-1}$ with two, 2 mm diameter bore holes separated by 7 mm center-to-center (5 edge-to-edge) as shown in fig 4-9 (a). The holes were filled with an absorbing media with an estimated absorption coefficient of $\mu_a = 5 \text{ cm}^{-1}$. The phantom was rotated counterclockwise through 360° with 2° increments using a motorized rotation stage (Velmex) and the transmitted TR curve was measured.

4.3 Results

4.3.1 Varying System Temporal Impulse Response Function with Multimode Optical Fibers

We first used the multimode fiber pulse dispersion method to vary the overall system temporal impulse response function (TIRF) as described. Other authors have shown previously that the degree of pulse dispersion obtained with this method is highly dependent on complete modal filing of the multimode optical fiber [56]. Therefore, for these measurements we placed a thin scattering material (white paper) in front of the detection fiber to mimic the intralipid media. Figure 4.3 (a) shows the expected broadening effect of increasing the source and detector fiber
lengths (in this case, when the SFL and DFL were equal and were increased from 1 m to 20 m) on the measured system TIRFs. We quantified this effect by measuring the TIRF full width at half maximum (FWHM) when the fiber length was altered from 1 to 20 m on the source, detector and source and detector sides as summarized in figures 4.3(b-d), respectively. As shown, increasing the fiber length increased the system TIRF by 5.6 ps/m on the source side, 11.6 ps/m on the detector side, and 14.5 ps/m when both the source and detector fiber lengths were increased equally. The longest TIRF FWHM we could produce with our method was 716 ps (for SFL = DFL = 20 m), which is on the upper end of instrument TIRF FWHMs reported in the time-resolved literature. The difference in dispersion observed on the source and detector sides was most likely due to sub-optimal mode filling on the source side; it was found that placing a white paper diffuser (versus the glass diffuser) resulted in a further ~50% increase in TIRF (although paper was not used in the experiments here since it resulted in excessive light loss through the system). Our results indicate less temporal dispersion than reported by [56] that was achieved using a similar method, although we attribute this difference to the lower numerical aperture of our fiber, specifically 0.37 versus 0.54. This effect can be estimated using the group delay relationship

\[
\frac{\Delta t}{L} = \frac{n}{c_0} \left( \frac{1}{\sqrt{1 - \frac{NA^2}{n^2}}} - 1 \right)
\]  

(4.3)

, where \(c_0\) is the speed of light in vacuum, \(n\) is the refractive index of the core material, \(NA\) the numerical aperture of the fiber, \(L\) the length of fiber, and \(\Delta t\) is the delay of highest order mode in comparison with the lowest order mode. Based on this relationship, use of an NA of 0.54 would have yielded about 2.25 times more dispersion for a fiber of identical length and core index of
refraction, consistent with the observed differences. In summary, we verified that it was possible
to use this approach to adjust the instrument TIRF without altering major system components
(i.e. light sources and detectors).

Figure 4.3(a) Measured system TIRFs for 1, 5, 10, and 20 m source and detector fiber lengths. The effect of fiber
length from 1 m to 20 m on system TIRF FWHM for varying (b) SFL, (c) DFL, and (d) SFL and DFL.
4.2.3 Measurement of Instrument Photon Density Sensitivity Functions

We then investigated the effect of the altered TIRF on experimentally measured time-dependent instrument photon density sensitivity functions (PDSFs). We first filled our 2 cm wide sample chamber with a tissue mimicking intralipid solution and measured the transmitted TR light through the media. As expected, increasing the length of the source and detector fibers (and therefore the instrument TIRF) resulted in significant broadening of the transmitted TR curve. Example curves are shown in figure 4.4(a) when the source and detector fiber lengths were equally increased from 1 m (TIRF FWHM = 163 ps) to 20 m (TIRF FWHM = 716 ps). Here, curves were normalized to their maxima, both in amplitude and in time. Temporal normalization to the peak was performed since the true starting point is difficult to accurately measure in practice.

We then translated a rectangular slit aperture through the media and measured the effect on the transmitted curve for each position as described in equation 4.1. Figures 4.4(b-d) show example PDSFs for the 50% time point on the rising edge, quasi-CW photons, and the 50% time point on the falling edge of the time-resolved curve, respectively, obtained for the fiber configuration corresponding to SFL = DFL = 1 m.
Figure 4.4(a) Example measured diffusely transmitted light through the liquid phantom for 1, 5, 10, and 20 m source and detector fiber lengths. Example measured photon density sensitivity function for 1 m source and detector fiber lengths (b) at the 50% point on the rising edge of transmitted time-resolved curve, (c) quasi-CW photons, and (d) and at the 50% point on the falling edge.

It is evident from the figures that the increase in photon scatter with time resulted in broadening of the measured instrument PDSFs. As expected, time points later than the meantime of the TR curve have PDSFs which are broader than the time-integrated, continuous wave case. For all configurations, we maintained identical photon count rate, laser power, detector gain and acquisition time to eliminate the possibility of artifacts due to these effects, although this resulted
in relatively minor differences in peak transmitted intensities in the time-resolved curve for different TIRFs as discussed below.

We repeated these measurements for all of the source and detector fiber length combinations as shown in figure 4.5 and measured the FWHM of the PDSF in the middle plane of the sample chamber as shown. Since we were principally interested in the use of this approach to improve tomographic imaging resolution, we considered the PDSF FWHM on the “early” rising edge of the measured TR transmitted curve, i.e. where PDSFs were narrower than the un-gated CW case. Measured PDSF FWHM are shown in figs. 4.5(a-c) as a function of fraction of peak (i.e. position on the rising edge of the TR curve) for the case where the SFL (fig 4.5(a)), DFL (fig 4.5(b)), and both SFL and DFL (fig 4.5(c)) were varied. Error bars are not shown on plots for clarity, but the measured FWHMs had uncertainty of less than ± 0.25 mm throughout the rising edge of the curve (N = 3 for all cases).
Figure 4.5 The FWHM of the middle plane of PDSF at early time points (fraction of the peak point) for varying (a) SFL, (b) DFL, and (c) SFL and DFL. (d) The FWHM of the middle plane of PDSF vs. the measured rise time for varying SFL and DFL.

As shown in figure 4.5, the PDSF breadth increased significantly with longer instrument TIRFs, and the most significant broadening was observed to occur at very early times. Considering the fiber configuration with the fastest TIRF (SFL = DFL = 1 m; TIRF FWHM = 163ps), the PDSF FWHM at the 10%-of-peak point on the curve was 5.8 ± 0.25 mm compared to the quasi-CW width of 10.5 mm. This represents a relative reduction by about 44.7%, which agrees with our
previous results of 40 to 60% over a wide range of conditions (chapter 2). In contrast, for the slowest overall TIRF, (SFL = DFL = 20m; TIRF FWHM = 716 ps) the PDSF FWHM at the 10%-of-peak point on the curve was about 7.6 ± 0.1 mm, which is equivalent to a relative reduction of only about 27.6%. For all fiber combinations, when considering the 10%-of-peak point on the transmitted curve, PDSFs broadened in the mid-plane by 0.2 ± 0.1 mm per 100 ps, 0.36 ± 0.06 mm per 100 ps and 0.29 ± 0.03 mm per 100 ps when the SFL, DFL, and both the SFL and DFL were varied, respectively. Similarly, when considering the 50%-of-peak point on the transmitted curve, the PDSFs broadened by 0.2 ± 0.1 mm per 100 ps, 0.24 ± 0.03 mm per 100 ps and 0.16 ± 0.07 mm per 100 ps when varying the SFL, DFL, and both the SFL and DFL, respectively. Therefore, our experimental data imply that altering the system TIRF by altering either the source or detector response (or both) results in approximately equivalent effect on the instrument PDSF breadth. We discuss the implications of this below. In the tomographic image reconstruction problem, this PDSF broadening due to instrument TIRF would directly result in loss of imaging resolution.

For figures 4.5(a-c), we chose to use the “fraction of peak” as the x-axis, as opposed to the absolute photon arrival time. The rationale was that these represent points on the measured TR curve with approximately equivalent signal-to-noise ratios (SNR), which, in practice are most relevant for comparison of different instrument configurations. (It is noted that the peak value on the TR curve was slightly less for configurations with longer TIRFs, but that this effect was minimal. Between all configurations studied here the SNR of the peak varied by 3dB or less). However, we note that the 10%-of-peak point on the TR curve is actually “later” on a slower system than the equivalent point on a faster system, i.e. since the rise portion of the curve is broadened. We therefore re-plotted the data from fig 4.5(c) on an absolute time scale in fig
4.5(d). As shown, PDSF broadening was still observed with the slower configurations (TIRF FWHM = 716 ps) relative to the faster configurations (TIRF FWHM = 163 ps). For example, PDSFs measured in the first 50ps were broadened by about 1.5 mm or 25%. Here, time curves were aligned at the 0.1%-of-peak point; experimentally, this represents a “very-early” point on the rising edge of the curve which was about 50 photon counts above background (assuming 5 x 10^4 photon count peak intensity). In principle, the very earliest points on the 716 ps curve should have PDSF widths that are equivalent to the fastest configuration, but in practice these time points have extremely low SNRs (or are below the system noise floor). Therefore, measurement of even the earliest detectable photons on the transmitted TR curve with the slower system would result in significant loss of tomographic imaging resolution at any early time-point.

4.3.3 MC analysis of Time-resolved Instrument PDSFs

We then considered the effect of system TIRF on instrument PDSFs computed with time-resolved Monte Carlo (MC) simulations. To consider the effect of finite system TIRFs, we performed point-by-point convolutions of the MC-computed PDSFs (due to an infinitely short laser pulse) with experimentally measured instrument TIRFs. Example results are shown in fig 4.6 (a) for the original output of the MC simulation, as well as convolutions with measured system TIRF FWHM of 163, 223, 375 and 716 ps (corresponding to source and detector fiber lengths of 1, 5, 10 and 20 m, respectively). In this figure the PDSF FWHM as a function of position on the rising edge of the TR curve is shown for each case. As indicated, the results of the MC simulations were qualitatively similar to our experimental results shown in fig 4.6, with the PDSF FWHM increasing in width with increasing instrument TIRFs. For example, at the 10%-of-peak point on the rising edge of the TR curve, the TR MC simulation predicted a PDSF
FWHM of 5.75 mm, representing a 45.2% relative reduction in width versus the un-gated CW case. When non-ideal system TIRFs were considered, this resulted in PDSF FWHM broadening up to 7.25 mm (for TIRF FWHM = 716 ps). Earlier time points resulted in even greater relative broadening; for example, at the 0.1%-of-peak-point, the PDSF FWHM broadened from 3.25 mm (MC) to 6 mm (for TIRF FWHM = 716 ps).

Figure 4.6a) Convolution of the output of time-resolved MC simulations with experimentally measured instrument TIRFs at different points on the rising edge of the transmitted curve. The effect of TIRF on broadening of the
instrument PDSF at the middle plane of the imaging chamber are shown for MC calculations, and experimental measurements at the (b) 10% and (c) 50% of maximum on the rising edge of the time-resolved curve.

Overall, time resolved MC computations yielded 0.2 ± 0.02 mm per 100 ps broadening of the PDSF FWHM for the 10%-of-peak point, and a 0.15 ± 0.01 mm per 100 ps broadening for the 50%-of-peak point as shown in figs 4.6(b) and 4.6(c). Generally this analysis agrees well with our experimental data (plotted on the same figures for clarity) where an average of 0.3 ± 0.02 mm and 0.17 ± 0.03 mm per 100 ps broadening of the PDSF FWHM was observed for the 10%- and 50%-of-peak points, respectively. We extrapolated the experimental best-fit curves in figures 4.6(b) and 4.6(c) to the respective y-axes (corresponding to infinitely short TIRFs) and obtained 5.45 ± 0.08 mm and 6.8 ± 0.1 mm for the 10% and 50%-of-peak points respectively. This was in good agreement with our original MC results (also corresponding to infinitely short TIRFs), of 5.75 and 6.75 mm for the two points. The slight discrepancy in slope of the 10%-of-peak curves may have been due to minor mismatch between the optical properties of our liquid phantoms and those used in the MC theory, or due to a slight mismatch in the measured system TIRFs with a white paper versus that with intralipid in place.

4.3.4 PDSFs measurement at early time points with APD

To quantify the effect of system TIRF on experimental reduction of photon scatter at early time points, we exchanged our 16-channel PMT with a fast commercial single photon APD.

As shown in fig. 4.7 (a) the overall TIRF FWHM was 59 ps. This is in good agreement with the manufacturer’s specified response time of the SPAD (40 ps) and the 20 ps timing jitter on the laser. This is also significantly faster than the instrument configuration in our previous work [6,7] and to faster than previously reported TR DOT or FMT instruments from other groups that
used, for example, fast PMTs, streak cameras, gated intensified CCDs or multi-anode-MCP detectors, where stated TIRFs were in the range of 150-500 ps.

Figure 4.7(a) Measured instrument TIRF. The FWHM was 59 ps, (b) Measured TR curve through diffusive liquid phantom. The SPAD diffusion tail is evident (arrow, blue curve) compared to the TR curve measured with a PMT through the same media.

The measured TR curve is shown in fig 4.7 (b), along with a TR curve measured with our previously instrument that used a multi-anode PMT and super-continuum (SC) laser combination and had an overall TIRF FWHM of 163 ps. As is evident, the SPAD TR curve has an extended decay portion when compared to the PMT curve (red arrow). This is a well-known effect and is caused by carrier diffusion processes in the SPAD material[69]. Therefore, for any application where the full-time curve is of interest – e.g. in TR measurement of optical properties or fluorescence lifetimes – this effect must be considered.

We then measured the instrument PDSFs (as described in sec 4.3.2) at early time points as well as quasi-CW photons. By analyzing the effect of the slit position on the TR curve we were able to measure the PDSF at its widest point (i.e. in the middle of the chamber). Each measurement took 1 s, and the maximum photon count rate was approximately $10^6$ photons/s. The early
portion of an example TR curve is shown in fig. 4.8 (a), along with the measured normalized PDSF profile at the center of the chamber (fig. 4.8 (b)) for selected time points (red arrows, fig 4.8 (a)). The average of 3 experimental trials is shown for each curve. As expected, the width of the PDSF increased at later times due to photon diffusion. The FWHM of the PDSF as a function of time is shown in fig 4.8 (c), and the dotted line indicates the width of the time-integrated, quasi-CW PDSF, which in this case was 8 mm. Overall, the rapid TIRF of the system allowed measurement of very narrow PDSFs on the order of 2-3 mm FWHM; this corresponds to a reduction by about 66% compared to quasi-CW. For comparison, we plotted the relative PDSF FWHM obtained with the SPAD along with data obtained from TR-MC simulations for the same geometry as we have done in section 4.3.3. Here we assumed $g = 0.85$, $\mu_s = 67 \text{ cm}^{-1}$, and $\mu_a = 0.1 \text{ cm}^{-1}$. For comparison, we have also plotted the corresponding curve measured with our previous system that used a SC source and multi-anode PMT that had an overall TIRF FWHM of 163 ps. To permit direct comparison of systems with different TIRFs (and also to minimize possible effects of small differences in media optical properties between experiments which were carried out months apart), the time axis of these curves were normalized to the “fraction of the peak” on the rising edge of the curve. As shown, the SPAD system (in combination with the Ti:Sapph laser) resulted in significantly better performance than our previous system, particularly at very early times. For example at 1% of peak, the SPAD system was 27% narrower than the PMT system and was extremely close to the theoretical TR-MC performance.
**Figure 4.8** (Colored online) (a) Measured TR curve through liquid phantom. (b) Measured PDSF profile in the center of the imaging chamber at 5 time points corresponding to the red arrows in (a), illustrating increasing photon scatter with time. (c) PDSF FWHM versus versus time. Error bars represent the standard deviation from 3 trials. (d) The relative PDSF FWHM (normalized to quasi-CW) as a function of time on the rising edge of the TR curve (normalized to fraction of peak) measured with the SPAD and obtained with TR-MC simulations. For comparison, the same curve measured with a PMT (TIRF FWHM = 163 ps) is shown.

The performance of the SPAD in measurement of early-arriving photons was also performed using cylindrical phantom measurement, which is shown in figure 4.9 (a). The transmitted TR curve was measured as shown in fig 4.9 (b). Example traces for selected time points along the early portion of the TR curve are shown in fig 4.9 (c), as well as the quasi-CW measurement.
Intensities at each time point were normalized to the maximum value of the scan, and the data shown is the average of 3 experimental trials. The reduced scatter of EPs is evident in the depth of the attenuation as the absorbers moved directly in front of the laser or detector (i.e. at 60°, 130°, 240°, 300°), and correspondingly by the extent of recovery as the gap between the holes passed in front of the laser or detector (90° and 270°). We used this ‘relative depth’ (i.e. the mean difference between local minima and maxima in fig 4.9 (c) as a quantitative metric of the EP measurement performance at a given time point. We simulated PDSFs using TR-MC for the phantom geometry, and the FWHM in the middle of the phantom is shown in fig. 3d as a function of time.
Figure 4.9 (Colored online) (a) Cylindrical cast resin phantom with two absorbing inclusions. (b) TR measurements were made as the phantom was rotated through 360°. (c) Normalized measured intensity as a function of rotation angle for 4 selected time points along with quasi-CW data.

4.4 Discussion and Conclusions

In recent years, there has been significant renewed interest in the fields of DOT and FMT in the measurement of early-arriving photons to improve imaging resolution, partially driven by the development of new, high speed light sources and detectors [31, 32, 34-36]. It was indicated in chapter 2 that a reduction in the relative instrument PDSF width by approximately a factor of 2 could be realized over a wide range of conditions by measuring photons that arrived at the 10% - of-peak point on the transmitted TR curve using our super-continuum laser and PMT.
combination. It was also determined that further reduction in PDSF width could not be obtained at earlier time points (<10%-of-peak), even though MC simulations predicted that a reduction of 3-4 times should be obtainable. In other words, we experimentally observed a “leveling out” of the early photon effect that was not predicted by theory. As such, the experiments described in this work were performed to quantify the relationship between system TIRF and time-dependent instrument PDSF widths over a range of response times that were generally representative of previously reported literature values (from 163 ps to 716 ps).

Two important conclusions from this work are noted. First, while it is generally understood that “faster is better” for time resolved DOT and FMT systems, our data indicate that substantial disagreement was observed versus TR MC models for all experimental instrument TIRFs considered. Further, the effect was larger at earlier time points, where up to 19%, 32% and 40% error were observed at the 50%-,- 10%- and 5%-of-peak points on the transmitted intensity curve, respectively. Experimental and Monte Carlo analysis showed that this broadening was fairly linear and was 0.15 to 0.3 mm per 100 ps for system TIRF (depending on the position on the time-resolved curve). As such, these results confirm that the “leveling out” of the early-photon effect observed in our previous work was most likely due to non-ideal system TIRF. Although systems with longer TIRFs resulted in temporally extending the rise portion of the transmitted intensity curve, in practice all usable points – in this case all points on the rising portion above the TR transmitted curve above 0.1% of the peak value - yielded broadened PDSFs versus faster systems. In a DOT or FMT imaging system, this would yield a corresponding loss in imaging resolution. Likewise, significantly narrower PDSFs and improved imaging resolution should be obtainable with faster systems, although this was not explicitly confirmed here. However, it is critical to re-iterate that the imaging resolution obtained in DOT and FMT is not only a function
of instrument PDSF width, but also the noise and sensitivity properties of the measurements, i.e. two systems with identical TIRF but disparate signal to noise ratios will yield different overall imaging performance.

Second, our data indicates that PDSF broadening was approximately equal when the instrument TIRF was increased on either the source or detector sides (or both simultaneously). In terms of the design of time-resolved DOT or FMT systems, this implies that no particular advantage would be obtained by using a faster pulsed laser or detector, so that the net effect on the overall system TIRF should be considered (again, this neglects effects of detector sensitivity and noise performance in the DOT inverse problem). As noted in the introduction, several groups have attempted to measure early photons with a range of detector types, including MCPs, PMTs, ICCDs, streak cameras and fast APDs. One evident issue therefore is the generalize ability of these results for systems with alternate component types. For example we cannot rule out the possibility that two detectors with identical TIRFs but with different sensitivity and noise properties would yield different instrument PDSF widths. However, the results of our studies with time-resolved MC data generally agree very well with our experimental data, and therefore our data indicates that simple convolution of the instrument TIRF appears to entirely account for the observed PDSF broadening effect. These data also underscore the importance of correcting PDSFs for instrument TIRFs via convolution when performing tomographic image reconstruction to ensure accurate data-model agreement.

As a complimentary, in combination with a Ti:Sapphire laser, the SPAD was extremely effective in measurement of EPs and yielded the narrowest EP PDSFs that we have been able to measure to date (i.e. a reduction of 63-66% versus the continuous wave case). For example, this is in
contrast to ~50% reduction in PDSF width as reported in chapter 2 where the overall system TIRF was 163 ps. Most significantly, EP PDSF measurements were close to the theoretical limit defined by TR-MC over the early time gates considered (~1% of peak or later) which we have not been able to achieve previously. In the context of DOT, potential disadvantages of this detector type include the “diffusion tail” effect (shown in fig. 4.7 (b)) which distorts the decay portion of the TR curve, and the relatively small detector area (~50 x 50 μm²), which limits the overall sensitivity. We next plan to build a small animal EP DOT imager using multiple APD detectors, 360 degree sample rotation and appropriate image reconstruction methods. We have also not yet tested the APD in measurement of fluorescence, which is typically of several orders of magnitude lower intensity than the excitation light. This is an ongoing area of research in our lab.
Chapter 5

Time-resolved Multispectral Fluorescence Imaging

5.1 Introduction

Fluorescence imaging is a powerful method for the study of cellular and molecular events in vivo. However, as noted the overlap of the emission spectra of fluorophores in a relatively narrow optical “diagnostic window” makes imaging and separation of multiple (3 or more) targets in deep tissues extremely challenging. In this chapter, multiplexed fluorescence imaging with multispectral and full-time data sets is described. Specifically, the goal was to image up to five fluorescent targets concurrently using joint spectral and temporal information in a multiplexed signal. As described in this chapter, this approach was implemented, in simulations in silico, in a tissue mimicking optical phantom and in a mouse. This work is complimentary to the image resolution improvement work described in the proceeding chapters.

Experimentally, we measured temporal and spectral data sets using a pulsed supercontinuum excitation laser and a time-resolved 16-channel spectrograph. The instrument was operated in time correlated photon counting mode. We measured transmitted time-resolved photons through the sample with 13 nm spectral and 8 ps temporal resolution. We used five distinct fluorophores with spectra emission in the range of red and near infrared wavelength. To be more specific, the maxima emission wavelengths were in the range of 700-810 nm and fluorophore lifetimes were in the range of 0.4-1.2 ns. We first embedded combinations of five fluorophores in a solid tissue mimicking diffusing phantom with 1.5 cm path length and then scanned the system with an x-y translation stage. The signal components were then analyzed and de-mixed using a set of three chemometric approaches. Our results show that joint use of spectral and temporal data decreases
the mean error in the estimated concentration by a factor of 6-7 times compared to temporal data and 1.2-1.5 times compared to spectral data alone. Preliminary results in mice were also validated with this method.

5.2 Theory

5.2.1 Analytical Calculation of Time Resolved Fluorescence Imaging

In this section, we review the theoretical background of time-resolved fluorescence imaging which has been comprehensively discussed in the literature [70-73]. A turbid medium with embedded fluorescent inclusion is considered. For time-resolved optical measurements, the medium is surrounded with at least a set of short pulse width laser and detector. The fluorescent intensity is given by

\[ U_{fl}(r_s, r_d, t) = \int_0^t \int_0^{t'} \int U_0 G^X(r_s, r, t'') G^m(r, r_d, t' - t'') d^3r dt'' \]  

Where \( r_s \) and \( r_d \) are the locations of source and detector, respectively; \( r \) is position in the diffusive media; \( \tau \) is fluorescence lifetime; \( t'' \) is an integration factor for the fluorescence lifetime; \( G^X \) and \( G^m \) are the source and emission green’s functions which depend on the absorption and scattering coefficients at the excitation and emission wavelengths; and \( \eta(r) \) is fluorescence yield, which is proportional to fluorescence quantum yield and fluorescent concentration.

If it is assumed that the optical properties of the diffused medium are equivalent at the excitation and emission wavelength, (5.1) can be re-written as given by

\[ U_{fl}(r_s, r_d, t) = \int_0^t \int_0^{t'} U_0(r_s, r, t'') G(r_d, r, t' - t'') d^3r dt'' \]  

(5.2)
By defining the measured time-resolved exit diffused photons at the detection site or “temporal point spread function” as given by

\[ TPSF_{\text{ex}}(r_s, r_d, t) = U_{SD}(r_s, r_d, t) = \int_0^t \int U_0(r_s, r, t'') G(r_d, r, t' - t'')d^3r \ dt'' \] (5.3)

where \( TPSF_{\text{ex}}(r_s, r_d, t) \) is the temporal point spread function at excitation wavelength.

Equation (5.2) is simplified to

\[ U_{fl}(r_s, r_d, t) = U_{SD}(r_s, r_d, t) * (\eta(r) e^{-\frac{t}{\tau}}) \] (5.4)

The form of fluorescent intensity measurement in (5.4) allows us to first measure the temporal point spread function of the medium at the excitation wavelength with our instrument, and then convolve the TPSF with the mono-exponential component of the fluorophore.

This approach relies on the fact that the decay rate of free single fluorophore is almost mono-exponential with a rate of \( \frac{1}{\tau} \) [74]. The time-dependent fluorescence emission of a mixture of n fluorophores when excited by an impulse light is the summation of respective fluorescence emission and is given by

\[ I_{fl}(t) = \sum_1^n C_n \exp(-t/\tau_n) \] (5.5)

The linearity of multiplex fluorescence intensity depends on the intensity of absorption light and the concentration of each fluorophore component. Increasing either of excitation light intensity or the number of fluorophore molecules can result in fluorescence quenching, i.e. reduction of fluorescence light intensity, through the system [75].

5.2.2 Chemometric Algorithm Using Pure Spectral Information

To illustrate the problem of multiplex imaging, we start with a simple mixed data that consists of n components, i.e. n fluorescent dyes. [76, 77]
\[ M(\lambda) = C_1 S_1(\lambda) + C_2 S_2(\lambda) + \ldots + C_n S_n(\lambda) = \sum_1^n C_x S_x(\lambda) \quad (5.6) \]

Where \( S_x \) and \( C_x \) are the spectral characteristic and concentration of fluorophore \( x \), respectively, and \( M \) is the measure signal.

For a multispectral image problem, equation 5.4 can be discretized into a matrix form as given by

\[ M_{m \times k}(\lambda) = C_{m \times n} S_{n \times k}(\lambda) \quad (5.7) \]

where \( M_{m \times k} \) is measured data matrix with \( m \) rows corresponding to \( m \) pixels in the image and \( k \) columns corresponding to \( k \) different wavelength (spectral channels). \( C_{m \times n} \) represents the matrix of fluorophore concentrations or component weight matrices with \( m \) rows corresponding to \( m \) pixels and \( n \) columns corresponding to \( n \) different fluorophore components. \( S_{n \times k} \) is pure spectral information of individual fluorophore with \( n \) rows corresponding to \( n \) fluorophore and \( k \) columns corresponding to \( k \) wavelength channels.

5.2.3 Chemometric Algorithm Using Joint Spectral and Temporal Information

As mentioned previously, the emission spectra of fluorescent dyes in red and NIR wavelengths are highly overlapped. In a multiplexed fluorescence imaging system with several fluorescence dyes, un-mixing using just spectral information is challenging. Our hypothesis is that if the unique life-time of each fluorophore is included in the chemometric analysis, then the accuracy of fluorophore separation will be improved. [78, 79]

Including joint spectral and temporal information of all fluorophore, the measured data (\( M \)) and spectra (\( S \)) matrices in equation 5.7 are related as given by:
\( M_{m\times k\times p}(\lambda, t) = c_{m\times n} s_{n\times k\times p}(\lambda, t) \) \quad (5.8)

where \( p \) is the number of time bins. In order to simplify this equation, each 3D matrix can be converted to a 2D matrix, and equation (5.8) will be simplified as given by

\[ M_{m\times q}(\lambda, t) = c_{m\times n} s_{n\times q}(\lambda, t) \] \quad (5.9)

where \( q \) is the multiplication of \( p \) (the number of time bins) and \( k \) (the number of spectral channels).

In this work, we define the reference library (RL) as joint spectral, lifetime, and intensity information of each fluorophore emission with is measured or calculated at specific concentration of the fluorophore. The multiplexed signal will be written as

\[ M_{m\times q}(\lambda, t) = c_{m\times n} RL_{n\times q}(\lambda, t) \] \quad (5.10)

Mathematically, the concentration matrix can be solved by:

\[ C = M \times RL^T \] \quad (5.11)

where \( RL^T \) is the transpose matrix of reference library information. A general approach for solving equation (5.11) is using least squares technique:

\[ e = M - \tilde{C} \times RL \Rightarrow \min(e) = \|M - \tilde{C} \times RL\|^2 \] \quad (5.12)

where \( \tilde{C} \) is an estimation of fluorophore concentration.
5.3 Methods and Materials

5.3.1 Instrumentation

The system used for time-resolved multiplexed imaging is shown in figure 5.2. A customized pulsed supercontinuum fiber laser with an 80 MHz repetition rate was used as the light source (Koheras Super K Power, NKT Photonics, Birkerod, Denmark). The laser was modified by the manufacturer so that it had a shortened fiber compared to standard models to reduce the pulse width to approximately 300 ps. The output of the broadband laser was passed through a visible-NIR splitter device (Spectra K Split; NKT Photonics) to isolate the 550 to 850 nm section of the pulse and then was further filtered with a 655 nm bandpass interference filter (50 nm FWHM; Chroma Technology, Bellows Falls, VT). A short-pass filter with 680 nm (2 nm FWHM) was placed in the illumination path to block out light with similar wavelength as emission spectra of fluorophores. The light was illuminated to a sample.

![Diagram](image)

**Figure 5.1** Schematic of the system used in time-resolved multiplexed imaging with solid phantom
A 1-mm pin hole was placed at the exit plane of the sample and the emission light was passed through a 700 nm long pass filter (HQ700LP, Chroma Technology, Bellows Falls, VT). This filter blocked out spectra of absorbing light. A 1 m fiber bundle (long silica core, multimode fiber, 0.22 NA and 1060 μm core diameter with almost ideal coupling efficiency) was used to transfer excited light to the entrance slit of a polychromator (MS125, Becker & Hickl). The exit plane of polychromator was attached to a 16-channel photo multiplier tube (PMT) array (PML-16-C, Becker and Hickl, Berlin, Germany). The gain on the multi-anode PMT was controlled with a detector control card (DCC; Becker and Hickl) that was operated at 90% of maximum. The output of the PMT array was amplified with internal preamplifiers and coupled into a time-correlated single photon counting card (SPC-130, Becker and Hickl) configured so that it had 8 ps temporal resolution. The polychromater grating was calibrated to record wavelength in the range of 704 nm to 912 nm with 13 nm spectral resolution. Therefore, the 16-channel detector measured 16 data sets with each spectral channel, each containing the time point spread function of the sample at a specific wavelength.

5.3.2 Tissue Mimicking Solid Phantom

A homogenous tissue mimicking solid phantom was designed with epoxy-resin to have optical properties similar to biological tissue at red and NIR wavelength. The solid phantom was prepared by a mixture of epoxy-resin (Castin’Craft), Titanium Oxide powder (Sigma-Aldrich), and India ink. The scattering property was controlled by Titanium Oxide (TiO$_2$) powder at a concentration of $2 \cdot \frac{g}{L}$, and the absorption property was controlled by adding 50 ppm India ink. The scattering and absorption coefficient of the phantom was approximately $\mu_s' = 17 \text{ cm}^{-1}$ and $\mu_a = 0.15 \text{ cm}^{-1}$ at 670 nm wavelength. In order to easily introduce fluorescent inclusion to the
custom made 12 cm × 12 cm × 1.5 cm solid phantom, two sets of holes were drilled on two sides of the phantom. The first set consisted of three holes with a wall-to-wall separation of 1.4 mm and internal diameter at 3.4 mm which were drilled in the mid-plane of the solid phantom. The second set consisted of two holes with diameter of 4 mm and wall-to-wall separation of 3 mm, one hole was drilled in the mid-plane and the other one was drilled 4 mm off the face of phantom. The depth of each hole was 4 cm. The set of three holes were used for multiplexed measurements, and the set of two holes was used for reference library measurement.

5.3.3 Fluorophores

Characterization of multiplexed imaging system was performed using a set of five commercially available fluorophores. The excitation and emission characteristic of these fluorophores were in the range of 680 nm to 782 nm and 702 nm to 810 nm, respectively. The spectral properties of these fluorophores are is given in table 5.1. These fluorophores were chosen based on three factors: i) emission and excitation spectra of each fluorophore were in the range of red and NIR wavelength that could provide relatively deep through tissue penetration, ii) distinct lifetimes, and iii) no biological toxicity were reported for each fluorescent dye. Using the aforementioned criteria, we chose Alexafluor-680, -700, -750, and -790 (Life technologies, USA) and ATTO-725-COOH (ATTO-Tec Gmbh, Siegen, Germany). According to the manufacture’s specification, the lifetime of these fluorophore is in the range of 0.4 to 1.2 ns. Before each experiment the fluorophores were dissolved with Dimethyl Sulfoxide (DMSO) and then diluted with 1% Intralipid at final concentrations between 1 µM to 10 µM. The lifetime of each mixed fluorophore-Intralipidsolution was measured with our 16-spectral channel system. The result is reported in section 5.4.1.2.
The spectral properties of the above mentioned fluorophores are highly overlapped and, as we show, un-mixing requires an instrument and algorithm that incorporates both spectral and temporal data. The use of quantum dot fluorophores with less spectra crossing has been reported in multiplexed imaging system [80, 81]. However, quantum dot were not used in this project due to growing debate related to human health and safety risk associated to quantum dot [82, 83].

<table>
<thead>
<tr>
<th></th>
<th>Maxima Absorption</th>
<th>Maxima Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexafluor-680</td>
<td>679 nm</td>
<td>702 nm</td>
</tr>
<tr>
<td>Alexafluor-700</td>
<td>702 nm</td>
<td>723 nm</td>
</tr>
<tr>
<td>ATTO-725</td>
<td>729 nm</td>
<td>752 nm</td>
</tr>
<tr>
<td>Alexafluor-750</td>
<td>749 nm</td>
<td>775 nm</td>
</tr>
<tr>
<td>Alexafluor-790</td>
<td>785 nm</td>
<td>810 nm</td>
</tr>
</tbody>
</table>

Table 5.1: The spectral properties of the fluorophores.

5.3.4 Modeling of Multiplexed Data

We first developed two sets of simulated multiplexed fluorescent (temporal and spectral) data. In the first simulated data set, we evaluated the performance of chemometric algorithms using joint spectral and temporal data on multiplexed fluorescence imaging. This was compared with the performance of conventional fluorescence imaging approaches where either purely spectral data or pure temporal data were used.

The total combinations from a set of n components, i.e. n=5 fluorophore solution, are:
\[
\text{Total } C^n_r = \sum_{r=1}^{n} \frac{n!}{r!(n-r)!}
\] (5.13)

where \( r \) is the number of objects that can be selected from \( n \) components.

Multiplexed data was simulated using combinations of five fluorophores (a total of 31 combinations), and the assigned concentration of each fluorophore was either 0 µM or 1 µM.

To achieve this, first the time-resolved and spectrally resolved emission of each fluorophore, i.e. Reference Library (RL), was measured with the system with fluorophores at the concentration of 1 µM using the experimental setup shown in figure 5.1.

The components of the measured RL were used in the generation of the simulated multiplexed data. In the multiplexed simulated data set, the concentration of each fluorophore was simply 0 µM, if the fluorescent dye was absent from the mixed data, or 1 µM, if the fluorophore was included in the mixed data. Simulated Poisson noise, corresponding to photon counting noise, was added to the mixed signal. As described in section 5.3.6 below a set of three un-mixing algorithms was applied to the simulated signals. The mean concentration error was calculated for each combination.

In order to investigate the impact of noise sensitivity on each individual analysis, we developed a second set of simulations. In the second set of simulated data, multiplexed data was generated based on the presence of all the fluorophores with random concentrations in the range of 0.1 µM to 5 µM. Similar to the above mentioned modeling, the simulated data was the summation of components of measured RL. Gaussian white noise was added to the multiplexed signal with a signal to noise ratio (SNR) value in the range of 19 dB to 70 dB in the steps of 5 dB. Each added noise was performed with \( k = 300 \) different realizations and each SNR value was irritated on 400 random concentration data sets.
5.3.5 Multiplexed Fluorescence Imaging

5.3.5.1 Phantom Measurement

We used a homogenous solid phantom with three embedded holes with diameter of 3.4 mm in the mid-plane of the phantom. The middle hole was filled with 1% Intralipid, and the side holes were filled each with three Tygon tubes. The inner diameter of Tygon tube was 0.02 inch. Based on the combination of fluorescent dyes, each tube was filled with either of the fluorescence solution or Intralipid.

All multiple fluorophore combinations, total 31 combinations with 5 fluorescence solution, were investigated here. Each measurement was performed by placing the Tygon tubes inside the hole in the solid phantom. The phantom was positioned on the translation stage, where the laser beam was emitted to the surface of the phantom. The laser power was set at 22 mW at 670 nm wavelength for these measurements. The solid phantom was scanned in x-y direction with the scan size of 30 mm × 10 mm in normal and vertical directions, respectively. The step size was 0.5 mm and 1 mm in normal and vertical directions, respectively. The emission signal was measured at each position with 2 s acquisition time.

The multiplexed signals were then analyzed and un-mixed with respect to the RL using a least squares approach. Each data set was analyzed with a set of three methods. i) joint spectral and temporal data, ii) spectral data only, i.e. all the temporal bins in each spectral channel were integrated, and iii) temporal data only, i.e. all the spectral channels in each time bin were integrated.
5.3.5.2 Measurements in mice

To demonstrate the performance of our time-resolved multispectral imaging system and un-mixing algorithm in small animals, initial measurements were performed on female nude mice (N=5). The mouse was first euthanized and the animal was placed vertically on the translation stage, and stage was translated in x-y direction with the scan size of 1.6 cm × 1.1 cm in normal and vertical directions, respectively with a 1 mm step size in both directions. The emission signal was measured at each position with 3.5 s acquisition time. The illuminated light power was set at 42 mW at 670 nm. Three glass capillaries filled with Alexafluor-680, Alexafluor-700 and Alexafluor-750 at final concentrations of 8 µM, 7 µM and 6 µM, respectively. The tubes were inserted subcutaneously.

5.3.6 Data Analysis

The multi-spectral and temporal fluorescence data was acquired and processed using custom-written Matlab software (The Mathworks, Natick, MA). Un-mixing of the fluorescence data was performed on each data set. As explained in section 5.2.3, the actual concentration of each fluorescent dye was estimated by a least squares approach with a non-negative constraint. As above, a set of three analyses were performed on each multiplexed dataset: i) joint spectral and temporal data, i.e. all spectral and temporal bins were analyzed individually ii) purely spectral data, i.e. the temporal bins of each spectral channel were integrated, and iii) purely temporal data, i.e. the spectral channel of each time bin were integrated.

5.3.6.1 Reference Fluorophore Library
In order to measure the reference library for the solid phantom, we first measured the spectra and lifetime data of each fluorophore which was embedded inside the phantom. The impact of fluorophore depth on the transmitted fluorescence emission was studied. As already explained, each fluorophore was diluted with 1% intralipid. Alexafluor-680, -700, and -750 were prepared at a concentration of 1 µM and ATTO-725 and Alexafluor-790 were prepared at concentration of 2.5 µM. We used very thin transparent Tygon tube (ID = 0.02 inch and OD = 0.06 inch) filled with the fluorophore solution. A total five Tygon tubes were fitted in the hole inside the solid phantom. To measure the emission spectra of each fluorophore, one tube was filled with one fluorophore at a time, and the other Tygon tubes were filled with 1% Intralipid solution. A complete set of measurements was performed by translation of solid phantom through 16 steps in the normal direction and through 1 step in the vertical direction. The step size in both directions was 0.5 mm, resulted in 8 mm × 0.5 mm scan size.

We limited the number of scan steps in vertical dimension because the positioning of fluorescent Tygon tubes were in the vertical axis, i.e. the fluorophore concentration was constant in this direction.

We also measured the background phantom auto-fluorescence by placing five Tygon tubes filled with Intralipid. The auto-fluorescence signal was then subtracted from fluorescent spectra for each of the 5 fluorophores. The measured spectral and temporal data at the position with maximum emission intensity was saved for each fluorophores, and this data set was included as one row of reference library matrix (RL). In total, the RL matrix contained five rows corresponding to five fluorophores.

5.3.6.2 Calculated Reference Fluorophore Library
Although it is feasible to measure the components of reference library in a homogenous medium, in general (e.g. in mice) this approach is not practical. In an *in vivo* measurement with a heterogeneous medium, it is necessary to estimate the RL based on the lifetime and spectra of each fluorophore. Therefore, the data characteristics of each fluorophore were analyzed based on one set of measured RL.

First, we measured the lifetime of each fluorophore with our system. Disposable plastic cuvettes with a window of 4.5 mm × 2.3 mm were filled with a mix of each fluorophore-Intralipid. We used a concentration of 1 µM for Alexafluor-680, -700, and -750, and the concentration of 2.5 µM for ATTO-725 and Alexafluor-790. Each fluorophore lifetime was measured by filling the cuvette with 1.5 mL of the fluorophore, which was then placed in front of the laser beam. The detector fiber bundle was placed at 90 degree of the path of excitation light and the fluorescence emission light was measured with our 16-channel detector.

In general the measured detected light with any optical system is the convolution of system temporal impulse response function (TIRF) with the time-resolved transmitted light. Therefore, the impact of system TIRF needed to be removed from the measured fluorescence emission light. The system temporal impulse response function (TIRF) was measured in a similar way by placing a cuvette which was filled with water in between source and detector.

\[
FI(t) * \text{system TIRF} = FI^{\text{meas}}(t)
\]  

(5.14)

As already described, the time-dependent fluorescence emission light is almost mono-exponential and equation (5.14) can be simplified as given by:

\[
\exp\left(-\frac{t}{\tau}\right) * \text{system TIRF} = FI^{\text{meas}}(t)
\]  

(5.15)
The system TIRF and “measured FI” were two components that could be measured within 16 individual wavelength channels using our spectrograph-PMT system. The lifetime of each fluorophore solution ($\tau$) was analyzed by a least squares approach at each spectral channel. As given in (5.4), the measured fluorescence emission from any scattering medium is the temporal convolution of system point spread function (TPSF) at excitation wavelength with fluorophore quantum yield.

In order to measure the temporal PSF of the system at excitation wavelength, system calibration was performed by: i) the 700 nm long pass filter in the detector arm was removed, ii) the spectrograph was calibrated with micrometer to detect the excitation light which was in the range of 605-680 nm. Then the scattering medium was placed on the stage, in the path of light beam and detector arm. The temporal point spread function of the diffuse media was measured at excitation wavelength ($TPSF_{ex}$) with our 16-spectral channel detector.

With this information, each component of RL was calculated by the convolution of $TPSF_{ex}$ the IESF with the mono-exponential component of the fluorophore.

### 5.4 Results

#### 5.4.1 Fluorophore Reference Library

#### 5.4.1.1 Measured Fluorophore Reference Library

As described above, each fluorescent dye was diluted with Intralipid at specific concentrations. One Tygon tube was filled with the fluorescent solution, and the tube was placed in the hole inside solid phantom. The fluorescence emission intensity was investigated by embedding the fluorophore inside one of the holes in the set of 2-holes different depths. First the impact of
fluorophore depth was studied on the measured transmitted TPSF of the system. The result showed that in transmission geometry, the emission fluorescence intensity is constant at different depths of embedded fluorophore. The theory of fluorescence imaging in equation (5.2) verifies this result. Therefore, we continued our measurements by placing fluorescence inclusion in the mid-plane hole.

The phantom was scanned and the emission light was recorded with the high resolution temporal spectrograph. Time dependent emission light with temporal resolution of 8 ps and total bins of 1024 was measured within 16 spectral channels. The spectral and temporal information converted into a vector data with the total bins of $1024 \times 16 = 16384$. We calibrated the spectrograph so that the 1st spectral channel was set to 704 nm. An example measured signal from the first spectral channel is shown in figure 5.3. Although our detection system was able to cover the spectral wavelengths in the range of 704 nm to 912 nm, the fluorescent dyes used in our measurements had a maximum emission wavelength of 810 nm, and therefore, the last five red shifted spectral channels, i.e. channel number 12 to channel number 16, were not used in our analysis. As shown in figure 5.4 each data vector of RL consists of the first 11 spectral channels. Since the excitation wavelength was in the range of 605-nm to 680-nm, the bluest shifted fluorescent dye, i.e. Alexafluor-680, triggered with the maximum excitation light power and the reddest shifted fluorescent dye, i.e. Alexafluor-790, absorbed the least excitation light intensity. This figure shows the normalized emission signal of each fluorescent dye based on the maximum light intensity among them.
Figure 5.2 The measured fluorophore RL used in time-resolved multiplexed imaging
5.4.1.2 Calculated Fluorophore Reference Library

We next calculated the reference library based on measured fluorescence spectral and lifetime data. We analyzed the fluorescence lifetime at each distinct spectral channel. Table 5.2 shows the mean value of measured lifetime on all the spectral channels as well as the lifetime reported by the manufacture. The measured lifetime in the emission spectra of each fluorophore was generally similar to the manufactures reported data, although the measured lifetime of ATTO-725 was about 1.8 times the reported value. We believe this discrepancy is most likely due to the binding of Intralipid molecules to ATTO-725 fluorescent dyes, since fluorescent lifetimes are known to be highly sensitive to their micro-environment [84].

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Measured lifetime (ns)</th>
<th>Manufacturer’s reported lifetime (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexafluor-680</td>
<td>1.25 ± 0.09</td>
<td>1.2</td>
</tr>
<tr>
<td>Alexafluor-700</td>
<td>0.97 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>ATTO-725</td>
<td>0.91 ± 0.24</td>
<td>0.5</td>
</tr>
<tr>
<td>Alexafluor-750</td>
<td>0.52 ± 0.05</td>
<td>0.6</td>
</tr>
<tr>
<td>Alexafluor-790</td>
<td>0.42 ± 0.04</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 5.2: The measured and manufacture’s data for lifetime of each fluorophore

In figure 5.4, the calculated reference library (blue line) was compared with the measured reference library (red line) for each fluorophore. Generally relatively good agreement was observed, and refining these estimates is a point of continuing optimization.
Figure 5.3 (colored line) Calculated fluorophore RL (blue line) compared with measured fluorophore RL (red line).
5.4.2 Multiplexed Fluorescence Imaging

5.4.2.1 Modeling of Multiplexed Data

In order to verify our experimental data, two sets of modeling were performed. Simulated signals were generated based on our measured RL as described above. The first set of mixed signal was simulated similar to the measured phantom data, i.e. 31 fluorophore combinations were examined. Each multiplexed signal was de-mixed with the chemometric algorithm with three methods. Figure 5.4 shows mean concentration error of the model multiplexed data for each fluorophore combination. The mean concentration error of the multiplexed model data, using spectral and temporal data, increased from $0.36 \pm 0.1\%$ to $2.1\%$ when the number of contributed fluorescents increased from one fluorophore to five fluorophores. The modeling of multiplexed data verifies that increasing the number of component in the multiplex data increments the mean concentration error. Similarly, the mean concentration error of the mixed signal increases from $1.9 \pm 1.04\%$ to $15.1\%$ and from $0.36 \pm 0.1\%$ to $2.1\%$ for only temporal data and pure spectral data, respectively.
Figure 5.4 Mean concentration error of different fluorophore combinations based on i) spectral and temporal data, ii) purely spectral data, and iii) purely temporal data for simulated multiplexed data.

As already described, the impact of noise sensitivity on each analysis method, i.e. joint spectral and temporal data, versus just spectral data versus just temporal data was investigated with the second set of simulated data. Again, the multiplex signal was simulated based on our measured RL. Gaussian white noise was added to the multiplexed signal with specific SNR value in the range of 19 dB to 70 dB. We calculated the experimental noise value in each data analysis, and the added noise value on simulated multiplexed data was based on our system noise sensitivity function. The system SNR values of our system was measured at 29 dB, 37.3 dB, and 55 dB for joint spectral and temporal data, only temporal data, and only spectral data respectively.
As shown in figure 5.5, the multiplexed imaging system that uses both spectral and temporal data has the lowest concentration error at different system noise sensitivity levels. Increasing the signal to noise ratio from 19 dB to 54 dB reduced the mean concentration error from 5.3% to 1.4% when joint spectral and temporal data were used in the un-mixing algorithm.

The simulated multiplexed data that uses joint spectral and temporal data in the un-mixing algorithm predicted the mean concentration error of 2%, when the noise power similar to our system noise value was added to the modeled multiplexed data. However, the mean concentration error of measured multiplexed data was 6.5 ± 0.5%.

Increasing the signal to noise ratio on multiplexed simulated data from 19 dB to 74 dB reduced the mean concentration error from 114.1% to 2.7% if only spectral data were used in the un-mixing algorithm. Based on the simulated data, the mean concentration error of a system that uses just spectral data with noise sensitivity function similar to our system was 4.1%. However, the measured mean concentration error of our system was 7.7 ± 1%.

Increasing the signal to noise ratio on multiplexed simulated data from 19 dB to 64 dB reduced the mean concentration error from 59.8% to 11.7% if only temporal data were used in the un-mixing algorithm. Based on the simulated data, the mean concentration error of a system that uses just temporal data with noise sensitivity function similar to our system was 15.3%. The measured mean concentration error of our system was 44 ± 0.3%.

In these three analysis methods, the un-mixing algorithm predicted lower error for simulated signals than our measured signals. This discrepancy is not surprising, and we believe results primary from the quenching effect in the measurement of mixed fluorescence signal due to spectral overlap between fluorescent dyes. This effect introduces nonlinear parameters to the measured multiplexed fluorescence signal. However, the simulated multiplex signal was
assumed as a linear summation of several fluorophor signals and the quenching effect was not modeled here. This is an area of potential improvement in the future.

**Figure 5.5** Effect of signal to noise ratio on the mean concentration error of joint spectral and temporal data, pure spectral data, and pure temporal data, with simulated data. The equivalent mean concentration error of the measured multiplexed data with phantom is shown in the figure.

**5.4.2.2 Phantom Measurement**

As we described previously, five fluorescent dyes used in our experiments. Separate Tygon tubes were used for each fluorescent dye, because, we wanted to eliminate the quenching artifact
which was observed by physical contacts of different fluorescent dyes. As shown in figure 5.6, three sets of Tygon tubes were placed in each side holes. The middle hole was filled with 1% Intralipid solution. Different fluorophore combinations were examined, each with at least three trials. The un-mixing algorithm was performed on the mixed signal, and the component concentration of each fluorophore was plotted in a 2D data. Figure 5.6 shows a sample of multiple fluorophore which is un-mixed to its components. The sample consisted of a set of Alexafluor-680, -700, -750 and a set of Alexafluor-790, ATTO-725, and Intralipid in the left and right sides of phantom, respectively. As shown in this figure, the algorithm un-unmixed the data into its components.

Figure 5.6 An example of measured multiplexed imaging with tissue mimicking solid phantom.

The accuracy of multiplexed imaging was quantified with the mean concentration error for each sample, i.e. the error concentration of each fluorescent component was calculated and the total error was analyzed based on the mean value. Total combinations of the five fluorophores, i.e. 31 combinations, were measured and each one repeated at least 3 times. The nonnegative least
squares were performed on i) spectral and temporal data, ii) spectral data, and iii) temporal data, and the mean concentration error was calculated for each. As shown in figure 5.7, generally use of spectral and temporal data resulted in significantly lower error than either spectral or temporal data types alone. The mean concentration error of the multiplexed imaging, using joint spectral and temporal data, increased from $2 \pm 0.7\%$ to $6.5 \pm 0.5\%$ when the number of contributed fluorescent increased from one dye to five dyes. Increasing the mean concentration error by incrementing of the number of fluorophores was due to two main factors: 1) higher spectral overlap 2) higher quenching effect which introduced nonlinear effects to the multiplexed system. The mean concentration error of the un-mixing algorithm using only temporal data, increased from $8.7 \pm 7.3\%$ to $44.5 \pm 0.4\%$ when the number of contributed fluorescent increased from one to five.

The mean concentration error of the un-mixing algorithm using only spectral data, increased from $1.8 \pm 0.6\%$ to $7.7 \pm 1\%$ when the number of contributed fluorescent increased from one to five.

As described above, purely spectral analysis was performed by integrating of all temporal bins in each spectral channel. This integration improved our system noise performance from 29 dB-joint spectral and temporal bins were analyzed- to 55 dB–where the integration of temporal bins in each spectral channel was analyzed-. Similar mean concentration error that was observed here by joint spectral and temporal data analysis versus purely spectral data analysis is most likely due this SNR discrepancy.
5.4.2.3 Mice Measurement

As we described previously, five fluorescent dyes used in mice measurements. Separate glass capillary tubes were used for each fluorescent dye, and the tubes were placed subcutaneously. Figure 5.8 shows an example of multiple fluorophore in mice which is un-mixed to its component. The sample consisted of a set of Alexafluor-680 and a set of Alexafluor-700 and 750 in the left and right side of the mice, respectively. The un-mixing algorithm with joint spectral and temporal data was performed on the mixed signal, and the component concentration of each fluorophore was plotted in a 2D data. Because of the heterogeneity of mice body,
preparation of measured reference library was not practical and the calculated reference library, as described previously, was used in the un-mixing algorithm.

**Figure 5.8** An example of measured multiplexed imaging in mice using joint spectral and temporal information.

The analysis of multiplexed mice data is under more analysis.
5.5 Discussion and Conclusions

Multiplexed fluorescence imaging is a powerful technique for studying multiple molecular or cellular events and is performed routinely in microscopy applications. However, fluorescent agents that are used for in vivo measurements require spectral emission properties in the red and NIR wavelength region due to relatively narrow “optical window”. Since organic fluorophores generally have broad emission spectra, use of multiple fluorophores is challenging because the emission spectra are highly overlapped. As such, un-mixing of several fluorescent dyes (3 fluorophores or greater) in whole animal or deep tissue imaging is highly challenging.

In this work we used both spectral and temporal information of each fluorophore in the un-mixing algorithm.

The relative mean concentration error of the fluorophores that used joint spectral and temporal data in the unmixed algorithm decreased by a factor of 6-7 times compared to the un-mixing method that used just temporal data.

By integrating the data of all time bins over each spectral channel, the signal to noise ratio of the system increased from 29 dB to 55 dB. This improvement of noise performance of the system had a direct result in the error of unmixed data. Specifically, the mean concentration error increased by a factor of 1.2-1.5 versus the use of both spectral and temporal data sets, which had inherently lower SNR. The effect of measurement noise on the performance of the un-mixing algorithm was verified in a simulated set of multiplexed fluorescence data. When the SNRs were match, joint use of spectral and temporal data significantly out-performed the spectral only data over all SNRs considered.

These experimental results imply that: i) the chemometric algorithms that use both spectral and temporal information of fluorophores result in accurate separation of fluorophore components.
(error < 7) ii) In a system with similar noise performance, the joint spectral and temporal data increases the accuracy of fluorophore un-mixing compared with only temporal data or only spectral data.
Chapter 6

Conclusion and Future Work

In summary, this PhD thesis was directed in two major paths. First, the impact of experimental factors such as physical and optical properties of diffuse media, photon arrival time, source-detector geometry, and instrumentation on the improved image resolution using time-resolved early arriving photons was quantified.

We then developed and validated a novel time-resolved imaging system that uses ultra-fast APDs to better approach the theoretical performance limits of EPs using current technology. Second, in the context of multiplexed fluorescence imaging, we have demonstrated that joint use of time-resolved and multi-spectral data in multiplexed fluorescence imaging allows concurrent imaging of up to five fluorescent dyes with significantly improved accuracy versus spectral or temporal data only.

In chapters 2-4 of this thesis, we quantified the impact of early arriving photons on optical image resolution experimentally. Time-resolved measurement of early arriving photons has been demonstrated as a method to improve the optical image resolution bulk diffusive media. However, the quantitative impact of early arriving photons had not been fully studied previously. In chapter 2, we studied the reduction of the width of photon density sensitivity functions using early arriving photons compared to the width of PDSFs using quasi-CW photons. This study was performed over a range of optical properties and path-lengths relevant to small animal imaging. The experimental results were verified against a set of theoretical modeling including the diffusion approximation to the Boltzmann Transport Equation, and time-resolved Monte Carlo simulations.
In chapters 3-4, we investigated the impact of instrumentation on measurement of early arriving photons. Specifically, the importance of temporal impulse response function on the system, detector sensitivity, and source-detector geometry were studied. Based on these results a time-resolved imaging tomography system was designed and implemented in our lab.

In addition to the quantification of time-resolved early photon measurements - i.e. the effect of temporal, media and instrument parameters - we studied time-resolved multispectral data in multiplexed fluorescence imaging system. The highly spectral overlap of near-infrared fluorophores is a true challenge in separation of several near-infrared fluorescent dyes. The relatively narrow “optical window” limits the number of fluorescent agents in an in vivo imaging and the accuracy of chemometric algorithm in a multiplexed fluorescence imaging.

In chapter 5, we focused on resolving current limitations in the field of in vivo multiplexed fluorescence imaging. A novel chemometric approach, i.e. using joint utilization of spectral and lifetime information of fluorophores, was demonstrated and could overcome the challenges of highly overlapped spectral window. The joint spectral and temporal method showed promising results compared with conventional pure spectral or pure temporal multiplexed imaging. Using this technique, we could separate up to five fluorophores with the maximum emission spectra in the range of 702 nm to 810 nm with an accuracy of more than 93% both in tissue-mimicking phantom and in nude mouse.

The results that were discussed in each section of this thesis can be utilized in the design of future optical and fluorescence imaging systems. Likewise, there are some potential avenues which require further study. Potential future studies are described in the following sections.
6.1 Future Work for Diffuse Optical Tomography

The first direction is an improved imaging system for time-resolved tomography measurements based on the work on this thesis. One of the principal goals of early photon measurement is the diagnosis of cancerous tumors at their early stage. The optical imaging of deep-seated tumors and agents requires a tomography system with several source-detector paths around the diffuse object.

The optical images in this thesis were mainly based on one set of source-detector. The diffuse media was scanned in two directions and 2D images were created. For a complicated diffusive media with a heterogeneous property, the medium needs to be surrounded by several source-detector paths. Having a tomographic imaging system also speeds up data acquisition, which is necessary for \textit{in vivo} measurements.

In this thesis, the relative improvement of early photon measurement in compared with un-gated photons was studied with a homogenous tissue-mimicking phantom. Increasing the complexity of diffuse phantom geometry or properties, i.e. heterogeneity and the shape of diffuse media, are the other paths that needs to be investigated.

The optical imaging system that was investigated in this thesis designed for the imaging in a transmission mode, because the goal was to develop a tomography system. The other direction that would be beneficial is by investigating the early-arriving photons for imaging in the reflection mode. In the expansion of clinical applications of time-resolved imaging system, it is crucial to design compact optical instrumentation that measures early-arriving photons in the reflection mode. Optical imaging has been used for the applications of cardiovascular,
intravascular, oral cavity, and endoscopy imaging system. The measurement of early-arriving photons for each of these applications is a merit.

6.2 Future Work for Multiplexed Fluorescence Imaging

Fluorescence imaging is a promising technique in studying cellular and molecular events. Each fluorophore has a unique chemical and physical characteristic. In order to investigate multiplexed fluorescence imaging with high accuracy, the fluorescence emission of each fluorophore should be in a linear relation to the number of fluorescent molecules as well as the excitation intensity. However, as discussed in chapter 5, there are several factors that introduced non-linearities in fluorescence emission intensity, i.e. excitation light intensity, fluorophore concentration, and quencher dyes, which bind to fluorophores. Through further fluorescence imaging testing, it would be beneficial to model the above mentioned factors in the un-mixing algorithms.

In addition, one of the quenching factors that we observed in chapter 5 was the presence of more than one type of fluorophore in a mixed data. Increasing the number of fluorophores resulted in incrementing the non-linearity effect of quenching. The impact of this non-linearity was not developed in the modeling of multiplexed data sets. The simulated multiplexed data predicted the accuracy of de-mixed data %5 - %15 better than the measured data.

Another direction in the area of fluorescence imaging is developing other un-mixing (chemometric) algorithms. In this thesis an algorithm based on least-squares approach was used and a reference library was created based on spectral, lifetime and intensity of each fluorophore with certain concentration. This is an accurate method if we have a priori knowledge of
exogenous fluorophores. In a complicated molecular imaging system, where the goal is to visualize several endogenous fluorophores little or no prior information is available. As a continuation of this research, it would be interesting to investigate other chemometric algorithms such as blind source separation.

The other future work in the area of multiplexed imaging project is the re-calibration of the instruments. Currently, the spectral range of detection system is in the range of 700-900 nm, however the emission spectrum of five fluorophores are in the range of 700-810 nm. Since the spectral resolution of the system is 13-nm, several spectral channels (5-6 channels) have not been used in the multiplexed imaging system. Therefore, our current system has the detection capacity of more fluorophores compared to other systems. Based on the application of multiplexed imaging system and the spectrum range of fluorophore agents, the system wavelength range can be re-calibrated to cover a whole range of ~200 nm. In a multiplexed imaging system with a limited excitation power, it is not practical to excite several fluorophores in this relatively long spectrum range. One approach would be dividing the 16-channels in two sets of 8-channels and each set would cover a range of ~100 nm wavelength. Then by adding another excitation channel, each source arm can excite several fluorophores with emission spectrum in a range of ~100 nm. Therefore, each excitation is suitable to excite several fluorophores within 8-spectral channels.

As discussed in chapter 5, preliminary in mice multiplexed imaging has been performed in this thesis. In all these measurements, fluorescent inclusion was inserted inside the animal using a glass capillary tube. The goal of this multiplexed imaging system is to validate three separate tumor models by labeling cells using fluorescent proteins or exogenous fluorophores. Spectra or
lifetime information of these fluorescent agents may be affected by binding to biological tissues. This effect would make some challenges in the chemometric analysis of multiplexed data.
Bibliography


