

Towards a Novel Optical Spectroscopy Technique Using Photon Absorption Remote Sensing

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Optical spectroscopy has shown great promise in the field of biomedical research. For example, works employing traditional spectroscopy approaches have demonstrated that analyzing a sample’s optical response to incoming light can effectively differentiate between healthy and diseased tissue. However, these techniques suffer from limitations due to the fact that they typically capture signals from only a single light-matter interaction type, such as absorption, scattering or fluorescence. Therefore, many traditional methods are constrained in terms of the types of samples they can feasibly analyze, as well as, potentially, the depth of their sample characterization, as they do not focus on capturing relevant information from other interaction modalities. This work employs photon absorption remote sensing (PARS) to overcome these limitations.

PARS is a novel all-optical imaging technique capable of capturing radiative and non-radiative relaxation processes following electronic photon absorption. This thesis explores the initial development of the first PARS system specifically designed and optimized for optical spectroscopy applications, aimed at studying wavelength-dependent relaxation processes to characterize a wide range of liquid samples.

The first step of this work was to build a non-radiative PARS spectroscopy system capable of accurately capturing the thermal and acoustic relaxation processes that arise from different ultra-violet (UV) excitation wavelengths. These signals were processed and used to construct a non-radiative PARS absorption spectrum for each sample of interest. These spectra were benchmarked against the absorption data collected from a NanoDrop spectrophotometer, which served as the ground truth in this work. This study revealed that for certain samples, such as eumelanin, which is highly absorbent to UV light and relaxes almost all absorbed energy non-radiatively, the non-radiative PARS spectroscopy system is capable of generating highly accurate absorption spectra. However, this system did not generate as close to ground truth spectra for samples that do not have as strong UV absorbing tendencies and are not as non-radiative in nature.

The second step of this work was to integrate a radiative relaxation arm into the developed non-radiative PARS spectroscopy system. This pathway was configured to collect fluorescence emission spectra, which represent radiative sample relaxation, simultaneously with the collected non-radiative data. Radiative PARS absorption spectra were generated for each sample. In this way, the developed PARS system combines absorption (monitoring both relaxation pathways) and fluorescence emission spectroscopy onto a single bench-top system. The radiative PARS absorption spectra were compared to the ground truth, which revealed that molecules that are highly fluorescent in nature are more appropriately studied through the radiative relaxation arm than the non-radiative pathway. Total absorption

spectra, which combine the non-radiative and radiative absorption data, were also generated, and it was determined that the absorption profiles of certain samples, such as NADH, are best studied using this approach.

The final step of this work was to use the collected total absorption and fluorescence emission data from the PARS spectroscopy system to identify the composition of different mixtures of craft red and blue ink samples. Traditional linear and generalized bilinear models were employed to perform this unmixing and the results from this study indicate that the combination of the absorption and fluorescence data collected on this system allows for a more accurate identification of a mixture’s components than either data source individually. This suggests that the PARS spectroscopy system provides an increased level of detail in sample characterization compared single-modality spectroscopy systems.

Ultimately, this research lays the groundwork for the development of a PARS spectroscopy system capable of being deployed in clinical settings to study samples and help inform diagnoses. This work demonstrates the feasibility of leveraging PARS for optical spectroscopy and presents a system design and framework that can be further iterated upon to enhance performance and enable a robust characterization of relevant and complex biological samples.

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Chapter 1

Introduction

1.1 Optical Spectroscopy

1.1.1 The Basics of Light-Matter Interaction

When incident light from the ultra-violet (UV) or visible portions of the electromagnetic spectrum is directed towards a sample, two primary types of interactions can occur: absorption and scattering [1]. Absorption events are an interaction type in which photons from the light source are absorbed by the electrons of particles in the sample. This increases their internal energy level and causes a transition from their ground state to an excited state [2]. Once excited, the particle may return to its ground state through radiative or non-radiative relaxation processes. Radiative relaxations typically occur in the form of fluorescence, where the excited particle emits photons to release energy [3]. In contrast, non-radiative relaxations involve dissipating the absorbed energy through pathways such as heat and pressure [4]. The ratio of how much absorbed energy is released through radiative and non-radiative means is an inherent property of the sample's molecular structure, known as the quantum yield (Φ) [5], and can be mathematically expressed as:

$$\Phi = \frac{\text{the number of photons fluoresced}}{\text{the number of photons absorbed}} \quad (1.1)$$

Scattering, on the other hand, is a light-matter interaction in which photons are redirected from their original path due to interactions with the particles in the sample. This process can occur elastically, where scattered photons are of the same wavelength and energy as the incident photons, or inelastically, where the scattered photons are of a longer

wavelength, and thus of a lower energy level, than the incident photons [6]. In practice, these interaction types often occur simultaneously, where some incident photons are scattered, some are absorbed (undergoing relaxation based on the quantum yield) and the remaining photons pass through the sample with no interaction.

1.1.2 The Promise of Optical Spectroscopy

Optical spectroscopy is a field of research focused on studying how matter interacts with incident light, specifically through the mechanisms of absorption, scattering and fluorescence [7]. The nature of these interactions are governed by the molecular structure of the sample. As such, the collected spectral data can serve as a distinctive “fingerprint” for characterizing a specific material.

An attractive feature of optical spectroscopy lies in its non-contact and non-destructive nature, allowing for samples to be studied without physically altering or damaging them [8]. This makes optical spectroscopy approaches particularly suitable for characterizing biological samples, which are often delicate and difficult to acquire. Furthermore, optical spectroscopy is widely applied in biomedical research due to its high sensitivity to small physiological and biochemical differences between samples which may not be detectable by other standard techniques [9]. For example, one of the key applications of optical spectroscopy lies in disease detection, where comparing the optical responses of biomolecules in healthy versus diseased samples has shown significant promise in aiding the diagnosis and monitoring of conditions such as cancer [10]–[20] and Alzheimer’s disease [21]–[27].

1.2 Thesis Motivations

A key limitation of conventional optical spectroscopy techniques is that they typically focus on collecting signals from only a single interaction type: absorption, fluorescence or scattering. While these single-signal approaches can provide detailed insights into a sample’s properties, they can also overlook complementary information from other interaction modalities that could enable a more comprehensive characterization. Furthermore, popular approaches are also limited in terms of the types of samples they can feasibly analyze. For example, an absorption-based spectroscopy system which specifically measures non-radiative relaxation processes is appropriate to use to study samples with a low quantum yield but is not ideal for analyzing highly fluorescent materials. Conversely, fluorescence

emission spectroscopy is effective for samples with a high quantum yield but cannot feasibly characterize non-fluorescent materials. The overarching limitations of existing single-modality optical spectroscopy systems provide motivation for developing systems capable of simultaneously studying multiple types of light-matter interaction.

1.3 Thesis Contributions

The impact of this work lies in the application of Photon Absorption Remote Sensing (PARS), which is a novel non-contact and all-optical imaging technique that captures both radiative and non-radiative relaxation pathways following an electronic absorption event. PARS has demonstrated great promise in the fields of histopathology [28]–[31] and in *in-vivo* functional and structural vasculature imaging applications [32], [33]. This work presents a novel application of this technology, developing the first PARS system specifically designed for optical spectroscopy. This research takes advantage of the multi-modal nature of PARS to effectively combine absorption and fluorescence emission spectroscopy into a single system. The development of this system overcomes the limitations associated with existing optical spectroscopy techniques by collecting multiple types of spectroscopic signals across many different excitation wavelengths. This offers a more detailed sample characterization and increases the range of samples which can be appropriately analyzed relative to single-signal systems.

An additional promise of this work lies in informing the design of future PARS microscopes. Existing imaging systems typically leverage fixed-wavelength excitation sources, where the excitation laser is chosen to be a wavelength which is known to be well absorbed by biomolecules in the sample. For example, PARS systems for histopathology applications commonly employ a 266nm excitation source, as UV light is well absorbed by DNA, resulting in excellent nuclear contrasts in the generated tissue images [28]–[30]. Similarly, PARS vasculature imaging systems typically use a 532nm excitation source, as hemoglobin is highly absorbent at this wavelength [32]. These design choices are crucial, as PARS relies on electronic photon absorption events to trigger the relaxation processes that generate image contrast. While selecting excitation wavelengths is straightforward for targeting well-characterized absorbers, such as DNA and hemoglobin, it is more challenging for targeting biomolecules with poorly studied absorption profiles. As such, the development of an in-house PARS spectroscopy system stands to serve as a valuable tool to determine the absorption spectra of these unstudied biomolecules, informing the optimal excitation wavelength choice for future imaging applications.

1.4 Thesis Objectives

The ultimate objective of this research was to develop the first PARS system specifically designed for optical spectroscopy. This work aimed to demonstrate the feasibility of using this technology to characterize a wide range of samples and lay the foundation for future research. In order to accomplish this, three main goals were identified:

1. Design a PARS system capable of capturing non-radiative relaxations simultaneously with radiative relaxations across many different excitation wavelengths and samples.
2. Explore the utility of capturing both relaxation pathways in developing accurate absorption spectra for the samples of interest.
3. Demonstrate the benefit of using the combination of absorption and fluorescence emission data to study and identify the make-up of unknown samples.

1.5 Thesis Organization

The organization of this thesis is as follows:

- Chapter 2 provides an in-depth review of classically used optical spectroscopy techniques, as well as some of the existing work which has been done in building multi-signal optical spectroscopy systems. This chapter also provides a technical background on PARS and its current applications.
- Chapter 3 presents a non-radiative PARS spectroscopy system, discussing the development of the system as well as presenting results to analyze its performance in studying the absorption profiles of a variety of samples.
- Chapter 4 presents a total absorption PARS spectroscopy system, capable of simultaneously capturing non-radiative *and* radiative relaxation processes. Similar to Chapter 3, this chapter describes the design process surrounding building this system and presents results.
- Chapter 5 explores the application of the fully developed PARS system in unmixing, or, correctly identifying the make-up of, a mixture solution. Results in using the absorption and fluorescence emission spectral data from this system to unmix mixtures of craft red and blue inks are presented.

- Chapter 6 summarizes the conclusions of this research and identifies relevant next steps for future work.

Chapter 2

Background and Literature Review

2.1 Review of Optical Spectroscopy Techniques

Many different optical spectroscopy techniques exist that aim to study interactions between a sample of interest and incident light. This section examines existing popular approaches, outlining the theory behind how each approach works and identifying relevant advantages and disadvantages where appropriate.

2.1.1 Absorption Spectroscopy Techniques

Absorption spectroscopy is a technique used to study how effectively a sample absorbs light of one or more wavelengths. To begin, it is important to provide some background on the theory behind molecular light absorption. Electrons in a particle typically exist in their ground energy state, however, they can transition to higher singlet or vibrational states upon excitation. Following excitation, the electron must return to its ground state and can do so by dissipating energy through a combination of non-radiative and radiative processes. These electronic transitions and associated relaxation pathways are commonly illustrated using Jablonski diagrams, as shown in Figure [2.1](#).

Each photon emitted from a light source contains a specific amount of energy, which is inversely proportional to its wavelength, as described by the equation:

$$E = \frac{hc}{\lambda} \tag{2.1}$$

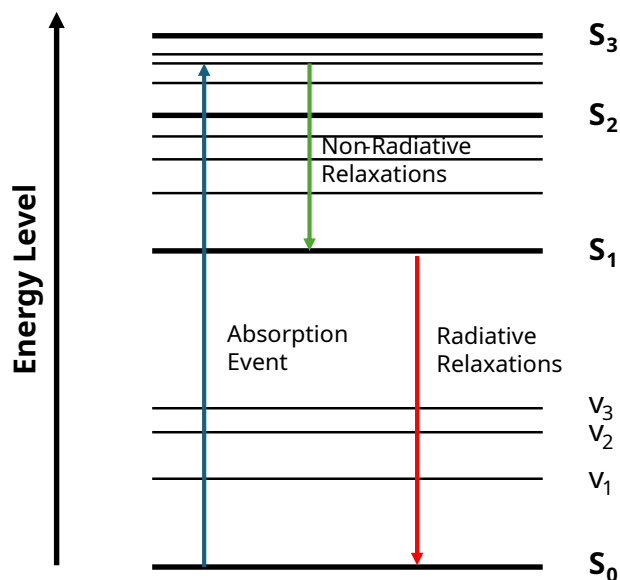


Figure 2.1: Basic Jablonski diagram illustrating a molecule’s singlet and vibrational energy states along with an absorption event and subsequent radiative and non-radiative relaxation processes.

where E represents the energy of one photon in this light source, h represents Planck’s constant, c represents the speed of light in a vacuum and λ represents the light source’s wavelength [34]. As such, an absorption event can only occur when the energy of an incident photon aligns with the gap between a particle’s ground state and an accessible excited state [35]. The arrangement and energy spacing of singlet and vibrational states are unique to each molecule, leading to molecule-specific tendencies to absorb light at particular wavelengths. As a result, each molecule exhibits a distinct absorption spectrum, which can be used as a fingerprint for identification and characterization purposes.

Photothermal Spectroscopy

Photothermal spectroscopy is a branch of absorption spectroscopy that measures the thermal effects that arise in a sample following the absorption of an incident photon [36]. These effects can be studied directly or indirectly. Direct measurement involves recording temperature increases in the sample that result from the absorbed energy being converted to heat. Indirect measurement is far more common and is done by studying the effects of localized heating within the sample. For example, temperature changes are known to alter

the refractive properties of samples [36], [37]. As such, most studies that use photothermal spectroscopy are based on probing these refractive index changes. Here, the magnitude of the changes to the refractive properties of a sample provides insight into how much heat was generated and, by extension, the magnitude of the initial absorption event.

There are several common ways to measure how a sample’s refractive index changes after an absorption event, most of which use a “pump-probe beam” configuration. In this approach, two laser beams are aligned and passed through the sample. The pump beam, also referred to as the excitation beam, is usually pulsed and set to a wavelength that the sample is likely to absorb. In contrast, the probe beam, also referred to as the detection beam, is continuous and chosen at a wavelength where the sample is transparent, ensuring minimal absorption. Figure 2.2 depicts a simple pump-probe configuration. Monitoring the properties of the probe beam as it travels through the sample reveals information about the nature of the initial absorption event.

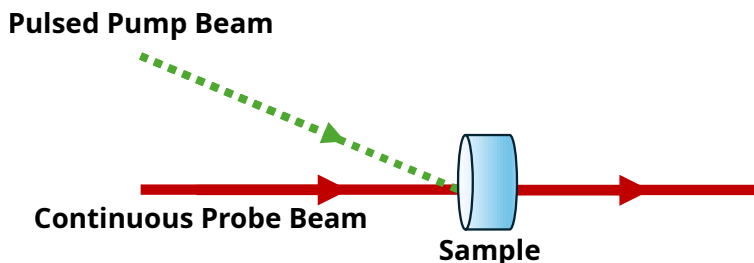


Figure 2.2: Simple pump-probe beam configuration.

Photothermal deflection studies how the probe beam is laterally deflected as it passes through the excited sample [38]–[41]. Photothermal interferometry monitors changes in the optical path of the probe beam due to induced refractive index shifts and has been shown to be especially effective in trace gas applications [42]–[45]. Finally, photothermal lens detection leverages the photothermal lensing effect, where localized heating causes a refractive index gradient to form in the sample, acting as a lens and slightly focusing or de-focusing the probe beam as it travels through [46]. This technique aims to monitor these focusing effects and has been successfully used to study various biomolecules, such as DNA and water [47]–[52].

Photothermal spectroscopy techniques are incredibly sensitive, as even small amounts of absorption are often sufficient to generate measurable photothermal signals [36]. However, a key limitation of these approaches lies in their reliance on solely measuring non-radiative relaxations, specifically heat generation. As a result, photothermal spectroscopy is not

the most suitable technique to analyze materials with high quantum yields that release significant portions of their absorbed energy through radiative processes [53].

Photoacoustic Spectroscopy

Photoacoustic spectroscopy measures the absorption of light by studying generated acoustic waves. When a sample absorbs a photon, the temperature of the material increases, causing thermal expansion and an increase in pressure within this localized region. This pressure increase generates acoustic waves, whose intensity is proportional to the magnitude of the initial absorption event [54]. Although these acoustic waves are produced by photothermal effects, photoacoustic spectroscopy is typically treated as a separate method from photothermal spectroscopy [36].

The generated acoustic waves can be detected directly through acoustic sensors [55]–[57], or indirectly using a probe beam. Modulations of pressure within a sample lead to mechanical stress, which also causes changes in the refractive index properties of the material, as described by the piezo-optical effect [58]. As such, similar to photothermal spectroscopy, studying how the probe beam travels through a sample after an absorption event can be used to measure and capture photoacoustic effects, as demonstrated in [59]–[61]. However, pressure effects tend to dissipate on time scales that are orders of magnitude faster than thermal effects. This makes their measurement through a pump-probe configuration more challenging but still feasible with sufficient absorption and high-speed signal digitization [62]. Furthermore, photoacoustic spectroscopy suffers from the same key disadvantage as photothermal spectroscopy, being that it only measures non-radiative relaxation processes. As such, this approach is not suitable for studying samples that primarily undergo radiative relaxation.

Direct Absorption Spectroscopy

Direct absorption spectroscopy is one of the simplest methods for characterizing a material’s optical absorption properties. It involves measuring the intensity of light transmitted through a sample relative to the intensity of the incident light source [1]. From this measurement, the sample’s absorbance value can be calculated using the Beer–Lambert Law:

$$A = -\log_{10} \left(\frac{I}{I_0} \right) \quad (2.2)$$

where A denotes the absorbance value at a specific wavelength, I_0 is the intensity of light directed *toward* the sample (incident light), and I is the intensity of light *transmitted* through the sample [63].

Direct absorption spectroscopy overcomes some of the limitations associated with photothermal and photoacoustic techniques, as it captures absorption events that lead to both radiative *and* non-radiative relaxation processes. However, this approach has a key limitation of its own, as it cannot differentiate between light lost due to true absorption and light lost due to scattering. When applying the traditional Beer–Lambert Law, any reduction in transmitted intensity is assumed to arise solely from photon absorption [64]. This limits its effectiveness for applications in biomedical research. However, developments have been made in extending the traditional Beer–Lambert Law to be capable of separating these absorption and scattering events, with current applications in accurately quantifying blood and tissue oxygenation levels [65]–[67].

Near-Infrared Absorption Spectroscopy

Near-infrared spectroscopy is a unique form of absorption spectroscopy that specifically uses near-infrared light. This differs from the methods described above, which typically rely on wavelengths in the UV to visible region of the electromagnetic spectrum. In the near-infrared region, photons do not contain sufficient energy to excite electrons. Instead, they are absorbed by vibrational overtones involving bonds such as O–H, N–H and C–H [68].

Near-infrared absorption spectra are commonly collected through a direct absorption spectroscopy mechanism [1] and can be used to provide insight into the chemical composition of a sample. This technique is commonly used in biomedical research, for example, in measuring hemoglobin oxygenation levels in patients with Alzheimer’s disease [23], [27].

2.1.2 Fluorescence Spectroscopy Techniques

Fluorescence spectroscopy specifically investigates the characteristics of the radiative relaxation processes that follow an absorption event. These techniques generally fall into two categories: fluorescence emission spectroscopy and time-resolved fluorescence spectroscopy.

Fluorescence Emission Spectroscopy

Fluorescence emission spectroscopy focuses on studying the spectral features of a sample’s radiative relaxations, such as the location of emission peaks and the intensity of the emitted light. These spectral characteristics are highly sensitive to the sample’s chemical structure and the magnitude of the absorption event. This sensitivity makes fluorescence emission spectroscopy a valuable tool in biomedical research, as physiological differences between samples can be identified by comparing their emission profiles [69]. An example of these spectral differences can be seen in Figure 2.3, which compares the fluorescence emission profiles of healthy mouse brain samples with the profiles of mouse brain samples with Alzheimer’s disease [24]. Other applications of this spectroscopic approach include the detection of cervical cancer, through the study of biopsied tissue samples [70] and urine analysis [71], as well as the identification of breast cancer from fresh tissue samples [72]–[74].

The main limitation of fluorescence spectroscopy in biomedical research lies in the challenge of extracting pure fluorescence signals from non-uniform samples such as biological tissues. In these complex environments, photons emitted from one biomolecule may be reabsorbed by another. This is a phenomenon known as “quenching” and ultimately alters the spectral characteristics of the measured emissions. Furthermore, scattering effects within the tissue further distort the emitted light, making it difficult to accurately isolate and interpret the true fluorescence emission spectrum of the sample [75].

Time-Resolved Fluorescence Spectroscopy

Time-resolved fluorescence spectroscopy is an approach that measures the temporal profile of the emitted fluorescence. This technique is commonly employed in combination with fluorescence emission spectroscopy. Each fluorophore exhibits a characteristic decay rate that determines the lifetime of its fluorescence emissions. Analyzing how the intensity of fluorescence decays over time can be used to understand the chemical and molecular structure of a sample [76]. A particularly useful feature of time-resolved fluorescence spectroscopy is that the lifetime of fluorescence from a molecule is independent of its concentration [77]. This approach has been shown to be effective for various biomedical applications, such as the study of cervical cancer, COVID-19 pneumonia and oral carcinomas [71], [76], [78].

Despite the promise of time-resolved fluorescence spectroscopy, a key drawback is the low signal-to-noise ratios, as background noise can often significantly distort the recorded decay profiles [79]. Furthermore, both emission and time-resolved fluorescence spectroscopy

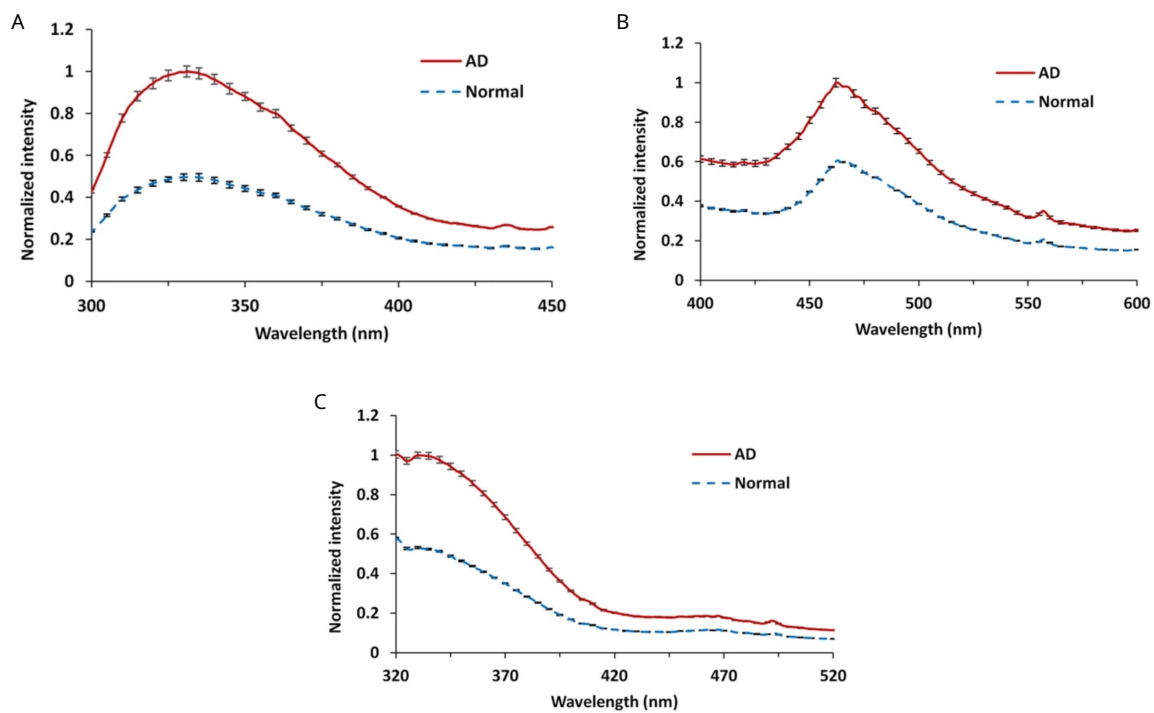


Figure 2.3: Fluorescence emission profiles of mouse brain tissue, comparing healthy (blue) and Alzheimer's disease (red) groups. Emission spectra were recorded following excitation at three different wavelengths: 266 nm (A), 300 nm (B), and 340 nm (C). In all cases, Alzheimer's samples exhibit higher normalized fluorescence intensity than healthy controls [24].

face the inverse limitation of photothermal and photoacoustic approaches, as these techniques strictly rely on probing radiative relaxation processes. This makes them unsuitable for studying samples with low quantum yields which exhibit minimal or no fluorescence.

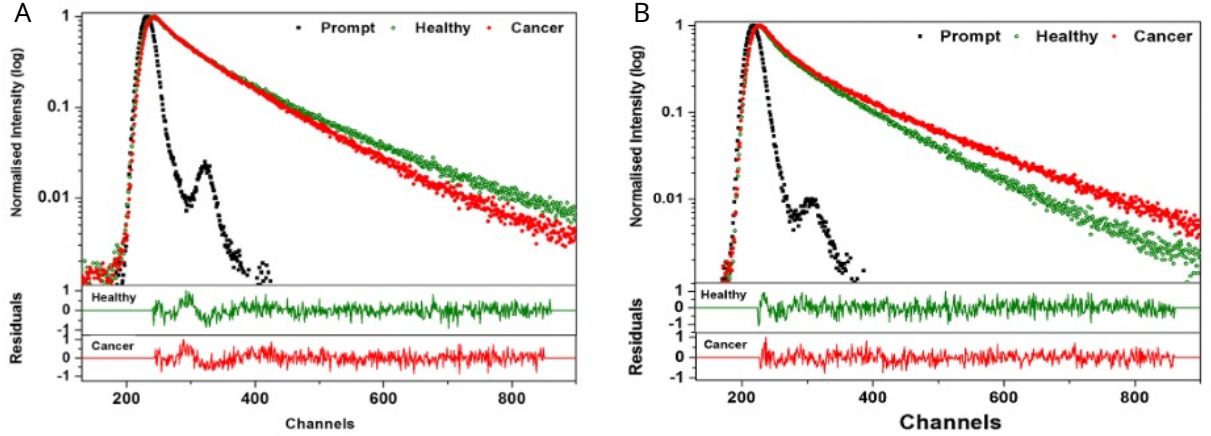


Figure 2.4: Time-resolved fluorescence decay profiles of urine samples from healthy individuals (green) and patients with cervical cancer (red), measured using a time-correlated single photon counting system. Emission was recorded at 390 nm (A) and 440 nm (B) following excitation at 280 nm. The sharp black peak labeled “Prompt” represents the instrument response function, which captures the timing and width of the excitation pulse [71].

2.1.3 Scattering Spectroscopy Techniques

Scattering spectroscopy is the last category of classical approaches. These techniques focus on characterizing samples through measuring their elastic or inelastic scattering interactions.

Diffuse Reflectance Spectroscopy

Diffuse reflectance spectroscopy, which is also referred to as elastic scattering spectroscopy, is a technique in which broadband light is directed towards a sample and the back-scattered light is collected and analyzed for spectral features. The wavelength-dependent intensity of the scattered light provides a spectral fingerprint of that specific material. Furthermore,

these reflectance spectra can also provide insight into the absorption characteristics of a material. For example, a sample's absorption spectrum can be indirectly estimated from its reflectance data through the equation:

$$A(\lambda) = -\log_{10} R(\lambda). \quad (2.3)$$

where $A(\lambda)$ represents a material's absorption of light at wavelength λ while $R(\lambda)$ is the reflection coefficient of the material at the same wavelength [75]. This can be seen in Figure 2.5

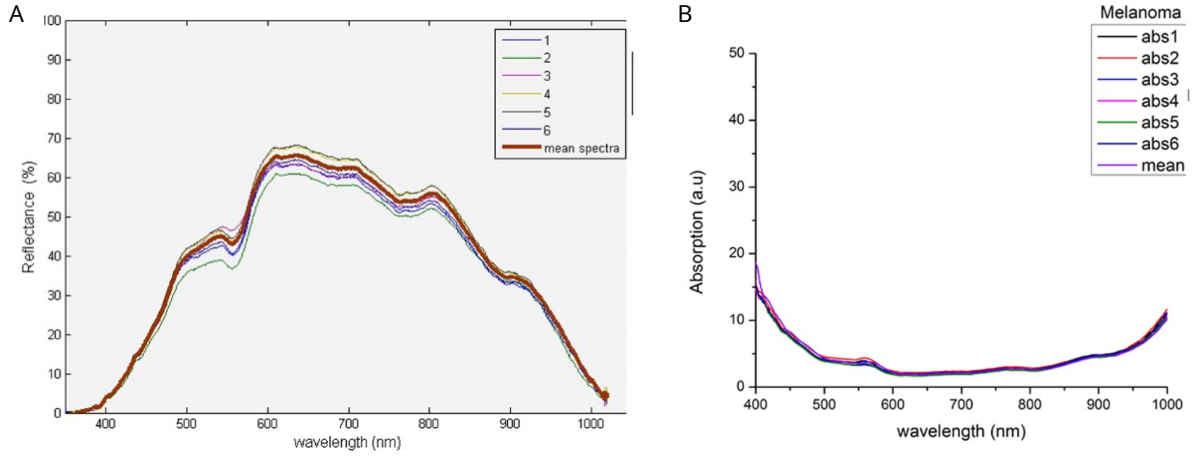


Figure 2.5: Diffuse reflectance and corresponding absorption spectra for melanoma cell line A375. Individual diffuse reflectance spectra from six melanoma samples, along with their mean spectrum (red), measured across 400–1000 nm (A). Calculated absorption spectra derived from the reflectance data (B) [10].

The effectiveness of using diffuse reflectance spectra to analyze biological samples has been demonstrated in various contexts. For instance, it has been applied *in-vivo* to study adenomatous colon polyps [80] as well as *in-vitro* to study brain and breast tissue samples [13], [21], as illustrated in Figure 2.6.

Raman Scattering Spectroscopy

Raman scattering is an inelastic process in which incident photons interact with the vibrational modes of molecules in a sample. This type of light-matter interaction typically

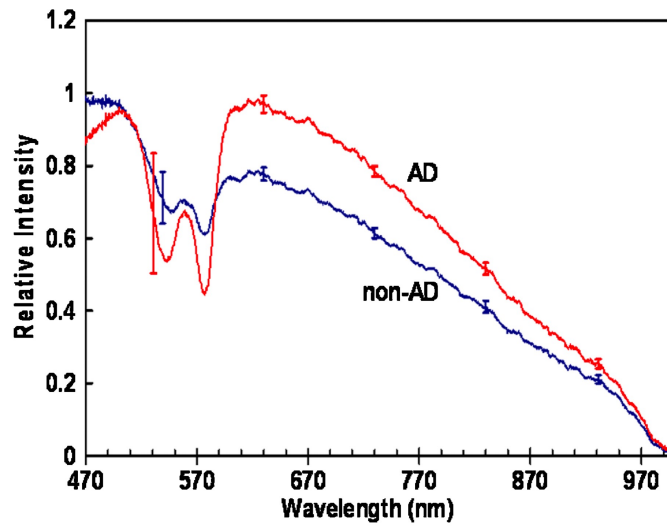


Figure 2.6: Diffuse reflectance spectra of temporal pole brain tissue from postmortem human subjects. The red curve corresponds to samples from individuals with Alzheimer’s disease, while the blue curve represents age-matched healthy controls. Measurements were taken in the 470–970 nm wavelength range [21].

occurs with infrared light and results in scattered light with wavelengths longer than the incident photons [81]. The difference between the wavelengths of the incident and scattered photons is referred to as the “Raman shift”, which is specific to the chemical structure of the sample. Analyzing the distribution of Raman-shifted wavelengths provides a highly sensitive spectral fingerprint for molecular identification. Due to its sensitive nature, Raman spectroscopy is one of the most commonly used approaches for biomedical research and has shown promise in informing cancer and pre-cancer diagnoses, both *in-vitro* [16], [18], [82] and *in-vivo* [83]–[85].

Raman spectroscopy is inherently limited by low signal-to-noise ratios, as a very small fraction of incident photons are Raman-scattered [19]. When comparing different spectroscopic approaches, a “cross section” refers to the likelihood of a sample experiencing a particular light-matter interaction type. Although these values tend to be sample specific, it has been reported that the cross section of Raman scattering is generally approximately 14 to 15 orders of magnitude smaller than that for UV or visible absorption [86]. To address this limitation, techniques such as Stimulated Raman Scattering (SRS) and Coherent Anti-Stokes Raman Scattering (CARS) have been developed. These methods use non-linear optical processes to amplify the Raman signal and suppress background noise, providing improved signal-to-noise ratios [87].

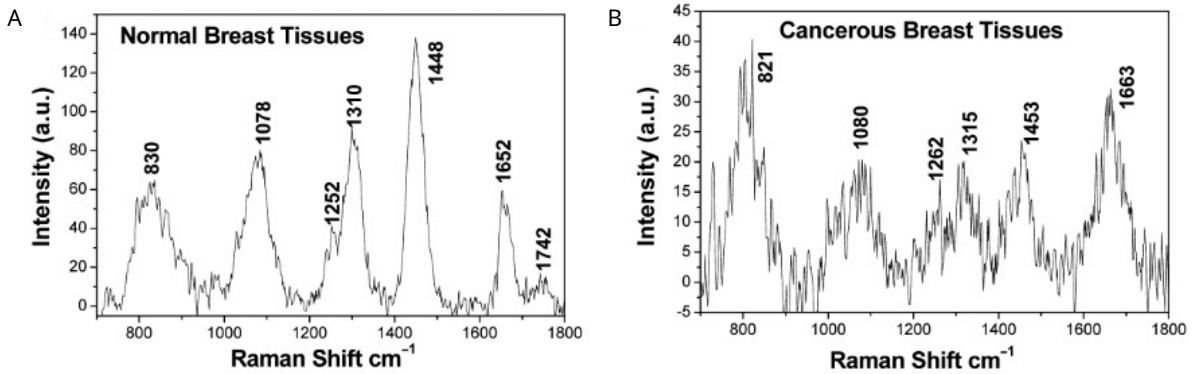


Figure 2.7: Raman spectra collected from breast tissue samples using 785 nm incident light. Spectrum from normal breast tissue, showing characteristic peaks at 830, 1078, 1252, 1310, 1448, 1652, and 1742 cm^{-1} (A). Spectrum from cancerous (invasive ductal carcinoma, IDC) breast tissue, with prominent peaks at 821, 1080, 1262, 1315, 1453, and 1663 cm^{-1} (B) [18].

2.1.4 Multi-Signal Optical Spectroscopy Techniques

While each of the reviewed traditional techniques have their own advantages and disadvantages, an overarching limitation is their focus on capturing spectroscopic signals from only a single interaction type. However, some work has explored the development of multi-modal optical systems, capable of capturing spectroscopic signals from multiple interaction modalities. For example, optical systems that collect both fluorescence emission and Raman scattering signals from a sample have shown promising results for biomarker identification in point-of-care applications [88], [89]. Furthermore, Nordstrom *et al.* has developed an optical tool capable of capturing diffuse reflectance and fluorescence emission signals from UV excitation wavelengths. Their results indicate that these modalities complement each other. In fact, the combined signal offers greater diagnostic power than the use of either of these signals individually in the context of Cervical Intraepithelial Neoplasia identification [90]. The encouraging results from existing work provide motivation for developing and testing other multi-modal spectroscopy systems.

2.2 Photon Absorption Remote Sensing

Photon Absorption Remote Sensing (PARS) is an innovative optical technique which captures the radiative *and* non-radiative relaxation processes that follow an electronic absorption event [28]. Traditional PARS systems follow a pump-probe configuration, where a pulsed excitation laser, typically with a UV or visible wavelength, and a continuous detection beam are co-aligned and directed toward a sample. Photons from the pulsed source are absorbed and excite a localized region of the sample, triggering both relaxation pathways.

Non-radiative relaxations occur in the form of thermal dissipations and, if the resulting thermo-elastic expansion is quick enough, induced pressure effects as well. The magnitude of the initial thermal rise in a sample is defined as:

$$\Delta T = \frac{\Phi \cdot \mu_a}{\rho \cdot C_v} \quad (2.4)$$

where Φ represents the fluence of the incident light source, μ_a is the optical absorption coefficient of the sample at the incident wavelength, ρ is the density of the sample and C_v represents the sample's specific heat capacity at a constant volume [28]. Similarly, the magnitude of the initial pressure increase is defined as:

$$p_0 = \Gamma \cdot \Phi \cdot \mu_a \quad (2.5)$$

where Γ is the Grüneisen parameter [91].

The lifetimes for the induced pressure and thermal effects are governed by the following equations, as derived by [92]:

$$\tau_t = \frac{\omega^2 \rho C_p}{4k} \quad (2.6)$$

$$\tau_p = \frac{2\omega}{v_s} \quad (2.7)$$

where τ_t and τ_p represent the thermal and pressure time constants of a sample, respectively, ω is the smallest radius of the excited region, C_p is the sample's heat capacity at constant pressure, k is the thermal conductivity and v_s is the speed of sound in the sample. Thermal relaxation occurs exponentially, following Newton's law of cooling, where τ_t is the time required for approximately 63.2% of the generated heat to decay, while $4 \cdot \tau_t$ describes the time for approximately 98.2% of the heat to dissipate [93]. Furthermore, τ_p represents the time required for the initially generated photoacoustic pressure to travel outside of the excited region.

In PARS, these non-radiative relaxations are captured by probing alterations in the refractive index of the locally excited region in the sample. Following an initial pressure increase, the refractive index of the excited region is altered according to the equation:

$$n(x, t) = n_0(x) \cdot \left(1 + \frac{\eta \cdot n_0(x)^2 \cdot p(x, t)}{2 \cdot \rho \cdot v_s^2} \right) \quad (2.8)$$

where $n_0(x)$ is the ground state refractive index of the sample, η represents the elasto-optic coefficient and $p(x, t)$ is the pressure field [91]. Thermal relaxation in the excited region results in refractive index changes depending on the material's thermo-optic coefficient, denoted as dn/dT [94]. These thermal and pressure induced refractive index changes modulate the intensity of the back-scattered light from the detection beam, which is monitored to derive a non-radiative PARS signal. The magnitude of the temperature and pressure rises dictates the amount of initially observed signal modulation, while the lifetime of the thermal effects governs the time required for the signal to return to its pre-excitation value. The steps for extracting non-radiative PARS absorbance values from these signals are outlined in Figure 2.8.

Radiative relaxations, in the form of fluorescence emissions, are captured by collecting the emitted photons from the excited sample. The total intensity of these emissions is

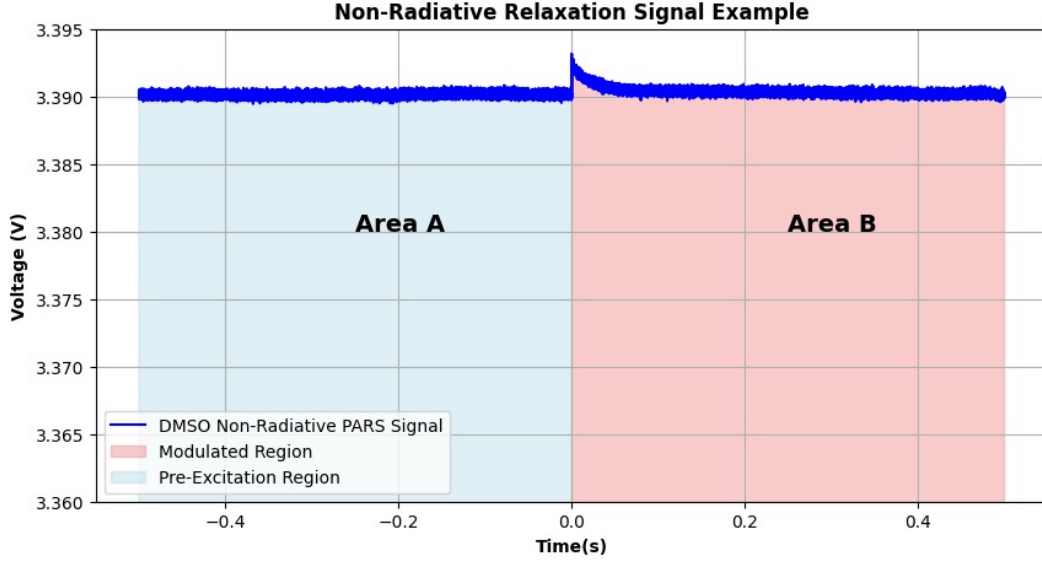


Figure 2.8: Mechanism for extracting non-radiative PARS absorbance values from non-radiative PARS signals where an excitation pulse occurs at 0s. The areas under the signal prior to excitation (A) and after excitation (B) are calculated. An absorbance value is calculated through the ratio of $(B-A)/A$. The absorbance value is then normalized by dividing by the energy of a single excitation pulse.

then normalized by the excitation pulse energy and used to represent a radiative PARS absorbance value for the sample. Furthermore, while previous PARS work has also included a scattering contrast, which arises from measuring the unperturbed back-scatter of the detection beam prior to an excitation event [28], capturing detection scattering signals falls outside of the scope of this work. Figure 2.9 summarizes the PARS mechanism.

PARS has been primarily applied for medical imaging applications and has shown particular promise due to its multi-modal nature. PARS is a point-scanning imaging technique in which relaxation signals are captured for every sample point. The calculated PARS absorbance values are then used as radiative and non-radiative pixel contrasts for this specific point. The sample is scanned in a raster pattern, ultimately yielding whole-sample radiative and non-radiative images. Furthermore, a “total absorption” image, which integrates both relaxation pathways, can be produced to enhance diagnostic capability. In histopathology, PARS has demonstrated the ability to generate whole slide images with diagnostic quality equal to existing gold-standard methods, which typically rely on

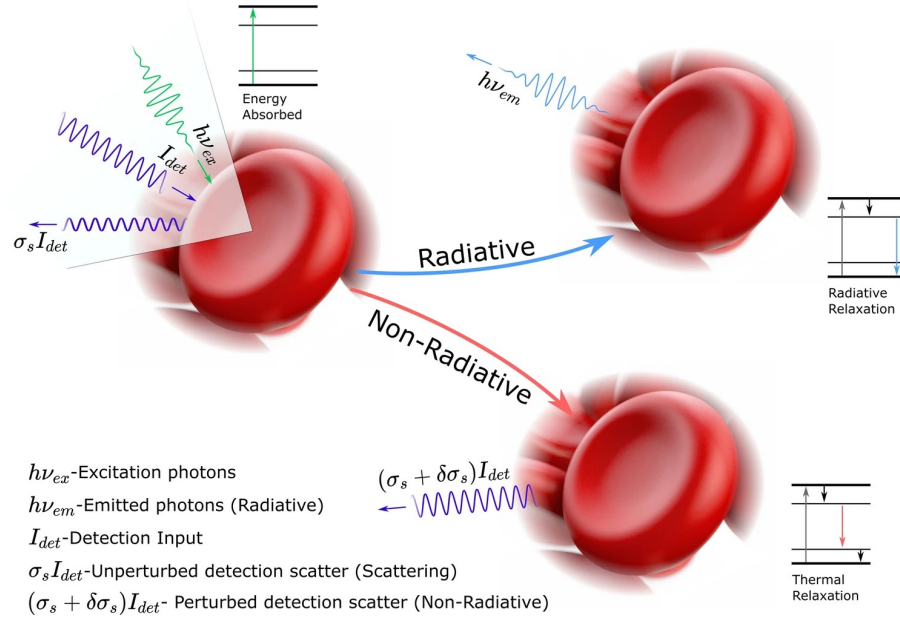


Figure 2.9: Traditional PARS radiative, non-radiative and scattering contrasts. Photons from the excitation source ($h\nu_{ex}$) are absorbed, resulting in the sample achieving an excited state and triggering radiative and non-radiative relaxation processes. Radiative relaxation processes are captured in the form of emitted fluorescence ($h\nu_{em}$). Non-radiative relaxations (ie: heat and pressure dissipations within the sample) lead to temporary alterations in the sample's optical properties, which cause modulations in the intensity of the back-reflected detection beam. This is denoted by a change in reflectivity multiplied by the power of the detection beam $(\sigma_s + \delta\sigma_s)I_{det}$ [29].

destructive and time-consuming processes such as Hematoxylin and Eosin (H&E) staining [29]–[31]. Figure 2.10 shows a comparison between a PARS total absorption image of human skin tissue and the same section stained with H&E. Furthermore, PARS has also shown strong potential for *in-vivo* vascular imaging, as illustrated in Figure 2.11, while also offering functional measurements related to blood flow and oxygenation levels of a sample [32], [33]. The multi-modal nature of PARS data provides rich insight into vascular health and holds promise for the diagnosis and treatment of various ophthalmic diseases.

While most PARS studies have employed fixed-wavelength excitation sources, some hyperspectral imaging work has also been explored with PARS. In this approach, a tuneable excitation laser was used to generate images using a few different wavelengths to target different absorbers in human adipose tissue, freshly resected murine tissue and chicken embryo membrane *in-vivo* [95]. This hyperspectral work further motivates this research, which introduces the first application of PARS in optical spectroscopy. The goal of this work is to explore how the PARS signals generated from a sample vary across many different excitation wavelengths and, subsequently, how these signals can be used to provide an improved level of detail in sample characterization. This work aims to address the limitations of traditional single-signal techniques by leveraging the multi-modal capabilities of PARS to analyze a wide variety of samples, serving as foundational work for future advancements in PARS spectroscopy research. A comparison of how a robust PARS spectroscopy system compares to the previously reviewed traditional techniques is presented in Table 2.1.

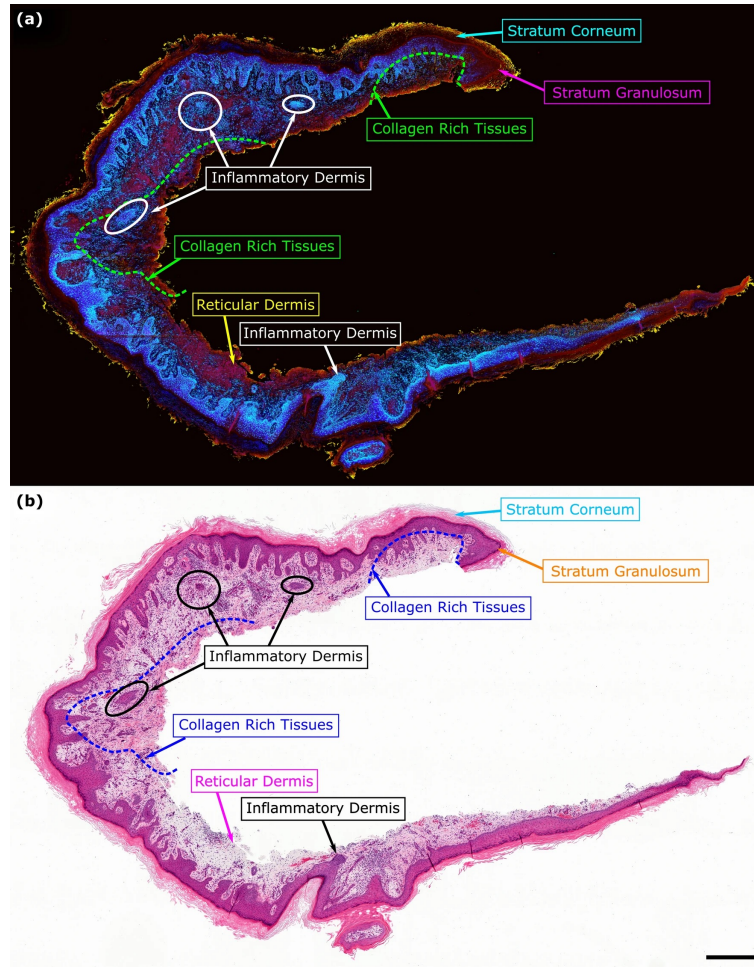


Figure 2.10: Total absorption PARS image, combining both PARS radiative and non-radiative contrasts, of a human skin tissue from a resection margin of squamous cell carcinoma (SCC) (a) and the corresponding image of the same section of human skin tissue after being stained with H&E (b). Scale bar for both images: 1mm [29].

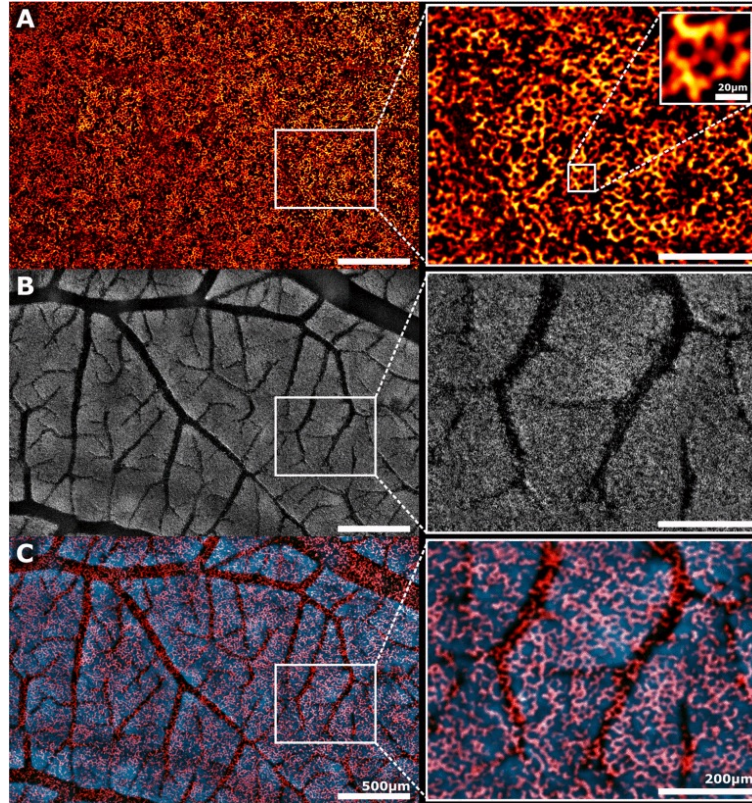


Figure 2.11: *In-vivo* PARS imaging of chicken embryo chorioallantoic membrane with a 3mm x 2mm field of view. The non-radiative contrast image shows a densely connected vascular network (A). The radiative contrast image depicts larger and deeper vascular structures (B). The total absorption image, which combines the non-radiative and radiative contrasts, captures a complete picture of the vascular structure (C) [32].

Table 2.1: Summary of classic optical spectroscopy techniques as well as a PARS-based optical spectroscopy system and the types of information they provide.

Technique	Category	Measures Non-Radiative Relaxations?	Measures Radiative Relaxations?	Measures Absorption?	Measures Fluorescence Emission Spectral Makeup?	Measures Scattering Effects?
Photothermal Spectroscopy	Absorption	Only Temperature	X	✓	X	X
Photoacoustic Spectroscopy		Only Pressure	X	✓	X	X
Direct Absorption Spectroscopy		✓	✓	✓	X	Not Explicitly
Fluorescence Emission Spectroscopy	Fluorescence	X	✓	Indirectly	✓	X
Time-Resolved Fluorescence Spectroscopy		X	✓	X	X	X
Diffuse Reflectance Spectroscopy	Scattering	X	X	Indirectly	X	✓
Raman Spectroscopy		X	Partially (via Inelastic Scattering)	X	X	✓
PARS Spectroscopy	Multi-Modal (Absorption & Fluorescence)	✓	✓	✓	✓	Only Excitation

Chapter 3

Non-Radiative PARS Spectroscopy System

3.1 Introduction

The first step of this work was designing and building a spectroscopy system capable of capturing induced non-radiative relaxation processes across many different excitation wavelengths, specifically in the UV range. The goal of this chapter is to demonstrate the feasibility of using wavelength-dependent non-radiative PARS signals to construct accurate absorption profiles for various liquid samples.

More specifically, this chapter outlines the design, development and experimental validation of this non-radiative PARS spectroscopy system, which employs a tuneable excitation source and a detection strategy optimized for capturing non-radiative relaxations. Results are presented to demonstrate the capability of the system in accurately capturing non-radiative relaxation processes and using this information to characterize a wide range of liquid samples.

3.2 Methods

This section describes the sample preparation processes and discusses the design and development of the non-radiative PARS spectroscopy system.

3.2.1 Sample Preparation

As mentioned above, the focus of this work was to study liquid samples. Specifically, the samples studied were dimethyl sulfoxide (DMSO), which is a commonly used solvent in biomedical research; methylene blue, which is a fluorescent dye that is used as a biomarker and indicator in various surgeries [96]; as well as eumelanin and the reduced form of nicotinamide adenine dinucleotide (NADH). Eumelanin is the biomolecule that provides the pigmentation of human skin [97], while NADH is a coenzyme involved in redox reactions within cells, playing a key role in energy production by transporting electrons during metabolic processes [98]. Eumelanin was chosen for this study as it is incredibly non-radiative in nature, relaxing over 99% of absorbed energy through non-radiative means [99]. This makes it an excellent target for studying the performance of the non-radiative PARS spectroscopy system. Furthermore, NADH is a biomolecule that is highly relevant in cancer diagnoses [100], [101] and has been extensively studied by other spectroscopy techniques in literature [102]–[104].

Stock solutions of DMSO (code: 472301) and methylene blue (code: 319112) were acquired from Sigma Aldrich. The stock solution of methylene blue was approximately 1.57mM in concentration and was diluted as needed using distilled water. Synthetic eumelanin (code: 8049-97-6) and NADH powders (code: 606-68-8) were purchased from ThermoFisher Scientific. The eumelanin was dissolved in DMSO while the NADH was dissolved in distilled water, due to its high water solubility. Solutions of these biomolecules were prepared in varying concentrations, as discussed in the future sections of this chapter.

3.2.2 Excitation Source Overview

Unlike traditional PARS imaging systems, which typically use fixed-wavelength excitation sources, this work employed an Ekspla NT200 laser as a tuneable excitation source. This enabled the study of induced non-radiative relaxation processes across many different excitation wavelengths. This laser produces 3-nanosecond pulses of light with target wavelengths specifiable from 210nm to 2600nm and repetition rates from 1Hz to 1kHz. This work was specifically interested in exploring UV excitation, as the absorption profiles of biomolecules such as eumelanin and NADH are commonly studied across this range of wavelengths [104], [105].

The Ekspla NT200 laser relies on non-linear optical interactions to convert a 1064nm “pump” source into the desired target wavelengths. Figure 3.1 provides a simple schematic illustrating how this laser produces light with target wavelengths in the region of 300nm to 380nm.

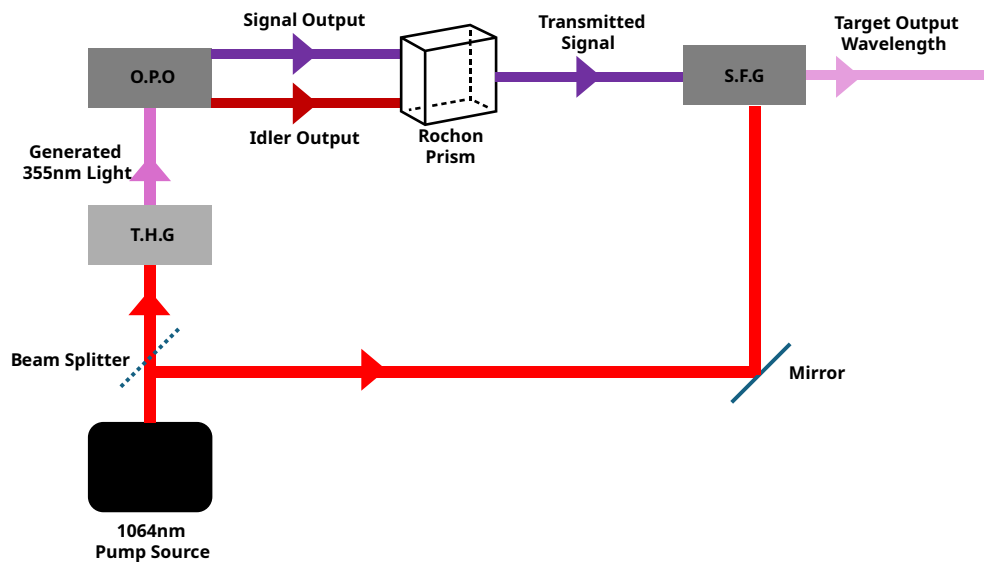


Figure 3.1: Simplified diagram outlining how the Ekspla NT200 laser generates various target output wavelengths from the 1064nm pump source. T.H.G: third harmonic generator, O.P.O: optical parametric oscillator, S.F.G: sum frequency generator.

Firstly, light from the 1064nm pump source is passed through a third harmonic generator, which produces light with a frequency three times greater than the input light frequency. This results in a 355nm output. This 355nm light is then directed through an optical parametric oscillator, which uses non-linear crystals to convert the 355nm input into two output beams: a signal and an idler. The specific wavelengths of the signal and idler are tuneable and controlled by the angle of the crystals. However, the following equation must be satisfied to ensure that energy is conserved:

$$\frac{1}{\lambda_{\text{signal}}} + \frac{1}{\lambda_{\text{idler}}} = \frac{1}{\lambda_{355}} \quad (3.1)$$

The idler is filtered out using a Rochon prism, while the transmitted signal is passed into a sum frequency generator alongside a portion of the initial 1064nm beam which was redirected from its original path using a beam splitter. This component works by outputting light with a frequency equal to the sum of the frequencies of the two inputs. In essence, this laser source works by tuning the angles of the crystals in the optical parametric oscillator so that the sum of the frequencies of the signal light and the 1064nm pump source yields the frequency of the desired target output wavelength.

Laser Bleed Through

While the Ekspla NT200 is a very useful laser to employ for this work, it has an inherent limitation in that the emitted light does not exclusively consist of the desired target wavelength. Instead, bleed through from the non-linear processes which convert the 1064nm pump source into the target wavelength is also present in the emissions. While the laser uses prisms to try and filter bleed through, this process is not entirely efficient, as trace amounts of light from the pump, signal and idler sources are also emitted. The wavelengths of the signal and idler bleed through are dependent on the wavelength of the target output light, and are derived from the non-linear processes shown in Figure 3.1. The relationship between a target output wavelength and the wavelengths of each bleed through source is presented in Figure 3.2. The pump source is constant at 1064nm, but for target wavelengths between 300nm and 380nm, the signal wavelengths lie between 417nm and 591nm, while the idler wavelengths are between 888nm and 2362nm.

3.2.3 System Design

A schematic of the non-radiative PARS spectroscopy system is presented in Figure 3.3. This system uses the Ekspla NT200 laser as its UV excitation source, outputting 3-nanosecond

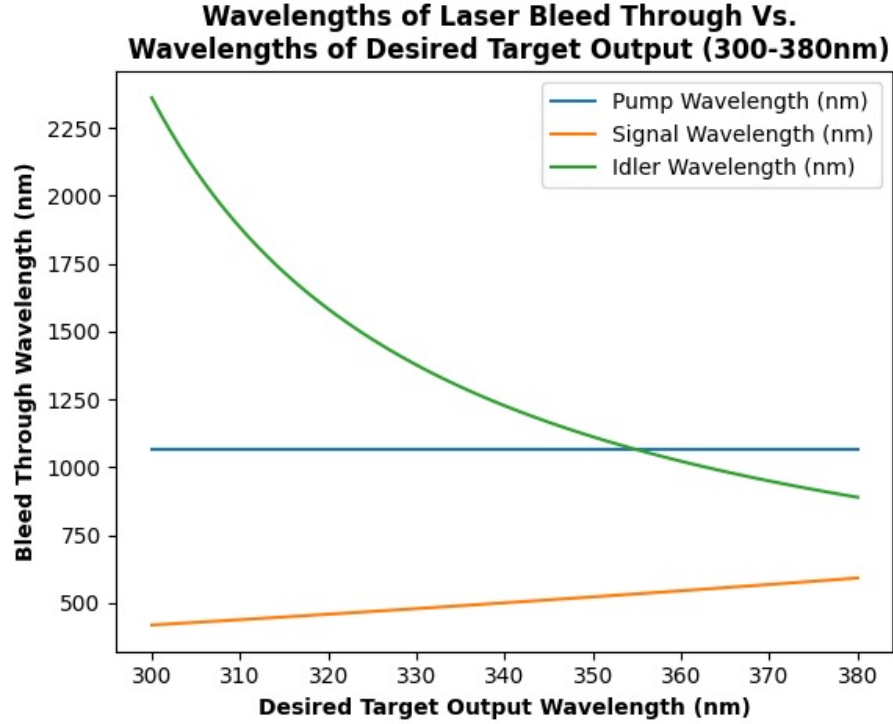


Figure 3.2: Associated bleed through wavelengths for each desired output target wavelength between 300 - 380nm from the Ekspla NT200 laser.

pulses at a 1Hz repetition rate. Excitation pulses are directed towards a harmonic beam-splitter, which is an optical component designed to reflect certain wavelengths of light and transmit others. This specific harmonic beam-splitter (H.B.S, HBSY134, Thorlabs) is highly reflective to light in the UV range but is highly transmissive to light in the signal, idler and pump ranges. Figure 3.4 summarizes the response of HBSY134 to different wavelengths of light.

The non-radiative PARS spectroscopy system takes advantage of the HBSY134 component's capability in separating these spectral bands, employing it as a filter to further purify the emitted excitation pulses. In this set-up, the target UV excitation light is reflected and directed towards the sample, while the bleed through sources are largely transmitted and captured in a beam trap (B.T, BT610, Thorlabs). Furthermore, a 970nm continuous beam (970nm Detection, SLD970P-A40W, Thorlabs) is used as the system's detection source. It is first collimated with an achromatic fiber collimator (Col, C40APC-B, Thorlabs) and

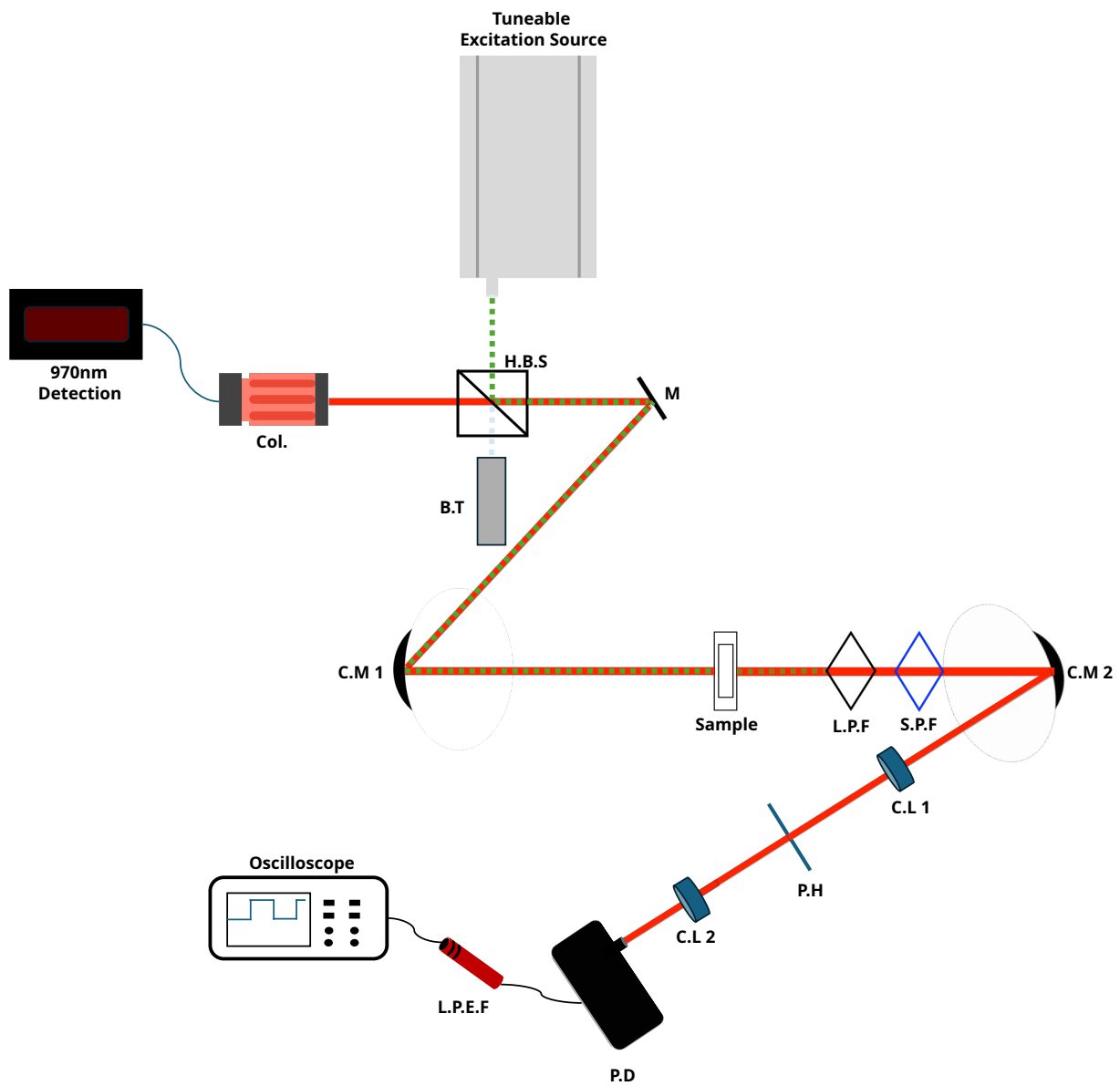


Figure 3.3: A simplified diagram of the non-radiative PARS spectroscopy system. Col.: achromatic fiber collimator, H.B.S: harmonic beam-splitter, B.T: beam trap, M: UV-enhanced aluminum-coated mirror, C.M: UV-enhanced aluminum-coated concave mirror, L.P.F: long-pass filter, S.P.F: short-pass filter, C.L: aspheric condenser lens, P.H: pinhole, P.D: photodetector, L.P.E.F: low-pass electrical filter.

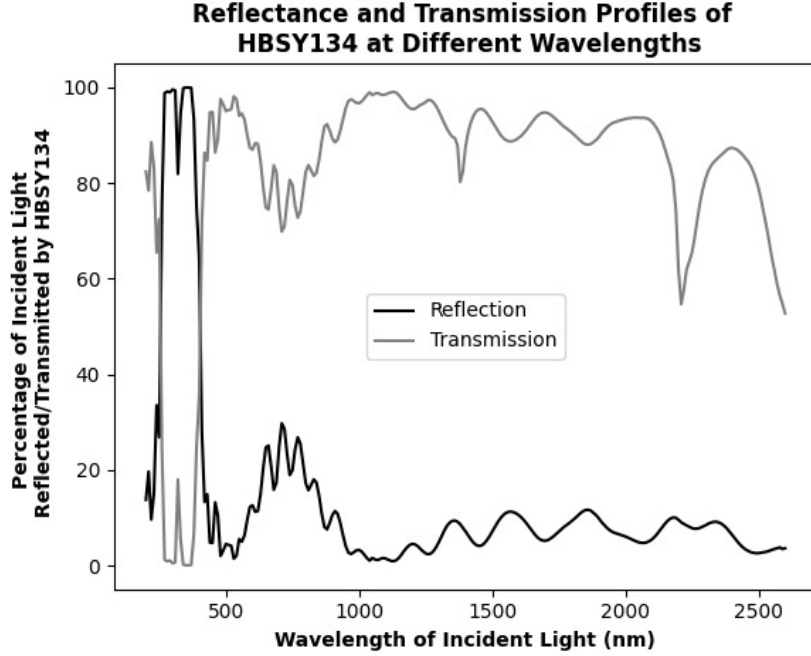


Figure 3.4: The reflection and transmission percentage of different wavelengths of light by the HBSY134 harmonic beam-splitter. Data from Thorlabs [106].

then transmitted through the harmonic beam-splitter to combine with the purified excitation path. Following this, both beams are reflected off of a UV-enhanced aluminum-coated mirror (M, PF10-03-F01, Thorlabs) and towards a UV-enhanced aluminum-coated concave mirror (C.M 1, CM254-500-F01, Thorlabs). Next, the combined beams are directed towards a liquid sample in a UV-transparent cuvette (Sample, Z637157, BRANDTECH). Here, some of the energy from the excitation pulses is absorbed by the sample, while the detection beam is transmitted with minimal interactions due to its longer wavelength. However, some light from the excitation beam is also transmitted through the sample, as not all of the photons are absorbed. This residual excitation light is filtered out using a 950nm long-pass filter (L.P.F, FELH0950, Thorlabs), which blocks the target excitation wavelength but allows the 970nm detection light to transmit through. A 1000nm short-pass filter (S.P.F, FESH1000, Thorlabs) is then used to further filter out any remaining bleed through from the pump and idler sources, preventing it from hitting the photodetector and distorting the recorded signals. The detection beam is then collimated using a second UV-enhanced aluminum-coated concave mirror (C.M 2, CM254-500-F01, Thorlabs)

and focused through a 1mm diameter pinhole (P.H, P1000K, Thorlabs) using an aspheric condenser lens (C.L 1, ACL25416U, Thorlabs). The beam is then refocused with another aspheric condenser lens (C.L 2, ACL25416U, Thorlabs) onto a fixed gain balanced amplifier photodetector (P.D, PDB425A, Thorlabs). This photodetector uses the photoelectric effect to generate voltage signals that are proportional to the number of incident photons hitting its sensor. Voltage signals from the photodetector are filtered using a 10MHz low-pass electrical filter (L.P.E.F, EF501, Thorlabs) and passed into a mixed-signal oscilloscope (InfiniiVision MSOX6004A, KEYSIGHT) for visualization and analysis.

3.2.4 Non-Radiative Signal Detection

This system is optimized to capture induced pressure and thermal effects through probing changes in the intensity of the detection light that reaches the photodetector. Pressure dissipations alter the sample’s refractive index properties, modulating the amount of the detection light that is back-scattered. Furthermore, thermal relaxations induce a refractive index gradient in the excited sample region, as described by the photothermal lensing effect. This gradient acts as a weak lens, slightly focusing or de-focusing the probe beam as it travels through the sample, and ultimately changing the amount of detection light which hits the photodetector. Photothermal lensing is a commonly used method for studying non-radiative relaxation processes in liquid biomolecular samples, as shown in [48]–[50]. This approach is particularly useful as photothermal lensing configurations are sensitive to both thermal and pressure effects [62].

The system is set up so that before excitation the collimated detection beam is almost perfectly focused through the pinhole using the first aspheric condenser lens (C.L 1), and then refocused onto the photodetector using the second aspheric condenser lens (C.L 2). In this situation, the entire detection beam hits the photodetector, yielding the maximum achievable voltage reading. However, after excitation, pressure effects modulate the amount of detection light that is transmitted through the sample. Furthermore, following the formation of the thermally induced refractive index gradient, the detection light from the second concave mirror (C.M. 2) is no longer collimated. As such, the first aspheric condenser lens (C.L 1) does not focus the detection light at the location of the pinhole. Therefore, part of the detection beam is blocked, resulting in fewer photons reaching the photodetector and subsequently a drop in the voltage reading. This mechanism is illustrated in Figure 3.5. As the thermal effects in the excited sample dissipate, its optical properties return to their pre-excitation state, and the detection beam correspondingly tends back to its original path. As such, measuring the modulation of the voltage readings

from the photodetector in-between excitation pulses can be used to provide insight into the nature and magnitude of the initial absorption event.

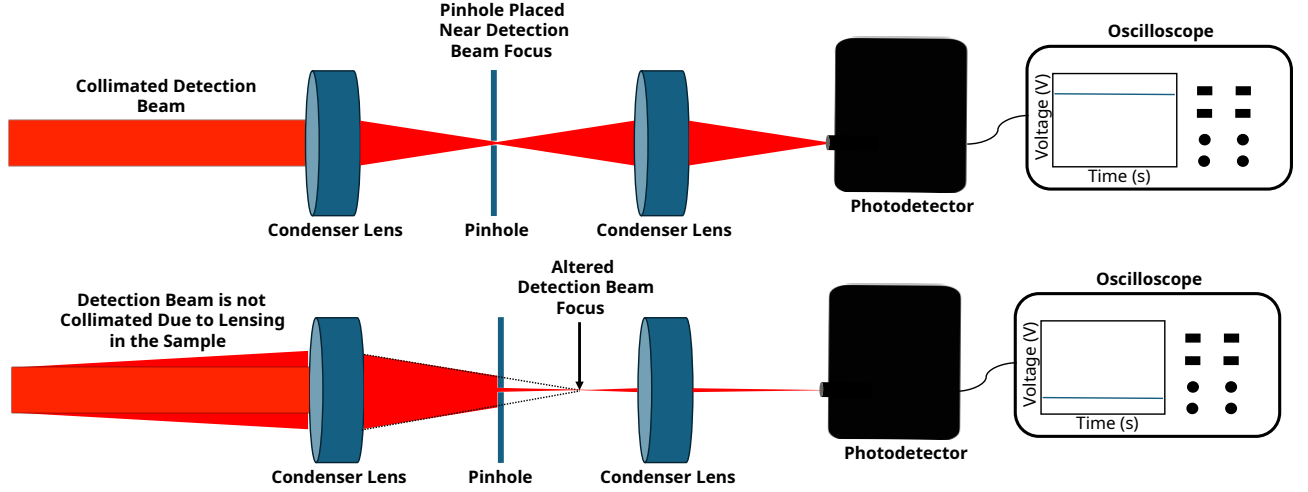


Figure 3.5: Photothermal lensing detection using a pinhole. System configuration before excitation (top) and immediately after excitation (bottom).

3.2.5 Photon Shot Noise

When photons from the detection source strike the photodetector’s sensor, they generate a photocurrent through the photoelectric effect. This current passes through an internal resistor, producing a voltage reading that is recorded over the time in-between excitation pulses. These voltage readings represent the non-radiative PARS signal and are directly proportional to the induced photocurrent and, by extension, to the number of photons that reach the sensor.

However, the arrival of photons at the photodetector is an inherently random process governed by a Poisson distribution [107]. Even in the absence of a sample or absorption event, the number of photons from the detection source arriving at the sensor fluctuates slightly over time. As a result, the recorded voltage signals inherently carry a baseline level of noise, known as photon shot noise, as illustrated in Figure 3.6.

Since the amplitude of the induced modulation in the non-radiative PARS signal is directly tied to the magnitude of the initial optical absorption event, sufficient absorption is required to produce a detectable modulation that rises above this noise floor. In cases

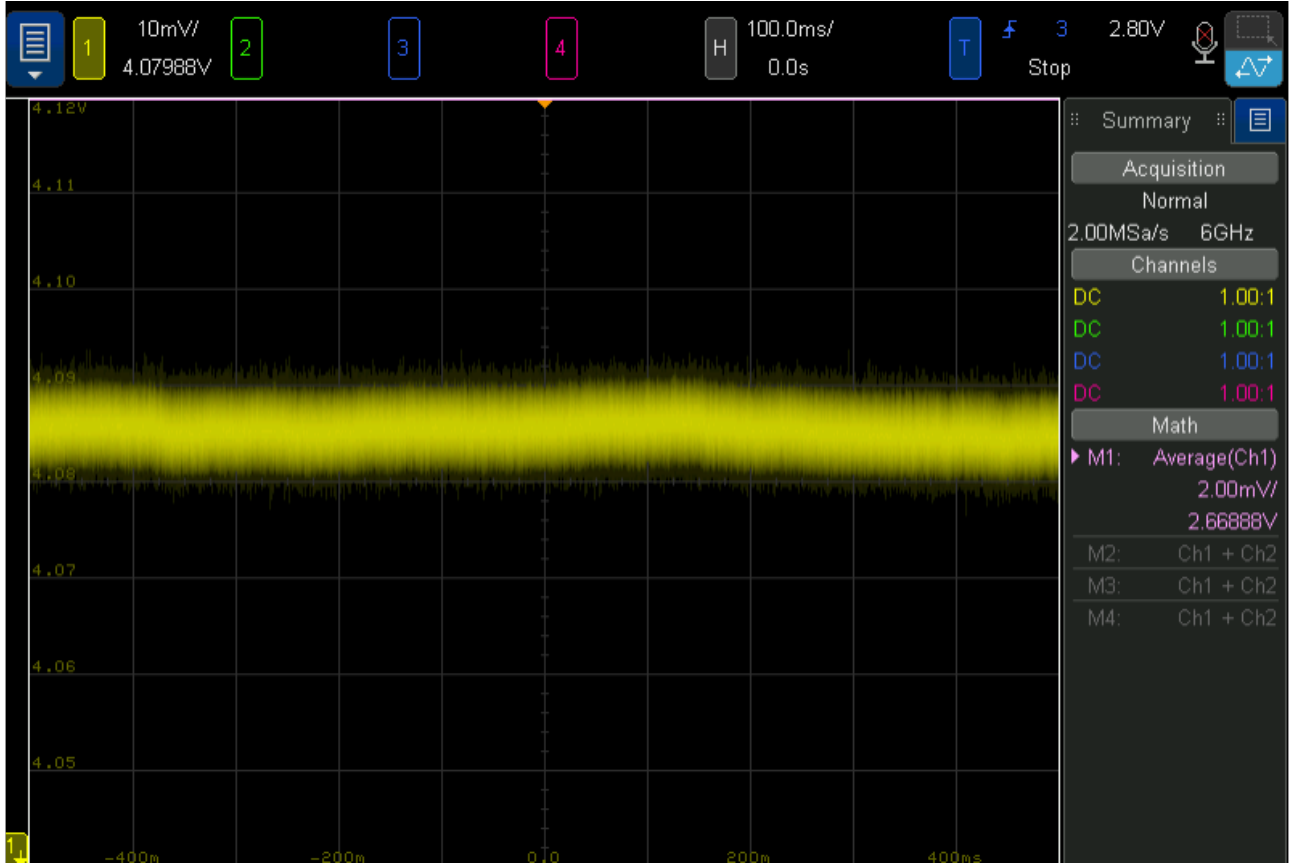


Figure 3.6: Signal from the oscilloscope with the detection laser on but the excitation source turned off and no sample present. Ideally, the signal should be completely constant with respect to time, however, fluctuations are present due to the inherently random arrival of photons from the detection source. This randomness results in a signal amplitude of approximately 10mV. Note that each box horizontally represents 100ms while each box vertically represents 10mV.

of weak absorption, the resulting signal may be completely masked by photon shot noise, making it undetectable on the system.

Furthermore, even if the signal amplitude does surpass the noise floor, photon shot noise can still compromise the accuracy and reliability of the extracted absorbance values, particularly in low absorbing samples. PARS measurements depend on capturing subtle modulations in the probe beam’s intensity as a result of non-radiative relaxations. Small modulations result in lower signal-to-noise ratios, making the calculated non-radiative PARS absorbance values more susceptible to distortion and a less precise quantification of true absorption. Note that in this work, the non-radiative PARS signals represent the averaged signal from 900 excitation pulses. This averaging was performed to reduce the impact of photon shot noise; however, it remains as an unavoidable source of error.

3.3 Results and Discussion

This section describes the validation of the non-radiative PARS spectroscopy system and its application in studying the absorption profiles of the previously described liquid samples.

3.3.1 System Validation

Relaxation Lifetimes

The first step in using this system was to ensure that the signals acquired from the photodetector are accurate representations of a sample’s non-radiative relaxation processes. This was accomplished by comparing the thermal and pressure relaxation times that were inferred from the collected non-radiative PARS signals with their theoretical values.

In this experiment, eumelanin was dissolved in DMSO in a ratio of 1mg/ml, yielding a 2mL solution with a concentration of approximately 3.15mM. Furthermore, a small alteration was made to the system diagram shown in Figure 3.3, changing the harmonic beam-splitter to a dichroic mirror (DMLP900, Thorlabs). Dichroic mirrors work in a similar manner to harmonic beam-splitters, as they are designed to reflect certain wavelengths and transmit others. However, the properties of this new component allowed for 516nm excitation. This was crucial as some of the required material parameter values for eumelanin that were found in literature assume a 516nm excitation source.

Following excitation, thermal effects tend to dissipate on the microsecond time scale, while pressure effects dissipate much faster, typically on the nanosecond time scale. As

mentioned in Chapter 2, the exact relaxation times depend on the sample material and are governed by Equations 2.6 and 2.7, which are stated again here for convenience:

$$\tau_t = \frac{\omega^2 \rho C_p}{4k}$$

$$\tau_p = \frac{2\omega}{v_s}$$

where τ_t and τ_p are the thermal and pressure time constants respectively, ω is the smallest radius of the excited region, ρ is the density of the sample, C_p is the sample's heat capacity at constant pressure, k is the thermal conductivity and v_s is the speed of sound in the sample.

The theoretical thermal and pressure time constants for this eumelanin solution can be determined using the above equations and the parameters found in Table 3.1. These calculations yield $\tau_t \approx 56.1$ ms, implying that the thermal effects should take approximately 224.4 ms to dissipate fully, and $\tau_p \approx 335.8$ ns. The theoretical lifetimes of these relaxation processes correspond very well with the values inferred from the averaged non-radiative PARS signal collected from the system, as seen in Figure 3.7. This agreement verifies the system's capability in accurately capturing induced non-radiative relaxation processes.

Table 3.1: Material properties of eumelanin-DMSO solution. Values from [28].

Parameter	Value
ω (μm)	250
ρ (kg / m^3)	1095
C_p ($\text{J} / (\text{kg K})$)	1968
k ($\text{W} / (\text{m K})$)	0.6
v_s (m / s)	1489

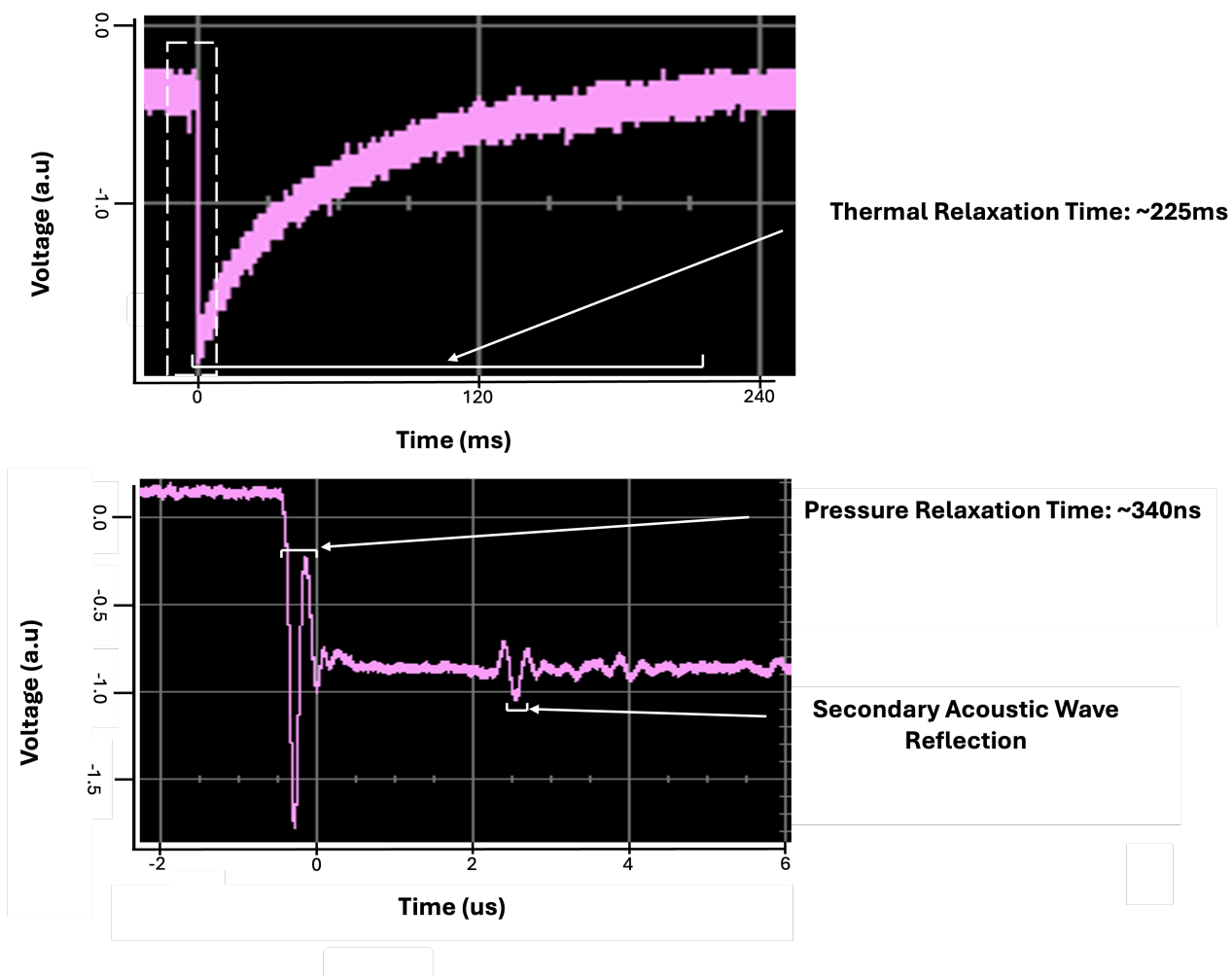


Figure 3.7: Thermal (top) and pressure (bottom) relaxation times for a 3.15mM eumelanin-DMSO solution based on the signal collected from the non-radiative PARS spectroscopy system after 516nm excitation. Note that the bottom plot represents a zoomed in version of the top plot in the field of view denoted by the dashed white rectangle. The bottom figure also captures an acoustic wave reflection, where the initially propagated acoustic wave reflected off of the walls of the cuvette, re-entered the excited region and was detected again via the probe beam.

Linearity of Absorbance Values

As mentioned previously, non-radiative PARS signals can be processed to provide insight on the magnitude of an initial absorption event. Specifically, non-radiative PARS absorbance values, which represent the amount of non-radiative relaxation that occurred, are extracted by calculating the ratio of the induced signal modulation to the pre-excitation signal level, similar to the process described in Figure 2.8. Furthermore, according to the Beer-Lambert Law, the absorption of a solution is linearly dependent on its solute concentration [108]. As such, the reliability of this system in capturing signals which are accurate representations of non-radiative relaxation processes was further validated through testing this linearity with various concentrations of eumelanin-DMSO solutions. The 3.15mM stock eumelanin solution was further diluted using DMSO to yield solutions of concentrations: 49.1 μ M, 24.6 μ M and 12.3 μ M. In this step, the DMLP900 dichroic mirror was replaced by the HBSY134 harmonic beam-splitter, allowing for UV excitation to be used. Non-radiative PARS signals were captured from each of these solutions, as well as a pure DMSO solution, using 330nm excitation. All of these samples were 2mL in volume. A non-radiative PARS absorbance value was extracted from each signal and plotted against its concentration. The results of this study are depicted in Figure 3.8. The strong linear relationship between eumelanin concentration and the calculated non-radiative PARS absorbance values at 330nm excitation is consistent with the expectations of the Beer-Lambert Law and reinforces the reliability of the non-radiative PARS spectroscopy system.

3.3.2 Non-Radiative PARS Absorption Spectra

The last section of this chapter outlines how the validated non-radiative PARS spectroscopy system was used to characterize the absorption profiles of the samples of interest. First, the DMSO solution was used. Second, the stock solution of methylene blue was diluted down to 3.13 μ M using distilled water. This concentration has been reported in literature for biomarker identification in ureteral imaging [109]. Third, the eumelanin solution was diluted to 383.5nM using a 90% distilled water and 10% DMSO solution, as the lower amount of eumelanin used in this study required less DMSO to fully dissolve in. Lastly, the NADH powder was dissolved in distilled water to produce a concentration of approximately 100 μ M, as was done in [104]. Each solution was 2mL in volume.

The energy of excitation pulses emitted from the Ekspla NT200 laser are wavelength-dependent. Every wavelength's average pulse energy was calculated using a PM100D Thorlabs powermeter. Here, the excitation laser's repetition rate was set to 1kHz and the powermeter was placed in front of the sample on the excitation path. The measured power

Absorbance Value at 330nm Excitation Vs. Eumelanin Concentration

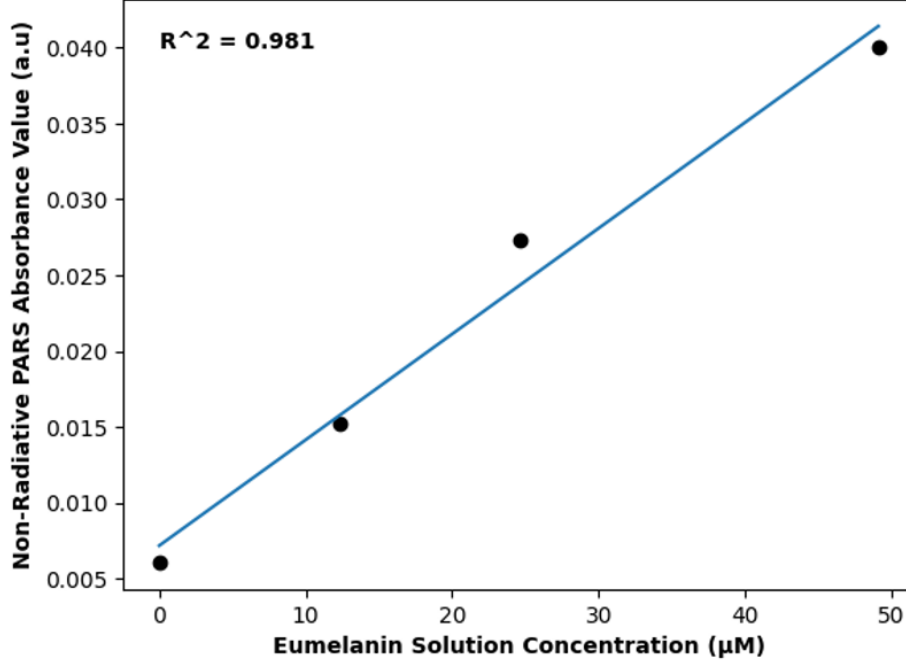


Figure 3.8: Extracted non-radiative PARS absorbance values from non-radiative PARS signals recorded from eumelanin-DMSO solutions with varying concentrations.

for each wavelength was recorded. As power is a measure of energy per unit time, these power readings were divided by 1000, which is the number of pulses per second, to yield the average energy of an excitation pulse for each wavelength. The calculated pulse energies are presented in Figure 3.9

In addition to normalizing for pulse energy variations, the spot size of the excitation beam needed to be considered as well, as this parameter also changes with respect to wavelength. With the Ekspla NT200 laser, the focal area of the emitted beam is proportional to the target wavelength. Since the initial temperature and pressure increases are dependent on the incident light's fluence, which is a measure of energy per unit area, a fluence correction factor of $C(\lambda) = E(\lambda)/\lambda^2$ was calculated for each excitation wavelength used in this work, as was done in [110]. The excitation wavelengths used in this study ranged from 310nm to 370nm, as pulses from the excitation laser with target wavelengths shorter than 310nm did not have sufficient fluence to generate any measurable signal. For each sample, the

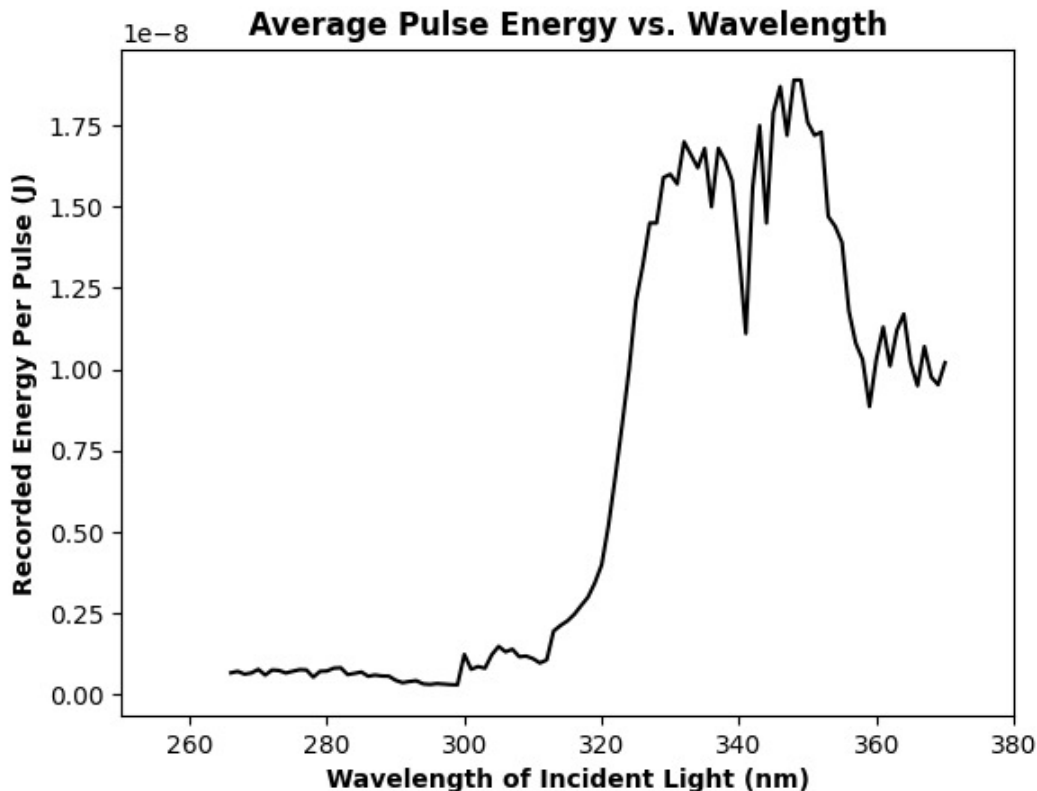


Figure 3.9: Measured average energy of excitation pulses. Measurements were taken using a PM100D Thorlabs powermeter placed immediately before the sample location on the excitation path.

wavelengths studied ranged from the shortest wavelength in this region that produced an observable signal on the system to the longest wavelength that did the same. These specific ranges varied slightly across samples, depending on their intrinsic absorption characteristics. The detection power for these experiments was 3.97mW, as measured by the same Thorlabs powermeter.

A total of 900 non-radiative PARS signals were captured for each sample and each excitation wavelength on the spectroscopy system and were averaged together to reduce photon shot noise. Non-radiative PARS absorbance values were extracted from each averaged signal. These values were divided by their corresponding fluence correction factor to account for wavelength-to-wavelength energy and focal area variations. The resulting non-radiative

PARS absorbance values for each wavelength in the methylene blue, eumelanin and NADH samples were further normalized by subtracting the wavelength’s absorbance value for the sample’s diluting agent. In optical spectroscopy this process is known as “blanking” and is required to remove absorption contributions from the solvent and capture an absorption spectrum of the molecule of interest only. This process ultimately yielded a non-radiative PARS absorption spectrum for each sample.

The spectra generated by the non-radiative PARS spectroscopy system were benchmarked against the absorption spectra collected from a Thermo Scientific NanoDrop 1000 spectrophotometer device. This NanoDrop device derives absorption spectra for liquid samples through a direct absorption spectroscopy mechanism, where 1-2 μ L of a sample is loaded onto the device’s pedals and broadband light is directed through it. The device captures the percentage of each wavelength in this light source that is transmitted and calculates an absorption spectrum based on these measurements. The NanoDrop device uses the same blanking procedure as described above, where a drop of the pure solvent is first loaded onto the device’s pedals and the corresponding absorbance values are subtracted from the sample’s spectral absorption data. The spectrum derived from the NanoDrop device was used as the “ground truth” for each sample in this work. Comparisons of the spectra generated by the non-radiative PARS spectroscopy system with the spectra from the NanoDrop device are presented in Figure 3.10. Note that the non-radiative PARS absorbance values were scaled to be between 0.0 and 1.0, for a more intuitive visualization, as the units of these values are arbitrary.

These results demonstrate varying levels of agreement between the spectra derived from the non-radiative PARS system and the ground truth spectra. Some differences between the two are expected due to the inherent limitations of direct absorption spectroscopy. Specifically, the NanoDrop device cannot distinguish between light lost from true absorption and light lost due to scattering. As a result, scattering events may artificially alter the measured absorption of a sample at a particular wavelength. In contrast, the proposed non-radiative PARS spectroscopy system exclusively detects non-radiative relaxation processes, providing a targeted measure of absorption that is not distorted by scattering events. Despite this difference, strong agreement is observed for samples with high absorption in this UV range that primarily exhibit non-radiative relaxations, such as eumelanin. In this case, the NanoDrop absorbance values are large, and as such, the effects of scattering are relatively less impactful. Furthermore, highly absorbent samples with very low quantum yields, such as eumelanin, generate a more pronounced signal modulation on the non-radiative PARS spectroscopy system. This leads to high signal-to-noise ratios and makes the extracted non-radiative PARS absorbance values more reliable representations of the sample’s true absorption profile. The small discrepancies present can likely be attributed

to unavoidable system noise or impurities in the excitation pulses, which may have contained trace amounts of pump, signal or idler bleed through that were not entirely filtered out by the harmonic beam-splitter component.

In contrast to eumelanin, the NADH sample shows more noticeable discrepancies between the non-radiative PARS absorption spectrum and the NanoDrop ground truth. While NADH does exhibit relatively strong absorption in the UV range, with NanoDrop absorbance values reaching approximately 0.080, it is still significantly lower than eumelanin's, which exceed 3.2 on the same scale. As a result, the influence of scattering on the NanoDrop measurements becomes relatively more pronounced, potentially contributing to the observed differences between the two spectra. Moreover, NADH is not nearly as non-radiative in nature as eumelanin, which relaxes almost exclusively through non-radiative pathways. This results in a smaller fraction of the absorbed excitation energy contributing to the non-radiative PARS signal, and ultimately, a lower signal-to-noise ratio. Together, these two factors account for the increased deviation observed in the NADH spectra compared to the high level of agreement with the eumelanin case.

The DMSO and methylene blue samples exhibit the largest discrepancies between the non-radiative PARS and ground truth spectra, which is consistent with their relatively low absorption in the UV range. Both samples show NanoDrop absorbance values below 0.017, indicating a weak tendency to absorb light in this range. At such low absorption levels, any scattering present in the NanoDrop measurement becomes a proportionally larger source of error and the extracted non-radiative PARS absorbance values are more susceptible to system noise. In the case of methylene blue, this effect is compounded by its highly radiative nature. Methylene blue is a fluorescent dye and is designed to dissipate a significant portion of absorbed energy through radiative pathways [96], resulting in even less signal modulation for this non-radiative PARS system to detect. These combined factors explain why the non-radiative PARS absorption spectra for both DMSO and methylene blue deviate more substantially from the NanoDrop ground truth compared to the higher-absorbing samples like eumelanin and NADH.

3.4 Limitations

The main limitation of this version of the PARS spectroscopy system is its sole focus on measuring a sample's non-radiative relaxation processes. Additionally, the time required to generate a sample absorption spectrum is another key limitation. Since this system relies on observing the entire non-radiative relaxation process, the repetition rate of the excitation laser is constrained by the thermal relaxation times of the samples. The repetition rate of

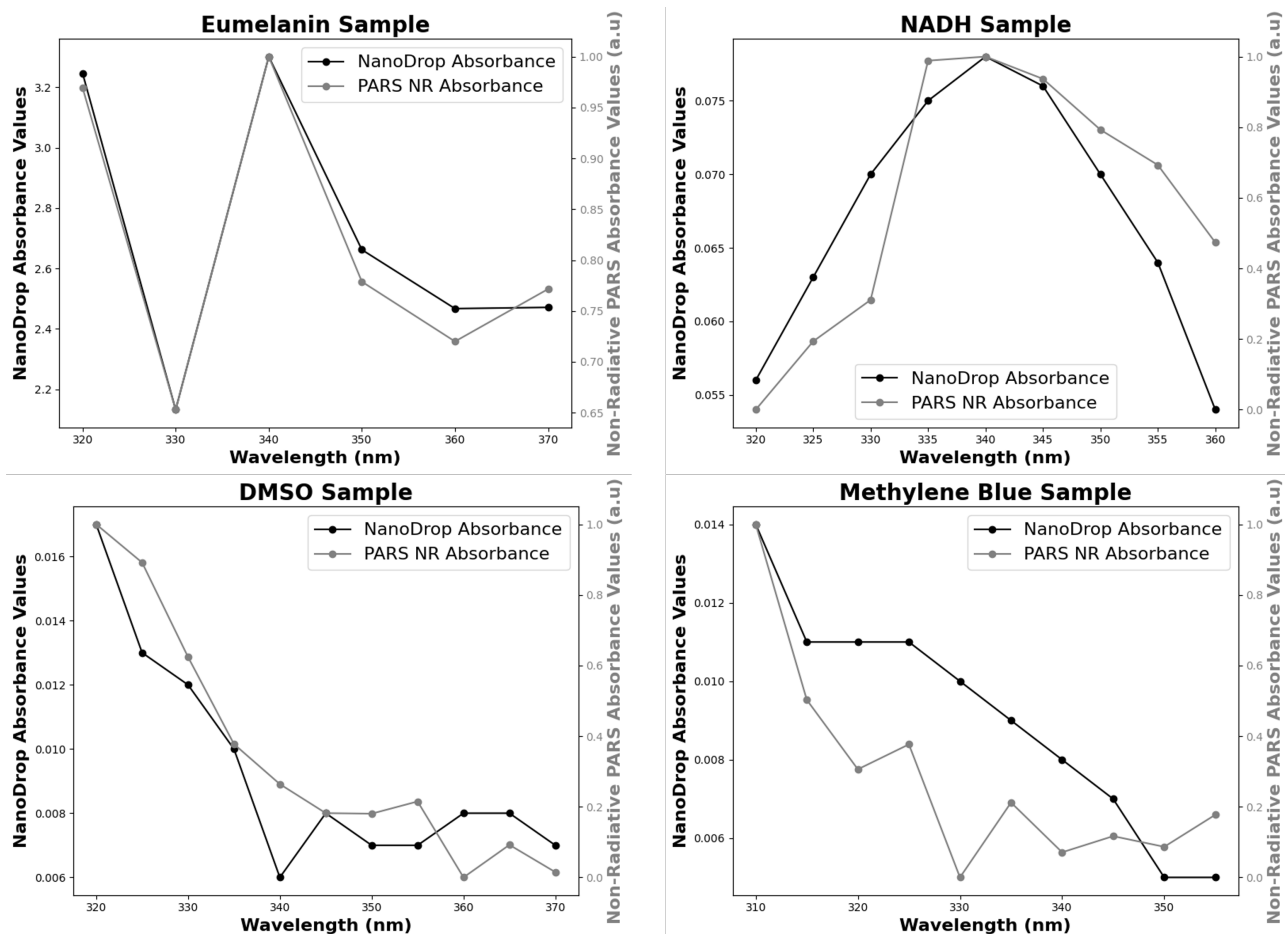


Figure 3.10: Non-radiative PARS absorption spectra (grey) benchmarked against the absorption spectra from a NanoDrop spectrophotometer device (black) for eumelanin, NADH, DMSO and methylene blue. The non-radiative PARS absorbance values were scaled from 0.0 - 1.0.

1Hz was a conservative choice to ensure sufficient time for thermal effects to completely dissipate for all samples in between excitation pulses. As non-radiative PARS signals from 900 excitation pulses were averaged together, this system operated at a speed of 15 minutes per wavelength. Furthermore, both laser sources were turned off for five minutes before switching excitation wavelengths to minimize the likelihood of damaging the sample. This resulted in the total spectral acquisition time being between 155 and 195 minutes, depending on the number of excitation wavelengths explored. Since the thermal relaxation time is proportional to the square of the excitation beam spot size, as described in Equation 2.6, this limitation could be partially addressed by using different lens components to reduce the excitation beam radius by orders of magnitude. This would make system set-up and alignment more difficult but would drastically reduce the lifetime of the thermal effects, allowing for a faster repetition rate to be used and ultimately, a decreased spectral acquisition time. An additional limitation lies in the measurement of the pulse energies from the Ekspla NT200 laser. Pulse power readings were taken with the laser set to a 1kHz repetition rate, while non-radiative signals were captured with a 1Hz repetition rate. The excitation energies per pulse may vary with repetition rate, making the normalization process described above slightly inaccurate.

Finally, the use of a photodetector to measure modulations of the detection light intensity introduces photon shot noise, which is an unavoidable source of error. This noise significantly constrains both the range of excitation wavelengths that can be explored, as sufficient fluence is required to generate detectable modulations above the noise threshold, and the concentrations of samples that can be measured. As previously discussed, the Beer-Lambert Law states that absorption is linearly dependent on sample concentration. Therefore, low-concentration samples may not produce enough absorption to overcome the system's noise floor. While the specific minimum concentration required for an observable signal varies between samples, as it depends on their inherent absorption at the excitation wavelength, photon shot noise ultimately sets the lower bound on system sensitivity.

3.5 Conclusions

In summary, this chapter detailed the design, implementation and validation of a non-radiative PARS spectroscopy system. The non-radiative PARS signals collected off this system were shown to accurately capture thermal and pressure relaxation processes. The accuracy of these signals were confirmed by comparing the measured relaxation lifetimes with their corresponding theoretical values and demonstrating that the extracted non-radiative PARS absorbance values scale linearly with sample concentration.

By integrating an Ekspla NT200 tuneable laser, the system captured non-radiative PARS signals across many UV excitation wavelengths in liquid samples of DMSO, methylene blue, eumelanin, and NADH. Absorption spectra were constructed from these measurements and compared to ground truth spectra obtained using a NanoDrop spectrophotometer. The findings indicate that this non-radiative PARS system can accurately characterize absorption profiles to varying degrees depending on the sample. For instance, eumelanin, which is highly UV absorbent and almost exclusively exhibits non-radiative relaxation, produced a non-radiative PARS absorption spectrum that very closely matches the ground truth. In contrast, samples with lower UV absorption or less prominent non-radiative relaxation tendencies showed greater discrepancies between their collected non-radiative PARS and NanoDrop absorption spectra.

The next chapter explores enhancements to this system, specifically the integration of a radiative detection channel, enabling the simultaneous capture of radiative and non-radiative relaxation pathways.

Chapter 4

Total Absorption PARS Spectroscopy System

4.1 Introduction

The next step of this work was to integrate a radiative relaxation pathway into the existing non-radiative PARS spectroscopy system. The goal was to upgrade the system presented in Chapter 3 so that it can simultaneously capture induced non-radiative *and* radiative relaxation processes. Since this system captures both relaxation pathways, it is referred to as a total absorption PARS spectroscopy system.

This work leverages the same tuneable excitation source as in the previous chapter and uses an Ocean Optics spectrometer device to capture the resulting radiative relaxations, which occur in the form of fluorescence. This chapter describes the design and validation of the radiative relaxation arm and presents results which demonstrate the benefit of capturing both relaxation pathways in studying the absorption profiles of certain samples.

4.2 Methods

This section discusses sample selection and preparation, the design of the proposed radiative relaxation pathway and the processing steps developed to minimize unwanted background and scattering effects captured in the radiative PARS signals.

4.2.1 Sample Preparation

Liquid samples were prepared using the same procedure as described in the Chapter 3. However, this chapter does not include analysis for eumelanin because, as mentioned previously, eumelanin dissipates over 99% of absorbed energy through non-radiative means. This makes eumelanin an unsuitable sample to use to evaluate the added radiative relaxation channel. As such, only the DMSO, methylene blue and NADH samples were used for this study.

4.2.2 System Design

This system integrates a pathway capable of capturing radiative relaxations from a sample into the system presented in the previous chapter, which solely focused on capturing non-radiative relaxations. This iteration of the system serves as a total absorption PARS spectroscopy system which simultaneously captures both sample relaxation pathways for every absorbed excitation pulse. An updated system schematic is presented in Figure 4.1. In this configuration, the non-radiative relaxations are captured in the same way as presented in Chapter 3. The radiative relaxations are captured by collimating photons from the excited sample using an off-axis parabolic mirror (O.A.P.M, 50328AL, Newport) and focusing them onto a spectrometer device (Spectrometer, USB4000, Ocean Optics) using a concave mirror (C.M 3, CM508-038-F01, Thorlabs) and an aspheric condenser lens (C.L 3, ACL25416U, Thorlabs). This spectrometer device generates a plot of the make-up of the captured photons according their wavelength intensity count. The plots from the spectrometer are the radiative PARS signals. These signals can be processed to extract radiative PARS absorbance values, representing the amount of sample relaxation that occurred radiatively. This combines with the non-radiative PARS absorbance values to provide total absorption insight. Furthermore, since the radiative relaxation arm captures not only the amount of fluorescence, but also the spectral breakdown of these emissions, this system fully integrates absorption and fluorescence emission spectroscopy.

4.2.3 Background Noise Correction

When collecting radiative PARS signals, the spectrometer also captures background noise in the forms of residual room light and dark current. In this system configuration, trace amounts of photons from background sources such as light bulbs in the lab may hit the spectrometer's sensor and be recorded in the signals. Another source of noise lies in the

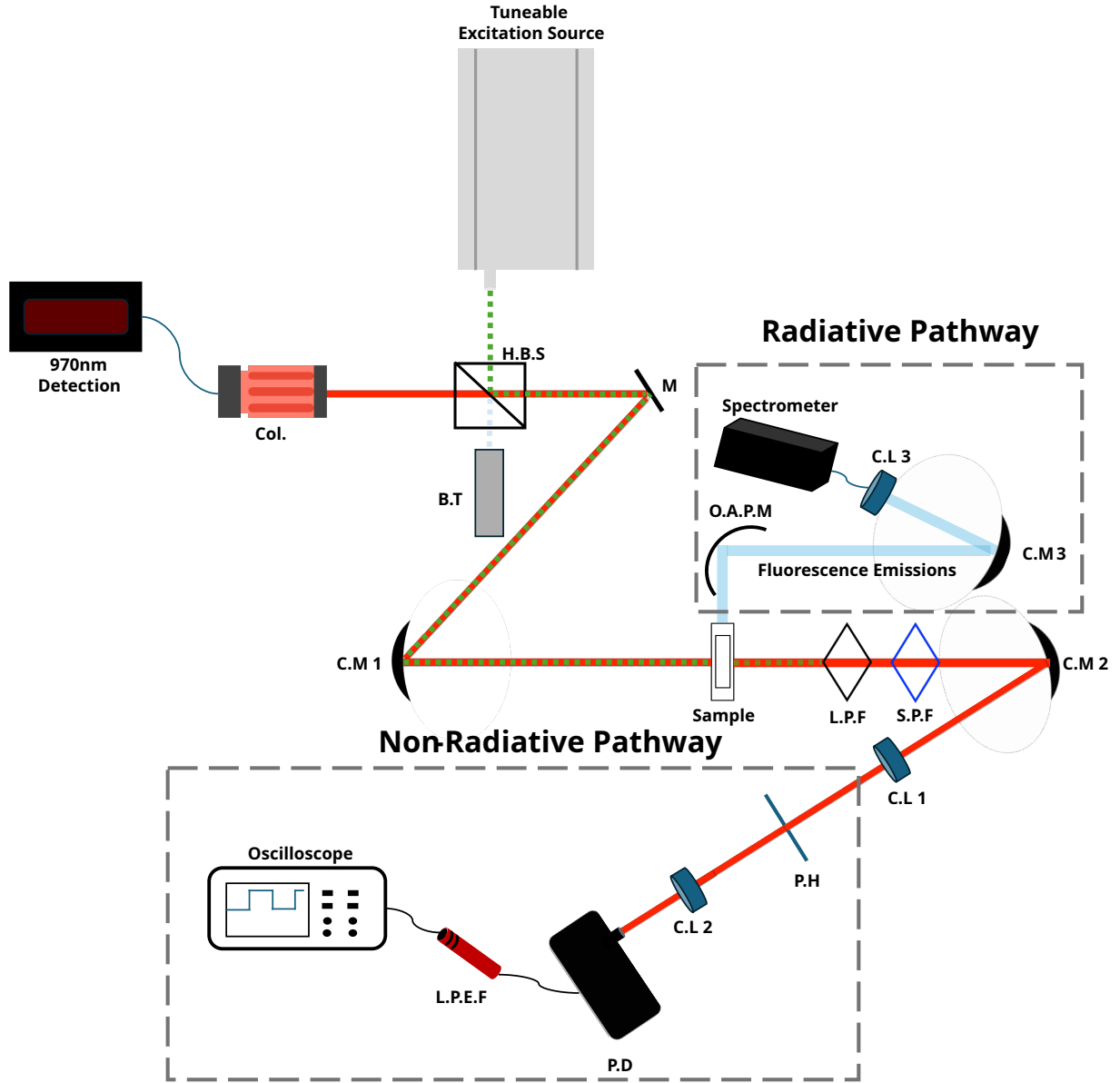


Figure 4.1: A simplified diagram of the PARS spectroscopy system with non-radiative and radiative relaxation pathways. Col.: achromatic fiber collimator, H.B.S.: harmonic beam splitter, B.T.: beam trap, M: UV-enhanced aluminum-coated mirror, C.M.: UV-enhanced aluminum-coated concave mirror, L.P.F.: long-pass filter, S.P.F.: short-pass filter, C.L.: aspheric condenser lens, P.H.: pinhole, P.D.: photodetector, L.P.E.F.: low-pass electrical filter, O.A.P.M.: off-axis parabolic mirror.

dark current of the device, which refers to the background signal in the recorded measurements that results from thermally induced currents in the detector of the spectrometer [111]. This background signal is present even when no light is reaching the device’s sensor. In order to account for these noise sources, the collected radiative PARS signals were corrected by subtracting a background noise reading, obtained from the spectrometer with no sample present and both the detection and excitation sources turned off. The background noise reading represents both residual room light and dark current noise sources. A plot comparing the collected radiative PARS signal from a methylene blue sample with its background noise is presented in Figure 4.2. Although this subtraction process should address both of these noise sources, in the work presented in this chapter the lab lights were turned off during signal acquisition whenever possible in order to completely eliminate the room light noise source. Furthermore, slight variations between the dark current present during background acquisition and that present during PARS signal capture may still serve as a source of error, particularly when the radiative signals are weak.

4.2.4 Scattering Effects

Light collected from the sample contains not only fluoresced photons, which represent radiative relaxations, but also scattered light from the detection and excitation laser. This scattering also includes trace amounts of bleed through from the signal, idler and pump. Since the goal of this system pathway is to capture radiative relaxations, it is crucial to minimize these scattering effects in the collected radiative PARS signals.

This specific spectrometer has a detection range of 176nm to 890nm, meaning that it cannot measure photons with wavelengths outside of this range. Since the wavelengths of the detection laser (970nm) and pump source in the excitation laser (1064nm) are constant and fall outside of this range, they are not captured by the spectrometer and thus not present in the radiative PARS signals. Furthermore, for the target excitation wavelengths studied in this work, which range from 310nm to 370nm, the corresponding idler wavelength is exclusively above 890nm as well. As such, the only sources of scattered photons that can be measured by the spectrometer are from the target wavelength and the signal bleed through from the excitation source. Figure 4.3 shows both scattering and fluorescence events present in a radiative PARS signal captured from a methylene blue sample. Note that, similar to the work done in Chapter 3, this data represents the average radiative PARS signal from 900 excitation pulses and after background noise subtraction.

In a fixed-wavelength system, filtering out these scattered photons could be accomplished relatively straightforwardly through placing a notch filter in front of the spectrometer. However, the multi-wavelength nature of this system makes this approach unfeasible,

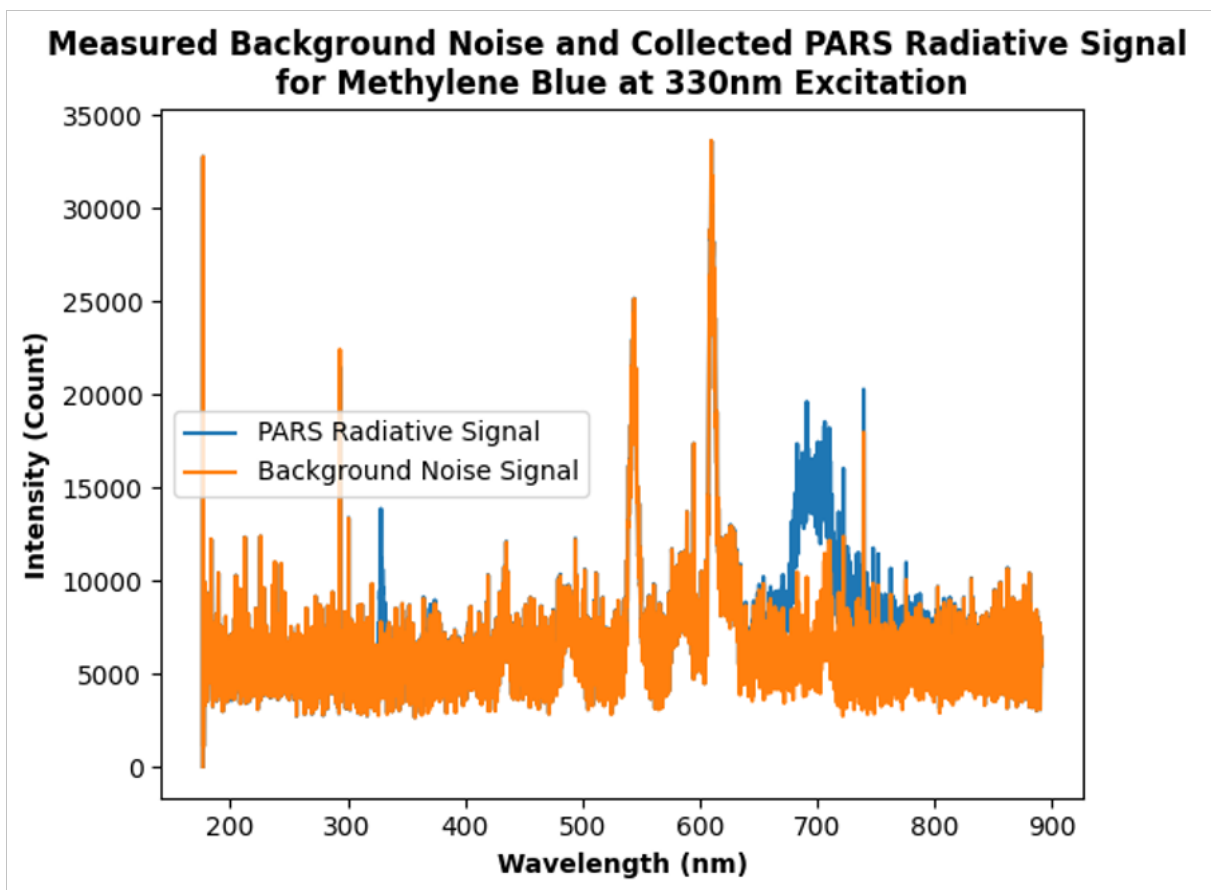


Figure 4.2: Radiative PARS signal captured from the spectrometer device (blue) and the background noise signal from the spectrometer device (orange). Differences in the two spectra represent scattering and fluorescence. Note that the shared peaks at approximately 550nm and 620nm represent residual room light captured by the spectrometer device. Since these peaks are captured in both signals, they are also removed through this subtraction cleaning processes.

Radiative PARS Signal For Methylene Blue Sample at 330nm Excitation

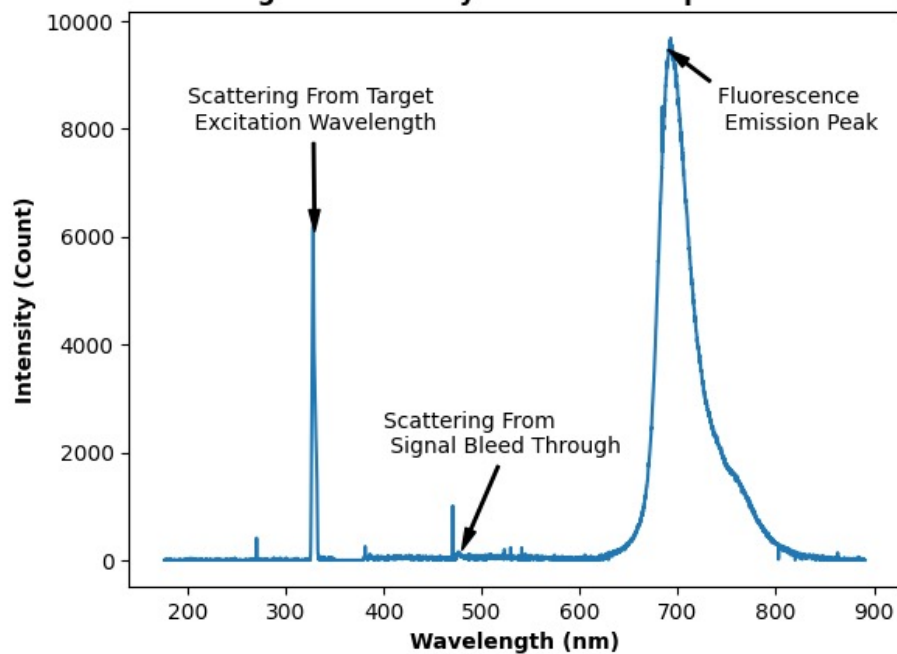


Figure 4.3: Radiative PARS signal captured on the Ocean Optics spectrometer device from a 3.13 μ M methylene blue sample at 330nm excitation. This signal includes fluorescence emissions, which represent the radiative relaxation of the sample, as well as scattering effects from the excitation laser at the target excitation wavelength of 330nm and at the signal wavelength of 478nm. These scattering effects do not represent radiative relaxations.

as the wavelengths of these scattering effects change with every new target excitation wavelength. As such, these effects were addressed using a post-processing technique. Eliminating scattering from the target excitation wavelength was accomplished by simply setting the intensities of wavelengths within a $\pm 10\text{nm}$ range of the target to zero. Scattering from the signal bleed through could not be handled with the same approach, as theoretically there could be overlap between the wavelengths of fluoresced photons and the photons from the signal bleed through. In this case, artificially eliminating the intensities of wavelengths in a local neighbourhood around the signal wavelength would also eliminate true fluorescence from the collected signal, serving to further distort it. As such, the scattering effects from the signal bleed through were reduced through linear interpolation of the measured intensity at the wavelength 10nm shorter than the signal and the measured intensity at the wavelength 10nm longer than the signal. Figure 4.4 depicts this approach on an arbitrary radiative PARS signal. This approach aims to eliminate any sharp peaks in the collected signals which may arise from signal bleed through scatter, while also preserving the presence of true fluorescence.

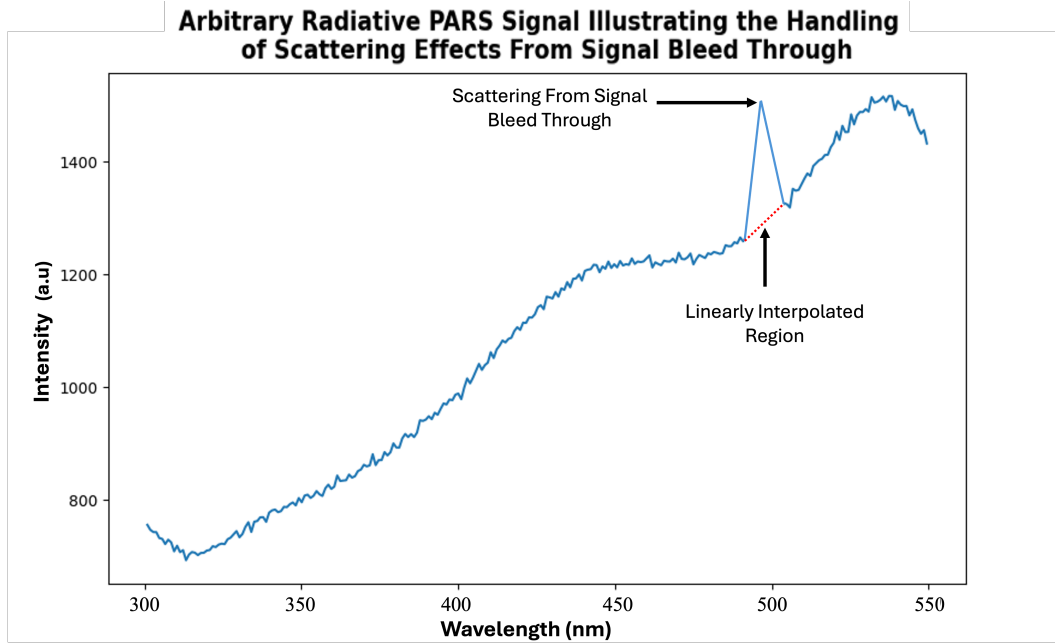


Figure 4.4: Linear interpolation process on a radiative PARS signal to minimize the scattering effects from signal bleed through.

Figure 4.5 illustrates the radiative PARS signal from the methylene blue sample after the steps to minimize collected scattering. The processed radiative PARS signals contain only fluorescence effects and represent the radiative relaxations of the sample. The area under these fluorescence emission signals can be calculated and normalized by the excitation wavelength's fluence correction factor to derive a radiative PARS absorbance value. Measuring and processing these signals across many different excitation wavelengths yields a radiative PARS absorption spectrum for the sample.

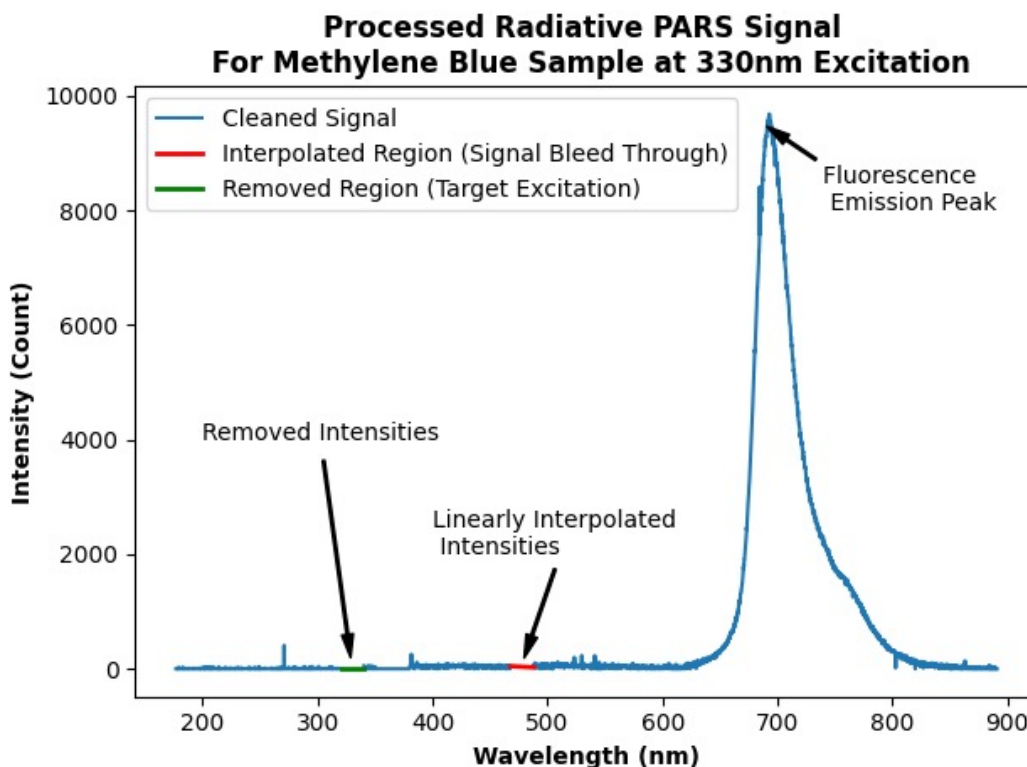


Figure 4.5: Processed radiative PARS signal from a methylene blue sample at 330nm excitation. Collected scattering has been minimized leaving only fluorescence effects in the signal.

4.3 Results and Discussion

This section outlines the validation of the radiative relaxation arm and presents results in leveraging the newly developed dual-signal system to accurately study the absorption profiles of the samples of interest.

4.3.1 System Validation

Verifying Sample Fluorescence Profiles

The first step in verifying the radiative relaxation arm of this system was ensuring that it captures accurate fluorescence emission profiles from liquid samples. This was tested using methylene blue and NADH samples as the fluorescence spectral features of these molecules are well studied and documented in literature, and thus can serve as a ground truth for these comparisons. As depicted in Figure 4.3, this system captures a fluorescence peak from methylene blue at roughly 688nm, which is consistent with values reported in literature [96], [112]. Furthermore, NADH has been reported to have a fluorescence emission peak around 480nm [113], which is also captured in the fluorescence emissions recorded by this system, as seen in Figure 4.6.

Linearity of Absorbance Values

Similar to the non-radiative signal case, the method of extracting radiative PARS absorbance values from the collected radiative PARS signals was verified by testing the linearity of these values with respect to sample concentration. In this study, four methylene blue samples were prepared with concentrations of 10 μ M, 8 μ M, 6.67 μ M and 5 μ M and an excitation wavelength of 330nm was used. For each sample, radiative PARS signals from 900 excitation pulses were captured on the spectrometer and averaged together. The collected background noise signal was then subtracted. Each purified signal was processed according to the steps described above to remove scattering effects, and a rolling average technique with a window size of 100 was applied to each signal for smoothing purposes. The processed signals for each methylene blue sample are presented in Figure 4.7.

The area under each of the fluorescence signals was then calculated and used as a radiative PARS absorbance value for each specific sample at 330nm excitation. Note that normalization for wavelength-to-wavelength energy and spot size discrepancies was not required here due to this being a single-wavelength experiment. The results illustrated

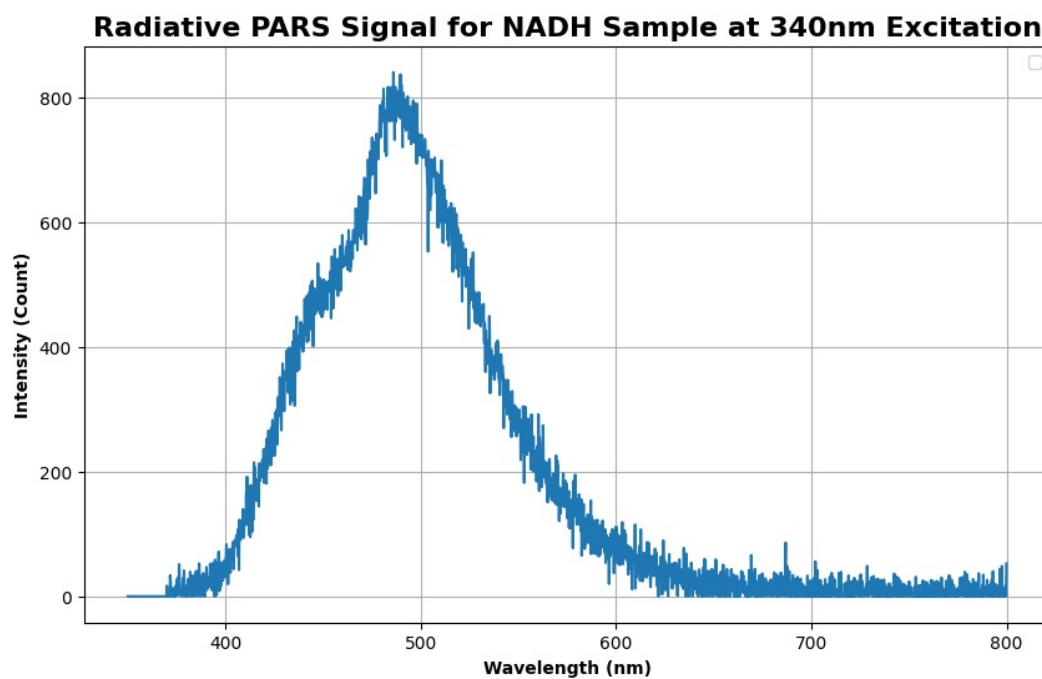


Figure 4.6: Processed radiative PARS signal from an NADH sample depicting an emission peak at roughly 480nm.

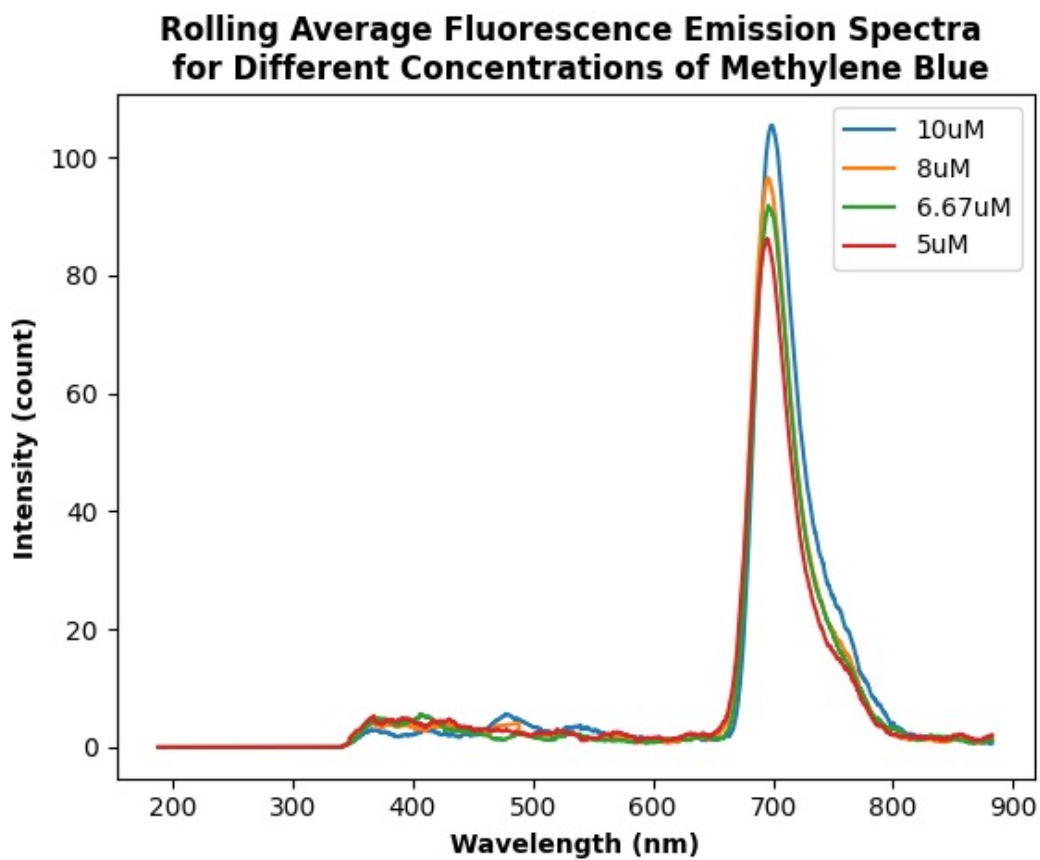


Figure 4.7: Processed radiative PARS signals from methylene blue samples with $10\mu\text{M}$, $8\mu\text{M}$, $6.67\mu\text{M}$ and $5\mu\text{M}$ concentration following 330nm excitation.

in Figure 4.8 show a highly linear relationship between the extracted radiative PARS absorbance values and the sample concentration, which is in line with the expected behavior of the system based on the Beer-Lambert Law. These results further reinforce the capability of the radiative arm in capturing accurate radiative relaxation signals and also validate the effectiveness of the proposed processing algorithms to remove unwanted effects and extract absorbance values from the processed radiative PARS signals.

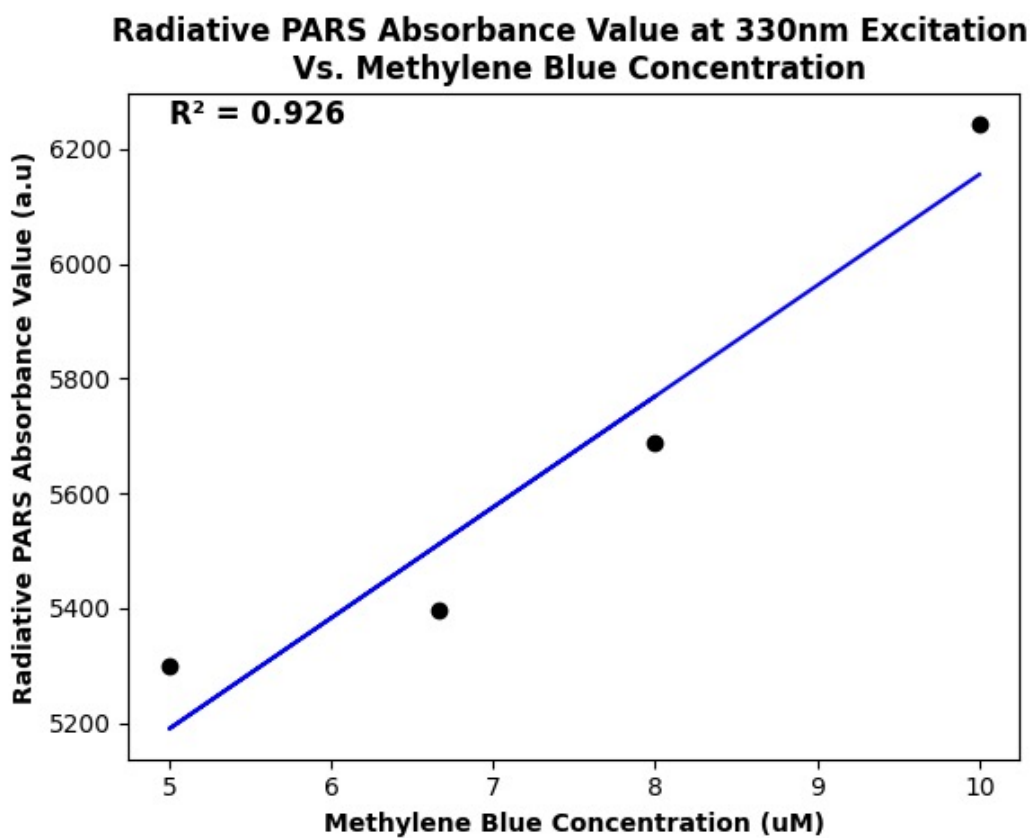


Figure 4.8: Extracted absorbance values from radiative PARS signals recorded from methylene blue solutions with varying concentrations.

4.3.2 Radiative PARS Absorption Spectra

The tuneable excitation source was used to explore the wavelength-dependent radiative relaxation processes which are induced following an absorption event. As mentioned above, the samples studied in this work were identical to the samples from Chapter 3, with the omission of the eumelanin sample. More specifically, the samples studied were: DMSO, a $3.13\mu\text{M}$ methylene blue solution and a $100\mu\text{M}$ NADH solution. All of these samples were 2mL in volume. The excitation wavelengths explored for each sample were the same as in the previous chapter, ranging from 310nm to 370nm. Radiative PARS signals were captured after each of 900 excitation pulses, set at a 1Hz repetition rate, for every sample and excitation wavelength. This data was captured simultaneously with the non-radiative data presented in the previous chapter. These 900 radiative PARS signals, which belong to a single sample and excitation wavelength, were then averaged together and corrected for background noise. Post-processing was performed on these averaged signals to remove scattering effects, and a rolling average technique with a window size of 100 was applied for further smoothing. The area under each processed signal was then calculated and normalized by the excitation wavelength’s corresponding fluence correction factor to generate a radiative PARS absorption spectrum for each sample. Similar to Chapter 3, these spectra were benchmarked against the data derived from the NanoDrop, which served as the ground truth. The results of this study are presented in Figure 4.9. Note that the radiative PARS absorbance values were scaled to be between 0.0 and 1.0, for a more intuitive visualization.

The absorption profile of methylene blue seems to be more appropriately characterized through the collected radiative PARS signals, as opposed to the non-radiative PARS signals from the previous chapter. This is in line with expectations, as methylene blue is a highly radiative molecule in nature [96] and therefore, is expected to be better studied through the radiative relaxation arm. Similar observations can be made for the DMSO sample, whose radiative PARS absorption spectrum seems to slightly better align with the collected ground truth than the non-radiative data collected previously. The performances of the radiative and non-radiative spectra for each sample are formally compared in the following section.

4.3.3 Total Absorption PARS Spectra

The spectral data presented thus far has been scaled such that all PARS absorbance values fall between 0.0 and 1.0. While this makes visualizing the recorded spectra more intuitive, it does make studying the performance of these spectra relative to the ground truth more

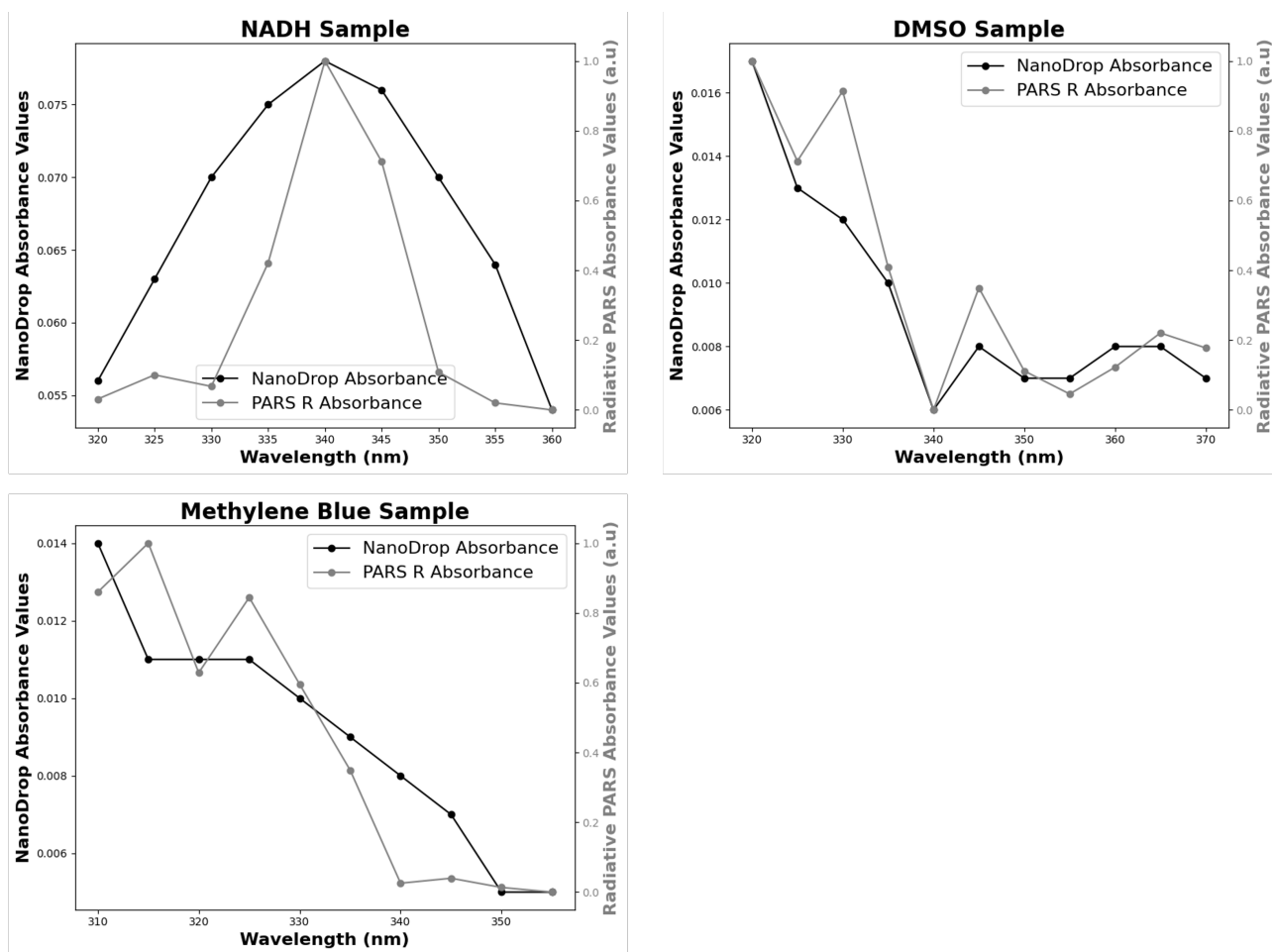


Figure 4.9: PARS radiative absorption spectra (grey) benchmarked against the absorption spectra from a NanoDrop spectrophotometer device (black) for NADH, DMSO and methylene blue. The radiative PARS absorbance values were scaled from 0.0 - 1.0.

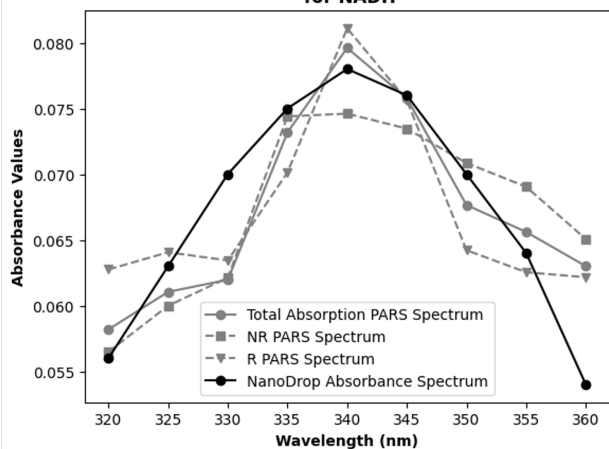
difficult, as the PARS and NanoDrop data operate on different scales. In order to compare the performances of the two PARS channels' absorption spectra, linear regression models were trained to map each sample's radiative and non-radiative absorption spectrum to the spectrum from the NanoDrop. This puts the non-radiative and radiative PARS spectra on the same scale as the ground truth, enabling direct comparisons of performance. In addition to the radiative and non-radiative spectra, a "total absorption" spectrum was also developed for every sample by taking the sum of the scaled radiative and non-radiative PARS absorbance values for each wavelength. Linear regression models were then also trained to map each of the developed total absorption spectra to their corresponding ground truth. Figure 4.10 shows how the mapped radiative, non-radiative and total absorption PARS spectra compare to the ground truth absorption profiles for the NADH, DMSO and methylene blue samples. The performance of each spectrum was quantified by calculating the percentage difference between the ground truth absorbance value and the mapped PARS absorbance value for each excitation wavelength and sample. The mean and variance of these differences are presented in Table 4.1.

Table 4.1: Comparison of non-radiative, radiative and total absorption PARS spectra accuracy using the mean and variance of the percentage differences to the ground truth NanoDrop spectra.

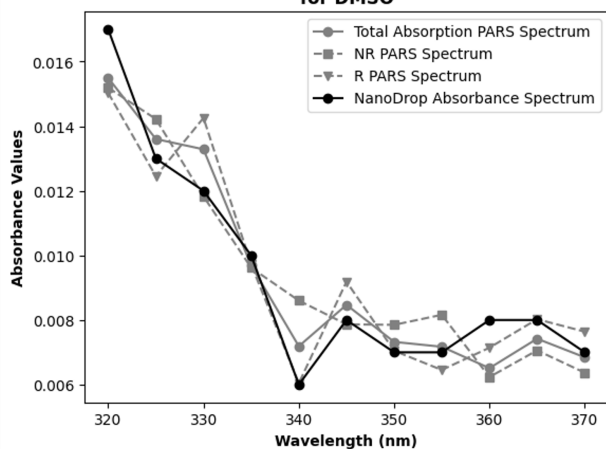
	NADH		DMSO		Methylene Blue	
	<u>Mean Percentage Difference to Ground Truth</u>	<u>Variance of Percentage Differences</u>	<u>Mean Percentage Difference to Ground Truth</u>	<u>Variance of Percentage Differences</u>	<u>Mean Percentage Difference to Ground Truth</u>	<u>Variance of Percentage Differences</u>
Non-Radiative PARS Spectra	5.99	0.314	12.9	1.27	20.4	4.50
Radiative PARS Spectra	6.55	0.200	7.41	0.353	12.6	0.628
Total Absorption PARS Spectra	5.00	0.233	8.01	0.342	12.7	1.00

These results indicate that this PARS system performed the strongest in characterizing the absorption profile of the NADH sample. This is expected as this sample expresses the highest tendency to absorb light in the UV range, as evidenced by its absorbance values from the NanoDrop being significantly higher than those from the DMSO and methylene blue samples. Furthermore, the non-radiative and radiative PARS absorbance values have, on average, a 5.99% and 6.55% difference to the NanoDrop absorbance values, respectively.

Linear Mappings of PARS Total Absorption, Non-Radiative and Radiative Absorbance Spectra to the NanoDrop Spectra for NADH



Linear Mappings of PARS Total Absorption, Non-Radiative and Radiative Absorbance Spectra to the NanoDrop Spectra for DMSO



Linear Mappings of PARS Total Absorption, Non-Radiative and Radiative Absorbance Spectra to the NanoDrop Spectra for Methylene Blue

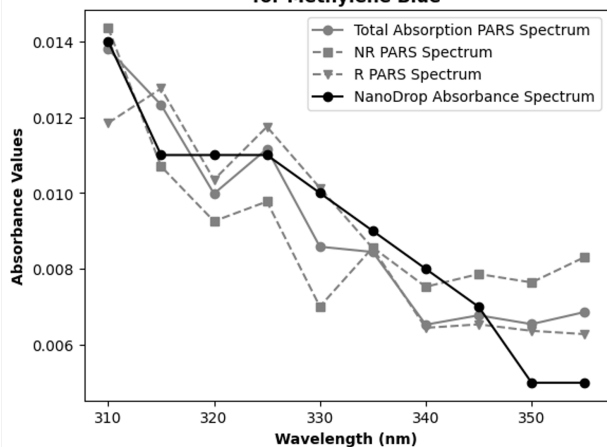


Figure 4.10: Results from linearly mapping the PARS total absorption spectra, PARS non-radiative spectra and PARS radiative spectra to the NanoDrop absorption spectra for the NADH, DMSO and methylene blue samples.

However, the total absorption PARS absorbance values are on average 5.00% away from their corresponding ground truth value. While the mean percentage difference to ground truth is relatively constant for each channel, there is a small improvement in the total absorption spectrum. This indicates that for NADH, the combination of radiative and non-radiative relaxation processes provides a slightly more accurate study of its absorption profile than either relaxation channel does individually.

In contrast to NADH, both the DMSO and methylene blue samples exhibit larger percentage differences between the PARS-derived absorbance values and the NanoDrop ground truth. This can likely be attributed to their intrinsically weaker absorption to light in the UV range. Furthermore, as mentioned in the previous chapter, the NanoDrop system operates on a direct absorption spectroscopy mechanism and is unable to distinguish between absorption and scattering effects. However, the developed PARS system directly measures absorption by probing relaxation processes, and is not distorted by scattering events. This fundamental difference becomes more pronounced in weakly absorbing samples, where the scattering may contribute a relatively larger portion of the reported NanoDrop signal.

Interestingly, for both DMSO and methylene blue, the radiative PARS spectra show slightly better agreement with the ground truth than the total absorption spectra. While these differences are marginal, this trend may reflect the inherent chemical composition of these samples. For example, incorporating non-radiative signals into the absorption profile of methylene blue, which is a highly fluorescent sample, may introduce more noise than meaningful information. Nonetheless, the total absorption spectra for methylene blue and DMSO still offer close approximations of the ground truth and remain particularly valuable for the NADH sample.

4.4 Limitations

While the system proposed in this chapter addresses a key limitation of Chapter 3 by incorporating a radiative relaxation pathway, it introduces a new constraint in that the radiative and non-radiative data are acquired using different types of detectors. As a result, the corresponding radiative and non-radiative PARS absorbance values exist on drastically different scales, making it difficult to infer sample parameters such as quantum yield with confidence. Furthermore, as a result of these different scales, the PARS absorbance values had to be scaled to fall between 0.0 and 1.0 before summing to form total absorption spectra. This artificially forces both channels to be represented equally in the total absorption data, whereas, ideally, they should contribute based on the actual proportion of relaxation that occurs through each pathway.

This limitation could potentially be overcome through studying a set of various samples with very well characterized and quantified quantum yield values at specific excitation wavelengths. A calibration curve could be constructed by collecting the ratio of the corresponding radiative and non-radiative PARS absorbance coefficients for these samples. This curve could be used to confidently deduce the quantum yield of other molecules based on their measured ratio of PARS radiative to non-radiative absorbance values and would provide insight into how the respective absorbance coefficients should be most appropriately weighted when building a sample’s total absorption spectrum.

4.5 Conclusions

This chapter described the design and validation of a radiative relaxation arm which was integrated into the previously developed non-radiative PARS spectroscopy system. This pathway relied on capturing radiative PARS signals and performing background noise and scattering correction. Radiative PARS absorbance values were extracted for each sample and excitation wavelength by calculating the area under the corresponding processed radiative PARS signal and normalizing by a fluence correction factor to account for excitation wavelength-to-wavelength energy and spot size differences. The system design and effectiveness of the processing algorithms were validated by ensuring that the location of fluorescence peaks present in the collected signals aligns with their expected values and that the extracted absorbance values from these signals scale linearly with the sample’s concentration.

The integration of a radiative relaxation pathway allows for the PARS spectroscopy system to simultaneously capture the non-radiative and radiative relaxation processes which arise following the same excitation pulse, giving it a “total absorption” outlook on an absorption event. Absorption spectra were developed for the NADH, DMSO and methylene blue samples using the processed radiative PARS signals as well as the sum of the collected radiative PARS and non-radiative PARS absorbance values. These are referred to as total absorption spectra. Comparing each of these spectra to their corresponding ground truth revealed that, for certain samples, such as NADH, the combination of radiative and non-radiative data allows for a slightly more accurate study of their absorption profile. However, for the DMSO and methylene blue samples, the inclusion of non-radiative data marginally decreased the accuracy of its reported absorption spectra. Ultimately, in all cases, the total absorption spectra provide accurate insight into the true absorption characteristics of a sample.

The addition of this radiative channel enables the study of the absorption profiles

of samples with a wide range of quantum yields, as highly non-radiative molecules can be analyzed through the non-radiative channel, while highly fluorescent materials can be studied through the radiative pathway. This provides a distinct advantage over other common pump-probe spectroscopy approaches, such as photothermal and photoacoustic spectroscopy, which specifically aim to study non-radiative relaxation processes, and thus are unsuitable techniques to employ in analyzing highly fluorescent samples.

Although this chapter did integrate a radiative relaxation pathway, it still focused exclusively on studying sample absorption profiles. This was done by calculating the cumulative emission intensity to provide a radiative PARS absorption contrast. The following chapter investigates how the spectral breakdown of the fluorescence emission signals can be used in combination with a sample's total absorption spectrum to provide improved insight into the composition of an unknown mixture solution.

Chapter 5

Sample Unmixing Using PARS Spectroscopy

5.1 Introduction

An important application of spectroscopy lies in using its data to identify the make-up of a mixture sample. This is particularly relevant in biomedical research, where researchers may want to quantify the presence of a specific chromophore in a non-uniform sample, such as a tissue section. In this case, the collected spectroscopic signals represent the optical response of the entire sample, with individual chromophores contributing to the signal based on their relative abundance. To address this, various “unmixing” algorithms have been developed to estimate the relative abundances of each chromophore based on the collective spectral data. For example, Arabul *et al.* demonstrated that multi-spectral photoacoustic signals can effectively unmix chromophores such as oxyhemoglobin, deoxyhemoglobin, cholesterol and collagen in human plaque samples [114].

This chapter serves as a proof-of-concept study aimed at exploring how the multi-modal signals captured by the PARS spectroscopy system can be used to accurately unmix liquid ink samples. This work lays the foundation for future applications in unmixing biomolecules. The mathematical algorithms used for unmixing are discussed and results are presented demonstrating that the PARS spectroscopy system outperforms purely absorption-based spectroscopy systems, such as the NanoDrop, in accurately determining the abundance ratios of inks in mixed samples.

5.2 Methods

This section describes how the ink mixtures were prepared, as well as the different unmixing approaches used in this study.

5.2.1 Sample Preparation

Red (Dr. Ph Martin’s Bombay Red India Ink) and blue (Dr. Ph Martin’s Bombay Blue India Ink) craft ink samples were purchased and used as stock solutions for this study. Initially, the concentration of ink in the stock solutions was so high that these samples completely absorbed incoming excitation pulses across the range of 320nm to 370nm. This is undesirable for studying the absorption properties of a sample, as ideally some amounts of each excitation pulse would transmit through the sample. This indicates that the absorption that occurs is a true reflection of the sample’s inherent properties and not limited by the amount of excitation light available for absorption. To overcome this, each stock solution was diluted using distilled water to a 1:454 ink-to-water ratio. This specific ratio was optimized experimentally, as each stock solution was continuously diluted until none of the excitation wavelengths were fully absorbed by the sample. Following this, mixture solutions were also prepared by combining the diluted red and blue inks in varying ratios. These served as the “target” solutions for this study, whose make-up was to be determined through the different unmixing approaches. Each target sample was 3mL in volume. This study specifically used craft ink samples because minimal chemical interactions are expected to arise when combined to form mixtures. This makes the unmixing process significantly more straight-forward for this proof-of-concept study.

5.2.2 Unmixing Approach

Two different unmixing methods were employed in this study. Both of these approaches used a least-squared error minimization fitting procedure and aimed to find the combination of the spectroscopic signals from the end-members (ie: the pure red and blue ink samples) that best approximate the signals collected from the target solutions. These techniques are particularly suitable and effective for a problem of this nature, where the end-members in the target solutions can also be easily studied in isolation [115].

The signals used in this unmixing work were the total absorption PARS spectra and the mean fluorescence emission spectra for the red, blue and target ink samples. A total absorption PARS spectrum was derived for each sample by summing the scaled non-radiative

and radiative PARS absorbance values for each excitation wavelength. The total absorption PARS spectra for the isolated red ink sample, isolated blue ink sample and a mixed ink sample, which was prepared in a 2:1 red-to-blue ink ratio, are presented in Figure 5.1, along with the corresponding ground truth data collected from the NanoDrop.

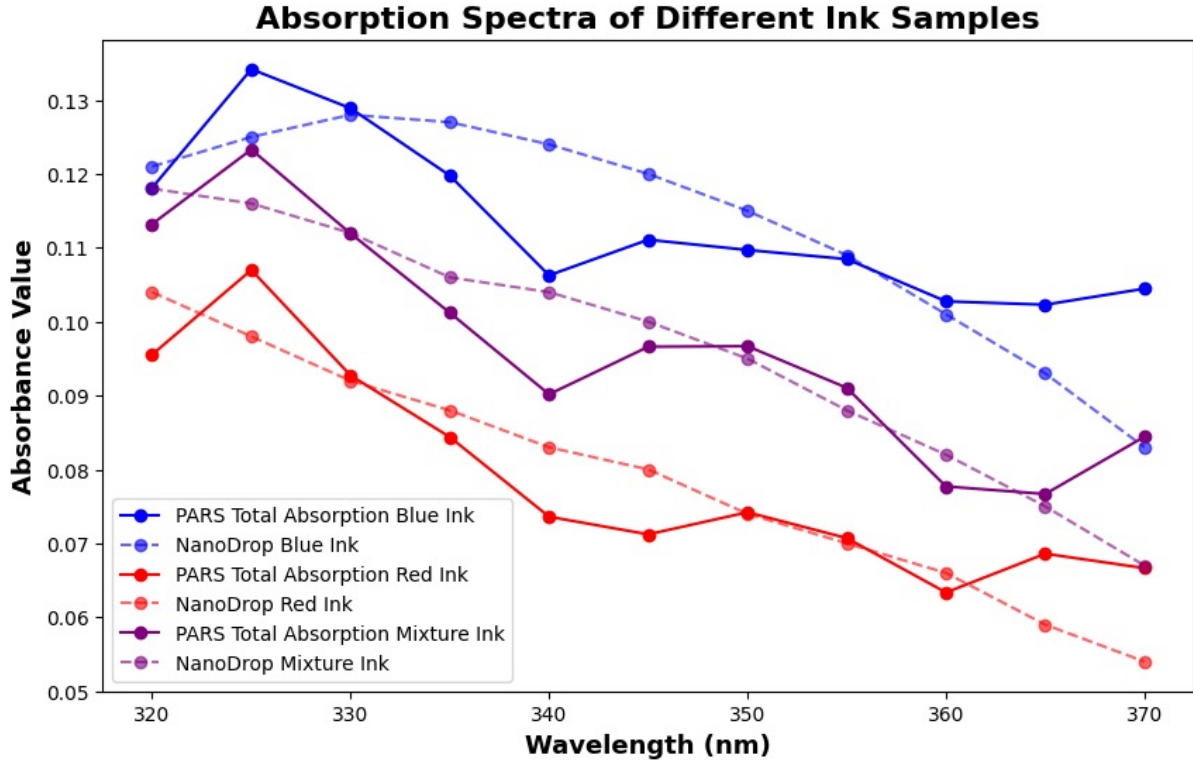


Figure 5.1: Comparison of the total absorption spectra of red ink, blue ink and mixed ink samples captured off of the PARS spectroscopy system and the NanoDrop system. Note that the total absorption PARS values were linearly mapped to the NanoDrop absorbance values for each sample to allow for easier visualization, as was done in the previous chapter.

The mean fluorescence emission spectrum for each sample was calculated by averaging the rolling means of the processed radiative PARS signal from each excitation wavelength. Each averaged rolling mean was then scaled so that all intensity counts also fall between 0.0 and 1.0. These mean fluorescence emission signals for the red, blue and 2:1 mixed ink samples are shown in Figure 5.2.

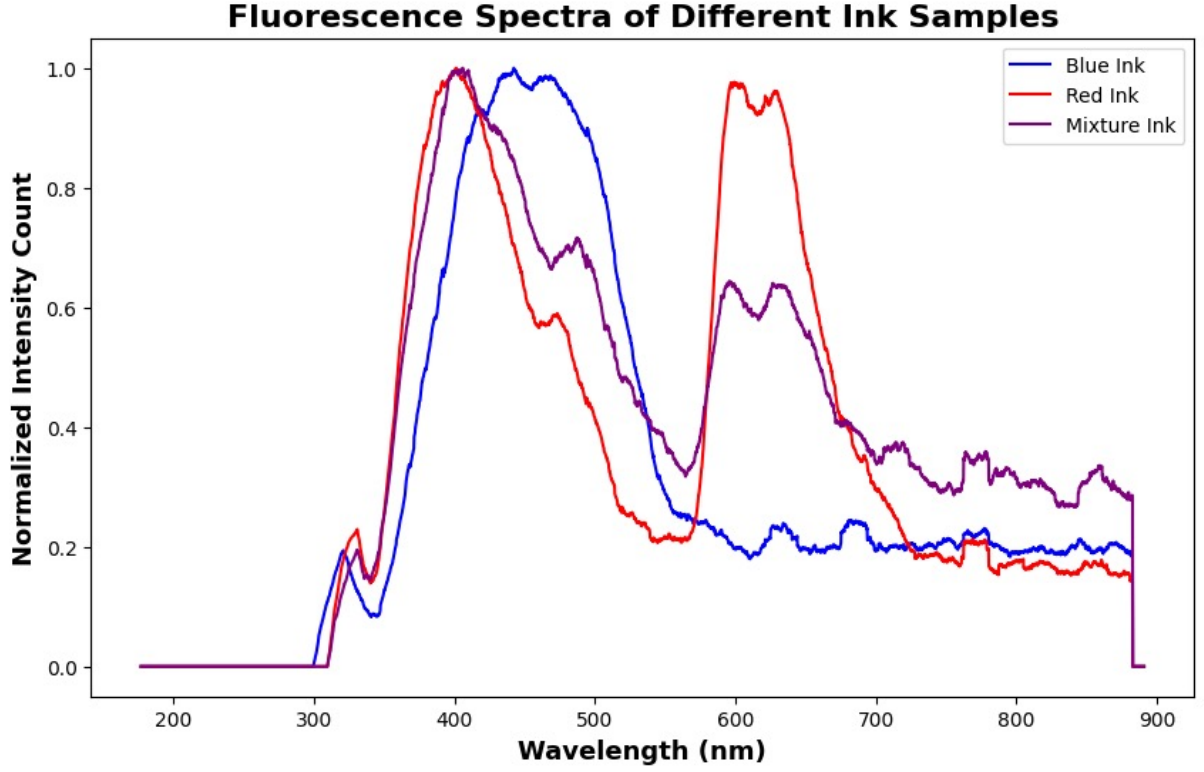


Figure 5.2: Comparison of the average fluorescence emission spectra of red ink, blue ink and mix ink samples captured off of the PARS spectroscopy system using the Ocean Optics spectrometer device.

Traditional Linear Model

The first unmixing method used was a traditional linear model. Here, a least-squares approach was used to find the optimal R and B coefficients which minimize the sum of the residual errors in the equations:

$$\mathbf{A}_T = R\mathbf{A}_R + B\mathbf{A}_B \quad (5.1)$$

$$\mathbf{F}_T = R\mathbf{F}_R + B\mathbf{F}_B \quad (5.2)$$

where R and B represent the fraction of red and blue ink in the target sample, respectively, and,

$$\begin{aligned}
\mathbf{A}_T &= \begin{bmatrix} \text{PARS Total Absorption Value of Target Ink Sample at 320 nm} \\ \text{PARS Total Absorption Value of Target Ink Sample at 325 nm} \\ \vdots \\ \text{PARS Total Absorption Value of Target Ink Sample at 370 nm} \end{bmatrix} \\
\mathbf{A}_R &= \begin{bmatrix} \text{PARS Total Absorption Value of Red Ink Sample at 320 nm} \\ \text{PARS Total Absorption Value of Red Ink Sample at 325 nm} \\ \vdots \\ \text{PARS Total Absorption Value of Red Ink Sample at 370 nm} \end{bmatrix} \\
\mathbf{A}_B &= \begin{bmatrix} \text{PARS Total Absorption Value of Blue Ink Sample at 320 nm} \\ \text{PARS Total Absorption Value of Blue Ink Sample at 325 nm} \\ \vdots \\ \text{PARS Total Absorption Value of Blue Ink Sample at 370 nm} \end{bmatrix} \\
\mathbf{F}_T &= \begin{bmatrix} \text{Fluorescence Emission Intensity Count of Target Ink Sample at 176.7 nm} \\ \text{Fluorescence Emission Intensity Count of Target Ink Sample at 176.9 nm} \\ \vdots \\ \text{Fluorescence Emission Intensity Count of Target Ink Sample at 890.9 nm} \end{bmatrix} \\
\mathbf{F}_R &= \begin{bmatrix} \text{Fluorescence Emission Intensity Count of Red Ink Sample at 176.7 nm} \\ \text{Fluorescence Emission Intensity Count of Red Ink Sample at 176.9 nm} \\ \vdots \\ \text{Fluorescence Emission Intensity Count of Red Ink Sample at 890.9 nm} \end{bmatrix} \\
\mathbf{F}_B &= \begin{bmatrix} \text{Fluorescence Emission Intensity Count of Blue Ink Sample at 176.7 nm} \\ \text{Fluorescence Emission Intensity Count of Blue Ink Sample at 176.9 nm} \\ \vdots \\ \text{Fluorescence Emission Intensity Count of Blue Ink Sample at 890.9 nm} \end{bmatrix}
\end{aligned}$$

This optimization problem can be formally stated as follows:

$$\begin{aligned}
&\arg \min_{R,B} \frac{1}{\text{len}(\mathbf{A}_T)} \|\mathbf{A}_T - (R\mathbf{A}_R + B\mathbf{A}_B)\|^2 + \frac{1}{\text{len}(\mathbf{F}_T)} \|\mathbf{F}_T - (R\mathbf{F}_R + B\mathbf{F}_B)\|^2 \\
&\text{subject to: } R > 0, \quad B > 0, \quad R + B = 1
\end{aligned} \tag{5.3}$$

Here, R and B are the optimization variables and the ratio of their optimal values should be as close to the ground truth composition ratio of the target solution as possible, as this would indicate an accurate unmixing process. This work employed a Sequential Least Squares Programming (SLSQP) optimizer implemented in Python. Each residual term in the equation above is normalized by the corresponding vectors' lengths. Without any weighting correction, an optimizer would be heavily biased towards simply fitting R and B coefficients to best approximate $\mathbf{F}_T$ using $\mathbf{F}_R$ and $\mathbf{F}_B$, as these vectors have lengths of 3648, compared to $\mathbf{A}_T$, $\mathbf{A}_R$ and $\mathbf{A}_B$ which have lengths of 11. In this work, the total absorption and mean fluorescence emission data were viewed as equally important, and as such, vector-length normalization was performed to eliminate this bias.

Generalized Bilinear Model

The second unmixing method which was employed in this work was a generalized bilinear model, which builds upon the traditional linear model presented above. This approach introduces an “interaction coefficient”, γ , into the reconstruction equation, as seen below:

$$\mathbf{A}_T = R\mathbf{A}_R + B\mathbf{A}_B + \gamma R B \mathbf{A}_R \odot \mathbf{A}_B \quad (5.4)$$

$$\mathbf{F}_T = R\mathbf{F}_R + B\mathbf{F}_B + \gamma R B \mathbf{F}_R \odot \mathbf{F}_B \quad (5.5)$$

This additional interaction term introduces an extra degree of freedom into the optimization process. In this case, R , B and γ are the optimization variables. While this approach has been previously applied in the unmixing of hyperspectral images [116], [117], this work extends the use of generalized bilinear models to the unmixing of liquid ink samples using PARS signals. This optimization problem can be formulated as:

$$\begin{aligned} & \arg \min_{R, B, \gamma} \frac{1}{\text{len}(\mathbf{A}_T)} \|\mathbf{A}_T - (R\mathbf{A}_R + B\mathbf{A}_B + \gamma R B \mathbf{A}_R \odot \mathbf{A}_B)\|^2 \\ & \quad + \frac{1}{\text{len}(\mathbf{F}_T)} \|\mathbf{F}_T - (R\mathbf{F}_R + B\mathbf{F}_B + \gamma R B \mathbf{F}_R \odot \mathbf{F}_B)\|^2 \quad (5.6) \\ & \text{subject to: } R > 0, \quad B > 0, \quad 0 < \gamma < 1, \quad R + B = 1 \end{aligned}$$

Similar to the traditional linear model, this work also employed a SLSQP optimizer and the same vector-length normalization procedure.

5.3 Results and Discussion

This section describes how the unmixing process was validated and presents results demonstrating the use of PARS data to accurately unmix ink samples.

5.3.1 Unmixing Validation

The first experiments ran in this study served as sanity checks for the system and the unmixing process. These experiments used pure red and blue inks as the target samples to unmix. In this study, total absorption PARS and mean fluorescence emission spectra were captured from isolated red and blue ink samples and then again from *different* isolated red and blue ink samples. Traditional linear and generalized bilinear models were used to unmix the two different target samples. The results of these experiments are shown in Table 5.1 and indicate that both unmixing models are capable of accurately identifying target samples as isolated end-members.

Table 5.1: Unmixing results from traditional linear and generalized bilinear models with isolated red and blue ink target samples.

Ground Truth		Traditional Linear Unmixing		Generalized Bilinear Unmixing	
<u>Relative Fraction of Red Ink In Target</u>	<u>Relative Fraction of Blue Ink In Target</u>	<u>Optimal R Coefficient</u>	<u>Optimal B Coefficient</u>	<u>Optimal R Coefficient</u>	<u>Optimal B Coefficient</u>
1.0	0.0	1.0	0.0	1.0	0.0
0.0	1.0	0.0344	0.9656	0.0001	0.9999

5.3.2 Unmixing Findings

Following the validation of the unmixing process, four different mixed ink samples were prepared to be used as targets. These mixtures had the following ratios of red-to-blue ink: 5:1, 2:1, 1:2 and 1:5. Total absorption PARS and mean fluorescence emission spectra were captured for each target sample and unmixed using the signals from the isolated red and blue ink samples discussed in the previous section and the two different unmixing methods.

Once the optimal R and B coefficients were determined through the traditional linear and generalized bilinear models, the optimal total absorption PARS and average fluorescence emission spectra were reconstructed and compared to the captured ground truth. Figure 5.3 presents this reconstruction process using the 2:1 red-to-blue ink target sample as an example.

The reconstructed spectra match the ground truth collected from the target sample quite well. However, there are some observable discrepancies. For example, the secondary fluorescence peak, located around 650nm, seems to be noticeably larger in the reconstructed averaged fluorescence emission spectra than in the signal collected from the actual target sample. This may be due to small light-matter interactions between the red and blue inks, such as quenching. For example, fluorescence emitted from the red ink chromophores at this 650nm wavelength may be slightly absorbed by the blue ink chromophores in the target sample. These interactions cannot be fully accounted for by either unmixing approach, potentially leading to the observed mismatch in this peak’s intensity. The small discrepancies in total absorption PARS values between the ground truth and reconstructed spectra can also likely be attributed to these unaccounted for sample interactions as well as system noise.

In addition to performing unmixing using the combination of the total absorption spectra and the mean fluorescence emission spectra, experiments were also run to see whether the use of either data source individually yielded more accurate results. Table 5.2 summarizes the findings of this study.

In three of the four target samples, the combination of the total absorption and mean fluorescence emission spectra provided the closest to ground truth unmixing results. However, in the case of the 5:1 red-to-blue ink target sample, the predicted mixture composition is most accurate when unmixing is performed using only the mean fluorescence emission spectra. Despite this, these results strongly suggest that both data sources provide useful information to be used in unmixing tasks, as in all other sample cases, the predictions from single-signal unmixing are significantly less accurate than the dual-signal results. Furthermore, both the traditional linear and generalized bilinear unmixing approaches yield very similar predicted sample composition ratios. This indicates that the additional “interaction term” in the generalized bilinear model does not provide a significant amount of utility in the unmixing of ink samples using the collected data.

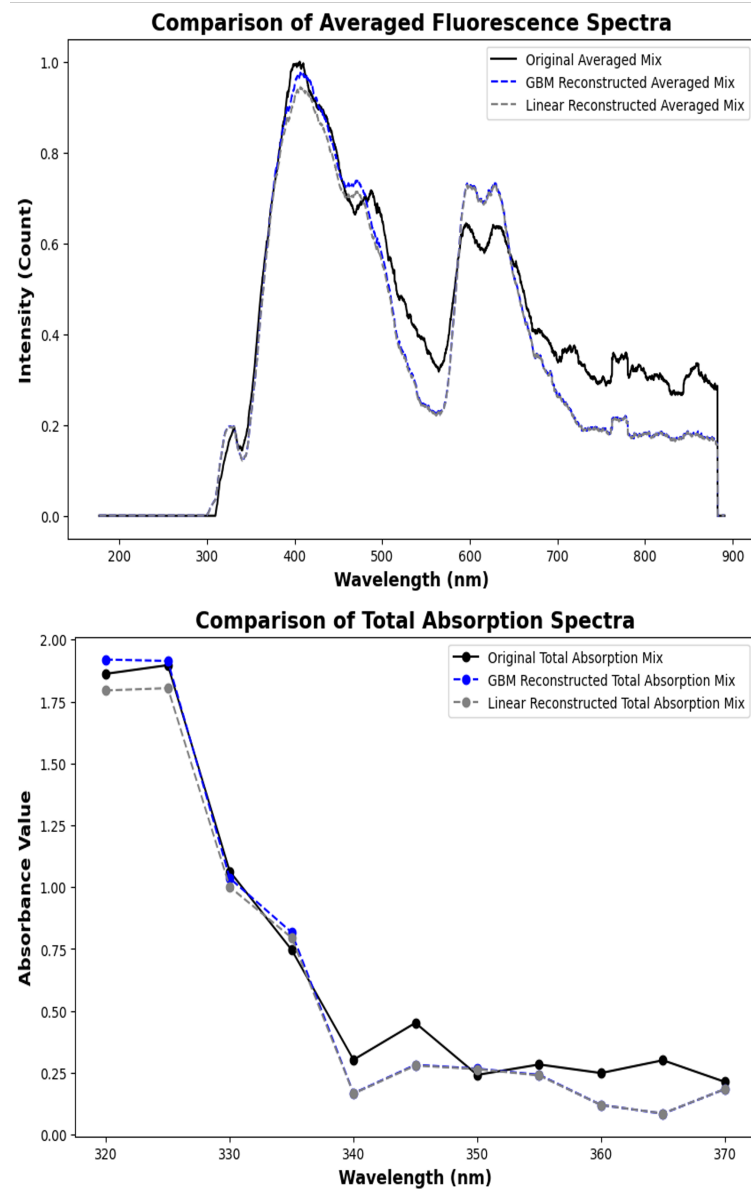


Figure 5.3: Reconstructed average fluorescence emission (top) and total absorption PARS (bottom) spectra using the optimal R and B coefficients found through a traditional linear model and a generalized bilinear model (GBM) for a target sample of a 2:1 mixture of red-to-blue ink.

Table 5.2: Comparison of unmixing results using traditional and bilinear models and different data sources. Green squares represent the approach which predicted the red-to-blue ink ratio closest to the ground truth composition of the target.

Ground Truth Ratio of Red-to-Blue Ink in Target	Traditional Linear Unmixing			Generalized Bilinear Unmixing		
	<u>Predicted R:B Ratio from Total Absorption Spectra</u>	<u>Predicted R:B Ratio from Average Fluorescence Spectra</u>	<u>Predicted R:B Ratio from Total Absorption + Average Fluorescence Spectra</u>	<u>Predicted R:B Ratio from Total Absorption Spectra</u>	<u>Predicted R:B Ratio from Average Fluorescence Spectra</u>	<u>Predicted R:B Ratio from Total Absorption + Average Fluorescence Spectra</u>
5:1	7.92:1	4.96:1	5.51:1	7.92:1	4.94:1	5.51:1
2:1	5.35:1	1.47:1	2.11:1	5.05:1	1.46:1	2.06:1
1:2	1:5.34	1:1.41	1:2.59	1:5.34	1:1.41	1:2.59
1:5	1:10.51	1:1.76	1:5.30	1:10.51	1:1.77	1:5.57

Unmixing Benchmarking

Spectral absorption data was also collected from the NanoDrop device for the isolated red and blue ink samples as well as each of the mixed target samples. This NanoDrop data was then used to unmix each of the target samples by employing the traditional linear and generalized bilinear models. More specifically, in the traditional linear unmixing case, this optimization problem can be stated as:

$$\begin{aligned} & \arg \min_{R,B} \quad \|\mathbf{N}_T - (R\mathbf{N}_R + B\mathbf{N}_B)\|^2 \\ & \text{subject to: } R > 0, \quad B > 0, \quad R + B = 1 \end{aligned} \quad (5.7)$$

and in the generalized bilinear unmixing case, the problem can be formulated as:

$$\begin{aligned} & \arg \min_{R,B,\gamma} \quad \|\mathbf{N}_T - (R\mathbf{N}_R + B\mathbf{N}_B + \gamma RB \mathbf{N}_R \odot \mathbf{N}_B)\|^2 \\ & \text{subject to: } R > 0, \quad B > 0, \quad 0 < \gamma < 1, \quad R + B = 1 \end{aligned} \quad (5.8)$$

where:

$$\begin{aligned} \mathbf{N}_T &= \begin{bmatrix} \text{NanoDrop Absorbance Value of Target Ink Sample at 320 nm} \\ \text{NanoDrop Absorbance Value of Target Ink Sample at 325 nm} \\ \vdots \\ \text{NanoDrop Absorbance Value of Target Ink Sample at 370 nm} \end{bmatrix} \\ \mathbf{N}_R &= \begin{bmatrix} \text{NanoDrop Absorbance Value of Red Ink Sample at 320 nm} \\ \text{NanoDrop Absorbance Value of Red Ink Sample at 325 nm} \\ \vdots \\ \text{NanoDrop Absorbance Value of Red Ink Sample at 370 nm} \end{bmatrix} \\ \mathbf{N}_B &= \begin{bmatrix} \text{NanoDrop Absorbance Value of Blue Ink Sample at 320 nm} \\ \text{NanoDrop Absorbance Value of Blue Ink Sample at 325 nm} \\ \vdots \\ \text{NanoDrop Absorbance Value of Blue Ink Sample at 370 nm} \end{bmatrix} \end{aligned}$$

The composition ratios predicted from using the data collected from the PARS spectroscopy system were benchmarked against the ratios predicted from using the NanoDrop data, as seen in Table 5.3. These results indicate that for every target sample, the combination of the total absorption PARS spectra and the mean fluorescence emission spectra allows for a more accurate identification of mixture components than the single-signal purely absorption-based data provided by the NanoDrop device.

Table 5.3: Unmixing results from using total absorption PARS and mean fluorescence spectra compared to the unmixing results from using the NanoDrop absorption data. Both traditional linear and generalized bilinear unmixing techniques were explored. Green squares represent the unmixing approach which predicted the red-to-blue ink ratio which was closest to the ground truth composition of the target.

Ground Truth Ratio of Red-to-Blue Ink In Target	Traditional Linear Unmixing		Generalized Bilinear Unmixing	
	<u>Predicted R:B Ratio from Total Absorption + Average Fluorescence Spectra</u>	<u>Predicted R:B Ratio from NanoDrop Spectra</u>	<u>Predicted R:B Ratio from Total Absorption + Average Fluorescence Spectra</u>	<u>Predicted R:B Ratio from NanoDrop Spectra</u>
5:1	5.51:1	3.16:1	5.51:1	3.24:1
2:1	2.11:1	2.42:1	2.06:1	1.12:1
1:2	1:2.59	1:3.17	1:2.59	1:3.02
1:5	1:5.30	1:5.88	1:5.57	1:5.41

5.4 Limitations

The primary limitation of this chapter lies in the unmixing approaches used in this work. These techniques rely on least-squares methods to find the optimal combination of the signals from the end-members to best approximate the target spectra. While these methods worked well in this proof-of-concept study, as minimal chemical interactions are expected

to occur when craft inks are combined, they would potentially struggle in the application of unmixing biomolecules. In this case, chemical interactions between molecules may result in the absorption and fluorescence emission spectra of the mixture not being adequately approximated using the spectra from the isolated end-members and the currently employed unmixing techniques.

Furthermore, this work relied on scaling the absorption and fluorescence emission data to fall between 0.0 and 1.0. This was done to try and minimize the impacts of small sample interactions in the target solutions, such as quenching. Although this approach yielded strong results in this work, it does make the unmixing process reliant on using relative spectral features, such as the locations of absorption and fluorescence peaks, as opposed to their absolute values.

5.5 Conclusions

This chapter described the application of the fully developed total absorption PARS spectroscopy system to predict the make-up of mixed ink solutions using traditional linear and generalized bilinear models. The data used for performing these unmixing tasks was the total absorption PARS and mean fluorescence emission spectra for the isolated red, blue and target ink samples. The results suggest that the combination of the two spectra allows for more accurate unmixing predictions than the use of either data source individually. Furthermore, the predicted ratios of red-to-blue ink in the target solutions using the total absorption PARS and mean fluorescence emission data were consistently closer to ground truth than the predicted ratios from using the absorption data from the NanoDrop device. These results indicate that the combination of total absorption PARS and fluorescence emission data provides an improved level of detail in sample characterization compared to a single-signal approach.

This chapter serves as a proof-of-concept study for employing the developed PARS spectroscopy system to unmix different solutions, providing motivation for leveraging this system to unmix biomolecules in the future.

Chapter 6

Conclusion and Future Work

6.1 Conclusion

In summary, this work presented the first application of PARS in the field of optical spectroscopy and achieved its overarching objective of demonstrating the feasibility of this technology for characterizing a variety of liquid samples. The developed system simultaneously captures the non-radiative and radiative relaxation processes that arise following sample excitation from different wavelengths in the range of 310nm to 370nm. This work also showed the capability of using these signals to study the absorption profiles of a wide range of liquid samples. By capturing both relaxations, the dual-signal nature of the PARS spectroscopy system broadens the range of samples whose absorption spectra can be analyzed compared other pump-probe spectroscopy configurations, such as photothermal and photoacoustic spectroscopy, which specifically rely on probing relaxations from non-radiative pathways. Furthermore, through the use of a spectrometer device, this system effectively combines absorption and fluorescence emission spectroscopy. This work also demonstrated that the combination of the fluorescence and absorption spectra is more effective in accurately unmixing liquid ink samples than either data source is individually. This ultimately suggests that the PARS spectroscopy system offers an improved level of sample characterization compared to single-signal spectroscopy systems such as the NanoDrop.

This research lays the groundwork towards developing a fully robust PARS spectroscopy system. The steps described in the next section serve as suggestions for future research which can be done to eventually see a PARS spectroscopy system used in clinical settings.

6.2 Future Work

The relevant limitations of this work were presented at the end of each chapter; however, this section aims to outline additional steps that can be taken to continue to push this project forward.

6.2.1 Improved System Performance Benchmarking

In this work, the performance of the PARS spectroscopy system in generating accurate absorption spectra was exclusively benchmarked against a NanoDrop device. As mentioned throughout this thesis, a key limitation of the NanoDrop is its inability to distinguish between scattering and absorption, making the absorption spectra generated by this device not fully accurate. While this work used the NanoDrop spectra as ground truth, due to the convenience of the device and its ability to generate spectra very quickly, an appropriate future step for this research would be to use a more sophisticated tool for benchmarking. For example, an integrating sphere coupled with a high-resolution spectrophotometer may be able to better account for scattering events. This would provide a more targeted reading of sample absorption and a more accurate ground truth to benchmark the derived PARS spectra against.

6.2.2 Exploration of Different Excitation Wavelengths

Although this work employed many different excitation wavelengths, the explored range exclusively fell within the UV region. As such, a relevant future step for this project would be to adapt the proposed PARS spectroscopy system to be able to explore sample interactions with excitation wavelengths in other regions of the electromagnetic spectrum, such as near-infrared.

In order to accomplish this, many of the optics in the system would need to be switched out, as the currently used components, such as the harmonic beam-splitter, mirrors and filters, were specifically chosen to allow for UV excitation in the system. Once a new desired excitation wavelength range is determined, appropriate component replacements can be found through suppliers such as Thorlabs or, potentially, the development of custom-built optics.

6.2.3 Inclusion of Scattering Contrasts

Another appropriate next step for this work would be the inclusion of excitation and detection scattering contrasts. Scattering from the excitation source is already captured in the raw radiative PARS signals; however, this work removed these effects to isolate the recorded fluorescence. Future work could explore the utility of including a channel that isolates the excitation scattering events, as quantifying the amount of scatter which occurs at every excitation wavelength may be useful information in further characterizing a sample.

Similarly, a detection scattering contrast could also be included. While this could not be measured using the spectrometer, because the wavelength of the current detection source falls outside of the measurable range of the device, a secondary photodetector could be placed beside the sample to collect scattered detection photons.

6.2.4 Studying Different Sample Types

The current work aimed exclusively at studying liquid samples; however, this research could be extended to study other types of samples as well, such as thin tissue sections. To accomplish this, parafin-embedded slides could be placed on a stage along the combined detection and excitation path, replacing the use of cuvettes in the current system. A benefit of working with liquid samples is that they are homogeneous, so the location of the beams on the sample is irrelevant. With tissue sections, which are non-homogeneous, the sample can be shifted horizontally and vertically using a stage controller to study the optical response of different sample areas, which can reveal physiologically useful information.

6.2.5 Developing a Robust Unmixing Algorithm

The final proposed next step revolves around improving the unmixing procedure presented in this work. The two unmixing models presented rely on the use of linear and bilinear combinations of captured spectra from isolated samples to predict the abundance ratios in a mixed solution. While these approaches performed well in identifying the make-up of mixed craft ink solutions, they may struggle in adequately unmixing biomolecules. When biomolecules are combined, chemical interactions between chromophores might occur that significantly change the absorption and fluorescence properties of the mixture solution. Here, the mixture's spectra could no longer be reasonably approximated through linear or bilinear combinations of the isolated end-members' spectra. As such, a feasible future

direction of this research could revolve around the application of different machine learning models to capture these potential sample interactions and allow for the accurate unmixing of biomolecular samples.

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Glossary of Key Terms

Non-Radiative Relaxations Processes in which an excited molecule releases absorbed energy without emitting a photon. Non-radiative relaxations consist of thermal and pressure/acoustic relaxations.

Radiative Relaxations Processes in which an excited molecule releases absorbed energy through the emission of photons.

Photon Absorption Remote Sensing (PARS) An optical technique which is capable of simultaneously capturing non-radiative and radiative relaxation processes.

Photothermal Signal A signal that captures the thermal relaxation processes which occur in a sample following excitation. In this work, these signals are derived by monitoring the evolution of the thermally induced refractive index changes in a sample with respect to time.

Photoacoustic Signal A signal that captures the pressure/acoustic effects in a sample following excitation. In this work, these signals are derived by monitoring the evolution of the pressure induced refractive index changes in a sample with respect to time.

Non-Radiative PARS Signal A signal that captures all measurable non-radiative relaxation processes.

Non-Radiative PARS Absorbance Value A value which is derived from the collected non-radiative PARS signal and represents the magnitude of non-radiative relaxation which occurred in a sample following an absorption event.

Radiative PARS Signal A spectral breakdown of captured photons from an excited sample. This signal is the combination of fluorescence and scattering events.

Fluorescence Emission Signal A radiative PARS signal in which scattering events have been minimized, leaving only the spectral breakdown of fluorescence emissions from a sample. This signal is representative of a sample's radiative relaxations. This is also referred to as a "processed radiative PARS signal".

Radiative PARS Absorbance Value A value which is derived from the fluorescence emission signal and represents the magnitude of radiative relaxation which occurred in a sample following an absorption event.

Total Absorption PARS The combination of the insight from the collected non-radiative PARS and processed radiative PARS signals. Total absorption consists of relaxation information from both pathways and provides a holistic outlook on an absorption event.

Unmix To determine the proportions of different isolated solutions present in a mixture.

Target Solution The mixture containing multiple solutions whose make-up is to be unmixed.

End-Member Each isolated, individual component present in the target solution.