Phasor analysis for nonlinear pump-probe microscopy

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Abstract: Pump-probe microscopy provides molecular information by probing transient, excited state dynamic properties of pigmented samples. Analysis of the transient response is typically conducted using principal component analysis or multi-exponential fitting, however these methods are not always practical or feasible. Here, we show an adaptation of phasor analysis to provide an intuitive, robust, and efficient method for analyzing and displaying pump-probe images, thereby alleviating some of the challenges associated with differentiating multiple pigments. A theoretical treatment is given to understand how the complex transient signals map onto the phasor plot. Analyses of cutaneous and ocular pigmented tissue samples, as well as historical pigments in art demonstrate the utility of this approach.

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1. Introduction

Nonlinear pump-probe microscopy (also known as transient absorption microscopy) is an emerging technique that achieves high molecular specificity of absorptive pigments [1,2]. To date, pump-probe imaging has been applied to identify oxy- and deoxy-hemoglobin [3,4], eumelanin and pheomelanin in pigmented lesions (such as melanoma) both in biopsies [5] and in vivo [6], pigments in historical artworks [7], nanostructures [8], and graphene [9]. Pump-probe microscopy signals (acquired as a function of the time delay between the pump and probe pulses) display positive and negative (i.e., bipolar) multi-exponential dynamics resulting from a broad range of physical mechanisms. This rich structure provides the sensitive molecular specificity of the method.

The primary challenges in pump-probe microscopy are to distinguish between multiple pigments with as few time-delay data points as possible to increase imaging speeds, and to do so without a priori information. Typically, signals are processed using principal component analysis (PCA) [5], however this processing method is inadequate when more than two pigments with non-orthogonal signatures are involved. Somewhat analogous challenges are encountered in fluorescence lifetime imaging microscopy (FLIM) [10], which contains signals with positive (i.e., unipolar) multi-exponential dynamics. In FLIM, these challenges have been addressed by using multi-exponential fitting; but this requires high signal to noise ratios for reliable separation of components [11], and a priori knowledge of the number of exponentials. Unfortunately, these requirements are often not met in pump-probe microscopy, and thus multi-exponential fitting is not always a feasible solution.

A remarkably simple, but powerful approach gaining popularity in FLIM is phasor analysis, which does not make any assumptions regarding the physical model (for example, number of exponentials), and does not involve fitting to determine the lifetimes of multi-exponential signals [10,12,13]. Here, we adapt phasor analysis to pump-probe imaging, characterizing the approach with respect to the unique concerns of pump-probe microscopy, and show that it provides a robust and convenient method to alleviate the challenges associated with differentiating multiple pigments. We demonstrate applications to pigment analysis of melanoma biopsies and historical artwork.

2. Experimental system and methods

To understand the origin of bipolar signals in pump-probe microscopy, we first give a brief overview of the experimental system and methods (illustrated in Fig. 1). A more detailed description may be found in Refs [1,3,5,6]. In short, the output of a mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics, 80 MHz), tuned to 810 nm, is split into two beams: the first beam pumps an optical parametric oscillator (Mira OPO, Coherent) and the second serves as the probe pulse. The output of the OPO, the pump beam, is tuned to 720 nm and modulated at 2 MHz with an acousto-optic modulator. Then, the modulated pump and unmodulated probe pulses are collinearly sent into a custom-built, laser-scanning microscope. Nonlinear interactions between the pump and the probe beams in the sample transfers the modulation onto the probe beam that is then detected using a photodiode and a lock-in amplifier. A variable time delay, t, between the pump and the probe beams allows detection of the transient absorption properties of samples. By convention, absorptive interactions, such as two-photon absorption (TPA) and excited state absorption (ESA), are considered positive signals; whereas gain in the probe, resulting from interactions like stimulated emission (SE),
3. Theory

In phasor analysis, signals are decomposed into two components, $g$ and $s$, that are related to the real and imaginary parts of the signals’ Fourier transform at a given frequency (see Eq. (1)). The components are then plotted against one another—this plot is known as the phasor plot. When the analysis is applied to signals consisting of unipolar multi-exponential dynamics, as is the case in FLIM, the resulting phasors are necessarily bounded by the universal semicircle (dashed black line in Fig. 2), defined as a semicircle in the first quadrant with radius 0.5, starting from the origin [10]. In pump probe imaging, however, the signals are a result of various physical processes that exhibit more complex transient behavior. As mentioned in section 2, these processes include instantaneous effects such as TPA and SRS, and time-delayed effects such as GSD, ESA, and SE which cause signals to be positive and/or negative. In turn, the resulting curves are mapped onto the phasor plot with bounds dictated by the unit circle, rather than by the more restrictive universal semicircle.

To understand how a bipolar transient signal, $I(t)$, maps onto the phasor plot, consider the components $g(\omega)$ (x-axis) and $s(\omega)$ (y-axis),

$$g(\omega) = \frac{\int I(t) \cos(\omega t) dt}{\int |I(t)| dt} \quad (1a)$$

$$s(\omega) = \frac{\int I(t) \sin(\omega t) dt}{\int |I(t)| dt}. \quad (1b)$$

Note that $I(t)$ is effectively zero for long negative times (i.e., when the pump has not yet overlapped with the probe). Equations (1a) and (1b) are an alternative definition to what is typically used (see [10,12,13]), where we add the absolute value in the denominator to avoid singularities in the event that the area under the curve of $I(t)$ is zero for bipolar signals. For near-zero (low intensity) signals thresholding or intensity weighting is used to suppress pixels without information content. Further, the frequency $\omega$ is a free parameter that may be tuned to optimize the analysis of a particular set of signals [10]. When $I(t)$ decays exponentially with a single lifetime, $\tau$, the components $g(\omega)$ and $s(\omega)$ yield $g(\omega) = (1 + (\omega \tau)^2)^{-1}$ and $s(\omega) = \omega \tau \cdot g(\omega)$, which define the universal semicircle [10]; however, for arbitrary functions, such as those resulting from pump-probe microscopy, $\{g(\omega), s(\omega)\}$ may lie anywhere within the unit circle. Figure 2 illustrates this by simulating typical pump-probe signals along with their corresponding phasors at different frequencies (experimental traces and corresponding phasors are illustrated and discussed later in section 4). Line 1 (blue), an even function, represents an instantaneous response such as TPA, and thus the phasors as a function of
frequency map onto a straight line along the x-axis. On the other hand, phasors of odd functions, such as line 2 (green) that may result from cross phase modulation (XPM) [14], map to a line along the y-axis (i.e., rotated 90 degrees), which clearly fall outside of the universal semicircle. Line 3 (yellow) decays exponentially with a negative starting point—resulting from, for example, SRS or GSD—and the phasors trace a semicircle along the fourth quadrant. Line 4 (red) is a combination of lines 1-3, and it resembles the eumelanin spectrum; here, the phasors trace a more complex path given by the combination of all contributing signals, which may extend outside of the universal semicircle. Here (and for the remainder of this study, unless explicitly stated otherwise), we choose $\omega = \pi/2$ THz since this falls between the optimal frequencies for the expected decays of eumelanin and pheomelanin.

Fig. 2. (a) Simulated transient absorption spectra: Line 1 (blue) represents an instantaneous response such as TPA. Line 2 (green) may result from XPM. Line 3 (yellow) decays exponentially with a negative starting point, resulting from, SRS or GSD. Line 4 (red) is a combination of lines 1-3, and it resembles the eumelanin spectrum. (b) Corresponding phasors at different frequencies ranging from $\omega = 0.01\pi$ to $2\pi$ THz. Each point is an increment of $0.01\pi$ THz.

One of the most advantageous features of phasor analysis when applied to FLIM is the fact that samples containing a mixture of fluorophores with different lifetimes map linearly across the phasor plot, thus providing an intuitive, fit-free method for analyzing and displaying the different fluorescent species. Mathematically, any multi-component signal may be described as,$I_{tot}(t) = \sum_i f_i I_i(t)$, where $f_i$ is the fraction (or relative concentration) of each independent species contributing to the total signal, and $\sum f_i = 1$. The resulting phasor of the multi-component signal may be described in terms of its independent species by

$$g_{tot} = \sum_i f_i \int \frac{|I_i(t)|}{I_{tot}(t)} dt \cdot g_i$$

$$s_{tot} = \sum_i f_i \int \frac{|I_i(t)|}{I_{tot}(t)} dt \cdot s_i,$$

where $I_i(t)$ is the delay trace from the $i^{th}$ independent species with phasor components $\{g_i, s_i\}$. From Eqs. (2a) and (2b) it can be shown that when two species are present, both with unipolar spectra, the phasors will form a line bounded by the independent components with slope $m = (s_{tot} - s_1)/(g_{tot} - g_1) = (s_2 - s_1)/(g_2 - g_1)$; in other words, the slope between any phasors originating from the mixture of two components is independent of the total spectra, $I_{tot}$, and the relative concentration of each species, $f_i$. In more complicated cases when $n$ species are present, the phasors will span an $n$-sided polygon. For bipolar signals, this linear geometric relationship no longer holds true due to the subadditivity property of the absolute value, thus causing linear mixtures of bipolar signals to map nonlinearly onto the phasor plot. Figure 3 illustrates...
this feature by linearly mixing two species: \( I_1(t) \) and \( I_2(t) \), and then applying phasor analysis at \( \omega = \pi/2 \) THz. Using the definition given in Eqs. (1) and (2), the phasors (Fig. 3(b) in the blue dashed box) take a curved path from one independent component to the other that depends on the specific shapes of the two traces. In comparison, using the conventional definition of \( g \) and \( s \) (i.e., without the absolute value in the denominator), the phasors form a line that begins at \( \{g_2, s_2\} \) and approaches \( \{\infty, -\infty\} \); once the area under the curve changes sign, the phasors rotate 180 degrees along the line axis and finally reach \( \{g_1, s_1\} \) from \( \{-\infty, \infty\} \). While this has desirable linear properties, it is much more susceptible to noise since small errors in the spectra can lead to significant errors in \( 1/\int I_\omega(t) dt \), particularly when the denominator approaches zero. On the other hand, using the definition given here (Eq. (1)), the phasors of mixed signals are bounded by a well-defined area, with the endpoints specified by the phasor of each independent component, free of singularities.

4. Results and discussion

To illustrate the utility of phasor analysis in pump-probe microscopy, we apply the approach to cutaneous and ocular pigmented tissue samples as well as historical pigments in art. Advantages of using this approach compared to PCA and multi-exponential fitting for these applications are discussed.

4.1 Pigmented lesions

Two inherent tissue chromophores are encountered in the analysis of pigmented lesions: melanin and hemoglobin (Hb); and often, surgical ink will also be present in excised samples. Figure 4(a) shows the typical experimental delay traces of these chromophores. While these pigments have distinct transient behavior, it is sometimes difficult to fully and/or efficiently separate them for quantitative analysis. For example, if the data are analyzed using orthogonal projections, such as in PCA, some contributions from Hb will typically be projected onto eumelanin, and similarly some contributions from surgical ink will be projected onto pheomelanin. This results from the fact that only small portions of the signals are actually orthogonal to one another. In contrast, phasor analysis utilizes the whole trace to identify its position in a two-dimensional space defined by \( g \) and \( s \), and does not require the signals to be orthogonal. Further, this method offers an intuitive display of the different contributing species. Figure 4(b) shows the phasors of eumelanin, pheomelanin, hemoglobin, and surgical ink, along with different simulated mixtures of eumelanin and pheomelanin. As observed, the four independent species are well separated in the phasor plot. We also note that the phasors from a linear combination of eumelanin and pheomelanin show a slight deviation from a line due to the fact that eumelanin is a bipolar signal.

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Fig. 4. (a) Experimental transient absorption spectra of hemoglobin (Hb), sepia eumelanin, synthetic pheomelanin, and surgical ink, along with different simulated mixtures of eumelanin and pheomelanin (eumelanin fraction of 75%, 50% and 25%). (b) Corresponding phasors. Each black dot indicates a eumelanin fractional increase of 1%.

Next, we apply phasor analysis to unstained biopsy slices (5 µm thick) of excised pigmented lesions. Images of cutaneous tissue samples were acquired using the system described in section 2. Before the phasors are computed, we remove long-lived signals resulting from both photothermal effects and long-lived excited states [15]. Figure 5 shows the cumulative histogram phasor plot of 42 excised pigmented lesions, including samples from benign nevi, compound nevi, dysplastic nevi, and melanomas, as classified by an expert pathologist. To compute the histograms, only pixels with intensities one standard deviation above the mean of each image are taken into account (we do not use intensity weighted histograms for tissue samples because the strong surgical ink component obscures features of interest). The results (Fig. 5) show several important features of the sample set: First, the phasors from eumelanin and pheomelanin, and mixtures thereof, are distributed along a line that is in good agreement with the simulated results shown in Fig. 4, without a large spread or deviation. This is important since it suggests that eu- and pheo-melanin, which are paramount to the diagnosis of melanoma [5], may be isolated from other pigments by generating a single mask in the phasor plot for all samples. Secondly, surgical ink produces phasors that are much more scattered, which indicates that it does not have a consistent transient absorption spectrum, further emphasizing the difficulty in eliminating these contributions using PCA (or any projection based method). Lastly, a small number of phasors are mapped between eu-/pheo-melanin and surgical ink, resulting from partial volume effects where ink and melanin are contained within the same diffraction limited spot (~0.4 µm).

Three representative cutaneous tissue samples are given in Fig. 6, including a dysplastic nevus (a-c), a malignant melanoma (d-f), and a benign sample from a patient diagnosed with malignant melanoma (g-i). The third column of Fig. 6 displays the histogram phasor plots of each sample. Again, the phasors clearly fall within the expected region for the distribution of eu- and pheo-melanin and are in good agreement with the cumulative histogram. Note that the phasor plots also provide an intuitive form of viewing quantitative results; specifically, the plots show that out of the three representative samples, the first row (a-c) contains the most pheomelanin and the second row (d-e) contains the most eumelanin. This is in good agreement with PCA analysis (with manual removal of surgical ink) which yield 31%, 50%, and 41% fractional eumelanin for rows 1-3, respectively, and also with the diagnosis of each sample, where the eumelanin fraction may be used as a diagnostic criterion [5].
Fig. 5. Cumulative histogram phasor plot of 42 cutaneous pigmented lesions. The color scheme used for Fig. 6 is also illustrated.

Fig. 6. Pump-probe images and corresponding phasor plots for a dysplastic nevus with atypia (a-c), malignant melanoma (d-f), and a benign sample from a patient with malignant melanoma (g-i). The first column includes all data point using a color scheme computed from the phasors as shown in Fig. 5. The second column omits surgical ink by applying a phasor mask. SC: stratum corneum; BL: basal layer.
The color scheme used for the pump-probe images in the first column of Fig. 6 is illustrated in Fig. 5, where mixtures of eumelanin and pheomelanin contain a hue ranging from red to green, respectively, using RGB color mapping, whereas the surgical ink is colored in cyan. The second column of Fig. 6 shows the same results but with the contributions from surgical ink removed using a single mask on the phasor plots (cyan region in Fig. 5 is removed). This can also be done using PCA, however, this tends to omit more data owing to the fact that these signals are not inherently orthogonal, and thus manual segmentation of the surgical ink is usually preferred over PCA.

Fig. 7. Cumulative histogram phasor plot of 17 ocular melanoma samples at frequency (a) $\pi/2$ THz and (b) $1.4\pi$ THz (Media 1).

As a last demonstration of the utility of phasor analysis in pigmented lesions, we examine biopsies of ocular conjunctival pigmented lesions, including conjunctival nevi, primary acquired melanosis, and conjunctival melanomas. A total of 17 images were analyzed from 9 different cases [16]. Figure 7(a) gives the cumulative histogram phasor plot of the 17 images at frequency $\omega = \pi/2$ THz, where contributions from eumelanin, pheomelanin, surgical ink, and also hemoglobin are clearly present. In these samples the surgical ink overlaps more with pheomelanin compared to the cutaneous samples (Figs. 5-6) due to the fact that these samples are more permeable and thus ink is found in deeper sample regions [16]. In addition, different types of ink are used for these ocular melanoma samples, adding to the heterogeneity of the ink transient spectra [16].

As previously noted in the theory section (section 3, Fig. 2), $\omega$ is a free parameter that may be tuned to provide better separation between different molecular species. This feature is experimentally illustrated in Fig. 7(b), which shows the phasors of the same data at a different frequency, $\omega = 1.4\pi$ THz, where the ink contributions are better differentiated from pheomelanin. At this frequency, however, the mapping between eu- and pheo-melanin is significantly less linear and the two species are clustered closer together. In either case, hemoglobin and eumelanin are clearly differentiated. Figure 7 (Media 1) shows the phasors at multiple frequencies ranging from $0.2\pi$ THz to $1.8\pi$ THz. Here it can be seen that at low frequencies the phasors cluster near $\{g = \pm 1, s = 0\}$ and at high frequencies the phasors approach the origin. Note that the optimal frequency for a particular signal, as defined in Ref [10], is the frequency which yields the largest change in the phasor plot with respect to a small change in frequency. Thus, the frequency, $\omega$, yields an additional dimension that may be utilized to optimize different signals, and hence provide better differentiation between molecules.

### 4.2 Historical pigments in art

Characterization of historical pigments in art is central to artwork conservation; specifically, it allows researchers to identify or determine authenticity, provenance, technology of manufacturer, state of preservation, and undocumented restorations. To this end, pump-probe
microscopy has been recently applied to characterize multiple pigments of important historical value [7]. For this particular analysis it was shown that bi-exponential fitting of the transient absorption spectra provided significant differences in pigments of similar visual appearance, however this analysis is not applicable for samples containing many pigments with unknown dynamics. Phasor analysis, on the other hand, may be readily applied for this purpose. To illustrate this, Fig. 8 presents a low-grade sample of lapis lazuli (Kremer # 10500, gray-blue), a semi-precious stone of significant importance in historical paintings. The bright field image (Fig. 8(a)) shows the presence of lapis (deep blue color) along with various undefined impurities. In the pump-probe image (Fig. 8(b), of a different region) lapis is depicted in cyan and the impurities are depicted in colors ranging form red to green, and purple (as demarcated by the colored circles and colorbar embedded in Fig. 8(c)). Interestingly, the cumulative, intensity weighted histogram phasor plot of five gray-blue lapis lazuli images (Fig. 8(c)) not only shows the presence of lapis, but also suggests the presence of at least three distinct impurities (highlighted by numbers 1-3). This type of analysis is difficult (if not impossible) to achieve with PCA or multi-exponential fitting and provides a promising tool for future work in this application.

Next, we directly compare multi-exponential fitting to phasor analysis for two lapis lazuli samples of different geographic origin (Afghan pure, #10530 and Chilean sky blue, # 10562). Since these samples only exhibit unipolar signals, they provide a best-case scenario for multi-exponential analysis of pump-probe signals. First, for each sample type, the phasors from five images are computed (Fig. 9(a) and 9(c)); then the same raw data are fitted to bi-exponential curves, and the resulting lifetimes are mapped onto the phasor plot (Fig. 9(b) and 9(d)) using their analytical solution, given in section 3. Note that the bi-exponential analysis is sensitive to its input parameters, including initial guesses of the lifetimes, a temporal cutoff that ignores the instantaneous response, and an arbitrary threshold that ignores signals with low amplitude. The cumulative, intensity weighted histograms for each method show that the computed phasor, and hence lifetime, distribution is much less spread out when the data are processed with phasor analysis. This clearly demonstrates that phasor analysis yields results with significantly better precision compared to bi-exponential fitting. In addition, the computational cost is significantly lower, and does not require any input parameters, which influence the accuracy of the results. Most importantly, qualitative differences between the Afghan and Chilean samples are readily apparent in the phasor diagram, while this is only possible with bi-exponential fitting when a great deal of averaging is applied [7].

Lastly, the cumulative histogram phasor distribution of three additional historical pigments of interest, previously analyzed in Ref [7], are plotted in Fig. 10, including caput mortuum, indigo, and vermillion. Note that all pigment are in different locations and have
different distributions, further demonstrating this method’s capability to differentiate multiple pigments of interest.

![Cumulative histogram phasor plot](image)

Fig. 9. Cumulative histogram phasor plot of Afghan pure lapis lazuli (Kremer # 10530) and Chilean sky blue lapis lazuli (Kremer # 10562) directly computed from the raw data (a, c) and from the analytical solution of lifetimes computed by bi-exponential fitting (b, d). The more compact distribution in (a) and (c) demonstrate the superior accuracy of phasor analysis compared to bi-exponential fitting.

![Cumulative histogram phasor plot](image)

Fig. 10. Cumulative histogram phasor plot of other pigments of interest: (a) caput mortuum, (b) indigo, and (c) vermillion. Note that all three are in different locations and show different distributions.

5. Conclusion

In this work we have demonstrated the adaptation of phasor analysis to pump-probe microscopy. A theoretical analysis reveals that the complex transient response of pump-probe signals are mapped onto the phasor plot with boundaries dictated by the unit circle rather than the more restrictive universal semicircle. Further, linear mixtures of independent molecular species do not necessarily map linearly onto the phasor plot, resulting from the alternate definition of the phasor plot components, $g$ and $s$ (given in Eq. (1)), which contains the absolute value in the denominator. However, this definition avoids singularities and unbound
results, with endpoints of mixtures given by the phasor of each independent component. Examination of cutaneous and ocular pigmented tissue samples indicate that the phasors from eumelanin and pheomelanin, and mixtures thereof, are contained within a well defined region, and are separated from ink and hemoglobin. This has important implications for efficient and accurate analysis of the diagnostically relevant melanin and hemoglobin species. Further, the frequency used to compute the phasor components provides an additional dimension that may be utilized to better differentiate between different molecular species. Finally, analysis of historical pigments in art shows the ability to identify different pigments with superior precision compared to multi-exponential fitting. The unique ability of phasor analysis to reveal unknown species, without a priori information, is an important feature for future studies in biomedical imaging and artwork conservation. We expect that phasor analysis will become an important tool for non-linear pump-probe microscopy.

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