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The Transition Dipole Moment Representation and Spectral Phasors

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ABSTRACT

Spectral phasors are currently based on fluorescence spectra in the wavelength domain. However, this is a problem when using spectral phasors to quantify emitters since wavelength represented spectra are not proportional to emitter population. Here we show the effect of unmixing using spectra in their transition dipole moment representation for a theoretical two component system and on a real seven component system.

Currently spectral phasors, are processed in the wavelength domain.[1,2] Spectral phasors are a sine and cosine transform based analytic method that can be used to quantify contributions to mixed spectra. Spectral phasors are defined by their *G* and *S* coordinates, the complete form of which has been addressed in detail elsewhere.[1,2] Spectral phasors are a useful unmixing tool because phasors are bound by linear combinations such that:

$$\begin{aligned} G_i &= aG_a + bG_b \\ S_i &= aS_a + bS_b \end{aligned} \tag{1}$$

Where the phasor (G_i, S_i) is a combination of the phasors (G_a, S_a) and (G_b, S_b) with percent contributions of *a* and *b* respectively.

While phasors are a useful unmixing tool, their ability to solve for a quantitative representation of emitters has room for improvement. While fluorescence is traditionally plotted as intensity with respect to wavelength this representation, referred to here as a wavelength representation, is not proportional to the number of emitters in a system and as such can not be accurately used to quantify emitters. However, wavelength represented spectra can be easily converted to the transition dipole moment representation which is proportional to the number of emitters in a system.[3,4] First, one must recognize that the upon conversion to wavenumbers, the spectrum is no longer sampled in equal intervals. This can be corrected by multiplying the spectrum in the wavelength domain by the wavelength squared $F(\tilde{\nu}) \propto \lambda^2 F(\lambda)$.[4] Second, to obtain the transition dipole moment represented spectra the fluorescence intensity should then be divided by a cubic frequency factor $F(\tilde{\nu}) / \tilde{\nu}^3$.[3,4] Here we show that the difference between the transition dipole moment and wavelength representations causes a significant difference in the unmixed percent contributions returned by spectral phasors. Although the traditional equations to transform spectra into *G* and *S* coordinates are based in the wavelength domain, they can easily be converted to a wavenumber domain by substituting the corresponding wavenumber for every wavelength in the equation.

We start our analysis with two area normalized spectra defined in the transition dipole moment representation, a red spectrum centered at 2.5×10^4 cm^{-1} (400 nm) and a blue spectrum centered at 1.8×10^4 cm^{-1} (570 nm) shown in Fig 1(a). These two spectra were combined in increments of 20%. The two pure spectra and four red-blue mixed spectra were converted into spectral phasors in a wavenumber domain. The six transition dipole moment represented spectra were converted into their wavelength representations and then converted into wavelength domain spectral phasors. These spectra and phasors are shown in Fig 1. The percent compositions resulting from using eq 1 to solve both the wavenumber and wavelength domain phasors are shown in Table 1.

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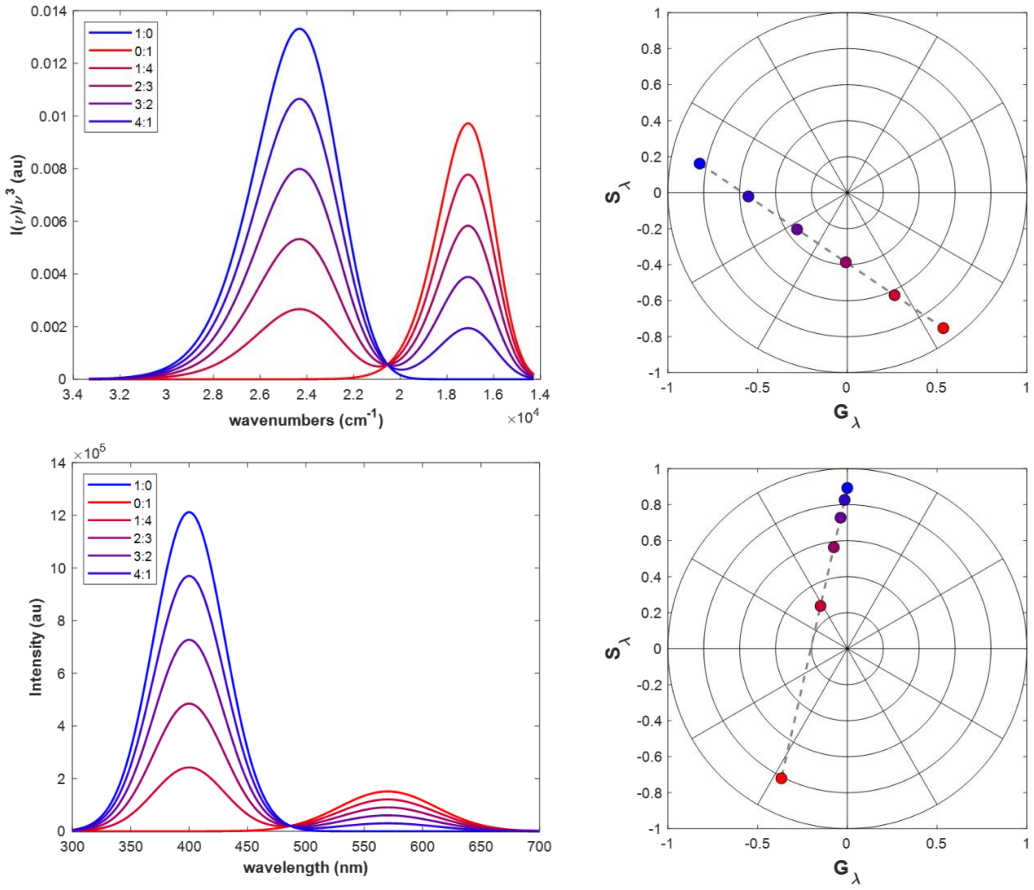

Figure 1. The mixed red-blue spectra and their corresponding phasors for the **a)** transition dipole moment representation spectra **b)** wavenumber domain phasors **c)** wavelength representation spectra **d)** wavelength domain phasors

The results in Fig 1 and Table 1 clearly show that spectral phasors in wavelength space are not proportional to emitter population, a necessary tenant if spectral phasors are being used for quantitative analysis. In the case of the mixed test spectra, wavelength domain spectral phasors overestimate the percent composition of the blue spectrum, and subsequently underestimate the precent composition of the red spectrum, by nearly 40%.

Table 1. Red and blue percent compositions returned by spectral phasor unmixing for both the transition dipole moment and wavelength represented spectra

Wavenumbers Domain		**Wavelength Domain**	
% Blue	*% Red*	*% Blue*	*% Red*
20	80	59	41
40	60	80	20
60	40	90	10
80	20	96	4

Recently super-phasor unmixing, a derivative of spectral phasors, was used to quantitatively analyze the composition of a mouse retina by retinal layer[5]. This analysis identified seven contributors: third harmonic generation (THG), second harmonic generation (SHG), elastin, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), melanin, and di-retinoid-pyridinium-ethanolamine (A2E) across the eleven retinal layers: nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner receptor layer (IRL), outer receptor layer (ORL), retinal pigment epithelium (RPE), choroid and sclera. The results of this analysis are summarized in Figure 2.

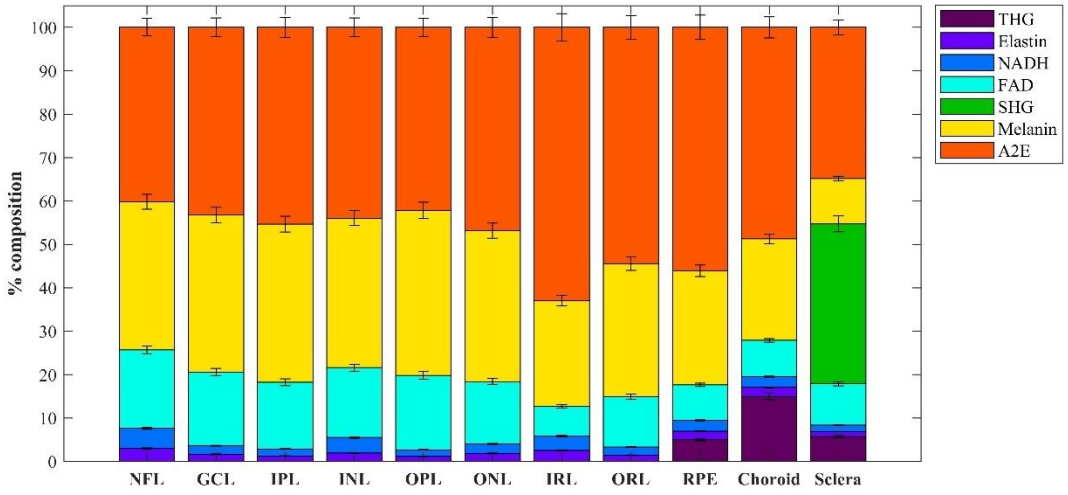

Figure 2. Quantitative analysis of a retina done by super-phasor unmixing in the wavelength domain. Reproduced from Ref 5 with permission

Figure 3 shows the super-phasor unmixing of the same data after it was converted into its transition dipole moment representation.

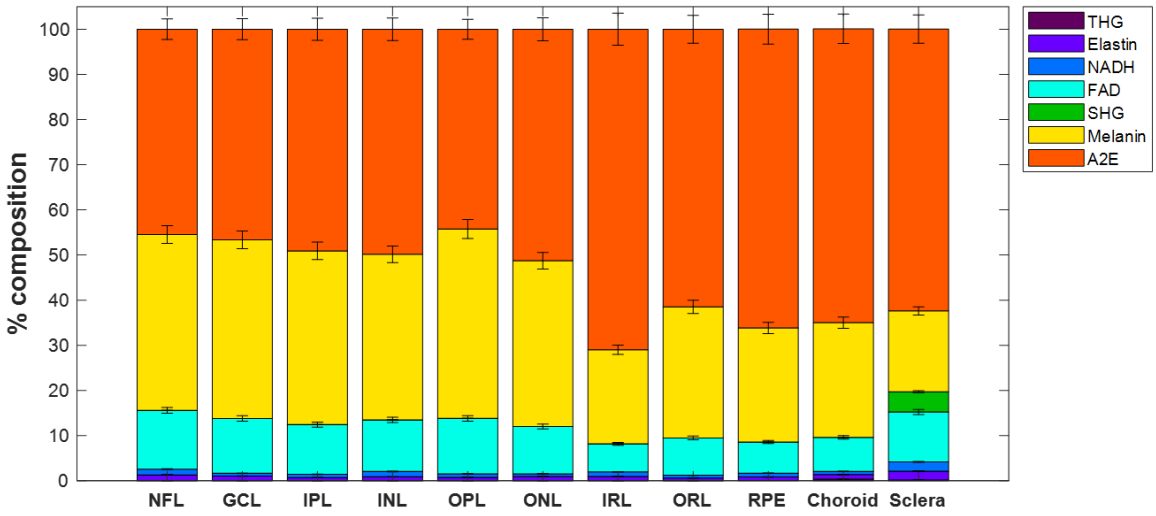

Figure 3. Super phasor unmixed retina composition data in its transition dipole moment representation

While the same general trends hold between the unmixed wavelength representation data and the unmixed transition dipole moment representation data, there are a few notable differences. The percent contribution of the spectra attributed to elastin, NADH and THG decrease by 1-2%, 1-3% and 5-15% respectively in the transition dipole moment representation. These decreases mean that when combined elastin, NADH and THG on average make up 2% of a retinal layer spectrum. This is not to say that elastin, NADH and lipid layers (THG is caused by a change in the refractive index, which in the retina indicates the presence of a lipid layer) only make up 2% of the retina combined. The percent composition returned by phasor unmixing is not an absolute concentration. Factors such as fluorescence quantum yield, two and three photon cross sections and second and third order susceptibilities must be taken into account after phasor unmixing to obtain absolute concentrations. The other notable differences include an 8-28% increase in A2E in the IRL-sclera, a 7-10% increase in melanin in the NFL-ONL and a 32% decrease in SHG in the sclera.

In conclusion, the correction from wavelength based spectral phasors to transition dipole moment representation based spectral phasors is key to the quantitative unmixing of spectra by phasors. In a simple two component system, wavelength based spectral phasors unmix percent compositions that differ from the real percent composition by forty percent. When the transition dipole moment representation is applied to data previously unmixed by super-phasors, there is a drastic difference in the percent composition attributed to fluorophores at short wavelengths and a significant increase in the percent composition attributed to fluorophores at longer wavelengths. These differences average near 20%, which is of a range than cannot be attributed to spectral noise-based error in the unmixing. Without correcting the spectra to their transition dipole moment representation, phasors will overestimate the contributions of short wavelength contributors and underestimate the contributions of longer wavelength contributors.

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