Intrinsic Nonlinear Microscopy: From Neuronal Firing to Historical Artwork

by

Prathyush Samineni

Department of Chemistry
Duke University

Date:_______________________
Approved:

___________________________
Warren S. Warren, Supervisor

___________________________
Jie Liu

___________________________
Martin C. Fischer

___________________________
John D. Simon

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

2012
ABSTRACT

Intrinsic Nonlinear Microscopy: From Neuronal Firing to Historical Artwork

by

Prathyush Samineni

Department of Chemistry
Duke University

Date:____________________________
Approved:

___________________________
Warren S. Warren, Supervisor

___________________________
Jie Liu

___________________________
Martin C. Fischer

___________________________
John D. Simon

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

2012
Abstract

Imaging based on nonlinear processes takes advantage of the localized excitation to achieve high spatial resolution, optical sectioning, and deeper penetration in highly scattering media. However, the use of nonlinear contrast for imaging has conventionally been limited to processes that create light of wavelengths that are different from the wavelengths used for excitation. Intrinsic nonlinear contrasts that do not generate light at distinct wavelengths are generally difficult to measure because of the overwhelming background from the excitation light. This dissertation focuses on extension of nonlinear microscopy to these new intrinsic processes by using femtosecond pulse shaping to encode the nonlinear information as new frequency components in the spectrum. We will present a pump-probe microscopy technique based on pulse train shaping technology to sensitively access nonlinear transient absorption or gain processes. This technique has recently been used to uniquely identify a variety of biological pigments with high spatial resolution. Here, we extend this technique to image and characterize several inorganic and organic pigments used in historical artwork. We also present a spectral reshaping technique based on individual femtosecond pulse shaping to sensitively access nonlinear refractive contrasts in scattering media. We will describe an extension of this technique to utilize two distinct wavelengths and discuss its application in biological imaging. This two-color implementation would allow the extension of widely employed phase contrast to the nonlinear regime.
# Contents

Abstract............................................................................................................................................. iv

List of Tables ......................................................................................................................................... ix

List of Figures ........................................................................................................................................ x

Acknowledgements ............................................................................................................................... xviii

1. Introduction to nonlinear microscopy .............................................................................................. 1

1.1. Linear optical microscopy techniques ........................................................................................ 1

1.2. Conventional multiphoton microscopy techniques ................................................................. 4

1.3. Novel intrinsic nonlinear microscopy contrasts ........................................................................... 7

1.3.1. Nonlinear absorption ............................................................................................................. 8

1.3.2. Self-phase modulation ........................................................................................................... 10

1.3.2.1. Z-scan technique ............................................................................................................ 11

1.3.3. Cross-phase modulation ....................................................................................................... 15

2. Spectral reshaping technique ........................................................................................................... 23

2.1. Frequency domain encoding ....................................................................................................... 23

2.2. Experimental setup ...................................................................................................................... 26

2.3. Comparison with Z-scan in scattering media ............................................................................ 30

2.3.1. Accuracy ............................................................................................................................... 34

2.3.2. Reliability ............................................................................................................................. 37

2.4. Mode-locked pulse shaper ........................................................................................................... 40

2.4.1. 4-f acousto-optic pulse shaper ............................................................................................. 40
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.2. Interferometric pulse shaper</td>
<td>43</td>
</tr>
<tr>
<td>3. Functional neuronal imaging</td>
<td>47</td>
</tr>
<tr>
<td>3.1. Introduction to neuronal imaging</td>
<td>47</td>
</tr>
<tr>
<td>3.1.1. Overview of current optical techniques</td>
<td>48</td>
</tr>
<tr>
<td>3.1.2. Neurobiology</td>
<td>50</td>
</tr>
<tr>
<td>3.1.2.1. Neuron</td>
<td>51</td>
</tr>
<tr>
<td>3.1.2.2. Hippocampal slice</td>
<td>52</td>
</tr>
<tr>
<td>3.2. Experimental design</td>
<td>53</td>
</tr>
<tr>
<td>3.3. Chemically activated neuronal signatures</td>
<td>58</td>
</tr>
<tr>
<td>3.3.1. Single neuron signatures</td>
<td>58</td>
</tr>
<tr>
<td>3.3.2. 2D self-phase modulation signatures</td>
<td>62</td>
</tr>
<tr>
<td>3.4. Patch clamp measurements</td>
<td>63</td>
</tr>
<tr>
<td>3.4.1. Single cell clamp technique</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2. Simultaneous single cell self-phase modulation and patch clamp measurements</td>
<td>68</td>
</tr>
<tr>
<td>4. Cross-phase modulation</td>
<td>73</td>
</tr>
<tr>
<td>4.1. Motivation</td>
<td>73</td>
</tr>
<tr>
<td>4.2. Two color spectral reshaping technique</td>
<td>74</td>
</tr>
<tr>
<td>4.2.1. Simulation of lock-in signal</td>
<td>79</td>
</tr>
<tr>
<td>4.3. Comparison with self-phase modulation</td>
<td>81</td>
</tr>
<tr>
<td>4.3.1. Experimental setup</td>
<td>81</td>
</tr>
<tr>
<td>4.3.2. Signal to background comparison</td>
<td>83</td>
</tr>
</tbody>
</table>
4.4. Nonlinear phase contrast measurements using mode-locked laser systems ........ 85
  4.4.1. Experimental setup ........................................................................... 85
  4.4.2. Design of two-color laser scanning microscope .................................... 87
  4.4.3. Detection and acquisition ................................................................... 90
  4.4.4. Noise and background scaling with pump and probe powers ................. 91
  4.4.5. Cuvette demonstration ......................................................................... 93
  4.4.6. Cell imaging ......................................................................................... 96
4.5. Spectral shifting implementation of cross-phase modulation ....................... 97
  4.5.1. Cuvette demonstration ......................................................................... 99
  4.5.2. Imaging skin biopsy slides .................................................................. 101
5. Pump-probe imaging of historical pigments ............................................... 105
  5.1. Introduction to characterization of pigments ............................................ 105
    5.1.1. Overview of conventional techniques .................................................. 106
    5.1.2. Overview of linear optical techniques .................................................. 109
    5.1.3. Overview of nonlinear techniques ....................................................... 113
  5.2. Pump-probe microscopy .......................................................................... 114
    5.2.1. Two-color modulation transfer technique ............................................ 114
    5.2.2. Experimental setup ............................................................................ 118
    5.2.3. Image acquisition .............................................................................. 122
    5.2.4. Data analysis ..................................................................................... 124
  5.3. Characterization of lapis lazuli ............................................................... 126
    5.3.1. Comparison of natural and synthetic ultramarine ................................. 129
5.3.2. Power scaling of the pump-probe signal in lapis lazuli ........................................ 132
5.3.3. Lapis lazuli in different binders ........................................................................ 134
5.3.4. Heterogeneity in the pump-probe signature ......................................................... 135
5.3.5. Various grades of lapis lazuli .............................................................................. 138
5.3.6. Wavelength dependence of pump-probe signal in lapis lazuli ......................... 142
5.3.7. Three dimensional imaging of lapis lazuli ......................................................... 143
5.4. Other blue pigments ............................................................................................... 145
5.4.1. Azurite ................................................................................................................ 145
5.4.2. Indigo .................................................................................................................. 146
5.5. Pigments of other colors ....................................................................................... 149
5.5.1. Vermilion .......................................................................................................... 149
5.5.2. Ochres ............................................................................................................... 151
5.6. Paint cross-section imaging .................................................................................. 154
5.7. Multi-layer imaging ............................................................................................... 159

References ...................................................................................................................... 162

Biography ....................................................................................................................... 180
**List of Tables**

Table 5.1: Parameters for bi-exponential fits of decay traces of natural and synthetic ultramarine. ........................................................................................................................................... 131

Table 5.2: Parameters of bi-exponential fits of lapis lazuli in casein and lime wash binders. ........................................................................................................................................... 134

Table 5.3: Heterogeneity in fit parameters of the pump-probe signature of lapis.......... 135

Table 5.4: List of bi-exponential fit parameters of the pump-probe signature for seven varieties of lapis lazuli. ........................................................................................................................................... 141

Table 5.5: List of bi-exponential fit parameters of the pump-probe signature of lapis at different pump wavelengths........................................................................................................................................... 143

Table 5.6: Heterogeneity in bi-exponential fit parameters of the pump-probe signature of lapis in the paint chip cross-section sample........................................................................................................................................... 157

Table 5.7: Set of parameters where only the corresponding pigment exhibits pump-probe response. ........................................................................................................................................... 160
List of Figures

Figure 1.1: Beam path for confocal laser scanning microscopy showing the rejection of out of focus rays using a pinhole before the detector (adapted from [15])..........................2

Figure 1.2: Comparison of single photon fluorescence (left) and two-photon fluorescence (right). Two-photon excitation is localized to the focal volume. .........................................4

Figure 1.3: Several nonlinear processes that generate light at different wavelengths than used for excitation. ........................................................................................................6

Figure 1.4: Illustration of several processes that can provide intrinsic nonlinear contrast. Single wavelength contrasts include two-photon absorption (TPA) and self-phase modulation (SPM). Two color contrasts include sum-frequency absorption (SFA) and cross-phase modulation.................................................................7

Figure 1.5: Illustration of open aperture Z-scan (left). On the right is a plot of transmitted power as a function of sample position for a Gaussian beam showing maximum attenuation at the center position........................................................................12

Figure 1.6: Illustration of closed aperture Z-scan (left). On the right is a plot of transmitted power as a function of sample position for a Gaussian beam showing the dispersive shape for SPM only case (blue) and the entangled profile when both TPA and SPM effects are present (green). .................................................................................13

Figure 2.1: Illustration of the principle of the spectral reshaping technique. Shows the effect of two-photon absorption (TPA) on a femtosecond pulse spectrum with a hole in the center.................................................................23

Figure 2.2: Illustration of the principle of the spectral reshaping technique. Shows the effect of self-phase modulation (SPM) on a femtosecond pulse spectrum with a hole in the center ........................................................................................................................25

Figure 2.3: The spectral shape of the pulse used in spectral reshaping technique. The local oscillator in the center of the spectrum has a time dependent phase. .........................26

Figure 2.4: Experimental setup used for SPM measurements using spectral reshaping technique and Z-scan technique........................................................................................................27
Figure 2.5: Sample design used for the experiments comparing spectral reshaping technique and Z-scan.

Figure 2.6: Relative linear transmission of the sample as a function of concentration of intralipid in water. The red line indicates the exponential fit. Also marked are the concentrations \( C_0 \), \( C_1 \) and \( C_2 \) used in Figure 2.7.

Figure 2.7: (a) Spectral reshaping scans showing the total signal as a function of focal position at concentrations \( C_0 \), \( C_1 \) and \( C_2 \). (b) Normalized Z-scan traces showing transmission as a function of focal position at concentrations \( C_0 \) and \( C_1 \). The red lines in the graphs are the corresponding fit traces. Concentrations \( C_0 \), \( C_1 \) and \( C_2 \) are defined in Figure 2.6. The spectral reshaping and Z-scan traces are averages of 9 scans performed at multiple transverse positions of the cuvette (to reduce the influence of possible sample impurities).

Figure 2.8: Relative self-phase modulation signals in the glass slide as a function of scattering strength of the intralipid solution. Shown are spectral reshaping (top) and Z-scan results (bottom). The values and error bars are best fit values and standard fit errors, respectively. The fit was performed on an average of 9 traces and the resulting fit values were normalized by the value for pure water. Also shown as black lines in each graph is the expected signal drop-off based on a focal intensity reduction due to scattering.

Figure 2.9: Ratio of best fit values for the nonlinear coefficient to the standard fit error for the spectral reshaping technique and Z-scan technique as a function of scattering strength of the intralipid solution. The fit was performed on a 9-trace average. Note that the incident power used for the Z-scan was about three times higher than the power used for the spectral reshaping technique.

Figure 2.10: Ratio of best fit values for the nonlinear coefficient to the standard fit error for the Z-scan technique with decreasing input power in comparison to increased scattering. The two x-axes are scaled to match the power reaching the sample at each point.

Figure 2.11: Illustration of the standard 4-f configuration. \( L_i \) and \( L_o \) are the input and output lenses of focal length \( f \). The spatial light modulator (AOM in our case) is placed at the Fourier plane.

Figure 2.12: Schematic of the acousto-optic modulator (AOM) based 4-f pulse shaper, which uses radiofrequency (RF) pulses to impart modulation on the spectrum of a laser pulse. The TeO\(_2\) crystal used is 4 cm in length. G: grating.
Figure 2.13: Illustration of the idea of the interferometric pulse shaper. The lower arm passes through a static pulse shaper. The upper arm passes through an AOM, which shifts the frequency of the beam such that the phase of the local oscillator rotates with respect to rest of the pulse spectrum. BS: beam splitter.

Figure 2.14: Experimental setup of the common-mode interferometric pulse shaper. The acousto-optic modulator (AOM) acts as the splitter and combiner for the interferometer arms. The local oscillator (LO) arm is frequency shifted by the AOM. DG: diffraction grating; CL: cylindrical lens; M: mirror. Adapted from [71].

Figure 2.15: Illustration of diffraction of beams during both the passes. The dashed lines correspond to the retro-reflected beams (vertically displaced by tilting the end mirror).

Figure 3.1: Schematic showing the structure of a neuron [90].

Figure 3.2: Image of a rat hippocampal slice showing information pathway [91]. DG: dentate gyrus; CA: cornu ammonis.

Figure 3.3: Experimental setup used for self-phase modulation imaging. BPF: bandpass filter; AOM: acousto-optic modulator; APD: Avalanche photodiode; PBSC: polarizing beam splitting cube.

Figure 3.4: (a) Sample setup showing the open flow cell. Glutamate can be injected into the flow at any point of time. ACSF: artificial cerebrospinal fluid. (b) Schematic of the hippocampal brain slice indicating the laser scanning direction in glutamate activated experiments.

Figure 3.5: Experimental results obtained with a 10X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations in the CA1 region. The average power used was about 400 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (c) show the signals when scanning away from the neuron layer. Column (b) shows the SPM spike when we scan on the cell body layer.

Figure 3.6: Experimental results obtained with a 20X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations in the CA1 region. The average power used was about 100 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (c) show the signals when scanning away from the neuron layer. Column (b) shows the SPM spike when we scan on the cell body layer.
Figure 3.7: Experimental results obtained with a 40X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations across a single neuron (brightfield image is shown at the top). The average power used was about 25 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (b) show the signals when scanning inside the neuron cell body. Column (c) shows the time course when we scan outside the neuron.

Figure 3.8: (a) Brightfield image of a neuron. The dashed line indicates the cell boundary. (c) Brightfield image without the dashed line. (b) SPM peak to baseline ratio image of the neuron shown in the brightfield image. (d) Corresponding linear transmission peak to baseline ratio image. All the scale bars are 10 µm.

Figure 3.9: Micropipette containing the silver electrode is sealed to cell membrane for recording ion channels [99].

Figure 3.10: Seal formation between the pipette tip and the membrane [101].

Figure 3.11: By applying a sharp suction the cell membrane is ruptured and provides access to the intracellular space [102].

Figure 3.12: Time course of the neuron potential in current clamp mode during an 80 Hz pulse train [96].

Figure 3.13: (a) Temporal dependence of the SPM change in a single neuron during stimulation by an electrical pulse train, averaged over many datasets and after application of a low pass 50Hz filter. The black and red graphs correspond to points outside and inside the neuron. (b) Corresponding transmission changes. (c) Sample electrical recording from the patch clamp amplifier in current clamp mode.

Figure 4.1: Pump and probe pulse spectrums used in the two-color spectral reshaping technique. The pump pulse train is amplitude modulated and a static phase shift is imposed onto the center of the probe spectrum. Modulation is transferred from pump to probe during nonlinear interaction.

Figure 4.2: Simulated XPM signal plot illustrating the effect of local oscillator (LO) position and width. X and Y axes indicate LO width and LO position relative to the bandwidth (FWHM) of the probe pulse.

Figure 4.3: Simulated pump-probe delay scans of cross-phase modulation signal with (a) no chirp and (b) third-order chirp.
Figure 4.4: Experimental setup for self- and cross-phase modulation measurements using the spectral reshaping technique. DC: dichroic mirror; BPF: band-pass filter; PD: photodiode; AOM: acousto-optic modulator. ................................................................. 82

Figure 4.5: Input power scaling of SPM and XPM signals in a glass slide. The nonlinear signal is background subtracted. XPM is plotted against the total laser power i.e. the sum of powers of both the pulses. Also plotted is the corresponding background of the nonlinear signals. ......................................................................................................................... 84

Figure 4.6: Experimental setup used for cross-phase modulation imaging. BPF: bandpass filter; LCM: liquid crystal modulator. ................................................................................................................................. 86

Figure 4.7: Scan system of the microscope showing the main components and formulation of the distances between them. The solid lines indicate the laser beam path and size. The dashed lines show the path of the beam during scanning. The scan mirror is imaged onto the back aperture of objective. Adapted from [107]. ................................................. 88

Figure 4.8: Pump power scaling of XPM signal, background and noise. The green lines illustrate the linear fits. Both XPM signal and noise (only at higher powers) scale linearly with pump power......................................................... 92

Figure 4.9: Probe power scaling of XPM signal, background and noise. The green lines illustrate the linear fits. Both XPM signal and noise scale linearly with probe power. The background is essentially constant with power.............................................................. 93

Figure 4.10: XPM measurements of Rhodamine 6G solution in a glass cuvette. $\theta$ is the static phase shift of the local oscillator (see Eq. 46). The total input power was about 22 mW. ......................................................................................................................... 94

Figure 4.11: Pump-probe delay scans of cross-phase modulation (XPM) signal in pure methanol and rhodamine 6G in methanol. The pump and probe powers were 12 mW and 13 mW, respectively. ......................................................................................................................... 95

Figure 4.12: (a) Linear transmission and (b) cross-phase modulation images of three fixed breast cancer cells. The scale bars are 10 µm. The total power impinging on the sample was about 21 mW. ......................................................................................................................... 96

Figure 4.13: XPM pump-probe delay scans with the spectral shifting technique in methanol with and without dissolved rhodamine-6G. A neutral density filter (10% transmission) was used to reduce the amount of probe power impinging on the balanced photodiode.......................................................... 99
Figure 4.14: Images of an H&E stained skin biopsy slide. (d) The first two principal components of the XPMSS delay stack. Red is the transient absorption component and green is the XPM component. (a) and (b) show the principal component images when only the corresponding (color in (d)) component is retained. (c) A zoomed-in view of the XPM component image in (a). All the scale bars are 50 µm.

Figure 4.15: Histogram plot (right) of phasors for all the pixels in the XPMSS delay stack of dermo-epidermal junction in a melanoma biopsy. RGB image (left) extracted from the delay stack (color corresponds to the regions indicated by circles in the phasor plot). The scale bar is 20 µm.

Figure 5.1: Various nonlinear processes of interest in the pump-probe microscopy experiments.

Figure 5.2: Nonlinear processes transfer modulation to the probe.

Figure 5.3: Simplified experimental setup used for pump-probe microscopy experiments. PBSC: polarizing beam splitting cube.

Figure 5.4: (a) Polished lapis lazuli rock [167]. (b) Commercially available good quality ultramarine pigment [168].

Figure 5.5: Linear absorption spectra of natural lapis lazuli.

Figure 5.6: Pump-probe images (zero delay) of natural lapis and synthetic ultramarine. On the right are the corresponding pump-probe decay traces averaged over the indicated region of interest (white rectangle).

Figure 5.7: (a) Pump and (b) probe power dependence of pump-probe signal in natural lapis lazuli.

Figure 5.8: Cumulative histogram phasor plot of 12 pump-probe delay stacks of natural lapis lazuli.

Figure 5.9: (a) Histogram plot of phasors for all the pixels in a delay stack of lapis lazuli. (b) RGB image of the lapis delay stack, where color corresponds to the region indicated in the phasor plot. The scale bar is 50 µm.

Figure 5.10: Zero delay pump-probe images of (b) grey-blue grade or low quality lapis and (d) pure lapis lazuli. Also shown are the brightfield camera images (taken at a
different location) of (a) low quality lapis and (c) pure lapis. All the scale bars are 50 µm.

Figure 5.11: Zero delay pump-probe images of (b) crystalline lapis [170] and (d) Chilean lapis lazuli. Also shown are the brightfield camera images (taken at a different location) of (a) crystalline lapis and (c) Chilean sourced lapis. All the scale bars are 50 µm.

Figure 5.12: Depth stack of zero delay pump-probe images of lapis lazuli. Z = 0 µm corresponds to the top layer.

Figure 5.13: (b) Pump-probe image of indigo at 500 fs delay. The scale bar is 50 µm. (a) Decay trace averaged over the region of interest shown as white rectangle in the image. (c) Decay trace with expanded time axis showing the signal peak around 400-500 fs. (d) Histogram plot of phasors for all the pixels in 10 pump-probe delay stacks of natural indigo.

Figure 5.14: Zero delay pump-probe image of indigo in casein binder with the pump wavelength at 810 nm. On the right is the corresponding decay trace averaged over the region of interest (white rectangle).

Figure 5.15: (b) Zero delay pump-probe image of vermilion. The scale bar is 50 µm. (a) Decay trace averaged over the region of interest (shown as white rectangle in (a)). (d) Histogram plot of phasors for all the pixels in 5 delay stacks of vermilion. (c) Representative decay traces of the impurities indicated in the phasor plot.

Figure 5.16: Zero delay pump-probe image of red iron oxide (hematite). On the right is the corresponding decay trace averaged over the region of interest.

Figure 5.17: Histogram phasor plots of (a) pure iron oxide (hematite), (b) Burgundy red ochre, (c) Burgundy yellow ochre and (d) Italian yellow ochre.

Figure 5.18: (a) A camera image of the 15th century Italian panel painting [183]. The real size of the painting is 34.8 x 22.2 inches. (b) A brightfield camera image of the paint chip cross-section taken from the robe of Madonna.

Figure 5.19: (a) Zero delay pump-probe image of the paint chip cross-section in the region of interest (shown as the yellow square in Figure 5.18(b)). (b) Histogram plot of phasors for all the pixels in a delay stack of the paint cross-section. (c) RGB image of lapis composition extracted from the delay stack (color corresponds to the regions indicated by circles in the phasor plot). (d) Decay traces averaged over the pixels in the regions shown in the phasor plot. All the scale bars are 20 µm.
Figure 5.20: Representative pump-probe decay signatures of (a) synthetic ultramarine at pump and probe wavelengths of 720 nm and 810 nm, respectively and (b) vermilion at pump and probe wavelengths of 810 nm and 720 nm, respectively.

Figure 5.21: Overlay of depth stacks showing 3-dimensional multi-layer information. Blue and red corresponds to ultramarine and vermilion pigments, respectively. Depth cross-sections in both X and Y directions (at the places indicated by white lines) are also shown.
Acknowledgements

It has been a pleasure to work with a number of brilliant people over the last 5 years. I am very grateful for all the support and guidance I have received during my graduate studies. I would like to acknowledge everyone who had a part in making this dissertation possible.

First, I would like to express my gratitude to my dissertation advisor Dr. Warren S. Warren for the guidance and encouragement he has provided me during all the turns in a variety of interesting projects. I appreciate his patience and vision in all aspects of research. I would like to thank Dr. Martin Fischer for his hands on guidance in every aspect of the research projects. I especially appreciate all the time Dr. Fischer spent in teaching me every tiny detail of everything. I am very grateful for his readily available counsel on any problem I have encountered in the lab. I would also like to thank my committee members for reading my dissertation.

It has been a wonderful experience to work in lab with such diverse expertise and helpful colleagues. I had many useful discussions with other graduate students and postdocs. I would like to thank the Warren group members for proof reading my works and providing me helpful comments on my presentations. I would like to thank Dr. Ivan Piletic for helping me through my first experimental setup in the lab. I would like to thank Dr. Henry Liu for guiding me to take over the neuron imaging project. I appreciate all the time Dr. Liu spent in teaching me patch clamp experiments. I would like to thank
Baolei Li for all his help in the lab. I am very grateful to Dr. Jesse Wilson for his constant assistance with various projects and insightful discussions. I especially appreciate his help in data analysis and programming. I would like to thank Tana Villafaña for her help with the pump-probe microscopy experiments. I would like to thank Dr. Francisco Robles for his help in phasor analysis.

I would also like to acknowledge our collaborators. I would like to thank Prof. Ryohei Yasuda for providing all the brain slice samples and helpful discussions on the neuron imaging project. I am grateful to Dr. Yasmin Escobedo-Lozoya and Dr. Hong Wang for their help in the patch-clamp experiments. I would like to thank Bill Brown and Adele deCruz of North Carolina Museum of Art, Raleigh for providing all the paint samples. I appreciate their help and guidance with historical pigment imaging project.

Finally, I would like to thank my family for their support, patience and belief in me throughout the course of my doctoral work. I would especially like to thank my wife Srujana for her encouragement and assistance.
1. Introduction to nonlinear microscopy

An optical microscope, in a basic sense, sends light through a system of lenses to magnify the object under study. The addition of spectroscopic capabilities to an optical microscope has made it an invaluable and widespread tool in molecular imaging. Modern optical microscopy techniques are capable of obtaining fast molecule-specific information with high spatial resolution. The fundamental issues in optical microscopy are still the tradeoffs between achievable contrast, resolution, and penetration depth. Optical microscopy techniques can be broadly divided into two categories: linear (one photon) techniques and nonlinear (multi-photon) techniques. In this chapter, we will discuss the conventional linear and nonlinear microscopy techniques and present novel intrinsic contrasts, which push the nonlinear imaging technology for molecular analysis forward.

1.1. Linear optical microscopy techniques

Conventional optical microscopy techniques use linear processes such as absorption [1, 2], reflectance [3-6], fluorescence [1, 5, 7-10], fluorescence lifetime [1, 11], and Raman [12-14] for contrast. They can be can be easily implemented with low cost apparatus. These linear techniques have been useful in obtaining specific molecular information at the surface of the target and in thin samples. However, the major
limitation of linear contrasts is the lack of depth resolved information in highly scattering media.

Figure 1.1: Beam path for confocal laser scanning microscopy showing the rejection of out of focus rays using a pinhole before the detector (adapted from [15]).

Limited depth selectivity can be achieved by implementing linear contrasts in a confocal scanning arrangement. The concept of confocal microscopy was originally developed by Marvin Minsky in 1955 [16]. The basic idea of confocal microscopy is to use a point illumination method to acquire information pixel-by-pixel and use spatial filtering to eliminate light generated or reflected from out of focal plane [17, 18]. The key idea of the confocal arrangement is the placement of a pinhole in the conjugate image
plane before the detector (see Figure 1.1). This detector pinhole acts as a spatial filter to block all the light coming from out of the focus planes. Thus the signal collected by the detector will mostly be from a well-defined focal plane. By using a motor to step the focal plane, axial sectioning can be achieved. An image is constructed by raster scanning the illumination spot using galvo scanning mirrors or acousto-optic devices. The spatial resolution obtained is dependent on the wavelength of light used and the numerical aperture (NA) of the objective. When imaging thick sections, the scattering properties of the material define the achievable resolution and depth. The temporal resolution achieved is dependent on mostly the signal-to-noise and sometimes on scanning speed of the mirrors.

After the development of lasers, confocal laser scanning microscopy (CLSM) was quickly adapted by the biological imaging community, because it could provide, for the first time, near diffraction limited information in three dimensions. Linear contrasts such as fluorescence, Raman and reflectance can be implemented in the confocal arrangement to generate high resolution depth resolved images. However, CLSM has some limitations in highly scattering media. In CLSM, excitation occurs throughout the axial direction, but only signals from the focal plane go through the pinhole. The rejection of signal photons leads to inefficient detection. Therefore, CLSM generally requires longer acquisition times. Also, light from the out of focus planes can be multiply-scattered and pass through the pinhole. All the photons passing through the
pinhole are interpreted as signal, therefore scattered photons contribute linearly to the background. As we go to higher depths in scattering media, the background eventually limits the penetration depth. In tissue, the strong scattering limits the imaging depth to about 100 µm.

1.2. Conventional multiphoton microscopy techniques

A powerful approach to combine high penetration depths with high spatial resolution is to take advantage of nonlinear optical processes. The invention of two-photon laser scanning microscopy in 1990 was one of the significant breakthroughs in imaging of scattering media [19].

Figure 1.2: Comparison of single photon fluorescence (left) and two-photon fluorescence (right). Two-photon excitation is localized to the focal volume.
In general, nonlinear microscopy has several advantages over linear confocal techniques. Because of the nonlinear dependence on laser intensity, signals are generated only at the focus. This leads to highly localized excitation, achieving inherent axial sectioning. Due to excitation being localized to the focal volume, no spatial filter is required to reject out of focus light. The out of focus contribution to the background from scattered light is negligible, because effectively there is no signal generated out of the focal volume. Unlike confocal microscopy, all the signal photons are collected; therefore the collection efficiency is higher. This leads to high signal to noise and greatly reduces acquisition times.

Two-photon fluorescence (TPF) based imaging is the most commonly utilized nonlinear imaging technique [20-26]. In TPF, a molecule is excited by absorbing two photons of half the energy required to make the transition. Therefore, a single photon excitation of 400 nm requires two photons of longer wavelength (800 nm) using the nonlinear mechanism (Figure 1.2). In tissue, these longer wavelengths have less attenuation, permitting deeper penetration [27, 28]. However, only molecules which fluoresce can be detected, so TPF needs either an exogenous or endogenous fluorophore to work. Also, light generated at shorter wavelengths suffers from strong re-absorption and scattering, thus having difficulty in reaching the detector.

Any nonlinear optical process that generates light of wavelengths that are different from the wavelengths used for excitation can be used as a microscopy contrast
by implementing laser scanning and spectrally filtering the signal (Figure 1.3). Apart from two-photon fluorescence, such contrasts used for imaging include nonlinear harmonic generation and coherent anti-Stokes Raman scattering.

Harmonic generation is an energy conserving process thus providing minimal energy deposition in the sample and lower photodamage. Second harmonic generation (SHG) is associated with second order nonlinear susceptibility, \( \chi^{(2)} \) and is non-zero only in non-centrosymmetric molecules [29-34]. SHG is generally used to get intrinsic structural contrast. In tissue, connective structures (collagen) are generally imaged using SHG. Third harmonic generation (THG) is associated with the real part of third order nonlinear susceptibility, \( \chi^{(3)} \) and is primarily generated at interfaces [32, 34-36]. Generally, severe symmetry constraints restrict the range of possible targets for harmonic generation imaging.

Figure 1.3: Several nonlinear processes that generate light at different wavelengths than used for excitation.
Coherent anti-Stokes Raman scattering (CARS) is a third order nonlinear optical process which enhances the Raman cross-section by coherently driving the vibration using a pump and stokes beam, whose difference frequency matches the Raman resonance. CARS provides a highly specific and intrinsic molecular contrast [37, 38]. However, large non-resonant background combined with generally weak Raman cross-sections present a major challenge in the usability of CARS contrast.

1.3. Novel intrinsic nonlinear microscopy contrasts

![Diagram of TPA, SPM, SFA, and XPM processes]

Figure 1.4: Illustration of several processes that can provide intrinsic nonlinear contrast. Single wavelength contrasts include two-photon absorption (TPA) and self-phase modulation (SPM). Two color contrasts include sum-frequency absorption (SFA) and cross-phase modulation.

In this thesis, we will focus on intrinsic nonlinear contrasts that do not generate light at distinct wavelengths (see Figure 1.4). Such contrasts can be broadly divided into two categories: non-parametric processes and parametric processes. Nonlinear
absorptive processes including both real-level based and virtual-level based contrasts are the non-parametric processes. Self-phase modulation (SPM) and its two-color equivalent, cross-phase modulation (XPM) are the energy conserving (parametric) refractive contrasts.

1.3.1. Nonlinear absorption

Many molecules have allowed multi-photon electronic transitions, but most of the molecules exhibit low quantum yields due to strong fluorescence quenching (from non-radiative relaxation effects or fluorescence reabsorption). For example, important biomarkers such as hemoglobin and melanin are excitable using near-infrared wavelengths, but do not fluoresce appreciably. However, the nonlinear absorption process itself is not affected by the quantum yield, therefore, provides a more general contrast than nonlinear fluorescence. Nonlinear absorption can technically be detected by measuring loss in the beam, but at physiologically acceptable power levels, it is very small and is masked by the losses due to other linear processes.

Previously, Warren and coworkers developed a pulse train shaping and detection technology to sensitively measure nonlinear absorption or gain processes. The basic idea is to force the nonlinear signal to be generated at frequencies not previously present in the beam. If we introduce an amplitude modulation at frequency $f_o$ on a pulse train; a nonlinear loss process (such as two-photon absorption) will create a frequency
component at $2f_0$ [39]. Linear processes such as scattering or absorption cannot create this new frequency component. In the two-color version of this technique, an amplitude modulation on the pump beam is transferred onto the probe beam of a different wavelength by nonlinear interaction, thereby generating a new frequency in the probe beam [40]. The two-color modulation transfer technique is similar to traditional pump-probe spectroscopy. By controlling the pump-probe delay, various transient processes such as excited state absorption, bleaching, and stimulated emission can be probed. This technique effectively combines the specificity of pump-probe spectroscopy with nonlinear optical imaging and is termed ‘pump-probe microscopy’. In recent work, the Warren group has applied this technique to image various biological targets without any labeling [41]. Eumelanin and pheomelanin have been differentiated and their distributions have been mapped in pigmented skin lesions [42-47]. Oxy-hemoglobin and deoxy-hemoglobin have been differentiated and blood vessels have been imaged in a live nude mouse ear [48, 49]. In chapter 5, we will discuss the application of pump-probe microscopy in imaging historical pigments used in artwork. We will characterize several important inorganic and organic pigments. We will also demonstrate multi-layer imaging and identify pigments in a paint chip taken from a 16th century painting.
1.3.2. Self-phase modulation

Self-phase modulation (SPM) is a nonlinear optical property which causes self-induced phase changes proportional to the instantaneous intensity in a propagating light pulse due to nonlinearity in the refractive index of the medium. It occurs due to the optical Kerr effect. The refractive index in Kerr medium can be written as

$$n = n_o + n_2 I.$$  

(1)

where $n_o$ and $n_2$ are defined as the linear refractive index and second order nonlinear refractive index, respectively. $I$ is the instantaneous pulse intensity.

SPM is well known in the physics community. For example, in optical fibers chromatic dispersion is generally balanced against the chirp from self-phase modulation to create optical solitons (pulses whose spectral shape remains constant during propagation in dispersive fiber). Fundamental solitons are very important in long-distance optical fiber communications due to their inherent stability [50].

The nonlinear refractive index (or nonlinear refraction coefficient) is known to be sensitive to several of material properties, such as resonant and non-resonant electronic effects or molecular reorientation effects [51]. Common transparent solvents can differ by more than two orders of magnitude in their nonlinear refraction coefficients. SPM is therefore an intrinsic material property that can depend on molecular content, local structure and anisotropy of the medium.
Phase contrast microscopy is widely used to investigate transparent specimens. Phase shifts are induced in transmitted light depending on the linear refractive index. These phase changes are generally invisible to eye and are quantified by conversion into intensity information. Measuring phase shifts occurring due to nonlinear refractive index can extend phase contrast to the nonlinear regime. However, nonlinear phase changes (or SPM effects) are generally small for the applicable powers and present themselves on an overwhelming background of incident light. Conventionally, SPM is measured using the Z-scan technique, which measures small changes in the transmitted beam profile by spatial filtering. In the next section we will describe the Z-scan technique and its limitations.

1.3.2.1. Z-scan technique

The Z-scan technique is the simplest and oldest method for measurement of nonlinear absorptive and refractive coefficients [52, 53]. It has been used to study nonlinear properties of a wide range of transparent optical materials, such as semiconductors, pure or doped glasses [54, 55], liquids, and liquid crystals [56]. It has also been used to study nonlinear refraction clear liquids such as vitreous humor [57].

The key idea of the Z-scan technique is that, when a focused laser beam interacts with a nonlinear medium it experiences changes in its spatial phase and amplitude profile. These changes cause variations in the far-field diffraction pattern which can be detected in two configurations, open aperture and closed aperture.
In the Z-scan experiment, the sample of interest is translated through the focus of a laser beam in the direction of beam propagation (the Z-direction) and the transmitted light is collected by a photodiode. In an open aperture Z-scan (Figure 1.5, left), the entire beam is collected, therefore, it is only sensitive to changes in transmitted power. Since two-photon absorption (TPA) has a higher order dependence on the light intensity, a transmission minimum (highest attenuation) occurs when the focus position is centered in the sample (Figure 1.5, right). The TPA coefficient can be extracted by fitting the transmission as a function of sample position. It is important to note that Z-scan traces are generally normalized by ‘low power’ traces to compensate for the linear transmission changes, such as those caused by sample distortions.
Figure 1.6: Illustration of closed aperture Z-scan (left). On the right is a plot of transmitted power as a function of sample position for a Gaussian beam showing the dispersive shape for SPM only case (blue) and the entangled profile when both TPA and SPM effects are present (green).

In the case of a Kerr medium without any two-photon absorption, the total transmitted power remains constant because SPM is a parametric process, but small phase shifts are induced on the high-intensity parts of the beam. For a Gaussian beam this effect leads to self-focusing or self-defocusing in the sample, resulting in changes in the far-field intensity pattern. Conventionally, these changes in the spatial profile are detected by measuring the power transmitted through an aperture placed in the center of the beam in far-field (Figure 1.6, left). In a closed-aperture scan, the transmission as a function of sample position shows dispersive shaped features, which can be fitted to extract the nonlinear refraction coefficient. If the material exhibits both TPA and SPM the two contributions can be disentangled by fitting the open- and closed-aperture scans [58].
Over the years various modifications have been introduced for the standard Z-scan technique to enhance its sensitivity and applicability [59-61]. In the closed-aperture measurements, the sensitivity to beam profile changes can be enhanced by eclipse-type measurements [62] or by numerically fitting complete beam profiles acquired with an imaging device [63]. However, a well characterized and clean input beam profile is still required for meaningful Z-scan measurements [58]. The stringent beam shape requirement can be partially relaxed by measuring the nonlinearity of the sample relative to a reference sample [64]. However, the idea of measuring changes in spatial profile is not applicable to scattering media. In strongly scattering media small changes in the beam profile are masked by the diffusive nature of multiple scattering. Beam profiles in such samples are completely destroyed or washed out. Therefore, Z-scan traces do not provide the information required to accurately and precisely extract nonlinear coefficients. Also, Z-scan is inherently a bulk measurement technique rather than a point measurement technique; therefore, it is not applicable to imaging.

Fischer et al. developed a spectral reshaping technique which can sensitively detect small spectral changes caused by nonlinear refraction and nonlinear absorption [65]. The key idea is that nonlinear processes can generate new frequency components in the pulse spectrum, whereas linear processes (such as scattering or linear absorption) cannot. Therefore, once the nonlinear signal is encoded in the spectrum, it is preserved and we can extract the required information with good sensitivity even in cases where
only a part of the beam manages to reach the detector. This means that even back-scattered (epi-detected) light can be used to extract the spectral information, making this technique suitable for thick, non-transmissive samples.

In chapter 2, we will describe the implementation of spectral reshaping technique and compare the performance of our technique with the traditional Z-scan technique in scattering media. We will show that the spectral reshaping by far outperforms the standard method, in regime where both methods can be used. We also demonstrate that spectral reshaping technique can extract nonlinearities even in highly scattering media that preclude the use of traditional Z-scan technique. We will also discuss the design of a novel interferometric acousto-optic pulse shaper that is capable of shaping each pulse coming out of a mode-locked system.

In chapter 3, we will discuss the application of spectral reshaping technique for non-invasive detection of neuronal activity in ex vivo hippocampal rat brain slices. We will present results showing SPM changes at a single neuron scale during glutamate-induced neuronal activation. We will also present results of simultaneous self-phase modulation and electrophysiological measurements in patch clamped neurons.

1.3.3. Cross-phase modulation

Cross-phase modulation (XPM) is a nonlinear optical property which causes phase changes in a propagating light pulse due to intensity dependent modulation of
the refractive index by a second pulse of a different wavelength. XPM is the two color analogue of self-phase modulation, and also occurs due to the optical Kerr effect.

Let us consider the co-propagation of two optical fields in a non-magnetic nonlinear medium. The wave equation which describes the propagation of optical fields can be derived from the Maxwell Equations [50].

\[ \nabla^2 \mathbf{E} - \frac{1}{c^2} \frac{\partial^2 \mathbf{E}}{\partial t^2} = \mu_0 \frac{\partial^2 \mathbf{P}}{\partial t^2}, \]  

(2)

where \( c \) is the speed of light in free space and \( \mu_0 \) is the permeability of free space. \( \mathbf{E} \) and \( \mathbf{P} \) are the electric field and induced polarization, respectively. In an isotropic medium the total polarization (neglecting terms higher than third order) can be written as

\[ \mathbf{P} = \mathbf{P}_{\text{Linear}} + \mathbf{P}_{\text{Nonlinear}} = \mathbf{P}^{(1)} + \mathbf{P}^{(3)}. \]  

(3)

Considering linearly polarized light and neglecting effects in the transverse direction (plane wave approximation) the wave equation takes the scalar form

\[ \frac{\partial^2 E(z, t)}{\partial z^2} - \frac{1}{c^2} \frac{\partial^2 E(z, t)}{\partial t^2} = \mu_0 \frac{\partial^2 P^{(1)}(z, t)}{\partial t^2} + \mu_0 \frac{\partial^2 P^{(3)}(z, t)}{\partial t^2}. \]  

(4)

In a Kerr medium, the first order and third order induced polarizations can be written as

\[ P^{(1)}(z, t) = \varepsilon_0 \chi^{(1)} E(z, t), \]  

(5)
Here, $\chi^{(1)}$ and $\chi^{(3)}$ are the first and third order susceptibilities, respectively. The electric field (omitting the conjugate term) describing both the optical fields can be written as

$$E(z, t) = A_1(z, t) \exp(ik_1 z - i\omega_1 t) + A_2(z, t) \exp(ik_2 z - i\omega_2 t),$$  \hspace{1cm} (7)

where $k_j$ and $\omega_j$ are the wave vector and the optical carrier frequency, respectively. $A_j(z, t)$ is the amplitude envelope of the pulse. The envelope is assumed to be slowly varying; therefore, higher order derivatives of the amplitude can be neglected. The subscript $j$ can be either 1 or 2, indicating pulse 1 or pulse 2, respectively. By substituting Eq. 7 in Eq. 6, we can obtain the following terms of induced third order polarization

$$P^{(3)}|_{\omega_1} = \frac{3\varepsilon_0}{4} \chi^{(3)} (|A_1(z, t)|^2 + 2|A_2(z, t)|^2) A_1(z, t),$$  \hspace{1cm} (8)

$$P^{(3)}|_{\omega_2} = \frac{3\varepsilon_0}{4} \chi^{(3)} (2|A_1(z, t)|^2 + |A_2(z, t)|^2) A_2(z, t),$$  \hspace{1cm} (9)

$$P^{(3)}|_{2\omega_1 - \omega_2} = \frac{3\varepsilon_0}{4} \chi^{(3)} |A_1(z, t)|^2 A_2^*(z, t),$$  \hspace{1cm} (10)

$$P^{(3)}|_{2\omega_2 - \omega_1} = \frac{3\varepsilon_0}{4} \chi^{(3)} |A_2(z, t)|^2 A_1^*(z, t).$$  \hspace{1cm} (11)

The terms oscillating at $2\omega_1 - \omega_2$ and $2\omega_2 - \omega_1$ correspond to the four wave mixing process. The four wave mixing terms can be neglected, since in our technique we spectrally filter to reject these components. By substituting Equations 5, 7, 8 and 9 in Eq.
4, and applying the slowly-varying-envelope approximation, we can obtain the simplified
coupled propagation equations for both the pulse amplitudes. The group velocity
dispersion effects can be included by expanding the wave vector in a Taylor series
around the carrier frequency and retaining first and second order terms [50]. The
resulting coupled equations can be written as

\[
\frac{\partial A_1(z,t)}{\partial z} + \frac{1}{v_{g1}} \frac{\partial A_1(z,t)}{\partial t} + \frac{\alpha_{o1}}{2} A_1(z,t) + \frac{i\beta_1}{2} \frac{\partial^2 A_1(z,t)}{\partial t^2} = \left(\frac{in_2\omega_1}{c} - \frac{\alpha_{21}}{2}\right) (|A_1(z,t)|^2 + 2|A_2(z,t)|^2) A_1(z,t),
\]

where \(\alpha_{oj}\) is the linear absorption coefficient, which corresponds to the imaginary part of
\(\chi^{(1)}\). The group velocity is defined as

\[
v_{gj} = \left(\frac{dk_j}{d\omega}\right)^{-1}\bigg|_{\omega_j},
\]

and the group velocity dispersion coefficient is defined as

\[
\beta_j = \frac{d^2k_j}{d\omega^2}\bigg|_{\omega_j}.
\]
$n_2$ and $\alpha_{2j}$ are the nonlinear refraction and absorption coefficients, respectively. They are related to the third order susceptibility as

$$n_2 = \frac{3}{8n_o} \text{Real}\{\chi^{(3)}\}, \quad (16)$$

$$\alpha_{2j} = \frac{3\omega_j}{4cn_o} \text{Imag}\{\chi^{(3)}\}, \quad (17)$$

where $n_o$ is the linear refractive index corresponding to the real part of $\chi^{(1)}$. For the definitions above, the subscript $j$ can be either 1 or 2, indicating pulse 1 or pulse 2, respectively.

We can shift the frame of reference such that it is stationary with respect to the pulse 1. Neglecting any linear and nonlinear absorption losses, we can write the coupled equations as

$$\frac{\partial A_1(z, \tau)}{\partial z} + i\beta_1 \frac{\partial^2 A_1(z, \tau)}{\partial \tau^2} = \left(\frac{in_2\omega_1}{c}\right)(|A_1(z, \tau)|^2 + 2|A_2(z, \tau)|^2)A_1(z, \tau), \quad (18)$$

$$\frac{\partial A_2(z, \tau)}{\partial z} + \left(\frac{v_{g1} - v_{g2}}{v_{g1}v_{g2}}\right)\frac{\partial A_2(z, \tau)}{\partial \tau} + i\beta_2 \frac{\partial^2 A_2(z, \tau)}{\partial \tau^2} = \left(\frac{in_2\omega_2}{c}\right)(|A_2(z, \tau)|^2 + 2|A_1(z, \tau)|^2)A_2(z, \tau). \quad (19)$$

where, $\tau = t - z/v_{g1}$. From this point, we will only consider the propagation analysis for pulse 1. The same analysis applies for the pulse 2. For simplicity, let us neglect the
second derivative term in Eq. 18 and also neglect any group velocity mismatch, then we can find an analytical solution for the propagation equation [66], which is

\[ A_1(z, \tau) = A_1(0, \tau) \exp(i\phi_1(\tau)), \]

(20)

where, \( z \) is the propagation length and

\[ \phi_1(\tau) = \frac{n_2\omega_1z}{c}(|A_1(0, \tau)|^2 + 2|A_2(0, \tau)|^2). \]

(21)

Here, \( A_1(0, t) \) and \( A_2(0, t) \) are the initial amplitude envelopes for pulses 1 and 2, respectively. Eq. 21 gives the phase acquired by the pulse due to nonlinear effects over a small propagation distance \( z \). The two terms in the nonlinear phase are two different types of contribution. The first term is the self-induced phase modulation (SPM), and the second term is the cross-induced phase modulation (XPM). We can write them separately as

\[ \phi_1(\tau) = \phi_{SPM}(\tau) + \phi_{XPM}(\tau), \]

(22)

where

\[ \phi_{SPM}(\tau) = \frac{n_2\omega_1z}{c} I_1(\tau) \]

(23)

and

\[ \phi_{XPM}(\tau) = \frac{2n_2\omega_1z}{c} I_2(\tau). \]

(24)
Here, the initial amplitudes of both the pulses (considering a case of two Gaussian pulses with no chirp) are defined as

\[
|A_1(0, \tau)|^2 = I_1(\tau) = I_1^0 \exp \left( -\left( \frac{\tau}{\tau_g} \right)^2 \right), \quad (25)
\]

\[
|A_2(0, \tau)|^2 = I_2(\tau) = I_2^0 \exp \left( -\left( \frac{\tau}{\tau_g} \right)^2 \right), \quad (26)
\]

where \(I_1^0\) and \(I_2^0\) are the peak intensities. The full width half maximum of the pulse is defined as

\[
\tau_{FWHM} = \sqrt{2 \ln 2} \tau_g. \quad (27)
\]

The time dependent phase corresponds to a frequency chirp across the pulse, which is given by

\[
\Delta \omega = -\frac{d\phi}{d\tau} \quad (28)
\]

An expression for the cross-induced chirp can be obtained as

\[
(\Delta \omega)_{XPM} = -\frac{4n_2 \omega_1 I_2^0 \tau}{\tau_g^2 c} \exp \left( -\frac{\tau^2}{\tau_g^2} \right) \quad (29)
\]

From Eq. 29, we can see that XPM causes spectral changes in a pulse. Similar analysis can be performed for self-phase modulation to show the induced spectral changes. Our nonlinear phase modulation measurement techniques are based on the
idea of detecting these spectral changes. In chapter 4, we will describe two different implementations of cross-phase modulation measurement. The first implementation uses femtosecond pulse shaping to detect the induced spectral changes and has the ability to quickly switch between nonlinear refractive and absorptive measurements by changing the pulse shape. In the second implementation we use an edge filter to measure the induced spectral shifts. This implementation does not require a pulse shaper. We will show that cross-phase modulation dramatically reduces the background associated with nonlinear phase measurements using self-phase modulation. We will also demonstrate biological imaging using nonlinear phase as contrast.
2. Spectral reshaping technique

In this chapter, we will describe a spectral reshaping technique based on frequency domain encoding of nonlinear signal to simultaneously measure nonlinear dispersion and absorption [65]. We will compare nonlinear spectral reshaping and Z-scan measurements of dispersive samples in a highly scattering environment and show that reliable spectral reshaping measurements can be performed even in a regime that precludes standard Z-scans [67].

2.1. Frequency domain encoding

![Illustration of the principle of the spectral reshaping technique. Shows the effect of two-photon absorption (TPA) on a femtosecond pulse spectrum with a hole in the center.](image)

Figure 2.1: Illustration of the principle of the spectral reshaping technique. Shows the effect of two-photon absorption (TPA) on a femtosecond pulse spectrum with a hole in the center.
To understand the principle of the spectral reshaping technique, first consider the effect of nonlinear absorption (here two-photon absorption, TPA) on a symmetric laser pulse with a ‘hole’ in the center of its spectrum as indicated in Figure 2.1 (top-left). By the definition of the Fourier transform, such a pulse needs to have zero area in the time domain for the center of the hole in spectrum to have zero intensity. Therefore, it consists of a short and intense pulse balance against a weak, negative, longer pulse (Figure 2.1, bottom-left). The areas of both these parts are equal. Generally, TPA is proportional to the square of the intensity; therefore, when such a waveform enters a nonlinear material which exhibits TPA, the intense portion will be absorbed much more than the weak portion. Therefore, the positive intense part is attenuated more than the negative weaker part of the pulse, causing an imbalance in the areas (Figure 2.1, bottom-right). After undergoing TPA, the net area of the pulse in the time domain is no longer zero and this corresponds to a non-zero component in the center of the pulse in frequency domain. Effectively the spectral hole is refilled (Figure 2.1, top-right) and since TPA is a loss process, the added field is negative (180° out of phase) with respect to the incoming pulse.

Now consider the effect of self-phase modulation (SPM) on a pulse with a ‘hole’ in the center of its spectrum (Figure 2.2). When the pulse enters a Kerr medium, the intense portion of the waveform will generate stronger SPM than the weak portion. SPM being a dispersive process, does not change the energy of the laser pulse, but only modulates the phase by an amount proportional to the instantaneous pulse intensity.
Therefore, a new frequency component appears on the imaginary axis, where the vector sum of this new component and the positive portion on the real axis is equal to the intensity of the initial intense portion. The areas of the two pulse components no longer exactly cancel because the intense part has greater phase shift. Consequently, the pulse in the frequency domain shows new frequency components on the imaginary axis (Figure 2.2, top-right). Effectively, the spectral hole is refilled by SPM and it being a dispersive process, the added field is 90° out of phase with the remainder of the pulse.

**Figure 2.2:** Illustration of the principle of the spectral reshaping technique. Shows the effect of self-phase modulation (SPM) on a femtosecond pulse spectrum with a hole in the center.

As described above, both TPA and SPM refill a spectral hole. However, the phase of the polarization induced is different: TPA is an absorptive process and adds a field that
is 180° out of phase, whereas SPM being a dispersive process adds a 90° out of phase component. The two contributions can be independently measured by a phase sensitive homodyne method [65]. This is done by adding a well-defined reference field (in the spectral hole) to serve as a local oscillator (Figure 2.3). The local oscillator interferes with the generated nonlinear signal depending on the relative phase and by rotating the phase of the oscillator we can determine the phase of the nonlinear signal. This homodyne scheme also provides a dramatic signal enhancement of the nonlinear signal by several orders of magnitude due to the amplification effect of the local oscillator [68].

Figure 2.3: The spectral shape of the pulse used in spectral reshaping technique. The local oscillator in the center of the spectrum has a time dependent phase.

2.2. Experimental setup

To compare the spectral reshaping technique with the conventional Z-scan technique, we performed a series of SPM measurements in glass using both methods and analyzed their precision and accuracy as a function of scattering strength. A
simplified schematic of the experimental setup used for these measurements is shown in Figure 2.4.

Figure 2.4: Experimental setup used for SPM measurements using spectral reshaping technique and Z-scan technique.

We used a regenerative amplifier (RegA 9000, Coherent) seeded by a mode-locked 80 MHz Ti:Sapphire oscillator (Vitesse, Coherent) with a center wavelength of 804 nm as our laser source. The RegA is pumped with 12.5 W of CW laser (Verdi-18, Coherent) power. The amplified laser system can technically operate at a repetition rate of 250 kHz. However, we currently run at a repetition rate of 20 kHz. This limiting factor comes from the maximum speed of BOXCAR integrator in the acquisition electronics.
The output temporal width was about 50 fs. The spectral shaping was performed using a standard 4–f acousto-optic pulse shaper [69]. For the spectral reshaping technique, the central portion of the pulse spectrum (about 3 nm) was phase rotated at 5 kHz to create the local oscillator (LO). For the Z-scan measurements, the pulse train was amplitude modulated at 5 kHz for better detection sensitivity.

The beam was then spatially filtered using a 25 µm pinhole to achieve near-Gaussian beam profile, which is required for the spatial domain measurements using Z-scan technique. Part of the beam was diverted by a beam splitter (BS) onto a photodiode (D1) for input power monitoring. The beam was then focused using a 100 mm focal length lens into the sample, which was mounted on a translation stage for scanning along the beam axis. The beam spot size at the focus was about 9 µm. For each sample, we performed the traditional Z-scans and spectral reshaping scans in succession. For the spectral reshaping trace, the beam was passed through a 1 nm band-pass filter (BPF, an angle-tuned interference filter) at 804 nm. The transmitted central portion of the LO was collected onto a photodiode (D3). The signal from the photodiode was time-gated with a boxcar integrator (SR250, Stanford Research Systems) and analyzed using a lock-in amplifier. A 5 kHz (LO rotation frequency) reference signal was provided to the lock-in amplifier by the pulse shaper electronics.

For the Z-scan traces, a 150 µm pinhole was placed in the far field and the light passing through this aperture was collected by a photodiode (D2). No spectral filtering was applied. The signal from the photodiode was also time-gated and analyzed using a
lock-in amplifier. A reference frequency of 5 kHz (same as the modulation frequency) was used to filter the output in the lock-in amplifier.

For the Z-scan measurements, we acquired two closed-aperture traces: a ‘high power’ trace and a ‘low power’ trace. With an assumption that the low power trace does not have any significant nonlinear component, it was used for normalization in order to compensate for linear sample distortions and variations in the input beam profile.

An illustration of the sample we used for these measurements is shown in Figure 2.5. The sample consisted of a 1 mm thick glass slide placed in a scattering medium (intralipid in water) contained in a 20 mm × 20 mm glass cuvette (Starna Cells). Varying the concentration of the intralipid solution changes the scattering strength of the medium. The incident power onto the sample was about 1.7 mW (pulse energy: 85 nJ) for the spectral reshaping measurements and about 4.9 mW (pulse energy: 245 nJ) for the Z-scan measurements.
2.3. **Comparison with Z-scan in scattering media**

To calibrate the scattering strength as a function of intralipid concentration, we performed a set of linear transmission measurements. For these measurements, we recorded only the unscattered light passing through the cuvette (the ballistic photons).

![Graph](image)

**Figure 2.6:** Relative linear transmission of the sample as a function of concentration of intralipid in water. The red line indicates the exponential fit. Also marked are the concentrations $C_0$, $C_1$ and $C_2$ used in Figure 2.7.

Figure 2.6 shows the linear transmission through the 20 mm cuvette as a function of intralipid concentration. The data plotted here is normalized by the no scattering case. The red line shows an exponential fit of the transmission data. Using Beer’s law, we can define normalized transmission as

\[ T = \exp(-a_0 CL), \]  

(30)
where $C$ is the concentration (in % volume) and $L$ is the length of the sample. For the fit, we can extract the exponential decay parameter $\gamma = a_0 L = 10.4$.

**Figure 2.7:** (a) Spectral reshaping scans showing the total signal as a function of focal position at concentrations $C_0$, $C_1$ and $C_2$. (b) Normalized Z-scan traces showing transmission as a function of focal position at concentrations $C_0$ and $C_1$. The red lines in the graphs are the corresponding fit traces. Concentrations $C_0$, $C_1$ and $C_2$ are defined in Figure 2.6. The spectral reshaping and Z-scan traces are averages of 9 scans performed at multiple transverse positions of the cuvette (to reduce the influence of possible sample impurities).
Figure 2.7 shows sample traces for the spectral reshaping and the Z-scan techniques at concentrations $C_0$, $C_1$, and $C_2$ of the intralipid solution. We can clearly see the qualitative differences between spectral reshaping (Figure 2.7(a)) and Z-scan (Figure 2.7(b)). The spectral reshaping technique provides a direct measurement of the nonlinear refraction coefficient [65, 68]. Therefore, the signal is proportional to the nonlinear coefficient at every position within the sample. From Figure 2.7(a), we can clearly see that glass exhibits a larger nonlinear refraction coefficient than water and the intralipid solution. We can also see that in a scattering sample, the SPM signal decays exponentially as a function of beam focus position, because scattering reduces the amount of light reaching the focal volume (the left side of the graph corresponds to the laser entrance side).

It is important to note that, even in the case of pure water ($C_0 = 0$) we observe a reduction in the SPM signal as we move the focus along the length of the cuvette. The effects contributing to this behavior are:

1) Dispersive broadening of the broadband femtosecond pulse in the solution.

2) Loss in laser power due to the linear refractive index mismatch at the solution/glass interfaces.

From Figure 2.7(b), we can see that the Z-scan traces exhibit the typical self-focusing/defocusing phenomena. In contrast to the absolute nature of the spectral reshaping signal, the Z-scan traces only show transmission changes at interfaces (air/glass and glass/solution). The changes are dependent on the relative change of
nonlinear refractive index of the materials. Note the asymmetry of the peaks at the air/glass and glass/air interfaces. Again, this is due to reduction in nonlinear signal because of refractive index mismatch and pulse dispersion.

Figure 2.8: Relative self-phase modulation signals in the glass slide as a function of scattering strength of the intralipid solution. Shown are spectral reshaping (top) and Z-scan results (bottom). The values and error bars are best fit values and standard fit errors, respectively. The fit was performed on an average of 9 traces and the resulting fit values were normalized by the value for pure water. Also shown as black lines in each graph is the expected signal drop-off based on a focal intensity reduction due to scattering.
2.3.1. Accuracy

To understand the effect of scattering on the two self-phase modulation (SPM) measurement techniques, we performed nonlinear least-square fits on the averaged signal trace (9 traces were averaged for each concentration). For the spectral reshaping technique, we approximated the trace within the cuvette as the sum of an exponential decay and a Gaussian peak centered at the position of the glass slide:

\[
\text{Signal}(z) = A_e e^{-\gamma z} + A_{\text{gauss}} e^{-\frac{(z-z_0)^2}{2\sigma^2}}.
\]  

(31)

With this model function, the Gaussian amplitude \(A_{\text{gauss}}\) measures the difference in nonlinear refraction coefficients between the glass and the surrounding solution. The traces in Figure 2.7 show the best fit curves as thin red lines. We extracted the values of the Gaussian amplitude for all the concentrations and normalized with the case of pure water (no scattering case). Figure 2.8 (top) shows the resulting data set (blue circles) plotted against the scattering strength.

Similarly, we extracted scattering dependence data from the Z-scan traces via nonlinear least-square fitting. The model function (assuming no nonlinear absorption) used in this case is (adapted from thick sample limit Z-scan in [58])

\[
T(z) = T_0 \left(1 - \frac{1}{4} \Delta \Phi \ln \frac{(1 + v_a^2)(9 + v_b^2)}{(9 + v_a^2)(1 + v_b^2)}\right),
\]  

(32)

where \(v_a\) and \(v_b\) are \(z\) dependent parameters corresponding to the entrance and exit points of the sample. They are defined as
Here, $z_R$ and $z_{aperture}$ are the Rayleigh length and distance of the pinhole from the sample, respectively. $L$ is the length of the sample.

We extracted the values the nonlinear refraction $\Delta \Phi$ for all the concentrations and normalized with the case of pure water. Figure 2.8 (bottom) shows the resulting data set (blue squares) plotted against the scattering strength. Please note that, for concentrations above $C_1$, we were not able to fit the Z-scan traces due to extremely low signal to noise ratios.

The black lines in Figure 2.8 indicate the expected decay calculated from a simple model accounting for scattering loss. The influence of scattering in the spectral reshaping case can be estimated as follows. If the sample is centered in the cuvette, the light reaching the focus is attenuated by half the length of the cuvette. Assuming the decay model in Eq. 30, the intensity of light reaching the sample can be written as

$$I_{sample} = I_{input} \exp(-a_o C L / 2).$$

(35)

Since the nonlinear signal scales quadratically with intensity [68], the signal at the focus is reduced by a factor $\exp(-a_o C L)$. The nonlinear signal generated is further attenuated
through half of the length of cuvette (from the sample to the exit surface). The output signal relative to no scattering case can be written as

\[
\frac{\text{Signal}_{\text{out}}}{\text{Signal}_{\text{pure water}}} = \exp(-a_o c L) \exp(-a_o c L/2) = \exp(-a_o c 3L/2).
\] (36)

Therefore, the exponential decay parameter for the spectral reshaping signal due to scattering can be written as \(\gamma_{\text{spectral}} = 3\gamma_o/2\), where \(\gamma_o\) is the decay parameter for linear transmission. An exponential curve with this parameter is plotted in the top panel of Figure 2.8. We can see that it matches the experimental measurements very well (note that this curve has not been obtained by fitting the SPM data). Therefore, the spectral reshaping technique can be described by a model that accounts for the loss of ballistic photons at the focal spot. The effect of scattering (other than the reduction of focal intensity) does not interfere with the actual measurement, indicating the accuracy of the spectral reshaping technique.

The influence of scattering can be estimated for the Z-scan measurements similarly. In a Z-scan trace the signal that is recorded is the power transmitted through the aperture divided by the transmission when the sample is out of focus (i.e. the linear transmission through the aperture). The absolute reduction of aperture transmission due to scattering scales similarly leading to a reduction of \(\exp(-3a_o c L/2)\). However, division by the linear transmission (scales as \(\exp(-a_o c L)\)) results in effective reduction of \(\exp(-a_o c L/2)\). The exponential decay parameter for the Z-scan signal due to scattering can be written as \(\gamma_{Z-\text{scan}} = \gamma_o/2\), where \(\gamma_o\) is the decay parameter for linear transmission.
An exponential curve with this parameter is plotted in the bottom panel of Figure 2.8. We can see that the Z-scan measurements decay differently from the expected values. Z-scan measurements of nonlinear refractive index are highly sensitive to the spatial profile of the transmitted beam. Any variation in beam profile will be recorded and cannot be disentangled from the nonlinear signal. In our experiments, we normalize the Z-scan traces with a corresponding ‘low power’ trace to account for the linear transmission changes, such as those caused by sample distortions. Even though the effect of scattering on the linearly transmitted beam can be accounted for, the nonlinear beam changes wash out equally, leading to a decrease in measured nonlinearity, therefore decreasing accuracy.

2.3.2. Reliability

To understand the reliability of the spectral reshaping technique and Z-scan technique in measuring nonlinear refraction, we plotted the ratio of fit value (nonlinear signal) to the statistical fit error (standard deviation) obtained by the nonlinear least-squares fit, for both measurement techniques in Figure 2.9. We can clearly see that the reliability of the fit decreases rapidly with increased scattering. We can also see that the scattering affects the measurement precision of the two techniques differently. While it is natural to expect an increase in uncertainty for increasing amount of scattering, the Z-scan method is substantially more susceptible to this effect. In the Z-scan technique, only very low concentrations (less than 0.03 cm$^{-1}$ scattering coefficient) allowed reliable
extraction of the nonlinear coefficient. In contrast, the spectral reshaping method showed an acceptable signal to fit error ratio even at high concentrations (where only about 5% of the input power is transmitted through the sample).

Figure 2.9: Ratio of best fit values for the nonlinear coefficient to the standard fit error for the spectral reshaping technique and Z-scan technique as a function of scattering strength of the intralipid solution. The fit was performed on a 9-trace average. Note that the incident power used for the Z-scan was about three times higher than the power used for the spectral reshaping technique.

In order to demonstrate that the increase in Z-scan’s uncertainty with scattering strength is not simply due to a loss of nonlinear signal, we investigated the direct influence of focal power on the Z-scan signal to fit error ratio. In a clean water sample, we varied the input power to reduce the focal intensity and compared the signal to error ratio with the scattering case (where the focal intensity reduction is due scattering losses).
Figure 2.10: Ratio of best fit values for the nonlinear coefficient to the standard fit error for the Z-scan technique with decreasing input power in comparison to increased scattering. The two x-axes are scaled to match the power reaching the sample at each point.

Figure 2.10 shows the ratio of nonlinear signal to fit error (standard deviation) for both the measurements mentioned above. The two x-axes are scaled to match the laser power reaching the focus in both cases. We can clearly see that the focal power reduction alone cannot account for the lower signal to error ratio in Z-scan measurements.

To further verify this finding we removed the far field aperture and recorded time-lapse images of the transmitted beam profile on a CCD camera. For scattering samples, we observed increasing fluctuations in the beam profile over a wide range of spatial and temporal scales. Since Z-scan measurements are dependent of the spatial
beam profile, they are washed out in high scattering cases. In comparison, for the spectral reshaping measurements in clean water cuvette the reduction of input power by a factor of 3 did not appreciably affect the measured signal to error ratio.

2.4. Mode-locked pulse shaper

The shaped pulse used in our spectral reshaping technique is shown in Figure 2.3. Such a pulse is created by amplitude and phase shaping in frequency domain. We use a 4-f configuration pulse shaper with an acousto-optic modulator (AOM) as a spatial light modulator [69]. A good discussion of various programmable pulse shaping methods is given in [70]. Here, we will summarize the 4-f acousto-optic pulse shaper and present an interferometric pulse shaper design which can limitedly shape each pulse coming out of a 80 MHz mode-locked laser system [71].

2.4.1. 4-f acousto-optic pulse shaper

Our frequency domain pulse shaper is setup in a 4-f configuration (illustrated in Figure 2.11). In the 4-f configuration, a diffraction grating is placed at the back focal plane of the input lens (L). The input beam is angularly dispersed by a diffraction grating into its frequency components; thereby, mapping color onto an angle. The input lens (of focal length $f$) collimates the frequency components effectively mapping angle onto a spatial axis position. Please note that each frequency component would be focused at the front focal plane (or Fourier plane) of the input lens. By placing a spatial light
modulator (fixed or programmable) at the Fourier plane, we can modulate the amplitude and/or phase of each frequency component of the input pulse with high resolution.

Figure 2.11: Illustration of the standard 4-f configuration. L_i and L_o are the input and output lenses of focal length f. The spatial light modulator (AOM in our case) is placed at the Fourier plane.

After modulation, the spatially dispersed frequencies are collected by an output lens (L_o) placed at distance f and mapped angularly onto a second diffraction grating. The second grating is placed at a distance f from the output lens (of focal length f) to recover the beam without any spatial and temporal distortions. If no modulation is introduced at the Fourier plane, this 4-f configuration should completely recover the input beam.

In our implementation, we use an all-reflective 4-f design (shown in Figure 2.12). This gives us two advantages:

1) No chromatic aberrations from the lenses.

2) Compact design due to folded optical path.
Figure 2.12: Schematic of the acousto-optic modulator (AOM) based 4-f pulse shaper, which uses radiofrequency (RF) pulses to impart modulation on the spectrum of a laser pulse. The TeO$_2$ crystal used is 4 cm in length. G: grating.

We use an acousto-optic modulator (AOM) as our spatial light modulator. In our AOM, a transducer is attached to a TeO$_2$ crystal. A radio-frequency (RF) pulse drives the transducer, which creates an acoustic wave that propagates through the crystal at a velocity of 4000 m/s. This creates a diffraction grating, which is effectively stationary with respect to femtosecond pulse. The momentary diffraction grating imparts a modulation onto the diffracted pulse spectrum. The undiffracted beam is rejected. The time profile of the RF pulse is mapped on the frequency axis of the femtosecond pulse. This allows arbitrary shaping of the femtosecond pulses by shaping the radio-frequency pulses.

Our acousto-optic crystal is 4 cm in length, which corresponds to 10 µs of acoustic wave transit time. The waveform transit time limits the update rate of the AOM.
Therefore, our current pulse shaper cannot shape pulses faster than 100 kHz. This fundamentally limits the data acquisition rate of our technique.

Using high repetition rate modelocked systems will improve the data acquisition rate of our spectral reshaping technique. Also, modelocked laser sources are relatively cheap and stable, easily tunable and require low maintenance. Therefore, we have developed an interferometric pulse shaper that is capable of shaping individual pulses from an 80 MHz modelocked laser system.

2.4.2. Interferometric pulse shaper

![Diagram of interferometric pulse shaper]

Figure 2.13: Illustration of the idea of the interferometric pulse shaper. The lower arm passes through a static pulse shaper. The upper arm passes through an AOM, which shifts the frequency of the beam such that the phase of the local oscillator rotates with respect to rest of the pulse spectrum. BS: beam splitter.

The basic idea of the mode-locked pulse shaper is to separately perform the phase rotation of the local oscillator and fixed pulse shaping of wings and combine the two fields interferometrically. Figure 2.13 illustrates the idea of the interferometric pulse shaper. The beam is split into two parts corresponding to the two arms of an
interferometer. The lower arm passes through a static pulse shaper, where a mask can be used to create a pulse shape with a hole in the center of spectrum. The upper arm passes through an acousto-optic modulator driven in a continuous mode to create the local oscillator. The frequency of this local oscillator beam is shifted such that the phase is continuously changing with respect to the wings of the beam (lower arm). The two arms are then combined to create the required pulse shape for the spectral reshaping technique.

**Figure 2.14:** Experimental setup of the common-mode interferometric pulse shaper. The acousto-optic modulator (AOM) acts as the splitter and combiner for the interferometer arms. The local oscillator (LO) arm is frequency shifted by the AOM. DG: diffraction grating; CL: cylindrical lens; M: mirror. Adapted from [71].

In our implementation (shown in Figure 2.14) the input 80 MHz pulse train is split into two arms by an acousto-optic modulator (AOM). The AOM is driven in the continuous mode and acts as a frequency shifter for the diffracted beam (local oscillator arm). Both the diffracted and un-diffracted beams are collimated and sent into a folded
4-f pulse shaper setup. Please note that the two beams are vertically separated. The beams are spectrally dispersed using a diffraction grating (DG) and focused using a cylindrical lens (CL) onto a large end mirror (M). The mirror is placed at Fourier plane to retro-reflected the beams to create a folded 4-f configuration. A mask for statically shaping the beams is placed close to the end mirror to obtain the highest spectral resolution. The undiffracted beam is shaped to create a hole in the center of the spectrum for the wings of the required pulse. Please note that both beams travel through the same optical components achieving interferometric stability.

![Diagram of a 4-f pulse shaper setup](image)

**Figure 2.15: Illustration of diffraction of beams during both the passes. The dashed lines correspond to the retro-reflected beams (vertically displaced by tilting the end mirror).**

The reflected beams are re-combined by the AOM (see Figure 2.15). At this point, the beams are diffracted for the second time. The combination of completely undiffracted and double-diffracted beams is picked off by a half-mirror. The end mirror is slightly titled to provide a vertical offset for the purpose of pick-off. If the AOM is driven at a frequency
\[ f_{RF} = m \cdot f_{Rep} + \Delta f, \]

where \( f_{Rep} \) is the pulse repetition rate, then the double-diffracted beam is frequency shifted by \( 2\Delta f \). Therefore, the phase of the local oscillator (double-diffracted beam) rotates at a frequency of \( 2\Delta f \) with respect to the wings (undiffracted beam) of the pulse spectrum. This creates the pulse shape required for the spectral reshaping technique without any AOM update rate restrictions.
3. Functional neuronal imaging

In the previous chapter, we discussed a spectral reshaping technique to measure self-phase modulation and demonstrated its robustness in scattering media. Here, we will discuss the application of this technique for non-invasive detection of neuronal activity in ex vivo hippocampal rat brain slices.

3.1. Introduction to neuronal imaging

The brain is the most complicated system known to us. Visualizing the information processing carried out by the central nervous system, and understanding simple processes in neurons (such as firing spikes and transmittance between neurons to generate the complicated cognitive functions of the brain) is of great value. For this purpose, we need a noninvasive technique that can map the rapid and complex dynamics of the neurons in the network with high spatiotemporal resolution and large field of view.

Neural activation patterns are generally studied by recording the associated electric potentials or currents using single or multiple electrodes. Electrophysiological recordings can provide high temporal and spatial resolution. However, this method is invasive and can only yield information locally at few specific points (which is a tiny fraction of the active population). Also, this technique cannot provide sufficient information about the interplay of signaling between different regions of the neural network.
At the other extreme, functional magnetic resonance imaging (fMRI) can non-invasively record indirect average activity of cortical regions. However, the spatial resolution of this modality is insufficient to observe neuronal activity on a cellular or sub-cellular level. In addition, since this method records the underlying slow hemodynamic response, it has limited temporal resolution of fractions of a second. In contrast to the above mentioned techniques, optical microscopy offers a combination of high spatial and temporal resolution economically.

### 3.1.1. Overview of current optical techniques

Linear near-infrared optical measurements of the oxygenation state of the tissue have been used for *in vivo* functional neuronal imaging [72]. These measurements make use of differences in linear absorption between oxyhemoglobin and de-oxyhemoglobin around the 800 nm spectral region. Like fMRI, near-infrared methods basically record the underlying hemodynamic response; therefore, they are limited in temporal resolution and essentially give an indirect measure of neuronal activity. Neuronal firing has been correlated with rapid structural changes, which change scattering and birefringence properties of the neuron on a sub millisecond time scale [73-75]. Measuring these changes can provide a direct measure of neuronal activity; but the linear response of these changes makes them less effective due to high scattering in the brain tissue.

Fast functional contrast for neuronal imaging can also be obtained by using exogenous dyes [76]. Calcium-sensitive dyes alter their fluorescence properties
depending on the intracellular calcium concentration. This makes it possible to visualize calcium ion flux within the neuron, which is a measure of action potentials in the neuron. Voltage-sensitive dyes are membrane-bound chromophores that change their optical properties in response to changes in membrane potential [77]. Neuronal activity is linked to changes in the electrical potential across their membranes. Therefore, voltage- and calcium-sensitive dyes give a relatively direct measure of neuronal activity. Also, simultaneous imaging of the activity of multiple neurons is possible using these dyes. However, the short wavelength light required to excite these dyes only penetrates a few tens of microns into tissue.

Nonlinear processes have greatly improved the applicability of dyes in monitoring neuronal activity. Nonlinear imaging methods take advantage of the quadratic dependence of signal on laser intensity for localized excitation and thus give higher spatial resolution [19, 21, 78]. Also, the longer wavelengths (typically in the near IR) can provide higher penetration depths due to lower tissue absorption and scattering [27, 28, 79]. Two-photon fluorescence microscopy of voltage-sensitive [80-82] and calcium-sensitive [83, 84] dyes has provided high resolution mapping of cortical activity in vivo and is widely used by neuroscientists. Second-harmonic generation microscopy has also been used to image the membrane potential using voltage-sensitive dyes [33, 85]. However, the use of external contrast agents often damages the neurons and may affect the excitability of neurons. Also, the field of view is restricted to an area around the injection pipette.
Using an intrinsic nonlinear contrast like self-phase modulation (SPM) or cross-phase modulation can give the advantages of nonlinear microscopy without using an external contrast agent. SPM is known to be sensitive to molecular structure, local anisotropy and chemical environment and thus can be significantly altered during neuronal firing because neurons undergo structural changes during firing events [74, 86-88]. For example, during action potential propagation molecular dipoles are known to re-orient across the membrane [89]. Also, it is well known that influx of molecules due to osmolarity changes across the membrane (corresponding to the firing events), lead to cellular swelling. Warren and coworkers have recently developed a spectral reshaping technique to sensitively measure SPM in scattering media (see chapter 2). Measuring nonlinear phase contrast signatures of neuronal activity opens up a new method for non-invasive functional neural imaging.

3.1.2. Neurobiology

The neuron is the basic building block of the central nervous system. Neurons are specialized cells that transmit information by electrical and chemical signaling. Signaling occurs through specialized connections with other cells called synapses.

During a chemical signaling event, the neuron releases a chemical called a neurotransmitter that binds to the receptors located in the membrane of the target cell. Depending on the receptor, the neurotransmitter may either excite or inhibit the
postsynaptic neuron. Glutamate is the most common excitatory neurotransmitter and GABA is the most prevalent inhibitory neurotransmitter.

Electrical signaling occurs by inducing voltage changes via gap junctions. The gap junction is a specialized channel connecting the cytoplasms of two neurons directly. Gap junctions are capable of passing ions and molecules rapidly and thus are used in situations that require fastest response possible.

3.1.2.1. Neuron

![Figure 3.1: Schematic showing the structure of a neuron [90].](image)

Various kinds of neurons with specialized responses exist in the nervous system. The three important parts of a typical neuron are described below:

- Soma: The central part of neuron which contains the nucleolus and various cytoplasmic organelles is called the soma (or cell body). The soma is not involved
actively in the transmission of the neural signals. It is usually about 10 to 25 μm in diameter.

- Axon: An axon is an extension of the cell body which transmits signals away from the cell body. Axons can sometimes extend thousands of times the size of the soma in length, but are only about a micron thick.

- Dendrites: They are thin structures that extend from the cell body forming a tree shaped projection. They receive information from other neurons via synapses and transmit it to the soma.

3.1.2.2. Hippocampal slice

![Hippocampal slice diagram]

Figure 3.2: Image of a rat hippocampal slice showing information pathway [91].
*DG: dentate gyrus; CA: cornu ammonis.*

The hippocampus is a major part of the brain, located in the medial temporal lobe. It plays major a role in memory (both short and long term) and spatial navigation. Figure 3.2 shows a stained hippocampal slice from a rat brain. The hippocampus contains two adjoint structures in a double C configuration: cornu ammonis (CA) and dentate
gyrus (DG). Information flows unidirectionally from the cortex to the DG via the perforant pathway, then to the CA3 region and then to the CA1 region via Schaffer collaterals. Neurons are packed in parallel along this pathway. In our work, we used cultured hippocampal rat brain slices. Most of the SPM imaging was performed on the pyramidal cells in the CA1 region.

### 3.2. Experimental design

The Warren group has previously demonstrated intrinsic SPM signal changes in CA1 region of rat hippocampal brain slices during glutamate induced neuronal activation using modest optical powers [92, 93]. SPM changes recorded in the pyramidal neurons were about several tens of percent during glutamate activation compared to only few percent changes away from the cell layer. However, these initial experiments were performed in bulk conditions using slow chemical stimulation. The microscope used for these experiments had limited spatial and temporal resolution. They were performed by scanning the sample, which is slow and also makes the system unusable in conjunction with electrophysiological techniques.

To validate these intrinsic signatures with established measurement techniques like electrophysiology, we developed a laser scanning microscope capable of sub-millisecond temporal resolution and sub-micron spatial resolution. With this improved setup, we tried to correlate the intrinsic nonlinear signatures with the single-cell patch
clamp technique [94, 95] (an invasive but established technique to record neuronal activity).

**Figure 3.3:** Experimental setup used for self-phase modulation imaging. BPF: bandpass filter; AOM: acousto-optic modulator; APD: Avalanche photodiode; PBSC: polarizing beam splitting cube.

Figure 3.3 shows a schematic of the experimental setup used for SPM imaging of neuronal activity. A complete description of the spectral reshaping technique [65] and its experimental setup is given in chapter 2. We use a continuous wave pumped regenerative amplifier (RegA 9000, Coherent) seeded by a mode-locked Ti:Sapphire oscillator (Vitesse, Coherent) with a center wavelength of 800 nm as our laser excitation source. These ultrafast pulses are shaped by an acousto-optic modulator based spectral
domain pulse shaper [69]. A folded prism pair arrangement is used for coarse dispersion compensation. The pulse shaper can be used for finer dispersion compensation by simply maximizing the nonlinear signal at the focus or reducing the pulse width (measured using a home built auto-correlator).

The shaped pulse with a rotating local oscillator (5 kHz) in the spectrum is guided into a laser scanning microscope. A complete description of this home built microscope can be found in [96]. The key features of the microscope are summarized below:

1) Raster-scanning of the beam at the focus is performed using a set of galvo scan mirrors (6210H, Cambridge technology). The line scan speeds can go up to 1.7 KHz. So frame rates (128 x 128) of about 13 fps are achievable technically, but we are limited by other factors like signal to noise and repetition rate of laser beam.

2) Scanning in the Z directions is performed by stepper motors attached to the focusing unit of the objective and the condenser. The sample is mounted on a XY motor stage.

3) Back-scattered photons can be collected by using a polarizing beam splitting cube (PBSC) without interfering the regular acquisition of the transmitted light. Though SPM imaging is possible in epi-mode, the experiments discussed here were performed in transmission mode for signal to noise concerns. Epi-mode detection was optionally used to collect fluorescence using a photo-multiplier tube (PMT). The light reaching the PMT (HC120-15, Hamamatsu) was filtered first using a dichroic (700 nm short pass, Omega Optics) and then using a 280 nm band pass filter (500 nm peak, BG39, Schott).
4) Micro-manipulators are mounted on the XY sample stage to control the electrodes used to perform the whole-cell patch clamp experiments. Simultaneous acquisition of SPM and neuron potentials is possible.

5) Proper illumination of the brain slice is critical to perform patch-clamp experiments. Neurons are very transparent; therefore it is difficult to identify the different CA regions of the hippocampal brain slices. We used Koehler illumination combined with an off-center mask to achieve gradient contrast (oblique illumination). The gradient contrast is necessary to see neuronal structure in the brightfield. Illumination setup needs to be carefully optimized every time to obtain the best contrast. A detailed explanation on the illumination setup is given in [96].

The transmitted light, collected by the condenser is directed into the detection setup. A glass slide is used to pick a part of the beam to measure linear transmission signals. Since neurons undergo transmission changes during firing events, the nonlinear signal changes are always compared against the linear changes. A tunable bandpass filter (1 nm) is used to reject the wings of the pulse and leave only the central part of the spectrum. We use an avalanche photodiode (APD) to collect the light containing spectral reshaping signal. The signal from the detectors is fed into a boxcar integrator (SR250, Stanford Research Systems) for individual time gated integration. It is important to note that the boxcar we used runs at 20 kHz, which is the real limitation of the acquisition speed in this system. The amplified laser system is capable of delivering pulses at 250
kHz rate. For the SPM imaging experiments a lock-in time constant of 1 ms was generally used.

The experiments for measuring SPM signatures of neural activation were performed in rat hippocampal brain slices. The slices with an average thickness of 300-400 μm were prepared and mounted on membranes and were cultured for 1–3 weeks. For each experiment, we mounted a slice on a glass cover slip within a horizontal open flow cell (flow rate ~1–2 ml/min). The sample setup and design is shown in Figure 3.4(a). The slice was completely immersed in artificial cerebrospinal fluid (ACSF) buffer bubbled with O₂ 95% /CO₂ 5% at all points of time.

Figure 3.4: (a) Sample setup showing the open flow cell. Glutamate can be injected into the flow at any point of time. ACSF: artificial cerebrospinal fluid. (b) Schematic of the hippocampal brain slice indicating the laser scanning direction in glutamate activated experiments.
During data acquisition the laser beam was repeatedly scanned across the CA1 neuronal layer to obtain the time course of the SPM signal at all points in the line (Figure 3.4(b)). At specific times, we switched the inlet of the flow cell from pure buffer solution to buffer solution containing glutamate (200 µM) to stimulate neuronal activity.

3.3. Chemically activated neuronal signatures

3.3.1. Single neuron signatures

Figure 3.5: Experimental results obtained with a 10X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations in the CA1 region. The average power used was about 400 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (c) show the signals when scanning away from the neuron layer. Column (b) shows the SPM spike when we scan on the cell body layer.
To reproduce the signatures seen in the previous work [92], we started out with a 10X objective and then moved on to higher NA objectives, while reducing the average power to avoid any photo damage to the tissue by maintaining similar peak intensities across various resolutions.

Figure 3.5 shows the time courses of SPM signal and linear transmission at three different locations across the CA1 neuronal layer in a hippocampal slice. The experiments were performed with a 10X water immersion objective (0.3 NA, Model: UMPLFLN, Olympus) using 400 µW (20 nJ per pulse) of average power. The beam spot size was about 4 µm. The measurements performed about 100 µm away from the cell body layer (the width of the cell body layer is about 200 µm) are shown in columns (a) and (c), and the measurement performed on the cell body layer is shown in column (b). When we measure the time course on the cell body layer, we can see a peak in the SPM signal after injecting glutamate. Glutamate is an excitatory neurotransmitter, therefore chemically activates the whole layer of neurons. The ratio of SPM activation peak height to the pre-activation baseline signal is about 29%. In contrast, the linear transmission only changes by 5%, indicating that the changes in SPM signal are not simply due to changes in linear scattering/re refractive index. Compare this to measurements outside the cell layer, where we do not see any significant changes in the SPM signal. Therefore, the nonlinear changes are localized to the neuronal layer.

Next, we performed the glutamate activation experiments using a 20X water immersion objective (0.5 NA, Model: UMPLFLN, Olympus). Figure 3.6 shows the time
courses of SPM signal and transmission at three different locations across the cell layer. The beam spot size was about 2 µm. Again, we can see a peak in the SPM signal after injection of glutamate, while scanning over the cell body layer (Figure 3.6(b)).

**Figure 3.6**: Experimental results obtained with a 20X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations in the CA1 region. The average power used was about 100 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (c) show the signals when scanning away from the neuron layer. Column (b) shows the SPM spike when we scan on the cell body layer.

With the 10X and 20X objectives, we were not able to see individual neurons. Therefore, line scanning across the cell body layer was performed. With the 40X objective (0.8 NA, Model: LUMPLFLN, Olympus), we were able to scan across the cell body of a single neuron and localize the signal changes to the cell body.
Figure 3.7: Experimental results obtained with a 40X objective, showing the time courses of SPM signal (top) and linear transmission (bottom) at three different locations across a single neuron (brightfield image is shown at the top). The average power used was about 25 µW. The bars denote the time when glutamate was injected into the flow cell. Columns (a) and (b) show the signals when scanning inside the neuron cell body. Column (c) shows the time course when we scan outside the neuron.

Figure 3.7 shows the time courses of SPM signal and linear transmission at three different locations across a single neuron in the CA1 layer. A brightfield camera image of the neuron is shown at the top. The black spot in the image indicates the center (for reference). The neuron is located on the left half of the image. The beam spot size was about 1 µm. Columns (a) and (b) show the signals inside the neuron cell body. We can
see changes in SPM signal after the injection of glutamate. In contrast, we cannot see any significant changes in the SPM time course outside the cell body (Figure 3.7(c)).

It is important to note the trend of SPM signal changes with increasing spatial resolution. From Figures 3.5 to 3.7, we can see that the smaller spot sizes correspond to smaller nonlinear signal changes relative to linear transmission. This would indicate that at least a part of the nonlinear signal is contributed by bulk effects occurring during chemical activation.

3.3.2. 2D self-phase modulation signatures

![Image](attachment:Figure_3.8.jpg)

**Figure 3.8:** (a) Brightfield image of a neuron. The dashed line indicates the cell boundary. (c) Brightfield image without the dashed line. (b) SPM peak to baseline ratio image of the neuron shown in the brightfield image. (d) Corresponding linear transmission peak to baseline ratio image. All the scale bars are 10 μm.

To further investigate the origin of the SPM signal changes i.e. to determine whether the nonlinear change is in soma, membrane, axon or some other part of the
neuron, we performed 2-dimensional image scans of the chemically activated neuronal response. Instead of line scanning across the neuron, we repeatedly acquired SPM images to obtained time course of the signal at each pixel of the image.

Figure 3.8 shows the SPM changes during chemical activation in 2 dimensions. Figure 3.8(c) shows a brightfield image of a neuron in the CA1 layer of the hippocampal slice. Figure 3.8(a) shows the brightfield image with a dashed line indicating the boundary of the cell. From the time courses we acquired for each pixel in the image, we calculated the ratio of the SPM peak after glutamate activation to the baseline. The baseline is defined as the average SPM signal before the glutamate injection. Figure 3.8(b) shows image of the SPM signal peak to baseline. The dashed line indicates the cell boundary. We can see that SPM changes near the boundary on one side of the cell. Figure 3.8(d) shows the corresponding changes in the linear transmission. We can see that the transmission changes are very small compared to the SPM changes.

3.4. **Patch clamp measurements**

In the previous section, we have demonstrated SPM signatures of neuronal activity with single cell spatial resolution. In these experiments, the neuronal activity was induced by massive chemical stimulation using glutamate. Such stimulation could lead to undesired effects such as cell swelling. Also, chemical activation is slow with the process taking several minutes. A better approach would be use direct electrical stimulation using electrodes. Using an electrical stimulation method will give us better control over
the location, strength and time scale of the activation. It would enhance our temporal resolution to sub-millisecond range. We synchronized the SPM acquisition with electrical stimulation and performed correlation experiments between the SPM response and simultaneously recorded patch clamp data. This correlation with established electrophysiological techniques will be an important step in investigating the origin of functional SPM contrast in neurons.

3.4.1. Single cell clamp technique

The single cell patch clamp technique has been widely used by neurobiologists to study single or multiple ion channels in the neurons [94, 97, 98]. It is considered the gold standard for intracellular recording of electrical activity. Several variations of the patch clamp techniques are used depending on the purpose of the study. We will be using the whole cell recording technique, in which the cell the membrane is ruptured to provide direct access to the intracellular space.

Patch clamping involves sealing a glass micropipette (open tip of diameter 3-5μm) as an electrode against the surface of the neuron (Figure 3.9). We use micropipette puller (PC-10, Narishige Scientific Laboratory Instruments) to make a glass pipette from standard capillary glass. The pipette tip diameter can be controlled by the temperature settings of the puller. The diameter at the end of the tapered tip needs to be in the 3 to 5 μm range to obtain the suitable resistance. The pipette resistance, $R_s$, should be around
3 to 8 MΩ for a good patch. We use a coarse pulling temperature set point of 62.7 °C and a fine pulling temperature set point of 42.8 °C.

**Figure 3.9:** Micropipette containing the silver electrode is sealed to cell membrane for recording ion channels [99].

The interior of the micropipette is filled with a solution matching the ionic composition of the cytoplasm for whole-cell recording. We use about 1.5 µL of the internal solution (recipe can be found in [100]). We also add a fluorescein dye (4 mM) to the patch pipette internal solution; so that we can simultaneously take fluorescence data to locate the position we are scanning. A chlorided silver wire is placed in contact with the internal solution to conduct electrical current to the patch clamp amplifier (Multiclamp 700B, Axon CNS, Molecular Devices). The pipette is mounted on a micromanipulator (MPC-200, Sutter) with XYZ control. A positive pressure (about 10 to 15 in H₂O) is generally applied to keep the tip clean from any debris when it is moved into the buffer solution. Pressure must be carefully selected, because applying too much
pressure would push the internal solution along with the dye into the buffer, whereas applying too low pressure would make the pipette tip susceptible to blockage.

The first step in a patch clamp experiment is to mount the slice in the open flow cell containing the buffer solution (ACSF). After that the neuron of interest is located using the brightfield camera. Then the pipette with the internal solution is moved into the camera field of view and set about a millimeter above the slice surface. While moving the pipette towards the target cell, the objective needs to be simultaneously moved along with it. Care should be taken to avoid scratching the slice surface. When the tip is near the slice surface, the pressure needs to be increased to about 35 (in H₂O). The high pressure is useful to clean tissue in the path to the cell membrane and also cleans the surface of the cell prior to contact.

![Figure 3.10: Seal formation between the pipette tip and the membrane [101].](image)

The micropipette is then pressed against the target cell membrane and gentle suction is applied to assist in the formation of a high resistance seal between the glass
and the cell membrane (Figure 3.10). If a seal starts forming successfully, the pipette resistance, $R_s$, goes up to a few hundred MΩ because the membrane acts as an insulator. When the resistance reaches about 1 GΩ, the required seal is established. After that, a sharp suction is applied to break through the cell. Consequently, a small patch of membrane in the electrode tip is ruptured, leaving the electrode sealed to the rest of the cell as seen in Figure 3.11. This provides electrical access to the intracellular space of the cell. After a successful patch, the cell currents and potentials can be monitored or recorded using a patch clamp amplifier in voltage clamp or current clamp mode.

![Diagram of cytoplasm and pipette interior](image)

**Figure 3.11: By applying a sharp suction the cell membrane is ruptured and provides access to the intracellular space [102].**

After the formation of patch, the dye (Fluorescein) in the internal solution starts diffusing into the cell. It generally takes up to 5 minutes for the dye to completely diffuse into the whole cell. At this point, it is advisable to take two-photon fluorescence images to locate the position of the cell relative to laser focus. After finding the position on the cell, simultaneous recording of electrical activity and SPM signals can be performed.
3.4.2. Simultaneous single cell self-phase modulation and patch clamp measurements

In the simultaneous SPM and patch clamp measurements, we scanned the laser beam across the patch clamped neuron (scan range: 25 to 35 μm) and at each pixel (10 to 20 pixels) we park the laser beam for about 1000 to 1500 ms to measure SPM signal while electrically stimulating the neuron by sending an electrical pulse train in the current clamp mode. A typical pulse train would have the following parameters: pulse width = 4 ms, amplitude = 2V, repetition rate: 80 Hz.

![Figure 3.12: Time course of the neuron potential in current clamp mode during an 80 Hz pulse train [96].](image)

The time course was divided into three blocks; no pulse is sent during the first (pre-activation) and the last (post-activation) parts, during these periods (generally 300 to 500 ms) the potential stays at the resting potential (-65 mV). These periods with no stimulation provide us the base line activity. In the middle block (activation period), we
send a pulse train for about 500 ms through the electrode. Figure 3.12 shows a typical electrical recording during the stimulation. The smaller spikes (potentials varying from -70 mV to 60 mV) shown in Figure 3.12 are direct electrical responses due to the pulse train and the larger spikes (potentials varying from -70 mV to about 80 mV or higher) are the actual firing peaks.

Figure 3.13 shows experimental measurements of simultaneous electrical and optical signals [103]. We used a 40X water objective (0.8 NA) for these experiments. We compared the data acquired inside the neurons with the data acquired outside the neurons. Figure 3.13(a) shows the time course of percentage SPM change (relative to the baseline) at points outside (black) and inside (red) the neuron during stimulation by an electrical pulse train applied using the patch electrode. Figure 3.13(c) shows a representative electrical recording corresponding to the stimulation region. When the beam is parked at points inside the neuron (red), there is a small SPM change (about 1%) that correlates with the stimulation response seen in the patch electrode recording. We cannot see any significant SPM changes when the laser beam samples outside the neuron (black). It is important to note that the data shown in Figure 3.13 is averaged over 114 traces for the signals outside the neuron and 43 traces for the signals inside the neuron. The traces were obtained from a total of seven different neurons. Also, Figure 3.13(b) shows that there are no changes in transmission corresponding to neuronal activation, both inside and outside the cells.
Figure 3.13: (a) Temporal dependence of the SPM change in a single neuron during stimulation by an electrical pulse train, averaged over many datasets and after application of a low pass 50Hz filter. The black and red graphs correspond to points outside and inside the neuron. (b) Corresponding transmission changes. (c) Sample electrical recording from the patch clamp amplifier in current clamp mode.

We performed various simultaneous SPM signal and electrical response recording experiments with a wide range of activation parameters. We used stimulation pulse
widths ranging from 1 ms to 500 ms and pulse repetition rates ranging from 80 Hz to 1 Hz. Different power levels were also used; however, it is important to note that the pulse energies above 1.5 nJ using a 40X objective generally damages the neurons and consequently breaks the patch.

We also performed a set of experiments using an extra-cellular stimulator, which stimulates a bulk of neurons at once. In these experiments, we placed a concentric bipolar electrode at Shaffer-collateral axons (about 200 µm away from the CA1 region). Schaffer collaterals are axon collaterals given off by the CA3 pyramidal cells which extend onto the CA1 dendrites making excitatory synapses. Information flows from CA3 to CA1; therefore, activating CA3 neurons induces action potentials in the CA1 neurons. We patched a neuron in the CA1 region and performed simultaneous measurements of SPM signal and electrical activity in response to activation by the extra-cellular stimulator. However, single-cell SPM responses remain inconclusive because of the low signal-to-noise ratio. Therefore, we have not yet validated the SPM signatures with the established measurement techniques.

In the next chapter, we will describe the implementation of a two-color version the spectral reshaping technique using modelocked systems, that reduces the unwanted linear background and improves the acquisition speed of our nonlinear phase modulation measurements. This implementation measures cross-phase modulation (XPM), the dual-color analogue of SPM. With this improved setup, we performed similar nonlinear phase modulation measurements and electrophysiology recordings using the
patch clamp technique in electrically activated neurons. However, thus far the improvement in sensitivity (specifically signal to noise) was insufficient to see conclusive correlations between nonlinear signatures and electrical recordings or to illuminate the underlying mechanism of previously observed SPM changes at a single neuron scale.
4. Cross-phase modulation

In chapter 2, we described a spectral reshaping technique to measure self-phase modulation using modest power levels. In this chapter, we will describe the extension of spectral reshaping technique to use two colors [104] and demonstrate biological imaging with this new technique.

4.1. Motivation

In the self-phase modulation (SPM) measurements using the spectral reshaping technique, we imprint a phase rotating local oscillator onto the spectrum of the pulse. After the pulse interacts with the material, we look for small changes caused by nonlinear interaction in the same region of the spectrum. Due to technical limitations i.e. limited spectral resolution of the acousto-optic pulse shaper and the bandpass detection filter, we observe a residual amplitude modulation resulting in signal even in the absence of SPM. This creates a linear background in our measurement system. The background can be taken into account by relative measurements. However, the noise associated with the background signal cannot be removed. Due to the linear scaling of the background (relative to the nonlinear scaling of the SPM signal), it eventually becomes insignificant at high laser powers. However, such high power levels are not acceptable in biological imaging. To suppress the background in nonlinear phase modulation measurements, we extended the spectral reshaping technique to use two colors and implemented sensitive detection using modulation transfer technique [40, 105].
4.2. Two color spectral reshaping technique

By using pulses of two different wavelengths, we can separate the pulse modulation (phase rotation in case of SPM measurements) from the spectrally filtered detection, such that the signal is nonlinearly transferred between the two colors. In the case of SPM, the phase modulation is self-induced. When using two colors, one pulse induces a phase modulation on the second pulse. This property is called cross-phase modulation (XPM), the dual-color analogue of SPM.

The XPM effect can be illustrated by using coupled pulse propagation equations [50, 51, 106]. In our implementation, we use two pulse trains of different wavelengths in a pump-probe configuration. The coupled pulse propagation equations for both the pump and the probe pulses (considered as plane waves) in slowly varying envelope approximation can be written as

\[
\frac{\partial A_{pu}(z, \tau)}{\partial z} + \frac{\alpha_{pu}}{2} A_{pu}(z, \tau) + \frac{i \beta_{pu}}{2} \frac{\partial^2 A_{pu}(z, \tau)}{\partial \tau^2} = \left( \frac{\alpha_2}{2} - \frac{i n_2 \omega_{pu}}{c} \right) \left( |A_{pu}(z, \tau)|^2 + 2 |A_{pr}(z, \tau)|^2 \right) A_{pu}(z, \tau),
\]

(38)

\[
\frac{\partial A_{pr}(z, \tau)}{\partial z} + \frac{\alpha_{pr}}{2} A_{pr}(z, \tau) + \frac{i \beta_{pr}}{2} \frac{\partial^2 A_{pr}(z, \tau)}{\partial \tau^2} = \left( \frac{\alpha_2}{2} - \frac{i n_2 \omega_{pr}}{c} \right) \left( |A_{pr}(z, \tau)|^2 + 2 |A_{pu}(z, \tau)|^2 \right) A_{pr}(z, \tau).
\]

(39)

Here, \( A_{pu}(z, \tau) \) and \( A_{pr}(z, \tau) \) are the pump and the probe electric field envelopes, respectively. \( \alpha_j \) and \( \beta_j \) are the linear absorption and group velocity dispersion coefficients, respectively; where, \( j = pu \) for pump and \( j = pr \) for the probe. The electric
field is written as function of time, \( \tau = t - z/v_g \), measured in the reference frame of group velocity \( v_g \). Please note that group velocity mismatch is neglected. \( n_2 \) and \( \alpha_2 \) are the nonlinear refraction and absorption coefficients, respectively.

In our cross-phase modulation measurement, we look for changes in the probe spectrum induced by the pump pulse. Therefore, we will only consider the evolution of the probe pulse. Neglecting linear absorption and group velocity dispersion, the effective propagation equation for the probe pulse can be written as

\[
\frac{\partial A_{pr}(z, \tau)}{\partial z} = \left( i\eta - \frac{\alpha_2}{2} \right) |A_{pr}(z, \tau)|^2 A_{pr}(z, \tau) + 2 \left( i\eta - \frac{\alpha_2}{2} \right) |A_{pu}(z, \tau)|^2 A_{pr}(z, \tau),
\]  

(40)

where, \( \eta = n_2\omega_{pr}/c \). The first term on the right side of Eq. 40 corresponds to the self-phase modulation and two-photon absorption effects occurring in the probe [65]. The second term corresponds to the cross-phase modulation and sum-frequency absorption processes.

Assuming a uniform nonlinearity, a first order expression for the approximate change in amplitude of the probe can be written as

\[
\Delta A_{pr}(\tau) \approx z \left( i\eta - \frac{\alpha_2}{2} \right) |A_{pr}(\tau)|^2 A_{pr}(\tau) + 2z \left( i\eta - \frac{\alpha_2}{2} \right) |A_{pu}(\tau)|^2 A_{pr}(\tau),
\]  

(41)

where \( z \) is the length of interaction. Considering frequency independent nonlinear coefficients, Eq. 41 can be Fourier transformed to obtain the change in the probe pulse spectrum. A small change in the probe spectrum can be written as
Here, tilde (~) denotes the Fourier transform.

\[
\Delta \tilde{A}_{pr} \approx \Re \left( i \eta - \frac{\alpha_2}{2} \right) |\tilde{A}_{pr}|^2 \tilde{A}_{pr} + 2 \Re \left( \eta - \frac{\alpha_2}{2} \right) |\tilde{A}_{pu}|^2 \tilde{A}_{pr}.
\]

(42)

Figure 4.1: Pump and probe pulse spectrums used in the two-color spectral reshaping technique. The pump pulse train is amplitude modulated and a static phase shift is imposed onto the center of the probe spectrum. Modulation is transferred from pump to probe during nonlinear interaction.

In our two-color spectral reshaping technique, a small part of the probe spectrum (about 2-3 nm in the center part) is phase shifted to create a static phase reference, called local oscillator (LO). Figure 4.1 shows the probe pulse spectrum with the local oscillator, whose phase is shifted by \( \theta \) with respect to the rest of the probe spectrum.

The phase of the local oscillator can be controlled by a pulse shaper. The static local oscillator is not large enough to generate any significant nonlinear component.

Therefore, it is assumed that the local oscillator does not contribute to the change in the spectrum \( \Delta \tilde{A}_{pr} \), defined in Eq. 42. However, the static local oscillator interferes with the
pump-probe induced nonlinear polarization. The probe spectrum after the interaction, can be written as

$$\tilde{A}_{out} = \Delta\tilde{A}_{pr} + \tilde{A}_{LO}e^{-i\theta}. \quad (43)$$

In our implementation, we impose an amplitude modulation at the reference frequency $f_o$ on the pump pulse train. This modulates the pump induced nonlinear polarization in the probe spectrum at the frequency $f_o$. The self-induced nonlinear polarization in the probe spectrum will not have a modulation at frequency $f_o$. We will filter our signal to only measure components at the reference frequency. Therefore, we will consider only changes in the spectrum at the reference frequency. The first term on the right side of Eq. 42 is the self-induced part. Neglecting the self-induced phase modulation, Eq. 43 can be written as

$$\tilde{A}_{out} = 2\pi \left(i\eta - \frac{\alpha^2}{2}\right) |A_{pu}|^2 A_{pr} + \tilde{A}_{LO}e^{-i\theta}. \quad (44)$$

This is the total electric field amplitude in the static local oscillator position of the spectrum. All the spectral components outside this local oscillator position are rejected using a band-pass filter, before sending the probe beam into a photodiode.

For simplicity, assume that $\tilde{A}_{LO}$ and $|A_{pu}|^2 A_{pr}$ are real. Then the total spectral intensity detected by the photodiode can be written as
The power detected by the photodiode is analyzed using a lock-in amplifier at the reference frequency $f_0$. The total signal measured by the lock-in (considering only the components at the reference frequency) can be written as \[ S_{\text{lock-in}} \approx -4z A_{\text{LO}} A_{\text{pu}} \sin \theta \frac{\alpha^2}{2} \cos \theta. \] (46)

Here, $\theta$ is the phase of the local oscillator (static reference). Choosing the reference phase as $\theta = \pi/2$ accesses the nonlinear refractive component (cross-phase modulation (XPM)), while a reference phase of $\theta = 0$ accesses the absorptive component (sum-frequency absorption). If both $A_{\text{LO}}$ and $A_{\text{pr}}$ are constant fractions of the input probe power, then $A_{\text{LO}} A_{\text{pu}} A_{\text{pr}} \propto P_{\text{pr}} P_{\text{pu}}$, where $P_{\text{pr}}$ and $P_{\text{pu}}$ are the probe and pump input powers, respectively. Therefore, the XPM signal scales as the product of the input powers.

Please note that, unlike the self-phase modulation (SPM) measurements (see section 2.1 for description), the phase of the local oscillator is kept static in the XPM case. Therefore, the probe pulse is untouched by the amplitude modulator. Consequently, the probe beam does not have any component at the reference frequency $f_0$ before the nonlinear interaction. This means that the entire signal measured by the lock-in is

\[
|\tilde{A}_{\text{out}}|^2 = |\Delta \tilde{A}_{\text{pr}}|^2 + |\tilde{A}_{\text{LO}}|^2 - 2z \left( i \eta + \frac{\alpha^2}{2} \right) |A_{\text{pu}}|^2 A_{\text{pr}} \tilde{A}_{\text{LO}} e^{-i\theta} \\
+ 2z \left( i \eta - \frac{\alpha^2}{2} \right) |A_{\text{pu}}|^2 A_{\text{pr}} \tilde{A}_{\text{LO}} e^{i\theta}. \] (45)
induced by the pump. Thus, the technique is able to achieve virtually background free detection.

4.2.1. Simulation of lock-in signal

Figure 4.2: Simulated XPM signal plot illustrating the effect of local oscillator (LO) position and width. X and Y axes indicate LO width and LO position relative to the bandwidth (FWHM) of the probe pulse.

To understand the effect of local oscillator position and size on the XPM signal, we simulated the XPM measurement. We numerically computed the lock-in signal shown in Eq. 46 with typical parameters. Figure 4.2 shows a plot of XPM signal dependence on local oscillator (phase reference) position and size (width). It is important to note that a detection band pass filter of size (spectral slice) equal to the width of local oscillator (LO) is assumed. XPM signal detected by lock-in is proportional to the size (spectral width) of the LO; therefore, the signal increases corresponding to increasing LO width. However, increasing LO width also decreases the power in the wings of the probe resulting in a
decrease of induced spectral changes. If we consider a single LO position, the lock-in signal increases up to an extent with increasing LO width and then goes down with further increase in the LO width. From Figure 4.2, we can also see that the LO position at the center of the spectrum gives the strongest signal. When the LO is located at the center of the spectrum, the signal peaks at the LO width of about 40% of the pulse bandwidth (FWHM). This is the optimal point for XPM signal.

![Figure 4.3: Simulated pump-probe delay scans of cross-phase modulation signal with (a) no chirp and (b) third-order chirp.](image)

We also simulated the pump-probe delay scans of XPM signal. Figure 4.3(a) shows the characteristic shape of the XPM delay scan. This is equivalent to the cross-correlation of pump and probe pulses. However, the shape of the trace is not a pure Gaussian. The shape of the trace originates from the shape of the probe pulse given by Fourier transform of the probe spectrum (a Gaussian with a central hole). Figure 4.3(b) shows a delay scan with positive third-order chirp added to the probe pulse. We see that,
apart from broadening the pulses, third-order chirp changes the shape of the delay scan non-symmetrically.

4.3. **Comparison with self-phase modulation**

To demonstrate the background suppression achieved by using cross-phase modulation (XPM) in nonlinear phase measurements, we compared XPM to self-phase modulation (SPM) in a standard glass slide [103].

4.3.1. **Experimental setup**

The experimental layout used for these comparison experiments is shown in Figure 4.4. As our laser source, we used a regenerative amplifier (RegA 9000, Coherent) seeded by a mode-locked Ti:Sapphire oscillator (Vitesse, Coherent) with a center wavelength of 804 nm. The pulse repetition rate used was 20 kHz. The 804 nm beam was split and about 80% of the power was used to pump an optical parametric amplifier (OPA 9450, Coherent) to generate the pump beam. The center wavelength of the pump beam was chosen as 740 nm. The remainder of the 804 nm beam (about 20%) served as the probe and was shaped using an acousto-optic pulse shaper [69]. In the pulse shaper, the beam is spectrally dispersed and mapped onto an acousto-optic modulator. A shaped radio-frequency waveform is used to drive the modulator and this imparts a modulation on the spectrum of the pulse, effectively transferring the shape onto the pulse. This gives us arbitrary computer control over the phase of the pulse.
Figure 4.4: Experimental setup for self- and cross-phase modulation measurements using the spectral reshaping technique. DC: dichroic mirror; BPF: band-pass filter; PD: photodiode; AOM: acousto-optic modulator.

For the XPM measurements, the central part of the 804 nm beam was statically phase shifted by $\pi/2$. The pump beam was amplitude modulated at 5 kHz reference frequency using an acousto-optic modulator. In the case of SPM measurements, a phase rotating local oscillator of about 3 nm width was imparted on the center of the 804 nm beam and the 740 nm pump beam was blocked. Dispersion compensation was performed on both the beams to minimize pulse chirp. The temporal width of each pulse was about 50 fs.
The two beams were then combined using a dichroic mirror and focused into a glass slide using a 0.25 NA objective (PLAN 10x, Olympus). After transmission through the sample, the pump beam and the wings of the probe beam were spectrally rejected using a 1 nm band pass filter (BPF). The transmitted part of the spectrum was focused onto a photodiode (DET 210, Thorlabs). The photodiode signal was time-gated with a boxcar integrator (SR250, Stanford Research Systems) and then filtered at 5 kHz reference frequency by a lock-in amplifier (SR830, Stanford Research Systems) with an integration time of 10 ms.

4.3.2. Signal to background comparison

Figure 4.5 shows experimental measurements of self-phase modulation (SPM) and cross-phase modulation (XPM) in a glass slide as a function of input power. The power scaling of SPM signal, measured using spectral reshaping technique is described in [68]. From Figure 4.5, we can see that SPM signal scales quadratically (linear fit slope: 1.93 ± 0.05). As shown in section 4.2, XPM signal scales as the product of the pump and probe powers. If the ratio of pump and probe powers is kept constant at a constant fraction, XPM signal should scale quadratically with the total power. Figure 4.5 confirms the quadratic scaling (fit slope: 2.03 ± 0.02) of the XPM signal measured using the dual-color spectral reshaping technique.
Figure 4.5: Input power scaling of SPM and XPM signals in a glass slide. The nonlinear signal is background subtracted. XPM is plotted against the total laser power i.e. the sum of powers of both the pulses. Also plotted is the corresponding background of the nonlinear signals.

The nonlinear signals plotted in Figure 4.5 are background-subtracted; where ‘background’ is defined as the lock-in signal measured when the laser focus is positioned outside of the sample (in air). The Rayleigh range of the beams was substantially smaller than the glass thickness. We can see that SPM exhibits as large linear background (linear fit slope: 1.00 ± 0.01). At a typical biological imaging power level of 20 µW (corresponding to a pulse energy of 1 nJ), the SPM background exceeds the real SPM signal by an order of magnitude. This overwhelming background degrades nonlinear phase modulation measurements in inhomogeneous media. In contrast, XPM has a
dramatically lower background at the relevant power levels, as shown in Figure 4.5. We can see an improvement in signal to background ratio of about 500. The background in the XPM measurement is independent of input power, suggesting that it is an electrical noise floor of the detection system. Therefore, better detection electronics could further reduce the background.

4.4. Nonlinear phase contrast measurements using mode-locked laser systems

The experiments described in section 4.3 were done using an amplified laser system operating at repetition rate of 20 kHz. The high peak powers delivered by the amplified pulses can give strong nonlinear signals. However, the low repetition rate limits the data acquisition rate. Therefore, amplified systems are not suitable for imaging dynamic process, especially on a sub-second timescale. Such acquisition speed is desirable to make XPM measurements applicable to microscopy of a wide range of biological samples. Also, the pulse energies generally used in biological imaging (about 1 nJ) can be easily delivered by modelocked systems. Additionally, modelocked laser sources are relatively cheap and stable, easily tunable and require low maintenance. Therefore, amplified systems do not have any advantages in our operating regime.

4.4.1. Experimental setup

We adapted out two-color spectral reshaping technique to use mode-locked systems and designed a laser scanning microscope for biological imaging experiments. The major constraint in adapting individual pulse shaping based techniques to high
repetition rate lasers is the update rate of the pulse shaper. In case of self-phase modulation measurements using spectral reshaping technique, we developed an interferometric pulse shaper (see section 2.4) to generate the phase rotation of the required local oscillator [65, 71]. In our two-color spectral reshaping technique, we only require a static phase shift in the probe. Therefore, we do not need to dynamically update the pulse shape at high repetition rate. This greatly simplifies the pulse shaper design.

Figure 4.6: Experimental setup used for cross-phase modulation imaging. BPF: bandpass filter; LCM: liquid crystal modulator.

In our new experimental setup (see Figure 4.6), we use a modelocked Ti:Sapphire oscillator (Tsunami, Spectra Physics, 80 MHz) with an 800 nm output, as our probe
source. The center part of probe spectrum is phase shifted using a liquid crystal
modulator based static pulse shaper to create our phase reference (local oscillator).
About ninety percent of the Tsunami output is used to synchronously pump a tunable
optical parametric oscillator (Mira OPO, Coherent, 80 MHz) to produce our pump beam.
The pump beam is intensity modulated (square wave) using an acousto-optic modulator
(AOM, Crystal technology, model 3200-124) at 2 MHz. More details of the experimental
setup are given in section 5.2.2. In the next section, we will discuss the design of the laser
scanning microscope used for cross-phase modulation imaging.

4.4.2. Design of two-color laser scanning microscope

Laser scanning microscopes are commercially available from a number of
microscope companies. However, they do not allow the customizability required for our
novel nonlinear imaging experiments. Therefore, we developed a custom laser scanning
microscope based on design described in [107].

The core of the laser scanning microscope is the scan system to deliver a
collimated laser beam at various angles onto the back aperture of an infinity corrected
objective, which maps the angle to position on the focal plane. By changing the angle,
the focal spot can be scanned across the sample. The main components of the scan
system are a scan mirror set, a scan lens, a tube lens and an objective. Figure 4.7 shows a
schematic of the scan system. The design of the system is based on a set of constraints
[107], which are discussed below.
The beam reaching the back aperture of the infinity corrected objective, needs to be collimated. Assuming the incoming beam is collimated, this condition is satisfied by setting the scan lens and tube lens in a telescopic configuration. This gives the condition

$$d_2 = f_1 + f_2.$$  \hspace{1cm} (47)

where $f_1$ and $f_2$ are the focal lengths of the scan and tube lens, respectively.

Figure 4.7: Scan system of the microscope showing the main components and formulation of the distances between them. The solid lines indicate the laser beam path and size. The dashed lines show the path of the beam during scanning. The scan mirror is imaged onto the back aperture of objective. Adapted from [107].

The surface of the scan mirror has to be imaged onto the back aperture of the objective to avoid any lateral motion during scan. Any spatial deviations would change the power delivered through objective. This would affect the uniformity of the field of view. The two scan mirrors (for x and y directions) are separated by a small distance, therefore, the back aperture of the objective should be imaged to a point halfway
between the mirrors. Thin lens equations are solved to satisfy the above mentioned imaging condition. This gives us relationship the between $d_1$ and $d_3$ as

$$d_1 = \frac{(f_1)^2}{f_2} + f_1 - d_3 \left(\frac{f_1}{f_2}\right)^2.$$

(48)

The diameter of our scan mirrors (6210H, Cambridge technology) was about 6 mm. The size of the incoming beam needs to be smaller than the scan mirrors to avoid losses at the edge. We modified our incoming beams (both pump and probe) to have a spot size of about 4.5 mm (FWHM). The average back aperture of our objectives is about 8 mm. We need to fill the objective completely to obtain complete NA. Therefore, the incoming beam needs to have a magnification of 1.8. This gives us the condition, $f_2 = 1.8 * f_1$. We used a standard Olympus tube lens, which has a focal length of 180 mm. As a result, the focal length of our scan lens is 100 mm. The scan lens need be well corrected for chromatic aberrations. This is critical in a two-color microscope to obtain a uniform field of view.

The distance between the tube lens and the objective, $d_3$ is chosen as 165 mm to accommodate for the optional epi-detection setup, which includes a polarizing beam splitting cube and a dichroic mirror. Using Eq. 48, we can calculate $d_1$ to be 104 mm.

Since we use two beams with different wavelengths, the focal spots are at different distances along the beam direction. Therefore, the beam spots need to be optimized for complete spatial overlap in the beam direction. This can be achieved by controlling the divergence of the incoming beams. We used a two lens system in
telescopic arrangement. The second lens is mounted on a translation stage and can be
optimized to give the best spatial overlap at the focus.

### 4.4.3. Detection and acquisition

As shown in Figure 4.6, the beams coming out of the condenser are diverted onto
a pump blocking filter. Then, a coverslip is used to direct a small part of the probe beam
onto the transmission photodiode. The signal from this photodiode can be used to
simultaneously collect linear transmission images. After that, a tunable band pass filter
(\(\sim 1\) nm) is used to reject the wings of the probe. Since, the band-pass filter is angle
dependent, the probe beam should be collimated before hitting the filter. The filter
should be placed at the minimum deviation position to obtain a uniform field of view for
the nonlinear signal.

The beam coming out of the microscope in transmission mode is not de-
scanned. Therefore, we need to image the back aperture of the objective onto the
detection photodiode (PDA55, Thorlabs) for minimum spatial deviations during scanning.
Additionally, the spot size of the beam hitting the detector needs to be minimized for
efficient collection. This represents a compromise between the spot size and deviation. A
combination of lenses is used to achieve optimized detection.

The photodiode signal is analyzed by a lock-in amplifier (SRS844, Stanford
Research Systems). The integrated signal from the lock-in is fed into a National
Instruments DAQ module (PXI-6259). The DAQ module features simultaneous 32 channel
sampling as well as four output channels; two of those are used for controlling the scan mirrors. Signal from the transmission photodiode is fed directly into the DAQ module. The typical sampling speed used is 20 kHz (for the signal from the lock-in). Sampling speeds up to 1.25 MHz are possible.

Custom image scanning software written in LabWindows/CVI (National Instruments) was used for data acquisition and instrument control. The main function of the program is to output waveforms to the scan mirrors to raster scan the beam and synchronously read out data from the DAQ module. We only read out data in the forward scan direction. The image display is updated in steps of 16 lines. The software also controls the XY sample stage, objective motor, condenser motor and the pump-probe time delay motion stage. While acquiring a Z stack of images, the condenser position is adjusted along with the objective, to keep the beam divergence out of the microscope constant.

4.4.4. **Noise and background scaling with pump and probe powers**

Figure 4.8 shows experimental measurements of cross-phase modulation (XPM) signal in a glass slide as a function of input pump power. The noise and background are also shown in the figure. Here, ‘background’ is defined as the lock-in signal measured when the laser focus is positioned outside of the sample (in air) and ‘noise’ is defined as the variance of the XPM signal in glass. XPM signal scale should scale linearly with pump power (see section 4.2). Figure 4.8 confirms the linear scaling of XPM signal with pump
power. We can see that, at lower powers (less than 2 mW) the noise is essentially independent of input probe power, suggesting that it is an electrical noise floor of the detection system. At higher powers (> 2 mW), the noise scales linearly with power indicating laser intensity noise.

**Figure 4.8:** Pump power scaling of XPM signal, background and noise. The green lines illustrate the linear fits. Both XPM signal and noise (only at higher powers) scale linearly with pump power.

Similar to noise, the background is constant at lower powers, dominated by electrical noise. At higher powers, the background scales linearly with pump power, suggesting a pump leak through (due to imperfect pump blocking).

**Figure 4.9** shows XPM measurements in a glass slide as a function of input probe power. The noise and background are also shown in the figure. We can see that the XPM signal scales linearly with probe power. We can also see that the noise scales linearly with probe power, suggesting laser intensity noise as the dominant noise source. Also,
we can see that the background is essentially constant with the probe power, indicating a pump leak through.

![Graph showing probe power scaling of XPM signal, background and noise. The green lines illustrate the linear fits. Both XPM signal and noise scale linearly with probe power. The background is essentially constant with power.]

**Figure 4.9:** Probe power scaling of XPM signal, background and noise. The green lines illustrate the linear fits. Both XPM signal and noise scale linearly with probe power. The background is essentially constant with power.

### 4.4.5. Cuvette demonstration

Figure 4.10 shows experimental measurements of cross-phase modulation (XPM) in a glass cuvette filled with a 30 mM Rhodamine 6G (R6G) solution in methanol. The pump and the probe wavelengths used for this measurement were 737 nm and 800 nm, respectively. The beams were focused into the sample using a 0.25 NA objective (PLAN 10x, Olympus). The lock-in time constant was 100 µs, which is the standard integration time used for the XPM imaging experiments. The total power used for this scan was about 22 mW, which corresponds to about 0.14 nJ per pulse.
Figure 4.10: XPM measurements of Rhodamine 6G solution in a glass cuvette. $\theta$ is the static phase shift of the local oscillator (see Eq. 46). The total input power was about 22 mW.

When the phase of the local oscillator in the probe pulse was set at $\pi/2$ to access the nonlinear refractive component both glass and the R6G solution showed XPM signals. The XPM signal in the solution is from methanol. We can clearly see the zero background in the measurement. As we can see from Eq. 46, adjusting the reference phase ($\theta$) to zero, accesses the absorptive component. Figure 4.10 shows that $\theta = 0^\circ$ gives a sum-frequency absorption trace, localized to the dye filled region of the cuvette. At $\theta = 0^\circ$, the technique is basically equivalent to pump-probe microscopy discussed in section 5.2. Therefore, band pass filter is not required during absorptive measurements.

Two-color spectral reshaping technique can quickly switch between refractive and absorptive measurements by selecting the phase of the local oscillator. The phase...
can be dialed in the software controlling the static pulse shaper. However, the phase needs to be calibrated to locate the zero level, which is dependent on the chirp on the pulse. This can be done by adjusting the phase such that the XPM signal in a glass slide is zero and setting that phase as zero.

![Graph](image)

**Figure 4.11:** Pump-probe delay scans of cross-phase modulation (XPM) signal in pure methanol and rhodamine 6G in methanol. The pump and probe powers were 12 mW and 13 mW, respectively.

To verify that we can extract XPM signal even in the presence of strong nonlinear absorption, we performed pump-probe delay scans of a 30-mM R6G solution in methanol and compared it with that of pure methanol [104]. The pump and probe wavelengths used for this scan were 672 nm and 802 nm, respectively. The lock-in time constant was 10 ms. Figure 4.11 shows the XPM delay scans with their characteristic shape. The shape of the traces originates from the shape of the probe pulse in time domain (see section 4.2.1). By comparing both the traces we can see that, in the pulse overlap region the dispersive signal component measuring XPM is essentially unaffected.
by the strong two-photon absorption of R6G. Please note that XPM measurements are generally performed at zero pump-probe time delay. Therefore, we are able to extract clean XPM signals even in highly absorbing samples.

4.4.6. Cell imaging

Figure 4.12: (a) Linear transmission and (b) cross-phase modulation images of three fixed breast cancer cells. The scale bars are 10 µm. The total power impinging on the sample was about 21 mW.

Figure 4.12 shows a linear transmission image (a) and an XPM image (b) of three fixed breast cancer cells in transmission mode. The pump and probe wavelengths used for acquiring these images were 712 nm and 804 nm, respectively. Lock-in integration time used was 100 µs, with a pixel dwell time of about 200 µs. While the linear transmission image in Figure 4.12(a) shows very little contrast, the XPM image provides a detailed structural contrast with substantially higher dynamic range and highlights different subcellular structures. Since, we are using 100 fs pulses, the contrast obtained should be mostly non-resonant electronic in origin.
4.5. Spectral shifting implementation of cross-phase modulation

As an alternative cross-phase modulation (XPM) measurement technique that does not require a pulse shaper we have implemented a detection configuration that detects pump-induced spectral shifting effects in the signal pulse [108]. Measuring induced phase changes in the spectral domain is robust in scattering media (see section 2.3); spectral shifting implementation retains this robustness. The major advantage of the spectral shifting implementation is that, individual pulse shaping is not required. This simplifies the experimental setup and makes the technique quickly interchangeable with pump-probe microscopy [42, 43, 48]. We refer to this implementation as cross-phase modulation spectral shifting (XPMSS) technique [109].

The spectral shifting effect can be well illustrated by using instantaneous frequency description. Consider the case of Gaussian pump and probe pulses of equal pulse widths. By positioning the peak of the probe pulse at the rising temporal edge of the pump pulse, the probe pulse experiences a refractive index that decreases with time (for $n_2 > 0$), corresponding to a decreasing phase with time. This increase in frequency is visible as a blue-shift of the signal spectrum. The situation reverses if the probe is positioned at the trailing edge of the pump pulse. From Eq. 29, we can write the pump ($I_{pu}^0 e^{-t^2/\tau_g^2}$) induced spectral shift at a pump-probe delay of $t_o$ as [66]

$$\Delta \omega|_{t_o} = \frac{4\eta z I_{pu}^0 t_o}{\tau_g^2} e^{-\left(\frac{t_o^2}{\tau_g^2}\right)} ,$$  (49)
where \( \eta = n_2 \omega_{pr}/c \) and \( \tau_g = \tau_{FWHM}/\sqrt{2 \ln 2} \). \( z \) is the interaction length and \( n_2 \) is the nonlinear refraction coefficient. We can see that the spectral shift depends on the pump-probe delay. The delay which gives the maximum spectral shift is be calculated from \( d(\Delta \omega)/dt_o = 0 \) as \( t_o = \tau_g/\sqrt{2} \). We can write the maximum spectral shift as

\[
(\Delta \omega)_{\text{max}} = \frac{4\eta z I_{pu}^o}{\tau_g \sqrt{e}}
\]

(50)

We can see that the induced spectral shift is proportional to the pump intensity. XPM-induced spectral shifts can be measured by detecting only a portion of the probe spectrum through a spectral filter, e.g. through an edge filter that symmetrically cuts off half the probe spectrum. In our implementation, we impose an amplitude modulation at the reference frequency \( f_o \) on the pump pulse train. We filter our signal using a lock-in to only measure components at the frequency \( f_o \). Therefore, the lock-in signal measured can be written as [109]

\[
S_{\text{lock-in}} = \int_0^{\Delta \omega} \frac{I_{pr}^o \tau_g}{\sqrt{2}} e^{-(\omega \tau_g/2)^2} d\omega = I_{pr}^o \sqrt{\frac{\pi}{2}} \text{erf}(\Delta \omega \tau_g/2).
\]

(51)

For small spectral shifts, signal measured can be approximated to the first order as

\[
S_{\text{lock-in}} \approx 2\eta z I_{pr}^o I_{pu}^o \sqrt{\frac{2}{\sqrt{e}}}.
\]

(52)

Therefore, XPMSS signal \( \propto P_{pr} P_{pu} \), where \( P_{pr} \) and \( P_{pu} \) are the probe and pump input powers, respectively.
It is important to note that intensity changes in the probe due to absorptive processes will also affect transmission through the edge filter. Since we are measuring changes in integrated spectral power going through the filter, the changes due to absorption will add to the lock-in signal. To get a pure measurement of XPM we can subtract out the absorptive component by using a reference beam, which does not go through the edge filter. In our implementation we use a balanced detector, with the beam rejected by the edge filter as the reference. Alternatively, a part of the probe beam can be picked off using a cover slip before the edge filter, to use a reference in the balanced photodiode.

### 4.5.1. Cuvette demonstration

![Figure 4.13: XPM pump-probe delay scans with the spectral shifting technique in methanol with and without dissolved rhodamine-6G. A neutral density filter (10% transmission) was used to reduce the amount of probe power impinging on the balanced photodiode.](image-url)
To demonstrate the spectral shifting based XPM measurement technique, we performed pump-probe delay scans in pure methanol using a 10x 0.25 NA objective. To demonstrate that the XPM measurement is not affected by residual nonlinear absorption we also performed pump-probe delay scans in 30 mM solution of R6G in methanol. The experimental setup used for XPMSS has few differences from the one shown in Figure 4.6. The pulse shaper is not required for the XPMSS measurements. The pump and the probe wavelengths used were 672 nm and 794 nm, respectively. The pump and probe powers used were about 12 mW and 13 mW, respectively. The band-pass filter (BPF) in the detection setup is replaced with a short-pass edge filter at 794 nm to separate the probe spectrum. The transmitted and reflected parts of the spectrum are directed onto the two photodiodes of the balanced detector. The pump amplitude modulation frequency in these experiments was 1 MHz, which is the maximum bandwidth of the balanced photo detector (Model 2307, New Focus).

Figure 4.13 shows the characteristic dispersive time-delay trace of XPMSS, since alignment of the pump with the leading or trailing edge of the signal pulse shifts the spectrum in opposite directions. We can also see that the XPM measurement in the R6G solution matches closely that of pure methanol. Even though R6G has a strong two-photon cross-section, the intensity loss in the probe is cancelled by the balanced detector. Please note that the cancellation works only when the absorption cross-section is frequency independent within the pulse bandwidth. In general, the absorptive component is subtracted cleanly only if both halves of the spectrum have equal loss.
Otherwise, we would see a residual absorptive component in the XPMSS measurement. In such cases, components need to be separated mathematically during post processing.

For comparison, we also performed XPM measurements using the two-color spectral reshaping technique under similar conditions (Figure 4.11). For these measurements the central part of the probe spectrum was statically phase shifted by $\pi/2$ and the edge filter was replaced by a 1 nm band-pass filter (BPF). Figure 4.11 and Figure 4.13 show that for the same amount of incident power, the spectral shifting signal amplitude is about 16 times greater than that of spectral reshaping technique (taking into account the 10% transmission neutral density filter). An additional factor of 2 could be obtained by measuring the difference between the two peak shifts.

4.5.2. Imaging skin biopsy slides

To demonstrate nonlinear phase contrast imaging using XPMSS technique, we acquired XPMSS delay stacks of skin biopsy slides. We used an edge filter to split the probe spectrum before detection to detect the spectral shift. A normal photodiode was used for these experiments. The pump and the probe wavelengths used were 720 nm and 810 nm. We acquired 512 x 512 image slices with a pixel dwell time of about 49 µs and 4 frames were averaged for each time slice.

Figure 4.14 shows the principal component images (512 x 512 pixels) of a hematoxylin and eosin (H&E) stained skin biopsy slide. A XPMSS delay stack of 50 slices was acquired using a 20x 0.7 NA objective. The total power impinging on the sample was
about 2.8 mW (split equally between pump and probe). Principal component analysis [43, 45] was performed to separate the refractive and absorptive components.

Figure 4.14: Images of an H&E stained skin biopsy slide. (d) The first two principal components of the XPMSS delay stack. Red is the transient absorption component and green is the XPM component. (a) and (b) show the principal component images when only the corresponding (color in (d)) component is retained. (c) A zoomed-in view of the XPM component image in (a). All the scale bars are 50 µm.

Figure 4.14(d) shows the first two principal components. The green component shows the characteristic dispersive line shape corresponding to the XPM signal. The red component shows a signal corresponding to the negative transient absorption (see section 5.2.1) in hematoxylin and eosin. Figure 4.14(a) shows the distribution of the
XPMSS signal and Figure 4.13(b) shows the transient absorption image. We can see that XPM signal provides some complementary structural information. Figure 4.14(c) shows a zoom-in of XPMSS image onto the epidermis. We can see that the XPM provides a structural context for the highly absorptive cells.

Figure 4.15: Histogram plot (right) of phasors for all the pixels in the XPMSS delay stack of dermo-epidermal junction in a melanoma biopsy. RGB image (left) extracted from the delay stack (color corresponds to the regions indicated by circles in the phasor plot). The scale bar is 20 µm.

Figure 4.15 shows an image (left) of dermo-epidermal junction in an unstained melanoma biopsy slide. A XPMSS delay stack of 49 slices was acquired using a 40x 0.8 NA water immersion objective (data taken from [109]). The total power impinging on the sample was about 7.5 mW (split equally between pump and probe). Phasor plot analysis (see section 5.2.4) was performed to separate the refractive and absorptive components.

On the right, Figure 4.15 shows a histogram plot of phasors (0.6 THz) for all the pixels in the XPMSS delay stack. The region indicated by the blue circle corresponds to
pixels with absorptive signal. The absorptive signal corresponds to eumelanin signature at these wavelengths. The region indicated by the red oval corresponds to the dispersive shaped XPMSS signal. On the left, Figure 4.15 shows an RGB image composed with colors corresponding to the region in the histogram plot. We can clearly see the pigmented regions in blue. We can see that the XPM (red in the image) provides a structural context for the highly absorptive cells. Also, structural details in dermal collagen can be seen, and boundaries of cells can be discerned.
5. Pump-probe imaging of historical pigments

In this chapter we will discuss the application of pump-probe microscopy in imaging pigments used in historical artwork. We will characterize several important inorganic and organic pigments. We will also demonstrate multi-layer imaging and identify pigments in a paint chip taken from a 16th century painting.

5.1. Introduction to characterization of pigments

Characterization of pigments used in historical artwork has long been of great value for authentication, restoration and conservation of art. Important additional information can be obtained by characterizing the composition of successive paint layers in three dimensions.

By identifying the method of paint application and understanding how the final effect was achieved, valuable information about the authenticity of an artwork can be deduced. Dating and authentication is extremely important in view of the immense value of historical artwork. Visualization of the pigments down to a single granule helps in determining the method used for preparation of the pigment, which in turn allows art historians to deduce the specific epoch. For example, distinguishing hand-ground from machine-ground pigment granules could help identify a 15th century original painting (with hand ground pigments) from a 19th century reproduction/copy (with machine ground pigments). A number of pigments, especially the recent synthetic alternatives have known date of first manufacture or use. Determining whether the pigment was
obtained from a natural source or was synthesized could also help deduce the epoch. Presence of a pigment with date of first manufacture later than the claimed period of artwork would indicate that the artwork is fake/copy or some retouching work was done at a later date.

In restoration, it is required to identify compatible alternatives that do not react with contiguous pigments and produce disastrous visual effects. Also, previous restoration work and changes in composition and structure over time (for example slow oxidation of azurite into malachite) need to be accounted for during repair.

In conservation, it is important to determine the spatial and chemical extent of degradation in paintings due to heat, light, humidity or interaction within adjacent pigments. In case degradation is identified before any significant visual changes have occurred, further damage might be prevented by changing holding conditions. In other cases, identifying degradation products of pigments is required to suggest possible treatments, whereby degradation processes may be stopped or reversed. Also, identifying degradation pathways can give information about the nature of the environment in which the artwork has been previously held.

5.1.1. Overview of conventional techniques

Unambiguous identification of pigments is possible using bulk chemical analysis. It is relatively simple to identify pigments and related species by removing samples from
paintings and performing direct chemical analysis. Important destructive techniques generally used for pigment analysis include:

- Chromatographic techniques like high-performance liquid chromatography (HPLC) and gas chromatography (GC).
- Mass spectroscopy techniques like laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) and matrix assisted laser desorption ionization–mass spectrometry (MALDI-MS) [110, 111].
- Laser-induced breakdown spectroscopy (LIBS): A high energy laser pulse is focused to generate a micro-plasma which atomizes and excites the material. The characteristic atomic emission from the excited species is used to determine the elemental composition [10, 112].

However, such destructive analysis is not an option in most cases (especially in historical artwork). Even in cases when samples/cross-sections from previously damaged parts or edges of the artwork are available, they may not be representative of the entire painting. Also, unlike paintings, pigment sampling from manuscripts is never allowed. Hence non-invasive techniques that can obtain required information without any direct contact are required. Some of such techniques are discussed below.

*X-ray based elemental analysis techniques,* such as x-ray fluorescence (XRF) and particle-induced x-ray emission (PIXE) [113-115] have been applied successfully for non-invasive and non-destructive analysis of paintings. However, they have limits in their applicability, since elemental composition cannot always unambiguously determine a
mixture of pigments. Also, lighter elements cannot be sensitively detected, thus these techniques exclude organic pigments. X-ray optics in confocal arrangement has recently enabled 3-D elemental mapping using XRF [116]. However, confocal XRF generally requires large and expensive instrumentation like synchrotrons for evaluating paintings in a reasonable time frame. Table-top confocal micro-XRF units have been constructed but do not have the sensitivity required for mapping minor elements [117]. High Z elements containing pigments like lead white (commonly used as ground) are highly x-ray absorptive and can swamp the signal from low Z elements. Recently, K-edge imaging using highly monochromatic x-rays has been able to record elemental maps through high Z elements containing overpaint [118]. Also, scanning XRF using monochromatic radiation was able to reveal metal distributions in pigments sandwiched between lead white ground layers and lead white overpainted surface layers [119]. However, such monochromatic x-rays are difficult to generate outside a synchrotron [120].

*Neutron radiography* has been used for non-destructive testing of paintings [121]. It uses the neutron attenuation properties of an atom as a contrast to generate X-ray like images. Element-specific imaging of paintings is done by neutron activation analysis. This is done by exposing a painting to a thermal neutron flux in an atomic reactor, which creates artificial radioisotopes of the elements and the characteristic decay by gamma radiation is used to map the elemental distribution [122]. However, only elements with practical half-life times can be detected. Also, neutron activation is affected by the shielding from lead containing pigments. Other major disadvantages to using neutron
radiography include the dangerous radiation exposure, need for an expensive atomic reactor and requirement of extremely long data acquisition times for proper image quality.

Terahertz (THz) imaging techniques have been recently used to identify pigments using spectroscopic information in the terahertz region of the electromagnetic spectrum [123, 124]. The THz spectrum can provide information about inter-molecular vibrational modes. An important advantage of terahertz imaging is good penetration depth through various materials and thus has been used to reveal hidden sketches and underdrawings [125]. Additionally, time resolved information can be used to determine relative thickness of the layers [126]. An advantage over the x-ray techniques is the use of non-ionizing radiation; however, terahertz imaging suffers from artifacts due to painting curvature, a major drawback due to the non-uniform layering in most paintings. Also, terahertz wavelengths fundamentally limit the spatial resolution, thus, the imaging resolution achieved is on the order of 0.25 mm to 0.75 mm.

5.1.2. Overview of linear optical techniques

Light microscopy has long been a simple yet powerful tool for characterizing pigments in art conservation at low cost. Polarized light microscopy uses differences in color, shape, refractive index and birefringence properties to characterize pigments and related species in dispersed samples and paint cross-sections [127].
UV-Vis reflectance spectroscopy gives a measure of light absorption in the ultra-violet through near-infrared part of the electromagnetic spectrum. The shape of this absorption spectrum and presence of unique features can be used to separate pigments from each other [3, 128, 129]. However, most of the pigments have broad featureless electronic absorption spectra and hence the low specificity does not enable unique identification of pigments.

Infrared spectroscopy is relatively specific but suffers from interference from binders and support. Also, spatial resolution achieved using infrared light is limited due to the wavelengths of light used. Fourier transform infrared spectroscopy (FTIR) has been used for studying a wide variety of cultural heritage materials [130] in transmission mode. Reflection mode infrared spectra depend highly on the heterogeneity in refractive index and surface structure [131]. Therefore, extensive characterization of cultural heritage materials is required for any useful interpretation of reflective infrared spectra from real paintings.

Fluorescence spectroscopy (both laser-induced and lamp-based) has been used to study a wide range of cultural heritage materials [10]. Spectrally resolved laser induced fluorescence life time techniques have been successful in differentiating between protein based binding media [132, 133]. However, very few historic pigments fluoresce and thus comprehensive identification of pigments is not possible. Another disadvantage is the attenuation due to absorption from non-fluorescent inorganic pigments.
Infrared imaging makes use of highly penetrating infrared light to reveal spatial features and underdrawings not visible to the naked eye [134, 135]. Infrared reflectography (0.8 - 2.5 μm) and thermography (7 - 14 μm) can quickly map large areas in contrast to micro-spectroscopy techniques. However, infrared imaging lacks chemical specificity and hence, pigment identification is not possible. Recently, multi-spectral imaging which combines infrared reflectance spectroscopy with digital imaging was demonstrated in paintings [136, 137]. This technique can simultaneously obtain both spatial and spectral information and thus is able to identify and map pigments rapidly in entire paintings. However, this technique has limited spatial resolution, generally suffers from binder interference, and does not give any depth information. Despite its shortcomings, multispectral/hyperspectral imaging is a useful tool and complementary to the scanning point measurement techniques [138]. For instance, it could be used as a guide for region selection during highly localized characterization of pigments to create distribution maps of individual particles and layers.

Raman scattering can be very pigment specific [12, 139] and use of visible/near-infrared probe wavelengths gives very good spatial resolution (< 1 μm). Raman spectroscopy has been widely used for pigment identification in manuscripts, paintings and other archaeological artifacts [14, 140, 141]. Large spectral libraries [142-144] have been established owing to success of Raman as a non-destructive in situ pigment analysis tool. Limitations include generally weak Raman cross-sections (excluding resonant enhancement) and background generated by the luminescence of binders and
varnishes. Even though micro-Raman spectroscopy can provide high resolution information it is limited to very thin samples or the surface.

Depth selectivity can be achieved by implementing Raman spectroscopy in a confocal arrangement. Three-dimensional information is obtained by scanning the laser beam across the sample and spatially restricting the detection to collect light only from the focal volume by using a pinhole. However, confocal detection is very inefficient and requires longer acquisition times. Highly scattering samples can introduce large out of focus background to the confocal measurements. Despite these limitations confocal imaging has substantially improved applicability and usability of Raman. Confocal Raman imaging is now recognized as a very important molecular imaging technique in cultural heritage [141, 145].

Optical coherence tomography (OCT) is an interferometric technique which uses scattering contrast to provide high resolution three-dimensional structural information in moderately transparent samples [146-149]. OCT is a well established diagnostic technique in ophthalmology and is commercially available. In art, OCT has been primarily used for examination of varnish and glaze layers of paintings and has been successful in layer thickness measurements [150]. However, OCT is generally limited to semi-transparent layers and since it does not provide any specific chemical information, pigment identification is not possible.
5.1.3. Overview of nonlinear techniques

Using nonlinear processes has several advantages over linear confocal techniques in highly scattering media. Nonlinear imaging techniques take advantage of quadratic (or higher) dependence on laser intensity for localized excitation, thereby achieving high spatial resolution. Using longer wavelengths (typically near-infrared) can provide higher penetration depths due to lower scattering and absorption. For example, two-photon fluorescence microscopy [19, 20, 22], a widely used biological imaging technique excites molecules with light of wavelength that is twice the wavelengths used for direct single photon excitation. Recently, two-photon fluorescence was used for detection of paint on an historic amphora (found at the Roman village of Isseo) and recovering the faded letters of the inscription allowed for dating of the amphora [151, 152]. Many organic binders fluoresce but most of the important historic pigments are non-fluorescent, severely limiting the capability of this technique in comprehensively identifying pigments.

Nonlinear harmonic generation can provide intrinsic high resolution structural contrast for examination of artwork. Harmonic generation is an energy conserving process thus providing minimal energy deposition in the sample and lower photodamage. Second harmonic generation (SHG) is associated with the second order nonlinear susceptibility, $\chi^{(2)}$ and is non-zero only in non-centrosymmetric materials. Third harmonic generation (THG) is associated with the real part of third order nonlinear susceptibility, $\chi^{(3)}$ and is primarily generated at interfaces. Recently, nonlinear harmonic
generation techniques have been used to study varnishes [36, 153] and lining glues [32]. Information extracted by these techniques includes layer thickness and types of lining glues. However, symmetry constraints severely restrict the range of possible targets for harmonic generation imaging.

Coherent anti-Stokes Raman scattering (CARS) is a third order nonlinear optical process which enhances the Raman scattering cross-section by coherently driving the vibration using a pump and Stokes beam, whose difference frequency matches the Raman resonance [37]. Recently, CARS microscopy has been used for imaging pigments and binders in paint layers [154]. However, large non-resonant background and fluorescence interference from synthetic organic pigments combined with generally weak Raman cross-sections limits the applicability of this technique.

5.2. Pump-probe microscopy

Recently, Warren and coworkers have developed a nonlinear optical pump-probe microscopy technique that uses excited state dynamics as a contrast for highly specific imaging of biological pigments. In this section we describe the pump-probe microscopy technique and its experimental implementation for imaging historical pigments.

5.2.1. Two-color modulation transfer technique

Nonlinear absorption is a very powerful intrinsic contrast mechanism for imaging. It provides molecular specificity due to the resonant absorption process and retains the optical sectioning capability of the nonlinear processes. However, most of the pigments
used in artwork have broad and generally featureless absorption spectra in the visible/near-infrared region. Therefore, two-photon absorption does not have the chemical specificity required for unique identification and separation of a comprehensive set of historical pigments.

Figure 5.1: Various nonlinear processes of interest in the pump-probe microscopy experiments.

By extending nonlinear absorption contrast to use two different colors, the Warren group has recently demonstrated nonlinear absorption dynamics as a contrast for imaging a wide variety of biological pigments. This is done by combining the concept of conventional pump-probe spectroscopy with nonlinear imaging [40, 41, 44]. The technique is termed ‘pump-probe microscopy’. In this technique, the pump laser pulse excites the ground state population and populates an excited state. A time-delayed probe pulse is used to monitor the temporal evolution of the ground state and the
excited state population, thereby measuring the molecular dynamics of the system. This can provide a specific molecular contrast since molecules that absorb similarly at particular wavelengths may have completely different excited state dynamics even when pumped at the same wavelengths. Various resonant nonlinear processes such as sum frequency absorption (SFA), excited state absorption (ESA), ground state depletion (GSD) and stimulated emission can affect the evolution of the system [155]. A schematic representation of these processes is shown in Figure 5.1. Sum frequency absorption is an instantaneous process involving a virtual state and occurs only when the two pulses are overlapped. ESA, GSD and stimulated emission involve a real state and thus have an associated decay time. Sum frequency absorption and excited state absorption cause an intensity loss in the probe pulse in the presence of the pump. In contrast, ground state depletion and stimulated emission processes cause an intensity gain in the probe pulse due to presence of the pump pulse. Effectively, the presence of pump pulse changes the transmission of the probe pulse through the material when any of the above mentioned nonlinear processes occur. By measuring the transmission of the probe at different pump-probe time delays the dynamics of these nonlinear processes are recorded.
Nonlinear processes like two-photon fluorescence or nonlinear harmonic generation generate light at frequencies different from the ones used for excitation and hence the generated signals are easy to separate from the excitation background. In pump-probe microscopy we need to measure changes in the transmission of the probe that are generally on the order of one photon per every $10^6$ photons in the probe light. To sensitively detect such a tiny loss/gain we force these changes to occur at a new frequency [40]. This is achieved by the modulating the amplitude of the pump pulse train at frequency $f_o$. When a nonlinear process involving both the pump and probe photons occurs in the sample, modulation is transferred onto the probe as shown in Figure 5.2. Thereby, transmission changes in probe caused by the pump appear as a component at frequency $f_o$, which was not previously present in the probe. Linear processes such as scattering and absorption cannot create such a frequency component. The probe is then filtered for this $f_o$ frequency component using a lock-in amplifier to achieve high sensitivity.
5.2.2. Experimental setup

A simplified schematic of our experimental setup used for pump-probe microscopy experiments is shown in Figure 5.3. The laser source is a modelocked Ti:Sapphire oscillator (Tsunami, Spectra Physics, 80 MHz) with an 810 nm output. The temporal width of each pulse is about 120 fs. About ninety percent of this beam is used to synchronously pump an optical parametric oscillator (Mira OPO, Coherent, 80 MHz) to produce our pump beam of wavelength 720 nm. The wavelength tuning range of our OPO is from 520 nm to 740 nm. The temporal width of pulses out of OPO is about 160 fs. The pump beam is intensity-modulated (square wave) using an acousto-optic modulator (AOM, Crystal technology, model 3200-124) at 2 MHz. The pump beam is focused onto the AOM by a 200 mm focal length lens. The collimation lens (125 mm) is mounted on a mechanical translation stage which can be used to adjust the divergence of the pump beam.
Figure 5.3: Simplified experimental setup used for pump-probe microscopy experiments. PBSC: polarizing beam splitting cube.

The remainder of the 810 nm beam (about 150 mW of average power) served as the probe in our experiments. The probe beam is sent through a motorized delay line (Newport MFA-CC 25 mm stage controlled by a Newport ESP300 motion controller) to control the pump-probe time delay. The delay line needs to be carefully aligned to ensure the beams going in and out are parallel. In case of misalignment, translating the stage would cause a spatial shift in the beam, which in turn changes the spatial overlap between pump and the probe beams in the far field and introduces an artifact in the signal decay profile. The delay line mirrors should be tweaked to make the beam profile...
stationary in the far field (> 6 meters). A beam profiler can be used to observe the beam spot in the far field while scanning the stage.

The two beams, modulated pump and unmodulated probe, are then combined using a dichroic mirror (DCXR760, Chroma) and sent into a home built laser scanning microscope (described in section 4.4.2). It is critical that the two beams are collinear and overlapped throughout their path. This can be ensured by optimizing spatial overlap right after the dichroic and in the far field (> 6 meters). The pump and the probe beams are focused onto a sample by a 20x 0.7 NA microscope objective (Olympus UPlanApo). For the transmission mode experiments the beams are re-collimated using a 1.1 NA condenser (a high NA condenser minimizes artifacts from thermal and Kerr lensing [41, 108]). The beams are then passed through a high quality band pass filter (HQ815/65m, Chroma) to reject the pump light and the probe beam is focused onto an amplified large area silicon photodiode (PDA55, Thorlabs). The signal from the photodiode is analyzed using a radio frequency lock-in amplifier (SRS844, Stanford Research Systems). As described in section 5.2.1 the probe gains a 2 MHz component due to nonlinear interaction in the sample. The lock-in amplifier filters the photodiode signal for this 2 MHz (reference frequency) component. The integrated signal from the lock–in is read out using a National Instruments DAQ module (PXI-6259) and an image scanning software written in LabWindows/CVI.

Pump-probe signal detection is achieved in epi-mode by using a polarizing beam splitting cube (10FC16PB.5, Newport). Back-scattered photons from highly scattering
media such as paint samples and panels have random polarization due to multiple scattering events. The polarizing beam splitter selectively reflects the multiply scattered light into the detection arm and rejects the polarization preserving direct back reflected light. Similar to the transmission mode the pump light is rejected using a high quality band pass filter and the probe light is focused onto an amplified photodiode (PDA55, Thorlabs) to be analyzed using a lock-in amplifier. The back reflected light which passes through the polarizing beam splitter is de-scanned and directed into a confocal detection arm using a coverslip. With this setup confocal reflectance can be simultaneously collected along with the pump-probe signal. Confocal reflectance images are used as a guide for pump-probe imaging. Unless specified, all the experiments mentioned in this chapter were done in epi-mode configuration, since paint samples are generally not suitable for transmission mode imaging.

Unless specified, the wavelengths used for the pump-probe experiments are 810 nm and 720 nm for the probe and the pump, respectively. Both the pump and the probe are pre-compensated for the dispersion in the microscope (mainly from the objective and the polarizing beam splitting cube). A folded prism pair arrangement is used for both pump and the probe separately to control the dispersion [156, 157]. Dispersion compensation is achieved by adjusting the prism insertions to minimize cross-correlation width in rhodamine 6G at the focus in the microscope. The cross-correlation width (full width half maximum) is generally optimized to about 240 fs.
5.2.3. Image acquisition

The first step in a pump-probe imaging experiment is calibration of the lock-in amplifier signal. Rhodamine 6G (R6G) is chosen for this purpose since it does not have any linear absorption above 600 nm, but has a large two-photon cross-section at our wavelengths (720 nm and 810 nm). Therefore, when the pump and the probe pulses are overlapped, R6G will exhibit only instantaneous response (sum frequency absorption). The phase of the lock-in amplifier is adjusted to make the signal positive in one channel and zero in the other. Since sum frequency absorption is a loss process and is set as a positive signal, the convention for the pump-probe signal is set as defined below:

- Positive signal corresponds to loss in the probe due to the presence of the pump. Since sum frequency absorption and excited state absorption cause loss in the probe beam, they show up as positive signal.
- Negative signal corresponds to gain in the probe due to the presence of the pump. Since ground state depletion and stimulated emission cause a gain in the probe beam, they show up as negative signal.

Alternatively, the phase of the lock-in can also be set by putting the pump beam directly on the photodiode and adjusting the lock-in signal to be negative and in one channel. Since a direct measurement of the pump modulation corresponds to a gain, the signal is set as negative to match our convention. This measurement is done by blocking the probe beam and removing the pump-blocking bandpass filter in front of the detection
photodiode. The pump needs to be heavily attenuated before the filter is removed so that it does not saturate the photodiode.

A pump-probe image (typically 256 x 256 pixels) is constructed by raster scanning the laser beam across the sample and simultaneously reading out the digitized signal from the data acquisition card. Typically, a laser dwell time of about 100 µs per pixel is used with a corresponding 30 µs lock-in amplifier time constant. The standard scan voltage used for the pump-probe images in this chapter is 1.0 V x 1.0 V, which corresponds to a field of view of 315 µm x 315 µm for the 20x 0.7 NA objective. Transient information is obtained by acquiring pump-probe images (also called transient absorption images) at different pump-probe time delays and the generated datasets are termed ‘pump-probe delay stacks’, meaning a stack of images taken with different pump-probe time delays. These image stacks yield pump-probe decay traces (pump-probe signal vs. time delay) for each pixel in the image. These decay traces contain chemically specific information which can be used to identify the composition of each pixel in the image. In this document both ‘decay trace’ and ‘delay trace’ refer to ‘pump-probe signal vs. time delay’.

Similarly ‘pump-probe depth stacks’ can be acquired by taking pump-probe images at different depths and a fixed pump-probe time delay. These datasets can be used to obtain 3-dimensional information for multi-layer pump-probe imaging.
5.2.4. Data analysis

Data analysis and visualization of pump-probe image stacks was done using custom MATLAB (MathWorks) software. Various analysis methods used for pump-probe microscopy are summarized below.

Excited state dynamics measured by pump-probe imaging are generally multi-exponential. The straightforward method of characterizing the pigments is to perform multi-exponential fitting to extract time constants (lifetimes) of the multiple components (short and long) of the decay traces. Multi-exponential fitting is useful to obtain quantitative characterization of the chemical signature of the pigments. In this work, multi-exponential fitting was performed using nonlinear least-squares method. Standard error for all parameters was computed using 95% confidence intervals.

In one procedure, the decay traces of all the relevant pixels in one or multiple pump-probe delay stacks are averaged to obtain a single decay trace. Only pixels containing the major component of the pigment are selected. The averaged decay trace was generally fitted using a bi-exponential function shown as Equation 53.

\[ A_s e^{-t/\tau_s} + A_l e^{-t/\tau_l} + B \]  \hspace{1cm} (53)

The parameters of interest are \( \tau_s \) (short time constant), \( \tau_l \) (long time constant) and \( A_s/A_l \) (ratio of short and long amplitudes). B (baseline) accounts for any background from the imperfect rejection of pump. Fitting of an averaged decay trace is useful to obtain characteristic decay parameters of the pigment.
Another procedure is to fit each pixel of the pump-probe delay stack individually. In this case, all the relevant pixels in one or multiple delay stacks are fitted individually one by one and the multi-exponential fit parameters obtained for each pixel are stored separately. Again, only pixels containing the major component are selected. The information obtained from this procedure is useful in visualizing and quantifying the distribution of the fit parameters in the pump-probe image, especially to determine heterogeneity. In both the above mentioned procedures signal thresholds were used to preferentially reject pixels with low signal to noise or no signal at all.

Phasor plot analysis is a simple and fast method to visualize the distribution of multi-exponential decay in transient absorption images [158, 159]. It is computationally much faster than nonlinear fitting. The application of phasor analysis for pump-probe microscopy is described in [160]. In phasor analysis, the pump-probe decay at each pixel is mapped to a coordinate pair \((g, s)\) called the phasor. For a pump-probe signal \(I(t)\), the phasor is calculated as:

\[
g = \frac{\int I(t) \cdot \cos(\omega t) \, dt}{\int|I(t)| \, dt} \tag{54}
\]

\[
s = \frac{\int I(t) \cdot \sin(\omega t) \, dt}{\int|I(t)| \, dt} \tag{55}
\]

where \(\omega\) is the phasor frequency that can be tuned to optimize the analysis. A two-dimensional histogram of phasors for all the pixels in a pump-probe image is called a phasor plot. Each pixel in the image corresponds to a point in the phasor plot. Thereby,
a phasor plot gives a visual representation of the distribution of lifetimes in the pump-probe delay stack. Since each pigment has a specific pump-probe signature, it corresponds to a specific position in the phasor plot. Therefore, we can potentially identify a pigment based on the position of the corresponding pixel on the phasor plot. Also, phasor plots are useful to visualize the heterogeneity in the pump-probe signature of the pigments.

5.3. Characterization of lapis lazuli

In this section we describe the pump-probe microscopy characterization of lapis lazuli. Lapis lazuli, a semi-precious rock, is the natural source of the prized historical royal blue pigment ‘ultramarine’ [161]. Lapis lazuli, also referred as ‘lapis’, has a rich and long past. In ancient civilizations of Mesopotamia [162] and Egypt [163] lapis was a popular gemstone used in jewelry. In medieval times, it was exported to Europe and has also been traded in the east. Lapis lazuli has been identified in Indian murals and Chinese paintings. In Europe, it was mainly used to make the ultramarine pigment. Genuine ultramarine was very valuable, since it provided an intense blue color unlike any other alternatives and was very stable. It was imported from Asia by sea, probably through Venice, the major trading port link to the east and hence the name ultramarine, which literally means ‘beyond the sea’. Imported lapis lazuli raw material was expensive and the further laborious extraction process required to produce pure quality pigment made ultramarine as expensive as gold. Ultramarine was extensively used in 14th and 15th
century Italian paintings (especially Venetian) and illuminated manuscripts [164].

However, due to its high cost, ultramarine was only applied to the most precious parts of the artwork like symbolic figures such as Madonna or Christ. Ultramarine was often underpainted with a cheaper blue pigment such as azurite to reduce the amount of ultramarine used and thus decreasing the total expense. Generally, lapis lazuli or lapis can refer to both the rock and the pigment made from the rock. In this document the pigment material will be referred as either lapis/lapis lazuli or ultramarine whereas the rock will only be referred as lapis lazuli.

In ancient times the only known deposits of lapis lazuli were in the Sar-e-Sang mines of Badakhshan, a north eastern province in modern day Afghanistan. These mines have been worked for more than 6000 years and still produce the best quality lapis lazuli. Lapis lazuli is formed by contact metamorphism in limestone. Very few sources of lapis lazuli exist due to the low probability of the geological conditions required for its formation. In the 19th century sizable deposits were found in Andes (Chile) and Lake Baikal (Russia). In smaller quantities, it has also been found in United States (Colorado and California), Tajikistan, Italy, Myanmar and Pakistan [165, 166].
The main component of lapis lazuli (Figure 5.4(a)) is the mineral lazurite, a complex sulfur containing sodium aluminosilicate (Na₈₋₁₀[Al₆Si₆O₂₄]₆S₂₋₄) belonging to the sodalite group. The blue color of lazurite is attributed to the sulfur radical anion [169]. Various accessory minerals such as calcite, pyrite, sodalite, diopside, hauynite, forsterite, nosean, muscovite and wollastonite are commonly found in the lapis lazuli rock [164]. These accessory minerals are removed by a lengthy purification process, which is repeated multiple to times to obtain lapis of various grades. Mineral pigments such as azurite are produced by a simple grinding, washing and sieving process. With lapis lazuli rock this simple technique results in a greyish-blue powder with high proportion of colorless particles. Ultramarine pigment of this kind was found in Byzantine manuscripts dating from sixth to twelveth centuries [161]. In the 13th century a new method of extraction was developed. A detailed description of this complex method was given by Cennino Cennini in the beginning of the 15th century. This is the most well known method and is still used commercially to obtain the best quality pigment (Figure 5.4(b)).
Figure 5.5: Linear absorption spectra of natural lapis lazuli.

Figure 5.5 shows the linear absorption spectra of lapis lazuli. We can see the absorption drop-off towards the near-infrared wavelengths. This makes lapis lazuli ideally suited for pump-probe imaging, since the typical near-infrared wavelengths (720-810 nm) used for pump-probe imaging [43, 46] can penetrate through a thick lapis paint layer and are also absorbed enough to generate a strong pump-probe signal.

5.3.1. Comparison of natural and synthetic ultramarine

In 1828, ultramarine was synthesized for the first time in Paris [161]. Jean Baptiste Guimet developed an economical industrial process to synthesize ultramarine, thereby reducing the cost of ultramarine pigment by more than 10 times. Synthetic ultramarine rapidly substituted the natural version in the artist’s palette, though the use of lapis lazuli as a gemstone has persisted. Differentiating natural and synthetic ultramarine could be helpful in identifying 19th century copies from 14th and 15th century originals.
Figure 5.6: Pump-probe images (zero delay) of natural lapis and synthetic ultramarine. On the right are the corresponding pump-probe decay traces averaged over the indicated region of interest (white rectangle).

We studied commercially available natural and synthetic ultramarine samples using pump-probe microscopy technique. The samples used are:

1. Natural lapis mixed in casein binder and painted on paper; Obtained from Kremer Pigments, New York (catalogue number: 10530).

2. Synthetic ultramarine mixed in acrylic binder and painted on paper; Obtained from Golden Artist Colors, New York (catalogue number: GMSA400).

Natural lapis is mixture of coarsely ground non-uniform particles, whereas synthetic ultramarine consists of uniform sized finely ground particles. We acquired pump-probe
delay stacks of both natural and synthetic ultramarine. Figure 5.6 shows pump-probe images of natural lapis and synthetic ultramarine taken at zero time delay [170]. The total power used for natural and synthetic ultramarine was about 3.1 mW and 2.5 mW, respectively. As expected, we can see that natural lapis has non-uniform particles whereas synthetic ultramarine has uniform sized particles. On the right, Figure 5.6 shows the corresponding pump-probe decay/delay traces averaged over the region of interest (shown as white rectangle in the pump-probe images). Both lapis and synthetic ultramarine show negative pump-probe signals, which according to our convention (see section 5.2.3) corresponds to either stimulated emission, ground state depletion or a combination of both the processes.

Table 5.1: Parameters for bi-exponential fits of decay traces of natural and synthetic ultramarine.

<table>
<thead>
<tr>
<th></th>
<th>Short time constant (ps)</th>
<th>Long time constant (ps)</th>
<th>Ratio of short and long components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural ultramarine</td>
<td>0.54 ± 0.01</td>
<td>6.9 ± 0.3</td>
<td>2.5 ± 0.05</td>
</tr>
<tr>
<td>Synthetic ultramarine</td>
<td>0.74 ± 0.01</td>
<td>4.5 ± 0.3</td>
<td>6.6 ± 0.35</td>
</tr>
</tbody>
</table>

From the decay traces in Figure 5.6 we can clearly see that both natural and synthetic lapis exhibit complex excited state dynamics at the standard wavelengths (720 nm pump and 810 nm probe). The resulting multi-exponential decay is different for both the pigments and this can be used to differentiate them. We performed bi-exponential fitting of decay traces averaged over all the relevant pixels in the pump-probe image for
both the pigments and the resulting fit parameters are listed in Table 5.1. We can see substantial differences in fit parameters of synthetic and natural ultramarine; hence they can be readily distinguished. Figure 5.6 shows that pump-probe imaging can extract structural differences along with specific chemical information at a spatial level down to an individual pigment grain.

In addition to the major component lazurite (negative signal) we can see some particles with positive signals in natural lapis (Figure 5.6). These could be a variety of accessory minerals found in natural lapis lazuli rock. These minor components can be collectively considered as impurities. Even after the lengthy purification process of lapis, some of the accessory minerals still remain and we are able to see a varying degree of these impurities in multiple pump-probe images of natural lapis. This is a significant differentiating factor from synthetic ultramarine. We can see that the synthetic ultramarine has virtually no impurities (minor components). We have acquired and analyzed multiple pump-probe delay stacks to confirm this trend. The presence of accessory minerals (impurities) can be used as an additional parameter in distinguishing natural and synthetic pigments.

5.3.2. Power scaling of the pump-probe signal in lapis lazuli

To confirm the nonlinear origin of the pump-probe signal we performed a power study of lapis in casein binder (#10530, Kremer Pigments). The pump-probe signal (transient absorption) is proportional to the product of pump and probe powers [41].
Hence, the signal should scale linearly with both the pump and the probe powers, separately.

![Graphs showing linear scaling of signal with pump and probe powers](image)

**Figure 5.7: (a) Pump and (b) probe power dependence of pump-probe signal in natural lapis lazuli.**

We acquired pump-probe delay stacks of lapis with increasing average powers of pump and probe pulses, separately. Probe power was kept constant at 0.7 mW during pump power scaling and the pump power was kept constant at 1 mW during probe power scaling. For each delay stack, the decay traces of all the relevant pixels (negative signal) were averaged to obtain a single decay trace. Peak signal thresholds were used to select only pixels with good signal to noise. Bi-exponential fitting was performed on this average decay trace to extract the fit parameters. Figure 5.7 shows the dependence of the short and long components of the pump-probe decay with the (a) pump and (b) probe powers. We can see that both short and long decay components of the pump-probe signal scale linearly within experimental error. This confirms the nonlinear scaling
of our pump-probe signal in lapis. We have also confirmed that the short and long decay
time constants do not vary with either the pump or the probe power levels.

5.3.3. Lapis lazuli in different binders

Casein is one of the earliest binders used in paintings. It was widely used until the
invention of the oil painting techniques. Around 15th century, oil mostly replaced the use
of casein (tempera style) in European paintings. Casein is one of the natural proteins
found in milk. It is made by precipitating milk, and mixed with an alkali to make binder.
Even though casein binder dries quickly, it takes a few months for the binder to cure
completely. Therefore, re-creating casein binder based paint samples is very time
consuming.

To study the effect of binders on the pump-probe signal of lapis we imaged
natural lapis in different binders. The samples used were lapis (#10520, Kremer Pigments)
in casein and lime wash binders.

Table 5.2: Parameters of bi-exponential fits of lapis lazuli in casein and lime wash
binders.

<table>
<thead>
<tr>
<th></th>
<th>Short time constant (ps)</th>
<th>Long time constant (ps)</th>
<th>Ratio of short and long components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>0.53 ± 0.01</td>
<td>6.8 ± 0.2</td>
<td>2.4 ± 0.06</td>
</tr>
<tr>
<td>Lime wash</td>
<td>0.49 ± 0.02</td>
<td>7.1 ± 0.3</td>
<td>2.5 ± 0.09</td>
</tr>
</tbody>
</table>

We acquired pump-probe delay stacks of lapis in casein and lime wash binders.
The total power impinging on the samples was about 2.8 mW (split equally between
pump and probe). We performed bi-exponential fitting of the decay traces averaged over all relevant pixels (negative signal) in 5 delay stacks for both the pigments. The resulting fit parameters are listed in Table 5.2. We can see that the characteristic fit parameters of the pump-probe decay signature for both lapis samples are equal within experimental errors. This indicates that the pump-probe decay signature of lapis is not affected by either of the two binders. We have also verified that the binders do not exhibit any pump-probe signal at the used wavelengths (720 nm for pump and 810 nm for probe).

5.3.4. Heterogeneity in the pump-probe signature

Pump-probe signature depends on the local molecular structure of the material. In a crystalline solid such as lapis, there can be considerable variation in the local environment. Therefore, it is important to understand the heterogeneity of pump-probe signature in lapis lazuli.

Table 5.3: Heterogeneity in fit parameters of the pump-probe signature of lapis.

<table>
<thead>
<tr>
<th></th>
<th>Mean time constant ± Mean fit error</th>
<th>Heterogeneity (sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short time constant</td>
<td>0.53 ± 0.128</td>
<td>0.123</td>
</tr>
<tr>
<td>(ps)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long time constant</td>
<td>6.8 ± 3.8</td>
<td>2.8</td>
</tr>
<tr>
<td>(ps)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We acquired pump-probe delay stacks from 12 different regions of the lapis (#10530, Kremer Pigments) paint layer sample. The total laser power impinging on the sample was about 2.5 mW. We performed bi-exponential fitting of each relevant
(negative signal) pixel in all the delay stacks. Selecting only statistically valid fits resulted in about 1100 sets of fit parameters. The variation (sigma) in the time constants of these 1100 pixels is defined as ‘heterogeneity’. Table 5.3 lists this sigma for both short and long time constants, along with their means. ‘Mean fit error’ is the average standard fit error calculated from the 95% confidence intervals of the corresponding parameter. The variation (heterogeneity) in the time constant can either be inherent variation in sample or due to unreliability of the fit. From Table 5.3, we can see that the average uncertainty of the fits is on the order of the heterogeneity (sigma) in the sample. Therefore, inherent heterogeneity in the lapis sample is smaller than variation due to unreliable bi-exponential fitting.

![Cumulative histogram phasor plot of 12 pump-probe delay stacks of natural lapis lazuli.](image)

**Figure 5.8: Cumulative histogram phasor plot of 12 pump-probe delay stacks of natural lapis lazuli.**
To visualize the heterogeneity in the lapis samples, we performed phasor analysis of all the delay stacks. Figure 5.8 shows a histogram of phasors (at $f = 0.15$ THz) for all the pixels in the 12 pump-probe delay stacks. We can clearly see the distribution of lapis lazuli in the negative quadrant and two different kinds of impurities in the positive quadrant. The spread in the lapis phasors indicates the heterogeneity in the pump-probe signature of the lazurite (the main component in lapis).

![Figure 5.8](image)

**Figure 5.8** shows a histogram of phasors (at $f = 0.15$ THz) for all the pixels in the 12 pump-probe delay stacks. We can clearly see the distribution of lapis lazuli in the negative quadrant and two different kinds of impurities in the positive quadrant. The spread in the lapis phasors indicates the heterogeneity in the pump-probe signature of the lazurite (the main component in lapis).

**Figure 5.9**: (a) Histogram plot of phasors for all the pixels in a delay stack of lapis lazuli. (b) RGB image of the lapis delay stack, where color corresponds to the region indicated in the phasor plot. The scale bar is 50 µm.

Figure 5.9(a) shows a histogram of phasors for a single delay stack of lapis. Two regions (blue and red circles) are selected across the spread of the phasors. The blue circle encloses the main component pixels, whereas the red region encloses the outliers. Figure 5.9(b) shows an RGB image in which the pixels are colored based on the region...
their corresponding phasor falls in. For example, all the pixels whose phasor falls in the region indicated by the blue circle in the phasor plot are colored blue in the image.

We performed bi-exponential fitting of decay traces averaged over all the pixels belonging to a region. The time constants (short and long) for the red region are about 15% different from the time constants for the blue region.

5.3.5. Various grades of lapis lazuli

Figure 5.10: Zero delay pump-probe images of (b) grey-blue grade or low quality lapis and (d) pure lapis lazuli. Also shown are the brightfield camera images (taken at a different location) of (a) low quality lapis and (c) pure lapis. All the scale bars are 50 µm.
The purification process of lapis lazuli used to remove various accessory minerals in the lapis rock is generally repeated multiple times, resulting in ultramarine pigment of different quality/grades. To investigate the pump-probe signature of various types of lapis, we studied seven different lapis varieties commercially available from Kremer Pigments, in casein binder. We acquired pump-probe delay stacks from 5 different regions for each of the lapis lazuli samples. The total power used for each of the samples was about 2.8 mW (split equally between pump and probe).

Figure 5.10 shows zero time delay pump-probe (transient absorption) images of (b) grey-blue grade or low quality lapis lazuli (#10500, Kremer Pigments) and (d) pure lapis lazuli (#10530, Kremer Pigments). For comparison, brightfield camera images of (a) low quality and (c) pure quality lapis from a random area in the sample are also featured. We can see clear differences in particles sizes and heterogeneity between low and pure quality lapis. From the bright field images, we can see that low quality lapis (Figure 5.10(a)) has smaller sized pigment grains (lazurite) and a higher percentage of impurities (colorless and brown particles). On the other hand, the pure quality lapis (Figure 5.10(c)) contains a high density of larger pigment grains and fewer impurities. We can clearly see that, pump-probe images also show this information. Moreover, pump-probe imaging can extract this kind of structural information in deeper layers where linear techniques would fail.

Figure 5.11 shows zero delay pump-probe images of (b) ‘crystalline, light’ lapis lazuli (#10540, Kremer Pigments) and (d) Chilean sourced lapis lazuli (#10560, Kremer
Pigments). For comparison, brightfield camera images of (a) crystalline and (c) Chilean lapis from a randomly selected area in the sample are also featured. We can see the distinct crystalline structure of lapis in Figure 5.11(b). Crystalline grade lapis contains larger crystals than other grades and gives a lighter hue.

Figure 5.11: Zero delay pump-probe images of (b) crystalline lapis [170] and (d) Chilean lapis lazuli. Also shown are the brightfield camera images (taken at a different location) of (a) crystalline lapis and (c) Chilean sourced lapis. All the scale bars are 50 µm.

The size, structure and quality of particles in the pigment depend on the extraction/purification process used to create the pigment. Accessing such chemically
specific structural information can give us an indication of the method used to prepare
the pigment. Various pigment preparation/extraction techniques are specific to certain
periods of time. Thus, information about the preparation technique can help art
conservators/historians in dating of the artwork.

Table 5.4: List of bi-exponential fit parameters of the pump-probe signature for
seven varieties of lapis lazuli.

<table>
<thead>
<tr>
<th>Pigment name (Kremer catalog no.)</th>
<th>Geological source</th>
<th>Short time constant (ps)</th>
<th>Long time constant (ps)</th>
<th>Ratio of short and long components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grey-blue grade (#10500)</td>
<td>Afghan</td>
<td>0.48 ± 0.01</td>
<td>7.0 ± 0.2</td>
<td>1.9 ± 0.04</td>
</tr>
<tr>
<td>Medium quality (#10510)</td>
<td>Afghan</td>
<td>0.53 ± 0.01</td>
<td>6.6 ± 0.1</td>
<td>2.3 ± 0.03</td>
</tr>
<tr>
<td>Good quality (#10520)</td>
<td>Afghan</td>
<td>0.53 ± 0.01</td>
<td>6.8 ± 0.2</td>
<td>2.4 ± 0.06</td>
</tr>
<tr>
<td>Pure (#10530)</td>
<td>Afghan</td>
<td>0.56 ± 0.01</td>
<td>6.6 ± 0.2</td>
<td>2.6 ± 0.05</td>
</tr>
<tr>
<td>Crystalline, light (#10540)</td>
<td>Afghan</td>
<td>0.49 ± 0.01</td>
<td>7.1 ± 0.2</td>
<td>2.4 ± 0.05</td>
</tr>
<tr>
<td>Chile (#10560)</td>
<td>Chile</td>
<td>0.69 ± 0.01</td>
<td>5.2 ± 0.2</td>
<td>3.2 ± 0.08</td>
</tr>
<tr>
<td>Chile, sky blue (#10562)</td>
<td>Chile</td>
<td>0.65 ± 0.01</td>
<td>4.5 ± 0.2</td>
<td>4.6 ± 0.14</td>
</tr>
</tbody>
</table>

We performed bi-exponential fitting of decay traces averaged over all the
relevant pixels in multiple delay stacks, for each of the seven lapis lazuli varieties. Table
5.4 lists the important fit parameters for all the varieties [170]. Two of the samples are
identified as having a Chilean source and the rest of the samples are identified as having
an Afghan source by Kremer Pigments. We can see that all the lapis samples of Afghan
source have similar fit parameters. Interestingly, we can see that Chilean lapis has clear
and substantial differences in characteristic fit parameters from the Afghan lapis. These differences in characteristic pump-probe signature can potentially be used for determining geological provenance of natural lapis [165, 166, 171, 172]. This would require a more comprehensive characterization of lapis samples of known geological origin, especially samples sourced from regions other than Chile and Afghanistan.

Also, characterizing the minor components (impurities) could give an important additional parameter for geo-sourcing of the lapis. Various studies have shown presence of accessory minerals in lapis, which are unique to particular geological regions [166, 171]. For example, wollastonite is considered to be exclusively found in Chilean sourced lapis lazuli [166, 173].

**5.3.6. Wavelength dependence of pump-probe signal in lapis lazuli**

To study the wavelength dependence of pump-probe signal in lapis, we compared the pump-probe signature of lapis at three different pump wavelengths. The evolution of various transient absorptions processes contributing to the pump-probe signal depend on the pulse wavelengths. Therefore, changing wavelengths can give different pump-probe signatures.

We acquired 5 pump-probe delay stacks at each pump wavelength, while keeping the probe wavelength constant at 810 nm. Bi-exponential fit parameters of the decay traces averaged over all relevant pixels (negative signal) in 5 delay stacks for each pump wavelength are listed in Table 5.5. We can see clear differences in the
characteristic fit parameters of lapis at different pump wavelengths. We have also seen
that the pump-probe signal strength increases with shorter pump wavelengths. This is
expected due to stronger linear absorption of lapis at shorter wavelengths.

Table 5.5: List of bi-exponential fit parameters of the pump-probe signature of
lapis at different pump wavelengths.

<table>
<thead>
<tr>
<th>Pump wavelength</th>
<th>Short time constant (ps)</th>
<th>Long time constant (ps)</th>
<th>Ratio of short and long components</th>
</tr>
</thead>
<tbody>
<tr>
<td>720 nm</td>
<td>0.54 ± 0.01</td>
<td>5.9 ± 0.2</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>690 nm</td>
<td>0.63 ± 0.03</td>
<td>5.2 ± 0.5</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>650 nm</td>
<td>0.56 ± 0.02</td>
<td>7.1 ± 0.7</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

5.3.7. Three dimensional imaging of lapis lazuli

To demonstrate the 3-dimensional spatial resolution capability of pump-probe
microscopy, we acquired zero time delay pump-probe images at different depths in a
lapis lazuli (#10530, Kremer Pigments) paint layer. The sample used is basically a layer of
paint (casein binder) on a thick piece of paper. This was obtained from the Kremer
historic pigments chart. The total power used for this study was about 4 mW. Figure 5.12
shows a depth stack of zero delay pump-probe images of lapis. Z = 0 is the top layer of
the paint sample. The negative Z direction corresponds to deeper layers. The stack of
images clearly shows distinct spatial information at different depths, thereby
demonstrating optical sectioning. We were able to image through the entire paint layer
of about 70 µm. Therefore, the imaging depth in this case was limited by the thickness of the paint sample, rather than signal to noise of the technique.

**Figure 5.12:** Depth stack of zero delay pump-probe images of lapis lazuli. Z = 0 µm corresponds to the top layer.
5.4. *Other blue pigments*

5.4.1. *Azurite*

Azurite is natural basic copper carbonate ($\text{Cu}_3(\text{OH})_2(\text{CO}_3)_2$) [174]. It is generally found along with the more abundant malachite ($\text{Cu}_2(\text{OH})_2(\text{CO}_3)$) [175] in copper ore deposits all over the world. Azurite is relatively unstable in atmosphere. It is sometimes pseudomorphically replaced by green malachite, which is the stable form. The process involves replacement of CO$_2$ units with H$_2$O, thereby dependent on the partial pressure of carbon dioxide [176].

Azurite was the most commonly used blue pigment in European paintings throughout the middle ages [177]. Mainly because of its wide-spread availability and low cost compared to the prized blue lapis lazuli. It was also widely used in the east, mainly in China and Japan. Synthetic azurite (called blue verditer or blue bice) was first manufactured in 17$^{th}$ century. It was cheaper and is known to be widely used until the invention of Prussian blue, a stable synthetic pigment.

We studied commercially available natural and synthetic azurite in casein binder, using pump-probe microscopy. However, azurite (both synthetic and natural) did not exhibit any pump-probe signal at our standard wavelengths. We did see pump-probe signals from various minor components (impurities) in natural azurite. We have noticed that for power levels greater than 10 mW, synthetic azurite turned black due to heat deposition. It is known that heating turns azurite into black copper oxide. Interestingly,
this black copper oxide exhibits a pump-probe decay, which can be mistaken as the signature of azurite.

### 5.4.2. Indigo

Organic pigments are generally difficult to characterize using conventional techniques such as x-ray based elemental analysis (due to low Z number) and Raman spectroscopy (due to fluorescence interference and weak scattering) [12]. Here, we characterize indigo (a historically significant organic pigment) using pump-probe microscopy.

Indigo is a blue-violet colored organic dye. A good review of its origin, history and use is given in [178]. Indigo has been used as a textile dye in many ancient civilizations. India is considered to be the earliest indigo production center. Indigo was relatively rare in Europe until the end of middle ages. After the establishment of a direct trade route to India in 15th century, significant amounts of indigo were imported into Europe. In Europe, indigo was mainly used as a pigment in paintings. At the end of 19th century, synthetic indigo was invented and successfully replaced the natural dye. Indigo is still heavily used as a textile dye, mainly for blue jeans.
Figure 5.13: (b) Pump-probe image of indigo at 500 fs delay. The scale bar is 50 μm. (a) Decay trace averaged over the region of interest shown as white rectangle in the image. (c) Decay trace with expanded time axis showing the signal peak around 400-500 fs. (d) Histogram plot of phasors for all the pixels in 10 pump-probe delay stacks of natural indigo.

We acquired pump-probe delay stacks of natural Indian indigo (#36000, Kremer Pigments) in casein binder. Figure 5.13 (b) shows a transient absorption image at 500 fs pump-probe time delay. The total power used for this image was about 2.1 mW (split equally between pump and probe). Figure 5.13(a) shows the corresponding pump-probe decay trace averaged over the region of interest (shown as white rectangle in (a)). We can see that indigo exhibits a long positive decay, indicating excited state absorption at the standard wavelengths. Figure 5.13(c) shows the decay trace with expanded time axis.
We can see that the pump-probe signal peaks around 500 fs. We have confirmed this to be a physical phenomenon, rather than an artifact due differences in pump and probe dispersion in indigo.

To understand the heterogeneity in the pump-probe signal of indigo, we acquired 10 pump-probe delays stacks and performed phasor analysis. Figure 5.13(d) shows a cumulative histogram of phasors (at 0.25 THz) for all the pixels in the 10 delay stacks. We can clearly see the distribution of indigo in the positive quadrant. The spread in phasors is very small, indicating virtually no heterogeneity in the pump-probe signature. The strong pump-probe signal of indigo could also contribute to the lack of spread. No other minor components (impurities) were found.

![Figure 5.13(d) showing a cumulative histogram of phasors for all the pixels in the 10 delay stacks.](image)

**Figure 5.14: Zero delay pump-probe image of indigo in casein binder with the pump wavelength at 810 nm. On the right is the corresponding decay trace averaged over the region of interest (white rectangle).**

Figure 5.14 shows a pump-probe image of indigo acquired with the pump and the probe wavelengths interchanged. The pump (810 nm) and the probe (720 nm)
powers used in this case are 5.5 mW and 3.5 mW, respectively. From the decay trace (on the right), we can see that indigo exhibits instantaneous pump-probe signal at these wavelengths, indicating sum frequency absorption. We can also see that the peak is not shifted from zero delay, indicating that there is no delay introduced due to differences in the dispersion of pump and probe wavelengths.

**5.5. Pigments of other colors**

We have studied other historically significant pigments of various colors using pump-probe microscopy. And have seen qualitative differences in their pump-probe decay signatures, which can be used to distinguish them in a mixture. Some of these pigments are discussed in this section.

**5.5.1. Vermilion**

Vermilion is an orangish red pigment of great significance through all the ages. Chemically, the pigment is mercuric sulfide, HgS. Vermilion is considered toxic due to presence of mercury. Its naturally form, called cinnabar has been used for thousands of years, especially by Romans and Chinese. Unlike other historical pigments, vermilion was synthesized fairly early. The synthesis of vermilion by fusing mercury and sulfur is believed to be invented in the 8th century by Chinese. Artificial vermilion was introduced to Europe in the 12th century. Vermilion has been the principle red in the paintings for centuries until it was replaced by cadmium red in early 20th century. A good overview of its origin, history and use is given in [179].

149
We investigated commercially available synthetic vermilion (#42000, Kremer Pigments) in casein binder using pump-probe microscopy. Figure 5.15(b) shows a zero delay pump-probe image of vermilion. We can see that the pigment is very finely ground. The total power used for this measurement was about 0.7 mW. Please note that vermilion has an extremely strong pump-probe response and can be damaged at powers greater than 8 mW. Figure 5.15(a) shows the decay signature of main component of vermilion averaged over the region of interest shown as a white rectangle in the image.
We can see that vermilion only shows instantaneous sum-frequency absorption at the standard wavelengths and does not exhibit any long lived signal. Therefore, it is necessary to investigate the wavelength parameter space to find wavelengths where vermilion exhibits a unique pump-probe signature.

Figure 5.15(d) shows a cumulative histogram of phasors (at 0.1 THz) for all the pixels in the 5 pump-probe delay stacks of vermilion. We can clearly see the distribution of the main component in the positive quadrant. There is a large spread in the phasors indicating significant heterogeneity. The phasor plot also reveals two minor components (impurities) in the delay stacks. Figure 5.15(c) shows representative decay signatures of the two characteristic impurities. The impurities are most probably byproducts of the synthesis procedure; therefore can be indicative of the synthesis method used. Also, by interchanging the pump and probe wavelengths i.e. pumping at 810 nm and probing at 720 nm, we have seen that the impurities do not give any pump-probe response, whereas the main component of vermilion exhibits instantaneous response.

5.5.2. Ochres

Ochres are one of the oldest natural pigments known to mankind. They have been used in pre-historic times for cave paintings. Ochres are made from the clay found in iron ore mines around the world. They have been part of the artist’s palette throughout the middle and modern ages. Ochres contain varying amounts of iron oxides (hematite) and iron hydroxides (goethite), along with various accessory minerals. When
iron oxide is the main component, red color is observed and increasing percentage of iron hydroxide gives it a yellow hue [180]. We studied commercially available red and yellow ochres (in casein binder) using pump-probe microscopy.

Figure 5.16: Zero delay pump-probe image of red iron oxide (hematite). On the right is the corresponding decay trace averaged over the region of interest.

Figure 5.16 shows a zero delay pump-probe image of pure red iron oxide (#48600, Kremer Pigments), taken with 1.5 mW of total laser power. On the right is the characteristic pump-probe decay trace averaged over the region of interest (white rectangle in the image). The pump-probe signature of iron oxide (hematite) indicates a combination of instantaneous sum frequency absorption and long exponential decay due to excited state absorption. We have investigated a variety of iron based pigments and they all exhibit qualitatively similar decay behavior consisting of instantaneous response with a long tail.
In Figure 5.17, we show cumulative histogram plots of phasors (0.2 THz) of all the pixels in 5 pump-probe delay stacks for various ochres. From Figure 5.17(a), we can see that pure iron oxide (#48600, Kremer Pigments) shows a symmetric distribution of main component in the positive quadrant and virtually no impurities. On the other hand, Burgundy sourced natural red ochre (#11574) shows relatively more heterogeneity, along with a clear presence of impurities. Red ochre is mostly iron oxide, but also contains a variety of accessory minerals. The shift in the position indicates small quantitative differences between natural red ochre and pure iron oxide. Also, from Figure 5.17(c) and

**Figure 5.17:** Histogram phasor plots of (a) pure iron oxide (hematite), (b) Burgundy red ochre, (c) Burgundy yellow ochre and (d) Italian yellow ochre.
(d), we can see that the yellow ochres, which contain larger percentage of iron hydroxide, are significantly different from the red. However, we do not see any substantial differences between the main component in yellow ochres sourced from Burgundy (#11752) and Italy (#40220). We can see some qualitative differences between the impurities in the two yellow ochres. Further investigation of the impurities is required to find a geological source specific distribution. It is well known that ochres sourced from different regions have distinct accessory minerals [180, 181]. For example, gypsum is found only in ochres sourced from Italy [180].

5.6. **Paint cross-section imaging**

To demonstrate pump-probe microscopy in real centuries-old historical pigments, we studied a paint cross-section taken from a 15th century painting. Paint cross-sections are made by embedding a paint chip in polyester resin and polishing until a flat cross-section of the multi-layers is visible [182]. Generally, a scalpel is used to remove the paint chip (300-400 µm in size). Considerable skill is required to remove a paint chip without causing visual changes. Therefore, paint chips are mostly taken from edges or previously damaged areas of the paintings.
Figure 5.18: (a) A camera image of the 15th century Italian panel painting [183]. The real size of the painting is 34.8 x 22.2 inches. (b) A brightfield camera image of the paint chip cross-section taken from the robe of Madonna.

We obtained a paint cross-section sample from North Carolina museum of art, Raleigh. The sample was taken from an oil painting (Figure 5.18(a)) titled Madonna and Child with Two Angels by Italian painter Francesco Francia. The painting is dated as 1495-1500 AD. Figure 5.18(b) shows a brightfield camera image of the paint chip cross-section taken from the robe of Madonna. Using conventional techniques (such as polarized light microscopy), it has been determined that the blue of the robe is lapis lazuli, along with an underpaint coating of azurite. We can see that the top blue layer (about 30 µm) has a different blue shade compared to the thicker second layer (about 70 µm). This is a typical example of using expensive ultramarine pigment as the top layer over a thicker coating of cheaper azurite to reduce the total cost. We have used Raman spectroscopy to confirm the presence of lapis lazuli by observing the characteristic 550 cm$^{-1}$ peak.
Figure 5.19: (a) Zero delay pump-probe image of the paint chip cross-section in the region of interest (shown as the yellow square in Figure 5.18(b)). (b) Histogram plot of phasors for all the pixels in a delay stack of the paint cross-section. (c) RGB image of lapis composition extracted from the delay stack (color corresponds to the regions indicated by circles in the phasor plot). (d) Decay traces averaged over the pixels in the regions shown in the phasor plot. All the scale bars are 20 µm.

Figure 5.19(a) shows a zero delay pump-probe image of the paint chip cross-section. The total power used for this acquisition was about 7 mW. We can see the presence of lapis lazuli particles (negative signal) and some impurities (indicated by positive signal) in the top layer. We performed bi-exponential fitting of the decay trace averaged over all the pixels with negative signal in the delay stack. The short and the long decay time constants were found to be $0.54 \pm 0.06$ ps and $5.2 \pm 1.0$ ps, respectively.
The ratio of short decay signal amplitude to long decay signal amplitude is $2.8 \pm 0.36$.

The fit parameters obtained from the paint chip are comparable (within experimental error) to the characteristic fit parameters of the pump-probe signature of the commercially available present-day lapis lazuli (see Table 5.4). This verifies the presence of lapis lazuli and the ability of pump-probe microscopy to identify pigments that are hundreds of years old. Lapis is considered to be very stable; therefore the close match of the pump-probe signatures is expected.

Figure 5.19(b) shows a histogram plot of phasors (at 0.15 THz) for all the pixels in the delay stack of the paint chip cross-section. We can clearly see the presence of the main component of lapis (blue circle) at the same position on the plot as the standard present day lapis samples (see Figure 5.8). We can also see the presence of two different kinds of impurities in the positive quadrant. Please note that the symmetric distribution across the center is due to noise pixels. From the phasor plot, we can also see that the heterogeneity of the main component of lapis is on the order of heterogeneity in the standard commercial samples (see Figure 5.8).

Table 5.6: Heterogeneity in bi-exponential fit parameters of the pump-probe signature of lapis in the paint chip cross-section sample.

<table>
<thead>
<tr>
<th></th>
<th>Mean time constant ± Mean fit error</th>
<th>Heterogeneity (sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short time constant (ps)</td>
<td>0.45 ± 0.140</td>
<td>0.103</td>
</tr>
<tr>
<td>Long time constant (ps)</td>
<td>5.4 ± 3.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>
To quantify the heterogeneity of the lapis signature in the paint cross-section, we performed bi-exponential fitting of all the relevant (negative signal) pixels in the delay stack, individually. Similar to section 5.3.4, the variation (sigma) in the time constants of all the pixels is defined as heterogeneity. Table 5.6 lists this sigma for both short and long time constants, along with their means. We can see that the heterogeneity of the lapis signature in paint chip is quantitatively similar to the heterogeneity in the standard sample (see Table 5.3). ‘Mean fit error’ is the average standard fit error calculated from the 95% confidence intervals. From Table 5.6, we can see that the average uncertainty of the fits is greater than the heterogeneity (sigma) in the sample. This is probably due to the low signal to noise of this acquisition.

From Figure 5.19(b), we can see the presence of a second negative component (red circle) apart from the main lapis component (blue circle). Representative decay traces of both these components are shown in Figure 5.19(d) in their corresponding color. Figure 5.19(c) shows an RGB image of the pump-probe delay stack in which the pixels are colored based on the region their corresponding phasor falls in. Thereby, the main component is colored blue and the minor negative component is colored red. From the image, we can see that the red component is present only in about 4 particles. Also, from Figure 5.19(c) and (d), we can see that the red component is significantly different from the main component (blue). Adding the fact that the signature of this red component is similar to that of some impurities found in azurite, we can say that the
source of the red component is most likely impurities in azurite (which forms the layer below lapis) rather than heterogeneity in the lapis signature.

5.7. Multi-layer imaging

![Multi-layer imaging graph]

Figure 5.20: Representative pump-probe decay signatures of (a) synthetic ultramarine at pump and probe wavelengths of 720 nm and 810 nm, respectively and (b) vermilion at pump and probe wavelengths of 810 nm and 720 nm, respectively.

One of the major benefits of pump-probe microscopy in imaging historical artwork would be the ability to provide chemically specific high resolution contrast in multiple layers. To demonstrate the multi-layer capability of the technique, we imaged a hand painted mock-up panel of ultramarine (blue) over vermilion (red). Synthetic versions of the pigments were used in oil binder.

At the standard pump (720 nm) and probe (810 nm) wavelengths, ultramarine (see Figure 5.20(a) and Figure 5.6) exhibits a negative multi-exponential decay signature, whereas vermilion (see Figure 5.15(a)) exhibits an instantaneous response. If we interchange the wavelengths i.e. pump at 810 nm and probe at 720 nm, ultramarine does
not have a pump-probe response, whereas vermilion (see Figure 5.20(b)) still shows an instantaneous signal.

**Table 5.7: Set of parameters where only the corresponding pigment exhibits pump-probe response.**

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Pump wavelength (nm)</th>
<th>Probe wavelength (nm)</th>
<th>Time delay (fs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultramarine</td>
<td>720</td>
<td>810</td>
<td>400</td>
</tr>
<tr>
<td>Vermilion</td>
<td>810</td>
<td>720</td>
<td>0</td>
</tr>
</tbody>
</table>

Using the above mentioned dependence of pump-probe signal on wavelengths, we can find a set of parameters where only one pigment exhibits pump-probe signal. Such sets of parameters for both ultramarine and vermilion are listed in Table 5.7. At a pump-probe time delay of 400 fs with our standard wavelengths, only ultramarine has pump-probe response. And at a zero time delay with interchanged wavelengths, only vermilion has a pump-probe response.

We independently acquired two pump-probe depth stacks with the two sets of parameters shown in Table 5.7. The total power used was about 7.5 mW. Each of these depth stacks shows the 3-d distribution of the corresponding pigment. Figure 5.21 shows an overlay image of these distributions. The ultramarine is colored blue and vermilion is colored red. For clarity, an exponential correction was applied, accounting for the power loss with depth. Such power loss with depth can be reduced by using optimized wavelengths with lower linear absorption. Also shown are the depth cross-sections in both directions showing multi-layer pigment specific information. We can
clearly see the separation of the two pigment layers and high resolution depth sectioning.

Figure 5.21: Overlay of depth stacks showing 3-dimensional multi-layer information. Blue and red corresponds to ultramarine and vermilion pigments, respectively. Depth cross-sections in both X and Y directions (at the places indicated by white lines) are also shown.
References


Biography