NONLINEAR MULTICONTRAST MICROSCOPY FOR STRUCTURAL AND DYNAMIC INVESTIGATIONS OF MYOCYTES

By

Catherine Ann Greenhalgh

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Physics

University of Toronto

© Copyright by Catherine Ann Greenhalgh 2009
Nonlinear Multicontrast Microscopy for Structural and Dynamic Investigations of Myocytes

Catherine Ann Greenhalgh

Department of Physics, University of Toronto

Doctor of Philosophy, 2009

Abstract

Nonlinear multicontrast microscopy is established in this study as an important tool for understanding biological structure and function of muscle cells. Second harmonic generation, third harmonic generation and multi-photon excitation fluorescence are acquired simultaneously in order to establish the origin of nonlinear signal generation in myocytes, and investigate myocyte structure and functionality during muscle contraction. Using structural cross-correlation image analysis, an algorithm developed specifically for this research, for the first time, third harmonic generation is shown to originate from the mitochondria in myocytes. The second harmonic, which is generated from the anisotropic bands of the sarcomeres, is further shown to be dependent on the crystalline order of the sarcomeres, thereby providing a potential diagnostic tool to evaluate disorder in muscle cells. The combination of the second and third harmonic provides complementary information that can be used to further elucidate the basic principles of muscle contraction.

Time-lapse nonlinear microscopic imaging showed structural and functional dynamics in the myocytes. The second harmonic contrast revealed nonsynchronized nanocontractions of sarcomeres in relaxed, non-contracting, cardiomyocytes and Drosophila muscle samples, providing insight into the asynchronous behaviour of individual sarcomeres. Furthermore, macrocontracting samples were found to exhibit a synchronization of nanocontractions, providing new evidence for how muscles contract. Dynamic image correlation analysis, another algorithm
developed specifically for this investigation, is used to reveal networks of mitochondria, which show fluctuations of multi-photon excitation fluorescence and third harmonic generation signals. The intensity fluctuations in the networks reveal both slow and fast dynamics; phase shifts of the slow dynamics between different networks are observed. Fast dynamics appear only in the inner networks, suggesting functional difference between interfibrillar and subsarcolemma mitochondria.

The groundwork for studying bioenergetics of mitochondria in cardiomyocytes with nonlinear multimodal microscopy is fully developed in this work. The origin of the nonlinear signals and the development of the image analysis techniques provide a solid foundation to further study of muscle contractility and bioenergetics.
Acknowledgements

A project of this magnitude would not be possible without the support of many individuals. First, I would like to acknowledge the support and guidance of Professor Virginijus Barzda, my supervisor. I appreciate all that he has done for me, both professionally and personally.

The project would also not have been a success without the many collaborators, group members and undergraduate students who I have had the pleasure of working with. Thanks to Richard Cisek, whom I’ve worked with the longest. His efforts with improvements and maintenance of the laser microscope system are one of the reasons I am able to write this thesis. Working with Nicole Prent on myocyte investigations and her efforts alongside Richard in the programming of the LabView interface are also appreciated. Nicole’s editing and cookies have also been a great help in finishing. I would like to also thank Arkady Major, not only for the development of the Yb:KGW laser used for some of the experiments but also for his insightful discussions on nonlinear optics and laser-matter interactions. Thanks to Donatas Zigmantas for his work in building the Ti:Sapphire laser. The work I did on cardiomyocytes could not have been possible without our collaborations with Jeff Squier, Jürg Aus der Au, Steven Elmore and Johannes HGM van Beek, thanks to all of you. Drosophila experiments would not have been possible without our collaboration with Professor Bryan Stewart. Thank you for the sample preparation and all your help.

The support I have received throughout graduate school extends beyond those within my group. The days at UTM would go much slower had it not been for the enthusiasm and spontaneity of the 5th floor research community, especially Sarah who has helped keep me sane through many late nights. Further thanks go to Sarah for all her help with editing.

Outside of campus life, the friendship of Christa means a great deal to me, and I appreciate her being there from the very start. My family, including my in-laws, nieces, nephews, aunts, uncles and cousins are all close to my heart, and they have helped in balancing home and school life. In particular, I’d like to thank my brothers and sisters (Ann Marie, Stephen, Paul, Martin, Richard, Robert, Michelle, James and Karen) and especially my mother, Sandra all whom have made me who I am today, have supported me through the years and have allowed me to learn through all of their experiences. Finally, I would like to thank Al, who has helped me in more ways than he can know; I love you and could not have done this without you.
# Table of Contents

**List of Tables**  viii
**List of Figures**  viii

**Chapter I - Overview**  1
  Section 1.1: Motivation  1
  Section 1.2: Thesis outline  2

**Chapter II - Nonlinear Microscopy, Contractility and Bioenergetics**  7
  Section 2.1: Introduction  7
  Section 2.2: Nonlinear mechanisms of light-matter interaction  8
    (a) Multi-photon Excitation Fluorescence  10
    (b) Second Harmonic Generation  13
    (c) Third Harmonic Generation  16
    (d) Multicontrast microscopy in biological systems  18
  Section 2.3: Contractility and bioenergetics  20
    (a) Structure of muscle sarcomeres  21
    (b) Sarcomere contractility  24
    (c) Current research in muscle contractility  27
    (d) Structure of mitochondria  28
    (e) Bioenergetics of mitochondria  29
    (f) Current research in bioenergetics of mitochondria  30
  Section 2.4: Harmonic generation microscopy of muscle  32

**Chapter III - Experimental Design and Methods**  35
  Section 3.1: Introduction  35
  Section 3.2: Microscopes used in this study  35
    (a) Two channel nonlinear multicontrast microscope:  35
    (b) Three channel nonlinear multicontrast microscope:  38
  Section 3.3: Specifications of lasers for this study  39
    (a) Ti:Sapphire laser (A and B):  39
    (b) Nd:glass laser:  40
    (c) Yb:KGd(WO4)2 laser:  40
  Section 3.4: Sample preparation  41
    (a) Cardiomyocyte preparation:  41
    (b) *Drosophila* preparation:  42
  Section 3.5: Image processing and analysis techniques  42
    (a) Image segmentation  43
    (b) Deconvolution  43
    (c) Filtering  45
    (d) Correlation spectroscopy  47
    (e) Volume rendering  48
    (f) Gaussian fitting  49

**Chapter IV - An Extended Description of Analysis Algorithms**  50
  Section 4.1: Introduction  50
Section 4.2: Structural Cross-correlation Image Analysis
(a) SCIA algorithm
(b) SCIA performed with fluorescing polystyrene beads

Section 4.3: Dynamic Image Correlation Analysis
(a) Dynamic Image Correlation Analysis (DICA) algorithm
(b) Using the standard deviation to highlight active regions in cardiomyocytes
(c) Demonstration of DICA with simple sinusoidal functions

Chapter V - Microcrystalline Origin of SHG in Myocytes
Section 5.1: Introduction
Section 5.2: SHG response in Drosophila myocytes
Section 5.3: Importance of actin-myosin organization for efficient SHG
(a) Effect of thick filament mutations on SHG efficiency
(b) Effect of Actin on SHG intensity
Section 5.4: Dynamic investigations to further elucidate origin of SHG
(a) Effect of induced structural changes on SHG intensity
(b) Effect of natural periodic contractions on SHG intensity
(c) Changes in the A-band width during contraction
Section 5.5: Conclusion

Chapter VI - Understanding Contractility Using SHG
Section 6.1: Introduction
Section 6.2: Nonsynchronized nanocontractions in myocytes
Section 6.3: Periodic contraction dynamics in myocytes
Section 6.4: Induced disruptions in periodic contractions
Section 6.5: Conclusion

Chapter VII - Origin of THG in Myocytes
Section 7.1: Introduction
(a) THG and NAD(P)H fluorescence
(b) THG and 1064 nm excitation autofluorescence
(c) THG and TMRM fluorescence excited with 1064 nm
(d) THG and SHG
(e) Linear image cross-correlation at different depths of cardiomyocytes
Section 7.2: Understanding the mechanism for THG generation in mitochondria
Section 7.3: Third harmonic generation in Drosophila samples
Section 7.4: Conclusion

Chapter VIII - Functional Dynamics of Mitochondrial Activity in Myocytes Observed with MPF and THG Microscopy
Section 8.1: Introduction
Section 8.2: Mitochondrial flickering dynamics in MPF
Section 8.3: Comparison of MPF and THG fluctuation dynamics
Section 8.4: Wave-like THG intensity propagation across cardiomyocyte
List of Tables
Table 1: Correlation Coefficients for Several Functions .......................................................... 58
Table 2: Parts of the Sarcomere ............................................................................................ 170

List of Figures
Figure 2.2-1: Jablonski diagram of two-photon excitation fluorescence and subsequent relaxation processes. ........................................................................................................... 11
Figure 2.2-2: Excitation regions for single and two-photon excitation fluorescence ....... 12
Figure 2.2-3: Schematic illustration of second harmonic generation ................................. 14
Figure 2.2-4: Schematic illustration of third harmonic generation ..................................... 16
Figure 2.3-1: Schematic Diagram of Composition of Muscle .............................................. 21
Figure 2.3-2: Structure of Myosin Head and Tail Domains .............................................. 23
Figure 2.3-3: Diagram showing the initialization of a contraction .................................... 26
Figure 2.3-4: Diagram of how myosin and actin interact to generate a contraction ....... 26
Figure 3.2-1: Schematic diagram of the multicontrast microscope ..................................... 37
Figure 4.2-1: Demonstration of SCIA on an imaged fluorescent polystyrene bead ....... 54
Figure 4.2-2: Comparison of traditional merged image technique to the SCIA technique ........................................................................................................................................... 55
Figure 4.3-1: Local activity of a cardiomyocyte observed with THG microscopy .......... 57
Figure 5.1-1: Schematic structural diagram of two sarcomeres. ...................................... 60
Figure 5.2-1: Microscopy images of larval and adult Drosophila muscles ...................... 62
Figure 5.2-2: Comparison of SHG and MPF signals from Drosophila larvae muscle.... 64
Figure 5.3-1: Analysis of SHG from IFM myocytes of different Drosophila mutants... 66
Figure 5.4-1: Effect of sustained contraction on the neighbouring sarcomeres .............. 69
Figure 5.4-2: The evolution of SHG intensity in Drosophila larval myocytes after addition of KCl. ................................................................................................................ 70
Figure 5.4-3: The evolution of SHG and THG in a cardiomyocyte .................................... 71
Figure 5.4-4: SHG intensity changes with contraction of Drosophila larval myocytes. 72
Figure 5.4-5: Influence of periodic contractions in the Drosophila myocyte on the SHG efficiency....................................................................................................................... 73
Figure 5.4-6: Original data and double peak Gaussian fits of a row of 4 sarcomeres at 2 different instances ........................................................................................................... 75
Figure 6.2-1: Nanocontractions in cardiomyocytes ............................................................. 81
Figure 6.2-2: Pearson’s Coefficient of the sarcomere length fluctuations and Z-line position for nanocontracting sample............................................................................................................................. 83
Figure 6.2-3: Length and standard deviation of length fluctuations dependence on the number of sarcomeres.................................................................................................................................................. 85
Figure 6.2-4: Nanocontractions in *Drosophila* larval myocyte.......................................................... 88
Figure 6.2-5: Length-tension relationship for sarcomeres.................................................................................. 90
Figure 6.3-1: Stress-velocity relationship for cardiomyocytes........................................................................ 92
Figure 6.3-2: Periodic synchronization of nanocontractions into macrocontraction in cardiomyocyte.................................................................................................................................................. 96
Figure 6.3-3: Pearson’s Coefficient evaluation of sarcomere length changes and Z-line position movement in time for contracting sample................................................................................................ 99
Figure 6.3-4: Length and standard deviation of length fluctuations dependence on the number of sarcomeres.................................................................................................................................................. 102
Figure 6.3-5: SHG image and time evolution of a row of sarcomeres during periodic contractions (*Drosophila* larval muscle)................................................................................................................ 104
Figure 6.4-1: SHG image of a cardiomyocyte and time evolution of a row of sarcomeres after the addition of FCCP.......................................................................................................................... 106
Figure 6.4-2: SHG image and time cross section of sustained contraction in *Drosophila* larval myocyte.................................................................................................................................................. 107
Figure 7.1-1 Comparison of NAD(P)H MPF and THG images in a typical cardiomyocyte excited with 837 nm laser pulses.................................................................................................................................................. 112
Figure 7.1-2: The comparison of autofluorescence and THG of a typical cardiomyocyte with 1064 nm excitation.................................................................................................................................................. 114
Figure 7.1-3 Comparison of MPF and THG images of TMRM labelled cardiomyocyte excited with 1064 nm laser pulses.................................................................................................................................................. 116
Figure 7.1-4 Comparison of SHG and THG images of cardiomyocytes excited with 1064 nm laser pulses.................................................................................................................................................. 118
Figure 7.1-5: Pearson’s coefficient (r) analysis of optical slices at different depths of typical cardiomyocytes. .................................................................................................................................................. 119
Figure 7.3-1: *Drosophila* larval muscle imaged with ~800 nm excitation.................................................. 121
Figure 7.3-2: Combined SHG-THG image of adult *Drosophila* indirect flight muscle. 122
Figure 8.2-1: Typical cardiomyocyte image in MPF and standard deviation of pixels’ intensity fluctuations.................................................................................................................................................. 126
Figure 8.2-2: MPF intensity flickering analysis of pixels inside and outside of the network in a cardiomyocyte .................................................................................................................................................. 128
Figure 8.2-3: MPF flickering activity networks in cardiomyocytes................................................................... 131
Figure 8.2-4: Correlation analysis of the network (c) and other networks as well as with the noise from within the myocyte area ................................................................. 133

Figure 8.3-1: Comparison of MPF and THG flickering dynamics of networks in cardiomyocytes ........................................................................................................... 140

Figure 8.4-1: Wave-like motion of a high intensity THG front ................................ 145

Figure 8.5-1: Effect of TMRM labelling on the flickering kinetics in a cardiomyocyte. 146

Figure 8.6-1: Inhibitor effect on the flickering of cardiomyocytes. ...................... 148

Figure AII.2-1: Schematic of the different subsections of the muscle. ............... 169

Figure AII.2-2: Cross section lattice structures of the different regions of the sarcomere .......................................................................................................................... 172

Figure AII.3-1: Different stages of contraction of an individual sarcomere .......... 173
Chapter I - Overview

Section 1.1: Motivation

This thesis is driven by multidisciplinary research in nonlinear optics and biology; it establishes the origin of nonlinear signals within muscle cells and explores how these nonlinear signals can be used to elucidate biological functions. In investigating second harmonic generation, third harmonic generation, and multi-photon excitation fluorescence within myocytes, this research expands the relatively new imaging technique of nonlinear multicontrast microscopy and provides a foundation to further explore these contrast mechanisms. Harmonic generation microscopy is advantageous because signals can be generated as a result of the inherent structural organisation of the biological sample and therefore does not require the addition of labels. Heat deposition is minimised for nonlinear microscopy versus traditional single-photon excitation fluorescence if the excitation wavelength of the laser radiation is chosen to be outside the linear and nonlinear absorption regions of the sample. An increased awareness and understanding of nonlinear imaging techniques will be beneficial for future research in medical and biological physics.

This thesis also focuses on utilizing these nonlinear microscopy techniques as a means of investigating the contractility and bioenergetics of muscle cells. The fast response of our microscope, combined with the non-invasive nature of nonlinear imaging provides an ideal means for imaging cells both in vitro and in vivo.

The simultaneous acquisition of several contrast mechanisms requires extensions to modern image analysis techniques in order to fully evaluate the underlying relationships between nonlinear signals. The use of this multicontrast nonlinear microscopy, combined with the development of several image analysis techniques and the subsequent achievement of new knowledge about cellular functions, will provide a stepping stone towards further understanding bioenergetics and contractility of the muscle cells.
Section 1.2: Thesis outline

This thesis is a comprehensive examination of both the structure and functional dynamics of myocytes using simultaneous nonlinear multicontrast microscopy. Chapter II provides a review of nonlinear optics and, more specifically, the nonlinear imaging techniques of second harmonic generation (SHG), third harmonic generation (THG) and multi-photon excitation fluorescence microscopy (MPF). This chapter also details contractility and bioenergetics and provides a thorough discussion on recent nonlinear microscopic biological investigations. By reviewing the current knowledge in nonlinear microscopy and bioenergetics and contractility, Chapter II provides a platform for the open questions remaining in this field, and how some of these questions are addressed in this research. The experimental designs and procedures as well as the sample preparations that are common to the ensuing chapters are described in Chapter III, with an extended description of the newly developed image analysis techniques in Chapter IV. These analysis techniques include structural cross correlation image analysis (SCIA), which is beneficial for understanding structural origin of microscopy signals, and dynamic image correlation analysis (DICA), which aids in comparisons of the time evolution between two imaged pixels in the time sequence of the frames.

The main results of this research are divided into several chapters, each based on either the structural or dynamic investigations of the myocytes. Chapter V and Chapter VI provide details on the structure and dynamics within the sarcomeres of myocytes, respectively, using second harmonic generation microscopy. Chapter V shows the differences in sarcomere structure between adult and larval *Drosophila melanogaster* muscle, but, more importantly, focuses on the importance of semicrystalline structure within the sarcomeres for efficient SHG. With this understanding, Chapter VI examines the principles of muscle contraction in myocytes through investigations of small scale, nonsynchronized, contractions of sarcomeres called nanocontractions. Periodic contractions are shown to have the synchronization of these nanocontractions. The loss of contraction periodicity with the addition of a mitochondrial uncoupler is also examined.
In Chapter VII, the origin of the third harmonic is shown, for the first time, to be generated from the mitochondria within cardiomyocytes. The origins of MPF and SHG are also confirmed in this chapter. Unlabelled *Drosophila* muscles are found to generate THG from the trachea, as well as the isotropic band of the sarcomeres.

Through an examination of the MPF and THG intensity fluctuations, termed flickering, within the myocyte mitochondria, Chapter VIII reveals several mitochondrial networks within the cardiomyocytes. Mitochondria appear to exhibit both slow and fast dynamics, and comparisons of the flickering networks in MPF and THG demonstrate that slow scale fluctuations follow similar kinetics. The wave like propagation of high intensity front observed in the third harmonic is also examined in this chapter.

Chapter IX provides a summary of the main conclusions and outlines the specific contributions I have made to this work. It also briefly discusses the benefits of this work for future studies, in particular to the area of biophysics of muscle contraction. Additionally, Appendix I provides a list of the abbreviations used, while Appendix II presents a more basic description of myocytes, for readers outside the area of biophysics. A description of the media files that accompany the electronic version of this thesis is provided in Appendix III.

The research I did on cardiomyocytes was conducted in collaboration with J. Squier, J. Aus der Au, S. Elmore and J. van Beek. The experimental data was recorded at the University of California at San Diego by the coauthors. I carried out the image processing and analysis of the data, which ultimately led to the main results of chapters VI-VIII. To aid in identifying the origin of the third harmonic in cardiomyocytes in Chapter VII, I designed and programmed the SCIA algorithm. I also developed and programmed the DICA algorithm, which I used in reaching the conclusions in Chapter VIII. Under the guidance of V. Barzda, I carried out the analysis for Chapter VI and discovered the synchronization of nanocontractions to form a macrocontraction. In collaboration with B. Stewart, experiments on the *Drosophila* samples were performed. The *Drosophila* samples were supplied by Stewart's group. I conducted the experimental work on the *Drosophila* samples with the help of R. Cisek, N. Prent, and the occasional
assistance of C. Green (Fig. 5.4-2). This work is reflected in several publications, proceedings, posters and oral presentations as listed below:

**Published Articles and Proceedings**


Seminars also given for this type of bullet

Additional Seminars


Posters


Articles in preparation

- C. Greenhalgh, N. Prent, R. Cisek, J. Aus der Au, S. Elmore, J.H. van Beek, J.A. Squier, V. Barzda "Synchronization of nanocontractions of sarcomeres to produce periodic contractions of cardiomyocyte".

- C. Greenhalgh, J. Aus der Au, S. Elmore, J.H. van Beek, J.A. Squier, V. Barzda "Visualization of flickering mitochondrial networks in cardiomyocytes with multicontrast harmonic generation and fluorescence microscopy".

- R. Cisek, C. Greenhalgh, N. Prent, V. Barzda, "Structural cross-correlation image analysis in multicontrast microscopy".
Chapter II - Nonlinear Microscopy, Contractility and Bioenergetics

Section 2.1: Introduction

Multidisciplinary research, combining physics with biology enables researchers to use light-matter interactions to explore biological structures and relate observations to the physiological function. The development of the microscope in the mid 17th century initialized this interdisciplinary combination of optics and biology, and has since then led to countless discoveries, from the first observation of a single cork cell to modern research studies on cellular dynamics. As developments in optical techniques progress, our biological and physiological understanding advances; laser scanning confocal microscopy, for example, has achieved high resolution 3-D imaging capability of different structures within the biological tissue (Brakenhoff, Vandervoort et al. 1988; Minsky 1988).

Over the last 15 years, another optical technique, nonlinear microscopy, has emerged as a powerful research tool. Understanding the physics of nonlinear light-matter interactions and applying these interactions as contrast mechanisms for cellular imaging investigations provides an excellent means for answering biological questions. The viability of using nonlinear interactions for imaging begins with a foundation in understanding the origins of nonlinear signals within biological samples. The contrast mechanism must also be shown to be advantageous over current techniques. Muscle structure and function has been studied for more than 50 years, and because it is now fairly well understood, it provides an excellent system to examine the origin of nonlinear signals within biological samples. Additionally, since several unanswered questions still remain regarding bioenergetics of muscle contraction, the practicality of nonlinear imaging can be demonstrated in helping to answer unresolved biological problems. A complete understanding of muscle contraction and bioenergetics, for example, would be invaluable in diagnosing muscular disorders, arrhythmia and fibrillation. The knowledge gained from developing nonlinear contrast mechanisms (through muscle cell
investigations) may also provide insight into understanding organelle functions within other cell types.

Nonlinear multi-photon effects occur as a result of a 2\textsuperscript{nd} or higher power dependence on the excitation intensity (Boyd 2003). Although there are numerous nonlinear effects that can be used to investigate biological systems, multi-photon excitation fluorescence (MPF), second harmonic generation (SHG) and third harmonic generation (THG) microscopy will be the focus here, as they are the techniques relevant to this thesis. This chapter provides details on the physical mechanisms of nonlinear microscopy and examines the current state of knowledge in contractility and bioenergetics of muscle contraction and mitochondria. The open questions in muscle structure and function are discussed alongside ongoing research in nonlinear microscopy of myocytes. Attention is concentrated on how our nonlinear multicontrast microscopy techniques can be employed to answer some of the standing questions in muscle contraction and bioenergetics.

Section 2.2: Nonlinear mechanisms of light-matter interaction

A thorough explanation of the principles of nonlinear microscopy begins with the linear interaction of light with matter. The linear response of a medium to the electric field can be written in terms of the induced dipole moment per unit volume, identified as the polarization $\vec{P}$, which is given by:

$$\vec{P} = \chi^{(1)} \vec{E}$$  \hspace{1cm} 2.2-1

where $\chi^{(1)}$ is the linear susceptibility and $\vec{E}$ is the electric field; it is assumed that the medium is both lossless and dispersionless (Boyd 2003). Linear light-matter interactions include reflection and refraction of light.

As the applied electric field becomes more intense, nonlinear effects emerge which can be seen by the ‘nonlinear’ relationship between the polarization, the electric field, and the material properties (susceptibility). Nonlinear effects can be described through the power series expansion of the aforementioned polarization equation:
\[ \tilde{\mathbf{P}}(t) = \chi^{(1)} \tilde{\mathbf{E}}(t) + \chi^{(2)} (\tilde{\mathbf{E}}(t))^2 + \chi^{(3)} (\tilde{\mathbf{E}}(t))^3 + \ldots + \chi^{(n)} (\tilde{\mathbf{E}}(t))^3 \]

\[ = \tilde{\mathbf{P}}^{(1)}(t) + \tilde{\mathbf{P}}^{(2)}(t) + \tilde{\mathbf{P}}^{(3)}(t) + \ldots + \tilde{\mathbf{P}}^{(n)}(t) \]

where
\[ \tilde{\mathbf{E}}(t) = E_1 e^{-i\omega_1t} + E_2 e^{-i\omega_2t} + \ldots + E_m e^{-i\omega_mt} + c.c. \]

2.2-2

Here, \( \chi^{(2)} \) is the second order nonlinear optical susceptibility and \( \chi^{(3)} \) is the third order nonlinear susceptibility, \( \mathbf{P}^{(n)}(t) \) is defined as the \( n^{th} \)-order nonlinear polarization and \( \mathbf{E}(t) \) may contain up to \( m \) different frequency components. For clarity, the above equation assumes both the polarization and field strength are scalar. Second order processes include second harmonic generation, while two-photon excitation fluorescence and third harmonic generation are both third order processes. The same excitation source can induce several nonlinear effects simultaneously.

Nonlinear effects are characterized by new components of the \( \mathbf{E} \)-field generated from the acceleration of charges as the nonlinear polarization drives the electric field. This can be seen through the inhomogeneous wave equation given by:

\[ \nabla^2 \tilde{\mathbf{E}} - \frac{n^2}{c^2} \frac{\partial^2 \tilde{\mathbf{E}}}{\partial t^2} = \frac{4\pi}{c^2} \frac{\partial^2 \tilde{\mathbf{P}}}{\partial t^2} \]

2.2-3

where \( n \) represents the linear refractive index and \( c \) is the speed of light in a vacuum (Boyd 2003).

Harmonic generation differs significantly from fluorescence; harmonic generation, including SHG and THG, are parametric processes, while fluorescence is nonparametric (Boyd 2003). Parametric processes are processes whereby the initial and final states are the same and in the momentary time between these states, the population can reside in a forbidden or ‘virtual’ level. In nonparametric processes, the initial and final states are different, so there is a transfer of population from one real level to another. Since parametric processes conserve photon energy, no energy is lost to the system. Nonparametric light-matter interactions, on the other hand, deposit energy into the system, making samples more prone to the effects of photobleaching and thermal
damage. Near the resonance transition of the molecule, parametric processes are resonantly enhanced, however, at these wavelengths, absorption of laser radiation leads to photobleaching of the sample. Optimal imaging requires a balance between excitation wavelength and emission intensity therefore, knowledge of the nonlinear absorption spectrum is beneficial in choosing the imaging parameters. Parametric processes are described by a real susceptibility while nonparametric processes have a complex susceptibility associated with them. Below, a more detailed theoretical description of each of the three aforementioned nonlinear processes (MPF, SHG and THG) is provided.

**(a) Multi-photon Excitation Fluorescence**

Multi-photon excitation fluorescence is an extension of single-photon (linear) excitation fluorescence. When light interacts with a sample, a single photon may be absorbed, exciting an electron from a vibrational state in the ground level to an excited singlet state. From this excited state, several processes may occur. The emission of a photon upon relaxation from the lowest excited state to the ground state is called fluorescence. The emitted photon generally has a longer wavelength than the absorbed photon due to a loss of energy via nonradiative processes such as internal conversion which results in heating of the sample. The difference between the absorption and emission wavelengths is known as the Stokes shift. Intersystem crossing (ISC) to the triplet state leads to other processes including phosphorescence and photobleaching. Although photobleaching is not fully understood, it is a reaction which prevents the molecule from undergoing further fluorescence.

Nonlinear multi-photon excitation fluorescence is very similar to linear fluorescence; the main difference is that 2 or more photons must be simultaneously absorbed (within $10^{-18}$ s) to create the excited singlet state. The consequence of multi-photon absorption is that the single photon that is emitted is at a higher energy than each of the absorbed photons. The longer excitation wavelengths generally used in multi-photon absorption have the additional advantage of allowing deeper penetration into the biological sample, mainly due to the reduced scattering (Chu, Chen *et al.* 2001). Figure 2.2-1 shows the Jablonski energy diagram for two-photon excitation fluorescence, as well as the process of intersystem crossing which leads to phosphorescence.
The simultaneous absorption of a multiple number of photons requires a large density of photons. In fact, the transition rate $R$ for two-photon absorption depends on the square of the intensity $I$ as given by (Boyd 2003):

$$R = \sigma^{(2)} I^2 / \hbar \omega$$

where $\sigma^{(2)}$ is the two-photon absorption cross section. The large intensities required for two-photon absorption necessitate the use of ultrafast lasers. The resulting low overall average power prevents damage to the system while the high pulse intensity facilitates nonlinear absorption. With the high peak power of femtosecond lasers combined with
the tight focusing using a high numerical aperture objective, multi-photon absorption is confined to the high photon density region of the focal volume as depicted in Figure 2.2-2. This confinement allows for intrinsic sectioning of the sample. In traditional confocal microscopy using linear excitation fluorescence, absorption occurs over the whole beam pass (see Figure 2.2-1(a)). For optical sectioning, a pinhole is then used in front of the detector to block the fluorescence from out of focus volume. The pinhole, however, does not prevent absorption and possible photodamage of the sample in the whole beam propagation volume (Denk, Strickler et al. 1990).

![figure 2.2-2](image)

**Figure 2.2-2: Excitation regions for single and two-photon excitation fluorescence.** Schematic diagrams showing an objective focussing the laser light into a ‘cuvette filled with fluorescing dye’ for single-photon excitation fluorescence (a) and two-photon excitation fluorescence (b). The excitation regions are indicated by arrows and shown in dark grey within the ‘cuvette’. The two-photon excitation is clearly confined to the focal volume providing inherent optical sectioning.

Two-photon excitation fluorescence is currently the most widely used nonlinear contrast mechanism for microscopic investigations. The first experimental demonstration of two-photon excitation fluorescence was provided in 1961 (Kaiser and Garrett 1961), even though the first theoretical description of two-photon excitation fluorescence stems back to 1931 (Göppert-Mayer 1931). As a microscopic imaging technique, multi-photon excitation fluorescence was first implemented in 1990 by (Denk, Strickler et al. 1990) who also demonstrated the advantages over traditional confocal microscopy.
Fluorescence imaging is advantageous in the specificity it has. Alongside the naturally occurring fluorophores, a wide variety of labels are available that enable a user to image specific organelles or molecules within biological samples including mitochondria, and numerous proteins. The two main disadvantages of imaging with fluorescence are: the deposition of heat which can damage the sample and the invasiveness of staining. The physiological function of the sample may be compromised with the addition of dyes. Unlike the harmonics described below, the fluorescence response is not coherent; there is no phase matching requirement imposed on the optical response. Therefore, the MPF signal can be directly related to the presence of fluorophores in the sample.

(b) Second Harmonic Generation

When the optical electric field is strong enough and the susceptibility ($\chi^{(2)}$) is non-zero, the second order polarization $\tilde{P}^{(2)}$ can be induced. Following equation 2.2-2, the second order nonlinear polarization (without considering the vector nature of the fields) is given by:

$$\tilde{P}^{(2)}(t) = \chi^{(2)} \tilde{E}(t)^2$$  \hspace{1cm} 2.2-5

If one considers a field with a single frequency component, the nonlinear polarization becomes:

$$\tilde{P}^{(2)}(t) = \chi^{(2)} \tilde{E}(t)^2$$

$$= \chi^{(2)} (E \cos \omega t)^2$$

$$= \frac{1}{2} \chi^{(2)} E^2 (\cos 2\omega t + 1)$$  \hspace{1cm} 2.2-6

where the $2\omega$ contribution, which can lead to the emission at the second harmonic frequency, is now clearly visible and is shown schematically in Figure 2.2-3. For fields with multiple frequency components, the second harmonic can be generated for each of the fields while the cross terms result in other nonlinear processes such as sum-frequency generation and difference-frequency generation.
Figure 2.2-3: Schematic illustration of second harmonic generation
Panel (a) shows the geometry of SHG and panel (b) is the energy-level diagram of SHG.

The generation of the second harmonic is possible only in non-centrosymmetric media. Systems with inversion symmetry have a zero second order nonlinear susceptibility; a non-zero $\chi^{(2)}$ is forbidden if one considers the equation for the second order polarization (see equation 2.2-5). For a system with inversion symmetry, a change in the sign of the applied electric field would result in a sign change in the induced polarization which would lead to:

$$\vec{P}^{(2)}(t) = \chi^{(2)} \vec{E}(t)^2$$
$$-\vec{P} = \chi^{(2)} [-\vec{E}]^2 = \chi^{(2)} E^2$$
$$\Rightarrow \chi^{(2)} = 0$$

Since equation 2.2-7 is only valid when $\chi^{(2)} = 0$, second harmonic generation is forbidden in centrosymmetric media.

At the surface of a material of interest, i.e. at the interface between two media, whether the bulk is centrosymmetric or not, the symmetry can be broken (Bloembergen and Pershan 1962). Molecules at the interface can generate detectable SHG, making surface-SHG a useful tool for surface science (Shen 1989). Florsheimer et al, for example, utilized surface second harmonic to not only quantify the second order

susceptibility tensor for different interfaces including the Langmuir monolayer at a water surface, but also find surface information not obtained with linear techniques (Florsheimer, Bosch et al. 1998).

Aside from having to be non-centrosymmetric, SHG is only efficient when phase matching conditions are met within the bulk. The most efficient conversion is achieved for orientations where the fundamental and harmonic propagate through the medium at the same speed. For angles not in this regime i.e. where there is a phase shift, or walkoff between the fundamental and second harmonic, the conversion efficiency and hence the observed signal is diminished (Armstrong, Bloembergen et al. 1962).

In microscopy, the light focused on the sample with the objective offers illumination of a large cone of angles, dependent on the numerical aperture of the lens. This multi-directional illumination results in phase matching and hence observable second harmonic in at least some of the rays. The efficiency of the second harmonic however is highly dependent on the structure and orientation of the sample itself. The orientation of the axes with respect to the direction of the beam propagation, for example, has been shown to affect the SHG efficiency in myocytes and thin layers of silicon crystal (Chu, Chen et al. 2004; Kolthammer, Barnard et al. 2005).

Although crystalline quartz was the first sample from which SHG was generated (Franken, Hill et al. 1961), since then SHG has been generated in a variety of nonlinear crystals as well as within biological samples including collagen (Freund, Deutsch et al. 1986; Guo, Ho et al. 1997) actin-myosin complexes (Guo, Ho et al. 1997), tubulin (Campagnola, Millard et al. 2002), and chloroplasts (Chu, Chen et al. 2001).

In microscopy, second harmonic generation was the first nonlinear response used as a contrast mechanism (Hellwart and Christen 1974). A large area of a sample was excited with a laser, and the SHG was observed in an optical microscope. In 1978, scanning SHG microscopy was implemented (Gannaway and Sheppard 1978) and 8 years later, the first biological sample (rat tendon) was imaged with SHG microscopy (Freund and Deutsch 1986).
(c) Third Harmonic Generation

Third harmonic generation (THG) is similar to SHG, although in this case, 3 photons of frequency $\omega$ are converted to one photon of frequency $3\omega$, which is shown schematically in Figure 2.2-4. The expansion of the $3^{rd}$ order polarization for a scalar field of a single frequency is given by

$$\tilde{P}^{(3)}(t) = \chi^{(2)} E(t)^3$$

$$= \chi^{(3)} (E \cos \omega t)^3$$

$$= \frac{1}{4} \chi^{(3)} E^3 \cos 3\omega t + \frac{1}{4} \chi^{(3)} E^3 \cos \omega t$$

The $3\omega$ component is evident in the first term of this expansion. As was the case for the second order polarization, applied fields with multiple frequency components could generate the third harmonic for multiple frequencies and may result in additional third order processes including Coherent Anti-Stokes Raman Scattering (CARS) and the optical Kerr effect.

![Figure 2.2-4: Schematic illustration of third harmonic generation](image)

Panel (a) shows the geometry of THG and panel (b) is the energy-level diagram of THG.

Homogeneous media can generate the third harmonic, although under tight focussing conditions the third harmonic vanishes despite having no symmetry restrictions like the second harmonic (Ward and New 1969). The loss of observable THG in the far
field in cases of tight focusing within a homogeneous media with normal dispersion can be examined by the paraxial wave equation. Derived from the inhomogeneous wave equation, the paraxial wave equation allows for the slow spatial variation of the electric field amplitudes along the direction of beam propagation (z direction). The amplitude of the third harmonic \( A_{3\omega} \) found in the solution of the paraxial wave equation is given by (Boyd 2003):

\[
A_{3\omega}(z) = \frac{i6\pi\omega}{nc} \chi^{(3)} A_{\omega}^3 J_{3\omega},
\]

where

\[
J_{3\omega}(\Delta k, z_0, z) = \int_{z_0}^{z} e^{i\Delta k z'} \left( \frac{2iz'}{b} \right)^2 dz',
\]

2.2-9

where \( A_{\omega} \) is the amplitude of the fundamental, \( J_{3\omega} \) is the phase matching integral, \( b \) is the confocal parameter, \( \Delta k = 3k_{\omega} - k_{3\omega} \) is the wave vector mismatch, and \( z_0 \) is the \( z \) value at the entrance of the nonlinear medium. The phase matching integral can be solved analytically for the case of a tightly focussed beam in a homogeneous medium. In this case, the integral in equation 2.2-9 is carried out over all space and the solution becomes:

\[
J_{3\omega}(\Delta k, z_0, z) = \begin{cases} 
0, & \Delta k \leq 0 \\
\frac{1}{2} \pi b^2 \Delta k e^{-\frac{b \Delta k}{2}}, & \Delta k > 0
\end{cases}
\]

2.2-10

For normally dispersive materials, the integral and therefore the amplitude of the third harmonic equals zero, even in the case of perfect phase matching where \( \Delta k = 0 \).

The lack of THG in the far field can be further explained by the Gouy shift, or phase anomaly that a beam undergoes when passing through the focus. Because of the phase shift the beam experiences, the third harmonic generated before the focus
destructively interferes with the third harmonic generated after the focus resulting in a diminished (or loss of) signal in the far field (Ward and New 1969; Boyd 2003).

In order to detect THG in the far field, the focal symmetry must be broken. Consequently, THG highlights the interfaces between two materials, within the same focal volume, with different third order nonlinear susceptibilities or refractive indices (Squier, Muller et al. 1998). However, the third harmonic is generated from the bulk media on either side of the interface, making it mainly a volume effect (Saeta and Miller 2001). Although the third harmonic was first generated and observed in calcite crystal in the early 1960s (Terhune, Maker et al. 1962)), the first efficient THG was generated at a dielectric surface in the 1990s (Tsang 1995). Tsang also showed that multilayer structures can significantly enhance the THG signal in the far field (Tsang 1995; Kolthammer, Barnard et al. 2005).

The sensitivity of THG as a result of interfaces was first exploited with the introduction of scanning THG microscopy in the late 1990s (Barad, Eisenberg et al. 1997). THG strongly depends on the orientation of the interface with respect to the beam propagation (Muller, Squier et al. 1998a). Multilayer structures with layers perpendicular to the beam propagation are shown to be most efficient in generating THG.

To date, several biological samples have been shown to efficiently generate the third harmonic. Rhizoids from green algae (Squier, Muller et al. 1998), chloroplasts (Muller, Squier et al. 1998a), human glial cells (Barille, Canioni et al. 2001), fixed epithelial, and neuron and muscle cells (Yelin, Oron et al. 2002) have all been investigated with THG microscopy.

**Multicontrast microscopy in biological systems**

Although each of the contrast mechanisms and some biological applications have been described above, it is important to return to the fact that the same excitation source can induce several nonlinear effects simultaneously. A sample can be imaged with multiple contrast mechanisms by using a single illumination source and several detectors. In addition to our research group, a multicontrast microscope that utilizes the simultaneous response of SHG, THG and MPF has been developed by (Chu, Chen et al. 2001).
Microscopes utilizing two contrast mechanisms are more widely used. Simultaneous SHG and MPF imaging was used to image labelled neurons (Moreaux, Sandre et al. 2001), labelled neuroblastoma cells (Campagnola, Wei et al. 1999), and muscle and tubulin structures (Campagnola, Millard et al. 2002). THG and MPF were collected simultaneously in glial human cell imaging (Barille, Canioni et al. 2001). Simultaneous SHG and THG microscopy has been used to monitor mitosis in a live zebrafish embryo (Chu, Chen et al. 2003) and study the nonlinear anisotropy of muscle cells (Chu, Chen et al. 2004).

The advantages of multicontrast microscopy far outweigh the difficulties that can arise due to the added level of complexity in the microscope design. Multiple structures within a system can be visualized simultaneously using multicontrast microscopy. This provides structural information of one part of the sample, relative to another part. Studies using multiple fluorescing dyes have enabled researchers to image more than one organelle at the same time, but the addition of multiple dyes increases the likelihood of photodamage and the adverse physiological effects could be compounded. In studies that use multiple dyes, the dyes are preloaded into the sample of interest. The dyes can be chosen such that their excitation wavelengths are the same but the different emission spectra allow one to separate the labels using different optical filters. Alternatively, several excitation sources can be used for dyes with different absorption spectra. In this case, fluorescence images are scanned and recorded separately with each excitation source and the images are combined digitally at a later time. Multiscan imaging, however, does not allow for fast dynamic comparisons between the organelles.

Dynamic visualization of multiple structures is likely the most beneficial aspect of multicontrast microscopy. Monitoring multiple structures in time allows for understanding of the dynamical relationships between these structures, which in turn leads to an understanding of the overall function of the system. In this thesis, multicontrast microscopy is used to examine the structure and functional dynamics of myocytes. Initially, background information into the contractility and bioenergetics of myocytes is needed, and is therefore provided in the next section.
Section 2.3: Contractility and bioenergetics

An understanding of the muscle structure is necessary before exploring its contractility function. It is assumed here that the reader has some knowledge of muscle structure and function, but a more basic description is provided in Appendix II – Structure and Function of Muscle, for readers outside this area of biophysics. There are three types of muscle cells: skeletal, cardiac and smooth. This thesis focuses on skeletal and cardiac muscle. Skeletal myofibrils are long chains of fused cells and, as such, are multinucleated. In cardiac muscle, however, cells are unfused, mono or binucleated, and are connected via intercalated disks. In general, both skeletal and cardiac muscles are comprised of numerous bundles of fibers, where each fiber is a bundle of myofibrils which are each in turn made up of myofilaments (see Figure 2.3-1). The myofilaments contain the most fundamental unit of both skeletal and cardiac myocytes, the sarcomere, which is the structure responsible for muscle contraction, and the structure that gives myocytes their characteristic striations. In between the myofibrils lie the mitochondria, the organelles that provide energy for muscle contraction. The mitochondria, along with the sarcomeres, are of interest to this work and are therefore discussed in more detail below.
Figure 2.3-1: Schematic Diagram of Composition of Muscle
Muscle composition consists of bundles of fibers that are in turn bundles of myofibrils, which are bundles of myofilaments. The myofilaments are made up of alternating isotropic and anisotropic regions and the fundamental unit of a muscle cell, the sarcomere, is defined from the middle of one isotropic band to the middle of the next. The diagram also shows the structures within a sarcomere. The different regions of the sarcomere are labelled in the figure. Additionally, cross-sections of the arrangements of myofilaments in the different regions of the sarcomere are shown: 1) the actin filaments, 2) the myosin filaments, 3) the M-line and 4) the overlap of the actin and myosin. (adapted from http://www.sci.sdsu.edu/class/bio590/). For additional description of the structure see the above text and Appendix II.

(a) Structure of muscle sarcomeres
Muscle structures and muscle contractility have been a source of investigation for more than a century, in fact muscle striations were first observed using a simple microscope in the late 1600s. Unfortunately, the use of a simple microscope was not commonplace and the resolving power of early compound microscopes, combined with the structures of the fibrils, did not allow for detailed or accurate measurements by researchers looking at living striated muscle. Thanks to chemical extraction studies, actin and myosin have been known to be part of muscle since the late 1800s, but it was not until later that the organization and structure of these proteins were studied. Even by 1950, the organization of these proteins was not known, although the anisotropic and isotropic bands (A-band and I-band respectively) had been defined, along with the H-zone (or H-band) and the Z-line (Huxley 2004) (see Figure 2.3-1).
The use of electron microscopy and the advent of phase contrast light microscopy in combination with X-ray crystallography led to significant improvements in understanding the structure and function of muscles. In 1953, a double filament model was proposed as the structural basis for the cross striations and, that same year, electron micrographs confirmed the presence of thin and thick filaments arranged in hexagonal arrays with a region of overlap (Hanson and Huxley 1953; Huxley 1953a). These arrangements are seen in Figure 2.3-1 (1-4).

The thin filaments were found to be the “actin filaments” through several studies, including tests involving the use of an actin-severing protein such as gelsolin which removes only the thin filaments. Although generally considered to be the “actin filaments”, the thin filaments are now recognised to contain troponin and tropomyosin in addition to the actin (Lodish 2000). These two proteins, which were discovered through biochemical investigations, are important for myocyte contraction. Capping proteins at each end of the actin filaments prevent depolymerization and stabilise the filament.

To maintain rigidity and structure, the actin filaments are also bound to the Z-disk, which is comprised mainly of α-actinin; the precise mechanism for how actin binds to the Z-disk is still unclear (Lodish 2000). The Z-disk is a meshwork that in some muscle cells enables the myosin filaments to slide from one sarcomere through the Z-line to the neighbouring sarcomere; this is known as supercontraction (Hoyle, McAlear et al. 1965).

The thick filaments were shown to be myosin through essentially two steps. Initially, salt extraction caused a loss of the anisotropic band and the thick filaments; the extracted molecule therefore was the major molecule in the thick filament. The molecule that was extracted was further analysed and found to be myosin, proving that the anisotropic band contained myosin (Lodish 2000).

In general, myosin is considered a motor protein for cellular movements, characterized by a head, neck and tail domain (Figure 2.3-2). Muscle myosin, the myosin that is found in the samples studied in this work, is a dimer known as myosin II which contains two globular head domains that bind to actin and also exhibit ATPase activity. The heavy chain of the globular head forms an α-helix as it extends through the neck.
domain which is also made up of two light chains. The light chains in the neck domains provide rigidity to the neck. The extra stiffness allows the neck to operate as a lever for the head. The neck domains join the head domains to the tail domains and regulate the function of the head domain. The α-helical heavy chains of each neck region coil together in the tail domain. The coil-coiled form of the tail is quite rigid. The protein structure of the head and neck domains can be seen in Figure 2.3-2.

Figure 2.3-2: Structure of Myosin Head and Tail Domains
The globular head domain made from the heavy chain is seen in blue. The heavy chain extends through the neck region. The neck region of myosin II has two light chains: the essential light chain (yellow) and the regulatory light chain (green). In muscle myosin, two of these head and neck domains are connected in a single tail domain via a coiled-coil structure of the heavy chains. This figure was created with RasMol using structure 1B7T from the protein data bank. (http://www.rcsb.org/pdb/home/home.do)

The myosin molecules are arranged into bipolar thick filaments in which the heads of the myosin are located at either end of the filament. The central region, which forms the H-zone, has no heads.

Thick myosin filaments are connected to the Z-disk via titin, a spring like protein. This protein is important in maintaining rigidity in the A-band. Its elasticity helps to restrain sarcomeres during contraction and relaxation. The titin and its role still being investigated, including work on the organization of the titin molecules within the
filaments and how this multidomained protein spans the region from the Z-line to M-line (Knupp, Luther et al. 2002).

The structure and arrangement of the individual molecules and proteins within the muscle is a key feature in a complete understanding of muscle function. More importantly, the basic organisation of the thin and thick filaments was imperative for an initial understanding of contractility. The discovery of the hexagonal arrangement of myofilaments provided a key element that enabled much of the research to shift from structural explorations to contractility investigations.

(b) Sarcomere contractility

The concept of sliding filaments was introduced by two separate groups at the same time in the early 1950s (Huxley and Niedergerke 1954; Huxley and Hanson 1954). Prior to this introduction, X-ray analysis showed differences between resting and rigor muscle, and it was suggested that two separate filaments interacted during contraction (Huxley 1953b). Aspects of the X-ray diffraction patterns however could not be explained by the popular belief that the filaments themselves contracted. To examine the possibility of a sliding model, H. Huxley and J. Hanson used phase contrast microscopy to measure band-pattern changes in contracting myofibrils (Huxley and Hanson 1954). They found that filaments remained constant in length but the overlap region changed. At the same time, similar results were discovered by A. Huxley and R. Niedergerke, who investigated muscle contraction with interference microscopy (Huxley and Niedergerke 1954). The sliding filament theory was not well received, as widespread belief was that the shortening of the filaments themselves were responsible for muscle contraction. Additionally, X-ray diffraction and EM techniques were at an early stage in their development, and as such, results based on these techniques, in particular results which contradicted previous theories, were generally mistrusted.

In the years following this seminal research, improvements of existing methods and further exploration led to the “swinging crossbridge model” of contraction whereby it was proposed that movement was a result of a change in tilt or shape of the myosin heads attached to actin during the ATP hydrolysis cycle (Huxley 1969). Further evidence of the sliding filament theory built upon this model, but it wasn’t until the mid-1980s that more
definitive evidence was realized via fluorescence microscopy (Sheetz and Spudich 1983). Specific details on the three-dimensional structure of myosin revealed with x-ray crystallography as well as the forces and step sizes involved in the movement of single myosin further supported the sliding filament theory (Finer, Simmons et al. 1994).

An overview of the functionality of muscle from neural signal to sliding of actin can be found in cell or neurophysiology text books (see for example Lodish 2000; Matthews 2001) but is outlined below and depicted in Figure 2.3-3 and Figure 2.3-4. Initially, a neural signal causes a depolarization of the muscle cell that in turn triggers the action potential which propagates into the transverse tubules (t-tubules). As the t-tubules depolarize, Ca\(^{2+}\) is released from the sarcoplasmic reticulum. The calcium binds to the troponin, altering the troponin and tropomyosin position to reveal the actin binding site for the myosin head. When the myosin binds to the actin, the cross bridge cycle begins (Figure 2.3-4). During this cycle the myosin hydrolyses ATP and then the myosin undergoes a conformational change to the head which now binds to the actin filament, forming the cross-bridge. The understanding of the cross-bridge cycle stemmed from both mechanical and structural investigations (Lodish 2000). As the excited myosin releases its energy, ADP and inorganic phosphate dissociates from the myosin and the relaxation produces the longitudinal sliding of the filaments. As the calcium level drops, the troponin and tropomyosin shift back to block the myosin from binding to the actin preventing further contraction.
Figure 2.3-3: Diagram showing the initialization of a contraction
The neural signal depolarizes the muscle cell triggering the action potential which propagates into transverse tubules (dotted arrows). When the transverse tubules depolarize, Ca\(^{2+}\) is released from the sarcoplasmic reticulum (solid arrows) and binds to the troponin causing a conformational change and revealing the actin to the myosin. The myosin then binds to the actin as described in the following figure. When the contraction has finished, the calcium is pumped back into the sarcoplasmic reticulum (dashed arrow) and the tropomyosin hides the actin binding site (Matthews 2001).

Figure 2.3-4: Diagram of how myosin and actin interact to generate a contraction
Here the steps of contraction, or myosin cross-bridge cycle, are shown. Initially, the relaxed myosin hydrolyses ATP and undergoes a conformational change to an energised state. Meanwhile, the calcium binds to the troponin altering the position of the tropomyosin. Once the myosin binding site is revealed on the actin, the energized myosin binds to the actin. The myosin releases its energy in the form of movement of the thin filament. This energy release returns myosin to its relaxed form and is released from the actin when it binds to another molecule of ATP. The cycle then repeats (Matthews 2001).
(c) Current research in muscle contractility

As mentioned in section (b) above, the confirmation of the sliding filament model was achieved through fluorescence imaging techniques. A description of the general process of contraction was also given. Specific details, including the gait of the myosin heads, however are still being understood, as is the mechanism of synchronization of the sarcomeres that leads to a full scale macrocontraction.

In discussing the myosin gait, it is useful to alter the naming convention for the parts of the myosin where now the heads are termed feet, and the necks termed legs (Kinosita, Ali et al. 2005). In myosin II, joints between the foot and leg as well as joints between the leg and tail (the hip joint) are free joints as revealed through optical anisotropy decay measurements (Kinosita, Ishiwata et al. 1984). These free joints are important in elucidating the mechanism of contraction and how myosin walks along the actin. In myosin II, the coiled-coil exhibits two-fold symmetry (Li, Brown et al. 2003), which in turn creates symmetry in the legs while the feet are the same because they are coded by the same gene (Kinosita, Ali et al. 2005). This symmetry implies that a rigid leg would force the feet to be pointing in opposite directions even though the binding sites of actin are unidirectional.

Understanding of the structure of myosin and its symmetry has stimulated the elucidation of how molecular motors walk. A hand over hand technique or inchworm mechanism were competing theories until recent studies revealed the myosin V walk as a hand over hand mechanism (Yildiz, Forkey et al. 2003). Myosin V is similar to myosin II, but has a longer neck region. By using single molecule fluorescence, one foot of the myosin V was labelled and its motion tracked and compared to the proposed mechanisms. In a single ATP hydrolysis cycle, the fluorescent marker was observed to move a distance equal to twice the motor step size, while during the following ATP hydrolysis cycle the marker remained still (since the other foot is moving during this phase) (Yildiz, Forkey et al. 2003); this observation is characteristic of a hand over hand technique. In an inchworm type motion, one foot would always lead, so that for every ATP hydrolysis cycle, the back foot would move forward one motor step and the front foot would do the same. Other myosin types have been shown to follow the hand over
hand type of motion (Okten, Churchman et al. 2004), and despite a lack of evidence, muscle myosin (myosin II) is likely to follow the same type of motion.

The myosin walk is still widely researched and not completely understood. Dynamic modelling, optical trapping and single molecule spectroscopy and microscopy are some techniques being used to investigate myosin gait. In 2006, myosin V was shown to exhibit a mechanical ratchet-like motion (Gebhardt, Clemen et al. 2006). Additionally, little work has been done to elucidate the orientation of the toes of the feet, which play a role in whether the myosin can step backwards (Kinosita, Shiroguchi et al. 2006).

The gait of the myosin is not the only active region of research in contractility. The underlying mechanisms of the cross bridge cycle are also not well understood, although recent single molecule observations confirm a two step process for the power stroke previously only known for non-muscle myosin (Capitanio, Canepari et al. 2006). The power stroke has been further explored, including the limiting factor of the power stroke velocity for different muscle myosin isoforms (Nyitrai, Rossi et al. 2006). The relationship between mitochondria and contractility is another aspect that is also being widely researched. Fluorescence confocal microscopy, for example, has provided evidence of changes in mitochondrial respiration with sarcomere contraction, when contraction is initiated by the addition of calcium (Anmann, Eimre et al. 2005).

(d) Structure of mitochondria

Mitochondria were first observed in the 1800s with light microscopy. Even then researchers could see the diversity in shape and number within a cell (Scheffler 1999). A more complete structural characterization of mitochondria could not be achieved until the advent of electron microscopy and significant improvements on sample preparation (Scheffler 1999). In 1952, an investigation of mitochondria with electron microscopy confirmed that the mitochondria are membrane bound and revealed the existence of both an inner and outer membrane (Palade 1952). Here, Palade also coined the term “cristae mitochondriales” for the inward foldings of the inner membrane.

The exploration of the morphology of mitochondria with electron microscopy further revealed the diversity of the organelle. The entire mitochondria can be several
microns long with a diameter of ~1µm and varies from cell to cell and even within a cell (Nicholls and Ferguson 1992). Myocytes, for example, have at least 2 types of mitochondria known as interfibrillar mitochondria and subsarcolemmal mitochondria, which have been shown to exhibit different biochemical properties (Stenger and Spiro 1961; Palmer, Tandler et al. 1977). Mitochondria within the heart tend to have more cristae, and thus an increased surface area of the inner membrane, when compared with the mitochondria extracted from liver. It is possible that this difference is related to the high respiratory activity needed in heart mitochondria (Nicholls and Ferguson 1992).

Two-dimensional images were not sufficient to fully understand the morphology of the mitochondria. Three-dimensional images constructed from serial sections of mitochondria as well as three-dimensional visualization with confocal laser scanning microscopy further enhanced the knowledge of mitochondria structure and organization. High resolution scanning electron microscopy (HRSEM) further added to the understanding of mitochondria ultrastructure. HRSEM revealed that striated rat muscle mitochondria exhibit both tubular and plate-like cristae, even in the same mitochondria (Lea, Temkin et al. 1994). These newly revealed tubular structures spanned across the mitochondrial matrix and connected with the inner membrane at both ends.

The spatial orientation and location of mitochondria within a cell type is potentially related to cellular function. The intermyofibrillar mitochondria within cardiac muscles are uniformly distributed in a crystal-like pattern within the myofibrils; this may be important in regulating function such as delivering of ATP to the sarcomeres to enable synchronous contractions (Vendelin, Beraud et al. 2005). Yeast cells, on the other hand, tend to have mitochondria close to the outside of the yeast; suggestions for the reason for this arrangement include maximizing the access to oxygen (Scheffler 1999).

(e) Bioenergetics of mitochondria

The contraction of sarcomeres requires energy. In general, the most important molecule in terms of transferring free energy and capturing free energy is adenosine triphosphate (ATP) (Lodish 2000). ATP is formed from the metabolism of certain energy rich compounds such as polysaccharides. The energy in the phosphoanhydride bonds of ATP can be released and coupled to other cellular functions through hydrolysis (Lodish
Mitochondria are the main source of ATP production in most eukaryotic cells. The inner membrane of the mitochondria is the energy transducing membrane associated with the synthesis of ATP and ion transport (Nicholls and Ferguson 1992). The inner membrane has low permeability to the ions and the folding of the cristae results in a large surface area. The inner membrane also has two different proton pump types, primary and secondary. The primary pump utilizes the energy from an electron-transfer chain to create a proton gradient, pumping protons from within the inner membrane space to the outside. This high gradient of protons causes the secondary pump to reverse its pump direction and synthesize ATP from ADP and Pi (Nicholls and Ferguson 1992). Therefore, the major source of the ATP production required for the conformation change of the myosin head is the mitochondria.

Endogenous fluorophores within mitochondria such as NADH and FAD as well as artificial dyes are commonly used in microscopy to study transmembrane potential changes in mitochondria (see for example (Duchen, Leyssens et al. 1998; Zorov, Filburn et al. 2000; O'Reilly, Fogarty et al. 2003; Brady, Elmore et al. 2004)). Quantitative three-dimensional microscopy and ratio imaging has also been used in order to investigate the membrane potential of individual mitochondria (Loew, Carrington et al. 1994). Current research in mitochondrial dynamics is further discussed in the following section (f).

(f) Current research in bioenergetics of mitochondria

As mentioned earlier, fluorescent dyes are regularly used for confocal, linear fluorescent imaging of mitochondria. More recently, studies are focused on imaging mitochondria with two- and three-photon excitation fluorescence microscopy. As well, the shift from the structural and spatial studies of mitochondria to investigations on cellular dynamics is becoming apparent.

The mitochondria within the myocytes play a significant role in the bioenergetics of muscle contraction. Aside from the aforementioned delivery of ATP, current studies are attempting to further expose the relationship between contraction and mitochondria function. Evidences have recently been presented that mechanical swelling of mitochondria plays a role in contractility (Kaasik, Joubert et al. 2004). Since the third
harmonic is sensitive to membrane spacing, this role could be further explored. With comparisons of dynamics in MPF and THG, the role of mitochondria in contractility may be better understood.

In cardiomyocytes, calcium sparks which are discrete events of $\text{Ca}^{2+}$ released from internal stores, have been observed along with larger signals suggestive of propagation of multiple sparks (Cheng, Lederer et al. 1993; Lipp and Niggli 1994). Using fluorescence microscopy, Duchen and collaborators investigated the relationship between calcium sparks, released from the sarcoplasmic reticulum and the uptake of calcium by the mitochondria (Duchen, Leyssens et al. 1998). Mitochondrial depolarizations observed from the fluorescence of tetra-methylrhodamine-ethyl ester (TMRE) resulted in discrete localized fluorescence intensity changes denoted fluorescence flicker (Duchen, Leyssens et al. 1998). Many additional studies have been carried out that support the observation of mitochondria depolarization and flicker (Collins, Berridge et al. 2002; O'Reilly, Fogarty et al. 2003; Falchi, Isola et al. 2005; Romashko, Marban et al. 1998). One study, however, carried out experiments with both two-photon excitation fluorescence and confocal microscopy of mitochondrial NADH and did not show any sign of the ‘flickering’ observed by Duchen (Blinova, Combs et al. 2004). The intensity fluctuations of NADH fluorescence observed in the experiments by Blinova et al were not above the background noise and the authors suggested biological processes do not influence the fluorescence signal. Alternatively, the lack of flicker could either be due to non-active samples or the possibility that the observed flicker is a consequence of an absorption of light by TMRE and production of reactive oxygen species (Blinova, Combs et al. 2004). Additional studies are required to aid in clarifying the adverse side effects of fluorescent labels on mitochondrial dynamics.

The results of Duchen’s work suggested that flickers are related to the release of calcium from the sarcoplasmic reticulum (Duchen, Leyssens et al. 1998). Changes in the membrane potential of the mitochondria, however, have been shown to occur even in the absence of calcium release from the sarcoplasmic reticulum (O'Reilly, Fogarty et al. 2004), further complicating the understanding of mitochondria function.
Many microscopic investigations involve the addition of dyes, or else the activation of natural fluorophores, which may affect mitochondrial function. Photoillumination of fluorescing dyes has already been shown to cause an increase in the concentration of reactive oxygen species, which may lead to mitochondrial depolarizations (Zorov, Filburn et al. 2000; Brady, Elmore et al. 2004). The side effect of fluorescence techniques emphasises the importance of finding minimally or non-invasive techniques for understanding mitochondrial bioenergetics.

Simultaneous depolarization over numerous mitochondria observed via the changes in fluorescence intensity is suggestive that the mitochondria are interconnected into synchronized networks (Amchenkova, Bakeeva et al. 1988; Diaz, Falchi et al. 2000). Other research has suggested the depolarizations in different mitochondria are independent (Collins, Berridge et al. 2002; O'Reilly, Fogarty et al. 2003). It has not been conclusively determined whether networks exist or not, or whether the formation of networks is dependent on cell type. Minimal or non-invasive techniques would be beneficial in understanding the networks, limiting the risk of false positives caused by the adverse physiological affects. Complementary information achieved through multicontrast imaging would provide an additional benefit, giving the opportunity to track the dynamics between multiple organelles.

Section 2.4: Harmonic generation microscopy of muscle

The anisotropy of the A-bands of sarcomeres lends itself well to SHG microscopy providing a novel contrast mechanism for biological investigations that does not require staining. Since 3-D images of living muscle cells can be achieved, SHG microscopy is advantageous over other structural imaging techniques such as TEM or SEM which require the samples to be fixed (Both, Vogel et al. 2004). Tomography studies first showed that the second harmonic could be generated from muscle (Guo, Ho et al. 1997), but it was not until 2002 that muscle cells were first imaged with SHG microscopy (Campagnola, Millard et al. 2002).

Mouse skeletal muscles imaged with SHG microscopy revealed bright SHG bands, with a periodicity on the same order of a sarcomere length. The bright bands in SHG were compared to the results from polarization microscopy and it was found that the
second harmonic was generated from the anisotropic bands of the sarcomere although the width of the second harmonic was larger than for the bands seen in polarization microscopy (Campagnola, Millard et al. 2002). Additionally, the SHG signal depicted the same characteristic faint line in the centre of the anisotropic band. Simultaneous SHG and two-photon excitation fluorescence of GFP labelled myosin in the M-line confirmed the faint line to be in the centre of the thick band, although the reason for the lack of second harmonic at this spatial location has only recently been determined (Prent, Green et al. 2008).

The orientation and symmetry of the muscle affects the second harmonic response, and it has been suggested that this dependency could be valuable in understanding muscular dynamics and intracellular regulatory processes, especially when combined with other complementary techniques such as multi-photon excitation fluorescence (Both, Vogel et al. 2004). The intrinsic structures of the biomolecules in A-bands were further explored by examining the SHG dependency on orientation between the linear polarization and the long axis of the myofilaments. The tensor elements of $\chi^{(2)}$ were deduced by measuring SHG at different sample orientations, leading to descriptions of the structural organization of molecules in a 3-D array (Chu, Chen et al. 2004). Other works have found the same pattern for the relationship between SHG intensity and the orientation of the polarization (Both, Vogel et al. 2004; Plotnikov, Juneja et al. 2006).

Live cardiac myocytes were investigated in 2004 to test SHG as a means of quantifying sarcomere length (Boulesteix, Beaurepaire et al. 2004). By using saxitoxin to block Na$^+$ channels and induce contraction, Boulesteix et al measured changes in sarcomere length to a 20 nm accuracy. The sarcomere length changes were attributed to the shortening of the isotropic band, consistent with the sliding filament model. Given the resolution, changes in the widths of the SHG bands were within the noise and the results were suggestive of the myosin heads in their crystalline arrangement being the source for SHG (Boulesteix, Beaurepaire et al. 2004). The origin of SHG from within the myosin was further investigated in 2006, and results were indicative of rod domains being the source for the second harmonic and (Plotnikov, Millard et al. 2006).
Additionally, results of the present study suggest that SHG is not dependent on the myosin heads.

The penetration depth for imaging muscle with SHG microscopy can be improved while maintaining the morphological structure of the sarcomeres. Optical clearing using glycerol led to a 2.5 fold increase in penetration depth in muscle (Plotnikov, Juneja et al. 2006). Glycerol is often used to extract myosin, but because glycerol reduces the cytoplasmic protein concentration it is also thought that absorption and scattering are diminished leading to increase in the penetration depth.

Muscle structures can also be observed with third harmonic generation microscopy. In multicontrast investigations, the I-bands observed in THG can be visualized alongside the A-band in SHG (Chu, Chen et al. 2004). The alternating refractive indices between the actin filaments and spacing between filaments likely breaks the symmetry about the focus, resulting in constructive interference of the waves and therefore generation of detectable third harmonic in the far field at a wavelength of 1230 nm. This means that while the second harmonic is produced in the anisotropic bands, the third harmonic may be observed in the isotropic bands. SHG microscopy enables one to obtain in-vivo, high-resolution and high-contrast images of sarcomeres; when combined with other contrast mechanisms such as MPF or THG, the multicontrast nonlinear microscopy provides a powerful, minimally invasive technique suitable for both dynamic and structural studies of myocytes.
Chapter III - Experimental Design and Methods

Section 3.1: Introduction

Many of the sample preparation methods and imaging tools are the same or similar for each set of experiments. To avoid redundancy, this chapter details the designs and methods that are common to the experiments discussed in later chapters. The lasers and microscopes are both described, as are the sample preparations for both Sprague-Dawley rat cardiomyocytes and *Drosophila melanogaster* myocytes. Additionally, alternative approaches for set-up of a multicontrast microscope are described for readers interested in developing their own system.

Section 3.2: Microscopes used in this study

Nonlinear microscopes are essentially descendents of traditional confocal laser scanning microscopes that utilize linear fluorescence in conjunction with a pinhole to filter out signal from the regions outside the focal volume. By coupling femtosecond lasers into an existing confocal microscope, a multi-photon excitation fluorescence imaging system could easily be achieved (Zipfel, Williams et al. 2003b). Additionally, since fluorescence is emitted isotropically the signal can be collected in either the forward or backward direction. Backwards collection does not require an extra collection objective or condenser lens. Further, more difficult modifications of a confocal system are necessary in order to implement the collection of harmonic signals, which mainly propagate in the forward direction. Simultaneous detection of three channels necessitates extensive modification of commercial microscopes (Sun 2005), or else a home built system becomes necessary. The work for this thesis was carried out on two similar home built systems that are described more thoroughly below.

(a) Two channel nonlinear multicontrast microscope:

Simultaneous imaging of cardiomyocytes with THG, SHG and MPF signals was accomplished by a home built microscope developed by the Squier group at University of California at San Diego. The basic outline of the microscope is presented in Figure 3.2-1. The Ti:Sapphire or Nd:Glass laser, described in Section 3.3: was employed for imaging.
For obtaining two-dimensional (2-D) images, the laser beam was scanned in a raster pattern by two closed-loop galvanometric mirrors (GSI Lumonics, VM2000 Series). Scanning the laser beam is generally faster than translating the sample with respect to the beam and does not induce any physical vibrations into the sample. The beam was appropriately relayed to the high numerical aperture objective via a 1:1 telescope as shown in Figure 3.2-1.

An IR-Achroplan 63x 0.9 NA water immersion objective (Zeiss) was used for the excitation and collection of the fluorescence signal. For 800 nm excitation, the forward generated harmonics were collected with 0.9 NA home built UV objective. A 0.65 NA air objective was used for collection of the harmonics generated with 1064 nm excitation. In order to obtain a complete three-dimensional (3-D) sectioning, specimen were translated axially using an encoded stepper motor stage (AF Optical). Filters and dichroic mirrors were used to separate the fluorescence in the backward direction and the harmonics in the forward direction; each signal was therefore collected with a different detector. Prior to this work, tests were done with filters in place in order to examine the spectrum of the sample and ensure proper signals were being acquired.

Detection of the nonlinear responses can be carried out by signal integration, lock-in, or photon counting methods. Signal integration, used in this set-up, is also the most common detection method among the commercial microscopes. The collected radiation was detected with photomultiplier tubes (PMT) (Hamamatsu model R5600) without using the pinholes, making it possible to obtain images from scattering as well as non-scattering samples. The detection system featured simultaneous dual channel recording. The PMT signals were sent to current-to-voltage converters that were simultaneously sampled by a 2-channel 12-bit digitizing board (National Instruments NI PCI-6111). This allowed comparison of images obtained with the different contrast mechanisms. Both the scanning and the detection were controlled with a LabView interface that was created specifically for this imaging system. In order to achieve the highest possible scanning rates, the data was first streamed directly to the hard drive and later recovered by the software. With this scheme acquisition rates up to 12 frames (128x128 pixels) per second were possible, only limited by the bandwidth of the analog-to-digital card (in our case 5 MHz) and maximum scanning frequency of the mirrors.
Due to the low yield of third-harmonic photons per fundamental laser pulse (1 third-harmonic photon per ~4–10 pulses) several frames were averaged to improve the signal-to-noise ratio. With averaging, the third harmonic exhibits noise values between 20% and 25% of the signal. SHG can achieve higher SNR, even on a single frame level. The error estimates given for intensity changes in time are based on intensity deviations in the region of interest, or in the case of single pixel evaluations, based on the noise signal in the background region outside of the myocyte. Typical acquisition times for the whole three-dimensional stack were on the order of several minutes and depended on the desired resolution.

Since the nonlinear signals are generated at the same time, detection systems employ simultaneous recording and hence enable direct comparison of the images in space and time.

![Figure 3.2-1: Schematic diagram of the multicontrast microscope.](image)

Figure 3.2-1: Schematic diagram of the multicontrast microscope. F-denotes filters, L-lenses, M-mirrors, DM-dichroic mirrors, and PMT stands for photomultipliers. *Taken from* (Barzda, Greenhalgh et al. 2005) *with permission.*
(b) Three channel nonlinear multicontrast microscope:

The three channel nonlinear multicontrast microscope developed by the Barzda Group at the University of Toronto has an optical design that is similar to the Squier Group microscope. Here a Ti:Sapphire or Yb:KGd(WO$_4$)$_2$ laser was used for excitation of *Drosophila* myocytes. For obtaining 2-D images, the laser beam was scanned in the same manner as in the Squier Group’s microscope. A Plan-Apochromat 20x, 0.75 NA air objective (Zeiss) was used for focusing the fundamental laser radiation. In some cases, a 1.3NA oil immersion objective (Zeiss) was used as the excitation objective to obtain higher resolution images. The harmonics were collected in the forward direction with a home built objective. In order to obtain complete 3-D sectioning, the specimen was translated axially.

The nonlinear responses were detected using a single-photon counting detection method. The low level intensity of the nonlinear signals generated from some biological samples makes photon counting a more appropriate choice when compared to the signal integration used in the previous set up. The photons were detected with photomultiplier tubes (PMT) (Hamamatsu model H5783P-01, 03 and -06).

The detection scheme of this microscope features a significant improvement compared to the previous microscope built at UCSD. The photon counting board (National Instruments NI-6602) allows simultaneous three channel detection, where harmonic signals (second and third harmonic) are captured in the forward direction, and multi-photon excitation fluorescence is detected in the backward direction. Both the scanning and the detection were controlled with a LabView interface that was created specifically for this imaging system. Acquisition rates were similar to that of the previous set-up. Due to the low yield of third-harmonic photons per fundamental laser pulse several frames were summed to improve the signal-to-noise ratio. As in the previous set up, typical acquisition times for the whole 3-D stack were on the order of several minutes and error estimates were based on the intensity fluctuations in the region of interest or the intensity level in the background region outside the myocyte. This microscope also enables sample rotation with respect to the laser beam direction, in order to maximise the signal strength based, as described in the work of Chu, Chen *et al.* 2004.
Section 3.3: Specifications of lasers for this study

For the nonlinear effects to be generated and imaged with the above microscopes, a high intensity excitation source is needed. At the same time, a low average power is required to prevent damage to the sample, which is particularly important for biological specimen. Typically for biological imaging, pulse energies are around 1 nJ. This reflects an approximate energy fluence on the order of $10^{10}$ W/cm$^2$, while the breakdown in transparent aqueous media occurs around two orders of magnitude higher. Ultrafast pulsed lasers are well suited for this purpose, but peak pulse powers must be low enough to prevent dielectric breakdown, which can cause visible structural changes and cavitation in the sample (Muller, Squier et al. 1998b). Further to this, optimization can be achieved by varying the repetition rate of the laser source. With nonlinear excitation fluorescence, it is useful to provide enough time between pulses to allow for complete relaxation of the excited states.

The laser can be further optimized by choosing an appropriate excitation wavelength. For biological samples, choosing a laser with infrared emission works well; by choosing a wavelength between 800 – 1300 nm, a balance can be achieved between a large penetration depth into the biological sample and prevention of water absorption. Excitations above 1 μm are also beneficial in that the third harmonic is in the visible region which is easy to detect. Excitations near 800 nm result in a third harmonic that is in the UV region and therefore requires special collection optics. Polarization of the excitation is also important for nonlinear microscopy; the third harmonic, for example is not generated with circularly polarized light (Fittinghoff, Aus der Au et al. 2005).

Four different lasers were used throughout this study; each sample type was investigated with two of the four lasers. Cardiomyocytes were studied using a Ti:Sapphire laser (A) and an Nd:glass laser, while Drosophila samples were examined with a different Ti:Sapphire laser (B) and a Yb:KGd(WO$_4$)$_2$ laser.

(a) Ti:Sapphire laser (A and B):

A: A home built femtosecond Ti:Sapphire oscillator with a tunable laser wavelength from 760 nm to 840 nm was used for capturing multi-photon excitation fluorescence of NAD(P)H in cardiomyocytes simultaneously with THG. The oscillator
was pumped by a Millenia V cw-laser from Spectra-Physics. The femtosecond cavity layout was based on a standard delta-cavity design with one of the cavity arms containing an additional focus to accommodate an acousto-optic Bragg cell. This allowed cavity dump of the laser beam and a convenient way to vary the repetition rate of the pulses from 200 kHz to 4 MHz. The base laser pulse repetition rate was 76 MHz. Dispersion compensation within the cavity was done by a fused silica prism pair. The pulse duration was approximately 25 fs at an average output power of 120 mW (output coupling 5 %, pump power 1.5 W).

**B:** A home built Ti:Sapphire oscillator tunable between 780 nm and 840 nm was used for imaging *Drosophila* myocytes. The pulse duration of this laser is approximately 25 fs and it has an output power of <200mW. The design of this laser is also based on the delta cavity design but with an additional intracavity telescope to reduce the repetition rate to 26.7 MHz.

**(b) Nd:glass laser:**

For imaging cardiomyocytes with 1064 nm excitation, a diode-pumped, Nd:glass laser was used. The home-built Nd:glass laser was based on the work of Kopf *et al.* (Kopf, Kartner *et al.* 1995). An inhomogeneously broadened silicate glass was cw-pumped by one diode laser (Spectra Diode Laboratories SDL-2472, 3.0 W). Self-starting was achieved with an output coupling of 3%. The pulse duration was ~150 fs, with an average output power of 100 mW at a repetition rate of 94 MHz. For stable mode-locking an intracavity semiconductor saturable absorber mirror (SESAM) (Keller, Weingarten *et al.* 1996) was used.

**(c) Yb:KGd(WO₄)₂ laser:**

For 1040 nm excitation of *Drosophila* samples, a diode-pumped, Yb-ion doped potassium gadolinium tungstate (Yb:KGd(WO₄)₂) laser was used (Major, Cisek *et al.* 2006b). This laser is based upon a previous Yb:KGd(WO₄)₂ laser with a 97 MHz repetition rate and a pulse energy of 24 nJ (Major, Cisek *et al.* 2006a) but includes the introduction of 2 intracavity telescopes to reduce the repetition rate to 14.6 MHz. A semiconductor saturable absorber was used for mode locking. The pulse duration of this
home built laser is on the order of 200 fs with an average power of 850mW, the equivalent of a 60 nJ pulse energy.

Section 3.4: Sample preparation

Muscle structure and function were studied in two different systems. Investigations were carried out on isolated cardiomyocytes from Sprague-Dawley rats as well as isolated Drosophila melanogaster muscles in the adult and larvae stages. Drosophila samples were studied both in fixed and non-fixed states, while cardiomyocytes were only examined in a non-fixed state.

(a) Cardiomyocyte preparation:

Institutional guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals were strictly adhered to. Cardiomyocytes were isolated as previously described (Li, Suzuki et al. 2002); they were generously provided by the laboratories of Wilbur Y. W. Lew (Cardiology Section, Department of Medicine, V.A. San Diego Healthcare System and UCSD) and of Wolfgang H. Dillman (Division of Endocrinology & Metabolism, Department of Medicine, School of Medicine, UCSD). Briefly, adult Sprague-Dawley rats were anesthetized with sodium pentobarbital; the heart was then excised and perfused with collagenase B and protease. Cardiomyocytes were plated on sterile custom-made dishes and maintained in Dulbecco’s modified Eagle’s medium at room temperature for 2 to 24 hours prior to experiments. Cardiomyocyte imaging was carried out at ambient environment in the same medium. With 1064 nm excitation, continuous imaging of cardiomyocytes over several hours produced no obvious toxicity and structural alterations were not observed during imaging unless or until the cell proceeded to hypercontraction. The likelihood of hypercontraction of imaged and non-imaged cells appeared the same. In some experiments, cardiomyocytes were labelled with the mitochondria-specific fluorophore TMRM (0.27 nM) to investigate dynamics and correlate mitochondrial fluorescence with the third harmonic.
(b) *Drosophila* preparation:

*Drosophila melanogaster* stocks were raised on Bloomington medium at 25°C. The *yw* strain was used as the control strain throughout the study. Third instar larvae or adults were selected and dissected under *Drosophila* saline HL3. Adult indirect flight muscles (IFMs) were lightly fixed in 4% formaldehyde for 3 minutes to aid dissection and visualization. For actin labelling, dissected IFMs were fixed, washed in phosphate buffered saline plus 0.1% Triton X-100 (PBT) and then incubated overnight in 1:1500 dilution of rhodamine-conjugated phalloidin or coumarin-conjugated phalloidin (Sigma) depending on excitation wavelength. Samples were washed in PBT, and mounted on glass slides in Vectashield. Confocal scanning images were acquired on a Zeiss LSM510 with the 543 line of a HeNe laser.

The following mutant strains were used (a gift of S. Berstein, San Diego State University): *Mhc*\textsuperscript{10}, is an allele in which no myosin heavy chain is produced in the adult indirect flight muscles (IFMs); *Y97*, is a transgenic line that expresses headless myosin heavy chain in the IFMs in a wild-type background; *Mhc*\textsuperscript{10}; *Y97* is a strain that expresses headless myosin heavy chain in the IFMs in the *Mhc*\textsuperscript{10} background.

**Section 3.5: Image processing and analysis techniques**

The images recorded with different contrast mechanisms can be compared pixel by pixel, although some difficulties may arise. Despite the fact that SHG, THG and MPF can originate from the same structure, the mechanisms are fundamentally different, as discussed in the previous chapter. Homogeneous structures cannot be visualized with the coherent processes, but could be observed in MPF. At interfaces, the maximum signal of both harmonics corresponds to the central position of the interface while the maximum signal in fluorescence occurs when the focal volume is entirely within the fluorescing sample. Therefore the central position of the interface corresponds to half the peak intensity in fluorescence. Additionally, the point spread function is different for each of the contrast mechanisms; this could lead to artifacts when comparing the signals, although deconvolution can aid in preventing such artifacts.

Image processing techniques such as deconvolution are generally used to improve the image quality by removing image artifacts, noise, blur, or bleaching effects.
Processing does not provide the quantitative values that analysis gives but rather enhances the image quality which in turn allows for improved analysis. Over-processing, however, can also lead to artifacts.

Once processing is complete, the image set can be analysed. Analysis techniques enable extraction of different parameters from the images. Analysis techniques include simple visual interpretation of an image as well as more complicated statistical analysis techniques such as fluorescence correlation spectroscopy. Some of the processing and analysis techniques used in this study are briefly outlined here. The novel analysis techniques, developed specifically for this project, are described in detail in the next chapter.

(a) Image segmentation

A traditional segmentation procedure was first performed for each 2-D optical slice of the 3-D images to separate the signal of interest from the background. Image thresholding and normalization was used to obtain standardized images that could be subjected for comparison. As an indicator for the threshold level, the image background (an image area unoccupied by the object) was used. Pixel intensities were normalized to the maximum pixel intensity of each image. Care was taken to analyze the intensity distribution of each image in order to avoid normalization of images to a high intensity glitch that can artificially appear during image recording.

(b) Deconvolution

Because of imperfections in the imaging system, the image collected is not a true representation of the original sample. A microscopic image is the convolution of the point spread function (PSF) of the light source with the object of interest. The point spread function is a result of the limitations of the optics. It is the resulting 3-D diffraction pattern that would be generated when the imaging system is used to image an ideal point source. Specific to each imaging system, the PSF is a reflection of the quality of the system. Mathematically, the image is given by the convolution function:

\[ I(X,Y,Z) = PSF(x,y,z) \otimes O(X,Y,Z) \]
where \( I \) represents the image and \( O \) represents the object being investigated.

The physical result of the convolution is that the imaged area or volume is blurred up to the resolution of the PSF. Convolution is the Fourier space equivalent to multiplication, and just as division is the inverse of multiplication in real space, deconvolution is the inverse of convolution. Deconvolution is an imaging processing technique that attempts to eliminate the PSF from the image, in order to view only the sample.

There are different types of deconvolution algorithms, but each one requires knowledge of an experimental or theoretical PSF. Since the PSF is the image of a point source, experimental PSFs are determined by imaging something close to a point such as a polystyrene bead or quantum dot. Theoretical PSFs require detailed knowledge of the imaging system. A simplified model PSF can be calculated using the wavelength of excitation, the numerical aperture (NA) of the objective and index of refraction of the immersion medium (air, water, oil). The calculation also requires the image size and the aspect ratio. A more complex theoretical PSF calculation requires additional information such as coverslip and sample thickness and their respective indices of refraction. An evaluation of the PSF in this way is described elsewhere by Gibson and Lanni (Gibson and Lanni 1991).

Once the PSF has been calculated or determined experimentally, the image or volume can be deconvoluted. Deconvolution algorithms can be categorized into two main types namely deblurring and image restoration. Deblurring algorithms attempt to remove the blur in a volume, by working on a slice by slice basis. The fast nearest neighbour method is one type of deblurring algorithm. Image restoration algorithms are applied to the entire 3-D volume simultaneously and try to redistribute the blurred light to a position in the volume where it will be in focus. One such image restoration algorithm is the maximum entropy method.

3D Dr Software (Able Software Corp.) was used for deconvolution throughout this work. This software utilises an iterative Maximum Entropy Method (MEM) of deconvolution. It is a constrained statistical iterative algorithm that functions by maximising the ‘image entropy’ of the system. Although the actual 3-D method used in
the 3-D Dr Software is proprietary, the basic description for restoring an image a maximum entropy method is as follows:

The MEM method of deconvolution attempts to restore the image by iteratively ‘guessing’ the set of intensities from sample and comparing them to the acquired image. This method is described in detail in (Burch, Gull et al. 1983) and is based on previous work by (Frieden 1972; Gull and Daniell 1978). The entropy \( S \) of an image is first given by:

\[
S = -\sum_{j=0}^{N-1} p_j \log p_j; \quad p_j = \frac{f_j}{\sum f}
\]

where \( f_j \) is the intensity of pixel \( j \) in the image containing \( N \) pixels; it is this data set \( f \) that represents the real sample. Since the PSF \( h \) is known, the data set \( f \) can be convoluted with the PSF to create a data set \( g \):

\[
g_i = \sum_{j=0}^{N-1} f_j h_{i-j} \quad (i = 0, 1, \ldots, N - 1)
\]

The statistical chi squared is a convenient comparison between the data set \( g \) and the data set \( d \) collected with the microscope:

\[
chi^2 = \sum_i \left( g_i - d_i \right)^2 / \sigma_i^2
\]

The deconvolution is completed through iterations, which maximise the entropy of a data set \( f \) and yield the user-specified chi squared value. At this point the data set \( f \) represents the deconvoluted image of the sample.

(c) Filtering

As mentioned earlier, one of the disadvantages of fluorescence imaging is bleaching. During dynamic imaging, the same two-dimensional region is repeatedly scanned by the laser, which often bleaches the sample. For dynamics to be properly analysed, this low frequency bleaching must be removed. The bleaching is not
necessarily uniform across the entire region, so each pixel must be treated separately. Two types of filtering processes to remove the bleaching were utilised in this work.

A LabView program was developed that enables filtering in the Fourier domain. Initially, the time evolution of each pixel was converted to Fourier space using the Discrete Fourier Transform (DFT) given by:

\[ X_k = \sum_{m=0}^{n-1} x_m e^{-i2\pi mk/n} \quad \text{for } k = 0, 1, 2, \ldots, n - 1 \]  

where \( X \) is the Fourier space representation, \( x \) is the input sequence, and \( n \) is the number of samples in the spatial and frequency domains.

Once the transformation of the time evolution of each pixel to Fourier space is complete, low frequency components can be removed. This removes the low frequency associated with fluorescence bleaching, while maintaining the faster dynamics that occur at higher frequencies are associated with cellular function. The filtered signal can then be transformed back to the spatial domain using the following equation:

\[ x_n' = \frac{1}{N} \sum_{k=0}^{N-1} X_k e^{i2\pi kn/N} \quad \text{for } k = 0, 1, 2, \ldots, N - 1 \]  

where \( x_n' \) is the new filtered signal in the spatial domain. This technique requires some knowledge of the dynamics to ensure that filtering eliminates only the bleaching effect and not relevant physiologically related dynamics.

More conventional filtering was also used whereby the time evolution of each pixel was fitted with a single exponential using a least squares fitting approach. An exponential function has the following form:

\[ y = Ae^{bx} \]  

where \( A \) and \( b \) are constants. The least square approach attempts to fit the data by minimising the difference between the exponential fit and the raw data, (i.e. the residues) using:
where $N$ is the length of $y$ and $y_i$ is the $i^{th}$ element of $y$ and $f_i$ represents the $i^{th}$ element of the best exponential fit.

Once the best fit has been established, the residuals become a representation of the fast scale dynamics that can be subjected to further analysis. The exponential fitting removes the effect of bleaching while keeping the dynamics of interest intact. This technique was more computationally intensive and resulted in poorer signal to noise ratios, although it did not require any prior knowledge of the muscle dynamics.

**Correlation spectroscopy**

Fluorescence Correlation Spectroscopy (FCS) and Image Correlation Spectroscopy (ICS) are the analysis techniques frequently used in fluorescence microscopy in order to investigate spatial and temporal fluctuations in samples of interest (Wiseman, Capani et al. 2002; Wiseman, Brown et al. 2004). Parameters such as local concentrations and diffusion rate constants are readily attainable with these highly sensitive techniques (Wiseman, Squier et al. 2000; Schwille 2001; Sengupta, Digman et al. 2005). In this thesis, we look only at temporal correlations which, with fast enough time scales, can lead to information into the molecular dynamics of the investigated system. More generally, temporal correlations depict whether or not two time evolutions are similar, if one leads or lags the other and by how much. In essence, how closely related two functions are in time are found by comparing the functions at different time delays.

Mathematically, the cross correlation of two functions $x$ and $y$ of length $N$ for a delay time $\tau$ is given by:

$$r_{xy}[\tau] = \frac{1}{N} \sum_{n=0}^{N-1} x(n)y(n-\tau)$$
where $r_{xy}$ is the cross-correlation coefficient. The autocorrelation is merely the cross correlation of the function with itself. The cross correlation can be normalized as follows:

$$\left| \frac{r_{xy}[\tau]}{\sqrt{r_{xx}[0]r_{yy}[0]}} \right| \leq 1$$

3.5-10

where $r_{xx}$ and $r_{yy}$ are the autocorrelations for functions $x$ and $y$, respectively.

In analysing dynamics, correlation spectroscopy is particularly useful in the way it handles noise. The correlation of a pixel containing real dynamics superimposed on noisy data with a pixel containing only noise will generally have a rapid decay as the time delay is shifted. When comparing 2 pixels containing real dynamics there is a slower decay of the correlation as the time is shifted. The autocorrelation can reveal information about a pixel as well. Slow and fast dynamics show slow and fast rates of decay of the correlation value respectively while periodic dynamics show periodic correlations.

**(e) Volume rendering**

The images acquired by the microscope are two-dimensional sections of the biological sample’s volume. A useful visualization technique, called volume rendering, is the recreation of the 3-D sample volume from the 2-D stacks. Three-dimensional rendering can be achieved in several ways; in this thesis a rendering program developed by collaborators in San Diego (called mu) combined with a Volume Pro graphics accelerator card (TeraRecon Inc.) was used for volume rendering. A volume rendering plug-in called Volume J (Abramoff and Viergever 2002) for the Image J software program was also used for visualizing the samples of interest.

The mu program employs an isosurface rendering algorithm. The volume can be manipulated in real time to enable viewing from any angle, and the levels of colour and transparency can be adjusted. Multiple isosurfaces can be rendered simultaneously, although this can introduce artifacts at the interfaces. Volume J can render using either a trilinear or nearest neighbour approach. The processing time for Volume J is slower than with mu, and real time manipulations cannot be done. This rendering algorithm is useful
however, in its ability to render RGB images. The RGB rendering does not result in the artifacts created by mu when rendering multiple isosurfaces.

Volume rendering is useful for visual inspection of the sample structure. It provides a means to look at the orientation and organisation of the acquired image in a 3-D environment, which is helpful in determining the sample origin. Mu and Volume J both aid in revealing the spatial relationship between multiple contrast mechanisms. Examination of volumes at different times reveals structural changes in the sample.

(f) Gaussian fitting

The double peak of the second harmonic signal can be examined for changes in peak to peak distance with changes in the sarcomere size. Peak to peak changes are useful in understanding the role of myosin in generation efficiency of the second harmonic. To evaluate the peak to peak distance, the double peaks of the second harmonic were fitted with double Gaussians according to formula:

\[
y = y_0 + \sum_{n} \frac{A_n}{w_n \sqrt{\pi/2}} e^{-\frac{(x-x_n)^2}{w_n^2}}
\]

where \(n\) is the Gaussian band number, \(x_n\) is the centre of the \(n\)-th peak, \(w_n\) is the bandwidth, \(y_0\) is the baseline offset and \(A_n\) is total area under the curve from the baseline for \(n\)-th band. Gaussian fits can also be used to analyse sarcomere length changes. By inverting a sarcomere profile in SHG and fitting single Gaussian peaks to each Z-line position, the sarcomere lengths given by the distance between two Z-lines can be calculated.
Chapter IV - An Extended Description of Analysis Algorithms

Section 4.1: Introduction

This chapter is taken in part from (Greenhalgh, Cisek et al. 2005) with permission. Image processing and image analysis are fundamental to understanding acquired microscopic data sets. Processing techniques enable one to manipulate images collected with a microscope in order to aid in image analysis. They allow researchers to extract only the data relevant for analysis from the entire data set. In this thesis, image processing and image analysis play an important role. After using the processing techniques described in the previous chapter, images can be analysed in a variety of means. Included in this project are two analysis algorithms developed specifically for the multicontrast microscopy investigations of myocytes. These analysis techniques have been termed Structural Cross-correlation Image Analysis (SCIA) and Dynamic Image Cross-correlation Analysis (DICA). This chapter provides more detailed descriptions of both of these algorithms.

Advancements in microscopy technologies open new possibilities for an increasing the number of signals that can be acquired during image recording. In addition to the three spatial variables, excitation and emission spectra, time, and polarization can be added to the multivariate data set acquired during imaging. Recently new multicontrast imaging techniques have opened the possibility of simultaneous generation of $m$-dimensional images with different contrast mechanisms originating from the same structure. The acquired $m$-dimensional multivariate images require new algorithms for qualitative and quantitative data analysis and visualization (for reviews on multivariate image analysis see (Bonnet 2004; Nattkemper 2004)). Presented below are some of the multivariate image analysis techniques and visualization methods that have been applied to multicontrast nonlinear microscopy data.

Multicontrast multivariate data sets enable one to compare different images acquired from the same structure. To colocalize structures in the different images, several approaches have been taken previously that involve: qualitative colour coded overlapping of images (Li, Lau et al. 2004), ratio imaging, and quantitative statistical
methods using different correlation functions (Manders, Verbeek et al. 1993; Costes, Daelemans et al. 2004). Colour coded overlapping, although quick and easy to use, is biased because varying intensities and shades makes it difficult to distinguish colors and determine the amount of overlap between structures. Ratio imaging can provide good estimates of structural overlap and is especially suited for spectral multivariate analysis. Unfortunately, ratio imaging of two images generated with different contrast mechanisms can be misleading because object to image transform functions are different for each imaging contrast mechanism. Statistical methods have also been employed to compare two images via Pearson’s coefficient (Manders, Stap et al. 1992), image intensity correlation plots (Demandolx and Davoust 1997) or image cross-correlation analysis (Wiseman, Capani et al. 2002). In all cases, analyses leads to a characterization of a whole image or its parts rather than detailed information about individual pixels.

Here we present an analysis technique called structural cross-correlation image analysis (SCIA), which compares images or volumes pixel by pixel in order to determine if and where two signals overlap. This type of analysis works well when multiple signals are being simultaneously collected during imaging. The result of SCIA is a three colour image or volume whereby two of the colours represent where the original signals do not overlap and the third shows the overlap. This technique is especially useful for characterizing structures from one imaging contrast by comparing to the known structures revealed by the other contrast mechanism. For example, determining which organelles within a cell generate a nonlinear signal (for example, THG) based on the fluorescence labelling, has emerged through this research as a very useful approach.

If nonlinear response signals have sufficient intensity, fast time resolved data can be obtained. The time resolved data can be analyzed in several ways. For example, image cross-correlation analysis can provide researchers with a correlation function which changes due to the movement of structures in a time series of images. Different parameters such as diffusion constants for random and directional movement can be obtained (Wiseman, Squier et al. 2000). In our studies we employed standard deviations to visualize active areas inside the image. Small regions with large fluctuations highlight the active structures. In addition, to evaluate similarities in time evolution of different pixels, the Pearson’s Coefficient was employed in a manner termed Dynamic Image
Correlation Analysis (DICA). By correlating the time evolution of one pixel intensity with a different pixel in the same image, a network of structures fluctuating with the same time dependency can be revealed. The same technique can also be used in examining the same pixel from two different contrast mechanisms.

Section 4.2: Structural Cross-correlation Image Analysis

(a) SCIA algorithm

Comparison of standardized 2-D images was performed on a pixel by pixel basis. Several correlation functions were investigated. For the purposes of comparing images generated with different contrast mechanisms the normalized intensity product $C_{i,k}$ of corresponding pixels $A_{i,k}$, $B_{i,k}$ from normalized images provided the best colocalization results:

$$C_{i,k} = \frac{A_{i,k} \cdot B_{i,k}}{(A \cdot B)_{\text{max}}},$$

where $A_{i,k} = \frac{a_{i,k}}{a_{\text{max}}}$ and $B_{i,k} = \frac{b_{i,k}}{b_{\text{max}}}$;

4.2-1

where $i$ and $k$ stand for pixel indices in the two-dimensional image data set and $a_{i,k}$ and $b_{i,k}$ stands for the intensity values in the original images at pixel $i,k$.

The correlated image is comprised of pixels calculated with equation 4.2-1. The thresholded pixels were assigned 0 (black) values. Pixels which had a correlation value of 0 (i.e. signal was generated in one mechanism and not the other) are said to be uncorrelated with each other. The two uncorrelated images were constructed from the $A_{i,k}$ and $B_{i,k}$ values, respectively, excluding the thresholded and correlated pixels. Each set was comprised of the correlated and two uncorrelated 2-D images which could be reconstructed into a 3-D image. The three 3-D images could be rendered separately, or combined together and rendered in a multicolour 3-D volume as described previously. The rendered 3-D data set provided a means to analyse the structural relations and spatial correlations of the investigated structure.
(b) SCIA performed with fluorescing polystyrene beads

Green fluorescent polystyrene beads with a 10.1 μm diameter, (Duke Scientific Corporation), were used for imaging. The beads were set in a small volume of agar gel sandwiched between two coverslips. Fluorescent polystyrene beads were imaged in 3-D with ~835 nm excitation. Third harmonic generation and multi-photon excitation fluorescence were simultaneously collected in two different channels. Figure 4.2-1 shows a montage of several slices at different depths of the bead for both contrast mechanisms. Panel A presents the MPF, while panel B shows the same slices in THG. In the fluorescence channel, the inner space of the bead appears homogeneous, while THG reveals several layers of the bead in a ring like pattern. This indicates that beads are likely built up in layers. The defined outer ring observed in THG shows the interface between the bead and surrounding medium. The central part of the bead has a low THG intensity suggesting a homogeneous structure in that area.

Two signal channels can be directly compared by overlapping images with different colors. The new colour appears in the combined image where the signals from both channels overlap (see Figure 4.2-2). However, the combined image gives only a qualitative view, and the varying signal intensities obscure a clear picture of the overlapping zones. More quantitative comparisons of the two images were done using SCIA. The correlated part of the images is shown in Figure 4.2-1C. The correlated signal retains intensity details of the THG variation inside the bead. Figure 4.2-1D and E shows uncorrelated parts of MPF and THG images, respectively. Figure 4.2-1D shows the ring of MPF where THG is absent. Most probably the doped dye diffused into the outer coating leading to an apparently larger fluorescent core diameter. The uncorrelated THG image (Figure 4.2-1E) enables us to separate the signal from the non-fluorescent outer coating of the bead. This example shows the elegant way of discriminating structures uniquely expressed by the different contrast mechanisms; a comparison of the merged image and the correlated results in colour are shown in Figure 4.2-2. The bead is a straightforward example and correlation behaviour could have been be predicted by the visual inspection of the initial images (Figure 4.2-1A and B). On the other hand, the
SCIA can help to discriminate different features of complex biological samples and provide a better understanding of investigated structures, as seen in later chapters.

**Figure 4.2-1: Demonstration of SCIA on an imaged fluorescent polystyrene bead.**
(A) depicts several depths of the bead in MPF. (B) depicts the same depths as in (A), imaged simultaneously in THG. (C) Results of a SCIA showing only the correlated regions. (D, E) Results of SCIA showing only the regions of MPF or THG which do not correlate with the other contrast mechanism, respectively. Each image represents the area of 20x20 µm.
Figure 4.2-2: Comparison of traditional merged image technique to the SCIA technique
Colour image comparing the merged image results (a) to the SCIA results (b) for polystyrene beads imaged simultaneously with MPF and THG. For (a), the MPF signal in red was merged with the THG signal in green. For (b), blue represents the region of overlap, green indicates regions with only THG, and red indicates pixels with only MPF. Each image represents the area of 20x20 µm.

Section 4.3: Dynamic Image Correlation Analysis

(a) Dynamic Image Correlation Analysis (DICA) algorithm

Time series of 2-D images obtained with one or more contrast mechanisms were analyzed. Initially, segmentation of all images in each time series was performed by thresholding pixels with background intensity levels. Time dependent changes in the image series were visually inspected by calculating the standard deviation of the intensity fluctuations at each pixel. The SHG and THG images were free from photobleaching, while fluorescence images experienced significant photobleaching. Filtering was carried out on the fluorescence images to compensate for the photobleaching effect as discussed previously. Subsequently, the normalized standard deviation was calculated from the photobleaching free fluorescence fluctuation kinetics. With this method, the magnitude of intensity fluctuations at each pixel can be observed. This qualitative method helps to distinguish active zones in the biological samples from inactive areas where intensity fluctuations are determined by the instrumental noise.

Statistical correlation analysis of time-resolved fluctuations between significant pixels (above threshold) of the image was performed based on a Pearson’s correlation coefficient which is a representation of how linear the relationship is between the two signals. The DICA coefficient is given by:
where $A_{i,t}$ represents the intensity of the $i$-th pixel and $A_{j,t}$ is the intensity of the $j$-th pixel. The index $t$ represents the time e.g. the image number in the image time series, $N$ is the total number of images in the image series. The output of Pearson’s coefficient for a comparison of the intensity kinetics of two pixels is a single number between -1 and +1 that represents the linear relationship between two samples. A coefficient of +1 represents a perfect correlation while -1 denotes a perfect negative relationship (anti-correlation). The DICA analysis provides information on the image areas that are connected into functional networks by means of similar time resolved fluctuation behaviour. This way, visualization of functional units in the biological samples can be achieved.

Additionally, a modified version of DICA, multicontrast-DICA, was also used whereby intensity fluctuations of each pixel from one contrast mechanism were visualized and correlated with the corresponding fluctuations of the same pixel from a different contrast mechanism.

(b) Using the standard deviation to highlight active regions in cardiomyocytes

Difficulties are met when trying to visualize or publish the changes in active areas of the investigated biological structures over time. Many researchers settle for showing several images at different time and indicating with arrows the regions of high activity. Here, the DICA algorithm employs a standard deviation analysis map to provide qualitative information about the areas of high physiological activity. Figure 4.3-1A shows a single cardiomyocyte imaged with THG microscopy. In Figure 4.3-1B, the standard deviation map is presented, where the bright regions correspond to higher structural activities in the cardiomyocyte. A simultaneous time series recorded with MPF (data not shown) exhibits similar regions of activity. The standard deviation map is very
useful for qualitatively discriminating localized repeatable structural/functional activities. If the whole structure exhibits large movements during imaging, the standard deviation map reflects this in its blurred appearance.

![Figure 4.3-1: Local activity of a cardiomyocyte observed with THG microscopy](image)

(A) the THG image averaged from the time series recorded at a rate of 2 frames per second for 800s. (B) represents the normalized standard deviation map and highlights some of the highly active regions in the sample.

(c) Demonstration of DICA with simple sinusoidal functions

To demonstrate the dynamic algorithm several sinusoidal functions are compared. The value of the coefficient does not depend on the amplitude of the function but on the way the function changes in time. Using a sine function as the reference ‘pixel’, DICA is applied to varying phase shifts. The chart below shows different sine curves and the corresponding coefficients when compared to \( \sin(x) \); the table is a summary of the phase shifts for several coefficients.
Table 1: Phase Shifts for Several Correlation Coefficients

<table>
<thead>
<tr>
<th>DICA Coefficient</th>
<th>Phase Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.7</td>
<td>π/4</td>
</tr>
<tr>
<td>0.5</td>
<td>π/3</td>
</tr>
<tr>
<td>0</td>
<td>π/2</td>
</tr>
<tr>
<td>-0.5</td>
<td>3π/4</td>
</tr>
<tr>
<td>-0.7</td>
<td>5π/4</td>
</tr>
<tr>
<td>-1</td>
<td>π</td>
</tr>
</tbody>
</table>

In analyzing real data with DICA analysis, a high coefficient highlights regions that behave in a similar manner, while large negative values show regions which are anticorrelated. When a coefficient has a value around 0, it could suggest a phase difference of $\pi/2$. Alternatively a zero value could suggest that relationship between the two pixels is undeterminable, perhaps due to noisy data, or some regions correlating while others anticorrelating. In demonstrating DICA just by tabulating values, however, the power of the technique is somewhat suppressed. The numbers shown in Table 1 are no more useful than the FCS technique evaluated for a zero time delay. The true power of the technique lies in the recreation of the 2-D image showing the coefficient for each pixel. This map, which is not shown here but used in later chapters, highlights the structural regions within the image that behave in the same manner and can be used to show which of these regions are anticorrelated or which are correlated (see for example Figures 8.3 -2 and -3).
Chapter V - Microcrystalline Origin of SHG in Myocytes

Section 5.1: Introduction

SHG microscopy has been used for several biological applications including muscle imaging (Campagnola, Millard et al. 2002; Boulesteix, Beaurepaire et al. 2004; Barzda, Greenhalgh et al. 2005). As mentioned previously, the second harmonic was found to be generated from the anisotropic bands of sarcomeres, excluding the bare region in the centre resulting in a double peak signal. Further investigation into the origin of the second harmonic from within the anisotropic bands is presented in this chapter, which is based on journal article published in a feature issue of Applied Optics on Topics in Biomedical Optics (Greenhalgh, Green et al. 2007).

The structure of the myocyte is described in Chapter II and in Appendix II. Recall, however that the fundamental unit of the myofibril is called the sarcomere (see Figure 5.1-1) which is made up of the isotropic band (I-band) and anisotropic band (A-band). The A-band consists of the overlapping zones of actin and myosin myofilaments that exhibit birefringence, while regions containing actin and titin have no birefringence and are labelled as isotropic bands (Figure 5.1-1). The thick myosin filaments are made up of ‘rods’ formed from the chiral myosin heavy chains (MHC) and ‘heads’ made up of the light chains. In the transverse direction, electron microscopy has shown that the formation of the sarcomeres exhibits a hexagonal symmetry (Hanson and Huxley 1953; Huxley 1953a). In the overlapping region between actin and myosin, each myosin filament is surrounded by 6 actin filaments, and each actin filament is surrounded by 3 myosin filaments. The sarcomere size, as measured between Z-lines is ~2-3µm for Drosophila melanogaster adults and ~5-8µm for larval muscle (Greenhalgh, Cisek et al. 2006). Hence, Drosophila larval sarcomeres are ideally suited for studying the origin of SHG from myocytes because they are 2-3 times larger than typical mammalian skeletal and heart myocytes. Furthermore, the variety of Drosophila mutants with different myosin content and altered structural integrity of A-bands provides a good model to study the influence of the A-band structure on the efficiency of SHG.
Several studies examining SHG in muscle have been carried out (Both, Vogel et al. 2004; Boulesteix, Beaurepaire et al. 2004; Chu, Chen et al. 2004; Barzda, Greenhalgh et al. 2005; Plotnikov, Millard et al. 2006). It was shown that mutations of myosin in Caenorhabditis elegans leads to a significant distortion of striated structure of myocytes, pointing out that myosin is involved in the structures generating SHG (Campagnola, Millard et al. 2002). It was further established that SHG requires myosin but not actin, and evidences were provided that SHG does not vary with the concentration or orientation of myosin heads (Plotnikov, Millard et al. 2006). The tensor elements of the second-order nonlinear susceptibility of myofibrils were elucidated by imaging the sample at different orientations with respect to the linear polarization of the laser excitation (Chu, Chen et al. 2004). The semicrystalline structure of the myosin filaments has been modeled using hexagonal symmetry. In a similar experiment it was found that the orientation of the harmonophore responsible for SHG appears to be similar to the pitch of the α-helix of the myosin rod along the thick filament axis (Plotnikov, Millard et al. 2006). In studying SHG during mechanical stretching of myofibrils in skeletal myocytes, a strong increase in SHG signal intensity was observed with the sarcomere
lengthening (Both, Vogel et al. 2004). Structural changes during contraction were also shown to effect the width of the SHG bands (Greenhalgh, Stewart et al. 2006).

This chapter demonstrates the importance of the microcrystallinity for second harmonic generation in sarcomeres. The influence of semicrystalline order on the SHG efficiency in the anisotropic bands of Drosophila melanogaster sarcomeres from larval and adult muscle has been investigated. Differences in the semicrystalline order were obtained by using wild-type and mutant strains containing different amounts of headless myosin. The reduction in semicrystalline order without altering the chemical composition of myofibrils was achieved by observing highly stretched sarcomeres and by inducing a loss of viability in myocytes. In all cases, the reduction of semicrystalline order in anisotropic bands of myocytes resulted in a substantial decrease in SHG. Second harmonic imaging during periodic contractions of myocytes revealed higher intensities when sarcomeres were in relaxed state compared to the contracted state. As the myocytes undergo a loss of crystallinity through hypercontraction or loss of viability, it is shown that the SHG signal diminishes, even though third harmonic still remains. This study demonstrates that an ordered semicrystalline arrangement of anisotropic bands plays a determining role in the efficiency of SHG in myocytes.

Section 5.2: SHG response in Drosophila myocytes

To visualize the structure of Drosophila myocytes, high resolution images were collected at 1042 nm excitation. Figure 5.2-1 presents a second harmonic image from both larval and adult muscle. In Figure 5.2-1(a), the larval muscle shows the periodic striated structure, which is characteristic of the sarcomere anisotropic bands. However, unlike the adult indirect flight muscles seen in Figure 5.2-1(b) the sarcomere size is much larger. Drosophila larval muscle has a sarcomere size ranging from 5µm to 8µm, whereas adult Drosophila indirect flight muscle have a typical sarcomere size of ~2µm, which is comparable to other myocytes, such as cardiomyocytes and skeletal muscles (see introduction for examples). The large difference in size is mainly due to the wider isotropic band observed in larval myocytes; the widths of the SHG bands are approximately equal for both specimens.
Figure 5.2-1: Microscopy images of larval and adult *Drosophila* muscles

SHG microscopy images of *Drosophila melanogaster* larval muscle ((a) and (c)) and adult indirect flight muscle ((b) and (d)). Two-dimensional images of the larval (a) and adult (b) muscle are presented. The stacks of images were rendered for the larval (c) and adult (d) myocytes. A conventional fluorescence image of (larval) muscle with actin labelled by rhodamine-conjugated phalloidin is shown in (e) for comparison. Images (a) - (d) were taken with 1042 nm excitation using a 1.3 NA oil immersion objective. Scale bar represents 5 µm in (a)-(d) and 100 µm in (e). (See media files 1 - 4)

The large sarcomere size of larval myocytes is advantageous for high resolution imaging. Two- and three-dimensional images of larval muscle in Figure 5.2-1(a) and (c) display more details than the corresponding adult IFM myocyte image (see Figure
5.2-1(b) and (d)) recorded under the same magnification. The large isotropic band in the larval muscle enables visualization of the interconnections of the myofibrils. Stair-case like structures, shown in Figure 5.2-1(b), are routinely observed in both larval and adult myocytes. Both et al. previously observed similar structures in mouse skeletal muscle cells (Both, Vogel et al. 2004).

Initially, the second harmonic from Drosophila larvae muscle was verified to originate from the anisotropic band (A-band, refer to Figure 5.1-1) by comparing images acquired simultaneously with SHG and MPF at 800 nm excitation. A similar technique has been successfully used to investigate SHG in skeletal and cardiac myocytes (Both, Vogel et al. 2004; Plotnikov, Millard et al. 2006). Multi-photon excitation fluorescence from actin was achieved by labelling the samples with coumarin-conjugated phalloidin. Figure 5.2-2 depicts the same region of myofibril imaged simultaneously with (a) SHG and (b) MPF. The most intense fluorescence signal corresponds to the Z-line of the isotropic band. The lowest MPF intensity is seen in the actin void region called the H-zone. The apparent MPF signal in H-zone comes into view due to point spread function broadening effects from the neighbouring actin filaments. Since the second harmonic is generated from specific endogenous structures, the signal is spatially confined, and therefore exhibits excellent contrast and signal to noise ratio. The second harmonic image shows periodic striations with double lines characteristic of the anisotropic bands of sarcomeres. In comparing the intensity profiles of SHG and MPF along the row of sarcomeres, the signal profiles are shown to anticorrelate (see Figure 5.2-2(c)); this provides evidence that observed second harmonic is being generated from the anisotropic bands of the Drosophila larval sarcomeres. Previous studies have explored the origin of SHG in further detail (Chu, Chen et al. 2004; Plotnikov, Millard et al. 2006). The remaining sections of this chapter focus on how the SHG intensity is affected by various structural alterations.
Figure 5.2-2: Comparison of SHG and MPF signals from *Drosophila* larvae muscle.
Typical images from *Drosophila* larvae muscle labelled with coumarin-conjugated phalloidin and recorded using (a) SHG microscopy and (b) MPF microscopy at 800 nm excitation. A profile of each signal across the row of sarcomeres (line shown in (a) and (b)) is presented in (c). The solid line with circles represents the SHG profile, while the dotted line with open triangles shows the MPF profile. Sample error bars based the distribution of intensity in the A-bands and I-bands of each signal is shown.

Section 5.3: Importance of actin-myosin organization for efficient SHG

(a) Effect of thick filament mutations on SHG efficiency

Adult *Drosophila* indirect flight muscles (IFMs) from wild-type and 3 different mutant strains were used to investigate the SHG response and determine how the structure of A-bands affects the SHG efficiency. One strain of mutant, *Mhc*\(^{10}\), develops indirect flight muscles that contain no myosin heavy chain which leads to a highly distorted sarcomere structure. Additionally, a mutant strain which has headless myosin in a wild-type (*Y97*) background and one with headless myosin in an *Mhc*\(^{10}\) background
(\textit{Mhc}^{10}; \ Y97) were also studied. Figure 5.3-1(a)-(d) compares the wild-type adult \textit{Drosophila} myocytes imaged with second harmonic generation microscopy (Figure 5.3-1(a)) with the SHG images from each of the 3 mutant strains (Figure 5.3-1(b)-(d)). For comparison, Figure 5.3-1(e) – (h) shows typical confocal images of the fluorescence (FL) from actin labelled with rhodamine-conjugated phalloidin for the 4 sample types. As expected, no significant SHG is generated in the \textit{Mhc}^{10} samples which do not contain myosin (Figure 5.3-1(d)); in addition, highly distorted actin structures can be seen in the fluorescence image (Figure 5.3-1(h)). This is in agreement with another study that observed a dramatic decrease in SHG when a disruption of the myosin filaments was induced by incubating myocytes with a low-ionic strength pyrophosphate solution (Plotnikov, Millard \textit{et al}. 2006).

Interestingly, SHG is generated in the two mutants that contain headless myosin (Figure 5.3-1(b), and (c)), however, the mutant with the \textit{Mhc}^{10} background exhibits a more distorted structure compared to the mutant with the wild-type background. As a control third harmonic generation from structures located in the myocytes were utilized (images not shown), however, this chapter will not directly address the THG imaging. In Figure 5.3-1 we see that in both the SHG and FL images, the structural disorder of the sarcomeres increases as the mutation becomes more severe. This agrees with transverse and longitudinal EM images of the same muscle types (Cripps, Suggs \textit{et al}. 1999).
Figure 5.3-1: Analysis of SHG from IFM myocytes of different *Drosophila* mutants.
Panels (a)-(d) correspond to SHG images: (a) wild-type, (b) Y97, (c) Mhc10; Y97, and (d) Mhc10. For comparison, confocal images of similar preparations of *Drosophila* myocytes labelled with rhodamine-conjugated phalloidin are also presented: (e) wild-type, (f) Y97, (g) Mhc10; Y97, and (h) Mhc10 mutants. The contrast has been adjusted in (a)-(h) for structural visualization. For intensity comparison, a bar graph (i) shows the average SHG intensities for the wild-type and the different mutants. The intensity of SHG was calculated by averaging the SHG signals from various scans. Note that the background was thresholded and zero values were ignored in calculating the average signals. The bar in panels (a) – (h) corresponds to 5 µm.

Although both Y97 and Mhc10; Y97 mutants generate the second harmonic, their SHG intensities are significantly different. Figure 5.3-1(i) shows the average SHG intensities for the different sample types. The wild-type muscles, which have the highest ordered structure, have the highest efficiency of the second harmonic generation. In looking at the 3 mutant strains, one sees that as the severity of the mutation increases the content of native myosin decreases and, consequently, the average SHG intensity
decreases. The absence of myosin in the Mhc\textsuperscript{10} mutant results in diminution of the ordered sarcomere structure and an almost complete loss of the SHG signal. Our observations correspond well with the previous study where mutations in MHC B of \textit{C. elegans} resulted in major distortion of striated structure and substantial decrease in the SHG intensity (Campagnola, Millard \textit{et al.} 2002).

There are three possible explanations for the variations in SHG intensity of different mutants. First, if the molecular hyperpolarizability $\beta$ of native myosin is higher than the headless myosin, a reduction in the concentration of native myosin could result in a decrease in SHG intensity. Secondly, the molecular hyperpolarizabilities might be similar for native and headless myosin, but the concentration of myosin is decreased with the severity of mutation. Consequently, a lower concentration of myosin would result in a weaker second harmonic signal. Third, the crystalline order is very important for bulk SHG generation. SHG is not observed in random suspension of non-centrosymmetric molecules even when they have a high hyperpolarizability $\beta$. Therefore, disorder in anisotropic bands would cause an SHG intensity decrease whether or not any changes to the molecular hyperpolarizability occurred. The images in Figure 2.2-1 combined with the original structural study of different mutations with electron microscopy (Cripps, Suggs \textit{et al.} 1999) provides clear evidence that crystalline structure gets increasingly distorted when comparing wild-type with Y97, Mhc\textsuperscript{10}, Y97, and Mhc\textsuperscript{10} mutants.

\textbf{(b) Effect of Actin on SHG intensity}

In examining the SHG double peaks, the majority of sarcomeres had one peak that was more intense than the other peak. See Figure 5.4-6 and Figure 5.2-2 for example. To examine this further, \textit{Drosophila} myocytes were labelled with cumarin-conjugated phalloidin, which binds to actin and two-photon fluoresces upon excitation with a Ti:Sapphire laser. Comparisons of the overlap between SHG and MPF indicate that the more intense SHG peak had less overlap between the MPF and SHG signals. The overlap between actin and myosin is therefore smaller on the more intense SHG peak (see Figure 5.2-2(c)). Notice that in the first two sarcomeres, the trough in MPF is much closer to the larger SHG peak in the double band meaning more overlap between actin and myosin where SHG is weaker. In the 3\textsuperscript{rd} sarcomere, the double peaks are almost
equal in intensity and the amount of overlap between actin and myosin is also similar for both bands. This difference in signal and overlap implies that the actin is affecting the crystallinity of the sarcomere in such a way as to decrease the $\chi^{(2)}$ of the anisotropic band.

**Section 5.4: Dynamic investigations to further elucidate origin of SHG**

(a) **Effect of induced structural changes on SHG intensity**

In order to estimate the influence of crystalline order on SHG efficiency in the anisotropic bands of sarcomeres, several experiments were performed where structural order in the anisotropic bands has been altered without alteration to the myosin thick filaments. Larvae muscles can be imaged for extended periods of time with no visible damage, and as mentioned previously, the large sarcomere size permits a more detailed study of the inner structure of the myocyte bands. The addition of 100 mM KCl changes the ion concentrations in the myocyte and induces contraction of the myofibrils, while excess of KCl can cause sample damage and loss of viability. These induced changes can be used to further understand the origin of the second harmonic intensity changes in myocytes.

Sustained contractions of many sarcomeres in a myofibril have been observed after the addition of KCl. Figure 5.4-1 presents 2-D SHG images over time showing the evolution of a sustained contraction. A localized shortening starting from one sarcomere and gradually engaging more than ten neighbouring sarcomeres can be seen. A large stretching and deformation of the neighbouring sarcomeres adjacent to the sustained contraction region can also be observed. The deformation of sarcomeres is induced due to the stress generated by the sustained contraction region. The SHG intensity of the A-bands is diminished around the sustained contraction region. Presumably, the myosin molecules are still present in the anisotropic bands, further suggesting that SHG intensity is largely determined by the crystalline order of the A-bands. The sustained contraction region also shows reduced SHG intensity compared to the region of unaffected sarcomeres. The disorder of crystalline structure induced by the large shortening could introduce a degree of randomness, which affects the efficiency of second harmonic generation.
Figure 5.4-1: Effect of sustained contraction on the neighbouring sarcomeres. The SHG images at various times during sustained contraction of *Drosophila melanogaster* larvae muscle are shown. The elapsed times for each image are indicated in the upper right corner. The region of sustained contraction is circled by the oval, while arrow points to the middle of the highly deformed region. (See media file 5)

Further evidence of the importance of a crystalline order in anisotropic bands of sarcomeres was examined during the loss of viability of larval muscle. After the addition of excess KCl, which can impact the viability, a small volume of sample was repeatedly scanned, approximately every 3 minutes. After removing the background intensity, the average SHG signal strength over the entire volumes were obtained and plotted in Figure 5.4-2(a). The signal decreases by ~70% over a period of 90 minutes, samples imaged under similar conditions without the addition of KCl do not change significantly (< 20%).
In examining a single optical section, initially (Figure 5.4-2(b)) and after 90 min (Figure 5.4-2(c)), one sees that 90 min after the KCl treatment, the myocyte image still shows similar muscle structure, despite the decreased SHG signal. The myofibrils do, however, appear broader after a loss of viability, and the staircase-like structures are no longer prevalent. In rendering the sample volumes both at $t = 0$ (Figure 5.4-2(d)) and at $t = 90$ min (Figure 5.4-2(e)) one can see the overall loss of order, which has lead to a decrease in SHG.

This result agrees well with previous observations of SHG signal loss due to disorder of crystalline structure during hypercontraction of cardiomyocytes (Greenhalgh, Barzda et al. 2005). With the addition of a mitochondrial uncoupler, the cardiomyocyte proceeds to hypercontraction. The resulting loss of SHG signal is shown in the Figure 5.4-3 below. One can see in the same figure, that the third harmonic signal remains quite bright. This indicates that the organelle/molecules responsible for the third harmonic remain intact after hypercontraction, while the sarcomeres lose their crystallinity. These
examples show that alteration of semicrystalline order without altering the chemical composition of myofilaments crucially impacts the second harmonic generation efficiency.

![Image of SHG and THG signals of a single cardiomyocyte at different times following the addition of a mitochondrial uncoupler.](image)

**Figure 5.4-3: The evolution of SHG and THG in a cardiomyocyte**

(a) Images of SHG and THG signals of a single cardiomyocyte at different times following the addition of a mitochondrial uncoupler. The panel (b) shows the corresponding plot of the average signal intensity in time for each signal. The myocyte proceeds to hypercontraction around 500s.

![Graph showing the evolution of SHG and THG intensity over time.](image)
(b) Effect of natural periodic contractions on SHG intensity

When myocytes undergo contraction, structural changes occur, including the conformational changes of the myosin heads. To study the changes in SHG intensity during contraction, spontaneous, rhythmic contractions of larval muscle were examined. Figure 5.4-4 shows the periodic SHG intensity changes over 2 minutes. As the anisotropic band size remains approximately the same, the intensities in Figure 5.4-4 represent the average signal for several A-bands. Individual images of a contracted and relaxed state at two separate times are also shown. The difference in intensity of the individual anisotropic bands is clearly visible.

![Figure 5.4-4: SHG intensity changes with contraction of Drosophila larval myocytes.](image)

The panel (a) shows the SHG intensity of a row of sarcomeres over time during rhythmic contraction, while SHG images depict a myocyte at the contracted (b) and extended (c) phases. The smaller width of the I-band in the first 2-D image is indicative of contraction. The arrows in
the upper graph depict the times at which the images (bottom) were recorded (see media files 7-10)

It was found that during spontaneous periodic contractions, the relaxed state exhibits a higher than average SHG intensity of a row of sarcomeres than when in the contracted state. Figure 5.4-5 shows the normalized average intensity of SHG over time along with the sarcomere length; the SHG intensity increases as the sarcomere length increases. A maximum of 20% change in sarcomere length was observed, while the maximum intensity variation was 10%. The SHG appears more efficient when the myocyte is in a relaxed state. Similar results on the dependence of SHG on sarcomere length were observed in mouse skeletal myocytes that were stretched mechanically (Both, Vogel et al. 2004). These changes can be attributed to a decrease in crystalline order during contraction. A more homogeneous structure can be envisioned in the relaxed state of the myocyte.

(c) Changes in the A-band width during contraction

The double peak SHG signal profile observed across a sarcomere can be fitted with two Gaussians as described in Chapter III. The contraction or relaxation of muscle leads to changes in the Gaussian bandwidths and spacing between the peaks. An SHG
profile along a row of four sarcomeres at two different instances was fitted with multiple Gaussian bands. The examination of these bands is useful in understanding the origin of the second harmonic generated from the anisotropic band. The fits for the two instances are shown in Figure 5.4-6. The average length of the 4 sarcomeres is longer in the second instance, although the first 3 sarcomeres increase in length, while the last one becomes shorter. Here, a sarcomere length is measured from the valleys on either side of the double peak (Z-line to Z-line). As the sarcomere length changes, changes are also observed in the SHG bandwidths. An increase in sarcomere length resulted in an increase in widths of one or both of the bandwidths for that sarcomere. In more than 50% of the sarcomeres examined, the peak-to-peak separation increased when the sarcomere length increased. As an example, Figure 5.4-6 has 3 sarcomeres which depict peak to peak changes with sarcomere length change; in the case of the second sarcomere, however, the increase in sarcomere size corresponded to a decrease in the peak-to-peak distance. This analysis shows that several factors influence the SHG band widths, and the SHG response during elongation of sarcomeres requires further investigation. However, the change in SHG band widths during contraction indicates higher elasticity of the myofilaments than previously reported (Both et al., 2004) although differences in sample type could also contribute to this discrepancy. The observed widths and intensities of the individual peaks are important to consider, especially with regards to the results of the previous section. It could be argued that the observed intensity changes with sarcomere length are merely a result of the muscle moving in and out of the focal plane during contraction. Examination of the individual SHG intensity peaks for a single time step however reveals that this is not the case. At a given time, there are differences in peak intensity of the sarcomeres. Furthermore, the intensity of the SHG peak was higher where less of the actin filament was overlapped with the myosin filament in the A-band. One can see the SHG peak intensity increases at the site where I-band is wider therefore showing that SHG strength depends on the sarcomere length. The increase in intensity can appear if the overlap of actin and myosin filaments decreases the crystalline order in the A-band and/or the $\chi^{(2)}$ of the band changes when amount of the actin is reduced. Both changes will affect the efficiency of the second harmonic generation in the A-band.
Figure 5.4-6: Original data and double peak Gaussian fits of a row of 4 sarcomeres at 2 different instances.
Note that with an increase in the sarcomere length, the intensity of the SHG peaks increases. The intensity of the peak also appears larger when beside the longer I-band.

Section 5.5: Conclusion

The second harmonic response from *Drosophila* myocytes was verified to be from the anisotropic bands of sarcomeres. Analysis of mutant strains provided strong evidence that a non-centrosymmetric semicrystalline arrangement has a crucial influence on the efficiency of SHG. Decrease in the semicrystalline order of anisotropic bands during mechanical stretching and without chemical alteration of myosin diminished SHG intensity. This presented further evidence that most significant factor in the second harmonic generation efficiency is the semicrystalline order of the anisotropic bands of sarcomeres. Results show that during functional contraction of muscle, the SHG efficiency changes likely due to small changes in the organization of the anisotropic bands. Additionally, the crystallinity and composition of the A-band is affected by the amount of overlap between actin and myosin, where a larger overlap results in a decreased SHG signal. Differences in the semicrystalline order of the anisotropic band observed via SHG microscopy may be useful as a diagnostic tool for muscular disorders.

By understanding the semicrystalline origin of the second harmonic in myocytes and how changes in the crystallinity affect the signal, SHG microscopy, in combination with other nonlinear techniques, can become invaluable tool for studying muscular function.
Chapter VI - Understanding Contractility Using SHG

Section 6.1: Introduction

The exploration of the origin of second harmonic generation in myocytes discussed in the previous chapter highlighted the suitability of Drosophila muscle cells for dynamic studies. Although the previous chapter examined myocyte dynamics as a means for understanding the structural organization of the filaments and the origins of the second harmonic generation, this chapter explores the potential application of SHG microscopy as an investigative tool for studying dynamics of muscle contractions.

The structural organization of myofilaments affects the second harmonic generation efficiency (Barzda, Greenhalgh et al. 2004; Both, Vogel et al. 2004; Greenhalgh, Aus der Au et al. 2005). Therefore it has been suggested that the SHG intensity dependence on structural organization could be a valuable tool for understanding muscular contraction dynamics. In combination with other imaging contrast mechanisms such as THG and MPF, the myocyte bioenergetics and dynamics of intracellular regulatory processes could be investigated in static and contracting myocytes (Barzda, Greenhalgh et al. 2004; Both, Vogel et al. 2004; Greenhalgh, Aus der Au et al. 2005). To date, most SHG muscle investigations have been limited to static investigations, or else to monitoring controlled changes of sarcomere length. Live cardiac myocytes, for example, were investigated in 2004 to test SHG as a means of quantifying sarcomere length (Boulesteix, Beaurepaire et al. 2004). By using saxitoxin to block Na⁺ channels, Boulesteix et al measured the effect of the inhibitor on changes in sarcomere length to an accuracy of 20 nm. The sarcomere length changes were attributed to the shortening of the isotropic band, consistent with the sliding filament model of myocyte contraction. Another study on isolated myocytes investigated the affect of sarcomere length on SHG intensity and showed that for a mechanical stretch of the muscle, SHG response increased when sarcomere length increased (Both, Vogel et al. 2004). In recent studies on myocyte stretching, the double peaked SHG of an A-band evolved into a single peak appearing in the center of the A-band. The dip in the SHG intensity profile at the centre of the A-band is attributed to destructive interference from
out of phase second harmonic radiating myosin molecules that, in the central region of myofilaments, are arranged antiparallel to one another (Prent, Green et al. 2008).

Here we highlight the capabilities of second harmonic generation by exploring the contractility mechanisms in myocytes. In particular, sub-video rate imaging of relaxed cardiomyocytes reveal nano-scale contractions of sarcomeres. The nanocontractions of sarcomeres do not induce significant change of the overall length of the cardiomyocyte, indicative of some sarcomeres contracting while others relaxing, which leads to the length compensation between the neighbouring sarcomeres. These antisymmetric nanocontractions are compared to the synchronized nanocontractions occurring during macro-contractions of cardiomyocytes. The changes of sarcomere length during muscle contraction as well as the contraction and relaxation rates are deduced. *Drosophila* larval muscle exhibits contraction dynamics similar to the dynamics observed in cardiomyocytes. The observed nanocontractions in both types of cells provide clear evidence that myosin nanomotors are continuously undergoing contraction cycles. However, macrocontractions occur only when the nanocontractions of many sarcomeres are synchronized. The observed nanocontractions in myocytes show the potential of using SHG microscopic imaging for dynamic investigations of myocyte contractility.

The capabilities of SHG microscopy as both a research and diagnostic tool for muscle contractility investigations are highlighted in this chapter. In addition, the exploration of contractility dynamics under different physiological conditions is performed. Changes in contraction dynamics are explored after the addition of a mitochondrial uncoupler. Since the mitochondria play an important role in muscle contraction, it is not unexpected that an uncoupler of electron transport will have an influence on the structure and contraction dynamics of myocytes. In this chapter, the loss of periodicity of the contractions under the influence of the mitochondrial uncoupler and how the muscle eventually proceeds to hypercontraction is examined. SHG is demonstrated as a useful tool to observe irregular contractions; likely, SHG microscopy will be an asset for diagnosing muscular dysfunction and fibrillation. Sarcomere dynamics observed in SHG of cardiomyocytes were also accompanied by the intensity fluctuations “flickering” of MPF and/or THG in the labelled cardiomyocytes, which will be described in more detail in Chapters VII and VIII.
Section 6.2: Nonsynchronized nanocontractions in myocytes

The imaging of cardiomyocytes with SHG microscopy at frame rates of almost 2 frames per second revealed, for the first time, small fluctuations of the position and length of individual sarcomeres. At the same time, the overall length of the myofibril did not change significantly. An SHG image of an optical section of a cardiomyocyte averaged over 50 frames is presented in Figure 6.2-1(a). A movie of this cardiomyocyte’s structural dynamics is presented in the supplemental material. It is particularly useful to view a row of sarcomeres at each of the individual time steps, enabling an examination of the structural dynamics of individual sarcomeres on a fast time scale. These fast frame rates revealed dynamics in a row of sarcomeres along the line AB (see Figure 6.2-1(a)) that can be visualized by creating a 2-D image with each vertical line representing the row of sarcomeres at a different time (Figure 6.2-1(b)). The brighter pixels in the image represent more intense SHG signal. This visualization can be created with Image J software by drawing a line along a row of sarcomeres and using the reslice function. Figure 6.2-1(b) shows a row of several sarcomeres observed in time. From the figure it can be seen that the position of individual SHG bands undergoes small as well as large fluctuations. The position fluctuations of the neighbouring sarcomeres are not synchronized. Moreover the fluctuations do not influence significantly the length fluctuations of the whole row of sarcomeres.

For analysis, sarcomere lengths were measured from one Z-line (middle of the valley in the I-band) to the next Z-line. The Z-line positions were found by inverting the profile of the row of sarcomeres at a given time and fitting each peak with a Gaussian profile. The peaks were fitted with OriginPro®, using the multiple peak fitter, and peak fitting wizard. The Gaussian peak-to-peak distance represents the distance from one Z-line to the next, which is equal to the sarcomere length. Figure 6.2-1(c) shows a sample of a row of sarcomeres at two different instances. The raw data is presented by a dotted line and the fits are shown by solid lines. For clarity, the profiles have been inverted back so that the Z-lines are once again the valleys. On average, with this fitting technique the position of the Z-line could be determined with a 50 nm accuracy, and the error in sarcomere length determination was approximately 70 nm. This measurement technique is similar to that used in other works (Boulesteix, Beaurepaire et al. 2004 and
Thompson, Larson et al. 2002). The technique applied here more closely resembles the Boulesteix’s work, where, as mentioned previously, sarcomere length was measured with an accuracy of up to 20 nm. The fast frame rate used to record the images in the present experiment, however, reduces this measurement accuracy due to apparent noise. The individual length fluctuations from the mean for each of the sarcomeres and for the entire row are shown in Figure 6.2-1(d). The mean length of each sarcomere is calculated from the measured lengths over the time span presented in the Figure 6.2-1(d). The sarcomere length changes over time are calculated from the fits of the AB line intensity profiles shown in Figure 6.2-1(b) and (c). It can be seen that the overall length fluctuations of the row of sarcomeres is similar to the fluctuations of individual sarcomeres (Figure 6.2-1(d)). The individual sarcomere length fluctuations have been denoted nanocontractions. In non-contracting myocytes, the nanocontractions represent nonsynchronized contractions of the individual sarcomeres. For a given instance, on average, 65% of the sarcomeres exhibit a measurable length change. The sarcomere length fluctuations reach more than 600 nm at some instances, which constitutes a change of approximately 30% of the average sarcomere length. One can see that at any time instance, some sarcomeres contract, while others relax or remain the same. This observation extends for the whole row of sarcomeres where contraction of some sarcomeres is compensated by the extension of the others, leading to little change in the overall length of the muscle fibril.
While sarcomeres are measured as the distance from one Z-line to the next, the Z-line positions can shift in different manners. A sarcomere shortening can be a result of the position of one Z-line moving closer to another Z-line which remains stationary, or else from both Z-lines moving towards each other. In the same manner, a sarcomere lengthening can be achieved by either one or both of the Z-line positions in a sarcomere shifting further away from the other. In cases where the sarcomeres do not change size, the Z-line pair can shift by the same amount and in the same direction, or else can remain quite still. Figure 6.2-1(e) shows the Z-line position fluctuations from the mean for each of the 8 Z-lines that make up the 7 sarcomeres that were analysed in Figure 6.2-1(d). By comparing the changes in a pair of Z-line positions, the way in which sarcomere
size changes can be deduced. One can see that some pairs of Z-lines appear coupled while others do not.

Chapter IV discusses the Pearson’s coefficient as a part of the dynamic image correlation analysis used in Chapter VIII whereby the intensity fluctuations of one pixel or region of interest are compared to that of another. In this chapter, the Pearson’s Coefficient can be used to examine the relationship between fluctuations of sarcomere lengths or Z-line positions of a reference sarcomere with neighbouring sarcomeres. To further support the observation of sarcomere length compensation, the Pearson’s Coefficient was calculated between the trajectories in Figure 6.2-1(d) for the length fluctuations of a reference sarcomere with those of its neighbours (see Figure 6.2-2(a)). As described in Chapter IV, the output ranges from -1 to +1, where +1 would indicate the sarcomeres are both contracting or relaxing at the same time and -1 would highlight two sarcomeres with one contracting while the other relaxing. The neighbouring sarcomeres generally display negative correlation with the reference sarcomere while sarcomeres that are further (2 or 3 sarcomeres) away from the reference have near zero correlation coefficients. This result indicates antisymmetric contraction/relaxation behaviour where immediate neighbouring sarcomeres appear strongly coupled, showing one sarcomere contraction at the expense of the extension of the nearest neighbours. Beyond the immediate neighbour, the contraction dynamics are not strongly correlated (See Figure 6.2-2(a)). The Pearson’s Coefficient was also calculated for the Z-line position of a reference sarcomere with its neighbours, shown in Figure 6.2-2(b). Here, the Z-line positions do not show as strong of a relationship as the observed negative correlation in the sarcomere length. Additionally, some cases show weak positive correlation, while other cases display weak negative correlation. The weak negative correlation in the Z-line positions of neighbouring sarcomeres results from the contraction of the reference sarcomere as well as extension of the neighbouring ones. The negative correlation of the Z-line positions are not influenced by the synchronized movement of the whole row of sarcomeres as in the case of the macrocontracting myocytes presented in the next section (see Figure 6.3-3(b)). The positive correlation shows that the whole sarcomere can shift its position without significantly altering its length. However, synchronized position shifts do not extend to more than two neighbouring sarcomeres.
Figure 6.2-2: Pearson’s Coefficient of the sarcomere length fluctuations and Z-line position for nanocontracting sample
(a) Pearson’s Coefficient between the length fluctuations of the reference sarcomere and those of its neighbours. (b) Pearson’s Coefficient between the Z-line position of the reference sarcomere and those of its neighbours. The error in the calculations of the coefficients is approximately 0.2. Each curve has a different reference sarcomere as indicated in the figure legend. The x-axis represents the number of sarcomeres away from the reference sarcomere. A point at +3, for example indicates the coefficient between the fluctuations of the reference sarcomere/Z-line and the sarcomere/Z-line 3 positions to the right while −2 is the coefficient between the reference and the 2nd sarcomere/Z-line to the left of the reference.
In analysing the nanocontractions, the total length vs. the number of sarcomeres was plotted for several time instances in Figure 6.2-3(a). Linear regression was used to make the best fit of the data. The average sarcomere length, as determined from the slope of the best fits in 6.2-3(a), was $1.77 \pm 0.02 \, \mu m$. Although there is some deviation in the lengths at the different instances, the variation is marginal. This is indicative of the approximately constant overall length of the row of sarcomeres showing that individual sarcomere length fluctuations do not add up. The fluctuations of the average length of a sarcomere are likely a result of contraction of some sarcomeres that are not fully compensated by its neighbours within the investigated region.

The standard deviation of the length fluctuation for different number of sarcomeres is shown in 6.2-3(b). The standard deviation calculations are based on the data from Figure 6.2-1(d); here the standard deviation for a single sarcomere is based on ‘Sarc 1’ while the row of 7 sarcomeres is calculated from the ‘Sarc All’ profile. The largest standard deviation of length fluctuation is observed for three sarcomeres, and the smallest value is obtained for the six sarcomeres. Overall the standard deviation oscillates, which indicates that individual sarcomeres undergo substantial fluctuations, but with the inclusion of a neighbouring sarcomere, there is a length compensation taking place, i.e. when one sarcomere contracts, the other tends to elongate. As the number of sarcomeres increases, fluctuations in the standard deviation arise when the length changes in one sarcomere are not compensated for by the other sarcomeres within the measurement. This provides further evidence that the sarcomeres are not involved in synchronized macrocontractions i.e. the overall lengths of the myofibrils are not changing. In the case where the contractions are synchronized, the standard deviation tends to increase for an increasing number of sarcomeres. This will be shown in section 6.3 on periodic contractions.
Figure 6.2-3: Length and standard deviation of length fluctuations dependence on the number of sarcomeres.

(a) The relationship between sarcomere length and number of sarcomeres for several different instances. From the Gaussian fits, sarcomere lengths could be determined to within 70 nm accuracy. Based on the slope of the best fits, the average sarcomere length was found to be 1.77 ± 0.02 μm.  

(b) The standard deviation of the length fluctuations for an increasing number of sarcomeres.
The observation of nonsynchronized nanocontractions is suggestive of a system where the work of myosin nanomotors continuously produces sarcomere length fluctuations that are not synchronized and therefore do not produce macrocontractions during the resting stage of the myocyte. Furthermore, it can be hypothesized that full-scale contraction can be obtained by synchronizing the nanocontractions. The synchronization can be achieved spontaneously or by the muscle cell receiving a neuronal signal, which would then lead to a full scale contraction of the whole myocyte. Although further investigation is required on the synchronization of contracting sarcomeres, the release of calcium into the T-tubules would likely trigger the synchronization.

For comparison, *Drosophila* larval muscle was also examined for nanocontractions. An SHG image of a non-contracting *Drosophila* larval muscle sample can be seen in Figure 6.2-4(a) as well as the evolution of the profile along the line AB in 6.2-4(b). Sample profiles and their fits can be seen in Figure 6.2-4(c). The individual length fluctuations from the mean for each of the sarcomeres and for the entire row are shown in Figure 6.2-4(d) and the fluctuations from the mean position of the Z-line is shown in Figure 6.2-4(e). The average sarcomere length determination accuracy in the *Drosophila* samples is 310 nm. This represents the same percentage of sarcomere length as observed in the cardiomyocyte samples. The length determination accuracy is more than four times larger than for cardiomyocytes. The main uncertainty comes from the greater structural details in the A-band of larva myocyte showing double or triple peaked structures, which hinder accurate fitting with the Gaussian shapes. Note also that much larger I-bands give flattop band shapes rather than Gaussians, which give larger error for the Gaussian fitting to the valleys of SHG profile (I-bands) compared to the case of the cardiomyocyte. Nanocontractions, however, have been shown to exist in both cell types and represent sarcomeres undergoing nonsynchronized contractions. In the next section, the way nanocontracting sarcomeres synchronize to lead to a macrocontraction is examined.
Figure 6.2-4: Nanocontractions in *Drosophila* larval myocyte
(a) SHG image of unlabeled *Drosophila larval* myocyte (average of 50 frames); (b) Time cross section of SHG intensity profile along the line indicated in (a), the x-axis represents time and the y-axis is the single frame intensity profile of a row of sarcomeres; (c) A sample profile of a row of sarcomeres at two different instances and the cumulative fit with the multiple Gaussians (profiles offset by 100 intensity units). (d) Sarcomere size fluctuations from the mean for 6 individual neighbouring sarcomeres and their total length fluctuations. Here ‘Sarc All’ represents the length change based on the sum of the individual changes. (e) Z-line position fluctuations from the mean for the 7 neighbouring Z-lines that make up the 6 sarcomeres in (d).
In order to examine the nanocontractions more closely, we first look at the imaged muscle fibril which is of constant length. By considering the work of Hill, a simplified model of muscle can be thought of as having a contractile component and an elastic element which are connected in series (Hill, 1938). For the case of the isometric contractions the length of the muscle does not change even though tension is building as is given by:

\[ L_m = L_{mc} + L_{me} \]  

where \( L_{me} \) is the length of the elastic element and \( L_{mc} \) is the length of the contractile element. As the contractile element of the muscle shortens, it stretches the elastic element of the muscle by an equal amount and therefore maintaining a constant length. Here, the force generated is related to the stretch of the elastic elements as given by:

\[ P_m = \alpha [L_{mc} - L_{me}(t = 0)] \]

where \( L_{me} \) is the length of the muscles elastic element of the muscle and \( \alpha \) is the ‘spring constant’ of the system.

While experiments here reveal imaged muscle lengths remaining unchanged and therefore the muscle may be exhibiting isometric-like contractions, the individual sarcomeres are, in fact, undergoing length changes. Passive stretching of the contractile element generates tension in the sarcomere; for increasing stretches, the tension increases dramatically. A typical graph showing the passive tension length relationship is seen in Figure 6.2-5; this figure is adapted from the literature, and created using Excel® (Lieber 1999). It should be noted that cardiac and skeletal muscle cells have significant differences in their length-passive tension relationship (Granzier and Irving 1995). Recent studies suggest titin is at least in part responsible for passive tension in muscle (Magid and Law, 1985; Granzier and Wang, 1993). It has also been shown that sarcomere lengthening can result from a separation of the central bare region of the myosin filaments (Prent, Green et al, 2008). Active tension is primarily a result of the cross-bridges formed between actin and myosin (Morgan, Claflin and Julian, 1991); a typical length-active tension relationship is seen in Figure 6.2-5. This figure shows that when the muscle is actively stretched to a point where the myosin and actin no longer overlap
there is no active tension (but a large amount of passive tension). From this point of no overlap, the tension increases as the sarcomeres shorten and the filaments slide along each other forming the actin and myosin cross-bridges. At the optimal sarcomere length, there is the maximum number of cross-bridges and the active tension is at its peak. The plateau region is a result of the lack of myosin heads in the H-zone resulting in a reduced length from the optimal, but no additional cross-bridges are formed. For sarcomere lengths well below the optimal length the tension is reduced as a result of the overlap of actin filaments reducing the number of cross-bridges. During nanocontractions, sarcomeres are near an optimal length and tension therefore is near maximum.

![Graph of Length-tension relationship for sarcomeres](image)

**Figure 6.2-5: Length-tension relationship for sarcomeres**
Here the dashed line indicates the passive force being generated as the sarcomeres are stretched, while the solid line represents the active length-tension relationship typical of muscle sarcomeres. This figure adapted from the literature (Gordon, Huxley and Julian, 1966; Leiber, 1999) and created in Excel®.

If the individual sarcomeres of nanocontracting samples can be treated as having an elastic and contracting element in the same manner as for the model of a muscle above, then it follows from above that the total resting length of sarcomere can be described as:

\[
L_s = L_{sc} + L_{sc}
\]  
6.2-3
where $L_{se}$ is the length of the elastic element and $L_{sc}$ is the length of the contractile element. It follows that the muscle length can now be given as:

$$L_m = \sum (L_{se} + L_{sc})$$

6.2-4

It is conceivable therefore that the shortening of the contractile elements in individual sarcomeres are compensated by the stretching of the elastic elements in other sarcomeres. However, as shown with the correlation analysis, the compensating stretching does not exceed more than two neighbouring sarcomeres (see Fig. 6.2-2). The troponin and tropomyosin enable contraction in the presence of calcium ions. Therefore, the sarcomere length changes along the fibril might be related to the concentration fluctuations of calcium ions along the fibril.

Section 6.3: Periodic contraction dynamics in myocytes

The nanocontractions in a non-contracting myocyte seen above are different from the synchronized nanocontractions observable in a row of sarcomeres of a macrocontracting myocyte. In this section, evidence is presented that during a myocyte macrocontraction, many sarcomeres shorten/lengthen synchronously by approximately the same amount. In addition, sarcomeres are displaced from their original position as a result of the synchronized contraction of many sarcomeres. In general, during muscle shortening, sarcomeres are undergoing active contraction. The force-velocity relationship for sarcomere contraction can be approximated using Hill’s model:

$$(P_0 - P)b = (P + a)V$$

6.3-1

where $P$ is the force generated stress, $P_0$ is the stress generated for an isometric contraction, $V$ is the velocity and $a$ and $b$ are constants. Differences in muscle type result in differences in $a$ and $b$; different muscle types also have different values for the maximum velocity and tension force. A typical stress-velocity curve for cardiac myocytes can be seen in Figure 6.3-1. This curve was generated with Matlab®, using the technique described in Holmes, 2006 and based on constants found in Daniels, Noble et al, 1984.
An example of the stress-velocity relationship for cardiomyocytes calculated using Hill's model described in the text. Values for the constants are estimates based on values found in Daniels, Noble et al, 1984 with $a = 9.52 \text{ mN/mm}^2$, $b = 1 \mu\text{m/s}$, $P_0 = 119.3 \text{ mN/mm}^2$ and $V_0 = 13.4 \mu\text{m/s}$.

In the nanocontraction case of the previous section, sarcomere length changes were on the order of 0.15 µm. These changes were observed in a time period of 0.75 s, resulting in contraction velocities on the order of 0.2 µm/s. These contractions are in the low velocity - high force regime of the curve. The total length of the fibril, does not exhibit length changes so the velocity approaches zero and the tension becomes $P_0$.

Now consider the case in of periodic contractions of the imaged region where the overall length of the row of sarcomeres changes. Figure 6.3-2(a) shows an SHG image of a cardiomyocyte (50 frames averaged) while Figure 6.3-2(b) shows the time series of an SHG intensity profile of the line AB drawn along a myofibril shown in (a), similar to the presentation of the data in Figure 6.2-1 for the myocyte undergoing nonsynchronized nanocontractions. The figure in 6.3-2(a) appears more blurred than 6.2-1(a) due to frame
average of a contracting cardiomyocyte. Periodic contractions of a row of several sarcomeres can be observed in Figure 6.3-2(b), where the myocyte was imaged with a frame rate of close to 5 frames per second. The presented cardiomyocyte undergoes small periodic macrocontractions. Much larger macrocontractions are also observed in the cardiomyocytes, however sarcomeres in that case are displaced out of the image field of view, and therefore the data of strongly contracting cardiomyocytes are not suited for the current contractility analysis. The macrocontractions of the presented cardiomyocyte are not as large as those of *Drosophila* larva shown later in this chapter. The displacement of an individual cardiac sarcomere during contraction for cardiomyocytes is about 2.5 μm, while the *Drosophila* larva sarcomeres exhibit the displacements of more than 12 μm. The macrocontractions of an observed cardiomyocyte occur at a rate of approximately 50 cycles per minute. In order to analyze the sarcomere length changes during contraction, profiles of the row of sarcomeres were fitted with Gaussians in the same manner as for the non-contracting sample (see Figure 6.2-1). Figure 6.3-2(c) shows the raw data and fits of the SHG intensity profile at two different instances: at the most extended phase and at the most contracted phase. Notice how the profile for the contracted sample contains one more sarcomere than the profile for the relaxed state. Also note how the Z-lines are shifted in the contracted case.

The length fluctuations in time for the row of sarcomeres are shown in Figure 6.3-2(d). As in the nonsynchronized nanocontraction case above, the length fluctuation from the mean for each of the sarcomeres as well as for the entire row of sarcomeres is shown in the graph. An arrow in the image indicates the point at which the myocyte is maximally shortened. It can be seen that in this case, at early times, sarcomeres behave in a manner similar to that of the nonsynchronized nanocontraction case, whereby contractions/extensions of the neighbouring sarcomeres are anticorrelated. The overall length of the row of sarcomeres is not changing and as with the non-contracting case, the force is equal to $P_0$. Individual sarcomeres contract by similar amounts as nanocontractions in the non-contracting case however the nanocontractions appear to be faster. It is possible that in the macrocontracting samples the nanocontracting velocities are higher, although, the non-synchronized contractions observed in the previous sample were imaged with a lower time resolution and most probably contain aliasing effect due
to faster contractions. As the time of the macrocontraction approaches, more sarcomeres become synchronized and ultimately most sarcomeres are shortening at the time between 1.2 s and 1.4 s, the peak of the macrocontraction. The synchronization of sarcomere shortening increases the tension force and induces macrocontraction. In the contraction of the myocyte the tension force decreases proportionally to the velocity of the contraction (see eq. 6.3-1). After the macrocontraction, the sarcomeres relax in a synchronized fashion, and then appear once again in a nonsynchronized state.

The fluctuations of the Z-line positions are shown in Figure 6.3-2(e). While in the case of the non-contracting sample there was little correlation between the Z-lines, here all of the Z-lines follow a very similar pattern. In the first few instances, there are small fluctuations which are similar to those observed in the non-contracting sample. Beyond this, the Z-line fluctuations synchronize, at first shifting in one direction while the overall length of the myofibril remains close to unchanged and then shifting in the other direction by an even larger amount which corresponds to the contraction phase where the sarcomeres synchronize and myofibril length shortens. It is possible that the shifting of the Z-lines prior to contraction, are part of the synchronization for contraction. During that phase, the contraction likely occurs within sarcomeres outside (above) the field of view of the image, and the sarcomeres in the imaged region do not contract but merely shift the positions.
Figure 6.3-2: Periodic synchronization of nanocontractions into macrocontraction in cardiomyocyte.

(a) SHG image of unlabeled cardiomyocyte (average of 50 frames). (b) Time cross section of SHG intensity profile along the line AB shown in (a). The nanocontractions of sarcomeres can be observed in time, the x-axis represents time and the y-axis is the single frame intensity profile of a row of sarcomeres. (c) A sample profile of a row of sarcomeres at two different instances and the cumulative fit with the multiple Gaussians. (d) Sarcomere size fluctuations for 7 individual neighbouring sarcomeres and their total length fluctuations. Here ‘Sarc All’ represents the length change based on the sum of the individual changes and offset values are given in μm. (e) Z-position fluctuations from the mean for the 8 neighbouring Z-lines that make up the 7 sarcomeres in (d). (See also media file 11)
The Pearson’s Coefficient was calculated for fluctuation traces from Figure 6.3-2(d) in order to examine the correlation of length fluctuations between sarcomeres (see Figure 6.3-3(a)). The resulting coefficients are similar to the non-contracting myocytes observed in the previous section. This appears because nonsynchronized nanocontractions are present approximately 2/3 of the time and synchronization occurs only briefly during macrocontraction. This sample, however, enables us to analyse the nanocontractions within the small time interval, as was mentioned previously. Therefore, the Pearson’s Coefficient represents a combination of a larger number of nonsynchronized nanocontractions and a few synchronized ones. The Pearson’s coefficient was also calculated for the Z-line positions as seen in Figure 6.3-3(b). Here the sample shows significant correlation, not only for neighbouring sarcomeres but also for sarcomeres much further away. The high correlation is a result of the translational movement of the Z-lines. As the myocyte contracts, the Z-lines shift towards the central part of the contraction region in the myofibril, whereas in the non-contracting case the Z-lines fluctuate around their central position. During the non-contracting stages of the periodically contracting sample (phase 1), the Z-lines shift likely as a result of the synchronized contraction of sarcomeres that are beyond the imaged region, pulling the observed sarcomeres. This could be a means to generate more force during contraction. During the brief synchronization (phase 2), the movement direction of Z-lines is reversed. In phase 3, synchronized relaxation/extension of the sarcomeres changes the direction of the Z-line movement. After the phase 3, sarcomeres enter the nonsynchronized phase 1 of the next macrocontraction period.
To further explore the synchronization of the contraction, the time series of sarcomere 1 was separated into the nonsynchronized phase (0 - 0.8 s inclusive), and the synchronized phase (1 - 1.6s inclusive) (see Figure 6.3-2 for time ranges). Figure 6.3-3(c) shows the Pearson’s Coefficient between the length fluctuations of sarcomere 1 and the length fluctuations for the sarcomere x positions away for the two different phases. In comparing the nonsynchronized phase the synchronization phase, 5 out of the 6 neighbouring sarcomeres show an increase in correlation, and 4 out of the 6 have significant correlation to be considered synchronized. One sarcomere shows a reduction in the Pearson’s Coefficient resulting in anticorrelation and is therefore not synchronized with the other sarcomeres. It is evident from Figure 6.3-3(c) that the synchronization of
nanocontractions in sarcomeres leads to a macrocontraction and highlights the importance of the continual nonsynchronized nanocontractions in muscle contraction.

Similar to the case of nonsynchronized nanocontractions, the total length versus the number of sarcomeres was plotted for several times in Figure 6.3-4(a). Linear regression was used to find the best fit of the slope, for each time step. The average sarcomere length based on the best-fit was plotted against time in Figure 6.3-4(b). One can see that at the time of contraction (1.4 s), the average length of sarcomeres has the smallest value. In addition to the length changes, the standard deviation of the length for rows of varying number of sarcomeres is shown in 6.3-4(c) as based on the data from Figure 6.3-2(d). As the number of sarcomeres is increased, the standard deviation also increases. Differences between the average length and the standard deviations are observable between the non-contracting sample and this sample. In terms of percentage of length, a single sarcomere in the non-contracting sample exhibits a larger fluctuation from length when compared with the contracting sample. While these small differences can be attributed to the variability in the samples, differences could also be expected as a result of environmental factors such as condition of the medium and time between plating and imaging.

The trend observed in Figure 6.3-4(c) presents additional evidence that the sarcomeres synchronize during macrocontractions and the overall myofibril length changes by a larger amount. Because there is a length shortening of many sarcomeres at the same time, the standard deviation of length fluctuations increases with the number of sarcomeres. Some variation from the linear increase occurs in the standard deviation dependence on the number of sarcomeres. The variation indicates that not all sarcomeres are synchronized during the macrocontraction or else do not contract by the same amount. For example, at the length of 4 sarcomeres, there is likely a sarcomere that is elongated during contraction, compensating partly for the shortening of the other sarcomeres in the myofibril.
The observations of the cardiac myocyte contraction dynamics can be compared with those observed in *Drosophila* larval muscle. The previous chapter showed that *Drosophila* myocytes can be imaged for a long period of time with no visible damage, and the strong SHG response combined with the large sarcomere size permits for a more detailed investigation than with the isolated cardiomyocytes. Several types of myocyte contractions were observed with the SHG imaging of the larval muscle. While the addition of 100mM KCl cause changes to the ion concentration in the myocyte and induces contraction of the myofibrils (see Figure 6.4-2), some periodic contractions also occurred spontaneously. These motions can be used to further understand the contraction mechanisms of myocytes using SHG microscopy.

Naturally occurring periodic contractions can be observed with SHG microscopy of *Drosophila* larva myocytes. Contraction and relaxation rates can be measured from

---

**Figure 6.3-4: Length and standard deviation of length fluctuations dependence on the number of sarcomeres.**

(a) The relationship between length and number of sarcomeres for several different times with dotted line showing best fit, (b) average sarcomere length based on the best-fit slope for each time step. (c) The standard deviation of the length fluctuations for an increasing number of sarcomeres.

---

![Graph showing standard deviation of length fluctuations](image-url)
SHG images. The periodic contractions show sarcomere displacements of more than 12 μm along the fibrils. This observed periodic motion, which highlights the usefulness of SHG for dynamic studies, could be part of the complex wave motion that larvae use to propel themselves. Figure 6.3-5(a) shows a single frame of a myocyte image that is undergoing contraction; here the entire fibril appears to contract simultaneously. The image shows a line (AB), which corresponds to the vertical axis in the Figure 6.3-5(b). The changes of SHG intensity in time along the AB line can be seen in Figure 6.3-5(b) (see also Figure 5.4-4(a)). The time evolution of periodic contractions is clearly represented by up and down movement of the SHG bands. The larger sarcomere size highlights this movement better than in the cardiomyocytes and the observed more than 12 μm fibril motion indicates that shortening of many sarcomeres is involved in this movement.

The single frame images recorded at approximately ten frames per second rate has substantial fluctuation of the SHG signal, which makes the H-band difficult to distinguish, but the width variation of the I-band is clearly visible. The individual sarcomeres were observed to have size variations of up to 1.6 μm or around 25 % of the average length of a sarcomere, a percentage similar to the changes in length that could be observed in the cardiomyocytes.

In the case of the larval muscle, data showed that contractions occurred at a rate of 4.5 cycles/min; more than ten times slower than observed in cardiomyocytes. From the most relaxed, to the most contracted state, the Z-line positions move along the fibril quickly, reaching velocities of nearly 7 μm/s, while, during relaxation, the shifting of the Z-line is slower, and averages to 3.5 μm/s.
Figure 6.3-5: SHG image and time evolution of a row of sarcomeres during periodic contractions (*Drosophila* larval muscle)

(a) Single frame of *Drosophila* larval muscle observed in SHG and (b) the evolution in time of the SHG profile along the AB line; the x-axis represents time and the y-axis is the single frame intensity profile of a row of sarcomeres (see media files 7-10)

The observed periodic contractions highlight the similarities and differences in contractility behaviour of the different cell types (skeletal vs. cardiac) and biological species (fruit fly vs. rat). Given the functionality of a heart cell compared to skeletal muscle, the faster contraction rate is not unexpected. The movements of sarcomeres along the fibril appear similar in both cases, although the large I-band of larval muscle makes this movement more easily observable. Therefore the larval myocytes can be used as a model system for further investigations of principles of muscle contractions.
Section 6.4: Induced disruptions in periodic contractions

Although the observed periodic contractions provide a baseline for ‘normal’ contraction behaviour, here we explore the viability of SHG imaging as a tool to detect disruptions or anomalous contraction behaviour in myocytes. By purposely inducing disruptions in the periodic contractions, contractility of sarcomeres can be analysed, while demonstrating SHG as a potential diagnostic tool for muscular diseases.

When carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, is added to a cardiomyocyte immersion medium, there is a disruption to the contraction periodicity. In Figure 6.4-1(a) an image of the myocyte sample prior to the addition of the inhibitor can be seen and Figure 6.4-1(b) shows the 2-D map of the row of sarcomeres along the line AB, similar to those seen for contracting and non-contracting samples, except observed over a much longer time duration. The ridge-like structures in this figure are actually a result of strong contractions causing sideways translational movements of the row of sarcomeres (see media files 12 and 13). Aside from the strength of contractions not presented previously for cardiomyocytes under normal conditions, the initial times show approximately periodic contractions. As time passes, after addition of the mitochondrial uncoupler, several changes arise: the periodicity becomes more erratic and the strong contractions last longer (the width of the ridges increases). Additionally, the time between these ridge-like structures tends to increase. As the time between contractions increases, one is able to see that in between contractions the sarcomeres appear to undergo the nonsynchronized nanocontractions discussed previously. The disruption in the periodicity of contraction shows the importance of mitochondria function for periodic contraction. Ultimately, however, the sarcomere undergoes hypercontraction and the SHG signal becomes lost (only noise remains in the right side of the image in Figure 6.4-1(b)).
Figure 6.4-1: SHG image of a cardiomyocyte and time evolution of a row of sarcomeres after the addition of FCCP.

(a) SHG image of a cardiomyocyte prior to the addition of FCCP (b) the evolution in time of the SHG profile at the AB line. Notice that as time passes after the addition of FCCP, the periodicity of contractions is disturbed. Between macrocontractions, the nanocontractions can be observed. (See media files 12 and 13).

The increase in time the sarcomeres stay contracted is reminiscent of the sustained contraction of many sarcomeres in a *Drosophila* larval myofibril that were observed after the addition of a potassium chloride solution (KCl), and discussed in the previous chapter (see Figure 5.4-1). Figure 6.4-2 shows the SHG images over time presenting the evolution of sustained contraction in *Drosophila* larval myocyte over 120 seconds. A localized shortening starting from one sarcomere at time t=75s and gradually engaging more than ten neighbouring sarcomeres is observed. The double peak band of SHG can be seen to disappear in some sarcomeres. The shrinking and disappearance of isotropic bands can also be seen. Only closely adjacent anisotropic bands remain visible in the sustained contraction region.
Figure 6.4-2: SHG image and time cross section of sustained contraction in *Drosophila* larva myocyte.

After the addition of the KCl solution, panel (a) depicts a single frame of *Drosophila* larval myocyte observed in SHG and panel (b) is the evolution in time of the SHG profile at the AB line. (See also Figure 5.4-1 and media file 5)

The effectiveness of SHG in imaging sustained contractions provides evidence for the reliability of SHG as a powerful diagnostic and imaging method at the subcellular level. Non-invasive imaging of sustained contractions with SHG will be beneficial for exploring muscular contraction disorders and atrophy. The erratic behaviour detected in cardiomyocytes with a mitochondrial inhibitor suggests that SHG could also be useful in examining fibrillations.

**Section 6.5: Conclusion**

This chapter explores structural dynamics of cardiomyocytes and *Drosophila* myocytes during contraction as well as in the relaxed, resting state. The sub-video rate imaging with SHG microscopy provides the possibility of following small length fluctuations of individual sarcomeres.

For the first time, this chapter demonstrates the nanocontractions in cardiomyocytes and *Drosophila* larva myocytes. These small scale contractions are present at all times and result in significant fluctuations of sarcomere length. The nanocontractions, however, are not synchronized in the resting myocytes and thus do not produce macrocontractions over the whole length of myofibril. When the
nanocontractions do synchronize for a short period of time, they result in macrocontractions of myofibrils in the periodically contracting myocytes. Therefore, a new way of understanding contractility is developed in this chapter. The macrocontractions can be viewed as the synchronization phenomena of nanocontractions rather than the initiation of the myosin contraction cycle upon arrival of the stimulus, for example, in the form of calcium release from T-tubules (Matthews 2001). The study of nanocontractions with SHG microscopy shows the potential for further elucidation of the myosin gait, contraction mechanisms, and bioenergetics during contraction. The nanocontractions are shown to be present under the influence of a mitochondrial uncoupler when periodic contractions are disrupted, highlighting the importance of the role of mitochondria in the synchronization of nanocontractions.

SHG microscopy is demonstrated as an effective means in examining sarcomere structural dynamics. Small contractions are shown to be observable in SHG, providing a suitable foundation for using SHG to examine muscular dysfunction and fibrillation. Through comparisons of dynamics in cardiomyocytes and Drosophila larval muscle, and by evaluating the disruptions in myocyte function, SHG is shown to be effective in measuring changes in contraction periodicity, length of contraction and SHG intensity changes during contraction of sarcomeres. The potential to further explore the bioenergetics of contraction through fast SHG microscopic imaging with improved signal to noise ratio is clearly evident and opens new perspectives for using the harmonic generation microscopy in contractility research and clinical diagnostics of muscular disorders.
Chapter VII - Origin of THG in Myocytes

Section 7.1: Introduction

In order to take full advantage of nonlinear microscopic imaging, the origin of the signals within the sample must be understood. Chapter II discussed the general features and restrictions for generating nonlinear signals at the focus of the microscope objective, while Chapter V detailed the importance of crystalline structure within myocytes for generating the second harmonic. In this chapter, findings are presented on the origin of THG specifically within cardiomyocytes (Barzda, Greenhalgh et al. 2005). The chapter elucidates the third harmonic origin through structural comparisons of THG images with those recorded simultaneously with MPF and/or SHG. By understanding where and why nonlinear signals are generated within cardiomyocytes, predictions can be made on the origin of the signals in other types of myocytes, and possibly other biological samples. Further to this, an understanding of the origin of the signal is a necessity for interpreting dynamic imaging results.

Co-localization of different intracellular structures and simultaneous monitoring of their dynamics is one of several very successful ways to investigate subcellular interactions and functional dynamics inside biological samples. Frequently, simultaneous investigations of several structures are accomplished by specifically labelling them with dyes that fluoresce at different wavelengths. Multi-photon excitation fluorescence microscopy is often employed for such studies, where several fluorescence labels can be efficiently excited with spectrally broad femtosecond pulses (Konig 2000). Often more than two fluorescence channels with different detection wavelengths are utilized. In analogy, simultaneous detection of SHG and THG together with fluorescence is a very efficient way of imaging and enables direct comparison of structures that are revealed by the different contrast mechanisms. In this study, we investigated isolated live cardiomyocytes with a simultaneous MPF, SHG, and THG detection microscope, and elucidated the structural origin of THG by comparing the structures with the MPF and SHG images. Cardiomyocytes were studied with MPF microscopy by recording NAD(P)H fluorescence as well as by obtaining fluorescence from tetramethylrhodamine
methyl ester (TMRM) labelled structures. Previous studies of myocytes with MPF microscopy showed that mitochondria can be visualized with NAD(P)H fluorescence (Huang, Heikal et al. 2002). TMRM labelling of the cells also revealed mitochondrial structures as well as labelled sarcoplasmic reticulum (Xu, Zipfel et al. 1996). In addition, THG of muscular myocytes was recorded (at 1230 nm), and the origin of the signal was assigned to isotropic bands of myofibrils (Chu, Chen et al. 2002; Chu, Chen et al. 2004).

This chapter examines multi-photon excitation fluorescent images of cardiomyocytes recorded with two different excitation sources. Using labelled and unlabelled samples, combined with the SCIA algorithm, comparisons are made between the fluorescing as well as second harmonic generating structures and those generating the third harmonic. It is revealed that very strong THG signal is generated in the mitochondria. Structural features of the mitochondria, which could be responsible for the enhancement of THG, are discussed.

(a) THG and NAD(P)H fluorescence

The 3-D imaging of live cardiomyocytes was performed by simultaneously collecting THG and MPF in two separate detection channels. Images were recorded with 837 nm peak excitation wavelength radiation from the cavity dumped Ti:Sapphire laser. The femtosecond laser spectral bandwidth was about 30 nm providing effective NAD(P)H excitation as well as flavin adenine dinucleotide (FAD) and lipoamide dehydrogenase (LipDH) excitation. The emission comprised mainly of NAD(P)H, FAD and LipDH fluorescence (Huang, Heikal et al. 2002). The NAD(P)H multi-photon excitation fluorescence is observed in the 400 – 600 nm region while FAD and LipDH fluorescence lies in the 460 – 600 nm range. For our study, the fluorescence was collected between 435 and 500 nm which originated predominantly from NAD(P)H (Zipfel, Williams et al. 2003a). The highest fluorescence of NAD(P)H is usually confined to mitochondria, where the tricarboxylic cycle takes place (Blinova, Combs et al. 2004). Figure 7.1-1(a) shows the NAD(P)H MPF of a single image slice while Figure 7.1-1(b) shows the corresponding image obtained with THG. The images are similar to each other. The intensity profiles of the line in Figure 7.1-1(a) and (b) is depicted in Figure 7.1-1(c). In general, both profiles follow a similar path. The 2-D SCIA result for the same non-processed slice is shown in Figure 7.1-1(d), where the majority of the
sample is correlated, shown in red. The cardiomyocyte structures obtained with 3-D fluorescence imaging (Figure 7.1-1(e)) revealed typical granular structures that can be attributed to mitochondria ordered in rows along the myofibrils (Eng, Lynch et al. 1989). The mitochondria are approximately 1-2 µm in diameter. The image generated from the third harmonic that was recorded simultaneously with the fluorescence is presented in Figure 7.1-1(f). The THG shows granular structures with periodicity and spatial location similar to those generated from the fluorescence.

For comparison THG and NAD(P)H MPF images were co-localized (see Figure 7.1-1(g)). The red coloured structure represents the correlated signal, while blue is uncorrelated THG, and green is the uncorrelated fluorescence. Figure 7.1-1(h) presents only the correlated volume of the cardiomyocyte. Structural cross correlation image analysis is performed only on pixels that have substantial signal in at least one of the channels. In this way correlation of the image backgrounds, which do not belong to the highlighted THG or MPF structures but would obscure the structural cross-correlation analysis could be avoided. The cross-correlation image (Figure 7.1-1(h)) once again shows an ordered granular pattern arranged along the myofilaments. There are only few pixels of uncorrelated THG (blue) visible in the co-localized image (Figure 7.1-1(g)). Those pixels have typically lower THG signal intensity. Some of the fluorescence signal is not correlated with the THG signal. The uncorrelated fluorescence (Figure 7.1-1(g)) appears to envelop the correlated structure, or shows up as a separate row of granular structures arranged along the myofilaments. The envelope structure emerges because the point spread function is broader for two-photon excitation than for THG. This blurs fluorescing structures in the image more than THG structures. The deconvolution procedure minimizes the blur; however some of it still remains giving envelope-like artifacts in the correlated image. The uncorrelated fluorescent granular structures, most probably, represent mitochondria that have their lamellae oriented predominantly parallel to the laser beam rendering unfavourable conditions for THG.

Unfortunately, around an 800 nm excitation wavelength, the live cardiomyocytes can only be imaged for several minutes before hypercontraction occurs. Damage of the cardiomyocytes is most likely inflicted by the multi-photon absorption of NAD(P)H, FAD and LipDH. The lowest phototoxicity with Ti:Sapphire excitation has been
achieved by tuning the oscillator to the lower absorption region of NAD(P)H around 837 nm wavelengths, and adjusting the pulse repetition rate to 3.1 MHz and pulse energy to 0.6 nJ at the sample while keeping the average laser power constant. With these conditions two-photon excitation fluorescence in the 435 - 500 nm range was still at a measurable level and excitation pulse energy was high enough to generate appreciable intensity of THG.

![Figure 7.1-1 Comparison of NAD(P)H MPF and THG images in a typical cardiomyocyte excited with 837 nm laser pulses.](image)

(a) A 2-D optical section of NAD(P)H MPF of a cardiomyocyte; (b) The same 2-D optical section simultaneously imaged with THG. The intensity profiles along the same line shown in (a and b) are plotted in (c), where the green diamonds indicate MPF and blue triangles THG intensities. (d) The 2-D SCIA output for the slice shown in (a) and (b), red color indicates the correlated region while green and blue are the uncorrelated MPF and THG, respectively. (e) The rendered NAD(P)H MPF. (f) The rendered THG image obtained simultaneously with the MPF. (g) Results of the SCIA where red represents the correlated signal, while green is the uncorrelated NAD(P)H MPF and blue is the uncorrelated THG signal. (h) The same SCIA as (g) but showing only the correlated volume in red. The laser beam propagation is parallel to the z direction while the origin of the axis is placed at the perimeter of the cardiomyocyte. The size of a pixel is 0.24µm. (see media file 14)

(b) THG and 1064 nm excitation autofluorescence

Live cardiomyocytes can be imaged for many hours with the Nd:glass laser excitation that peaks at 1064 nm. At this excitation wavelength NAD(P)H as well as FAD and LipDH are not excited (Huang, Heikal et al. 2002) i.e. the deposition of the excitation energy into the cardiomyocyte is drastically reduced. This enables higher laser
powers, which in turn, generate intense THG and SHG signals. The \(\sim20\) mW excitation power at the sample was used, that resulted in 0.2 nJ energy per pulse with the 94 MHz repetition rate laser. Figure 7.1-2(a) shows a single raw image slice revealed by autofluorescence emitted at 630-700 nm while the 3-D structures of the cardiomyocyte are revealed in the deconvoluted rendered volume displayed in Figure 7.1-2(c). Only a few bright spots spanning through the cardiomyocyte perpendicularly to the myofibrils are visible. Similar structures have been observed by (Huang, Heikal et al. 2002) with 900 nm excitation. The origin of the revealed structure and the fluorophore is unknown.

The 2-D and 3-D THG images recorded simultaneously with the MPF are shown in Figure 7.1-2(b) and (d), respectively. The structure reveals characteristic granular rows of mitochondria aligned along the myofibrils. The THG image shows similar structures to those recorded at 837 nm. The structural cross-correlation analysis is presented in Figure 7.1-2(e). It is clearly visible that fluorescing structures overlap with corresponding structures revealed by THG (overlap coloured in red). The remaining structures in Figure 7.1-2(e) originate from the uncorrelated THG signal and are depicted in blue. Uncorrelated fluorescence structures are coded in green and are practically absent in the co-localized image. The correlated structures are presented separately in Figure 7.1-2(f). It is interesting to note that correlated structures appear as continuous oval shape volumes with the longest axes oriented perpendicular to myofibrils and parallel to the direction of the laser beam propagation. The structures are several times larger in axial direction (\(~8\) \(\mu\)m) than the diffraction limit of the imaging system. THG would not be detected inside an isotropic media, suggesting that the observed structures contain a multi membrane arrangement. The correlated structures appear elongated laterally in the scanning direction. The elongated shape most likely is an artifact that comes from lateral dragging of the structure optically trapped by the laser during scanning. Apparently, those structures are much more susceptible to trapping compared to mitochondria.
Figure 7.1-2: The comparison of autofluorescence and THG of a typical cardiomyocyte with 1064 nm excitation.

(a) A 2-D slice of the autofluorescence emitted at 630-700 nm; (b) The THG generated simultaneously with the MPF. (c) The 3-D rendered image of autofluorescence; (d) the rendered image of simultaneously generated THG. (e) The SCIA where red is the correlated signal, while green, which is barely noticeable, is the uncorrelated autofluorescence and blue is the uncorrelated THG signal. (f) The same SCIA as (e) but showing only the correlated volume in red. The laser beam propagation is parallel to the z direction while the origin of the axis is placed at the perimeter of the cardiomyocyte. The size of a pixel is 0.24 µm.

(c) THG and TMRM fluorescence excited with 1064 nm

We also compared structures revealed by MPF and THG from TMRM labelled cardiomyocytes. TMRM is a lipid membrane permeable cationic dye. It accumulates in the compartments with high negative potential according to the Nernst equilibrium. This dye accumulates preferentially in energized mitochondria and endoplasmic reticulum, revealing them in the fluorescence image (Farkas, Wei et al. 1989). TMRM labelled cardiomyocytes were imaged with 1064 nm excitation pulses by simultaneously recording THG and MPF into two separate channels. The TMRM MPF raw data image and rendered volume are shown in Figure 7.1-3(a) and (e), respectively. The image reveals spherical structures organized in a grid like pattern that has a characteristic spatial arrangement of mitochondria in cardiomyocytes (Romashko, Marban et al. 1998). The 2-
D and 3-D THG images recorded simultaneously with fluorescence are shown in Figure 7.1-3(b) and (f), respectively. The THG images are similar to the previous images in Figure 7.1-2(b) and (d). Note that the images in Figure 7.1-3 have more than twice the magnification of Figure 7.1-2. The THG image has the same structural similarities as the fluorescence image. The intensity profile of the line in Figure 7.1-3(a) and (b) is shown Figure 7.1-3(c); as with Figure 7.1-1(c), there are similarities in the profiles of THG and MPF.

The same structural cross-correlation analysis that had been done with THG and NAD(P)H fluorescence was carried out on the images of THG and TMRM fluorescence. The co-localized 2-D and 3-D images are shown in Figure 7.1-3 (d) and (g), respectively. The uncorrelated THG is presented in blue and uncorrelated fluorescence is presented in green. The correlated part is shown in red in Figure 7.1-3(d) and (g), and presented separately in Figure 7.1-3(h). Only partial correlation between images of TMRM fluorescence and THG were found. The correlated image shows oval structures arranged in rows positioned along myofibrils. As viewed from the side of the three-dimensional image, the correlation appears in the upper part of the cardiomyocyte. The lower part of the cardiomyocyte, (part that is closest to the plated surface), is mostly dominated by the THG signal. The correlation provides evidence of the THG signal being generated in the mitochondria. More uncorrelated signals are observed between TMRM fluorescence and THG images than between NAD(P)H fluorescence and THG. The uncorrelated fluorescence appears possibly due to accumulation of TMRM not only in mitochondria, but also in the sarcoplasmic reticulum. The other two factors of THG dependence: (i) the membrane orientation with respect to the laser beam, and (ii) the differences in MPF and THG point spread functions, contribute to the amount of uncorrelated fluorescence signal in the co-localized image Figure 7.1-3(g). The uncorrelated THG can originate from membranous cellular structures other than mitochondria. The uncorrelated THG can also appear due to non-uniform staining that might have occurred in our sample, because less fluorescence was observed from the structures located closest to the plated side of the cardiomyocytes where access of dyes is more restricted. An extremely low TMRM concentration of 0.27 nM was used. Furthermore, the TMRM excitation may produce oxygen radicals leading to depolarization of connected groups of mitochondria.
Therefore, scanning of upper layers may depolarize the mitochondria in the lower layers of the cardiomyocytes prior to imaging. In addition to TMRM fluorescence, autofluorescence contributed to the MPF and correlation images as described in the previous section (Figure 7.1-2).

**Figure 7.1-3 Comparison of MPF and THG images of TMRM labelled cardiomyocyte excited with 1064 nm laser pulses.** (a) A 2-D optical section of TMRM MPF of a cardiomyocyte collected at 630-700 nm; (b) The same 2-D optical section simultaneously imaged with THG. The intensity profiles along the same line shown in (a and b) are plotted in (c), where the green diamonds indicate MPF and blue triangles show THG intensities. (d) The 2-D SCIA output for the slice shown in (a) and (b), red color indicates the correlated region while green and blue are the uncorrelated MPF and THG, respectively. (e) The rendered 3-D image of TMRM MPF. (f) The rendered THG image obtained simultaneously with the MPF. (g) Results of the SCIA where red represents the correlated signal, while green represents uncorrelated NAD(P)H MPF, and blue is the uncorrelated THG signal. (h) The same SCIA as (g) but showing only the correlated volume in red. The laser beam propagation is parallel to the z direction while the origin of the axis is placed at the perimeter of the cardiomyocyte. The size of a pixel is 0.12 µm. (See media file 15)

(d) THG and SHG

Three-dimensional images of cardiomyocytes were obtained with a simultaneous recording of THG and SHG into two separate detection channels. For imaging, the mode-locked Nd:glass laser with 1064 nm excitation was used. The SHG image of the cardiomyocyte is presented in Figure 7.1-4(a) with the rendered structures shown in Figure 7.1-4(c). Myofibrils of the cardiomyocyte are clearly visible. The THG image of a cardiomyocyte is presented in Figure 7.1-4(b) with the rendered volume shown in
Figure 7.1-4(d). The granular structures in the THG image appear along the myofibrils. The image closely resembles features of the THG image recorded with the Ti:Sapphire laser (Figure 7.1-1) and can be assigned primarily to mitochondria.

The image of co-localized SHG and THG structures is presented in Figure 7.1-4(e). The uncorrelated SHG structures are presented in green and uncorrelated THG structures are presented in blue. The correlated structures that appear very seldom in the image are shown in red. There is almost no visible correlation between structures revealed by SHG and THG (Figure 7.1-4(e)). However, it should be mentioned that there is weak correlation from the SHG and THG signals generated at the sarcolemma (see Figure 7.1-5) where low correlation accumulates over the whole image area to a significant value of Pearson’s coefficient but is thresholded in the pixel by pixel correlation analysis. This shows that SHG and THG are generated from completely different formations.

The co-localized image (Figure 7.1-4(e)) showed that oval shape structures revealed by THG were preferentially positioned at the maximal SHG intensity of sarcomeres. The SHG highlighted myofibrils appeared in a central part of the cardiomyocyte. In contrast, the mitochondria revealed by THG were localized mainly along the sides of the cell where the subsarcolemmal mitochondria dominate, rather than in between the myofibrils where the interfibrillar mitochondria are located. The interface orientation dependence of THG efficiency most likely resulted in this preferential visualization of subsarcolemmal mitochondria with lamellar membranes oriented perpendicular to the laser beam propagation.
Figure 7.1-4 Comparison of SHG and THG images of cardiomyocytes excited with 1064 nm laser pulses.
Panel (a) depicts a 2-D optical section of SHG of a cardiomyocyte; (b) The same 2-D optical section simultaneously imaged with THG. (c) The rendered 3-D image of SHG. (d) The rendered THG image obtained simultaneously with the SHG. (e) Results of the SCIA where red, which is barely visible, represents the correlated signal, while green represents uncorrelated SHG, and blue is the uncorrelated THG signal. (f) The same SCIA as (e) but showing only the minute correlated volume in red. The laser beam propagation is parallel to the z direction while the origin of the axis is placed at the perimeter of the cardiomyocyte. The size of a pixel is 0.24 µm. (See media file 16)

(e) Linear image cross-correlation at different depths of cardiomyocytes

Pixel-by-pixel analysis of SCIA used in the previous paragraphs renders one correlated and two uncorrelated 3-D structures that give visual information about morphology of the structure. The traditional image cross-correlation analysis of the optical sections obtained with two different signals is also useful to perform for quantitative comparison. Image cross-correlation analysis of optical sections at different depths of cardiomyocytes was carried out using Pearson’s coefficient. Pearson’s coefficient provides a measure of the linear relationship between two images. The two signals that are being generated within the same structure will result in a positive correlation. If, however, a structure generates one signal and not the other then the
correlation will be negative. Positive correlation also occurs for regions with background intensities in both images. By excluding pixels that have background intensity for both channels, we are able to eliminate correlation of the area around the cardiomyocyte as well as regions inside the cardiomyocyte that do not generate appreciable signal. The results of this analysis, shown in Figure 7.1-5, reveal that THG and MPF show mainly a positive correlation while THG and SHG appear negatively correlated. This agrees with the initial pixel-by-pixel structural cross-correlation of THG, SHG, and MPF. The cell to cell variations of structural cross-correlations revealed by different contrast mechanisms varies by about 10 to 15%. Figure 7.1-5 also shows that the correlation coefficient approaches zero in the middle of the cardiomyocyte, showing the presence of both correlated and anticorrelated structures in the image.

![Figure 7.1-5: Pearson's coefficient (r) analysis of optical slices at different depths of typical cardiomyocytes.](image)

THG and MPF are correlated ($r > 0$) through most depths of the sample, while THG and SHG are anticorrelated ($r < 0$) for much of the sample. In the middle of cardiomyocyte, the coefficient shifts closer to 0, where correlation is difficult to determine. The error is the coefficient is, on average 0.1.
Section 7.2: Understanding the mechanism for THG generation in mitochondria

The third harmonic can be generated as a result of any structural interface and depends on the differences in the refractive index of the two media and differences in the third order nonlinear susceptibility of the materials (Tsang 1995). In addition, the THG signal generation efficiency depends on the orientation of the interface with respect to the excitation beam (Muller, Squier et al. 1998a). The largest THG signal is generated when an interface is positioned perpendicular to the propagation of the beam.

There are many interfaces inside cardiomyocytes that could potentially allow for far field detection of THG signals. Besides mitochondria, trachea, outer membranes, sarcoplasmic reticulum, myofibrils and other membrane containing structures are good candidates for visualization by THG. This data indicates that a large part of the THG signal generated in the cardiomyocytes origins from mitochondria. In fact, a very low intensity THG signal reveals isotropic bands of myofibrils that appear as a striped pattern anticorrelating with SHG (see also (Chu, Chen et al. 2004)). Our analysis involves thresholding the THG. This eliminates very low intensity signals, which in turn enables discrimination of the mitochondria that generate substantially higher THG intensity. The main reasons for THG enhancement in mitochondria most likely appears due to the multilamellar structure of the densely folded cristae, and outer and inner membrane in the mitochondria. Tsang in his work showed this effect for dielectric multilayer structures (Tsang 1995). In multilayer structures, in addition to the orientation of the membranes with respect to the laser propagation direction, the spacing between layers plays a significant role in the THG process. THG wave fronts generated at each membrane may interfere constructively or destructively depending on the distance between the membranes. It is therefore not surprising that some mitochondria observed in NAD(P)H or TMRM labelled MPF image do not appear in THG. The most intense THG comes from subsarcolemmal mitochondria that are along the sides of the cardiomyocyte. The mitochondria along the sides have their cristae oriented approximately perpendicular to the laser beam propagation resulting in significant enhancement of THG, while the mitochondria, which are situated between the myofibrils, the interfibrillar mitochondria,
are likely to have a less favourable cristae orientation or spatial periodicity. Although there are no known structural differences between subsarcolemmal and interfibrillar mitochondria (Skarka and Ostadal 2002), some may still exist that could also affect the THG efficiency. In any case, the biochemical properties of subsarcolemmal and interfibrillar mitochondria from rat cardiac muscle appear to differ significantly (Palmer, Tandler et al. 1977). It is very likely therefore that we are experiencing the most significant THG enhancement from the multilamellar structure of subsarcolemmal mitochondria. Therefore THG appears to be a practical tool for mitochondrial visualization. THG does not require staining, which can influence structural organization of mitochondria, ion distribution, and free radical generation during laser scanning.

Section 7.3: Third harmonic generation in Drosophila samples

In examining Drosophila larval muscle with ~800 nm excitation, it was found that significant THG was generated both in the trachea interconnected with the muscle as well as in the muscle region itself (see Figure 7.3-1) (Greenhalgh, Cisek et al. 2006). The origin of THG from within the trachea was determined based on structure, location and comparison with white light images of the same regions. The origin of THG from within the muscle was not determined at this wavelength, although it was presumed to be partially from the mitochondria along the edges of the myocyte, the more uniform distribution from the sarcolemma and possibly from the I-bands.

Figure 7.3-1: Drosophila larval muscle imaged with ~800 nm excitation. Drosophila myocyte (unlabelled) imaged at 800 nm, where left is the imaged with MPF, centre with SHG and the right is imaged with THG.
Further investigations were carried out at an excitation of 1040 nm. The images revealed an increase in THG, in both adult and larval muscle. Figure 7.3-2 shows the merged image of SHG and THG for an adult IFM imaged with 1040 nm excitation. There is some THG between the rows of myofibril revealed by SHG, likely originating from the mitochondria. Some THG signal is generated in the isotropic bands of the sarcomeres. THG has been previously observed from the isotropic band of sarcomeres in myocytes (Chu, Chen et al. 2004). The presence of THG from the isotropic region was not observed in all cases. Since the THG is dependent on the layer spacings and thicknesses, it follows that in myocytes, THG efficiency may depend on the sarcomere size and contraction state of the myocyte.

![Figure 7.3-2: Combined SHG-THG image of adult Drosophila indirect flight muscle](image)

*Figure 7.3-2: Combined SHG-THG image of adult Drosophila indirect flight muscle*

*Drosophila* myocytes imaged at 1040 nm, (green SHG, purple THG)


**Section 7.4: Conclusion**

The development of a multicontrast MPF, SHG and THG microscope has enabled simultaneous visualization of different subcellular structures, as well as spatial correlation and reconstruction into 3-D images. The new structural cross-correlation analysis helped to construct the correlated and uncorrelated 3-D images and showed if the signals originate from the same or two different structures. By applying multicontrast MPF, SHG, and THG microscopy for imaging isolated live cardiomyocytes, we were able to assess the structural origin of THG and SHG signals. A large part of the THG signal was generated from the mitochondria possibly due to the signal enhancement from the multilayer arrangement of the cristae. The SHG signal was generated from anisotropic bands of myofibrils, consistent with the literature (Chu, Chen et al. 2004) and the findings of Chapter V. This investigation has shown that a simultaneous imaging technique offers new possibilities in obtaining complementary information on cellular structures and colocalization between cellular organelles.
Chapter VIII - Functional Dynamics of Mitochondrial Activity in Myocytes Observed with MPF and THG Microscopy

Section 8.1: Introduction

With the understanding gained in the previous chapter on the origin of third harmonic generation from within myocytes, this chapter focuses on mitochondrial functional activity and the role of mitochondria in contractility dynamics. Time dependent intensity changes of THG and MPF signal observed in mitochondria are presented, with a focus on explaining the underlying physical and physiological processes reflected in these changes. An understanding of the intensity changes could be valuable in characterizing mitochondrial disorders or in providing insight into other diseases that mitochondria play a role in. In investigating these time dependent changes, the importance of nonlinear imaging techniques is also exposed. Third harmonic generation imaging is shown to be particularly attractive for non-invasive studies of bioenergetics in mitochondria, enabling investigations without labelling.

Mitochondrial function of ATP production is well understood, however the dynamics of ATP production rates i.e. how the ATP production is regulated and how it depends on the cell activity as well as the interaction with other organelles inside the cell is not fully understood. Recent studies, have also attempted to elucidate the additional roles that mitochondria may have, aside from the ATP production (Kaasik, Joubert et al. 2004; Anmann, Eimre et al. 2005). Several imaging studies have investigated the relationship between the intensity fluctuations observed in fluorescence (commonly referred to as flicker) and the physiological activity of mitochondria (Loew, Tuft et al. 1993; Duchen, Leyssens et al. 1998; Vergun, Votyakova et al. 2003; O'Reilly, Fogarty et al. 2004; Vergun and Reynolds 2004). This chapter presents findings from dynamic investigations of isolated cardiomyocytes imaged with MPF and THG at an excitation wavelength of 1064 nm. The fluorescence flickering in mitochondria were studied with MPF microscopy in TMRM labelled rat cardiomyocytes. The fluorescence intensity fluctuations were recorded by time lapse imaging for a prolonged duration of 10 to 15 minutes.
Furthermore, intensity fluctuations in the third harmonic from labelled and unlabelled myocytes were simultaneously recorded and compared to changes observed with MPF imaging. The study of these fluctuations was carried out with dynamic image correlation analysis (DICA) as described in Chapter IV. By analysing the fluctuations of one pixel in an image with the other pixels within the same image, or by comparing the fluctuation of one pixel with the corresponding pixel from an image taken with a different contrast mechanism, synchronized networks of mitochondria have been demonstrated. Fluorescence correlation spectroscopy of the intensity fluctuations showed the differences in time scales of the flickering dynamics for various networks. Additionally, cross-correlations of the fluorescence fluctuations revealed phase shifts between the intensity fluctuations of neighbouring networks showing propagation of the flicker signal across the myocyte. The spatio-temporal investigation of these active networks is very beneficial for further understanding of mitochondrial physiology and bioenergetics during muscle contraction.

The observed multi-photon excitation fluorescence in TMRM labelled cardiomyocytes reveals fluorescence flickering demonstrating the MPF imaging as a useful tool for studying the activity of mitochondria. However, in exposing differences in the third harmonic generation response before and after labelling cardiomyocytes with TMRM, a mitochondrial staining dye, this chapter also highlights the disadvantages of adding labels to a biological system and demonstrates the need for less invasive staining-free imaging techniques. Because the third harmonic is generated within the mitochondria and without the addition of labels, (see Chapter VII), THG could serve as a valuable contrast mechanism for dynamic imaging of mitochondrial activity. It has been suggested, however, that observed fluctuations are only an artefact of the fluorescing dye interacting with the sample (Blinova 2004). In contrast, our studies revealed THG dynamics in unlabelled cells showing that flickering is not a staining artefact and relates to the functional activity of mitochondria. The non-invasive studies of flicker in mitochondria with THG microscopy present a new method for investigating physiological activity of \textit{in vivo} myocytes.
Section 8.2: Mitochondrial flickering dynamics in MPF

The characteristic intensity fluctuations of the MPF signal, the flickering, at individual pixels could be observed when a cardiomyocyte is imaged for prolonged period of several minutes. Localized flickering kinetics of MPF were related to the biological function of mitochondria in cardiomyocytes (Duchen, Leyssens et al. 1998); therefore, the active areas larger than one pixel could exhibit flickering and be functionally synchronized. Due to permeability through membranes and cationic nature, the dye redistributes according to the Nernst potential across the membranes. At low concentration limit, which is the case in our experiments, the fluctuations in the dye concentration appearing due to depolarization and/or physiological activity of the mitochondria lead to the observed fluctuations in fluorescence intensity (Duchen, Surin and Jacobson, 2003). The presence of flickering activity in the sample can be visualized by calculating the standard deviation of the intensity fluctuation at each pixel, as discussed in Chapter IV. Figure 8.2-1 (a) and (b) shows the MPF image of a cardiomyocyte at the initial time and the standard deviation map, respectively. Figure 8.2-1 (b) reveals several active regions within the sample where the standard deviation is substantially higher than in other areas inside the myocyte. The outside edges of the cardiomyocyte, where subsarcolemmal mitochondria are situated, show particularly large standard deviations.

Figure 8.2-1: Typical cardiomyocyte image in MPF and standard deviation of pixels’ intensity fluctuations.
The panel (a) shows the unprocessed MPF image of a cardiomyocyte at time 0s. The image (b) shows the standard deviation map of the residuals obtained from the exponential fit to the intensity fluctuation traces (filtering for the photobleaching) for each pixel. Brighter regions
indicate more intense fluctuations. By dividing image (b) by image (a) we find that active areas of the filtered image fluctuate up to ~10%. For comparison, non-active regions within the sample fluctuate by no more than ~1%. The scale bar is 10 µm. For details on constructing the standard deviation map see the text and Chapter IV.

Using dynamic image correlation analysis, the mitochondrial flickering can be further examined for the presence of networks, where a network is considered to be comprised of pixels which have a minimum Pearson’s Coefficient of +0.7 with the reference pixel at zero time delay between the two. More details on DICA analysis method can be found in Chapter IV. Results from the DICA analysis demonstrate several active networks of mitochondria. Figure 8.2-2 examines the pixels within one such network. Figure 8.2-2(a) shows the network in white superimposed on the standard deviation image from the Figure 8.2-1(b) to provide some perspective. Unfiltered data for several pixels is shown in Figure 8.2-2(b). The MPF intensity evolution traces contain bleaching as well as flickering signature. Before performing correlation analysis of flickering, the traces were filtered from photobleaching kinetics by performing exponential fitting and subtracting the fitted exponential decays from the data. The filtered data for the reference pixel is shown in Figure 8.2-2(c). To examine the dynamics within this network, comparisons between several pixels in and outside of the network with respect to a pixel within the network were made using correlation analysis, similar to the procedure used in fluorescence correlation spectroscopy (FCS) (Wiseman, Capani et al. 2002). This comparison was done on the filtered data to eliminate correlating the low frequency component related to the bleaching kinetics as described in Chapter III. The results of this comparison are shown in Figure 8.2-2(d). Pixel 2 is highly correlated to the reference pixel 1, and is in fact a part of the same network. Pixel 3, which is from a different network than the reference pixel 1 appears highly anticorrelated. The noise from within the myocyte, but outside of the active area (pixel 4) does not correlate with the reference pixel 1.
Figure 8.2-2: MPF intensity flickering analysis of pixels inside and outside of the network in a cardiomyocyte.
(a) A network of pixels with correlation coefficients greater than +0.70, is superimposed on top of the standard deviation image from Figure 8.2-1(b). (b) The MPF intensity evolution of several pixels before filtering, the pixel locations are indicated in (a); (c) the MPF intensity evolution for pixel 1 after filtering of the low frequency bleaching component; (d) auto and cross-correlation of pixel 1 with the pixels (shown in (a)) for various time delays in seconds with an error in coefficient of ±0.08.

For this sample, the dynamic image correlation analysis (DICA) reveals at least 5 networks presented in Figure 8.2-3(a)–(e) with the overlap of all of the networks displayed in Figure 8.2-3(f). Although the networks are quite distinct, due to the high threshold value of the Pearson’s coefficient (the threshold value that denotes whether a pixel is part of a network) and nature of the DICA analysis method, some pixels can be found to belong to two networks. It is interesting to note that some networks span across the entire myocyte, while others localize mainly along a row of mitochondria, corresponding well with the previous results (Brady, Elmore et al. 2004). Interestingly,
the structures revealed by network (c), have similar shape to the unidentified, autofluorescing structures seen in Figure 7.1-2.

The corresponding intensity fluctuation traces for the five networks is presented in Figure 8.2-3(g). It can be clearly seen that the network (a) closely follows the fluctuations of network (b), including a few pixels which overlap, while network (d) corresponds well to network (e). The related networks (a) and (b) exhibit opposite kinetics to the networks (d) and (e). The MPF fluctuation traces of the networks were autocorrelated to reveal the temporal range of the flickering (Figure 8.2-3(h)). The flickering had approximately half of the period around 90s. It should be noted however that the transition between low and high fluorescing state occurred much faster, generally in less than 1.0 seconds as observed from the non-averaged, single frame data. The inner networks seem to have slightly larger periodicity than the outer networks.

The MPF fluctuation trace of network (a) from Figure 8.2-3(g) was cross correlated to each of the other networks; the results are shown in Figure 8.2-3(i). One can see from the correlations that networks (a) and (e) on the opposite edges of the sample are anticorrelated. Additionally, inner networks (b) and (d) match closely with the outer networks (a) and (e), respectively, following a similar correlation decay. Only small differences in the correlation decay and shift in the correlation maxima and minima delay times for the matching inner and outer network can be observed. The differences are indicative of flickering phase delays between the matching networks that might be due to slight dissimilarities in response between interfibrillar and subsarcolemmal mitochondria, which are known to have distinct biochemical properties (Palmer, Tandler et al. 1985).
Figure 8.2-3: MPF flickering activity networks in cardiomyocytes
Each (a) to (e) image represents a separate flickering network in the same cardiomyocyte overlaid on top of the standard deviation image for perspective. Initially, a reference pixel was selected and the Pearson’s Coefficient was calculated for each pixel in the entire image area. A network is considered when a significant number of pixels in the image area have a Pearson’s coefficient $>+0.7$ with the reference pixel. A new reference pixel was randomly selected from pixels not within the network. Panel (f) shows combined image of all five networks. (g) MPF filtered flickering data for each of the networks shown in (a-e); offset intensity values are indicated in the legend. (h) Autocorrelation for each network, where the slower dynamics in the inner networks (b) and (d) are clearly visible. (i) Cross correlation spectrum of flickering networks. Network (a) was used as the reference network. The y-axis in the correlation graphs is the Pearson’s correlation coefficient and the x-axis is the delay in seconds. The estimated error in the correlation coefficient is ±0.08. Scale bar represents 10µm. (See media files 17 and 18)
The dynamics of network (c) are quite different from those of the other networks. In looking at the dynamics of network (c), as observed in Figure 8.2-3(g), the presence of both a slow component, which is similar to the dynamics of the other networks, and a faster component can be determined. Figure 8.2-4(a) shows the cross-correlation of network (c) with each of the other 4 networks. The cross-correlation shows the highest correlation with networks (a) and (b) at a shift of approximately -50s, while the maximum correlation for the networks (d) and (e) occurs around +50s. The slow kinetic of the network (c) is phase shifted with respect to the other networks. The phase shift of network (c) fluctuations might be related to the wave like propagation of THG intensity front described in section 8-4. The fast dynamics seen in network (c) reduce the cross-correlation amplitude of the slow component.

To verify that the faster dynamics did not reflect the noise, autocorrelation of the network (c) and the autocorrelation of a pixel from within the cardiomyocyte but outside of any of the networks, representing the noise inside the sample, are shown in Figure 8.2-4(b). The autocorrelation of the noise decays slightly faster than the fast component of the autocorrelation from the network (c), indicating the presence of fast flickering kinetics distinct from the noise. The cross-correlation of these two fluctuation traces is also presented in the Figure 8.2-4(b). One can see from the cross-correlation that network (c) does not exhibit similar dynamics to the noise. The dynamics of the network (c), therefore, reflects physiological activity that contains faster component than the flickering dynamics in the other networks.
Figure 8.2-4: Correlation analysis of the network (c) and other networks as well as with the noise from within the myocyte area
The autocorrelation of network (c) and cross-correlation with networks (a), (b), (d) and (e) are presented in panel (a); the network notation is the same as in Figure 8.2-3. The autocorrelation of a pixel from within the myocyte that is not part of any network and as such represents noise, and the cross correlation of flickering in network (c) and the noise are presented in panel (b). The estimated error in the correlation coefficient is ±0.08

The observed fluorescence intensity changes seen in each of the networks were analysed to verify if the changes were due to physiological effects and not due to imaging artefacts. The anticorrelating symmetry between networks (a) and (e) required particular
attention. Small axial or lateral structural movements of cardiomyocyte could produce a similar effect. Initially, images were examined visually to verify that no obvious shift occurred in the sample. The anticorrelated networks appear distanced from each other (Figure 8.2-3(f)) indicating that lateral shifts did not occur. More quantitative examinations were also performed. Subtraction of images before and after the flickering transition did not show the sign change of intensity at adjacent pixels, confirming that there were no lateral shifts. Similarly slices before and after the transition were normalized and subtracted to probe for possible axial shifts. If the curved outer membrane of a mitochondrion were to translate out of the focal plane, the image area occupied by the organelle would change. This effect was not observed indicating that axial shifts on the order of the PSF did not occur. The fact that the inner networks are broadly scattered across the myocyte area, and pixels with temporally different flickering kinetics are present inside the myocyte, provides the evidence that flickering cannot be explained due to simple lateral or axial shifts. The inhomogeneous nanocontractions of sarcomeres described in Chapter VI could induce a flicker like effect in THG. However, the time scales for the nanocontractions and flickering are very different, indicating that flickering did not occur due to the movement artefacts.

Although mitochondria association into networks have been suggested by others (Amchenkova, Bakeeva et al. 1988; Diaz, Falchi et al. 2000), this study convincingly demonstrates the presence of several distinct networks within a cardiomyocyte. Moreover, the networks are revealed based on the dynamic flickering data that relates to physiological activity inside the myocyte. The synchronization of mitochondria into networks, agrees well with other work on cardiomyocytes (Zorov, Filburn et al. 2000) although it contradicts work on smooth muscle (O'Reilly, Fogarty et al. 2003). The networks will likely play an important role in future investigations of the physiological mechanisms of mitochondrial depolarization as well as the bioenergetics of muscle contraction.

The mitochondrial membrane potential ($\Delta \Psi$) and proton gradient across mitochondrial membranes ($\Delta pH$) are important in cellular function by providing the necessary energy for ATP production. The mitochondrial membrane potential plays a determining factor in the uptake of calcium ions, positively charged dye molecules, such
as TMRM, as well as influences proton and phosphate currents. $\Delta \Psi$ collapses during mitochondrial depolarization. The depolarization introduces the TMRM concentration fluctuations in mitochondria, which manifests in the fluorescence flickering observed in our studies.

The diverse roles of mitochondria in cellular function have stimulated interest in investigating mitochondrial depolarizations with fluorescence microscopy. A complete understanding of the fluorescence signal is imperative in understanding cellular function and bioenergetics. Experiments on TMRE labelled cardiomyocytes relate changes in fluorescence intensity caused by transient mitochondrial depolarisations to the release of calcium from the sarcoplasmic reticulum (Duchen, Leyssens et al. 1998). Investigations in fluorescence intensity changes of imaged isolated brain mitochondria suggested that calcium ions trigger the fluctuations in the mitochondria membrane potential, while adenine nucleotides modulate the fluctuations (Vergun and Reynolds 2004).

The observed fluorescence intensity fluctuations in mitochondrial networks could also occur due to reactive oxygen species (ROS) produced from the excitation of label dye and this increase in ROS production subsequently would lead to a depolarization of mitochondria membrane potential and an increase in the mitochondrial ROS production. This process has been identified as ROS-induced ROS-release (RIRR) (Zorov, Filburn et al. 2000). In previous results, the RIRR induced loss of $\Delta \Psi$ led to complete loss of fluorescence signal (Zorov, Filburn et al. 2000). In our work, however, the sensitivity of using two-photon excitation enabled us to visualize mitochondria after $\Delta \Psi$ depolarization and then after recovery. The use of a two-photon excitation process allowed for imaging over a long duration, capturing several depolarization/repolarization periods. Although, the RIRR could play a role in the observed depolarization of mitochondria, the flickering in THG of unlabeled cardiomyocytes presented in the following section indicates that ROS are not the major factor inducing depolarization.

Small differences in maxima and minima delay times for the matching inner and outer network are indicative of either phase delays between the networks (possibly on a faster time scale) or slight differences in response between interfibrillar and subsarcolemmal mitochondria which differ in biochemical properties (Palmer, Tandler et
Kinetics of MPF could be directly related to the biological function of cardiomyocytes; therefore, the active areas could be functionally synchronized. The anticorrelation observed between the two outside networks is indicative of an induced wave propagating along the surface of the cardiomyocyte where the subsarcolemmal mitochondria are located. The flickering of the inner network indicates that waves also propagate across the inner volume of the cardiomyocyte. The apparent scattered islands of the inner networks represent the areas where networks cross the focal plane of the image. If the 3-D structure of the myocyte is pictured, the networks constitute a three-dimensional web spanning the entire volume of the myocyte. Therefore, the wave-like propagation across the myocyte has to be envisioned in the three-dimensional context.

**Section 8.3: Comparison of MPF and THG fluctuation dynamics**

The observed flickering kinetics in MPF and observed mitochondrial networks present a new question as to whether the same networks can be revealed in the third harmonic. Although the signal to noise ratio of THG was too low for flickering correlation analysis of single pixels to be performed, the observed MPF flickering of an entire network could be used as a basis to compare the MPF response with that of THG.

By summing all the intensity fluctuation traces of pixels belonging to a network and calculating the averaged intensity fluctuation trace, rather than comparing individual pixels, the signal to noise ratio is improved, and comparisons can be made between the intensity fluctuations of a network in MPF and the same spatial region in THG. The averaged intensity fluctuations of the network (a) for THG and MPF signals are shown in Figure 8.2-4(f). Here image processing enables a better comparison between the two signals; analysis of the non-processed data shows the fluctuations in MPF to be significantly larger than those observed in THG. Panels (a) to (e) show the THG autocorrelations and cross-correlations with MPF for the average fluctuations of corresponding networks (the same network notation is used as in Figure 8.2-3(a)–(e)). The networks (a) (b) and (e) show appreciable cross-correlation between THG and MPF signal fluctuations, while cross-correlations of networks (c) and (d) are not distinct. The autocorrelation of networks (c) and (d) drops rapidly due to the noise in the third harmonic signal. Network (d) however still exhibits the expected cross-correlation trends.
with the MPF network. Network (c), however does not show significant cross correlation with the MPF signal.

As mentioned previously, network (c) differs from the other networks in the cardiomyocyte. The THG and MPF signals do not correlate in the network (c) which indicates either the low signal to noise ratio of THG masking the correlation of the dynamics, or absence of similar kinetics in both data sets. The network (c) may correspond to autofluorescing structures revealed in unlabeled cardiomyocytes (see Figure 7.1-2), and may not originate from TMRM labelled mitochondria. Therefore, THG, although spatially correlated with these structures, may not exhibit the same fluctuation dynamics. Higher signal to noise ratio is required to determine the relation between THG and MPF fluctuations in the network (c). Since the slow dynamics of networks (a), (b), (d) and (e) are observed in both MPF and THG and the slow dynamics of network (c) are not observed in THG, it is evident that noise at least partially contributes to the lack of correlation.

The THG intensity fluctuations of networks (a), (b), (d) and (e) have some similarities to the flickering kinetics in MPF. The THG autocorrelations of the four networks have similar temporal characteristics as autocorrelations of MPF (compare Figures 8.2-5 and 8.2-3). The period of the fluctuations is around 170s. The cross-correlations for all the networks reach an approximate value of 0.4. Even though the dynamics are similar, the lower coefficient could indicate temporal differences between the two responses, the existence of additional underlying dynamics in the third harmonic not present in MPF, or dynamics in MPF not revealed by THG. The presence of networks in THG provides the new avenue for studying bioenergetics of muscle contraction and using THG for non-invasive studies of functional dynamics in unlabeled myocytes.
Figure 8.3-1: Comparison of MPF and THG flickering dynamics of networks in cardiomyocytes

Corresponding images of the networks in MPF can be seen in Figure 8.2-3. Panels (a)-(e) show the autocorrelations of THG fluctuations (squares) and cross-correlations between MPF and THG (diamonds) for the averaged fluctuations over the area of corresponding networks (a) to (e). The example of THG and MPF traces (panel f) obtained by averaging the fluctuation traces from all pixels constituting the network (a). Note that the THG signal has been processed and as such values have been modified from their original values. The correlation coefficients have an estimated error of 0.2.
To effectively use THG as a monitor for functional dynamics in mitochondria, the origin of the changes in THG signal strength must be considered. The THG intensity changes might follow the MPF intensity changes caused by changes in the concentration of TMRM dye, which accumulates in mitochondria according to the Nernst potential. The effect will depend on the hyperpolarizability of the TMRM molecule. The TMRM could also affect the THG, causing an increase in signal through a multi-photon resonant enhancement process. Although changes in TMRM concentration might influence the THG intensity, it will be demonstrated in the next section that fluctuation dynamics in THG can also be observed in unlabeled cardiomyocytes, indicative of THG fluctuations originating at least partially from something other than TMRM concentration changes.

In the previous chapter, it was suggested that the multilayer structure of the inner cristae enhances the third harmonic. Since evidence suggests that mechanical swelling of mitochondria plays a role in contractility (Kaasik, Joubert et al. 2004), it is possible that altered spacing in the cristae changes interference conditions for signals emitted from the different membranes leading to a modulation of THG intensity in the far field. Therefore, functional activity of mitochondria may induce the structural changes (Anmann, Eimre et al. 2005) which in turn affect the third harmonic generation. During state III, mitochondria cristae and inner matrix each have an approximate thickness of 50 nm. The index of refraction differs in these two regions by about 0.15, and as a result, the mitochondria can be seen as a multilayer structure, with alternating high and low indices (Thar, Kuhl, 2004). The difference in refractive index is due to the difference in protein and metal ion concentration between the two regions. The total THG response from this type of structure would be largely due constructive interference of the THG generated as a result of differences in the index values. In state IV, however, the multilayer structure changes. Protein concentration equalizes in the two regions, resulting in equal indices of refraction. The structure, however also changes; the cristae are now thin structures of about 10 nm while the matrix has widened to approximately 90 nm (Thar, Kuhl, 2004). Under these conditions there is still a possibility of multilayer enhancement of THG; however the THG response would only be due to the alternating changes in nonlinear susceptibility and not index changes.
Not only could the structure of the mitochondria influence the THG response, THG is also sensitive to changes in electric field, and as such is a good candidate for monitoring changes in transmembrane potential. It has already been demonstrated that forcing changes in intercellular calcium results in changes to the average THG signal (Canioni et al 2001). Continuing on this work, the same group provided insight into the correlation between the changes in THG and MPF signals by imaging in vivo glial cells (Barille et al 2001). It is most likely that both changes: the transmembrane potential and the structural change of mitochondria induce the THG signal changes in cardiomyocytes. The possibility of monitoring transmembrane potential changes without labelling the cell could open new perspectives for non-invasive research in bioenergetics and contractility, as well as neurophysiology.

To estimate the potential THG response under the different conditions, the theory of focused Gaussian beams can be used (Boyd 2003). Following this theory, the power of the third harmonic beam is given by (Barad, Eisenberg et al. 1997):

\[ P_{3\omega} = k_{3\omega}^{3} \left( \frac{4\pi}{n_{3\omega}^{2}n_{\omega}^{2}c} \right)^{2} P_{\omega}^{3} |J|^{2} \]  

with

\[ J = \int_{z'_{L}}^{z'_{R}} \chi(z') e^{i \Delta k b z'} \left( 1 + 2iz' \right)^{-2} dz' \]  

where \( \chi \) is the third order nonlinear susceptibility, \( n_{\omega} \) and \( n_{3\omega} \) are the refractive indices at the fundamental and third harmonic wavelengths, \( k_{\omega} \) and \( k_{3\omega} \) are the wavenumbers of the fundamental and third harmonic beams and \( b \) is the confocal parameter. Following the work by Schins et al, the integral \( J \) can be expanded to account for multilayer structures as follows:

\[ J = \sum_{j=1}^{N} e^{i\Delta \phi_{j}} \int_{q_{j}}^{r_{j}} \chi^{(3)} e^{i \Delta k_{j} z} \left( 1 + \frac{iz}{z_{R,j}} \right)^{-2} dz, \]  

where

\[ \frac{q_{j+1}}{n_{j+1}(\omega_{j})} = \frac{r_{j}}{n_{j}(\omega_{j})}, \]  

\[ r_{j} - q_{j} = \text{Layer Thickness}, \]  

\[ \Delta \phi_{j+1} - \Delta \phi_{j} = \Delta k_{j} r_{j} - \Delta k_{j+1} q_{j+1} \]  

8.3-3
Note here that the efficiency of the third harmonic is sensitive to the position of the interface relative to the beam waist. This means that small shifts of the myocyte in and out of focus would alter the third harmonic response. Since out of focal plane myocyte movements were not observed in MPF, and the sample was imaged simultaneously in THG, it can be assumed that the THG response is not from shifts in the myocyte.

Considering mitochondria in state IV in which there is no change in refractive index from the interface, a single thin infolding of the cristae is surrounded on either side by the matrix. Using the above theory, this physical description is similar to a thin film in a homogenous media and the power of the generated third harmonic beam is related to the change in susceptibility as follows (Barad, Eisenberg et al. 1997):

$$P_{3\omega} \propto \delta \chi^2 \left( \frac{t}{b} \right)^2 \left( 1 + 4 \frac{z_{\omega}^2}{b^2} \right)^{-2} P_{\omega}^3$$

Following estimates of $\chi^3$ for proteins and polypeptides and those of ionic solutions, similar in susceptibility to what would be in the mitochondria (Debarre, Beaurepaire, 2007), the difference in $\chi^3$ would be approximately 0.05, leading to little third harmonic power being generated. Taking into consideration the multilayer structure however, constructive interference as a result of each interface would increase the signal significantly.

When considering mitochondria in state III, there is not only a change in susceptibility, but also there is a significant change in the index of refraction. The width of each region is now the same, so the system no longer represents a thin film in an isotropic media. Here, the power of the third harmonic due to far field detection due to an interface would be different than the previous case, resulting in changes of the observed THG response.

Although these two cases represent specific states of mitochondria, one can imagine that ion flux induced structural variations within the mitochondria would play a role in the efficiency of the third harmonic. Changes in ions concentrations would also alter the THG power generation. Moreover, because the transmembrane potential and the mitochondria structure changes would vary in time, the THG response would also change in time, giving the type of fluctuations that we have observed. Further investigations
beyond the scope of this research would be necessary to quantify the mechanism for THG fluctuations in mitochondria.

**Section 8.4: Wave-like THG intensity propagation across cardiomyocyte**

In examining time series imaging data of myocytes, wave-like propagation of THG intensity across the cardiomyocyte was observed. The high THG intensity initially appeared at one edge of the cardiomyocyte, likely generated from a networked row of mitochondria. The high intensity line propagated across the cardiomyocyte with a direction perpendicular to the myofibrils. Figure 8.4-1 shows the intensity evolution over time for three different regions of a cardiomyocyte. Around 100 seconds, the peak of the third harmonic occurred in region 1. Then, at ~200s, the THG peak has shifted to region 2. The peak has shifted to the third region by ~300s. The propagation of this THG high intensity front arose spontaneously and propagated across the cardiomyocyte with a speed of less than 1 μm/s. The origin of THG high intensity front can occur due to changes in transmembrane potential, or due to movement of cardiomyocyte area in and out of the plane of focus. Both phenomena could be related and appear at the same time.

THG is known to be sensitive to changes in transmembrane potential and are closely associated with calcium currents across the neuronal membrane (Barille, Canioni *et al.* 2001; Canioni, Rivet *et al.* 2001). Calcium waves can propagate within cardiomyocytes both in the transverse and longitudinal directions. Therefore calcium related activity is most probably reflected in the changes of THG intensity. Calcium waves are known to propagate with very slow speeds (nm/second) to very fast speeds (~100cm/second), the observed dynamics represent slow to ultra slow wave propagation (Jaffe, Creton. 1998). The current time step resolution does not permit us to verify that faster reoccurring waves are not present in the sample. The presented THG intensity front propagation demonstrated, for the first time, the possibility of following signal propagation dynamics with THG contrast mechanism. This lays perspectives for further research of bioenergetics and signal transduction in cardiomyocytes with THG microscopy.
Figure 8.4-1: Wave-like motion of a high intensity THG front
Image (a) shows three regions across a myocyte imaged with THG. Image (b) shows the intensity evolution of each of these regions, (error in intensity is about 20%). Intense THG appears in region 1 at the earliest time. As the THG wave propagates across the sample, the most intense THG region moves appearing in region 2 after some delay, and then, at an even later time, in region 3. The y-axis is offset by 70 %. (See media file 20).

Section 8.5: Effect of TMRM labelling on mitochondrial dynamics
Cardiomyocyte flickering dynamics are investigated before and after labelling. The influence of dyes on the flickering kinetics was studied in order to better understand the origin of flickering in cardiomyocytes as well as investigate whether or not TMRM labelling induces or influences the flickering. The THG image of an unlabelled cardiomyocyte sample and map of standard deviations is presented in Figure 8.5-1(a) and (b), respectively. Very intense flickering is observed in the THG images; flickering can be observed without the use of labels, and is therefore intrinsic to the sample and not an artefact. Labelling of cardiomyocytes with TMRM dye affected the flickering. Figure 8.5-1(c) shows the THG image of a cardiomyocyte after labelling, while Figure 8.5-1(d) shows the standard deviation map for this series. For comparison, Figure 8.5-1(e) and (f) shows the image and standard deviation map of MPF, after loading the myocyte with TMRM. The flickering in THG and MPF can be seen. However, it is easily observed
that after labelling, flickering of THG in the cardiomyocyte is reduced. The highly active areas revealed by the standard deviation maps (intense spots in the middle of the cardiomyocyte in Figure 8.5-1(b)) disappear in Figure 8.5-1(d) and the standard deviation map becomes more homogeneous with lower standard deviation values. Some active areas in the MPF standard deviation map (Figure 8.5-1(f)) could still be observed.

Long standing debate on the origin of flickering formed the opinion that radical species are produced due to the excitation of label dye that leads to depolarization of mitochondria. This experiment demonstrates strong evidence that without a labelling dye, and at the wavelength of excitation, where there is no autofluorescence observed, active flickering of mitochondria is still present, suggesting the existence of an additional mechanism for depolarization of mitochondria. Although we can not exclude that non-resonant generation of the third harmonic does not produce reactive oxygen species (ROS), the amount of the reactive oxygen in such cases would be substantially reduced leading to less flickering in unlabeled cardiomyocytes. Our observations however show the opposite result.

![Figure 8.5-1: Effect of TMRM labelling on the flickering kinetics in a cardiomyocyte.](image)

THG image of cardiomyocyte and standard deviation map obtained with THG before addition of TMRM ((a) and (b), respectively). (c) and (d) show the THG image and standard deviation map after the addition of TMRM. Panel (e) shows the TMRM fluorescence image taken simultaneously with the image in (c), and (f) is the standard deviation map for this MPF series. The myocyte moved slightly during addition of TMRM, however flickering was present at different focal planes of the myocyte, therefore the standard deviations of THG can be compared.
Section 8.6: Effect of inhibitors on THG and MPF responses

To test if flickering can be hindered with an uncoupler, ~1 μM of FCCP was added to the sample. FCCP initiated hypercontraction shortly after the addition to the sample. Figure 8.6-1(a) and (b) shows the THG and MPF images just after the addition of FCCP. The standard deviation map is shown in Figure 8.6-1(c) for THG and Figure 8.6-1(d) for MPF. Functional activity is clearly visible in MPF, while THG shows variation over the entire scanned area, with only slightly higher variations in the myocyte regions. Addition of the uncoupler induced structural changes as seen in Figure 8.6-1(e) (THG) and Figure 8.6-1(f) (MPF). The standard deviations are again presented in Figure 8.6-1(g) and (h) respectively. The flickering is no longer distinguishable from the noise in the standard deviation map and examining the time series visually slice by slice, shows no significant deviations either. Loss of flickering was followed by the structural changes leading to loosening of the whole structure of the cardiomyocyte. The addition of FCCP also affected the strength of the third harmonic generation, indicative of the structural changes in the mitochondria cristae (see Figure 8.6-1(e)), although, slight movement of the whole myocyte may have affected the THG intensity as well. In the final step cardiomyocytes evolved into hypercontraction.

The addition of FCCP diminished the flickering in both THG and MPF, suggesting that dynamics of flickering is, at least in part, related to the transmembrane potential and depolarization of mitochondria. The sensitivity of the THG to the mitochondrial uncoupler provides additional evidence that MPF and THG flickering is related to the physiological activity of mitochondria. Therefore, THG can potentially be explored as a non-invasive imaging tool for dynamic investigations of mitochondrial activity in relaxed as well as contracting myocytes.
Figure 8.6-1: Inhibitor effect on the flickering of cardiomyocytes.
Image of THG and MPF just following the addition of FCCP ((a) and (b), respectively) with the corresponding standard deviation maps for THG and MPF in (c) and (d). The panels (e) and (f) represent the THG and MPF images evolving into hypercontraction of the myocyte due to the FCCP. Standard deviation maps of the cardiomyocyte for the images evolving into hypercontraction are shown in (g) for THG and (h) for MPF.

Section 8.7: Conclusion

The MPF dynamic investigations demonstrate an association of mitochondria into functional networks in cardiomyocytes. The networks appear mainly along the rows, parallel to the myofibrils, but can span over many rows in a cardiomyocyte. The flickering dynamics of the inner and outer mitochondria networks indicate a slightly slower activity of interfibrillar compared to the sarcolemmal mitochondria. The appearance of mitochondria in synchronized networks is important for understanding cellular function. This investigation also shows that at least some of the networks observed in MPF are also highlighted in THG. The correlation indicates that THG flickering is also related to the changes in transmembrane potential, or else to physical changes that occur at the same time, such as changes in the spacing between the cristae membranes. Therefore THG imaging can be used for dynamic investigations of mitochondria physiology without sample labelling.

Through the variation in signal strength, it is shown that there is a significant flicker in the myocyte, but the flicker is no longer evident after the addition of the uncoupler FCCP. The sensitivity of the flickering to FCCP provides additional evidence that flickering areas are related to the functional activity of mitochondria, where the addition of FCCP depolarizes the mitochondrial transmembrane potential. The observed
flickering in THG is sensitive to the addition of TMRM dye showing that labelling affects the mitochondrial activity. The flickering in THG is seen to exhibit a wave like propagation across the sample. This could be attributed to the propagation of calcium waves across the myocyte. The results presented here provide the basis for further investigations of physiological activity of mitochondria and interactions of different organelles inside of living cells with the non-invasive multicontrast nonlinear microscopy.
Chapter IX - Conclusions and Future Work

Section 9.1: Conclusions

This work has culminated not only in a better understanding of the generation of nonlinear signals within biological species, but also in a better understanding of the functional activity of myocytes. Second harmonic generation is shown to be sensitive to the crystalline-like arrangement of the thick filaments in myocytes. The crystalline changes during contraction and relaxation of the sarcomeres affect the SHG response, presenting a new research and diagnostic tool for studying muscle contractions that might be valuable for understanding muscular disorders such as fibrillation, arrhythmia and muscular atrophy.

Contractility in Drosophila skeletal myocytes and Sprague-Dawley rat cardiomyocytes studied here further highlights the potential for SHG as a viable imaging tool. Nanocontractions in cardiomyocyte sarcomeres are revealed for the first time. When these non-synchronized contractions become synchronized, macro-sized periodic contractions occur. Drosophila contractility experiments reveal small changes to the SHG band width during contraction. In addition, periodic contractions of Drosophila sarcomeres show periodic changes in SHG intensity, correlating the intensity increase with the increase in the size of the sarcomeres. The dynamic study of nanocontractions on a single sarcomere level provides the basis for further investigation of fundamental principles of contractility in myocytes.

THG microscopy of cardiomyocyte mitochondria was demonstrated for the first time. Exploration of dynamics in mitochondria imaged with THG and MPF microscopy revealed synchronized networks of mitochondria. Analysis of mitochondrial flickering dynamics further revealed differences in function between interfibrillar and subsarcolemmal mitochondria. The ability to distinguish differences in dynamic activity and determine relationships between mitochondria with MPF and THG contrast mechanisms demonstrates the power of nonlinear multicontrast microscopy as an imaging technique.
The wave-like propagation of intensity increase observed in the third harmonic occurred in cardiomyocyte samples in which periodic contractions were evident. Although the periodic contractions and THG intensity front propagation occurred at different time scales, the results demonstrate the possibility of a simultaneous study of contractility and functional activity of mitochondria with multicontrast MPF, SHG and THG microscopy. Finally, this work demonstrates that nonlinear multicontrast imaging microscopy is a valuable tool for studying biological structures and intracellular dynamics of physiological processes.

Section 9.2: Summary of the main contributions

1. Developed several novel algorithms to aid in (nonlinear) multicontrast imaging.

2. Demonstrated that the order of the microcrystalline-like structure in myocytes is the major factor in determining the efficiency of the second harmonic generation.

3. Demonstrated that *Drosophila* myocytes have different sarcomere sizes for the larval and adult stages. The *Drosophila* larva sarcomeres had approximately 7μm length compared to average of 2μm length for the indirect flight muscle and cardiomyocyte sarcomeres.

4. Investigated contraction dynamics in myocytes with SHG microscopy, and revealed that cardiomyocytes as well as larval myocytes exhibit nanocontractions while in a resting state.

5. Revealed active samples of cardiomyocytes and larval myocytes showing periodic contractions that occur when nanocontractions become synchronized into a macrocontraction.

6. Demonstrated how periodic contractions exhibit a loss of periodicity upon the addition of the mitochondrial uncoupler FCCP.

7. Demonstrated the first use of THG microscopy for imaging mitochondria in myocytes.

8. Demonstrated the existence of functionally active networks of mitochondria in cardiomyocytes with both MPF and THG contrast mechanisms.

9. Revealed differences in mitochondrial function between interfibrillar and subsarcolemmal mitochondria using MPF imaging.
10. Showed that intensity fluctuations in MPF and THG from mitochondria are present in non-labelled as well as labelled myocytes and therefore provides evidence that ROS production is not the only cause of mitochondrial depolarizations.

**Section 9.3: Future work**

The increased understanding of mitochondrial depolarization and sarcomere contraction are in themselves already beneficial to the research community. The main disadvantage in this current research is the need to average numerous frames for increasing the signal to noise ratio of images collected with THG, in particular for the cardiac samples. Although the work in this thesis has shown that sarcomere contraction and mitochondria respiration are related, the improved signal to noise ratio of the current microscope at the University of Toronto as well as imaging with a new fast scanning microscope, which is currently under development, will provide a wonderful opportunity to image the mitochondria dynamics simultaneously with sarcomere contraction. By imaging the two different organelles within the same sample at the same time, it is possible to further investigate muscle contraction dynamics and the delivery of ATP to the contraction sites of sarcomeres. Understanding the relationships between organelles can then be useful for characterizing different muscular disorders at a single sarcomere level. The relationship between the wave-like motion observed in THG and the contractions observed in SHG could be further explored in this advanced manner. The exploration between SHG and THG in myocytes would benefit from further investigations on the origins of THG in mitochondria. While this work develops the importance of the microcrystalline structure for efficient SHG and how the response changes during contraction, little is known about the origins of THG intensity changes in time. A better understanding of the relationship between changes in mitochondria physiology and changed in the THG response would be useful in further understanding the bioenergetics of muscle contraction.

Since the third harmonic was shown to be generated from the mitochondria, THG imaging has the potential to extend beyond myocytes, to other cellular types. Dynamic imaging in different cells could provide new information for understanding the
physiological activity of mitochondria and other organelles inside the cells. Already, we have shown THG imaging of yeast mitochondria (data not shown). Therefore the processes of yeast budding or cell division could be characterised with THG microscopy. The SHG generated from the inherent yeast structures could also aid in understanding the structure and interaction between cellular organelles.

To our knowledge the synchronization of nanocontractions within cardiomyocytes has yet to be observed by other methods. Physiological or neurobiological studies using SHG microscopy can be employed to aid in further understanding how sarcomeres synchronize to full-scale muscle contractions.

The development of SCIA has been useful not only in this research but in research of other group members. Currently SCIA is only beneficial for the comparison of 2 signals while the current microscope set-up allows for the simultaneous recording of three signals. A modified or extended version of SCIA would be beneficial for images acquired with 3 contrast mechanisms. The modified version would require not 3, but 8 ‘output colours’. For 3 contrast mechanisms A, B and C the following outputs would be required: (1) signal A only, (2) signal B only, (3) signal C only, (4) signals A and B, (5) signals A and C, (6) signals B and C, (7) signals A, B and C, and (8) no signal.

The advantage of using a longer wavelength laser is that it enables deeper penetration into biological samples, and minimizes the potential for absorption and subsequent thermal damage. Currently, multi-photon excitation fluorescence provides a slight advantage over THG and SHG in its wide variety of available fluorophores to label different organelles. The development of harmonophores, new labels for harmonic microscopy, could provide an increased selectivity for THG and SHG imaging, while still maintaining some advantages including the reduction of bleaching. Already, some research has begun in this area with investigations of SHG-labels (Moreaux, Sandre et al. 2000; Salafsky 2001).
Chapter X - References


## Appendix I – List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>A-band</td>
<td>anisotropic band</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DICA</td>
<td>dynamic image correlation analysis</td>
</tr>
<tr>
<td>DFT</td>
<td>discrete Fourier transform</td>
</tr>
<tr>
<td>f-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>g-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>HRSEM</td>
<td>high resolution scanning electron microscopy</td>
</tr>
<tr>
<td>I-band</td>
<td>isotropic band</td>
</tr>
<tr>
<td>IFM</td>
<td>indirect flight muscle</td>
</tr>
<tr>
<td>MEM</td>
<td>maximum entropy method</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>MPF</td>
<td>multi-photon excitation fluorescence</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OPD</td>
<td>optical path difference</td>
</tr>
<tr>
<td>PSF</td>
<td>point spread function</td>
</tr>
<tr>
<td>RIRR</td>
<td>ROS-induced ROS release</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCIA</td>
<td>structural cross-correlation image analysis</td>
</tr>
<tr>
<td>SHG</td>
<td>second harmonic generation</td>
</tr>
<tr>
<td>SNR</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T-tubule</td>
<td>transverse tubules</td>
</tr>
<tr>
<td>THG</td>
<td>third harmonic generation</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine methyl ester</td>
</tr>
</tbody>
</table>
Appendix II – Structure and Function of Muscle

Section AII.1: Introduction

As the majority of this thesis explores the nonlinear microscopy of myocytes, it is useful to review the physiology of skeletal and cardiac myocytes. This introduction to the structure and basic functions of myocytes is useful for physicists unfamiliar with biology. The first part of this appendix provides some details about the structure of striated muscle cells. Following this comparisons are made between rodent cardiomyocytes and Drosophila larval skeletal myocytes. The final section of this appendix discusses the physiology of muscle contraction.

Section AII.2: Basic structure and function of striated and cardiac muscle

Striated or skeletal muscle is composed of many muscle fibers each surrounded by a membrane called the sarcolemma. These muscle fibers are bundles of even smaller fibers called myofibrils which are surrounded by a thin membrane. Myofibrils are made up of myofilaments that form the sarcomeres of muscle. Figure AII.2-1 shows a schematic of the different parts of the muscle (see also Figure 2.3-1, Figure 2.3-2, and Figure 5.1-1). The striations are the result of alternating isotropic (I-bands) and anisotropic bands (A-bands); these dark and light bands make up the basic units, the sarcomeres, of the fibre and allow the contraction to take place. One sarcomere length is measured from one Z-line to the next Z-line, or equivalently from M-line to M-line. The mitochondria are located in between the myofibrils along the sarcomeres.
Figure AII.2-1: Schematic of the different subsections of the muscle.

Here a section of muscle fiber is shown. The muscle fiber is made up of myofibrils and is surrounded by the sarcolemma. The transverse tubules (t-tubules) are extensions of the sarcolemma that propagate the action potential to the myofibrils. The sarcoplasmic reticulum (SR) surrounds the sarcomeres of the myofibril and stores calcium needed for actin myosin interaction until it receives the signal being propagated down the t-tubule. The mitochondria lay in between the myofibrils, close to the sarcomeres in order to provide the ATP needed for contraction. The regions of the sarcomere are also labelled in this diagram. (Rogers 1983)

The isotropic bands are made up of thin filaments that are primarily composed of globular actin (g-actin) that is polymerized into actin filaments (f-actin). The thick filaments are made up of myosin filaments and span the A-band. Additionally, sarcomeres also contain troponin, tropomyosin and titin. The Z-disk, which provides rigidity, is made up of additional proteins. The M-line connects the myosin filaments and is made up of other proteins. The large multidomained titin, emerging as an important protein in the sarcomere, connects the Z-line to the M-line and interacts with the thick filaments to help maintain the myosin lattice structure (Knupp, Luther et al. 2002). Descriptions of the molecular components of the sarcomere and their location within the sarcomere are described in Table 2.
# Table 2: Parts of the Sarcomere

<table>
<thead>
<tr>
<th>Sarcomere Component</th>
<th>Description</th>
</tr>
</thead>
</table>
| Actin filament      | - made from g-actin which forms a right handed double helix  
|                     |   - 7-9 nm in diameter depending on where in the twist you look at  
|                     |   - length dependent on sample, but same for all filaments in the same sample  
|                     |   - ATP binding site is in the same direction in all actin subunits of the filament  
|                     |   - bound to the Z-disk at one end  
|                     |   - two actin filaments per half myosin filament  |
| Myosin Molecule     | - two globular heads that interact with actin during contraction  
|                     |   - heads contain ATP binding sites  
|                     |   - generate the force required to cause the muscle to contract  
|                     |   - a coiled-coil tail that interacts with other tails to form myosin filaments  
|                     |   - neck domain which joins the head and tail domains  
|                     |   - approximately 150 nm in length  |
| Myosin Thick filament | - formed from bundled myosin II molecules  
|                     |   - approximately 1.5 um in length and 12-15 nm in diameter  
|                     |   - bipolar  
|                     |   - about 180 myosin molecules per filament  
|                     |   - filaments are about 50 nm apart  |
| Thin filament        | - filament in the I-band that contains, actin, tropomyosin and troponin  |
| A-band               | - anisotropic band of the sarcomere  
|                     |   - defines the region containing thick (myosin) filaments  
|                     |   - includes the overlapping region of actin and myosin as well as the H-zone which contains only myosin  
|                     |   - does not change size during contraction  |
| I-band               | - isotropic band of the sarcomere  
|                     |   - defines the region containing only the thin filaments and the Z-disk  
|                     |   - changes size during contraction and relaxation  |
| H-zone               | - region of sarcomere containing thick filaments that do not overlap with thin filaments  
|                     |   - includes the central M-line  
|                     |   - changes size during contraction and relaxation  |
### Table 1: Components of the Sarcomere

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
</table>
| **Z-disk (or Z-line)** | - binds the thin filaments to maintain organisation  
- mesh-like structure that enables supercontraction in some systems  
- Z-disk to Z-disk defines the length of one sarcomere |
| **M-line** | - a connective tissue network  
- helps maintain hexagonal lattice structure of myosin filaments |
| **Tropomyosin** | - runs in the groves on either side of the actin filament chain  
- one troponin molecule for each tropomyosin molecule |
| **Troponin** | - prevents myosin from binding to actin during resting state  
- made up of troponin – C, -I and T  
- troponin –C is regulated by calcium |
| **Titin** | - spring like protein that adds rigidity to the A-band by anchoring myosin filaments to the Z-line  
- multidomain protein  
- connects Z-line to M-line  
- 6 titin molecules for half myosin filament |

The arrangement of actin and myosin within the sarcomeres forms a microcrystalline structure. A cross section of a sarcomere in the region of overlap between actin and myosin shows that each myosin is surrounded by 6 actin filaments and each actin filament is surrounded by 3 myosin filaments. The cross-section lattice structures are shown for the different bands of the sarcomere in Figure AII.2-2. The change in lattice structure of the thin filaments is accomplished by an equal displacement change of each of the filaments from the Z-line to A-band (Squire 1974).
Cardiac muscle cells, or cardiomyocytes, exhibit features different from skeletal muscles, allowing efficient pumping of blood to the body. Although still striated, cardiomyocytes exhibit branching between myofibrils not present in skeletal muscle cells. Cardiac muscles are also composed of single cells, each with a single nucleus (Katz, 1992).

The rat and mouse cardiomyocytes and Drosophila myocytes examined in this thesis differ in several ways. One difference is in the sarcomere size. Larval muscle has a sarcomere size nearly 3 times as long as both adult Drosophila myocytes and rat cardiomyocytes (see Chapter V). Drosophila myocytes, unlike rat cardiomyocytes, also have the ability to supercontract. Supercontraction is a contraction whereby the Z-disk changes its form to allow thick filaments to slide through into adjacent sarcomeres resulting in a contraction well below the rest length (Herrel, Meyers et al. 2002). Skeletal Drosophila larval muscle cells generally require an external stimulation to bring about contraction while cardiomyocytes generate their own trigger enabling them to beat without an external signal from the nervous system except to control the rate and strength of the beat. The density of mitochondria is also different for rat cardiomyocytes and
Drosophila muscle. Unlike skeletal muscles, cardiomyocytes have a high dependency on respiration i.e. production of ATP, and thus have a larger number of mitochondria than skeletal muscles. Differences in contractility dynamics are discussed in Chapter VI.

Section AII.3: Physiology of muscle contraction / bioenergetics

The sarcomere is the basic unit of myofibrils where contraction takes place. For an entire muscle to contract, many sarcomeres must contract simultaneously, that is, there is a synchronization of the sarcomere contraction. A schematic representation of a sarcomere at different stages of contraction is given in Figure AII.3-1. Details into sarcomere contraction such as more information on the cross-bridge cycle and the mechanism for the myosin walk are discussed in Chapter II.

![Figure AII.3-1: Different stages of contraction of an individual sarcomere](image_url)

A diagram showing the changes to the bands as the sarcomere undergoes contraction. During contraction, the Z-lines become closer together and therefore the sarcomere length shortens. The amount of overlap between thin and thick filaments increases during contraction as the myosin heads walk along the actin. This increase in overlap results in a decrease in the size of the isotropic band and decrease in the H-zone. The A-band remains unchanged.
The schematic in Figure AII.3-1 can be somewhat misleading when extending the contraction of a single sarcomere to the contraction of many. The schematic shows the Z-lines moving towards each other while the positions of the thick filaments remain unchanged. For synchronized contractions of many sarcomeres this type of behaviour is only possible for one sarcomere. In neighbouring sarcomeres, both Z-disks would move in the same direction and the thick filament would have to move with it.
Appendix III – Index for Electronic Files

Both the structural and dynamic investigations benefit from additional media files. 2-D stacks that are rendered into a 3-D volume are useful for structural observation. A sample area imaged in time is useful for dynamic study and the addition of movies of these time series provides an additional benefit of allowing the reader to better observe what is being discussed.

(a) List of Additional Media Files (*.avi)

The media files attached in the electronic version are as follows. File names are numbered in the same manner as below. Captions below highlight the content of the movie. The letters show which figure(s) in the text the file refers to.

1) 3-D rendered image of Drosophila adult muscle in SHG (rendered in mu- image area 24 μm)
   a. Figure 5.2-1: Microscopy images of larval and adult Drosophila muscles

2) 3-D rendered image of Drosophila larval muscle in SHG (rendered in mu- image area 24 μm)
   a. Figure 5.2-2: Comparison of SHG and MPF signals from Drosophila larvae muscle.

3) Low resolution 2-D sample area of Drosophila larval muscle in SHG imaged at different depths (movie frames represent different depths; movie shows depth 1-n followed by depth n-1)
   a. Figure 5.2-1: Microscopy images of larval and adult Drosophila muscles

4) High resolution 2-D sample area of Drosophila larval muscle in SHG imaged at different depths (movie frames represent different depths; movie shows depth 1-n followed by depth n-1)
   a. Figure 5.2-1: Microscopy images of larval and adult Drosophila muscle

5) Sustained contraction of Drosophila larval muscle in SHG
   a. Figure 5.4-1: Effect of sustained contraction on the neighbouring sarcomeres.
   b. Figure 6.4-2: SHG image and time cross section of sustained contraction in Drosophila larva myocyte.
6) *Drosophila* muscle (3-D volume) before and after excess KCl as observed in SHG
   a. Figure 5.4-2: The evolution of SHG intensity in *Drosophila larval* myocytes after addition of KCl.

7) *Drosophila* natural rhythmic contractions observed in SHG (averaged low res.)
   a. Figure 5.4-5: Influence of periodic contractions in the *Drosophila myocyte* on the SHG efficiency.
   b. Figure 6.3-5: SHG image and time evolution of a row of sarcomeres during periodic contraction
   c. Figure 5.4-4: SHG intensity changes with contraction of *Drosophila* larval myocytes.

8) *Drosophila* natural rhythmic contractions observed in SHG (averaged high res.)
   a. Figure 5.4-5: Influence of periodic contractions in the *Drosophila myocyte* on the SHG efficiency.
   b. Figure 6.3-5: SHG image and time evolution of a row of sarcomeres during periodic contraction
   c. Figure 5.4-4: SHG intensity changes with contraction of *Drosophila* larval myocytes.

9) *Drosophila* natural rhythmic contractions in SHG (non-averaged low resolution)
   a. Figure 5.4-5: Influence of periodic contractions in the *Drosophila myocyte* on the SHG efficiency.
   b. Figure 6.3-5: SHG image and time evolution of a row of sarcomeres during periodic contraction
   c. Figure 5.4-4: SHG intensity changes with contraction of *Drosophila* larval myocytes.

10) *Drosophila* non-contracting muscle observed in SHG (non-averaged)
    a. This file is not represented by a figure but is related to Figure 5.3-6 and 6.2-4 in that it shows how non-contracting muscle appears in SHG in time

11) Periodic contractions of a cardiomyocyte observed in SHG
    a. Figure 6.3-2: Periodic synchronization of nanocontractions into macrocontraction in cardiomyocyte.
12) Disruption of rhythmic contractions of a cardiomyocyte after uncoupler agent has been added (SHG)
   a. Figure 6.4-1: SHG image of a cardiomyocyte and time evolution of a row of sarcomeres after the addition of FCCP.

13) Loss of SHG (left) signal in a cardiomyocyte with hypercontraction while some THG (right) remains present
   a. This file is not represented by a figure but is related to Figure 6.4-1: SHG image of a cardiomyocyte and time evolution of a row of sarcomeres after the addition of FCCP. in that it also shows the effect of hypercontraction on SHG signal.

14) 3-D SCIA results of THG – NAD(P)H MPF comparison with 800 nm excitation
   a. Figure 7.1-1 Comparison of NAD(P)H MPF and THG images in a typical cardiomyocyte excited with 837 nm laser pulses.

15) 3-D SCIA results of THG - TMRM MPF comparison with 1064 nm excitation
   a. Figure 7.1-3 Comparison of MPF and THG images of TMRM labelled cardiomyocyte excited with 1064 nm laser pulses.

16) 3-D SCIA results of THG - SHG comparison with 1064 nm excitation
   a. Figure 7.1-4 Comparison of SHG and THG images of cardiomyocytes excited with 1064 nm laser pulses.

17) MPF mitochondria flickering
   a. Figure 8.2-3: MPF flickering activity networks in
   b. Figure 8.2-1: Typical cardiomyocyte image in MPF and standard deviation of pixels’ intensity fluctuations.

18) Overlay of mitochondria networks in MPF
   a. Figure 8.2-3: MPF flickering activity networks in cardiomyocytes

19) THG mitochondria flickering
   a. Although not directly related to a specific figure, Figure 8.2-1: Typical cardiomyocyte image in MPF and standard deviation of pixels’ intensity fluctuations. relates to the flickering in MPF. Here a sample is shown to flicker in THG. Mitochondrial networks discussed in Chapter VIII - Functional Dynamics of Mitochondrial Activity in Myocytes Observed with MPF and THG relate to the simultaneous flickering of multiple mitochondria

20) Wave-like motion observed in THG
   a. Figure 8.4-1: Wave-like motion of a high intensity THG front.