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Two-photon microscopy and its application to live cell imaging

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TWO-PHOTON MICROSCOPY AND ITS APPLICATION TO LIVE CELL IMAGING

A Thesis

Presented to

The Faculty of the Department of Chemical Engineering

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

John Shoemaker

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ABSTRACT

TWO-PHOTON MICROSCOPY AND ITS APPLICATION TO LIVE CELL IMAGING

By John Shoemaker

Two-photon fluorescent microscopy is emerging in many areas of science as a premier method of exciting fluorescence in biological samples. This technology enables noninvasive study of biological specimens in three dimensions with improved resolution over conventional imaging methods. Two-photon excitation produces a higher-contrast image and is a novel method to trigger localized photochemical reactions.

In this study, constitutively expressed eGFP in the nucleus of live HeLa cells is monitored over a period of hours. Other conventional imaging methods are analyzed versus a two-photon generated image. An experimental matrix of two-photon settings is used to determine functional settings for two-photon fluorescent microscopy and compare its resolution versus other conventional imaging techniques.

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INTRODUCTION

History

Live fluorescent microscopy is an emerging research tool in the biomedical industry. Within the last decade, the development of noninvasive fluorescent markers and light sources which can illuminate fluorescent markers in living cells without affecting their function has allowed fluorescent microscopy use to grow exponentially. Fluorescent markers used in live cells can either be inserted into a cells genome and thereby expressed constitutively or via monoclonal antibodies that attach to specific extra-cellular markers. Fluorescent microscopy has always been an important aspect of scientific research since the late 1950's, but its application in live cells was not feasible until recently because of the toxicity and specificity of the fluorescent markers and the toxicity induced by the applied light source required for fluorescence [1]. However, recent advances in genetically engineered fluorophores and more biocompatible light sources are accelerating the development of live cell fluorescent microscopy.

Currently, biologists and scientists use fluorescent dyes to tag molecules via specific antibodies or directly through strong chemical affinities between a dye and substrate (protein) [2-4]. These target substrates are often monitored or measured using fluorescent quantification software [2]. The excitation of the fluorescent probe is usually delivered by a single photon laser or an arc lamp light at a specific range of wavelengths. However, these short wavelength light sources (400 nanometer-600 nanometers) can cause several undesired side effects [5-7]. Short wavelength sources can create a toxic wave that can induce a live cell into apoptosis [6]. Secondly, single-photon lasers and

mercury arc lamps excite fluorescence in various three-dimensional directions, which reduces the contrast that images can be acquired [4,6]. The sensitivity of fluorescent microscopy is important to its effectiveness, as well as its efficacy in a live cell assay. For example, in drug development frequently new cancer targets can be validated and screened through live cell microscopy, by fluorescing the known protein or drug of interest, and interrupting its function through the cell cycle [8]. Therefore, a better means to excite a fluorophore in a live biological sample is worthy of further research. An emerging technology that improves both these aspects in live fluorescence microscopy is fluorescence with a two-photon laser.

BACKGROUND

Fluorescent Microscopy Light Sources

Conventional fluorescent microscopy is traditionally done using either a single-photon laser or a mercury arc lamp in order to illuminate a given cells fluorescent features. Mercury arc lamps are one of the oldest forms of inciting fluorescence [1]. Arc lamps are a dynamic source of light in that they produce enough power to excite fluorescence in wavelengths from 350 nanometers to 700 nanometers. In Figure 1, a typical high-pressure mercury arc lamp is shown with its corresponding fluorescent light emission curve.

Figure 1: A typical mercury arc lamp and a graph of its emission spectra at a particular wavelength vs. fluorescence intensity at that specific wavelength.

In microscopes, the entire light spectrum generated by the arc lamp is passed through an optical filter, which selects a specific bandwidth, while absorbing the other wavelengths. A microscopist will have a collection of optical filters to select the appropriate bandwidth for the excitation of a selected fluorophore. By this means, mercury arc lamps are used to excite fluorescence in microscopy.

Single-photon lasers are a more advanced fluorescent source than a mercury arc lamp.

Figure 2: 488 nanometer argon gas laser.

A laser is considered an advanced tool in microscopy for two main reasons. Lasers can reach higher power levels and their wavelengths are specific. Depending on the inert gas used in the plasma-generating hub of a laser, a single wavelength is produced with a laser. For instance, an argon laser typically produces a 488 nanometer wavelength, while a krypton laser will produce a 567 nanometer light source, and a

krypton/argon mixed gas laser will produce a 647 nanometer band. A mercury arc lamp's light is passed through an optical filter to separate its wavelengths however these filters cannot select single wavelengths. In fact, the emission of light passing through a typical filter usually looks like a parabolic curve encompassing 7-10 wavelengths [9]. Consequently, a mercury arc lamp's broader spectrum of light is refracted through a biological sample to a greater degree due to the varying wavelengths. Power is the second reason why lasers are preferred over a mercury arc lamp for fluorescent excitation [9]. Mercury arc lamps light disperse in every direction while lasers have coherent light that is completely in phase. Therefore, laser light can be transmitted over considerable distance while continuing to maintain its power. The power of a laser can be adjusted by specifying the electric current applied to the plasma tube where the laser is lased. An arc lamp has a fixed wattage, governed by the voltage applied to the bulb.

The option of adjusting the power of a light source is critical, because eventually a light source's power can be too high and the fluorophore will not fluoresce. These phenomena's are referred to as quenching and bleaching [5]. Quenching occurs when a fluorophore is physically excited to jump to another higher energy level and release a substantial amount of energy in the form of heat. Another similar phenomenon is bleaching. Bleaching is the irreversibly switch to a non-fluorescent species by a non-radioactive transition releasing a oxidative free radical, usually due to high excitation power [5]. Quenching is not the same as bleaching a fluorophore, although the end results are the same. Therefore, a microscopist is always limited to a specific power range with a given fluorophore. A microscopist rarely has to worry about quenching or

bleaching with mercury arc lamps because their wattage is relatively low. There are some very sensitive fluorophores that can even be bleached or quenched with a mercury arc lamp, but the majority of popular fluorophores are physically stable with arc lamps. Conversely, arc lamps are challenged to generate enough power to incite fluorescence [9,10]. Mercury arc lamps are generally reduced to 10-15 milli-Watts when they reach the biological samples in their shorter wavelengths. This is mostly due to the fact that an arc lamp light is diverging in every direction and they are passed through optical filters, which select their specific wavelength and generally reduce their power by 25-30% [9]. The limiting power of a mercury arc lamp causes various problems for the fluorophore and the biological sample. Since an arc lamp's power is lower than a laser, its interval of exposure to the fluorophore must be longer to collect the same amount of fluorescence as a higher power light source. The objective used to collect the image must have a larger lens to collect more light than an objective that will be used for laser excitation sources. Lower excitation power also limits the depth of penetration of the fluorescent source within a sample. These two limitations could either cause increased damage to the surrounding sample or limit the applications of fluorescence to relatively thin samples. Conversely, a single photon laser can cause technical problems because of its excessive power. Typical single photon lasers may cause a mutation or quench or bleach a biological material as its power is increased. Therefore, each of these light sources has its limitation to use of certain fluorophores and biological materials.

Two-Photon Lasers

The first report of a two-photon laser was in 1990, by applied physicist Watt Webb and his colleagues at Cornell University in Ithaca [5]. Six years later, Bio-Rad Laboratories, Hercules, Ca, developed and patented the first commercial two-photon laser [1]. The first generation of two-photon lasers were extremely expensive, and only until the last couple of years has the price dropped to the level where more companies and institutes can realistically begin purchasing and applying a two-photon laser for microscopy [6]. Consequently, there has been an exponential increase in scientific publications where two-photon microscopy was identified as a tool to analyze biological samples. According to PubMed, one National Database of Scientific Journals, there are hundreds of articles published in scientific journals that have “two-photon microscopy” in their abstract, and the vast majority of them were printed from 1998 to the present. Therefore, it can be inferred that two-photon microscopy is a relatively new and exciting research tool in the realm of scientific research.

Topics that are related to two-photon microscopy include any fluorescent microscopy, molecular biology, drug development and live-cell imaging. Two-photon microscopy has been implemented in many of these fields and continues to be tested in other areas of micro to nanometer-scale fluorescent optical detection [7]. This research tool is so new; that there are few areas where similar research has overlapped each other. And the investigation of two-photon microscopy in live cell assays will be analyzed in this study.

Laser Properties

Single photon lasers are generally continuous wave lasers and two-photon lasers are pulsed lasers. In order to deliver a high density of photons per absorption events the two-photon lasers are pulsed. The two-photon lasers pulses are regulated through a high-speed electronic shutter. Pulses for two-photon lasers are usually in the pico to femto-second range (10^{-12} – 10^{-15} sec) and a 100 MHz repetition rate [3-4,6]. A femto-second may seem like a relatively short amount of time, however the period a fluorescent molecule fluoresces is on the order of 10^{-8} seconds [9]. Therefore, the fluorophore will “see” the photons for relatively long periods of time. It is reported that using a standard two-photon laser will fluoresce 80% of a sample within 700-1000 nanometers of their 3-dimensional focal point using a lens of between 1.0 to 1.4 numerical apertures [11,12]. Thus, it will limit background fluorescence as shown in Figure 3.

Figure 3: The left objective shows a two-photon laser that fluoresces only at the three-dimensional point where the two-photons center and create enough energy to excite the fluorescent gel, while the right-side shows a one-photon laser as it fluorescence's the entire optical path.

The fact that a two-photon laser is pulsed is beneficial because the laser is not continuously irradiating the biological material as an arc lamp or single photon continuous laser would. Two-photon lasers have two major beneficial technical properties for fluorescence microscopy, longer wavelengths and pulsed waves.

The longer wavelength of a two-photon laser allows a microscopist to probe deeper into a biological sample [13]. Since a two-photon laser is a longer wavelength than a single photon laser, it doesn't scatter as it penetrates material. It only has enough collective power to fluoresce at a specific three-dimensional point. Moreover, it maintains its energy further through the material than a single photon laser [13-15]. Scientific literature has agreed on this fact, and for some applications the depth of penetration is the primary reason why two-photon laser microscopy is being developed [9-10]. For example, the trafficking of specific cells in intact organs *in vivo* has been recently reported with two-photon laser microscopy to better understand *in vivo* biological systems [8]. Whereas, a single-photon laser or mercury arc lamp source would not be able to penetrate the 500 microns of tissue to excite fluorescence in the lymph node.

Fluorescence

Fluorescence is a consequence of a fluorophore being excited from its resting ground state to a higher energy level and back to the initial ground state.

Figure 4: A schematic showing the path of a fluorescent molecule. Circle 1 refers to the excitation from a given ground state S_0 to a higher energy level S_1' . Circle 2, signifies the Stokes Shift, or release of heat. Circle 3, is the emitting fluorescent light at a lower energy level than the exciting source.

After excitation, a fluorophore will return to its ground state and emit a small amount of heat and specific spectra of fluorescence. The amount of heat released after excitation is the same quantity whether a given fluorophore is excited with either a single photon or a two-photon source [9].

Figure 5: Jablonski diagram simulating the excitation processes of two ($\lambda_A + \lambda_B$) and one (λ_1) photon absorption and the resulting fluorescence (λ_2).

However, the emitted quantity of heat specific to the fluorophore is rarely a cause of undesired side effects [4]. What is a concern to a microscopist is if the light source that excites the fluorophore also mutates other biological material that it passes through in order to get to the fluorophore or excites the fluorophore into a higher energy level above fluorescence [6]. This is where the benefit of having a longer wavelength laser to excite

your fluorescence is realized. The energy of a photon is inversely proportional to its wavelength as shown in Table 1.

Table 1: Standards for Two and One Photon Excitation.

τ =pulse time, δ =volume of focal plane, σ_1 =area of focal plane, f =frequency, I =intensity, P =laser power.

	Two photon	One photon
Excitation rate	$N_2 \propto \delta I_e^2$	$N_1 \propto \sigma I_e$
Cross section	$\delta_2 \sim 10^{-50} \text{ cm}^4 \text{ s/photon}$	$\sigma_1 = 10^{-16} \text{ cm}^2$
Laser	$P_e \sim 100 \text{ mW mode-locked}$ $\tau_p \sim 100 \text{ fs}$ $f_p \sim 100 \text{ MHz}$ $P_{\max} \sim P_e / \tau_p f_p \sim 10^4 P_e$	$P_e \sim 1 \text{ mW continuous}$ $P_{\max} = P_e$
Focusing 1 mm laser beam to 1 μm spot at specimen	$I_e \sim 100 \text{ mW/mm}^2$ $I_{e\max} \sim 10^5 \text{ W/\mu m}^2$	$I_e \sim 1 \text{ mW/cm}^2$

Therefore, a longer wavelength laser will transmit less energy to material that it passes through. Since the energy of a photon is an inverse function of its wavelength, in theory two photons of double the wavelength must be delivered to a fluorophore to excite it as opposed to a single photon at half the wavelength. The two photons must be absorbed within a short time period and spatially, on the order of $\sim 10^{-18}$ seconds of each other, in order to deliver the necessary energy to excite the fluorophore [9]. These limitations of the spatial and kinetic requirements for two-photon excitation are the reasons why fluorescence is only excited at the focused 3-dimensional plane of focus for a given objective. Due to the simultaneous adsorption of the two photons, the fluorescence emission is dependent by the square of excitation intensity. In experimental practice the two-photon excitation intensity is reported to be a very highly non-linear process [4]. Whereas, single photon's emission level is linearly dependent on its excitation intensity and it has sufficient energy to excite fluorescent material above and below the 3-dimensional point of focus. Only at the point where a two-photon laser is

focused to where both photons adsorb within 10^{-18} seconds, will fluorescence occur. Due to the fact that two-photon excitation is highly non-linear, the photon density at the point of fluorescence must be one million times greater than with one photon excitation [9]. The increase in photon density is feasible for two-photon excitation because the two photon lasers are pulsed. The focusing of the two-photon laser is dependent on the numerical aperture (NA) of the lens, which the two-photon laser must pass through before entering the biological material [4]. The fluorescence is then collected through the same objective. Since, the sample will only fluorescence at the 3-dimensionsional point where the two-photons are focused and were they arrive at the same time (10^{-18} sec), background fluorescence is eliminated.

Green Fluorescent Protein

Green fluorescent protein, GFP, is an activated fluorescent protein isolated from marine invertebrates. Green fluorescent protein was discovered by Shimomura et al. in 1962 when their laboratory found the green companion of the already famous chemiluminescent aequorin protein from *Aequorea* jellyfish [10]. Shortly after GFP's discovery its excitation and emission spectra were published. In its wild-type form, GFP can be excited at two wavelengths, 395 and 475 nanometers. At an excitation of 395 or 475 nanometers the emission spectra is nearly identical, with a peak around 505 nanometers [10, 15]. Until the early 1990's the use of GFP in molecular biology was limited because of the double excitation peaks and also the fact that the wild type form would unfold at temperatures above 30 degrees Celsius. However, with the explosion of

molecular genetics in the 1990's, different groups began genetically modifying various nucleotides in the wild type GFP genome in order to create a stable form at 37 degrees Celsius and a single excitation peak. In 1995 the laboratory of Tsien replaced the wild type form of GFP's 65th amino acid of serine with threonine and consequently, effectively altered the natural phenol structure that accounts for the 395 nanometer excitation peak, while enhancing the anion that accounts for the excitation peak at 480 nanometers by five-fold [10]. Moreover, the additional substitution of leucine for phenylalanine at the 64th amino acid position stabilized the folding of GFP at 37 degrees Celsius. This popular mutation of GFP, S65T and F64L, is referred to as eGFP. eGFP has a maximum excitation peak at 488 nanometers and maximum emission at 509 nanometers, while maintaining its native physical structure at temperatures well above 37 degrees Celsius [10]. Numerous other GFP mutants have also been generated that present different spectra.

Fluorescent GFP has been inserted and expressed through well-described molecular biology techniques in various organisms including: bacteria, yeast, slime mold, plants, drosophila, zebra fish, and in mammalian cells [16]. GFP can function as a protein tag, because it can tolerate either an N- or C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function [16]. The fact that GFP can be inserted into a protein or RNA's sequence and the biology of the genetic product will continue to function normally has led to its success as a fluorescent marker. GFP has been inserted into many different cellular locations including the nucleus, mitochondria, secretory pathway, plasma membrane, RNA and cytoskeleton via fusions

both to whole proteins and individual targeting sequences [15]. Since GFP has been used for almost a decade in fluorescent analysis it has widely defined molecular genetics techniques. Also, the different mutations of GFP's have spurred various experiments that capitalize on GFP's different excitation/emission spectra's to allow a researcher to monitor simultaneously two distinct GFP markers as they co-localize throughout a single cell. This enormous flexibility as a noninvasive marker in living cells allows for numerous other applications such as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions.

Figure 6: The overall shape of wild type green fluorescent protein, which had been referred to as a “β-can”.

Histone

Histones are proteins that are present in all eukaryotes and serve as part of the structural framework in the nuclear compartment of all eukaryotic cells. The histone protein family is comprised of 32 different proteins [17]. One of the main functional purposes of histones in the nucleus is organizing chromosomal structure. The histone family contributes to virtually all chromosomal processes, such as gene regulation, chromosome condensation, recombination, and replication [17]. A subset of histones, histone 2B.1, is key protein in the structural adaptation of the chromosomal matrix in mammalian cells during the cell cycle [18]. The protein, histone 2B.1 is systematically condensed and replicated during the mitotic cell cycle, before cellular division [19]. Histone 2B.1 is required throughout the cell cycle however it plays its especially key structural role during mitosis as it frames the various chromosomes during mitosis [19]. For the purpose of visualizing the various structural confirmations in a mammalian cell cycle, the histone family serves as an attractive candidate to fluorescently tag with GFP.

Drug Development Using Fluorescent Light Microscopy and GFP Labeling

The recent technical advances in noninvasive sources for fluorescent excitation and the development of *in vivo* fluorescent markers for various protein and RNA intracellular markers has inserted fluorescent light microscopy into a standard for drug development companies. One example of how fluorescent light microscopy is being used presently is the understanding the viability of a drug or a drug targets function in parallel with the use of interference RNA. Briefly, a specific sequence of sRNA, silencing RNA,

is genetically generated and infected via enzymes into a cell which recognizes the artificial sRNA's and silences all mRNA, messenger RNA, that possesses its identical sequence. The RNA silencing method was developed because it exploits the natural response by mammalian cells when they are infected with viral RNA [20]. Virus's use double-stranded RNA, dsRNA, to send their genetic information, so mammalian cells are programmed to attack and silence any dsRNA that they can recognize. By specifically, selecting an RNA sequence, as small as 20 nucleotides, that transcribes a genetic message, a RNA knockdown assay can briefly, up to 48 hours, silence a gene [20]. Through a mammalian cells natural mechanism of recognizing viral RNA, sRNA was designed. This assay gives microscopy the ability to witness functional consequences from specifically silencing a RNA product.

RNA knockdown technology has recently developed at the same time as the fluorescent microscopy, and their use in parallel is frequently being applied throughout the pharmaceutical and biotechnology industry. Fluorescence microscopy is an attractive technology to evaluate the possible functional side-effects of the RNA knockdown. Using a cell-line that has specific structural fluorescent features or using an RNA target sequence that has a fluorescent tag, a researcher can visually observe functional, structural, and positional effects of silencing different genes, one by one. Data can be generated real-time through microscopy as the cell will continue to function without the translation of a specific genetic message. For example, consider a pharmaceutical company that is designing a small molecule drug that will inhibit a specific protein that is inferred to be necessary for cancer cells to replicate. Furthermore, the protein is thought

to be critical in the mitotic phase of cell division or cell cycle. By introducing a sRNA sequence that is specific for the protein in question on a cancerous cell-line, that has a GFP-tag to the chromosomal structure, a pharmaceutical company can view the function of the cancerous cells without the protein [18]. By this method it's possible to visualize where and if the cancerous cells will exit replication and enter apoptosis. This process is called "target validation". Cancer related protein targets are only one example of a possible scientific application of this technique. For drug development purposes this is a powerful, high-throughput, method to acquire a global picture of the structural, functional, and positional of specific genes.

LITERATURE REVIEW

Two-Photon Reviews

Recent papers have explored the combination of two-photon microscopy and live cell imaging. Tong-Sheng Chen analyzed the rate at which a green fluorescent protein (GFP) in HeLa cells is bleached in a live cell assay [21]. HeLa cells are an effective tool to optimize the operating parameters of live cell two-photon microscopy because of their popularity and defined cell function. Chen used the excitation power of the two-photon laser as the only varying parameter, and increased the excitation power until the photo bleaching of the GFP was reached. This is an important aspect of using a two-photon laser which has only begun in the last three years, because it begins to define the limitations of two-photon microscopy. Chen concludes that a microscopist has to be very careful when using two-photon microscopy because it can easily bleach the biological sample and alter the fluorescent emission and cellular function. Moreover, Chen defines the relationship between two-photon's intensity and the bleaching rate of GFP, as a function to the 4th power instead of to the 2nd power as the theoretical value suggests. Chen used a femto-second pulsed Titanium:Sapphire laser with a laser power intensity of 24-38 milli-Watts, and the photons were focused with a 0.6 numerical aperture lens. The two-photon laser was set to 780 nanometers and fluorescence from the GFP was collected in an emission filter set from 510-570 nanometers.

The relationship between intensity and bleaching for Chen's group is different than a paper by Hopt and Neher, who estimate the function of intensity and bleaching is raised to the power of ~2.5 [11]. However, there are some major differences between

these two groups. Hopt and Neher are using bovine adrenal chromaffin cells and they are measuring the fluorescence of an indicator dye, Flura-2. Flura-2 is not a naturally secreted fluorescent marker as GFP is, but it is a artificial chemical fluorophore that can pass through live cells and bind to there nuclear envelope. In addition to using a different fluorophore, Hopt and Neher use a two-photon laser were the pulses were maintained at 190 femto-seconds and the wavelength was set to 840 nanometers. Hopt and Neher also use an objective with a higher numerical aperture of 0.9. Hopt and Neher do not specify the exact laser power that they use throughout their bleaching experiment, however they do estimate tolerable two-photon intensity is less than 3 milli-Watts. Piston and Patterson conducted a similar study of bleaching with two-photon microscopy [21]. They determined that the rate of photo bleaching as a function of laser intensity was on the order of approximately 3. Piston and Patterson also concluded that the thickness of the sample would decrease the rate of photo bleaching, and therefore thin samples would have a greater chance of bleaching at a constant laser power. They used a 150 femtosecond, 710 nanometer, and two-photon laser with a 1.3 numerical aperture objective lens. It can be inferred from these to papers that photo-damage is proportional to the several physical parameters; however the bleaching rate seems to be a dependent on multiple parameters, and not just one parameter. One parameter that seems to be consistent to increased bleaching is the pulse width of the two-photon laser.

Table 2: Summary of Literature Describing the Relationship of Photo Bleaching vs. Two-Photon Intensity.

	Wavelength (nm)	Pulse width (10-15 secs)	Numerical aperture	Cells	Photobleaching exponent vs. Laser intensity
Tong-Sheng Chen et al.	780	70	0.6	HeLa	~4
Patterson and Piston	710	150	1.3	fluorophore (no cells)	~3
Hopt and Neher	840	190	0.9	adrenal chromaffin	~2.5

Not only can bleaching effect the visual emission of your fluorophore but it can also affect the toxicity of a cell [12]. It is critical when probing a live cell for fluorescence, that you do not affect its biological machinery.

Cahalan, Miller, and Parker have developed a series of methods that aim to prove that two-photon microscopy can be the premium way to image cellular functions and mobility without impairing their native function [3]. For the first time in an intact whole animal mammalian system, two-photon imaging was used to monitor immune cell-to-cell interactions in a lymph node of a mouse [8, 23]. Using the non-invasive properties of the two-photon laser and its greater depth of penetration, a mouse lymph nodes T and B cells were fluorescent-tagged and their interactions were monitored in various immune challenges. Cahalan reported highly random T-cell movements that were an average of 6 times greater in velocity than the corresponding B cells in naïve lymph nodes [23]. Whereas, given an antigenic challenge, T-cells exhibited a coordinated effort to swarm and organize around an inferred APC (antigen-presenting cell) [23]. This technique allowed scientists a first glimpse into the cell-to-cell interactions that begin an immune response within an intact lymph node in a whole animal. Malone also recently showed

two-photon microscopy, to be effective in diagnosing and monitoring leukemic cancerous cells in humans [2].

Barely a decade old, two-photon laser applications have already made a significant impact on science. The opportunity for scientists to monitor and test fluorescent-tagged small molecule drugs or monoclonal antibodies is being developed. The realization of testing drugs and cellular mechanics in a native system instead of the artificially environment of a petri-dish creates an improved use of live cell fluorescent microscopy.

HeLa Cell Reviews

HeLa cells were the first cancerous cell line to be discovered in 1951 [24]. The name HeLa, is an abbreviation for Henrietta Lacks, who was the patient that the HeLa cell-line was generated from [24]. HeLa cells have been used for various imaging assays and continue to be a standard in cancer-related assays. HeLa cells have also shown to be easily transfected with different genetically modified proteins and retain their viability. With the amplification of research involving oncogenes and the dependences of various biological factors within cancerous cells, the HeLa cell line has emerged as the premium standard to study cell-based oncology research.

In 1998, Kanda, Sullivan, and Wahl, fused an eGFP-structural gene with the constitutively expressed human histone-2B.1 gene within a stable HeLa cell-line [25]. Kanda selected the histone-2B.1 protein as a site to attach GFP because it is expressed throughout the chromosomal matrix. Kanda used a single-photon laser to excite the GFP

within the HeLa cells. Therefore, with this cellular vector, live fluorescent microscopy can be used to monitor and investigate chromosomal alignment throughout the mitotic cycle. Since the GFP-histone complex is inserted into the DNA of the HeLa cells, and it will be passed along to daughter cells after replication with the same fluorescent intensity of the parent cells. Kanda has created a high-resolution imaging cell-line that can be used to investigate multiple oncogene knock-outs or protein-mediated factors that specifically affect the chromosomal cell cycle, within a well-described cancerous cell-line.

Other HeLa cell-line/GFP vectors have been used to monitor components of the HeLa life cycle. Falk and Lauf used eGFP-tagged HeLa cells to image different gap junction channels and determine their dependence on various tagged proteins [26]. Howell and Truant used single-photon microscopy and HeLa cells to investigate protein shuttling and trafficking within the nuclear compartment during mitosis [27]. Howell and Truant describe the previous labor-intensive methods of monitoring protein trafficking *in vitro*. Using single-photon microscopy, they derive an effective assay that allows the researcher to monitor protein trafficking with HeLa cells *in vivo*. Although Howell and Truant run into some technological barriers with the cell viability, they demonstrate that live cell microscopy can be used to monitor proteins. This is a powerful tool in the sense that various potential protein drug targets can be easily monitored as to their specific role in the cell cycle of a cancerous cell line. Theoretically, a pharmaceutical or biotechnology company could validate the best protein candidate by watching, through fluorescence, numerous proteins that are essential to the cancerous cells proliferation. With the recent emergence of knock-out gene cell-lines and interference RNA techniques

which silence specific genes, the intra-cellular localization and function of these vectors is extremely important to research and industry. The importance of an accurate live cell assay can be the key to validating the most promising drug target.

RESEARCH OBJECTIVES AND SUMMARY

Two-photon microscopy is a new, exciting way of monitoring and analyzing biological substrates that can be tagged with fluorescent probes without impairing their native function *in vivo*. Since two-photon fluorescence is relatively new, further research is necessary to establish its benefits versus existing technology in order to realize its full potential. In this study, light microscopy was used to monitor eGFP-tagged HeLa cells through their cell cycle using either a mercury arc lamp, single-photon laser, or a two-photon laser. The two-photon laser fluorescent light microscopy was evaluated alongside other more conventional well-defined light sources of a mercury arc lamp and a single-photon laser. The two-photon laser set-up was varied throughout an experimental matrix based on different literature review results. The experimental parameters were studied to determine if they affect the quality and quantity of the fluorescent image. Through variation of these parameters, the two-photon light excitation was evaluated to determine if it will provide a clearer image of the eGFP-tagged HeLa cells than the traditional light source of mercury arc lamps or single-photon lasers. The physical settings for a two-photon laser light microscopy derived from this study could then be translated to other applications involving *in vivo* HeLa cell-eGFP fluorescent microscopy.

Following the literature, several experimental parameters for the two-photon laser will be adjusted to analyze the vitality and exposure to photo bleaching and biologic activity. The parameters to be varied are the wavelength and magnification/numerical aperture of the objective. The effect of these parameters on the efficacy of the HeLa cells and the relative transient fluorescence intensity of the eGFP protein over time will be

monitored. The resolution of the chromosomal matrix with in the histone-tagged HeLa cells with various light sources will also be subjectively evaluated. The HeLa cells will be imaged transiently through their mitotic cell cycle and the proper two-photon laser properties will be varied to attain the most desirable settings for imaging and cell efficacy. HeLa cells will also be analyzed with a single-photon laser and a mercury arc lamp as a light source. The optimal physical settings for both the mercury arc lamp and the single-photon laser will be taken directly from the manufacturer. Through these three standard light sources, the hypothesis of the two-photon laser as a superior excitation light source for eGFP for *in vivo* light microscopy will be evaluated.

EXPERIMENTAL APPARATUS AND ANALYSIS

Microscope Set-up: Single Photon Laser and Two Photon Laser

A Leica DM_IR standard inverted microscope was used for the microscope bench for the single and two-photon laser. The entire microscope was surrounded by a temperature controlled cube, set at 37 degrees Celsius. The image scanning was done with a scan head galvometer designed by Leica Microsystems. Each picture was generated as an eight-bit, 1024x1024 .tif file. A computer used the Leica Microsystems TCS SL version 2.0 acquisition software to view and record each sample. During the time-lapse experiments a three-minute interval for each image for the HeLa cells was acquired over a time period of 4 to 6 hours continuously. During each experiment the same HeLa cells will be monitored throughout the experiment. Before each experiment the microscope will be aligned using standard Kohler illumination protocol as described in Appendix A.

Microscope Set-up: Mercury Arc Lamp

The mercury arc lamp light source 50 milli-Watts, came from an OSRAM Mercury Short Arc Photo Optic lamp. A Zeiss Axiovert S 100 microscope was used as the microscope bench for the mercury arc lamp source. A 37°C heating chamber was installed around the HeLa cells and a 5% CO₂ incubation unit. The images were acquired with a Hamamutsa Orca-ER digital camera attached to the microscopes optical bench. The mercury arc lamp was set to pass through an optical filter set that selects

wavelengths between 480-492 nanometers while omitting an average wattage of 20 milli-Watts.

Laser: Two-Photon

The two-photon laser has several components as shown in Figure 7.

Figure 7: Spectra-Physics Millennia solid-state pump 532-nanometer argon laser and the Spectra-Physics Tsunami two-photon laser.

The initial light source for the two-photon laser is a Spectra-Physics Millennia solid-state pump 532-nanometer argon laser set to 5.5 Watts. The light from the Millennia laser is passed through a Titanium:Sapphire prism within the casing of the Spectra-Physics Tsunami two-photon laser. The laser light is split into two wavelengths of equal frequency, wavelength, power, and pulse width. The two pulses of laser light are delivered to the microscopes objective via a single-mode fiber optic wire with a fiber

optic frequency coupler. The combination of a single-mode fiber optic wire with a fiber optic frequency coupler increases the spatial sectioning and reduces the number of bulkier optical components [28]. Two-photon laser intensity was measured with a Coherent power meter at the point of laser interrogation. The two-photon laser will be between 18 and 20 milli-Watts depending on the wavelength selected. A Tektronix TDS 200 Digital Real-Time Oscilloscope measured the two-photon laser's wavelength. This oscilloscope is equipped with two channels for verification that each photon is at the same wavelength and frequency. The frequency was kept constant at 80MHz and the pulse width was maintained at 2 pico-seconds for each wavelength used in order to allow the two photons necessary time to simultaneously excite the eGFP.

Laser: Single-Photon

The single-photon laser is a solid state argon 488 nanometer laser. It is manufactured by Spectra-Physics and it connects via a fiber optic cord to the same Leica DM_IR microscope that is used for the two-photon laser. The acquisition software is the same as with the two-photon laser experimental design. The single-photon laser power will be set at 20 milli-Watts.

HeLa Cells

Live histone-2B.1 eGFP tagged-HeLa cells were plated on a two-well glass chamber cover-slip slide. The histone-2B.1 eGFP tagged-HeLa cells were acquired through American Type Culture Collection (ATCC, Manassas, VA). The thickness of the

cover-slip will be 0.17 micrometers for either laser application and approximately 0.5 micrometers for the mercury arc lamp. These cover-slip dimensions are required for the objectives used in this experiment as well as the limitations of the excitation source. A media of D-MEM with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% L-glutamine were used to stabilize the live HeLa cells and promote growth. The media was covered in a thin layer of light mineral oil to reduce the loss of CO₂ over the experimental time period. The entire chamber will be kept at 37°C. Cells were selected from a field that has some cells beginning the metaphase to mitosis cell cycle stage of cell cycle kinetics. Imaging for all three light sources, a mercury arc lamp, single photon and two-photon laser, was acquired at a time interval of three minutes between each time point. Each experimental time-lapse was acquired for at least two hours and a maximum of six hours.

Experimental Matrix

Two-Photon Laser	
Wavelength	Magnification/Numerical Aperture
820	100x/1.4
820	63x/1.32
820	40x/1.2
840	100x/1.4
840	63x/1.32
840	40x/1.2
860	100x/1.4
860	63x/1.32
860	40x/1.2
Single-Photon Laser	
Wavelength	Magnification/Numerical Aperture
488	100x/1.4
488	63x/1.32
488	40x/1.2
Mercury Arc Lamp	
Wavelength	Magnification/Numerical Aperture
482- 490	40x/0.4
482- 490	32x/0.32
482- 490	20x/0.3

- Each excitation source and objective setting was run three times.
- Images were acquired every three minutes for at least two hours.
- 6 cells per two-photon wavelength/objective were analyzed for greatest mean fluorescent intensity for the two-photon laser set-up.

Data Analysis

The data analysis for the different excitation sources is both quantitative and qualitative. Quantitatively, fluorescence was measured as an average mean fluorescence over time and at specific time points for the various experimental set-ups. This measurement investigates the possible degradation over time of the eGFP emission through quenching, bleaching, or over-exposure to the fluorescent excitation sources. Moreover, a qualitative analysis of the data is necessary to explore the greatest resolution and quality of the fluorescent image. These two methods of interpreting the data will result in a conclusion that will identify an experimental set-up that is superior to analyze cell division without impairing cell cycle.

The intensity of the fluorescence for each image for the two-photon experimental set-up was analyzed with the analysis software provided by Leica. When a cell is selected for analysis, a region of interest (ROI) will be defined around the cell and measure the average intensity level of the cell within that ROI. The two-photon lasers image will then be analyzed along side the single photon and mercury arc lamps time-lapse files. The Leica software gives three parameters; area of my ROI, mean channel intensity, and sum of channel intensity's.

Mean channel intensity	$\mu(I) = \frac{1}{N_{Pixel}} \sum_i I_i$	Equation 1
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Average channel intensity	$\mu(I) = \sum_i I_i$	Equation 2
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These three values were recorded at each time point throughout each samples acquisition period. The fluorescent intensity values were plotted versus time to interpret the stability of the eGFP within the HeLa cells over time.

Each experimental set-up was evaluated for its fluorescent resolution. The time-lapse images were decomposed into .tif files, and then compressed into live streaming video format, .avi files. The compression software for .tif to .avi conversion will be Microsoft RLE. After conversion to .avi files, the different time-lapse .avi files were analyzed for superior resolution. The qualitative results were captured in streaming movie format with Windows Media Player version 9.0 and image format in .bmp images.

RESULTS AND DISCUSSION

The results from this study are discussed in this section. The initial set of data was generated with the Zeiss mercury arc lamp microscope. This is the general baseline technique used commonly to analyze live cell fluorescent microscopy. These first experiments with the Zeiss mercury arc lamp microscope set a minimum required resolution and quality of the fluorescent HeLa cells. Three different objectives were used to evaluate the HeLa cells under the fluorescence of the Zeiss microscope. Secondly, the Leica microscope was used with a single-photon laser to monitor the development of the HeLa cells. As with the Zeiss microscope, three different objectives were varied to analyze the fluorescence during the Leica single-photon excitation experiments. The quality of fluorescence was determined over time with the single-photon set-up versus the two-photon experimental set-up. Thirdly, the two-photon laser and Leica microscope were used to with the same objectives as the single-photon set-up to fluoresce the live HeLa cells. The objectives and wavelength of the two-photon laser were adjusted throughout the experiment.

- ❖ Mercury Arc Lamp

- Objectives

- ❖ Single-Photon Laser

- Objectives

- ❖ Two-Photon Laser

- Objectives
- Wavelength

The same HeLa cell was used to evaluate each quantitative fluorescent value for each objective used in the two-photon set-up. All three excitation sources used HeLa cells derived from the same cell background. Each experimental set-up imaged HeLa cells at three-minute intervals for a total of up to six hours at 37 degrees and 5% CO₂. Together these three methods in studying fluorescent live-cell biology are discussed for their positive and negative effects on fluorescence and resolution of the cellular structural components over time.

Mercury Arc Lamp Results

The first general experimental set-up was done using a Zeiss AxioVert S 100 microscope with a mercury arc lamp. The mercury arc lamps fluorescent power was measured at 20 milli-Watts at the stage of excitation. The wavelengths of 480 to 492 nanometers were selected with a Chromalogic optical filter before the light passes through the objective. A six-well dish with a thickness of 0.5 microns was used to plate the HeLa cells. The HeLa cells are prepared as shown in Appendix B. A typical fluorescent field of HeLa cells and a bright-field image is shown below imaged with a 32x/0.32 numerical aperture objective.

Figure 8: Images of HeLa cells under the Zeiss microscope with a 32x/0.32 numerical aperture objective. Picture A represents a phase contrast image. Picture B represents the fluoresce generated by the mercury arc lamp and the resulting eGFP expressing the fluorescent H2.B in the nucleus. Picture C is an overlay of the two channels together.

Figure 8 highlights the need to have an additional channel of fluorescence to frame the nucleus within the cell as compared to just the phase contrast image. The phase contrast images are imaged independently from the fluorescent excitation source via a 25 milli-Watt halogen light. HeLa cells were imaged at three minute intervals over time periods of four to six hours. Figure 9 is an example of a specific HeLa cell undergoing cell division.

Figure 9: HeLa cells imaged with the 32x objective. Each image is taken 6 minutes after the previous one. The box in image i selects a HeLa cell that is about to undergo cell division.

In Figure 9 the 32x/0.32 numerical aperture objective in combination with the mercury arc lamp gives a substantial field of HeLa cells and still allows the researcher to witness some degree of resolution as the nucleus condenses and separates throughout its normal cell cycle. Also, there is no degradation of the eGFP fluorescence over time.

Shown below in Figure 10, are the fields of view with a 20x/0.3 numerical aperture and 40x/0.4 numerical aperture objective.

Figure 10: HeLa cells imaged with the 20x/0.3 numerical aperture and 40x/0.4 numerical aperture objectives and a mercury arc lamp.

Through Figure 10, it clearly shows the limitations of detail observed at a lower magnification, 20x. And at the higher objective, 40x, the definition of the image is comprised for other technical reasons that will be explained later. The lower magnification, 20x, loses the detail of the various cell cycle phases of division because the magnification is not great enough. Conversely, the 40x magnification begins to lose the definition around the edges of the fluorescent nucleus because of the physical limitations of the experimental set-up.

When using a 20x objective along with the Zeiss microscope the field is large enough to view over 200 HeLa cells in one image. This view is expectable if you are analyzing cell growth on a global level; however you begin to lose the structural definition of the fluorescent nucleus during the cell cycle period. Details of how the

chromosomes condense and separate are important to most cell cycle dependent therapeutics, and establishing the phenotype of how the chromosomes structural change is inadequate with this objective. Moreover, if live-cell based growth (live vs. dead) are the main goals of a studies then there is likely no need to use fluorescent channels to image cells in order to count them on the basis of cell vitality. Cells can be counted over time with a phase contrast or differential interference contrast (DIC) image which can be acquired with a halogen lamp.

Conversely, the 40x/0.4 numerical aperture objective suffers from the physical limitations of using a mercury arc lamp excitation source in combination with a bright-field microscope. With bright-field microscopes a researcher must be careful that the numerical aperture of the objective is sufficient to accommodate a higher magnification with mercury arc lamp fluorescence. Objectives used for bright-field microscopy have inherently larger lens surface areas in order to collect more light, but that limits their numerical aperture. And an arc lamp's wide spectrum of wavelengths and unfocused beam of light make certain higher magnification objectives more difficult to focus in this physical set-up. The by-product of combining a higher magnification objective and a mercury arc lamp excitation light source is a blurry image around the edges as seen in Figure 10 with the 40x/0.4 numerical aperture objective. It should be noted that a phase contrast or DIC image using the same physical parameters and halogen light instead of mercury arc lamp will provide a clear image with all three objectives. The physical limitations of the fluorescent light source are specific key variables why a 40x/0.4

numerical aperture objective is ineffective while a 20x/0.3 numerical aperture objective is too low of a magnification to define the cells eGFP fluorescent nucleus.

Single-Photon Laser Results

A Leica DM_IR standard inverted microscope is used with a solid-state 488 nanometer Argon laser in this section to acquire images. The live HeLa cells were plated in two-well chamber-slip slides that have a thickness of 0.17 microns. The 488 nanometer laser will be measured to be 20 milli-Watts at the stage of intersection. The objectives were varied from 40x/1.2 numerical aperture, 63x/1.32 numerical aperture, and 100x/1.4 numerical aperture. These objectives are all oil-immersion objectives. The images shown below in Figure 11, represents the three various objectives fluorescence of the live HeLa cells.

Figure 11: HeLa cells excited with a single-photon 488 nanometer laser and imaged with a 40x/1.2 numerical aperture, 63x/1.32 numerical aperture, and a 100x/1.4 numerical aperture objective.

From Figure 11, it is clear from the definition of the fluorescent nucleuses that the single-photon laser images are of better quality than the mercury arc lamp acquired images in the previous section. The nucleus has a visible three-dimensional texture with the single-photon laser. At higher magnifications the edges of the nucleus do not blur as with the mercury arc lamp. The single wavelength property of lasers attributes to this

improved optical condition, whereas the abbreviations from the multiple wavelength arc lamp sources cause the blurring at the fluorescent edges at higher objectives.

The advantage of the combination of using a single-photon laser with the ability to use an oil immersion objective allows the user to image a clearer picture of the fluorescent nucleus as compared to the mercury arc lamp and a dry objective. Depending on the field of view required for the assay, any of these three objectives used with the single-photon laser will produce functional images.

One area of improvement for this experimental set-up can be seen when cells that are undergoing mitosis, and they physically condense their chromosomes into the center of the cell and form what is called a metaphase plate. At this point the chromosomes are stacked in the three-dimensional plane and with a single-photon laser you can see more blurring of the fluorescence as opposed to when the nucleus is spread out in a flat, relatively two-dimensional configuration. This is illustrated further in Figure 12, where a time-lapse movie was acquired over an eighteen-minute time period.

Figure 12: Live HeLa cells are excited with a 488 nanometer single-photon laser and imaged with a 63x/1.32 numerical aperture objective. The fluorescent image and DIC image are paired together to enhance the significance of the cell division occurring within the red box shown in image i/ii. Each pair of images (i/ii, iii/iv, and v/vi) were acquired at 6 minute time intervals from the previous pair.

In Figure 12, the cell labeled with the red box has just undergone cell division. And during its division the nucleus is condensed in a three-dimensional stack that is typical of any eukaryotic cell-cycle process. The cell division phase of the cell cycle represents an important functional part of the nucleus, and represents the paramount reason why a cell-line like these fluorescent HeLa cells would be used analyze effects on environmental factors. More specifically, the clarity of the imaged nucleus throughout this cell cycle phase is extremely important. Ideally, the image only at the plane of focus would be fluorescence, and thereby eliminating background fluorescence. This scenario presents a significant obstacle for the excitation source to fluoresce a fluorescent nucleus at one plane while not exciting the fluorescent nucleus in either z-directions. These factors are addressed further in the next section. Figure 9, also demonstrates that there is no degradation of the eGFP over time with the single-photon laser.

Two-Photon Laser Results

A Leica DM_IR standard inverted microscope was used with a Spectra-Physics Titanium:Sapphire two-photon laser to acquire images. This is the same microscope bench as the single-photon set-up. The two-photon laser wavelengths were varied from 820, 840, and 860 nanometers for a given objective. Each wavelength was set and maintained using the procedure in Appendix B. The Wattage of the two-photon laser is measured in the range of 18 to 20 milli-Watts at the stage of intersection. Along with the three separate wavelengths selected, the objective used to focus the two-photon laser was varied. Three objectives were used; 40x/1.2 numerical aperture objective, 63x/1.32

numerical aperture objective, and a 100x/1.4 numerical aperture objective. All the objectives are oil immersion objectives. The procedure to set-up an oil immersion objective is given in Appendix C. An oil immersion objective is needed to obtain a numerical aperture over the value of one.

In the first set of data the 40x/1.2 numerical aperture objective images are given. The wavelength of the two-photon laser is varied from 820, 840, and 860 nanometers. The same field of *in vivo* HeLa cells is shown below in Figure 13 at various wavelengths of excitation.

Figure 13: Images of *in vivo* HeLa cells imaged with the two-photon laser via a 40x/1.2 numerical aperture objective at various two-photon wavelengths and the appropriate single-photon wavelength, 488 nm.

Two things are apparent from the images in Figure 13. Firstly, the images in Figure 13 portray the greatest amount of definition of any of the previous images analyzed to this point. For instance, the HeLa cell in the center images in Figure 13 is aligned in its metaphase plate at the time that these images were acquired. The three-dimensional aspect of this cell demonstrates the benefits of excitation of the two-photon

laser. In general, the HeLa cells eGFP signal is clearer when excited with the two-photon laser as opposed to the single-photon laser and the mercury arc lamp. The background levels of light versus the expressing eGFP fluorescence within the nucleus at the plane of the objectives focus are clear, especially when compared to the mercury arc lamp.

Secondly, Figure 13 shows that the two-photon wavelengths of 840 and 860 nanometers have stronger eGFP fluorescence than the 820 nanometers wavelength. Another example of the greater excitation of the higher two-photon wavelengths, 840 and 860 nanometers is in Figure 14.

Figure 14: Images of *in vivo* HeLa cells imaged with the two-photon laser via a 40x/1.2 numerical aperture objective at various two-photon wavelengths.

Secondly, the 63x/1.32 numerical aperture objective was used to fluoresce the HeLa cells. The same field of *in vivo* HeLa cells is shown below in Figure 15 at various wavelengths of excitation.

Figure 15: Images of *in vivo* HeLa cells imaged with the two-photon laser via a 63x/1.32 numerical aperture objective at various two-photon wavelengths and the appropriate single-photon wavelength, 488 nm.

From Figure 15, it is clear through the 63x/1.32 numerical aperture objective that the higher wavelengths, 840 and 860 nanometers, produces the strongest level of fluorescence in the two-photon experimental matrix. Furthermore, similar to the 40x/1.2

numerical aperture objective, the two-photon wavelengths bring a clearer eGFP signal compared to the single-photon wavelength under the same conditions. The definition and resolution between the background and the eGFP fluoresce within the nucleus is impressive at these settings, especially at the higher two-photon wavelengths of 840 and 860 nanometers.

Thirdly, a 100x/1.4 numerical aperture objective was used to image HeLa cells. The same field of *in vivo* HeLa cells is shown below in Figure 16 at various wavelengths of excitation.

Figure 16: Images of *in vivo* HeLa cells imaged with the two-photon laser via a 100x/1.4 numerical aperture objective at various two-photon wavelengths and the appropriate single-photon wavelength, 488 nm.

Again some parallels between the other objectives given in Figure 14 and 15, can be seen through Figure 16. The two-photon excitation gives a clearer image of the eGFP emission than the single-photon fluorescence, especially when looking at cells aligned in their metaphase plate configuration. Secondly, and a bit more pronounced at this objective, is the increased level of two-photon fluorescence at 860 nanometers as compared to the other wavelengths used for the two-photon laser. This could be due to the fact that as the image continues to be enlarged through higher objectives magnifications and minor differences between fluorescence levels are more pronounced for wavelengths used. The level of fluorescence at different wavelengths at a fixed objective will be quantified later.

An *in vivo* division of HeLa cells is shown in Figure 17 below. The wavelength was set to 860 nanometers and the objective is a 100x/1.4 numerical aperture lens. The cell in the middle of this series of images undergoes the entire cycle of cell division from the metaphase plate in the image i, from Figure 17. It is important to visualize the definition and resolution of the individual chromosomes as they align and separate through this process. And due to the fact that this is a very high magnification objective and the excitation source is a two-photon laser the chromosomal separation is extremely well defined here. It should also be noted that there is no appreciable bleaching or degradation of the eGFP signal over time with these 3-minute time intervals of image acquisition. The images shown in Figure 17 are at 6-minute intervals in order to limit the number of images shown.

Figure 17: Live HeLa cells are excited with an 860 nanometer two-photon laser and imaged with a 100x/1.4 numerical aperture objective. Each image, i-vi, was acquired at a 6-minute time interval from the previous image.

In order to quantify the fluorescence levels for the two-photon set-ups, 6 different cells were selected for each two-photon wavelength and objective. At a given objective, one cell was selected per image, and their mean fluorescence values for a region of interest, ROI, were calculated for the three different two-photon wavelengths. An example is shown in Figure 18.

Figure 18: Images acquired at various two-photon wavelengths of the same cell with the 63x/1.32 numerical aperture objective, and the corresponding mean channel fluorescent values that are recorded in the second column of the Area based Analysis window.

Figure 18 shows the mean fluorescent values for one cell at various wavelengths for the 63x/1.43 numerical aperture objective. Each wavelength and objective has a total

of 6 different cells analyzed. In an effort to limit variation, the same cell is analyzed with the three different two-photon wavelengths for the same objective. The 6 cells for each setting were divided up between 4 non-cell cycle phase HeLa cells, and 2 cells aligned in their metaphase plate. These 6 mean channel fluorescent values were added up and divided by 6 in order to come up with a mean channel intensity value for a two-photon wavelength and objective. The results are shown in Figure 19.

Figure 19: Graph of the mean fluorescent values for each two-photon laser set-up and wavelength.

Some general points can be made from Figure 19. First, the same specific cells were chosen for each objective and the various wavelengths for that objective, therefore Figure 19 can be used to evaluate an objectives dependence on a two-photon's wavelength. However, it is not possible to specifically compare mean channel intensity values for different objectives, because the cells analyzed are different between

objectives. Although the exact cells between objectives may not be the same, the overall trend can be analyzed between objectives. And that overall trend points to higher mean fluorescence values at 860 nanometers for each two-photon set-up. Also, 840 nanometers has the second highest mean fluorescence values, followed by 820 nanometers totaling the lowest fluorescence values for each separate experimental set-up. Together, it can be assumed in this experiment that for two-photon excitation of eGFP, 860 nanometers will excite the highest amount of fluorescence for any of the experimental set-ups used in this study.

Degradation or bleaching of the fluorescent signal was reported in previous literature as a concern for two-photon excitation; however it was not seen in these experiments. One reason for this might be that previous experiments were done under different conditions and shorter periods of time between excitation. However, in this experiment eGFP was excited every three-minutes for up to six hours, and there was absolutely no degradation of the eGFP signal over time for any of the nine different experimental set-ups for two-photon excitation.

No appreciable difference in quality of eGFP resolution and definition was witnessed for the various objectives at the optimal two-photon wavelengths. The objective that is chosen for analysis with the two-photon laser can depend strictly on the information that is needed by the researcher and not by any technical constraints. For instance, if an analysis of various cells is required, then the 40x/1.2 numerical aperture objective with an 860 nanometer two-photon laser could image up to 50 cells in one image field. Whereas, a cell-specific analysis could require the 100x/1.4 numerical

aperture objective with an 860 nanometer two-photon laser could image up to 10 cells in one image field, but include more detail in its image since the cells are larger. The key points here being;

- ❖ The wavelength of the two-photon laser should remain at 860 nanometers for any objective.
- ❖ The selection of an objective should depend on the needs of the experiment.
- ❖ Bleaching or quenching of the eGFP was not an issue at these settings.

A general demonstration of the three objectives imaging eGFP in HeLa cells is shown below in Figure 20.

Figure 20: Images of *in vivo* HeLa cells imaged with an 860 nm wavelength two-photon laser via three different objectives.

FUTURE RESEARCH

Areas of future research include; thicker tissue or whole animal fluorescence, and alternative fluorophores. As seen in this study, two-photon excitation gives a spatially clearer image than a single-photon or mercury arc lamp excitation source. This is especially apparent, when a fluorescent source is excited in a three-dimensional field of excitable fluorophores. Moreover, the application of this technique could be extended to models of fluorescence that are thicker in tissue and have multiple levels of fluorescent material. The longer wavelength of the two-photon laser also gives for a less evasive tool and allows deeper penetration to excite fluorescence [29]. This could be tested with analysis of thicker tissue or cell-based samples. The application of whole animal fluorescence is already being reported by researchers using two-photon excitation [8, 29]. For example, research with eGFP and derivatives of GFP, as a versatile source of a bright, non-toxic fluorophore in whole animals would be very interesting and applicable to future bio-medical research. Together, these could be a very exciting new area of research for two-photon microscopy.

Other non-toxic, chemical fluorescent sources need to also to be studied for two-photon fluorescence. Some new fluorescent dyes available commercially are Molecular Probes Alexa-fluorophores and smaller nano-scale inorganic crystals referred to as Quantum Dots. Inorganic semiconductor nanocrystals, Quantum Dots, have been reported to last longer than organic chemical fluorophores and have a broader excitation spectrum, while maintaining relative small emission spectra [30]. Both Alexa dyes and quantum dots could provide a more selective and less burdensome application for

fluorescent labeling. Instead of constructing cell-lines or whole animals that have a GFP derivative constitutively expressed in the genetic background, a researcher could insert specific fluorescently-labeled antibody that target proteins. The technological advance in smaller, less intrusive chemical fluorescent dyes to survive *in vivo* is a very aggressive area of research. However, their durability in two-photon excitation would need to be analyzed further, as far as the appropriate wavelength for excitability and there endurance over time. For instance, the ability of new chemical fluorescent dyes to withstand bleaching and degradation from the two-photon excitation would need to be investigated. The two-photon laser is shown to be the superior tool to excite specific fluorescence, and now with the field of generating stable, non-toxic fluorophores soon to be another area of improvement, the future of fluorescent microscopy is definitely bright.

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APPENDIX

Koehler Alignment

- Turn to the 10x objective into the light path and focus on any specimen.
- Engage the condenser disc to the BF position (bright field position).
- Close the field diaphragm.
- Adjust the height of the condenser until the edge of the field diaphragm is sharply in focus.
- Center the image with the two centering screws on the field diaphragm.
- Open the field diaphragm until it just disappears from the field of view.
- Change objective to your desired objective for study and re-adjust the field diaphragm to focus the specimen.

Setting the Two-Photon Wavelength

- Turn on the two-photon laser for at least two hours to allow the multiple mirrors to reach steady state.
- Simultaneously walk the two-photon pulses to the desired wavelength by turning the Spectra-Physics bi-refrident filter and watching the pulses move on the Tektronix digital real-time oscilloscope.
- Now to get the two-pulses to reach mode-lock phase, you simultaneously adjust the GTI position dispersion compensation controller and the Output Coupler M_{10} knobs (horizontal and vertical) on the Spectra-Physics two-photon housing while watching the pulses on the oscilloscope.
- Continue to adjust these knobs till the highest pulse width is created on the oscilloscope.
- Then adjust the M_1 GTI knobs on the Spectra-Physics two-photon housing to stabilize the two pulses.

Oil Immersion Objectives

- Make sure the objective has the oil symbol on its base to signify it is an oil immersion objective.
- Place the desired objective in the light path.
- Place one drop of immersion oil on the lens of the objective.
- Gently raise the objective until it is in contact with the slide of interest.