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Photo- and Bio-Physical Characterization of Novel Lipophilic Fluorescent Dyes for Multicolor Neurotracing and Lipid Transcellular Diffusion Measurements

By

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A THESIS

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Abstract

Lipophilic fluorescent dyes are used to trace neuronal connections because of their ability to insert into and diffuse laterally within cellular membranes. These dyes successfully define neuronal tracts for normal and mutant development no matter how aberrant the molecular processing of cells. Simultaneous delineation of multiple pathways requires multicolor labeling with a set of diffusion matched and spectrally resolvable dyes. To achieve more than 3 distinct colors within a single sample, imaging strategies using two-photon excitation are necessary. The efficient utilization of two-photon excitation is dependent on the knowledge of the two-photon excitation action cross section for the set of dyes. The first specific aim of this thesis was to measure the two-photon excitation action cross-section spectra for a set of neurotracing dyes to facilitate the design of multicolor imaging strategies. Two-photon excitation action cross sections were measured using a standard ratiometric approach comparing the neurotracer dye fluorescence to a well-known dye. The results indicated that all fluorescent dyes measured can be efficiently two-photon excited with a Ti:S laser. The acquisition of the two-photon excitation spectra for the neurotracer set allowed dyes exhibiting fluorescence emission cross talk to be spectrally resolved, and thus achieve multicolor neurotracing.

Recently certain lipids have been implicated as signaling molecules that travel between cells. Although their effects are fairly well-characterized, the mechanism for their diffusion across the aqueous gap between cells is unknown. This mechanism might be affected by variations in the length of their hydrocarbon chains. Because lipophilic fluorescent dyes consist of a head group and two variable-length hydrocarbon chains analogous to naturally occurring lipids, they can be used to investigate lipid diffusion between living cells. The second specific aim of this thesis was to investigate the hydrocarbon-chain-length dependence on the mechanism of lipid transcellular diffusion. Dye diffusion was examined by labeling single cells in a connected network and measuring the spread of dye into the network using laser scanning microscopy. Additionally, FRAP measurements within individual cells were performed. The results demonstrated that for both transcellular and intracellular diffusion, the length of the hydrocarbon chain influences the mechanism of lipid diffusion in living cells.
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The research that I conducted for this thesis was funded by a N.I.H. SBIR II grant R44 MH079805-04. The objective of this grant was to build a set of lipophilic fluorescent dyes to maximize the total number of discrete neuronal connections that can be simultaneously traced in a single specimen.

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Chapter 1

Introduction

Lipophilic fluorescent dyes have been powerful tools for the study of cellular structure and dynamics. These dyes incorporate into and diffuse laterally along cell membranes, and are easily visualized with laser scanning microscopy (LSM). Because of these properties, lipophilic fluorescent dyes are used to trace neuronal connections and probe lipid dynamics, among other applications. For this thesis, the photophysical and biophysical properties of novel lipophilic fluorescent dyes were explored to extend the number of discrete colors in multicolor neurotracing and to elucidate the mechanism of lipid transcellular diffusion.

1.1 Towards Multicolor Neurotracing

The nervous system consists of billions of cells interacting with each other in complex networks to perform and integrate autonomic, motor, and sensory functions. Understanding the development, genetic factors, as well as repair mechanisms for neuronal circuits requires the dissection of the neuronal topology and connections in mutant and transgenic animal models. During development, neurons use various cues to establish connections. Mutating these cues and analyzing the resulting connections allows the determination of the molecular basis for the development of neuronal circuits.

To discover the genetic basis for neuronal connections, mutations have to be breed into animal models. Because mutations that affect neuronal connections often cause death after birth, it becomes time consuming to obtain animals with the desired mutation, especially for
complex mutants with double, triple, or conditional mutations. The difficulty in producing complex animal models has to be matched with efficient methods to map the development of neuronal connections in a small number of animals. Multicolor labeling allows for tracing of multiple pathways to maximize the information obtained from a single specimen.

Additionally, multicolor tracing can dissect the complex patterns of connections in the brain that are not accessible via single-color analysis. The brain consists of two broad categories of networks. One contains fast, precise connections relaying information in a one-to-one manner between neurons (e.g., mapping frequencies of sound onto the auditory pathways from the ear to the brain). The other set of networks consists of more diffuse connections conveying information to multiple targets (e.g., type II sensory neurons that project to the inner ear and extend several hundred microns along the outer hair cells of the cochlea). If the fibers of each type of network have different targeting, then they must have different molecular cues to establish the different types of connections during development. By selective labeling of multiple locations along the cochlea, the precise patterns of connections of the type II neurons can be defined.

There are several techniques used today to label multiple neuronal connections. Two recently developed techniques that have garnered excitement are Diffusion Tensor Magnetic Resonance Imaging (DTI) and Brainbow. By measuring the diffusion of water molecules, which preferentially flow in the direction of neuronal connections, DTI can be used non-invasively to map out the connections in the human brain [1]. The main disadvantage to DTI is that its spatial resolution is limited to large nerve bundles. However, Brainbow can achieve images of individual neurons [2]. This technique labels neurons by genetically inserting green fluorescent protein (GFP) and 3 other GFP color variants such that only neurons produce the fluorescent proteins. Each protein is produced in random amounts in each neuron, creating a rainbow of different colors. The main problem with the Brainbow technique is that it is dependent on properly functioning neurons, and when many genes involved in neuronal development cause problems in the molecular processing of neurons,
Brainbow can fail to label neurons. To obtain the resolution of individual neurons and to label neurons with aberrant molecular processing, lipophilic fluorescent dyes are used because all cells have a lipid membrane.

DiI (1,1'-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate) was the first lipophilic fluorescent dye used as a neurotracer [3] and has revolutionized single color analysis of neuronal connections. To trace neuronal pathways between two brain regions, like from auditory neurons in the brain to sensory neurons in the ear, specimens are first formaldehyde fixed, and then lipophilic fluorescent dye is inserted into one region (e.g., the brain) where the dye only incorporates into the cell membrane of neurons at the insertion site. The specimen is then incubated for a couple of days to allow the dye to diffuse along the cell membrane, tracing the long projections extending from the brain to the ear. When the dye has reached the ear, the specimen is imaged using LSM. The resulting images define the detailed connections between auditory neurons in the brain and sensory neurons in the ear.

Despite the power of DiI to dissect neuronal circuits, multicolor analysis has been limited by time consuming and cumbersome protocols largely due to poorly matched spectral and diffusional properties. A pair of blue-absorbing dyes, DiO and DiA, are available for multicolor labeling, but fail to match the spectral and diffusional properties of DiI [4]. DiO diffuses much slower than DiI, and DiA cannot be completely separated spectrally from DiI. Two-color tracing can be performed with the near-infrared dye, DiD, which has spectral and diffusional properties comparable to DiI. Despite this, more than two colors are needed to dissect complex diffuse connections and to efficiently use animal models.

A set of lipophilic fluorescent dyes, called NeuroVue (NV) dyes, have been developed for the specific purpose of multicolor neurotracing by Molecular Targeting Technologies, Inc. (MTTI). Three dyes from this set have been recently characterized, showing favorable spectral and diffusional properties for three-color neuronal tracing [5], NV Maroon (NVM), Red (NVR), and Jade (NVJ), which emit in the far-red, red, and green, respectively. The
complete set of NV dyes also contains NV Orange (NVO) and Burgundy (NVB), which have comparable diffusion rates, but exhibit significant spectral overlap with NVR and NVM, respectively. Additionally, to extend the set to 7 dyes, violet and near-infrared candidate dyes have been produced [6]. With absorption in the UV, the violet candidate dyes are not accessible to the conventional lasers available in standard microscopy facilities. However, the violet candidate dyes can be two-photon excited using a tunable, near-infrared Ti:S laser. Furthermore, two-photon excitation (2PE) provides a method to separate spectrally overlapping fluorescent dyes. Therefore, 2PE can be used to increase the number of spectrally distinct colors in a single specimen.

To plan 2PE imaging protocols, the efficiency for 2PE of each fluorescent dye over the wavelengths emitted by the Ti:S laser is required. The 2PE action cross section, which is a quantitative measure for the probability that a fluorescent dye will generate two-photon excited fluorescence for a given laser intensity, provides the needed information. Therefore, for this thesis, measurements of the 2PE action cross section for commercial and violet candidate NV dyes over the tuning range of a Ti:S laser were measured to design imaging protocols for multicolor neurotracing.

1.2 Mechanism for Transcellular Diffusion of Lipids

For many years, amino acids and nucleic acids (e.g., DNA and RNA) were considered the only information-carrying molecules in cells, and lipids were regarded as having only structural or energy-storage capacities. However, more and more evidence has been discovered implicating lipids as more than just structural components of cells. Lipids directly modulate the function of ion channels, ease membrane fusion and fission, create discrete membrane microdomains facilitating signal transduction, as well as serve as precursors for signaling molecules and as information-carrying molecules themselves [7]. Signaling lipids have been shown to move between connected, living cells [8], adding a new component to intercellular communication.
Lipids have been shown to contribute to cell-cell communication by retrograde signaling between neurons in the brain [7]. Through connections called synapses, neurons send electrical signals from the presynaptic neuron to the postsynaptic neuron. When a specific type of postsynaptic neuron in the brain is stimulated by a presynaptic neuron, the lipid 2-Arachidonoyl-sn-glycerol (2-AG) is produced in the postsynaptic neuron, diffuses through the aqueous gap between neurons, and incorporates into the membrane of the presynaptic neuron. Within the cell membrane, 2-AG diffuses laterally to receptors producing a transient inhibition of the signal sent to the postsynaptic neuron. This retrograde signaling (retrograde because the lipid signal is moving backwards relative to the electrical signal) is thought to be involved in learning and memory [9, 10]. Although the effects of 2-AG on the electrophysiology of neurons are well known, it is unknown how 2-AG diffuses from one neuron to another. It appears that most signaling lipids in the brain operate like 2-AG by traveling short distances from their sites of production to neighboring cells.

The mechanism by which lipids diffuse between cells might be due to variations in the physiochemical properties common to many lipids such as the length of their hydrocarbon chains. A consequence of these variations could be different preferential routes for diffusion between cells. To study this, a series of lipophilic fluorescent dyes consisting of the same head group, but differing in the length of their hydrocarbon chains was used because the dyes insert into and diffuse laterally along cellular membranes analogous to naturally occurring lipids. In addition to measuring transcellular diffusion, the diffusion of the chain-length-variant dyes was measured within individual cells to explore additional temporal and spatial scales of lipid diffusion.

1.3 Overview of Thesis

This thesis investigated the photophysical and biophysical characteristics of lipophilic fluorescent dyes for multicolor neurotracing and transcellular lipid diffusion by addressing two specific aims. Chapter 2 discusses the photophysical and biophysical properties of
lipophilic fluorescent dyes needed to address each specific aim.

A major obstacle to multicolor neurotracing is the spectral cross contamination exhibited by fluorescent molecules because of their broad emission spectra. One route to overcome this obstacle, and obtain multicolor labeling, is 2PE. To implement 2PE imaging protocols for multicolor labeling, specific aim # 1 was addressed:

**Specific Aim #1: Measure the 2PE action cross-section spectra for commercial and violet candidate NV dyes.**

Chapter 3 discusses the materials and methods used to determine the 2PE action cross-section spectra for fluorescent molecules. Chapter 4 Section 4.2 presents the 2PE action cross-section measured over the tuning range of a Ti:S laser for commercial and violet candidate NV dyes. Section 4.3 discusses imaging protocols based on the 2PE spectra to produce spectrally uncontaminated multicolor labeling.

Contrary to a long held belief, lipid molecules have functions other than forming cellular structures. These new roles for lipids include operating as signaling molecules, which diffuse between neighboring cells facilitating intercellular communication. The exact mechanism by which specific lipids diffuse between cells is not known. Therefore, specific aim #2 addresses a particular aspect of lipid transcellular diffusion by using lipophilic fluorescent dyes:

**Specific Aim #2: Investigate the hydrocarbon-chain-length dependence on the mechanism of lipid transcellular diffusion.**

Chapter 5 discusses the materials and methods used to measure lipophilic fluorescent dye diffusion between living cells. Chapter 6 Section 6.1 presents the transcellular diffusion measurements of lipophilic fluorescent dyes with hydrocarbon chains of 10, 18, and 28 carbons long. Furthermore, to examine the hydrocarbon-chain-length dependence on lipid diffusion at a shorter spatial and temporal scale, diffusion measurements of lipophilic fluorescent dyes with chain lengths ranging from 6 - 28 carbons within single, living cells are presented in Section 6.2.
Chapter 2

Photo- and Bio-Physical Properties of Lipophilic Fluorescent Dyes

Because lipophilic fluorescent dyes insert into and diffuse laterally along cell membranes, they have been used to trace neuronal connections, highlight cellular membrane structure, and probe lipid dynamics. Knowing the specific photophysical and biophysical properties of lipophilic fluorescent dyes allows researchers to better utilize them. The photophysical and biophysical characteristics of lipophilic fluorescent dyes are based on their chemical structure, which consists of a head group and two hydrocarbon chains. The biophysical characteristics can be influenced by the entire chemical structure of the dye, both the head group and hydrocarbon chains. The hydrocarbon chains impart a lipophilic character to fluorescent dyes, while the head group determines their photophysical properties. The general structure for a common family of lipophilic fluorescent dyes, called indocarbocyanine dyes, is shown in Figure 2.1. These dye molecules consist of two indole rings, with various chemical side groups attached, connected by a carbon linker, and two hydrocarbon chains. For the chain-length-variant dyes studied in this thesis, the $R$ chain was set to a length of 3 carbons and $R_1$ was varied from 3 - 28 carbons long. Because the $R$ chain was small and constant for all the dyes in the series, the chain-length-variant dyes are identified by the number of carbons in their $R_1$ chain. By comparison, most lipids in cells have hydrocarbon chain lengths between 14 and 20 carbons long.

For this thesis, the photophysical properties of a set of lipophilic fluorescent dyes called
NeuroVue (NV) dyes were investigated to enable the simultaneous tracing of multiple neuronal connections. Specifically, a photophysical measurement for the amount of fluorescence generated by the simultaneous absorption of two photons at a given laser intensity, called the 2PE action cross section was measured. This important quantity is described in this chapter along with an explanation of the processes of 1PE and 2PE fluorescence and a description of the protocols for imaging multiple dyes with different colors in a single specimen.

![Figure 2.1: The chemical structure of a generic indocarbocyanine dye. The head group consists of two indole rings on either side of a carbon linker. The W, X, Y, Z and the corresponding W₁, X₁, Y₁, Z₁ indicate variable chemical side groups. To vary the photophysical properties of the dye, the length of the carbon linker is changed and/or the chemical side groups are modified. The two hydrocarbon chains are represented by R and R₁. These chains bestow the dye molecules with a lipophilic nature.](image)

Additionally, the biophysical properties of a set of prototype lipophilic fluorescent dyes consisting of a constant head group and various hydrocarbon chain lengths were examined in this thesis. These dyes were used to study the hydrocarbon-chain-length dependence on the rate of diffusion between and within living cells. In this chapter, the diffusion of lipophilic molecules, in general, is described with particular attention to the effect of hydrocarbon chains. Furthermore, general methods for measuring the rate of diffusion of dye molecules is discussed.

## 2.1 Photophysical Properties of Lipophilic Fluorescent Dyes

### 2.1.1 Molecular Fluorescence

Lipophilic fluorescent dyes are intrinsically fluorescent because their head group (Figure 2.1) is a fluorophore; a functional chemical group that is responsible for the efficient absorption and emission of light by molecules. As shown in Figure 2.2, fluorescence induced by
1PE is a process by which a fluorophore absorbs a single photon and subsequently emits a photon. When a fluorophore absorbs a photon, it is excited from the ground state \( (S_0) \) into a higher energy state \( (S_1) \). The fluorophore will transition into any one of its vibrational energy levels within the higher energy state. After photon absorption, the fluorophore will quickly transition, through internal conversions, to the lowest vibrational energy level within the higher energy state, called the equilibrium excited state. The energy of the absorbed photon must be approximately equal to the energy difference between the two energy states to be absorbed by the fluorophore. When the excited fluorophore transitions back down into the ground state, the fluorophore releases the absorbed energy by emitting a photon of fluorescence or by transitioning via a non-radiative process. The percentage of the number of fluorescence photons emitted to the number of photons absorbed is the fluorescence quantum yield. The duration of time for which a molecule is in a higher energy state is called the fluorescence lifetime. When the fluorophore emits fluorescence, the fluorophore can transition into any one of its ground state vibrational energy levels, before quickly returning to its original lowest-energy ground state. The discrepancy between energy levels transitioned during the absorption step compared to the emission step results in a red-shifted emission spectrum compared to absorption spectrum.

A fluorophore can also simultaneously absorb two photons in a process called 2PE (Figure 2.2). In 2PE, the fluorophore is excited into nearly the same the higher energy state \( (S_1) \) where the two photons combined equal the energy difference between the electronic levels. The excited fluorophore transitions back into its original, lower energy ground state \( (S_0) \), releasing a photon of fluorescence that is indistinguishable from fluorescence produced by 1PE. To absorb two photons, the fluorophore must pass through a virtual intermediate state \( (S_i) \). Based on the energy-time uncertainty principle (Equation 2.1), the two photons must be absorbed within a small temporal window, \( \Delta t \) (the lifetime of the virtual intermediate state), in order to preserve conservation of energy. The duration of the temporal window depends on the energy difference between the intermediate state and the ground
For a fluorophore with an energy difference between its ground state ($S_0$) and first excited state ($S_1$) equal to the energy of a blue photon, excitation can occur via absorption of a single photon (blue arrow) or via the simultaneous absorption of two, lower energy photons (red arrows). Between the simultaneous absorption of two photons, the fluorophore passes through a virtual intermediate state ($S_i$). For both 1PE and 2PE, a photon is emitted (green arrow) with an energy less than that of the absorbed photon(s). Fast, non-radiative transitions between vibrational energy levels (gray lines) are indicated by yellow arrows.

The 2PE cross section, $\sigma_2$, is a quantitative measure of the probability of a 2PE event occurring. The 2PE cross section consists of the product of the 1PE cross section for the absorption of the first photon, $\sigma_{0,i}$, the duration of the intermediate state, $\Delta t_i$, and the state of the fluorophore, $\Delta E$,

$$\Delta E \Delta t \geq \frac{\hbar}{2}. \tag{2.1}$$

For example, if the energy difference, $\Delta E$, is $1.5eV$ (energy for 500 nm, green, light), then the fluorophore can simultaneously absorb two photons, each with approximately half the energy (NIR light, 1000 nm), in $10^{-16}$ s or less. Because of this short time window for two photons to interact with the fluorophore and a 2PE event to occur, high peak illumination intensities are needed to increase the probability of two photons interacting simultaneously with the fluorophore.
1PE cross section for the second photon absorption, $\sigma_{i,1}$.

$$\sigma_2 = \sigma_{0,i} \Delta t_i \sigma_{i,1}. \quad (2.2)$$

The 2PE cross section has units of $cm^4$ per photon absorbed. Because 2PE cross sections are such small numbers, the number $10^{-50}$ $cm^4$ photon$^{-1}$, which is the 2PE cross section for the typical molecule, is defined as 1 G"oppert-Mayer (GM). This is named in honor of Maria Göppert-Mayer, who theoretically predicted 2PE in 1931 [11].

When an approximately transparent sample (e.g., living cell) is labeled with lipophilic fluorescent dye at a concentration, $C(\vec{r},t)$, the amount of photons absorbed per unit time by the sample within the focal volume, $V$, is proportional to the intensity of the excitation source raised to the number of photons simultaneously absorbed by individual fluorophores, $I_p(\vec{r},t)$:

$$N_{\text{abs}}(t) = \int_V C(\vec{r},t) \sigma_p I_p(\vec{r},t) \, d^3r, \quad (2.3)$$

where $\sigma_p$ is the $p$-photon excitation cross section and the integral is over the excitation volume, $V$.

The amount of fluorescence from a fluorophore-labeled sample, collected and counted by a detection system, is decreased by non-radiative transitions and inefficiencies in the detection system. After photon absorption, fluorescence emission is only one of the possible routes back to the ground state. There are many non-radiative transitions that primarily depend on interactions between the fluorophore and its environment. For example, an excited fluorophore could lose its excited-state energy via collisions with its neighbors. Non-radiative transitions decrease the fluorescence yield of a fluorophore. Because of non-radiative transitions and inefficiencies in detection systems, the amount of $p$-photon excitation fluorescence detected per unit time is given by,

$$F(t) = \frac{1}{p} \phi \eta N_{\text{abs}}(t), \quad (2.4)$$

where $\phi$ is the fluorescence detection efficiency and $\eta$ is the fluorescence yield.
2.1.2 Fluorescence Imaging

By plotting the fluorescence intensity as a function of wavelength, a fluorescence emission spectrum is obtained for the fluorophore. Similarly, the absorption spectrum can be obtained by graphing absorption intensity versus wavelength. Figure 2.3 shows the absorption and emission spectra for the lipophilic fluorescent dyes from MTTI, NV commercial and violet candidate dyes. The NV dyes have broad absorption and emission spectra that is representative of all fluorophores. Spectral broadening is due to electronic transitions from the excited state to a variety of ground state vibrational energy levels.

The broad spectra of lipophilic fluorescent dyes makes it a non-trivial process to image multiple dyes in a single sample. As seen in Figure 2.3 for the NV dyes, spectrally adjacent dyes often have fluorescence emissions that overlap. To avoid cross contamination from spectrally overlapping dyes, specific multicolor imaging strategies must be designed. Complete spectral separation can be achieved either by using an appropriately narrow-band emission filter, which collects only light from a single dye, or by sequential excitation of each dye individually with appropriately selected excitation wavelengths. Because emission spectra are invariant with excitation wavelength [12], excitation wavelengths for imaging protocols can be chosen to minimize fluorescence cross contamination. However, the available emission filters and excitation wavelengths are limited. For the purposes here, the resources of a typical imaging facility, like the Integrated Biological Imaging Facility at Creighton University, with a Zeiss LSM 510 NLO microscope (Carl Zeiss, Inc., Thornwood, NY), are considered for planning imaging strategies.

Figure 2.4 shows a theoretical success and failure of multicolor imaging strategies using 1PE to achieve separation of the fluorescence emission for NV dye pairs. In the NV dye set, the spectral neighbors, NVR and NVM, exhibit spectral overlap caused by the long red-tail of NVR emission spectrum overlapping with NVM emission. Complete segregation of NVM fluorescence from NVR contamination is achieved by individual excitation with a wavelength specific to each dye. When NVM is excited by a 633 nm HeNe laser (red...
Figure 2.3: The one-photon absorption (dotted lines) and fluorescence emission (solid lines) spectra for NeuroVue dyes. The NV Violet candidate dyes PTIR 334, PTIR 335, PTIR 336, and NVR and NVM are displayed in top figure and NVJ, NVO, and NVB in bottom.
Figure 2.4: 1PE imaging strategies to separate spectrally overlapping NV dyes. Complete spectral separation of NVR and NVM (top) can be achieved by selective excitation of NVR with a 543 nm HeNe laser (green line) and NVM with a 633 nm HeNe laser (red line). The emission filter for NVR (green region) collects only NVR fluorescence. Even through the NVM emission filter (red region) could collect fluorescence from both dyes, only NVM fluorescence is collected when sequential excitation is performed. Because NVO and NVR exhibit considerably more spectral overlap than NVR and NVM (bottom), complete spectral separation is not possible using 1PE imaging strategies. The excitation wavelength 514 nm from an Argon laser is indicated by green line. The emission filters for NVO and NVR are indicated by the green and red regions, respectively.
line), NVR fluorescence is not generated, and thus only NVM fluorescence is collected in a 650-710 nm band pass filter (red region). Similarly for NVR, a 543 nm HeNe laser (green line) will excite only NVR and a 565-615 nm band pass filter (green region) will collect only NVR fluorescence.

The one-photon absorption and fluorescence emission spectra for NVO and NVR overlap too much to achieve complete spectral separation using 1PE imaging strategies. The best available imaging protocol is illustrated in Figure 2.4. The 514 nm laser line (green line) from an Argon laser generates both NVO and NVR fluorescence emission. A reasonable emission filter, which collects only NVO fluorescence, is possible (535-575 band pass filter (green region)), but none is possible for NVR fluorescence without NVO contamination (600-665 band pass filter (red region)). Similar problems with multicolor imaging occurs for the NV dye pairs of the violet candidates with NVJ and NVM with NVB. Therefore, another imaging strategy is needed to achieve spectrally distinct labeling with the complete set of NV dyes.

2PE offers an alternative method for imaging multiple fluorophores in a single sample. Because two-photons are absorbed during 2PE compared to one in 1PE, the quantum mechanical selection rules are different [13]. Many fluorophores have different 2PE spectra compared to 1PE spectra. Often times, 2PE is stronger at wavelengths less than twice the 1PE peak [14]. For example, the 1PE at 400 nm for rhodamine B is weaker than at 500 nm, but 2PE is stronger at 800 nm than at 1000 nm. This is advantageous for 2PE of NV dyes, because for most of the NV dyes, twice the 1PE peak is beyond the tuning range of a Ti:S laser, the conventional laser used for 2PE. Furthermore, the shape of 2PE spectra often allows for simultaneous excitation of multiple fluorophores using a single wavelength [14]. To fully utilize 2PE for imaging multiple NV dyes, the 2PE spectra was measured for the NV dye set.
2.1.3 Measuring Two-Photon Excitation Fluorescence

To find the 2PE spectra and plan 2PE imaging protocols, the 2PE action cross section, a quantitative measure for 2PE of a fluorophore, is required. This quantity can be determined by measuring the fluorescence generated in a sample by a pulsed laser. By combining Equations 2.3 and 2.4 with \( p = 2 \) for 2PE fluorescence and separating the laser intensity into its temporal \((I(t))\) and spatial components \((S(\vec{r}))\), an equation for the time-averaged fluorescence detected as a function of laser intensity is obtained,

\[
\langle F(t) \rangle = \frac{1}{2} \phi \eta \sigma_2 C \frac{g^{(2)} g^{(2)}}{f \tau} \langle I(t) \rangle^2 \int_V S^2(\vec{r}) \, d^3r 
\]

where the dye concentration is assumed to be constant, \( g^{(2)} \) is the second-order temporal coherence factor, and \( f \) and \( \tau \) are the laser frequency and pulse width, respectively. The temporal and spatial components of the laser intensity can be rewritten in terms of the refractive index of the dye solvent, \( n \), the laser wavelength, \( \lambda \), and the laser power, \( P(t) \) [15],

\[
\langle F(t) \rangle = \frac{1}{2} \phi \eta \sigma_2 C \frac{g^{(2)}}{f \tau} \frac{8 n}{\pi \lambda} \langle P(t) \rangle^2 .
\]

Typically a quantity combining the 2PE cross section with the fluorescence quantum yield, called the 2PE action cross section \((\sigma_2 \eta)\) is measured and is sufficient to plan 2PE imaging protocols. Equation 2.6 was used to determine the 2PE action cross-section of NV dyes over the tuning range of a Ti:S laser, and the materials and methods are discussed in Chapter 3. The resulting 2PE action cross-section spectra are presented in Chapter 4.

The second part of this thesis, addresses the biophysical properties of a set of prototype lipophilic fluorescent dyes used to investigate lipid diffusion between and within living cells. Therefore, the next section discusses the biophysical characteristics of lipids and lipophilic fluorescent dyes.

2.2 Biophysical Properties of Lipophilic Fluorescent Dyes

The biophysical properties of lipophilic fluorescent dyes are determined by the physiochemical structure of their head group and hydrocarbon chains. Lipophilic fluorescent dyes
incorporate into cell membranes with their head group facing the hydrophilic aqueous environment outside the membrane, and their hydrocarbon chains pointing into the lipophilic regions of the membrane. The electrostatic and van der Waals interactions between the dye molecule and its neighbors determine the biophysical properties of the dye such as the rate of diffusion and the strength of association with the lipophilic regions of the membrane. In this thesis, the effect of the shape of the dye molecule, specifically the hydrocarbon chain length, on these biophysical characteristics was explored.

To investigate the influence of the hydrocarbon chain length, a series of dyes was studied that contain the same head group (NVM), but have varying hydrocarbon chain lengths. The hydrocarbon chains were saturated, consisting of a sequence of carbons connected by single covalent bonds (i.e., no double or triple bonds in the chain). The series of lipophilic fluorescent dyes, studied in Chapters 5 and 6, generally fall into the category of dye molecules called indocarbocyanine dyes (Figure 2.1). Diffusion measurements of the series of dyes were performed in connected, living cells. To interpret the diffusion measurements, molecular diffusion of lipids in the crowded cellular membrane and the processes capable of transporting lipids across the aqueous gap between cells are examined.

2.2.1 Molecular Diffusion

A molecule in solution will exhibit Brownian motion due to collisions with its neighbors. This thermal agitation leads to the random movement of the particle through its environment. The spread of an average molecule after time, \( t \), is given by the mean-squared displacement,

\[
\langle r^2 \rangle = \Gamma t^\alpha = 2d D(t) t.
\]  

The constant of proportionality, \( \Gamma \), is called the transport coefficient, \( D \) is the diffusion coefficient, \( \alpha \) is the time-scaling exponent, and \( d \) is the number of dimensions available for diffusion. For three-dimensional diffusion, the mean-squared displacement becomes \( \langle r^2 \rangle = 6 D(t) t \). If the mean-squared displacement scales linearly with time, the diffusion is normal or Fickian, corresponding to an \( \alpha = 1 \), and a diffusion coefficient that is not a function of
time. Otherwise, the diffusion is called anomalous.

For anomalous diffusion, the time-scaling exponent does not equal one, and thus the mean-squared displacement follows a power law with time. Diffusion with an $\alpha > 1$ is called anomalous superdiffusion, and $\alpha < 1$ is anomalous subdiffusion. In general, anomalous subdiffusion is caused by the transient trapping of the diffusing molecules in potential wells. Anomalous superdiffusion is caused by forced or directed diffusion. The type of diffusion a molecule undergoes depends on the environment and interactions with that environment. To understand the factors that influence lipid diffusion in cellular membranes, the simple case of a molecule diffusing in solution is considered first.

The rate of diffusion for an individual molecule in a homogeneous, hydrodynamic solution is given by the fluctuation dissipation theorem,

$$D = \frac{k_B T}{\xi},$$  \hspace{1cm} (2.8)

where $T$ is the absolute temperature, $k_B$ is the Boltzmann constant, and $\xi$ is the viscous friction coefficient. For a spherical particle, $\xi = 6 \pi \eta r$, where $\eta$ is the viscosity of the solvent and $r$ is the radius of the particle. Thus the diffusion of a molecule is dependent on its shape and the viscosity of the solution. For a lipid molecule in solution, lengthening the hydrocarbon chain increases the size of the molecule and thus decreases its rate of diffusion. However, lipids do not stay in solution; they aggregate into lipid bilayer membranes, which are not homogeneous, are crowded with molecules, and these molecules interact via forces other than collisions. One of these forces, which is dependent on hydrocarbon chain length, affects the viscosity of cell membranes.

Lipids interact with other lipids via the London dispersion force. The London dispersion force is an induced dipole-induced dipole attraction that occurs between any two molecules in close proximity, but has a significant influence in cell membranes. The strength of the force increases with size and decreases with distance by $1/r^6$. Lipids with saturated hydrocarbon chains, like the lipophilic fluorescent dye series studied in Chapters 5 and 6, can pack closer together than unsaturated lipids. By packing close together, a strong London
dispersion force occurs between the saturated lipids, creating localized regions within the cell membrane with high viscosities, called lipid rafts [16] [17]. Therefore, depending on the lipid composition of the cellular membrane and the location within the membrane, a lipophilic fluorescent dye molecule will experience a different environment and thus different rates of diffusion.

2.2.2 Diffusion in Cells

Cellular membranes are heterogeneous mixtures of lipids and proteins, where membrane proteins make up 50% or more by volume of the membrane. The plasma membrane is compartmentalized into corrals by membrane proteins interacting with the cytoskeleton [18]. Lipids and membrane proteins are transiently trapped within these corrals, which can lead to anomalous subdiffusion. Inside eukaryotic cells (e.g., animal cells), there are cellular membrane structures called organelles. Figure 2.5 shows the major components of a eukaryotic cell. The lipid and protein species within each cellular membrane are different and are actively regulated to maintain a particular composition. Therefore, lipophilic fluorescent dye diffusion may vary when located in different cellular membranes.

Figure 2.5: The major components of a eukaryotic cell shown in cross section. The process of endocytosis is illustrated in the inset.

After staining a cell with lipophilic fluorescent dye, the dye molecules incorporate into
the cell’s plasma membrane. Through a process called endocytosis (illustrated in inset of Figure 2.5), lipophilic fluorescent dyes are transported into the cell and to the other lipophilic environments (organelles). Endocytosis is a protein-mediated process by which a piece of the plasma membrane is pinched off and forms a vesicle. Vesicles diffuse through the intracellular space (cytosol) and are, at times, actively transported by molecular motors to organelles or recycled back to the plasma membrane. In addition to being transported into a cell, lipophilic fluorescent dyes can be transported out of the cell and through the aqueous gap to neighboring cells.

2.2.3 Diffusion between Cells

Lipids can be transferred between neighboring cells by micelles, extracellular proteins, or at cell-cell contact points. Figure 2.6 illustrates the processes by which lipophilic molecules can diffuse from cell to cell. The exact mechanism by which lipids transfer via cell-cell contact points is not known, but the process is facilitated by the close proximity of the adjacent cells. Micelle and protein-facilitated diffusion involves fluctuations in the membrane leading to lipid molecules disassociating from the cell membrane and either forming or binding to water soluble objects that can diffuse to neighboring cells.

Lipids aggregate in water due to the hydrophobic effect, an entropic force that minimizes the free energy of the system (i.e., solvent + lipophilic molecules) by clumping hydrophobic molecules together. Because hydrophobic molecules disrupt the hydrogen-bonding network between water molecules, there is a hydrophobic force that keeps lipids associated with the cell membrane. The hydrophobic force is stronger for lipids with longer chains because the larger molecule disrupts more water molecules than shorter chain length molecules. For a short chain length molecule, thermal fluctuations can knock/disassociate the lipid from the membrane, and often times the lipid simply returns to the membrane. However, if the rate of lipid disassociation is high enough, then a group of lipids can aggregate in solution, forming a micelle. In the form of a micelle, lipids become water soluble and thus can diffuse through the aqueous medium to another cell.
Figure 2.6: Cartoon illustrating the possible processes capable of transporting lipophilic fluorescent dye molecules between cells. Each process that transports lipophilic fluorescent dyes from cell A to cell B is indicated by arrows (closed arrow heads). Lipids, along with lipophilic fluorescent dyes, can disassociated from the membrane of cell B, form a micelle, and diffuse to cell B. Gap junction formation pulls adjacent cells closer together, allowing lipophilic fluorescent dye molecules to diffuse to cell B, possibly through lateral diffusion along transient lipid bridges (not shown). Water soluble proteins that are capable of binding lipids, like bovine serum albumin (BSA), can carry lipophilic fluorescent dye molecules from cell A to B. Membrane proteins, other than connexins, have not been illustrated for clarity.
Water soluble proteins can also transfer lipids to neighboring cells. There are extracellular proteins with lipid binding sites that can pick-up a disassociated, or weakly associated, lipid and transfer it to another cell through the aqueous medium. For example, bovine serum albumin (BSA) is a blood protein contained within cell culture media that has the capability of binding and transferring lipids between cultured cells.

Lipids can be transferred between cells at cell-cell contact points. Network forming cells create gap junctions that connect two adjacent cells and are made of membrane proteins called connexins. Connexins span the plasma membrane of both cells, pulling the two cells closer together. Intracellular molecules [19] as well as lipids [8] have been shown to transfer between cells connected by gap junctions. It is not known precisely how lipids transfer between cells at gap junctions, but it has been proposed that transient lipid bridges form allowing lipids to diffuse laterally to the adjacent cell [8].

2.2.4 Measuring Diffusion

To measure the diffusion of a large number of molecules, a common method is to create a concentration gradient and monitor the resulting dispersion of the gradient over time. This diffusion is quantified by calculating a diffusion coefficient from a solution to the diffusion equation.

Through diffusion, molecules spread out from regions of high concentrations to regions of lower concentration. The flux, $J$, of molecules through an area (per unit time) across a concentration gradient, $C$, is given by Fick’s first law,

$$J = -D \nabla C,$$

where $D$ is the diffusion coefficient. If the molecules do not undergo any chemical reactions (i.e., no sinks or sources of molecules), then the time rate of change of the molecules in a region is equal to the sum of the molecular flux into and out of the region. This statement is expressed mathematically by the continuity equation,

$$\frac{\partial C}{\partial t} + \nabla \cdot J = 0.$$
By combining Fick’s first law (Equation 2.9) and the continuity equation (Equation 2.10), the diffusion equation (Fick’s second law) is obtained:

$$\frac{\partial C}{\partial t} = \nabla \cdot (D(C) \nabla C).$$  \tag{2.11}

The diffusion equation describes the spatial-temporal dissipation of a concentration gradient.

By starting from the diffusion equation, an expression can be obtained that models the molecular diffusion for a particular situation and can be used to calculate the diffusion coefficient. The expression derived from the diffusion equation depends on the specific geometrical dimensions of the diffusion, as well as additional parameters used to characterize the distinct diffusion for a given situation. For example, the diffusion of fluorescent dye molecules into a cylindrical volume of fixed radius was modeled by Equation 5.1. This expression was used to calculate the rate of diffusion of dye molecules within living cells (Chapter 5 Section 5.8 for specific technique and Chapter 6 Section 6.2 for results).

Sometimes the specific situation for a diffusing molecule is too complicated and/or not well understood enough to formulate a suitable expression from the diffusion equation. In these cases, the rate of diffusion of molecules can be estimated using Equation 2.7. This formula says that the area ($\langle r^2 \rangle$), which the molecules spread out from the location of initial high concentration, is proportional to the duration of time for which the molecules diffuse, and the constant of proportionality is the diffusion coefficient. This estimate was used to obtain a relative measure of the rate of diffusion of dye molecules between living cells (Chapter 5 Section 5.5 for technique and Chapter 6 Section 6.1 for results).
Chapter 3

Experimental Technique for Measuring Two-Photon Excitation Action Cross Sections

Because fluorescent dyes have broad fluorescence emission spectra, it is difficult to image multiple dyes each with a different color within a single specimen, while obtaining complete segregation of all the colors. Although, there are techniques using 1PE to separate dyes with overlapping emission spectra, they are not sufficient to obtain images of 4 or more colors. The number of discrete colors can be extended by using 2PE. The efficient use of 2PE as a multicolor imaging strategy requires knowledge of the 2PE action cross section, a measure of the probability that a fluorescent dye molecule will be two-photon excited and emit a photon of fluorescence. By measuring the 2PE action cross section of fluorescent dyes over the tuning range of a Ti:S laser, the standard 2PE laser, multicolor imaging protocols can be designed to maximize the number of distinct colors in a sample. This chapter describes the method used to determine the 2PE action cross section for fluorescent dyes as a function of excitation wavelength.

3.1 Approach Overview

2PE action cross sections were determined by systematically varying the intensity of the excitation laser beam directed into the dye solution for various wavelengths and recording the amount of fluorescence generated. To calculate the 2PE action cross section, the in-
tensity and the spatial-temporal shape and frequency of the laser pulses have to be taken into account along with the detection efficiency of the optical collection and detection apparatus, the dye concentration, and the index of refraction of the solvent. To avoid the requirement for accurate measurements of the spatial-temporal characteristics of the laser pulses, a standard ratiometric approach was used [15], comparing the fluorescence generated from the NV dyes to fluorescein, a well-characterized fluorescent dye [15]. Another well-characterized fluorescent dye, Lucifer Yellow, was measured along with the NV dyes and served as an internal control [15]. The 2PE action cross sections were measured for NeuroVue Jade (NVJ), Orange (NVO), Red (NVR), Burgundy (NVB), and the violet candidates PTIR334, PTIR335, and PTIR336. All commercial and candidate dyes were provided in 1 mM solutions in ethanol by MTTI (West Chester, PA) holding proprietary rights to novel fluorescence-based technologies from PTI Research Inc. (Exton, PA). The lipophilic neurotracer dyes were prepared in ethanol and diluted to approximately 50 µM. Fluorescein and Lucifer Yellow were obtained from Invitrogen (Carlsbad, CA) in powdered form, prepared in ultra-pure water, and diluted to approximately 10 and 50 µM, respectively. All dye solutions were placed in sealed quartz cuvettes (Starna Cells, Atascadero, CA) for all measurements.

By taking the ratio of the fluorescence, given by Equation 2.6, of a dye with unknown 2PE action cross section \((U)\) relative to a dye with a known \((K)\) 2PE action cross section, the unknown 2PE action cross section can be determined from:

\[
(\sigma_2 \eta)_U = (\sigma_2 \eta)_K \left( \frac{\phi_K}{\phi_U} \right) \left( \frac{C_K}{C_U} \right) \left( \frac{n_K}{n_U} \right) \left( \frac{a_{2,U}}{a_{2,K}} \right).
\]

(3.1)

where \(\sigma_2\) is the 2PE cross section, \(\eta\) is the fluorescence quantum yield, \(\phi\) is the fluorescence detection efficiency, \(C\) is the dye concentration, \(n\) is the refractive index of the solvent at the excitation wavelength, and \(a_2\) is the quadratic rate of increase of fluorescence with laser intensity. The 2PE action cross section for the reference dye, fluorescein, was obtained from the literature [15]. The refractive indices for water [20] and ethanol [21] were determined from the literature. All other quantities were measured independently.
3.2 Determination of Quadratic Rate of Increase of Fluorescence with Laser Power

As described in Chapter 2, fluorescence, produced by 2PE, is proportional to the excitation laser power squared. The quadratic coefficient was used to calculate the 2PE action cross section (Equation 3.1), and was determined from fluorescence intensity versus laser power measurements.

The fluorescence intensity was measured by placing the dye solution within a sealed cuvette in the experimental apparatus shown in Figure 3.1. The cuvette was placed at the focus of a Plan Archomat 10x/0.25 NA objective (Olympus Corp., Central Valley, PA). The sample was excited at wavelengths ranging from 720 - 1040 nm using the mode-locked pulse train of a femtosecond Ti:S laser (Chameleon XR, Coherent, Inc., Santa Clara, CA). The excitation beam was expanded from 6 mm to 2.4 cm by a telescopic beam expander and then directed into the objective using a 700 nm short pass dichroic (700 DCSP, Chroma Technology Corp., Rockingham, VT). The resulting fluorescence was collected using the same objective, passed back through the dichroic and filtered using a Schott BGG22 blue-glass filter before being detected by a photomultiplier tube (PMT) (HC120-03, Hamamatsu Corp., Bridgewater, NJ). Photons arriving at the PMT were counted using a SR400 photon counter (Stanford Research Systems, Inc., Sunnyvale, CA) controlled by a LabView program (Section 3.3), which simultaneously recorded the average excitation laser power measured by a power meter (S20MM, Thor Labs, Newton, NJ). For each dye, the mean count rate of three trials was determined as a function of excitation laser power, adjusted by attenuation with a neutral density filter wheel (5215, New Focus, San Jose, CA). Due to excited state saturation and practical limits to acquisition time, laser powers were restricted to between 2 - 20 mW as measured at the power meter.

Additionally, the experimental apparatus (Figure 3.1) was modified to accommodate the far-red fluorescence emission of NVM and NVB. The Schott BGG22 blue-glass filter was replaced with a 840-nm short pass filter (Chroma, Bellows Falls, VT). The 700 DSCP dichroic
Figure 3.1: Experimental apparatus used to measure 2PE action cross sections for commercial and violet candidate NV dyes. Fluorescence intensity versus laser power measurements were made by varying the power of the femtosecond pulse train of a Ti:S laser using a neutral density (ND) filter and recording the fluorescence generated in the dye solution within a cuvette placed at the focus of an objective. The excitation beam was expanded by a telescopic beam expander to fill the back aperture of the objective. The 700 nm short pass dichroic (700 DCSP) reflected the excitation beam to the sample while only allowing the transmission of dye fluorescence to the PMT. A Schott BGG22 blueglass filter was used to block stray background and laser light from entering PMT while permitting dye fluorescence to enter. The fluorescence counts determined by the SR400 photon counter were recorded, along with the laser power, by a LabView program. Image obtained from [6].

was removed, and the cuvette and objective were placed such that the PMT detected the resulting fluorescence at ninety degrees.

To find the quadratic rate of increase of fluorescence with laser power, a 4th-order polynomial (Equation 3.2) was fit to the fluorescence intensity versus laser power data using the Levenberg-Marquardt algorithm [22], a standard nonlinear least squares curve fitting technique. The polynomial accounts for sources of detected photons other than from 2PE ($a_2$),

$$F = a_0 + a_1 \cdot P + a_2 \cdot P^2 + a_3 \cdot P^3 + a_4 \cdot P^4. \quad (3.2)$$

The constant term, $a_0$, was included when there was significant background or electronic noise in the detector. When 1PE occurred, the linear term, $a_1$, was included. The higher order terms, $a_3$ and $a_4$, were required to account for 3- or 4-photon excitation, or more complicated photophysical events, such as multiple, sequential excitation events (either 2+1 or 2+2 photon excitation).
Log-log plots of fluorescence intensity versus laser power were used as an initial guide to determine the appropriate terms to fit to the fluorescence intensity versus laser power. The slope of a line fit to the log-log plots, $p$, is the excitation photon order, which is the average number of photons involved in the molecular excitation. The excitation photon order was determined with Equation 3.3,

$$\log \langle F(t) \rangle = p \cdot \log \langle P(t) \rangle + \log (B),$$  \hspace{1cm} (3.3)

where $B$ is $1/2 \phi \eta \sigma_2 C \left[ g^{(2)}/(f \tau) \right] \left[ 8 \, n/(\pi \lambda) \right] \langle P(t) \rangle^2$ from Equation 2.6. For pure 2PE, the slope is 2. For the 2PE case, two curves were fit to the data: one with only the quadratic term and the second with the quadratic term along with the constant term. When the linear fit to the log-log plot yielded a slope of less than 2 (e.g., 1.8) or greater than 2 (e.g., 2.2), then additional fits were explored including linear terms or higher order terms, respectively. For each fit, $\chi^2$ values were calculated to obtain a measure of the “goodness of the fit”. An F-test [23] was performed to determine if additional terms improved the fit by a statistically significant amount. For the original setup (Figure 3.1), typically only the quadratic term was necessary to fit the data with very few exceptions. In contrast, the data obtained from the setup modified for the far-red emitting dyes often required the $a_4$ term for fluorescein, NVB, and Lucifer Yellow, and the $a_1$ term for NVM in addition to the quadratic term.

### 3.3 LabView Program used to Control Detection System Hardware and Record Measurements

A LabView (National Instruments, Austin, TX) program was written to control the photon counting hardware, and to acquire and store data from fluorescence versus laser power measurements. The LabView program conducted measurements for several fluorescent dyes at multiple laser intensities and over many excitation wavelengths. The code for the program is displayed in Appendix A. A flow chart illustrating the logical flow of the program is presented in Figure 3.2 and the virtual instrument front panel is shown in Figure 3.3.
Figure 3.2: Logical flow of LabView program. This flowchart illustrates the sequence of steps in the LabView program used to control the photon counter and to record dye fluorescence versus excitation laser power measurements.
Figure 3.3: The virtual instrument front panel for LabView Program. The LabView program was used to control photon counter and to record dye fluorescence versus excitation laser power measurements.
To begin the program, the user enters the photon counter settings: discriminator voltage level, acquisition time, and delay time between trials. The discriminator voltage level was set, based on oscilloscope readings, to a threshold such that only photons were counted, not electronic noise. The acquisition time was set to obtain on the order of a million counts per second for the brightest dye in the set. When there are no errors in configuring the photon counter, the measurement parameters are entered by the user: data points to average, as well as the number of solutions and laser powers in the measurement. The user places the sample in the holder (Figure 3.1) and initiates the measurement sequence. The LabView program calculates the mean and standard deviation for the sample photon counts and laser powers at each wavelength.

At times, faulty communication between the photon counter and LabView program resulted in errors in sample collection. Therefore, the user is given the opportunity to repeat the previous measurement with the ‘accept data’ or ‘repeat measurement’ buttons. The LabView program repeats the readings for as many samples and laser powers the user has entered. When measurements are completed, the LabView program saves the recorded data to a text file.

3.4 Determination of Fluorescence Emission Spectrum

The fluorescence emission spectrum for each dye was determined by ninety degree reflectance measurements. By placing the dye solution in a sealed cuvette in the path of the output of a pulsed xenon light source (OceanOptics PX-2, Dunedin, FL), fluorescence was generated and collected by a fiber-optically coupled, converging lens located ninety degrees to the optical axis of the excitation source. The collected fluorescence was sent into a spectrometer (OceanOptics SD2000) and the fluorescence emission spectrum for the dye was recorded by OOIBase32 software (OceanOptics). The fluorescence emission spectra for NV dyes is presented in Figure 2.3. The fluorescence emission spectrum was measured to calculate the fluorescence detection efficiency of the experimental system.
3.5 Measurement of Fluorescence Detection Efficiency

As dye fluorescence passes through the experimental apparatus (Figure 3.1), photons are lost due to wavelength-dependent scattering, reflection, and absorption by the optics. Because each dye has a different fluorescence emission spectrum, the photon loss through the optics yields preferential detection of certain dyes over others. This can result in one fluorescent dye appearing artificially dimmer than another, simply because the apparatus is less efficient at detecting its particular fluorescence emission spectrum. If this discrepancy is not taken into account, an artificially low 2PE action cross section will be calculated.

The fluorescence detection efficiency is the ratio of the number of detected to the number of emitted fluorescence photons. The efficiency was calculated using Equation 3.4,

\[ \phi_{dye} = \frac{\int F(\lambda) \cdot \phi_{sys}(\lambda) d\lambda}{\int F(\lambda) d\lambda \int \phi_{sys}(\lambda) d\lambda}, \]

where \( F(\lambda) \) is the fluorescence emission spectrum of the dye, and \( \phi \) is the detection efficiency of the system. The fluorescence emission spectrum for each dye was measured as described in Section 3.4. The system detection efficiency was calculated from the ratio of the detected photon count rate to the incident photon count rate using a stable, tunable, narrow-band (10 nm) beam of light. The tunable light source was produced by passing the emission of an air-cooled quartz-tungsten-halogen lamp through a home-built Czerny-Turner monochromator. The output of the monochromator was fiber-optically coupled and passed through the collection optics of the experimental apparatus (Figure 3.1). The design and construction of the tunable light source is described in Appendix B.

The fluorescence detection efficiency determined for each round of measurements is summarized in Table 3.1. Two rounds of measurements were taken with the setup using a Schott BGG22 blue-glass filter (Figure 3.1), and for measurements of the far-red emitting NV dyes, a modified setup with a red short pass (RSP) filter was used (Section 3.2).
Table 3.1: Summary of Dye Fluorescence Detection Efficiency Measurements

<table>
<thead>
<tr>
<th>BGG22 filter (1)</th>
<th>BGG22 filter (2)</th>
<th>RSP filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTIR334</td>
<td>80% NVJ</td>
<td>55% NVM</td>
</tr>
<tr>
<td>PTIR335</td>
<td>60% NVO</td>
<td>3% NVB</td>
</tr>
<tr>
<td>PTIR336</td>
<td>71% NVR</td>
<td></td>
</tr>
<tr>
<td>LY</td>
<td>59% LY</td>
<td>39% LY</td>
</tr>
<tr>
<td>FL</td>
<td>79% FL</td>
<td>56% FL</td>
</tr>
</tbody>
</table>

LY = Lucifer Yellow, FL = fluorescein

3.6 Measurement of Dye Concentration

The dye concentration was also required to calculate the 2PE action cross section using Equation 3.1. Dye concentration was determined by measuring the optical density (OD) of the sample, calculated using the Beer-Lambert law,

\[ OD \equiv \log \left( \frac{I_0}{I} \right) = \epsilon C d, \]  

(3.5)

where \( I_0 \) is the intensity of the incident light, \( I \) is the intensity of the transmitted light, \( \epsilon \) is the molar extinction coefficient of the fluorescent dye in \( M^{-1} \cdot cm^{-1} \), \( C \) is the concentration of the fluorescent dye in \( mol \cdot L^{-1} \), and \( d \) is the optical path length. Dye solutions in sealed cuvettes were placed in the path of the output from a quartz-tungsten-halogen lamp (OceanOptics PX-2, Dunedin, FL). The transmitted light was fiber-optically coupled into a spectrometer (OceanOptics SD2000). By comparing the transmitted light through the dye solution to light transmitted through the solvent only, the absorption spectrum for the dye was obtained using OOIBase32 software (OceanOptics). NV dye absorption spectra are displayed in Figure 2.3. The optical path length of the quartz cuvettes (Starna Cells) was 3 mm. The molar extinction coefficients for fluorescein and Lucifer Yellow were obtained from Acros Organics (Fair Lawn, New Jersey) and Invitrogen (Carlsbad, CA), respectively. The molar extinction coefficients for the NV dyes were obtained from MTTI. The range of concentrations for the NV dyes and Lucifer Yellow were approximately \( 50 \pm 5 \ \mu M \), while the range for fluorescein was approximately \( 10 \pm 5 \ \mu M \). Over time the concentration of the samples decreased, on average \( 0.5 \ \mu M^{-1} \) per day. The concentration decreased, instead of increasing as expected with solvent evaporation, because as the solvent evaporated, dye was
deposited on the cuvette cap. Therefore, every two days the concentration was measured and the value was used in the calculations for the most recent measurements.

3.7 Summary of Experimental Technique

This chapter described the materials and methods used to determine the 2PE action cross section for NV dyes. A standard ratiometric approach, comparing the fluorescence from NV dyes to a well-characterized dye, fluorescein, was used. The dye fluorescence was detected as a function of laser power and fit to a polynomial to determine the quadratic rate of increase of fluorescence with laser power. The quadratic coefficient, along with the detection efficiency of the experimental system, dye concentration, and refractive index of dye solvent were determined and substituted into Equation 3.1 to calculate the 2PE action cross section. The results of this method and their implementation for multicolor labeling are presented in Chapter 4.
Chapter 4

Measurement of Two-Photon Excitation Action Cross Section for Novel Lipophilic Fluorescent Dyes

Multicolor tracing of multiple neuronal connections provides neurobiologists with the means to effectively use and maximize the information obtained from rare, complex mutant animal models. Moreover, multicolor neurotracing can dissect the complex patterns of connections in diffuse neuronal networks where individual neurons send information to multiple targets. To perform multicolor tracing of neuronal pathways, a set of tracers is needed that is spectrally distinct and diffusion matched. Molecular Targeting Technologies, Inc. (MTTI) has produced a set of lipophilic fluorescent molecules designed for multicolor neurotracing, called NeuroVue (NV) Dyes. Using confocal, 1PE LSM alone, only 2 - 3 colors can be imaged without fluorescence emission cross contamination. To extend the number of spectrally distinct colors, 2PE LSM can be used, provided the 2PE action cross-section spectra are known. This important quantity was measured for NeuroVue Jade (NVJ), Orange (NVO), Red (NVR), Burgundy (NVB), and the violet candidates PTIR334, PTIR335, and PTIR336. 2PE action cross sections were calculated using a ratiometric approach with a well-characterized fluorescent dye as a reference (Section 3.1). Knowing the 2PE action cross-section spectra, 2PE imaging strategies can be designed to separate spectrally overlapping fluorophores and thus obtain multicolor labeling of neuronal connections.
4.1 Two-Photon Excitation of NV Dyes

Dye fluorescence versus excitation laser power measurements were made to determine if NV dyes could be efficiently two-photon excited. As described in Chapter 3, the measurements were performed by placing NV dye solution in a sealed cuvette at the focus of an objective. Fluorescence generated by the femtosecond pulse train of a Ti:S laser was detected by a PMT. Photon count rates as a function of laser power were recorded for excitation wavelengths ranging from 720 - 1040 nm. From the fluorescence versus laser power data, the excitation photon order was determined. The excitation photon order is the average number of photons simultaneously absorbed per dye molecule at an excitation wavelength. For 2PE, the excitation photon order is 2.

The excitation photon order was obtained by producing a log-log plot of fluorescence intensity versus laser power and fitting a line to the data (Equation 3.3). As demonstrated in Figure 4.1, NVJ, NVO, NVR, and all violet candidate NV dyes can be two-photon excited with the output of a modelocked Ti:S laser. All the fluorescent dyes measured in the first two rounds of measurements (BGG22 filter setup, Figure 4.1 A and B) exhibited pure or near-pure 2PE with a few exceptions. When excited by 800 and 820 nm Ti:S light, NVO exhibited excitation photon order between 1.0 and 1.5 indicating that a majority of the photons detected were due to 1PE. NVO also produced very small photon count rates (large uncertainties), indicating that NVO is poorly excited at these wavelengths.

The results are not as straightforward for the far-red emitting dyes (Figure 4.1 C). A systematic trend of the excitation photon order increasing from 880 nm, peaking at 940 nm, and decreasing to 1040 nm appears for all fluorescent dyes measured in this 90° configuration. The trend appears to be primarily dependent on excitation wavelength of the Ti:S laser and not fluorescence emission wavelength. All the 90° fluorescence emission measurements were used to calculate 2PE action cross sections, except the 940-nm measurements because of the unusually high excitation photon order.
Figure 4.1: Excitation photon order of NV dyes as a function of wavelength. Two rounds of measurements were made with the original setup (Figure 3.1) (A and B), and a third round was performed (C) with 90° fluorescence emission detection (Section 3.2) to measure the far-red fluorescence of NVM and NVB dyes. An excitation photon order of 2 indicates pure 2PE of the dye. LY stands for Lucifer Yellow.

4.2 Measurements of Two-Photon Excitation Action Cross Section

2PE action cross sections for fluorescent dyes were determined using a standard ratiometric method, comparing fluorescence from dyes with unknown cross sections to a reference dye with a known cross section, fluorescein. From dye fluorescence versus excitation laser power measurements, the quadratic rate of fluorescence increase with laser power was calculated. By determining the quadratic coefficient, detection efficiency of the fluorescence detection system, dye concentration, and refractive index of the dye solvent, the 2PE action
cross sections were calculated using Equation 3.1. 2PE action cross sections were measured over the range of a Ti:S laser, 720 - 1040 nm.

4.2.1 Experimental Control

Lucifer Yellow, a well-characterized dye, was used as an experimental control for the method of determining 2PE action cross sections of fluorescent dyes. Figure 4.2 presents the measurements for each round in this thesis (black symbols) along with the literature values (white symbols) [15]. For both the measurements in this thesis and from the literature, the peak 2PE action cross section for Lucifer Yellow appear to be around 1 GM at an excitation wavelength between 820 and 840 nm. Additionally, the general features of the spectra are very similar, including a constant dip in the value at 860 nm. Therefore, the 2PE action cross section measurements in this thesis are consistent with published measurements performed by another group and can be consider reliable.

![Figure 4.2: 2PE action cross-section spectra for Lucifer Yellow. The measurements in this thesis (black shapes) are compared with those obtained from the scientific literature (white shapes) [15]. Three independent measurements were performed in this thesis: two with a (BGG22) blue glass filter (Figure 3.1), and the third using a modified setup with a red short-pass filter (RSP). The three sets of data from Xu and Webb (1996) were obtained using different mirror sets to cover the entire tuning range of the Ti:S laser. Error bars are standard deviations; errors are not shown for literature values.](image-url)
4.2.2 Two-Photon Excitation Spectra for NV Dyes

The 2PE action cross-section spectra for the commercial and violet candidate NV dyes are displayed in Figure 4.3 and peak values are summarized in Table 4.1. The violet candidate dyes (PTIR334, 335, 336) have similar 2PE action cross sections, with peak values ranging from 0.05 - 0.20 GM at wavelengths roughly twice their 1PE peaks. These are the least bright of the set but are comparable to regularly imaged biological fluorophores [24]. NVJ has a modest peak value at twice its 1PE peaks. NVO and NVR are very bright two-photon fluorophores with peak cross sections comparable to the bright fluorophores fluorescein and rhodamine [15]. The 2PE peaks for NVO and NVR are blue-shifted with respect to their 1PE peaks. NVM and NVB, similar to NVO and NVR, have blue-shifted and large two-photon peaks.

Figure 4.3: 2PE action cross sections for commercial and violet candidate NV dyes. 1 Göppert-Mayer (GM) = $10^{-50}$ cm$^4$ s photons$^{-1}$. Image modified from [6].
Table 4.1: Summary of Two-Photon Excitation Action Cross Section Peak Values for NV Dyes

<table>
<thead>
<tr>
<th>Name</th>
<th>abbrev</th>
<th>TP Cross section peak (GM)</th>
<th>@ λ peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTIR 334</td>
<td>334</td>
<td>0.183(4)</td>
<td>880</td>
</tr>
<tr>
<td>PTIR 335</td>
<td>335</td>
<td>0.108(8)</td>
<td>820</td>
</tr>
<tr>
<td>PTIR 336</td>
<td>336</td>
<td>0.056(7)</td>
<td>840</td>
</tr>
<tr>
<td>NeuroVue Jade</td>
<td>NVJ</td>
<td>2.0(3)</td>
<td>920</td>
</tr>
<tr>
<td>NeuroVue Orange</td>
<td>NVO</td>
<td>258(30)</td>
<td>720</td>
</tr>
<tr>
<td>NeuroVue Red</td>
<td>NVR</td>
<td>28.3(6)</td>
<td>760</td>
</tr>
<tr>
<td>NeuroVue Maroon</td>
<td>NVM</td>
<td>15(1)</td>
<td>1040</td>
</tr>
<tr>
<td>NeuroVue Burgundy</td>
<td>NVB</td>
<td>468(52)</td>
<td>900</td>
</tr>
</tbody>
</table>

Uncertainties in the least significant digits are given in parentheses.

4.3 Discussion

With the knowledge of 2PE action cross-section spectra, 2PE imaging strategies can be designed for multicolor labeling. For example, 900 nm would be good choice to simultaneously excite all NV dyes with a single wavelength. However, this protocol would contain fluorescence cross contamination by spectral neighbors in the NV dye set. As discussed in Chapter 2, a few pairs of NV dyes, like NVO and NVR, cannot be completely separated spectrally by 1PE imaging strategies. 2PE strategies can be used to achieve complete spectral separation. Figure 4.4 compares the 1PE and 2PE spectra for NVO and NVR. To minimize the cross contamination of NVO fluorescence collected in the NVR emission channel, the excitation wavelength should be chosen to maximize the ratio of NVR to NVO absorption. One photon excitation (bottom and right axes) can achieve a maximum ratio of 100 by exciting NVR over NVO with an excitation wavelength in the range of 580 - 600 nm. With 2PE (left and top axes), the excitation ratio is increased to approximately 700 when exciting NVR at 800 nm. This wavelength is easily accessible to a Ti:S laser and near the peak 2PE wavelength for NVR. By contrast, 1PE of NVR with 580 - 600 nm light reduces its brightness by 5 - 10 fold from its peak, and the typical lasers available with most commercial confocal microscopes do not emit in this range.

Using the 2PE action cross-section spectra as a guide (Figure 4.3), similar protocols can be designed to achieve spectral separation of the violet candidates from NVJ and the far-red
emitted dyes, NVM from NVB, all which are difficult to segregate using 1PE. Because there might be slight spectral differences for each dye when used in cells or tissue compared to pure ethanol, some tissue-specific optimization of the imaging protocols may need to be performed. Nevertheless, using a combination of 2PE and 1PE in multi-laser sequential imaging, six-color images of fixed nervous tissue have now been demonstrated [6].

### 4.4 Conclusions

2PE action cross sections for NV dyes were measured over the tuning range of a Ti:S laser using a ratiometric method. The 2PE action cross section indicate that all violet candidate and commercial NV dyes can be two-photon excited efficiently enough to use in 2PE microscopy. Additionally, NV dyes with fluorescence emission cross-contamination can be separated using 2PE imaging strategies. Knowledge of 2PE spectra of NV dyes allows neurobiologists to label multiple neuronal pathways, extending the power of lipophilic fluorescent dyes to dissect neuronal topology and connections.

After investigating the photophysical properties of lipophilic fluorescent dyes, the bio-
physical characteristics of lipophilic fluorescent dyes in living cells are explored. Labeling and probing membrane dynamics is another common use of lipophilic fluorescent dyes. Chapters 5 and 6 explore the influence of hydrocarbon chain length on the diffusion of lipophilic fluorescent dyes within individual and between connected living cells.
Chapter 5

Experimental Techniques for Measuring Fluorescent Dye Diffusion

To investigate the mechanism of lipid transcellular diffusion, the diffusion of lipophilic fluorescent dye molecules was measured in living cells. A series of prototype lipophilic fluorescent dye molecules with hydrocarbon chains of varying lengths was used to examine lipid diffusion within a cellular network and within individual cells. To measure dye diffusion, a concentration gradient was established between cells by incorporating a chain-length-variant dye into a single cell within the network. To explore the resulting transcellular diffusion, a reliable technique was needed to label a single cell (donor cell) within an established network and then monitor the subsequent diffusion into the unlabeled cells (acceptor cells). For this purpose, three methods were explored: cell dropping, soft lipid-assisted microinjection (SLAM), and whole-cell photobleaching. LSM was used to monitor the diffusion of fluorescent lipophilic dye molecules from initially labeled cells into the surrounding cellular network. The rate of transcellular diffusion was estimated by the spread of dye into surrounding cells. Additionally, the diffusion of the lipophilic fluorescent dyes within single cells was investigated using fluorescence recovery after photobleaching (FRAP). FRAP establishes a concentration gradient by partially “unlabeling” a small region within a cell, and by monitoring the dissipation of the gradient over time, the rate of diffusion can be calculated using a solution to the diffusion equation.
The materials and methods involved in the preparation and culture of living cells, the staining of living cells with fluorescence dyes, the collection of fluorescence images via LSM, and the processing of the collected images are described. Then the three labeling techniques used to investigate the mechanism of lipid diffusion in living cells are discussed.

### 5.1 Cell Culture

All diffusion studies were conducted using the MC-2T3 immortalized mouse cell line [25]. Cells were grown in Minimum Essential Alpha Media (GIBCO-BRL, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (GIBCO-BRL, Grand Island, NY) and 5000 units/mL Penicillin-Streptomycin antibiotics. Cells were cultured at 37°C and 5% CO₂ before staining with fluorescent dyes (Section 5.2). Additionally, cells were fixed by incubation with 4% formaldehyde prepared from paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and phosphate buffer saline (PBS) solution for 20 minutes at room temperature (20°C) before staining with fluorescent dyes (Section 5.2).

### 5.2 Staining Living Cells with Fluorescent Dyes

Cells were labeled with lipophilic fluorescent dye molecules obtained from MTTI (West Chester, PA). A series of lipophilic fluorescent dyes were synthesized with the same head group (NeuroVue Maroon, NVM) but with hydrocarbon chain lengths of various lengths (Figure 2.1). Solutions of the NVM chain-length-variant dyes were prepared by dilution in Diluent C, a solution patented by MTTI designed for cell labeling. Cells, either suspended or adhered to culture dishes, were incubated with 3 µM dye and Diluent C mixture for 10 minutes at 37°C. After labeling, cells were washed with PBS three times to remove excess dye. Cells were counter-stained with the cytosolic hydrophilic fluorescent dye, Calcein AM (Invitrogen, Carlsbad, CA), which was initially dissolved in dimethyl sulfoxide (DMSO). The Calcein staining solution was prepared by dilution in a modified Tyrodes buffer supplemented with 25 µM of D-glucose (Sigma-Aldrich, St. Louis, MO) and 0.5% albumin.
from bovine serum (Sigma-Aldrich, St. Louis, MO). Living adherent cells were incubated in Calcein solution at 6 µM concentration for 20 minutes at 37°C and then washed twice with PBS to remove residual dye.

### 5.3 Cell Imaging by Laser Scanning Microscopy

Fluorescence images of living and fixed cells were acquired with a Zeiss LSM 510 NLO microscope and a 40x/0.80 NA water immersion objective (Carl Zeiss, Inc., Thornwood, NY). Living cells were imaged in a Tyrodes buffer supplemented with 25 µM of D-glucose (Sigma-Aldrich, St. Louis, MO) and 0.5% albumin from bovine serum (Sigma-Aldrich, St. Louis, MO). Fixed cells were imaged in phosphate buffer saline (PBS). All cells were imaged at room temperature (20°C). NVM and its chain-length variants were imaged using confocal LSM, while Calcein was imaged using two-photon LSM. Imaging protocols for each fluorescent dye are presented in Table 5.1. NVM was one-photon excited by 633 nm light from a HeNe laser, and the emission was collected through a pinhole of 1 Airy unit (i.e., pinhole size is equal to the diameter of an Airy disk) and a 650-710 nm band pass filter. Calcein was two-photon excited by 780 nm light from a Ti:S laser, and the emission was collected through a 500-550 nm band pass filter. The same dichroics were used for both NVM and Calcein excitation and emission detection. However, the notch filter UV/488/543/633 nm should have been used for the primary dichroic for NVM imaging. The inappropriate dichroic caused significantly less fluorescence signal to be collected by the detector (< 3% incident on dichroic). Despite this decrease in signal, fluorescence images were produced that were suitable for the diffusion studies.

**Table 5.1: Microscope Settings for NVM and Calcein Fluorescence Imaging**

<table>
<thead>
<tr>
<th>Dye Name</th>
<th>Em. filter</th>
<th>Excitation λ nm</th>
<th>Primary dichroic</th>
<th>Secondary dichroic</th>
<th>Pinhole size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVM</td>
<td>BP 650-710</td>
<td>633 (1PE)</td>
<td>HFT KP 650*</td>
<td>NFT 545</td>
<td>1</td>
</tr>
<tr>
<td>Calcein</td>
<td>BP 500-550 IR</td>
<td>780 (2PE)</td>
<td>HFT KP 650</td>
<td>NFT 545</td>
<td>Max.</td>
</tr>
</tbody>
</table>

* NVM primary dichroic should have been UV/488/543/633 (see text for explanation).
5.4 Image Processing to Determine Labeling Status of Cells

Processing of fluorescence images, to determine the intensity of dye fluorescence within each cell, was conducted with ImageJ [26]. The image containing only the Calcein signal was contrast enhanced to clarify cell boundaries, when necessary. Because different excitation laser powers were used depending on the brightness of the NVM signal, the pixel values of the NVM image channel were normalized by the percentage of the excitation laser power.

A threshold on the pixel values and an image processing operation were performed on the normalized NVM images to remove the background signal and to produce a binary image mask indicating the location of fluorescence dye. The threshold value was determined based on control images. The settings for the image processing operation (Open, which is a composite operation composed of an Erode followed by a Dilate operation) were set based on control images, obtained by labeling cells with only NVM and collecting images with the various microscope settings used during experiments. After processing, cells containing any signal in the NVM image channel were considered labeled with NVM chain-length-variant dye.

5.5 Cell Dropping

The first method used to label a single cell within a cellular network and investigate the mechanism of lipid diffusion between living cells was cell dropping. This technique involves labeling a population of donor cells suspended in solution with a lipophilic fluorescent dye and then dropping them onto a counter-labeled network of acceptor cells. After dropping, the donor cells settle within the cellular network and over time establish connections to the acceptor cells. The cells are incubated for a specified period of time and then imaged using LSM. Image processing of the fluorescence images is performed to create a binary mask indicating whether or not cells are fluorescently labeled.
Figure 5.1: Unprocessed image obtained from cell dropping experiments. The red cell was labeled with the NVM chain-length variant, MTTI109, (18, 3) and then dropped onto an established network of connected cells (green) counter-labeled with a cytosolic fluorescent dye (Calcein). The cells were incubated for 0.5 hour and then imaged with LSM to determine the spread of the NVM chain-length-variant dye into the cellular network after 0.5 hours of incubation. Scale bar is 50 µm and the height in the z-direction is 35 µm.

5.5.1 Materials and Method

To label single cells within a connected network by cell dropping, a population of suspended cells was labeled with a NVM chain-length variant as described in Section 5.2 and pipetted at a low cell concentration, typically on the order of 1000 cells per mL, onto a counter-labeled network of cells. This second population of cells was plated onto a 60-mm round cell culture dish, allowed to adhere and form networks (at least 24 hours prior to labeling), and counter-stained with Calcein as described in Section 5.2. After dropping the suspended cells, the combined cell populations were incubated in culture media for 0.5, 2, 4, 8, or 12 hours and then imaged as described in Section 5.3. Only areas containing one donor cell in the immediate vicinity of the image region were selected for imaging. Figure 5.1 shows a raw, unprocessed image of a NV-labeled, donor cell on top of a Calcein-labeled network of living cells 0.5 hours after dropping. In the front and side views of Figure 5.1, the donor cell is spherical and does not have connections to its neighbors. Over time the donor cell flattens out and forms processes extending to its neighbors, integrating itself into
the cellular network. To determine if an acceptor cell within the network was labeled with NVM chain-length-variant dye, image processing was performed on the fluorescence images as described in Section 5.4. The image processing generated a binary mask overlaid on top of the fluorescence image of the counter-labeled, cellular network. The mask indicated red for NVM chain-length variant positive labeling and black for negative labeling. Qualitative observations of transcellular diffusion were made comparing the spread of the NVM chain-length-variant dyes at various time points for living and fixed cells (Section 6.1).

The mechanism of dye diffusion between living and fixed cells is difficult to determine when the connections of the donor cell changes drastically over time or even never forms. This process occurs with living cells in the cell dropping technique. After dropping, the donor cells are initially not connected to the network, but over time formed connections with the surrounding, acceptor cells. Moreover, fixed donor cells within a fixed network labeled by cell dropping never form connections with their neighbors. These variations in cell connections confounds the investigation of the mechanism of intercellular lipid diffusion between living cells. Therefore, to further investigate the mechanism of lipid diffusion between cells, other methods were explored. These other methods, which are capable of labeling a single cell within an established network, are soft lipid-assisted microinjection (SLAM) and whole-cell photobleaching.

5.6 Soft Lipid-Assisted Microinjection

SLAM involves introducing foreign material into a cell, not by the conventional stab microinjection technique, but by forming a lipid bridge between the micropipette and cell membrane to gently introduce material into the cell [27]. This drastically reduces the damage to the cell as well as makes it possible to transfer lipids to the cell membrane. To form the lipid bridge, the micropipette is coated with a phospholipid and then placed into gentle contact with the lipid bilayer membrane of the cell. The original technique was slightly modified to transfer a NVM chain-length variant into an individual cell within an
established network.

Figure 5.2: Schematic diagram of the cell-labeling technique, soft lipid-assisted microinjection (SLAM). The tip of a micropipette backfilled with NVM chain-length-variant dye (blue) was lipid coated (A) and then placed into gentle contact with a living cell (B). Upon contact and a positive pressure (70 mbars), fusion occurred between the lipid-coated tip and the lipid bilayer allowing the transfer of dye into the cell membrane.

5.6.1 Materials and Methods

The SLAM protocol performed in these studies is illustrated by the cartoon in Figure 5.2. Cells were plated onto 60-mm gridded cell culture dishes at least 24 hours before injection to allow for the formation of connected networks. SLAM was performed on cells in a modified Tyrodes buffer solution. A micropipette (World Precision Instruments, Sarasota, FL) was pulled to form a tip with a 3 µm inner diameter and was back-filled with a 1 mM solution of NVM chain-length-variant dye. A 1 mM solution of the phospholipid, palmitoyl-oleoyl-phosphatidyl-choline (POPC, Sigma-Aldrich, St. Louis, MO) was prepared from a dry powder and dissolved in chloroform (Sigma-Aldrich). The tip of the micropipette was dipped into the phospholipid solution and allowed to dry in air for 30 seconds. The micropipette was mounted to a hydraulic micromanipulator oriented at approximately 45° to the culture dish and lowered into the aqueous medium bathing the cells. By placing the tip into the aqueous medium, the lipid seal is rehydrated and forms a lipid coat. Cells and the micropipette tip were visualized by transmitted light microscopy through a 10x/0.25 NA Olympus objective for course movements and a 40x/0.55 NA Olympus objective for fine movements. The lipid-coated tip was lowered into gentle contact with the membrane of a cell using the hydraulic micromanipulator. When a positive pulse of air at approximately
70 mbars (1 psi) for a duration of 0.1 seconds was applied by a PicoSpritzer (General Value Corp., Pine Brook, NJ) to the micropipette, fusion between the lipid coat and lipid membrane allowed the NVM chain-length-variant dye to diffuse into the cell membrane. Three cells were labeled with dye before recoating the tip with the phospholipid solution. The resulting cell-labeling and diffusion was monitored over time using LSM (Section 5.3). Processing of the recorded images was performed using ImageJ to determine the labeling status of cells (Section 5.4).

5.6.2 Testing and Initial Experiments with SLAM of Living Cells

The first step in testing the SLAM technique was to verify the formation of a lipid coating at the end of the micropipette tip. The microscope used in the SLAM technique did not have fluorescence capabilities so verifying the lipid coat via fluorescence labeling could not be implemented. Instead, a lipid seal was verified by the ejection of NVM chain-length-variant dye from the pipette upon applying a modest pulse of air. A pulse at 70 mbars (1 psi) for a 10 second duration was applied to a micropipette back-filled with dye. Using a 40x/0.55 NA objective, dye ejection was not observed from a micropipette dipped into the phospholipid solution. However dye ejection was observed from a micropipette that was not dipped into the phospholipid solution. If a pulse of 415 mbars (6 psi) for a duration of 10 seconds is applied to a micropipette dipped in the lipid solution, then dye release into the aqueous solution was observed. Therefore this suggested that a lipid seal was formed at the tip of the micropipette and ready to perform SLAM labeling of cells.

The modified SLAM technique was successfully implemented to label a single cell within a connected network, but technical difficulties were encountered. Using SLAM, several individual cells were labeled in their entirety, including extended processes. However, these cells showed cellular damage and inconsistent labeling determined by fluorescence imaging. It was suspected that the pulse pressure was too great (1 psi) and resulted dye being forced into the cytosol of the cell rather than gently diffusing into the membrane as intended. Additionally, the SLAM technique often resulted in no fluorescence labeling of the injected...
cell or inconsistent amount of dye observed within SLAM-labeled cells.

The inconsistent labeling was thought to be caused by an air bubble formed between the NVM chain-length-variant dye and lipid coat. A suggested solution to remove the bubble is, after forming a lipid coat on the tip, apply a high pressure pulse (2000 mbars, 30 psi) for a short duration (0.5 second) to force the dye to the tip. After the pulse, the lipid coat should reform.

To prevent cellular damage, a column of NVM chain-length-variant dye was used, instead of a pulse of air, to provide the positive pressure needed to transfer dye into the cell membrane. This solution was cumbersome and wasteful of dye, and is not recommended for future SLAM experiments. A suggested alternative is to back-fill a cytosolic solution into the micropipette and the column to provide the pressure required for SLAM, while incorporating the dye into the lipid coating for transfer.

Through testing the SLAM technique, many complications were encountered. If these complications could be overcome, the SLAM would be an excellent method to investigate the mechanism of lipid diffusion between living cells. However, because of these difficulties, another method to label a single cell within a connected network was explored, called whole-cell photobleaching.

5.7 Whole-Cell Photobleaching

To avoid the technical difficulties of SLAM, but also label a single cell integrated within a network, a technique called whole-cell photobleaching was developed. In whole-cell photobleaching, an entire cell within a fluorescently labeled network was photobleached. If a high intensity laser beam is focused onto a sample, fluorescent molecules within the illumination volume can be photobleached, losing their ability to emit fluorescence due to changing their chemical structure via covalent modification. When performed on a whole cell, photobleaching partially “unlabels” the cell allowing for the fluorescent molecules diffusing into the bleached cell from surrounding cells to be monitored. This fluorescence
recovery of the photobleached cell was monitored over time. Whole-cell photobleaching is similar to FRAP (Section 5.8) but no quantitative analysis, which was able to obtain a diffusion coefficient, was developed. Therefore, for this thesis, only qualitative analysis of the fluorescence recovery was conducted.

5.7.1 Materials and Methods

Cells were plated onto 60-mm gridded cell culture dishes at least 24 hours before photobleaching, and then stained with a NVM chain-length-variant dye (Section 5.2). Photobleaching was performed using the Region of Interest (ROI) feature of the Zeiss LSM confocal software, selecting a cell located within a NVM chain-length-variant dye-labeled network, and photobleaching the entire cell including its processes. NVM chain-length-variant dyes were photobleached with 960 nm light from a Ti:S laser at an average laser power of 50%. The initial fluorescence intensity was acquired by taking an image of the selected cell and its connected neighbors. Using LSM, the fluorescence recovery of the photobleached cell was recorded through a series of images at specific time increments after photobleaching. The intensity of each cell was determined using ImageJ and the change in the photobleached cell’s intensity was plotted over time.

5.7.2 Initial Experiments with Whole-Cell Photobleaching of Living Cells

Whole-cell photobleaching was performed on living cells labeled with the NVM chain-length variants MTTI103 (10, 3), PTIR284 (14, 3), MTTI109 (18, 3), and MTTI114 (28, 3). Cells were two-photon photobleached with 960 nm light at 25% laser power for 5 scans and a pixel dwell time of 3.2 µs. Initial fluorescence recovery scans of cells labeled with MTTI109 were conducted with the Time Series feature of the Zeiss microscope software and taken once per minute. No discernible fluorescence recovery of the cell was observed after 5 minutes so scans were made at 1, 3, and 10 hours after photobleaching. Partial fluorescence recovery of a photobleached cell labeled with MTTI109 (18, 3) was observed 10 hours after photobleaching. Taken together, these initial experiments suggest that a full recovery for
an entire cell labeled with MTTI109 will be on the order of several hours (possibly up to a couple days). During this long recovery time, dye was transported to internal cellular membranes and degraded to a non-fluorescent form. This degradation of dye molecules decreases the amount of fluorescence available for recovery, which will artificially lower the fluorescence recovery in the photobleached cell. In cell dropping studies, significant degradation of MTTI106 (24, 3) in living cells was observed to occur between 12 and 16 hours after labeling cells. Therefore, it will be difficult to measure transcellular diffusion that occurs on time-scales longer than 12 hours due to loss of fluorescence by degradation of the dye molecules.

Despite some success, the most common outcome for photobleaching an entire cell was cellular apoptosis or loss of cell from the grid where it was photobleached when returned to for imaging. Cell death was suspected to be due to photodamage caused by the high intensity of the bleaching beam. In future whole-cell photobleaching experiments, it is recommended that a lower laser power be used to photobleach cells. The disappearance of photobleached cells from their initial grid was probably due either to cell death and release into solution or to the high mobility of cells within small networks (< 4 cells per network on average). Over time, new cells were observed to enter an image region and form connections with a photobleached cell several hours after photobleaching. This movement of MC-2T3 cells within sparse networks is a possible problem with all the transcellular diffusion investigation techniques, including cell dropping and SLAM. This cell mobility was not observed in cell dropping or SLAM experiments because only confluent or near-confluent networks were used. In a confluent network, the cells cover the entire surface of the culture dish and have no room to move or grow. Therefore, it is recommended that confluent or near-confluent networks be used for future whole-cell photobleaching experiments.

After labeling cellular networks with NVM chain-length-variant dye, it was observed that there was a significant amount of dye labeling areas without cells. When an entire cell was photobleached, a portion of the dish not containing a cell was also bleached. After
photobleaching, the fluorescence “background” signal in the photobleached regions, lacking a cell, decreased in intensity. This loss of signal was interpreted as the photobleaching of dye labeling the surface of the dish or the matrix secreted by 2T3 cells. This deposit of dye could contribute to the fluorescence recovery of photobleached cell, which would confound results. Therefore in future studies, it is recommended to decrease the staining time or to stain suspended cells and then plate them onto culture dishes to avoid dye deposited onto the culture dish contributing to the fluorescence recovery of a photobleached cell.

Unlike FRAP measurements (Section 5.8), the whole-cell photobleaching technique only yielded qualitative results. The rate and extent of the fluorescence recovery of the cell depends on the number of lipophilic fluorescent dye molecules initially photobleached. Thus, future studies need to standardize or at least take into account the amount of photobleaching. Despite these complications, the whole-cell photobleaching technique is a useful method to investigate the mechanism of lipophilic fluorescent dye diffusion in living cells in combination with FRAP measurements.

5.8 Fluorescence Recovery After Photobleaching

To investigate the mechanism of lipophilic fluorescent dye diffusion in living cells from a different perspective, FRAP was used to measure the diffusion coefficient for NVM chain-length-variant dyes within a single living cell. FRAP begins by imaging a region of interest within a fluorescently labeled cell. By focusing an intense laser beam onto the region of interest, a fraction of the fluorescent molecules are photobleached, losing their ability to emit fluorescence. After photobleaching, the fluorescent molecules from outside the region diffuse into the photobleached region according to the diffusion equation (Equation 2.11). This fluorescence recovery is monitored over time using a laser beam of lower intensity. By measuring the rate and extent of the fluorescence recovery, the mobility of the fluorescent molecules can be determined using the appropriate equation modeling the diffusion of molecules into the photobleached region. The results of the FRAP measurements are
5.8.1 FRAP Experimental Technique

FRAP measurements were made using the Region of Interest (ROI) and Time Series features in the Zeiss LSM confocal software. A small circular region of interest (average 3 µm) was selected in the cell as shown in Figure 5.3 A. The initial fluorescence intensity of the region was determined from the mean of 5 scans prior to bleaching. The cell was photobleached by scanning 780 nm light from a pulsed Ti:S laser at 25% average laser power over the region. This procedure bleached a region in the shape of a uniform disk of fixed radius. The fluorescence recovery of this region was monitored using 633 nm light from a HeNe laser at 20% average laser power. The recovery sequence was recorded through a series of 120 scans, on average, separated by 1 second intervals (Figure 5.3 B).

![Figure 5.3: Illustration of the FRAP technique.](image)

The recovery curves were fit to an equation modeling the diffusion of fluorescent molecules into the photobleached region. This equation was derived from the diffusion equation (Equation 2.11) allowing for specific diffusion and photobleaching parameters. It is often observed in FRAP experiments that the fluorescence recovery signal does not recover to the initial
value. This non-recoverable fraction of fluorescent molecules is interpreted as a subset of the molecules are immobile over the duration of the measurement. Thus an immobile fraction term is included in the fluorescence recovery model, when necessary. Based on single-particle tracking and single-molecule imaging, it was found that membrane proteins and lipids exhibit anomalous diffusion. Thus an anomalous diffusion term is included in the recovery model. Because multiphoton excitation is inherently localized to a small 3D Gaussian illumination volume, photobleaching via multiphoton excitation is advantageous over single photon excitation for measuring three dimensional diffusion of fluorescent molecules. Therefore, the fluorescence recovery model allows for multiphoton excitation and photobleaching.

Starting from the diffusion equation (Equation 2.11), a fluorescence recovery model was derived for a uniformly bleached disk to match the specific method of photobleaching [6]. For $b$-photon photobleaching and $p$-photon excitation by the probe beam ($b$ and $p$ are integers), the time-dependent fluorescence recovery signal is given by,

\[
F_{b,p}(t) = F_0 f_{nr} + (1 - f_{nr}) F^0 \times \left[ 1 + \left( \frac{t}{\tau_1} \right)^\alpha \sqrt{2p(t/\tau_2)^\alpha} \times \sum_{m=0}^{\infty} A_m((\tau_1/t)^\alpha) \times \sum_{l=1}^{\infty} \frac{(-\beta)^l}{l!} (1 + 2lb(t/\tau_3)^\alpha)^{-1/2} \left[ 1 + 2p(t/\tau_2)^\alpha - \frac{1}{1+2lb(t/\tau_3)^\alpha} \right]^{-1/2} \right],
\]

where,

\[
A_m(x) = 1 - e^{-x} \left( \sum_{n=0}^{m} \frac{x^{m-n}}{(m-n)!} \right),
\]

$F^0$ is the fluorescence prior to photobleaching, $F_0$ is the fluorescence of the bleached region immediately following photobleaching, $\beta$ is the bleach depth, and $f_{nr}$ is the non-recoverable fraction. Equation 5.3 presents the characteristic diffusion time for the radial component ($\tau_1$) of the fluorescence recovery, as well as the characteristic diffusion time of the axial direction for the probe ($\tau_2$) and bleaching beams ($\tau_3$):

\[
\tau_1 = \left( \frac{w_x^2}{4\Gamma} \right)^{1/\alpha}, \quad \tau_2 = \left( \frac{w_yp^2}{4\Gamma} \right)^{1/\alpha}, \quad \tau_3 = \left( \frac{w_z^2}{4\Gamma} \right)^{1/\alpha},
\]

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where \( \Gamma \) is the transport coefficient, \( w_r \) is the radius of the ROI, and \( w_z \) and \( w_{zp} \) are the Gaussian axial radius of the bleaching beam and probe beam, respectively. All the NVM chain-length-variant dyes were two-photon photobleached \((b=2)\) and monitored via one-photon excitation \((p=1)\).

Equation 5.1 was fit to the fluorescence recovery curves using the Levenberg-Marquardt algorithm \([22]\) to optimize the fitting parameters, \( \alpha, \beta, \Gamma, \) and \( f_{nr} \). The size of the bleached region was calculated using the relation, \( w_r = \text{image scaling factor} \times \text{pixel size of ROI} \).

Theoretical values for the axial radii were estimated using high numerical aperture pseudoparaxial approximations \([28]\) with the axial full width half maximum given by,

\[
\delta_z = \frac{0.32\lambda}{n_{\text{medium}} \times \sin^2(\frac{1}{2}\theta)} \tag{5.4}
\]

where \( \theta \) is the half-angle of the light cone, and

\[
w_z = \frac{\delta_z}{\sqrt{2\ln(2)}} \tag{5.5}
\]

The best fit was determined by calculating a \( \chi^2 \) for each fit and using an F-test \([23]\) to decide if additional parameters significantly improved the fit. A nonzero immobile fraction was included only when justified by the F-test. The diffusion coefficients were calculated from the best-fit parameters using Equation 2.7 at a time of 180 seconds.

**5.8.2 Statistical Analysis of FRAP Measurements**

Statistical tests were required to determine if the measured diffusion parameters were reliably different. A between subjects t-test \([29]\) was performed to compare two independent means following the statistical decision tree shown in Figure 5.4. The decision tree was used to determine whether a pooled or non-pooled variance t-test should be performed to compare two means. If there was an unequal number of data points in the data set and the variances were determined to be reliably different, based on the between subjects variance test \([29]\), then a pooled variance between subjects t-test was performed. Otherwise a non-pooled variance between subject t-test was used to compare means. The difference in the means was determined to be statistically significant for a p-value less than 0.05.
5.9 Discussion of Experimental Techniques

To investigate the mechanism of diffusion for NVM chain-length-variant dye molecules in living cells, a suitable method with the ability to probe all aspects of transcellular diffusion of NVM chain-length-variant lipophilic dyes was desirable. Three different techniques capable of labeling a single cell within an established network were developed, cell dropping, SLAM, and whole-cell photobleaching. Additionally, FRAP was used to explore diffusion of dyes within a single cell rather than a cellular network. Table 5.2 presents the primary advantageous and disadvantageous for each technique.

Because these studies were performed with living cells, significant damage or disruption to the cells or their connections was a concern. For the cell dropping technique, NV-labeled cells are dropped onto an established, cellular network after which the dropped cells flatten out from a spherical shape and form connections to their neighbors. This process could affect the diffusion of NVM chain-length-variant dye molecules both within and between the living cells. If performed ideally, SLAM would gently introduce dye molecules into the plasma membrane of living cells, while the cell within the established network would be
<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantageous</th>
<th>Disadvantageous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Dropping</td>
<td>• Easily performed with available materials</td>
<td>• Cellular connections change drastically over time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No control over placement of donor cell in network</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unable to label fixed cells with connections to neighbors</td>
</tr>
<tr>
<td>SLAM</td>
<td>• Ability to label single cell integrated within a network</td>
<td>• Difficult to perform with available materials</td>
</tr>
<tr>
<td></td>
<td>• Ability to label fixed cells within a network</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Control over selection of donor cell in network</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Perturbs cells the least amount when performed correctly</td>
<td></td>
</tr>
<tr>
<td>Whole-Cell</td>
<td>• Easily performed with available materials</td>
<td>• Possibility of photodamage and phototoxicity</td>
</tr>
<tr>
<td>Photobleaching</td>
<td>• Ability to “label” a single cell integrated within a network</td>
<td>• Possibility of dye adhering to culture dish and then contributing to fluorescence recovery of photobleached cell</td>
</tr>
<tr>
<td></td>
<td>• Control over selection of donor cell within network</td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>• Probes spatial and temporal scales not measured by other techniques</td>
<td>• Does not directly probe spatial or temporal scales large enough for transcellular diffusion</td>
</tr>
<tr>
<td></td>
<td>• Easily performed with available materials</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.2: Measurement Techniques.* This table lists the major advantageous and disadvantageous for the techniques used to investigate the mechanism of lipophilic fluorescent dye diffusion in living cells.
minimally perturbed. However, this was not the case during the initial development of the SLAM technique. Oftentimes, the micropipette tip penetrated the cell causing parts of the cell to stick to the tip or the dye would be forcefully sprayed into the cytosol of the cell. FRAP and especially whole-cell photobleaching have the possibility of generating photodamage or phototoxicity in the cell that is photobleached with a high intensity laser beam. This damage could cause a change in the physiological state of the cell that could confound the diffusion measurements. Living cells photobleached with a very high intensity laser beam were observed to drastically change shape over a period of several seconds in whole-cell photobleaching experiments.

As part of the whole-cell photobleaching technique, living cells, adhered to a culture dish, are incubated with NVM chain-length-variant dye to label their membranes. By photobleaching the culture dish, it was observed that dye molecules adhere to the dish or the matrix secreted by 2T3 cells. These dye molecules could contribute to the fluorescence recovery of the photobleached cell confounding the investigation of the mechanism of transcellular diffusion of dye molecules.

The cell dropping and SLAM techniques directly probe the transcellular diffusion of NVM chain-length-variant dye molecules from a single cell into its neighboring cells. Whole-cell photobleaching also directly examines dye diffusion between cells but in the reverse direction, diffusion from neighboring cells into a single cell. FRAP provides a different perspective of dye molecule diffusion: diffusion within a single cell.

Unlike SLAM and whole-cell photobleaching, cell dropping has no control over the selection of how the donor cell is connected within the established network. By selecting how the donor cell is connected to its neighbors, different aspects of transcellular diffusion of lipophilic fluorescent dyes can be dissected.

Each technique has its advantages and disadvantages, but the major factor in the decision, for which technique to use, was time. Despite their many advantages over cell dropping, SLAM and Whole-Cell were developed too late in these studies to produce results. The
results presented in Chapter 6 were obtained from cell dropping and FRAP measurements. The two techniques probe different temporal and spatial scales of NVM chain-length-variant dye molecule diffusion. Therefore their results were combined to hypothesize a mechanism of dye molecule diffusion between and within living cells.
Chapter 6

Measurements of Lipophilic Fluorescent Dye Diffusion Between and Within Living Cells

Recently some naturally occurring lipids in the brain are suspected to play an important role in the communication between neurons, particularly in networks involved in learning and memory. However, the mechanism by which these lipids diffuse across the aqueous gap between neurons is unknown. To investigate the mechanism for the transcellular diffusion of lipids, the diffusion of a series of lipophilic fluorescent dyes was measured in living cells that form connected networks. All the lipophilic fluorescent dyes in the series had the same head group (NVM), but differed in the length of their hydrocarbon chains. This difference was expected to affect the dyes’ preferred route of diffusion as well as their rate of diffusion between cells. To explore a wide range of diffusion, short (6 or 10 carbons), medium (16 or 18 carbons), and long chain-length (24 or 28 carbons) dyes were studied. The transcellular diffusion of NVM chain-length-variant dyes was studied by labeling single cells within an established network using cell dropping (Section 6.1) and monitoring the spread of dye into the network over time using LSM (Section 5.3). Furthermore, the rate of diffusion of NVM chain-length-variant dye molecules within individual cells was measured using FRAP (Section 5.8). FRAP measures shorter spatial and temporal ranges than the transcellular diffusion study. It was expected that these alternate spatial and temporal scales would provide additional information on the hydrocarbon-chain-length dependence.
for the transcellular diffusion of lipophilic molecules. In addition to studying the effect of chain length on dye diffusion within single cells, FRAP measurements were performed at various time intervals after labeling cells to examine the temporal affects on dye diffusion.

6.1 Influence of Hydrocarbon Chain Length on Dye Transcellular Diffusion

The cell dropping labeling technique and LSM were used to investigate the mechanism of NVM chain-length-variant dye diffusion between living cells. As described in Section 5.5, cell dropping involved staining a suspended population of cells with a NVM chain-length variant and then dropping them onto a counter-labeled network. The network was grown to confluency and counter-labeled with an intracellular hydrophilic fluorescent dye. After incubating at 37°C for a specified period of time, the cells were imaged using LSM, and the images were processed to determine the labeling status of each cell. Images were collected for living cells labeled with MTTI103 (10, 3), MTTI109 (18, 3), and MTTI114 (28, 3) at time points ranging from 0.5 to 12 hours after dropping cells.

Additional experiments in formaldehyde fixed cells were performed to isolate the influence of active cellular processes on lipid transcellular diffusion from passive processes. To conduct cell dropping experiments with fixed cells, suspended cells and cellular networks were stained with 10-carbon or 28-carbon dyes, fixed with 4% formaldehyde, and then the fixed, suspended cells were dropped onto the fixed network. After incubation at 37°C for a specified time interval, fluorescence images of the fixed cells were collected and processed just like living cells.

6.1.1 Measurements of Dye Diffusion in Living and Fixed Cellular Networks

Representative images of living and fixed cells labeled with either 10-carbon or 28-carbon dye using the cell dropping technique are presented in Figure 6.1. The 10-carbon dye (MTTI103), is shown in living cells 0.5 hours after dropping (Figure 6.1 A) and 2
hours after dropping (Figure 6.1 B). At 0.5 hour, the immediate neighbors of the donor cell was labeled with 10-carbon dye, and the dye diffused approximately 50 µm away from the source. Furthermore, the 10-carbon dye had diffused to the edge of the image region approximately 150 µm from the donor cell 2 hours after dropping. As the dye diffuses into living cells within the network, it starts by labeling only perinuclear regions and over time labels the entire cell. Therefore, it appears that this dye quickly diffuses radially into a confluent network of living cells. In contrast to the short chain-length dye (10 carbons), medium (18 carbons) and long chain-length dyes (28 carbons), which associate with the cell membrane more strongly, were also measured.

Living cells labeled with the 28-carbon dye (MTTI114) are shown four hours after dropping (Figure 6.1 C) and eight hours after dropping (Figure 6.1 D). At 4 hours after dropping, a small amount of fluorescence appears in two cells adjacent the donor cell. At 8 hours after dropping, a larger amount of fluorescence appears in two acceptor cells and a small amount in a third cell. Interestingly, one of the two, highly-labeled cells was not immediately adjacent to the donor cell. Presumably, the dye has diffused from donor cell to an adjacent cell and then to another neighboring cell.

Transcellular diffusion of the medium chain-length variant, 18-carbon dye (MTTI109), was qualitatively indistinguishable from the 28-carbon dye. Over the 12 hours when 18- or 28-carbon dye diffused into the cellular network, they only labeled the perinuclear regions of living cells. Even though there was some transcellular diffusion of 18- and 28-carbon dye at all time points, there were still a subset of images showing no transcellular diffusion 12 hours after dropping (images not shown). Therefore, it appears that unlike the short 10-carbon dye, the longer variants, 28-carbon dye and 18-carbon dye, diffuse much slower into a confluent network of living cells and appear to label only specific cells that are not necessarily the cells closest to the donor cell.

Cell dropping experiments were also performed with fixed cells. 28-carbon dye (MTTI114) did not show any fluorescence signal within acceptor cells even 63 hours after dropping (im-
Figure 6.1: Representative processed images of transcellular diffusion in living and fixed cells. Donor cells (red) labeled with 10-carbon dye (MTTI103) or 28-carbon dye (MTTI114) were dropped onto a counter-labeled, established network (green), incubated for a certain time interval, and then imaged. Raw images were processed using ImageJ to yield a binary mask indicating the dye-labeling status for each cell. Living cells labeled with 10-carbon dye are shown 0.5 hours (A) and 2 hours (B) after dropping donor cells. Living cells labeled with 28-carbon dye are presented 4 hours (C) and 8 hours (D) after dropping with labeled acceptor cells indicated by white arrows. Additionally, cells were labeled and then fixed before dropping onto the counter-labeled network. 28-carbon dye-labeled fixed cells showed no diffusion into surrounding cells even 63 hours after dropping (images not shown). 10-carbon dye, also showed little to no diffusion 1 hour after dropping (E). However, 24 hours after dropping, 10-carbon dye-fluorescence signal was observed in the surrounding, fixed network (F). Scale bars = 50 µm.
ages not shown). Yet, 10-carbon dye (MTTI103) did show fluorescence signal in acceptor cells after incubation. One hour after dropping 10-carbon dye-labeled, donor cells, showed little to no dye transferred to neighboring cells (Figure 6.1 E) in most images. However, 24 hours after dropping, the 10-carbon dye fluorescence signal spread into the fixed, cellular network (Figure 6.1 F). This diffusion appears to be at the same distance from the donor cell as 10-carbon dye in living cells only 0.5 hour after dropping. Therefore, there appears to be a delay in 10-carbon dye diffusion between fixed cells compared to living cells, and hence a different mechanism of diffusion for 10-carbon dye in fixed cells compared to living cells.

6.1.2 Discussion of the Results

To interpret the results of transcellular diffusion experiments, three biophysical processes, which are known to transfer lipids between cells (Section 2.2.3), were considered for the mechanism of diffusion for each chain-length variant. The three processes are micelle formation, binding to water-soluble proteins, and transfer mediated by cell-cell contact points. The mechanism by which a NVM chain-length-variant dye diffuses between cells can be due to one or all of these processes. The goal for these preliminary studies was to eliminate some diffusion processes and determine a primary mechanism of diffusion for each chain-length-variant dye.

In living cells, the short chain length, 10-carbon dye, quickly spread radially from the source into the surrounding cellular network. By contrast, the rate of transcellular diffusion for the 10-carbon dye in fixed cells was substantially slower, but with a similar radial spread into the network. During incubation in which most of the dye transcellular diffusion occurred, the living cells were bathed in cell culture growth media, while fixed cells were immersed in phosphate buffer saline (PBS). Cell culture growth media, but not PBS, contains BSA, a lipid-binding protein, which can transport lipids between cells. Because the dye radially diffused into the network indiscriminate of cell-cell connections, then the greater rate of diffusion of the 10-carbon dye in living cells compared to fixed cells is hypothesized to
be primarily caused by lipid-binding proteins rather than cell-cell contact points or micelle formation. This hypothesis could be tested by performing a cell dropping experiment with fixed cells using cell culture growth media as incubation solution rather than PBS.

For the medium, 18-carbon and long, 28-carbon chain length dyes in living cells, transcellular diffusion was much slower than for the 10-carbon dye, but also diffusion only occurred between specific cells. Because no discernible transcellular diffusion occurred, even after 63 hours, for the 18 or 28-carbon dyes in fixed cells, which could not form cell-cell connections, then transcellular diffusion in living cells is hypothesized to be mediated by (mature) gap junctions formed between adjacent cells. There are two tests that are needed to test this hypotheses. First, a cell dropping experiment with living 2T3 cells using a fluorescent dye that labels gap junctions can be performed to verify gap junction formation between adjacent cells. This study would investigate whether transcellular diffusion is proportional to the total number and density of gap junctions between adjacent cells. Second, a similar study employing a mutant 2T3 cell line, which does not produce the protein (connexin43) that forms gap junctions between 2T3 cells, would test the role of gap junctions in transcellular diffusion. If the hypothesis that diffusion of the 18- and 28-carbon dye between cells was mediated by gap junctions, then it is expected that no transcellular diffusion will be exhibited in networks of the mutant 2T3 cell line.

To study the influence of hydrocarbon chain length on lipid diffusion at small temporal and spatial scales, the mobility of chain-length-variant dye molecules in living cells was measured using FRAP. The FRAP measurements were carried out in parallel with the cell dropping experiments to match experimental conditions so that the results of the two approaches could be combined to define the mechanism of lipid diffusion.

6.2 Influence of Hydrocarbon Chain Length on Dye Diffusion Within Cells

FRAP measurements were performed to investigate NVM chain-length-variant dye diffusion from the perspective of a single cell rather than a cellular network. FRAP was
conducted by selecting a small circular region within a dye-labeled cell, typically the bright perinuclear area (Figure 5.3), and photobleaching a cylindrical volume with a 3 \( \mu m \) radius and a height of 3 \( \mu m \) within a cell. This method probed dye diffusion within the plasma membrane as well as within internal cellular membranes. As fluorescent dye molecules diffused into the volume from various cellular membranes, the fluorescence recovered over time, and this recovery was monitored for, on average, 120 seconds after photobleaching. The recovery curves were fit to an equation modeling the diffusion of fluorescent dye molecules into the photobleached region (Equation 5.1), allowing for 3D anomalous diffusion, multiphoton excitation and bleaching, and an immobile fraction. FRAP measurements of NVM chain-length variants were taken at time points ranging from 0.5 to 24 hours after staining living cells. The NVM variants studied had chain lengths of MTTI102 (6, 3), MTTI108 (16, 3), MTTI109 (18, 3), MTTI106 (24, 3), and MTTI114 (28, 3). Not all time points were measured for each variant because FRAP measurements were conducted in conjunction with cell dropping experiments and performed as time permitted. The best-fit parameters, time-scaling exponent and transport coefficient, were used to calculate the diffusion coefficient using Equation 2.7. The diffusion parameters are presented as means weighted by the uncertainty in the fitting parameters. Two to five measurements were taken for each variant at each time point.

An immobile fraction was best fit, as determined by an F-test comparing \( \chi^2 \) values (goodness of fit), to all recovery sequences, except for three: one for 16-carbon dye at 2h and measurements for 24-carbon dye at 8h and 24h. Two recovery sequences contained a drastic jump in fluorescence signal in the middle of the recovery. These curves were truncated to remove the jump and then best-fit parameters were obtained from the shortened sequence. Occasionally the transport coefficient obtained from the best fit had a large uncertainty (\( > 50\% \) relative error). Because the transport coefficient was used to calculate the diffusion coefficient, a better fit with a lower uncertainty was desired. An additional fit was obtained by fixing the immobile fraction at the best value and refitting the recovery curve. If this
new fit was a better fit, as determined by a statistically significant increase in the $\chi^2$ value, then the newly acquired fitting parameters were used to calculate the diffusion coefficient.

To explore the temporal as well as the chain-length effects on dye diffusion in living cells, FRAP results are compared for a constant chain length over various incubation times and for various chain lengths for a single incubation time.

### 6.2.1 Temporal Measurements of Dye Diffusion within Living Cells

The FRAP measurements for the 6-carbon and 24-carbon dyes, at all time points were grouped to investigate how the diffusion parameters change over time within living cells. Representative recovery curves for the 6-carbon dye at 0.5, 2, and 8 hours after staining living cells and 24-carbon dye at 2, 8, 16, and the 24 hours after staining are displayed in Figure 6.2 A and B, respectively.

All the time-scaling exponents are significantly different ($p < 0.05$) for both the 6-carbon dye (Figure 6.2 C) and the 24-carbon dye (Figure 6.2 D). The 6-carbon dye exhibits near-Fickian diffusion at the 0.5 hour time point ($\alpha = 0.937 \pm 0.004$), but is subdiffusive at the 2 and 8 hour time points. The 24-carbon dye has a time-scaling exponent of $1.04 \pm 0.01$, and thus also obeys Fickian diffusion at the 2 hour time point. The time-scaling exponent becomes subdiffusive and steadily decreases over time until the 24 hour time point where it has a value of $0.128 \pm 0.001$.

The transport coefficients for 6-carbon dye (Figure 6.2 E) and 24-carbon dye (Figure 6.2 F) are all reliably different ($p < 0.05$), except when comparing the 24-carbon dye at the 2 and 8 hour time points. For the 6-carbon dye, the transport coefficient appears to decrease with incubation time from 0.5 to 8 hours. The transport coefficient for the 24-carbon dye appears to decrease over time just like the 6-carbon dye except at the longest time point (24 hours). Surprisingly, the 24 hour time point for the 24-carbon dye has the largest transport coefficient compared to the other time points.

All the diffusion coefficients are significantly different ($p < 0.05$) for the 6-carbon dye (Figure 6.2 G) and the 24-carbon dye (Figure 6.2 H) except when comparing the 8 and
Figure 6.2: FRAP measurements of NVM hydrocarbon chain-length-variant dyes at various intervals of incubation time after labeling living cells. Representative recovery curves for the 6-carbon dye (A) at 0.5 (n=2), 2 (n=5), and 8 (n=2) hours and for the 24-carbon dye (B) at 2 (n=5), 8 (n=3), 16 (n=3), and 24 (n=3) hours are displayed. Time-scaling exponents (C, D) and transport coefficients (E, F) were determined from the best fit of Equation 5.1 to the recovery curves. All time-scaling exponents and transport coefficients are reliably different (p<0.05) except for the transport coefficient for 24-carbon dye at 2 hour compared to the 8 hour time point. Diffusion coefficients (G, H) were calculated from the time-scaling exponents and transport coefficients using Equation 2.7. All diffusion coefficients are significantly different (p<0.05) except for 24-carbon dye at 8 hour compared to 16 hour time point. Error bars represent the weighted standard error in the mean.
16 hour time points. The diffusion coefficient for the 6-carbon dye decreases from the 0.5 hour to the 8 hour time point. Moreover, the diffusion coefficient for the 24-carbon dye also decreases from the 2 hour to the 8 hour time point, but the diffusion coefficient at the 8 hour time point is not significantly different ($p < 0.05$) from the 16 hour time point. The 24 hour time point for the 24-carbon dye has the smallest diffusion coefficient, and thus, overall the diffusion coefficient for the 24-carbon dye, as well as for the 6-carbon dye, exhibits a trend decreasing with time.

Altogether, the FRAP measurements of NVM chain-length variants exhibit similar temporal diffusion patterns within living cells. Time-scaling exponents and diffusion coefficients for a short, 6-carbon chain-length variant and a long, 24-carbon variant decrease over time. This could be due to internalization of dye molecules as they are transported into the cell from the plasma membrane to internal cellular membranes with different lipids, proteins, and morphological features. Additionally, multimers of the dye molecules may form in the plasma membrane or within inner cellular structures in which these dye aggregates experience restricted diffusion due to their increased size. In the next section, NVM chain-length-variant dye diffusion within living cells is further explored by comparing dye with different chain lengths at a single time point.

6.2.2 Measurements of Chain-Length Variation on Dye Diffusion in Living Cells

To investigate the effect of the hydrocarbon chain length on the diffusion of NVM chain-length-variant dyes, all the NVM variants measured using FRAP at the 2 and 4 hour time points were grouped for comparison. These time points were used because diffusion of at least three chain-length variants was measured. Short 6-carbon, medium 18-carbon, and long 28-carbon chain-length variants were compared at the 2 hour time point. Typical recovery sequences are shown for measurements taken 2 hours after labeling cells (Figure 6.3 A). All the time-scaling exponents (Figure 6.3 C), transport coefficients (Figure 6.3 E), and diffusion coefficients (Figure 6.3 G) are significantly different ($p < 0.05$).
The diffusion measurements at the 2 hour time point are summarized in Table 6.1. The 24-carbon dye exhibited Fickian diffusion while the 6-carbon and 16-carbon dyes were subdiffusive. It is interesting that at the same time point the 24-carbon dye was Fickian, the 16-carbon dye was significantly subdiffusive given that the only difference between the 24-carbon and the 16-carbon dye is 8 hydrocarbon units. The transport coefficients appear to be inversely proportional to the time-scaling exponents. The longest chain-length variant, 24-carbon dye, has the largest time-scaling exponent, while 16-carbon dye, a medium length variant, has the smallest transport coefficient. The diffusion coefficients reflect the same pattern as the time-scaling exponents. The longest chain-length variant has the largest diffusion coefficient while the medium length variant has the smallest and the smallest variant, 6-carbon dye, has an intermediate value. It is interesting that the medium chain-length variant has the smallest diffusion coefficient compared to both a long and short chain-length variant.

At the 4 hour time point, two medium chain-length variants, 16-carbon and 18-carbon dyes, were compared to a long chain-length variant, 28-carbon dye. Representative recovery curves are presented for measurements of the 16-carbon, 18-carbon, and 28-carbon dyes (Figure 6.3 B). All the time-scaling exponents (Figure 6.3 D), transport coefficients (Figure 6.3 F), and diffusion coefficients (Figure 6.3 H) are reliably different ($p<0.05$). The diffusion measurements are summarized in Table 6.1. All three of the variants exhibited subdiffusion 4 hours after labeling. The time-scaling exponent increases with hydrocarbon chain length from 16 to 28 carbons. The transport coefficient for the 16-carbon dye is large enough to overcome its very small time-scaling exponent relative to the 18-carbon dye such that the diffusion coefficient for the 16-carbon dye is significantly larger ($p<0.05$) than for the 18-carbon dye. Moreover, as seen at the 2 hour time point, the longest chain-length variant has the largest diffusion coefficient.

Taken together, the chain-length FRAP study indicates some interesting patterns for NVM chain-length-variant dye diffusion within living cells. The time-scaling exponents and
2 hour time point  

4 hour time point

Figure 6.3: FRAP measurements of NVM hydrocarbon chain-length-variant dyes at 2 and 4 hours of incubation after labeling living cells. Colors indicate dyes with different hydrocarbon chain lengths. Typical recovery sequences of the 6-carbon, 16-carbon, and 24-carbon dyes at the 2 hour incubation time (A) and of the 16-carbon, 18-carbon, and 28-carbon dyes at the 4 hour incubation time (B) are presented. By fitting Equation 5.1 to the recovery sequences, the time-scaling exponents (C, D) and transport coefficients (E, F) were obtained. With the time-scaling exponents and transport coefficients, the diffusion coefficients (G, H) were calculated using Equation 2.7. All time-scaling exponents, transport coefficients, and diffusion coefficients are reliably different (p<0.05) comparing all chain-length variants. Error bars represent the weighted standard error in the mean.
diffusion coefficients appear to exhibit similar patterns: starting relatively large at short 6-carbon chain-length variants, dipping to a low point with medium 16- and 18-carbon length variants, and increasing to the largest value with long 24- and 28-carbon chain-length variants. Not enough information was known about the chain-length variant diffusion in living cells to hypothesize a mechanism, but the factors involved in the diffusion exhibited by each chain-length-variant dye are discussed in Section 6.2.3.

### 6.2.3 Discussion of the Results

To investigate the mechanism of NVM chain-length-variant dye diffusion in living cells from the perspective of a single cell, FRAP measurements were conducted. To match experimental conditions and thus combine results to formulate possible mechanisms, FRAP measurements were performed in parallel with cell dropping experiments of living cells labeled with NVM chain-length-variant dye molecules. Time-scaling exponents, transport coefficients, and diffusion coefficients were measured for several chain-length variants and compared as a function of time and of hydrocarbon chain length. The results of these measurements are summarized in Table 6.1.

#### Table 6.1: Summary of Diffusion Coefficients and Time-Scaling Exponents for Lipophilic Fluorophores

<table>
<thead>
<tr>
<th>Lipophilic Fluorophore</th>
<th>Chain Lengths (carbons)</th>
<th>Measurement Obtained in</th>
<th>Measurement Technique</th>
<th>Time-Scaling Exponent</th>
<th>Diffusion Coefficient (×10⁻³µm²s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTTI102</td>
<td>6, 3</td>
<td>2T3 cells (2h)</td>
<td>FRAP</td>
<td>0.808 ± 0.002</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>MTTI106</td>
<td>24, 3</td>
<td>2T3 cells (2h)</td>
<td>FRAP</td>
<td>0.496 ± 0.001</td>
<td>1.66 ± 0.02</td>
</tr>
<tr>
<td>MTTI108</td>
<td>16, 3</td>
<td>2T3 cells (4h)</td>
<td>FRAP</td>
<td>1.04 ± 0.01</td>
<td>2.1 ± 1</td>
</tr>
<tr>
<td>MTTI108</td>
<td>16, 3</td>
<td>2T3 cells (4h)</td>
<td>FRAP</td>
<td>0.507 ± 0.009</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>MTTI109</td>
<td>18, 3</td>
<td>2T3 cells (4h)</td>
<td>FRAP</td>
<td>0.739 ± 0.004</td>
<td>2.98 ± 0.09</td>
</tr>
<tr>
<td>MTTI114</td>
<td>28, 3</td>
<td>2T3 cells (4h)</td>
<td>FRAP</td>
<td>0.821 ± 0.007</td>
<td>14.0 ± 0.5</td>
</tr>
<tr>
<td>DiI-C18</td>
<td>18, 18</td>
<td>GUV’s*</td>
<td>FCS*</td>
<td>1.00</td>
<td>700 ± 100</td>
</tr>
<tr>
<td>DiI-C12</td>
<td>12, 12</td>
<td>RBL cells†</td>
<td>FCS†</td>
<td>0.74 ± 0.08</td>
<td>40 ± 20</td>
</tr>
</tbody>
</table>

See text for details: * [30] and † [31].

From the analysis in Section 6.2.1, it appears that the time-scaling exponent and diffusion coefficient for both long (24-carbon) and short (6-carbon) chain length NVM variants within living cells decrease with incubation time. The 6-carbon dye at 0.5 hours and
24-carbon dye at 2 hours exhibited Fickian diffusion whereas all other time points and all other variants exhibited subdiffusion. Subdiffusion by these lipophilic dye molecules was expected because previous measurements of fluorescent lipid analogues in living cell membranes exhibited anomalous subdiffusion [31]. Diffusion measurements of lipophilic molecules in model membranes and living cell membranes are summarized in Table 6.1. The mobility of DiI-C18, a carbocyanine fluorophore similar to NVM, was measured in synthetic, model membranes called giant unilamellar vesicles (GUV) [30] using a technique called fluorescence correlation spectroscopy (FCS) [32] at room temperature (23°C). DiI-C18 within GUVs exhibited Fickian diffusion and had a diffusion coefficient 30 times larger than the largest diffusion coefficient measured in these studies. Furthermore, the diffusion of DiI-C12 was measured in living rat basophilic leukemia (RBL) cells [31] using FCS at room temperature (23°C). As opposed to the synthetic membranes, DiI-C12 in living cells exhibited subdiffusion and had a diffusion coefficient similar to NVM chain-length variants measured in these studies.

Additionally, the chain length appears to influence the time-scaling exponent and diffusion coefficient of NVM variants in living cells (Section 6.2.2). Both diffusion parameters tend to obtain their lowest values for medium, 16-carbon and 18-carbon chain-length variants and obtain their largest values with the longest, 24-carbon and 28-carbon chain-length variants. The results suggest that within living cells the NVM variants with chain lengths greater than 24 carbons have a larger rate of diffusion than variants of shorter chain lengths. Furthermore, NVM variants with a chain length of 6 carbons long appear to have an intermediate rate of diffusion compared to medium and long chain-length variants.

Formulating a hypothesis for the chain-length dependence on lipophilic fluorescent dye diffusion within individual living cells was complicated because dye diffusion was measured at several locations simultaneously within cells and each location could contribute differently to the measured diffusion. The examined location was determined by the size and placement of the photobleached region within the cell. The FRAP region of interest (ROI),
studied in this thesis, included the plasma membrane of the cell as well as internal cellular membranes like the endoplasmic reticulum (Figure 2.5). The diffusion coefficient and time-scaling exponent calculated were ensemble averages of dye diffusion in each location within the cell.

A hydrocarbon chain-length dependence on the plasma membrane component of the diffusion, measured by FRAP, was found in the transcellular diffusion study (Section 6.1). In this study, medium (18 carbons) and long (28 carbons) chain-length dyes exhibited drastically slower transcellular diffusion and thus stronger association with the plasma membrane compared to a short chain-length dye (10 carbons). Because of this stronger association with the plasma membrane, the medium and long chain-length dyes experience restricted diffusion, which increases the rate of diffusion for the medium and long chain dyes compared to the shorter chain dyes when measured using FRAP.

Moreover, the transportation of lipophilic fluorescent dyes to internal cellular membranes has been observed to be dependent on the hydrocarbon chain length of the dye [33]. In the studies in this thesis, short chain-length dyes exhibited different labeling of internal cellular membranes compared to the long chain-length dyes (images not shown). The long chain-length dye preferentially labeled small compact membrane structures with the cell, probably lysosomes and/or mitochondria, whereas the short chain-length dye appeared to label all the membranes within the cell. Thus, depending on the length of their hydrocarbon chains, dyes are internalized to different internal membranes. Because each internal membrane has different lipid and protein compositions as well as different membrane geometries, each chain-length-variant dye could experience different interactions, and thus chain-length dependent rates of diffusion.

A specific aspect of the photobleaching protocol increased the error in the diffusion parameters that were determined. NVM variant dyes were photobleached to 25 ± 14 percent of the initial fluorescence. This is significantly lower than what is typically performed for FRAP measurements (70% of initial fluorescence is typically recommended). By photo-
to bleach a large percentage of fluorophores, it produces an initial steep slope of the recovery curve and contains relatively fewer data points. This is more difficult to fit to than a recovery curve generated from more limited photobleaching.

Because the measurements presented in Table 6.1 are means weighted by the uncertainty in the fitting parameters, the final value is primarily determined by the best fit curves. To see if cell variation would affect the trends observed in the results, the mean and standard deviation for each NVM chain-length-variant dye was also calculated (data not shown). For both the temporal study and the chain-length study, the same trends appeared, but because of large variations between measurements, the mean values were not reliably different ($p > 0.05$). The one exception to the trends observed in the FRAP measurements was the diffusion coefficient in the chain-length study. Instead of the 16-carbon dye exhibiting the smallest rate of diffusion, the 6-carbon dye had the smallest rate and the 16-carbon dye displaying an intermediate value, while the 24-carbon dye once again exhibited the greatest rate of diffusion.

Because of the small number of measurements collected, these results should be considered preliminary. For all FRAP results, only two to five recovery curves were collected for each dye at various time points. Based on the variability from recovery curve to recovery curve for the same dye at the same time point, two to five measurements was probably not enough to be confident in the exact values and trends observed. Therefore additional measurements are suggested for future studies of NVM chain-length variant diffusion in living cells (Chapter 7).

### 6.3 Conclusions

Preliminary measurements of NVM chain-length-variant dye diffusion between cells and within single cells were used to investigate the hydrocarbon-chain-length dependence on the mechanism of lipid diffusion in living cells. The cell labeling technique, cell dropping, and LSM were used to examine dye diffusion between living cells within a connected network.
The analysis in Section 6.1 suggests that transcellular diffusion of NVM chain-length variants is dependent on the hydrocarbon chain length. Dye molecules with short chain lengths quickly diffuse radially out from the initial cell, while the medium and long chain length dyes slowly diffuse only to a subset of surrounding cells. Additionally, FRAP was conducted in individual cells to dissect the diffusion of NVM chain-length-variant dyes within living cells. FRAP measurements of NVM chain-length-variant dyes in Section 6.2 suggest that the diffusion coefficient for NVM chain-length-variant dyes is dependent on the length of their hydrocarbon chains. The FRAP results suggest that the longest chain-length dyes have the fastest rate of diffusion, while the medium chain-length dyes have the slowest.
Chapter 7

Conclusion

Lipophilic fluorescent dyes are effective instruments in illuminating neuronal connections and lipid dynamics in living cells. The goals for the study of these topics include obtaining understanding of the molecular/genetic basis for neuronal development as well as the lipid signaling component of intercellular communication. To fully explore these goals, the characterization of certain photophysical and biophysical properties of lipophilic fluorescent dyes is required. The purpose of this thesis was to extend the number of colors resolvable in a single sample and to investigate the effect of the hydrocarbon chain length on lipid transcellular diffusion.

7.1 Multicolor Neurotracing

Multicolor neurotracing is required to efficiently use difficult to obtain and rare, complex mutant animal models for neurodevelopment studies, as well as to delineate complex patterns in neuronal connections not revealed via single-color analysis. A set of lipophilic fluorescent dyes, called NeuroVue (NV) dyes, with fluorescence emissions ranging from violet to near-infrared have been produced for multicolor neurotracing. However, conventional 1PE is not capable of extending the number of distinct colors within a single specimen to the amount desired by neurobiologists, even with the NV dye set. Thus, with the recent increase in availability of the near-infrared emitting Ti:S laser, 2PE was proposed as an additional tool to achieve multicolor neurotracing. To effectively use 2PE in multicolor
labeling studies, the 2PE action cross sections for commercial and violet candidate NV dyes were measured over the entire tuning range of a Ti:S laser. The 2PE action cross section is a quantitative measure for the probability of a fluorescent dye to be two-photon excited. Using a standard ratiometric technique comparing the fluorescence generated from the NV dyes to a well-characterized fluorescent dye, the 2PE action cross sections were calculated using Equation 3.1.

The results in Chapter 4 showed that all the commercial and violet candidate NV dyes can be efficiently two-photon excited, and have 2PE action cross sections ranging from very dim (on the order of 0.1 GM for violet candidate dyes), but comparable to commonly imaged biological fluorescent molecules, to very bright (on the order of 100 GM for NVO and NVB) and similar to rhodamine, a popular fluorescent dye. Additionally, the 2PE action cross-section spectra of commercial and violet candidate NV dyes (Figure 4.3) revealed that the spectral neighbors with fluorescence emission cross contamination in the set can be distinctly resolved using 2PE. For example, in Section 4.3 it was shown that NVR can be selectively excited over NVO by 2PE at 800 nm. Similar imaging protocols can be used to spectrally resolve other NV dyes including selectively exciting NVJ over the violet candidates and NVB over NVM. Recently, a combination of 1PE and 2PE imaging strategies have been used to demonstrate 6-color labeling of neuronal connections with commercial and violet candidate NV dyes [6].

7.2 Mechanism of Lipid Diffusion in Living Cells

After years of assuming lipids served simply structural functions in cells, it now appears that lipids perform many more diverse roles including as signaling molecules in the brain. The effects of the signaling lipid, 2-AG, on neuron electrophysiology are well known, however the mechanism by which 2-AG and many other signaling lipids in the brain diffuse across the aqueous gap between neurons is unknown. The mechanism of lipid transcellular diffusion might be influenced by the length of their hydrocarbon chains leading to different
preferential routes between cells for different lengths. Because lipophilic fluorescent dyes insert into and diffuse laterally along the cell membrane analogous to naturally occurring lipids, a series of lipophilic fluorescent dyes varying only in the length their hydrocarbon chain were used to investigate the hydrocarbon chain-length dependence on the mechanism for transcellular diffusion of lipids. The transcellular diffusion of the chain-length-variant dyes was measured by labeling a single cell, dropping it onto a counter-labeled cellular network, and observing the spread into the surrounding network. The distance of dye from the source and the area of spread were taken as indicators of the rate of diffusion, and along with the structure of the cellular networks and specific experimental conditions, as indicators for the mechanism of transcellular diffusion.

The results in Section 6.1 indicate that for the chain-length-variant dyes, the length of their hydrocarbon chains affected the mechanism by which transcellular diffusion occurred. The short, 10-carbon dye quickly diffused radially into the surrounding cellular network, traveling approximately 150 microns in 2 hours (Figure 6.1). Because diffusion into fixed networks, containing no free proteins in solution, was substantially slowed compared to living networks (Figure 6.1), the mechanism for the transcellular diffusion of the 10-carbon dye was hypothesized to be due to transport between cells by water soluble, lipid-binding proteins. In contrast, the medium, 18-carbon, and long, 28-carbon, dyes diffused slowly into the surrounding network and labeled only a subset of adjacent cells (Figure 6.1). Slow transfer between cells at gap junctions could account for the medium and long chain-length-variant dye diffusion. Moreover, additional studies using a fluorescent dye to label the gap junctions within the cellular networks would help illuminate the relationship between gap junction formation between cells and chain-length-variant dye diffusion.

To further explore lipid diffusion in living cells, the rate of diffusion of chain-length-variant dyes within single cells was measured using FRAP. To perform the FRAP technique, a small region within the cell was photobleached and the fluorescence recovery, due to fluorescent dye molecules diffusing into the photobleached region, was monitored over
time. The recovery curve was fit to Equation 5.1 that allows for an immobile fraction, 3D anomalous diffusion, as well as multiphoton excitation and photobleaching. By optimizing the fit, values for the time-scaling exponent and transport coefficient were obtained and used to calculate the diffusion coefficient.

The results in Section 6.2 demonstrate that the rate of diffusion depends on both the time within a living cell and the length of the hydrocarbon chains. In the temporal study, the diffusion coefficient, as well as the time-scaling exponent, decreased with time in living cells for both a short, 6-carbon, and a long, 24-carbon dye. At early time points, both dyes exhibited Fickian or near-Fickian diffusion, but became more subdiffusive over time. Concentration-dependent multimer formation of dye molecules within internal cellular structures could lead to the observed anomalous subdiffusion. In the chain-length study, the longest chain-length dyes, 24 and 28 carbons, exhibited the fastest rate of diffusion and Fickian or near-Fickian diffusion. In contrast, the medium chain-length dyes, 16 and 18 carbons, had the slowest rate of diffusion and the most subdiffusive behavior, while the short chain-length dye, 6 carbons, displayed intermediate values. This chain-length dependence on diffusion in living cells was probably due to their selective transportation to different internal cellular membranes, which have different lipid-protein compositions and membrane geometries, leading to different environments for dye diffusion.
Appendix A

LabView Program for Hardware and Software Control

This appendix contains the complete LabView block diagram code for the program controlling the photon counter and recording data for measuring the fluorescence versus laser power discussed in Section 3.3.

Figure A.1: The virtual instrument front panel for LabView Program.
Figure A.2: Code for the Entire LabView Program. Colored areas indicate different functional parts of the program. Green area configures the photon counter (Figure A.3). Light blue area records the laser power and photon count readings (Figures A.4 - A.8). Pink area saves the collected data to a text file (Figure A.9).
Figure A.3: Configures measurement parameters of the SR400 photon counter.
**Figure A.4:** Wait for user to press take fluorescence readings button.
Figure A.5: Starts DAQ Assistant, which interfaces with the data acquisition card.
Figure A.6: Tell SR400 photon counter to collect data, wait for photon counter to finish, transfer data from photon counter to computer, calculate mean and standard deviation of data.
Figure A.7: Close communication between DAQ Assistant and data acquisition card.
Figure A.8: Wait for user to accept data or request a repeat measurement.
Figure A.9: Saves data to a text file.
Appendix B

Design and Construction of a Narrow-Bandwidth Tunable Light Source

In this appendix, the design and construction of a tunable light source is described. The device was designed to serve as a standard light source for calibrating the detection efficiency of the apparatus used to measure 2PE action cross sections (Figure 3.1). The basic design plan consisted of a Czerny-Turner grating monochromator layout with a white light source input. To determine the dimensions of the device, theoretical analysis for diffraction gratings and grating monochromators was performed. To characterize the completed device, the output intensity as a function of wavelength was measured. Additionally, the typical procedure for initial alignment of the device is presented.

B.1 Design Requirements

For the device to serve as a standard photon source for determining the detection efficiency of the cross section apparatus, it must satisfy certain requirements. First, the device must produce enough photons such that a large number of counts could be achieved by the photon detector to produce good counting statistics. It was anticipated that 100 µW would be sufficient and practical for the setup (Section B.2). Second, the device must have an output with a small wavelength band (5 - 10 nm) so that the detection efficiency can be determined as a discrete function of wavelength. Next, the output of the device must be
tunable to the entire range of wavelengths making up the emission spectra of the fluorescent dyes being studied. For the NeuroVue dyes, the range of emission spectra is approximately 500 nm. Finally, it was desired that the device be as small as possible so it can be used in a variety of experiments and enable ease of mobility. The narrow-bandwidth tunable light source was constructed with these design requirement in mind.

B.2 Basic Design Plan

The narrow-bandwidth tunable light source consisted of a grating monochromator and a white light source, each contained within its own housing. The basic design plan for the grating monochromator was the Czerny-Turner optical layout, as illustrated in Figure B.1.

![Figure B.1: A schematic diagram of a Czerny-Turner grating monochromator.](image)

In a Czerny-Turner grating monochromator, an external light source is focused on the entrance slit, which is located at the focal point of a spherical mirror. This collimating mirror directs light toward a diffraction grating, which splits the white light into its component wavelengths. The diffracted light is collected by a spherical mirror and focused on the exit slit, which allows only a small wavelength band of light to exit. To tune the wavelength exiting the grating monochromator, the grating is rotated, changing the wavelength reflected
from the center of the focusing mirror.

The filament of an incandescent bulb imaged by a converging lens served as the external light source for the grating monochromator (Figure B.2). To increase the intensity of the image, a spherical mirror was placed opposite the lens to reflect additional light through the lens into the monochromator. To determine the precise dimensions of the device, theoretical analysis of diffraction gratings and grating monochromators was performed.

Figure B.2: Schematic diagram of White Light Source from Side and Top Views.
B.3 Theoretical Analysis

The primary purpose of a diffraction grating is to disperse light spatially by wavelength. Dispersion is a measure of the separation (either angular or spatial) between diffracted light of different wavelengths. The spatial dispersion is given by the equation, $D = d\lambda/dx$. This property of diffraction gratings was used to produce the narrow-bandwidth tunable light source.

For light incident at angle $\alpha$ on a diffraction grating with the number of grooves per mm, $d$, the $m^{th}$ diffraction order is diffracted from the normal by angle $\theta$ given by the grating equation,

$$d \cdot (\sin \alpha - \sin \theta) = m \cdot \lambda. \tag{B.1}$$

Considering the geometry of the Czerny-Turner layout (Figure B.3), namely $\alpha + \phi = \delta$ and $\delta = \phi + \theta$, then Equation B.1 can be rewritten in the following form:

$$2 \cdot d \cdot \cos \delta \cdot \sin \phi = m \cdot \lambda, \tag{B.2}$$

where $\delta$ is half the angle formed by the incident rays from the collimating mirror and the diffracted ray directed toward the focusing mirror. The grating angle, $\phi$, is the angle at which the grating is rotated from the line bisecting $2\delta$.

![Figure B.3: A schematic diagram of Czerny-Turner grating monochromator with illustrated geometry.](image)

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Considering the optical arrangement shown in Figure B.3 and looking at the change in wavelength as the length of the micrometer, $x$, is varied, equation B.2 can be written in the following form:

$$\Delta \lambda = \frac{2 \cdot d \cdot \cos \delta}{m \cdot L} \cdot \Delta x,$$  \hspace{1cm} (B.3)

where $L$ is the length of the grating rotation arm. With this expression, all the dimensions of the tunable light source, except the exit slit width, can be determined.

An expression for the exit slit width, $w$, was obtained by differentiating equation B.1 and using the geometry of the grating monochromator arrangement (Figure B.3). After differentiating Equation B.1 at constant incidence angle, the angular substitution, $\delta = \phi + \theta$, was made such that trigonometric substitutions from Figure B.3 could be used.

$$\Delta \lambda = \frac{w \cdot d}{m \cdot r} \cdot \cos \delta \cdot \left( \frac{\sqrt{L^2 - x^2}}{L} \right) + \sin \delta \cdot \frac{x}{L}.$$  \hspace{1cm} (B.4)

Note, $w = r \cdot d\theta$ was used to obtain a function of the slit width, where $r$ is the optical path length from the diffraction grating to the exit slit.

**B.4 Final Construction and Results**

Given the design requirements listed in Section B.1, the narrow-bandwidth tunable light source was constructed based on the geometry and diffraction expressions described in Section B.3. The final construction of the device consisting of a white light source and grating monochromator is presented in Section B.4.1 and B.4.2.

**B.4.1 White Light Source**

The purpose of the white light source was to send light into the grating monochromator by focusing the filament of a bulb on the entrance slit. Because the height of the entrance slit was approximately 4 times the height of the filament, the optics of the white light source needed to magnify the filament by 4 times at the focus. Using the Gaussian Lens Formula, $1/f = 1/s_o + 1/s_i$, and the equation for the transverse magnification, $s_i/s_o = |M| = 4$, a
lens with a 10 mm focal length and diameter of 1 inch was chosen. A spherical mirror with
a 25 mm focal length and 1 inch diameter was selected to improve throughput.

The white light source, shown in Figure B.4, consists of an incandescent light bulb (LS),
converging lens (L1), and a spherical mirror (M1) (Table B.1). The light bulb mount
is secured to the base by two mounting screws. To facilitate the ease of alignment four
adjusting screws (arrows in Figure B.4) were installed. A 2 inch electric fan (not shown in
Figure) was attached to the outer shell of the white light source housing to cool the device.

Figure B.4: External White Light Source. The filament of a light bulb (LS) is focused
by a lens (L1) onto the entrance slit. The mirror (M1) reflects additional light from the light
source into the lens. Arrows indicate adjusting screws for focusing filament on entrance slit.
B.4.2 Grating Monochromator

The grating monochromator was constructed using Equation B.3 and the equipment listed in Table B.1. A reflection diffraction grating with 1200 grooves per mm, which was most efficient at the 1st diffraction order, was used. Given this diffraction grating, a micrometer with a 1 inch maximum displacement, and the wavelength spectrum needed ($\Delta \lambda = 500 \text{ nm}$), the two spherical mirrors and the angle between them, $\delta$, were determined along with the length of the grating rotation arm, $L$.

Two spherical mirrors with focal lengths of 15 cm and 2 inch diameters were selected for the collimating and focusing mirrors. A 200 $\mu$m slit was placed at the entrance slit to keep the image of the filament from overfilling the collimating mirror. Using Equation B.4, the exit slit width was calculated to be 3 mm. This slit was later removed because it was discovered that the optical fiber was sufficient to act as the final slit. Finally, the white light source was attached to the grating monochromator by a L-shaped connecting bar.

<table>
<thead>
<tr>
<th>Table B.1: Equipment Used to Construct Light Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FROM THORLABS, INC.</strong> (NEWTON, NJ)</td>
</tr>
<tr>
<td>Diffraction Grating GR25-1205</td>
</tr>
<tr>
<td>Spherical Mirrors</td>
</tr>
<tr>
<td>focal length 15 cm</td>
</tr>
<tr>
<td>diameter 2 inches</td>
</tr>
<tr>
<td>Entrance Slit</td>
</tr>
<tr>
<td>slit width 200 $\mu$m</td>
</tr>
<tr>
<td><strong>FROM OSRAM</strong> (BELLAPHOT, GERMANY)</td>
</tr>
<tr>
<td>Light Bulb i849 64628 12V</td>
</tr>
<tr>
<td>power output 100 W</td>
</tr>
</tbody>
</table>

B.4.3 Narrow-Bandwidth Tunable Light Source

After construction, the narrow-bandwidth tunable light source was aligned, characterized, and the results are displayed in Table B.2. Overall, all the parameters measured met the requirements for use as a standard light source for measuring the detection efficiency of the cross section apparatus.
Figure B.5: Narrow-Bandwidth Tunable Light Source. Light from the white light source (LS) is focused (by lens, L1) into the entrance slit (Slit), collimated by a spherical mirror (M3), reflected off the grating, and imaged by another spherical mirror (M2) onto the exit slit. This image is directed by a lens into a fiber optic cable. By adjusting the micrometer, the wavelength entering the fiber optic cable can be tuned.
Table B.2: Characteristics of Narrow-Bandwidth Tunable Light Source

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power Output</td>
<td>≈ 100 µW</td>
</tr>
<tr>
<td>Full Width Half Maximum (FWHM)</td>
<td>8 nm</td>
</tr>
<tr>
<td>Wavelength Range</td>
<td>490 nm</td>
</tr>
<tr>
<td>Dispersion, $\Delta \lambda / \Delta x$</td>
<td>19 nm·mm⁻¹</td>
</tr>
</tbody>
</table>

B.5 Method for Measuring the Output Intensity of the Narrow-Bandwidth Tunable Light Source

To measure the output intensity of the narrow-bandwidth tunable light source, the light directed through the exit slit was collected into a fiber optic cable (OceanOptics P600-1-SR, Dunedin, FL) by a converging lens. The other end of the cable was connected to a spectrometer (OceanOptics SD2000 fiber optic spectrometer) connected to a computer which recorded the resulting spectrum using software (OceanOptics OOIBase32) controlling the spectrometer. A spectrum was collected every 10 nm and integrated to determine the photon counts per wavelength.

![Example output spectra at different peak wavelengths for the narrow-bandwidth tunable light source.](image)

**Figure B.6:** Example output spectra at different peak wavelengths for the narrow-bandwidth tunable light source.
B.6 Typical Alignment

Below is the typical procedure used to align the narrow-bandwidth tunable light source so that the image of the filament is split into its component wavelengths and focused at the exit slit, as well as obtaining the desired wavelength range.

1. **Set the “fixed” components into place and secure them.** For the grating monochromator, adjust the collimating and focusing spherical mirrors such that their focal points are located at the entrance and exit slits, respectively. For the white light source, adjust the spherical mirror, bulb filament, and lens such that they are co-linear.

2. **Align the white light source such that the direct image of the filament overlaps with the mirror image.** Move the bulb base forward or backward and raise or lower the 4 adjusting screws (loosening the 2 larger mounting screws as needed) until the image formed directly from the filament is the same size as the image formed by the light reflected from the mirror and is in focus.

3. **Attach white light source to grating monochromator and align with collimating mirror.** Attach the white light source to the L-shaped connecting bar then raise or lower the white light source housing and move the connecting bar forward or backward until the image formed by the lens is at the focal point of the collimating mirror, producing parallel light illuminating the grating.

4. **Align mirrors and grating such that an image of the filament is formed at the exit slit.** Orient the collimating mirror until the image of the filament completely covers, but does not over-fill, the grating. Rotate the grating until green light (color arbitrarily chosen for alignment purposes) is centered on the focusing mirror. Orient the focusing mirror until green light is imaged at the exit slit.

5. **Attach fiber optic cable and converging lens, and align with grating monochromator.** Attach a fiber optic cable and converging lens to grating monochromator. Attach the other end of the cable to a spectrometer to determine the alignment that maximizes photon counts. Adjust the converging lens until light is optimally focused.
into the cable resulting in the greatest count reading on the computer.

6. Re-orient the grating and micrometer such that the desired wavelength range is obtained. Move the micrometer to 24.5 mm (extreme end of the micrometer, for this particular micrometer any movement farther than 24.5 (e.g., 25) mm has no effect on bar displacement (length)), which will correspond to the blue end of the wavelength range desired. Rotate the grating until the blue extreme wavelength is achieved at the output (indicated by the computer reading). Adjust the micrometer to the lower end (i.e., 0 or -2.0 mm) to ensure that the desired red extreme wavelength is obtained.

B.7 Conclusion

A narrow-bandwidth tunable light source was designed and constructed to be used as a standard light source for determining the detection efficiency of the apparatus used to measure 2PE action cross sections. Starting with the Czerny-Turner optical layout, the dimensions of the narrow-bandwidth tunable light source were determined using the design requirements and two derived diffraction equations. Summarized in Table B.2, the constructed narrow-bandwidth tunable light source satisfied all the design requirements and served as a capable standard light source for determining the detection efficiency of the cross section apparatus.
Bibliography


