Enhanced Control and Characterization of Complex Molecules with Ultrashort Lasers

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Abstract

Quantum control employs ultrashort-pulse laser fields to observe and guide atoms and molecules by manipulating the wave-like interference between quantum pathways, generating constructive interference favoring a target product state. As technology has developed, especially tools for femtosecond pulse generation and shaping, the range of systems investigated by quantum control has grown from simple dilute atomic gases to complex condensed phase molecules. This thesis applies the tools of quantum control to optically-sensitive biochemical protein sensors and switches.

A primary focus of this work is the enhanced control of Optogenetic switching by non-linear pulsed excitation. Optogenetics enables optical control of \textit{in vivo} biological functions by inserting light-sensitive switches into cells. However, spectral cross-talk between switch states (their overlapping steady-state absorption and emission spectra) prevents full-range control. We introduce a novel nonlinear optical technique to overcome cross-talk by exploiting differences in the excited state dynamics of the active and inactive switch states. We effectively demonstrate the feasibility of this control mechanism and begin characterizing the dependence of the enhancement factor on laser control parameters to discover the laser controls which most effectively maximize the dynamic range of the optogenetic switch. This proof of principle work is a first step toward multiplexed control of multiple optogenetic switches.

We also quantitatively characterize mixtures of Fluorescent Proteins (FPs) suffering from cross-talk by employing Optimal Dynamic Discrimination (ODD), which amplifies differences in the excited state dynamics of quantum systems using optimally tailored control fields. ODD enables accurate concentration determination of mixtures of Enhanced Blue and Enhanced Cyan FPs, advancing the technique toward the simultaneous monitoring of many biochemical processes by multiplexed ODD of several FPs, leading
to the ultimate goal of implementing ODD to control multiple sensors and switches for multiplexed control and observation of biochemical processes.

This thesis also presents a novel capability to map concentrations of intermixing fluids in colliding micro-droplets using planar laser induced fluorescence of a chemically-sensitive dye, revealing details of early-stage mixing; as well as an evaluation of new and developing ultrafast optical technologies to suggest tools for future development of optimal quantum control experiments.
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Dedicated to my Mother,
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Chapter 1

Introduction and Overview

Since the advent of the laser it has been a goal of physicists and chemists to use it as a source for controlled, selective photochemistry, particularly the selective breaking of specific bonds and control of molecular conformation by vibrational pumping. Classical chemistry manipulates macroscopic variables like temperature, pressure, or species concentration; altering the environment or energetics to favor reaction pathways that yield desired products and obstruct reaction pathways that yield unwanted bi-products. Where tuning these bulk parameters can not yield desired specificity, catalytic chemistry introduces specially constructed molecular species that interact and combine in such a way as to drive reactions in a given direction. Reaction Control in these fashions changes the entire energy landscape of the system, it is difficult or impossible to deposit energy into specific bonds or atoms within molecules. Lasers could deliver precisely defined energy into specific degrees of freedom of molecules, and it was speculated that these monochromatic, narrow bandwidth sources would be able to selectively deposit energy into targeted bonds and drive chemical kinetics [1]. However, this mode-selective photochemistry failed due to intra-molecular vibrational energy redistribution: the initially well defined excitation is rapidly redistributed to all degrees of freedom in the molecule by an-harmonic coupling amongst the vibrational modes [2]. In a very short time all modes of a molecule have been energized and the laser excitation is essentially equivalent to external heating.
With the advent of femtosecond lasers there was a way to deposit energy faster than vibrational motion, finally allowing delivery of energy to specific modes in a molecule. However, generation of these ultrashort pulses required broad bandwidth sources (this relationship is described in Chapter 2) and rather than exciting a specific vibrational mode with a well defined laser frequency, these sources now excite a vibrational wave packet on the excited state surface: a coherent super-position of all vibrational modes within the spectral range of the laser. This wave packet undergoes unique dynamics depending on the energies and couplings of the individual modes as well as the phase relationship of the components of the wave packet. The description of these dynamics can not be expressed in classical terms, and must be described by a quantum mechanical time dependent Hamiltonian. This quantum mechanical treatment is the foundation of Quantum Coherent Control: the coherent manipulation of the phases and amplitudes of the wave-like modes of an excited molecule to modify and steer the dynamics of the quantum system toward a desired product state.

Early coherent control experiments were able to manipulate the dynamics of simple quantum systems like dilute atomic vapors or diatomic or constrained molecular systems with relatively simple control schemes. Early experimental applications of few-parameter coherent control include the Pump-Dump experiments of Tannor and Rice [3,4] in which a triatomic molecule was pumped to a coherent wave-packet on an excited energy surface, where the complex amplitude of the wave-packet would oscillate in time. After a variable time delay $\tau$, the system is de-excited by a dump pulse. The final product of the transition is dependent on the excited state dynamics and the timing of the dump pulse. Brumer and Shapiro developed Phase control [5,6], in which two phase-locked CW lasers excite a sample into a target state via two distinct, resonant pathways (i.e., single photon or three photon absorption). Chirping of the laser, imparting a positive or negative linear time dependence to the instantaneous frequency of the pulse, has been successfully applied for control of numerous atomic and molecular processes, including control of vi-
brational wave packets [7–9], control of population transfer between atomic states [10] and molecular vibrational levels [11], control of electronic excitations in molecules [12,13], and enhancement of Coherent Anti-Stokes Raman Scattering (CARS) spectroscopy [14, 15]. In all of these examples, through variation of a few parameters it is possible to control the quantum dynamics of molecular systems. But these techniques are of limited applicability and require advanced knowledge of the complete molecular Hamiltonian for the system of interest (the quantum mechanical operator containing the energies and couplings of all nuclei and electrons making up the molecule, a fundamental description of the time-evolution of the molecule and the way the molecule responds to stimuli). To control more complex systems a different technique is required that does not necessitate knowledge of the molecular Hamiltonian.

Since we can not always use the known molecular Hamiltonian to calculate an interaction field that drives the time-evolution of the system to some preferred product state, we can apply a potentially effective interaction field to the system letting the molecule solve its own Hamiltonian, then measure the state of the system to evaluate the effectiveness of the field and use that information to design new controls. This is the foundational principle of Adaptive Feedback Control (AFC) or Optimal Quantum Control (OQC) as proposed in the benchmark paper by Judson and Rabitz [16]. OQC experiments first define the fitness function - some observable of the system under control that is related to the population of the target product state. The control objective is to steer the dynamics of the system toward this desired product state through application of a tailored control field designed by an adaptive feedback, learning algorithm. The control field is typically a shaped femtosecond laser pulse, defined as a function of a set of control parameters which are set by the algorithm. The system is exposed to a series of tailored control fields and the fitness of each is measured and passed to the algorithm, which designs new control fields incorporating the successful controls into the design and throwing out unsuccessful ones. This process is repeated in an Adaptive Feedback Loop until the fitness
of the controls converges to an optimal value. The benefit of this technique is that it requires little to no knowledge of how the controls and system are interacting, the algorithm will optimize the field based solely on measured effectiveness of previous controls. This broadens the field of potentially controllable systems to much more complex quantum systems. Experimental implementation of OQC experiments was enabled by a confluence of technological and theoretical advances: the development of stable, high repetition-rate femtosecond lasers [17], accurate and fast pulse shaping capabilities [18,19], and the development of evolutionary algorithms that can navigate the high-dimensional space ascribed to the controls and discover optimal solutions [20,21]. Each of these is a field unto itself, a functional description of these experimental tools sufficient for understanding the material in this dissertation is given in Chapter 2. The interested reader is encouraged to study the listed references for further details.

The first experimental implementation of this optimized quantum control scheme was by Wilson and Bardeen, who optimized the fluorescence of laser dyes in solution [22]. Since then optimal quantum control has grown into a burgeoning field that has been successfully applied to a broad array of experiments and theoretical conclusions [23–25]. OQC experiments enhance a number of optical techniques including photo-dissociation [26, 27] and photo-ionization [28], coherent vibrational dynamics [29, 30], high-harmonic generation [31], coherent energy flow in photosynthetic molecules [32,33], and detection of complex, spectroscopically indistinguishable molecules in solution [34, 35]. In this thesis we present several projects that cover a range of applications, all are united by the goal of improving the sensitivity, selectivity, and fidelity of information extracted from a system or control over the manipulation of a system by exploiting ultra-fast optical interactions between ultra-short pulse lasers and the unique dynamic non-linear optical response of molecules. In these experiments we apply the tools of optimal quantum control to enhance conventional characterization and control techniques. The projects presented in this thesis are briefly summarized below.
Chemically-Sensitive LIF Imaging of Colliding Micro-Droplets

The first project, presented in Chapter 3, uses the frequency doubled ultrashort pulses from the Ti:Sapphire laser to excite Laser Induced Fluorescence (LIF) from a chemically sensitive dye within a pair of colliding micro-droplets of different chemical composition. The imaging of the emission of this dye in the dynamically varying environment of the mixing droplets allows a simple, but powerful tool for characterizing the fluid dynamics of mixing over significant length and time scales. Information about the macroscopic flow fields and the advective transport of material resulting from the initial kinetic energy and momentum of the droplets is gained by observing the bulk movement of the dye over successive laser exposures. Meanwhile, the color shift of the dye gives valuable information about the molecule scale diffusion of the dye. Direct measurements of mixing over these vastly different length scales is valuable for calculation of fluid mechanical properties of materials and increasing the accuracy of more complex fluid dynamic simulations. It is a unique quality of the particular dye used in these experiments, Pyranine, that the chemically sensitive color shift straddles the boundary between the Green and Blue color filters used in common commercial color digital cameras. This greatly simplifies the experimental setup and expands the potential transfer of the technique by enabling imaging of the mixing systems with only a single camera with no additional filtering or specialized construction. Several images of streams of micro-droplets colliding and mixing at different collision geometries are imaged and analyzed, with unique perspectives gained by the chemically sensitive imaging discussed.

Optimal Dynamic Discrimination of Spectrally Similar Bio-Sensors

The second body of work presented in Chapter 4 demonstrates the capability of Optimal Dynamic Discrimination (ODD), a novel non-linear optical interrogation method, to quantitatively characterize mixtures of Fluorescent Proteins (FPs) with substantial spectral overlap that would prohibit their simultaneous use in conventional fluorescence analysis. FPs are a preferred tool for monitoring living cells and synthetic biochemical reactions.
The conventional experimental setup for measuring the filtered excitation/emission of
the sample is simple to construct and measure, and the proteins are expressible by
the biological system through genetic encoding. This not only means the sample does
not require preparation by dosing with exogenous and potentially toxic tags such as
organic dyes or quantum dots, but more importantly that the synthesis of the FP can be
accomplished by the cell being studied to mark the progress of a reaction as it progresses
in real time. As a result, FP sensors are ubiquitous in modern biological laboratories.
However, these FP reporters suffer from spectral cross-talk: their broad, featureless
absorption and emission spectra makes it difficult to distinguish more than 4-5 FPs in a
single experiment.

ODD overcomes this spectral cross-talk by using series of optimally shaped femtosec-
ond laser pulses to coherently excite unique wavepackets on the excited state surfaces
of the different molecules within a sample. These unique excited state wave packets in
the different molecules are designed by feedback algorithm to follow maximally distin-
guishable pathways on the excited state energy surface, enabling the different species to
be identified by their unique excited state dynamics even when their static absorption
spectra are inseparable.

**Enhanced Coherent Control of Optogenetic Switches**

The final (and most developed) project, presented in Chapter 5, uses coherent non-linear
optical interaction to enhance the control of a class of optogenetic switches. Optogenetics
is a revolutionary new technique which inserts light sensitive proteins into cellular sig-
nalling pathways to control biological functions in living cells using light. However, as
with the FP sensors in the previous work, spectral cross-talk limits the number of these
switches that can be independently controlled. For unidirectional photoswitches (switches
remain active only when illuminated) this cross-talk severely limits the number of fluo-
rescent reporters that can be used to monitor the system. Worse, for photo-reversible
optogenetic switches (switches latch in an activated or deactivated state depending on
last illumination condition) cross-talk prevents independent control of the two states of the single switch. The same light signals that turn off the switches also turn them back on, leading to a photoequilibrium threshold that restricts the maximal dynamic range of the switch and impedes its functionality. In the present work we successfully implement a non-linear optical control technique, Stimulated Depletion Quenching (SDQ), which overcomes this spectral cross-talk. SDQ shifts the photoequilibrium threshold of the optogenetic switch by selectively emptying the excited state of one of the photoswitching reaction pathways through stimulated emission while allowing the other reaction to continue unaffected. The unique, coherent, ultra-fast excited state dynamics of the chromophore molecules in the switches enable the differentiation of the two states even when their static optical responses are inseparable.

In our present work we have experimentally demonstrated the effectiveness of the SDQ technique in the phytochrome based optogenetic protein Cph8, which is used to control cellular signalling and gene expression in E. Coli bacterium [36]. The Cph8 protein controls cellular signaling by switching from the biologically in-active $P_{FR}$ state to the activated $P_R$ state (reverse photoswitching reaction) by exposure to ‘far-red’ light ($\lambda > 720$ nm), but can not be fully switched off (forward photoswitching reaction) as both states absorb red light (red light initiates simultaneous forward and reverse photoswitching reaction). We observed enhanced quenching of the reverse photoswitching reaction (inactive-to-active) compared to the forward (active-to-inactive), which should reduce the unwanted persistent activated population under red-light illumination. We have begun characterizing the dependence of the SDQ enhancement on the power, wavelength spectrum, and timing of the excitation and depletion pulses. To guide our experiments and aid in interpreting our measurements we have also developed a model that incorporates the measured optical characteristics and excited state dynamics of optogenetic switches to simulate the non-linear optical SDQ interaction and predicts the equilibrium threshold enhancement. This work is an initial feasibility study and few-parameter survey of the control space
to establish the effectiveness of the proposed mechanism. The success of this experiment lays the foundation for forthcoming experiments incorporating optimally tailored pulse shaping of the excitation and depletion pulses.

Finally, chapter 6 concludes the thesis with a projected outlook of the experiments presented herein as well as an outlook for the optimal quantum control field as a whole. An important aspect of this section is an evaluation of the role of technology and the prospective experiments enabled by these new an improved light sources and other experimental equipment. Final Concluding remarks are given in Section 6.4
References


Chapter 2

Background and Fundamentals

This chapter presents a brief overview of a range of fundamental background information that will aid in understanding the research presented in the following chapters. We begin with a basic introduction to the ultrafast laser pulses employed in the following experiments beginning with the mathematical formalism for describing the electromagnetic radiation in Section 2.1.1, followed by a presentation of the optical processes involved in generating the pulses in the sources used in the lab in Section 2.1.2. Next, Section 2.2 gives an overview of the mathematics and practical aspects of shaping the ultrafast pulses. Finally, in Section 2.3 there is a discussion of the interaction of the ultrashort pulsed light with matter. The subjects of ultrashort pulse lasers and ultrafast optics are broad fields unto themselves, and it is beyond the scope of this work to give a full review of the history and physics of the fields. There are several thorough and well written books and reviews that more comprehensively cover the subjects introduced in this chapter. The works of Boyd [1] and Mukamel [2] are the canonical books for nonlinear optics and spectroscopy. Weiner’s *Ultrafast Optics* [3] focuses on aspects related to ultrashort pulses, while Tannor’s *Interaction of Light with Matter* [4] highlights the role of coherence, both are well written and offer unique perspectives. Rulliere provides a good introduction to the generation and manipulation of femtosecond laser pulses [5].
state of optimal quantum control theory and experiments is found in reference [6]. The interested reader is directed to these works for additional information.

2.1 Ultrashort Pulsed Lasers

This section presents a basic introduction to sources of coherent ultrashort light pulses. Section 2.1.1 provides the mathematical formalism to describe the pulses, while Section 2.1.2 describes important features of the Ti:Sapphire Laser and the Optical Parametric Amplifier sources used in this work.

2.1.1 Mathematical Description of the Laser Pulse

The propagation of light is governed by the differential wave equation derived from Maxwell’s equations [7], which can be solved by the complex exponential wave function solution:

\[
e(\vec{z}, t) = \left[ A_0 e^{i(\omega t - \vec{k} \vec{z} + \phi_0)} + e^{-i(\omega t - \vec{k} \vec{z} + \phi_0)} \right] \hat{x},
\]

where \(A_0\) denotes the amplitude of the wave, \(\omega\) defines the monochromatic frequency, the wave propagates in the \(\hat{z}\)-direction and is polarized in the \(\hat{x}\)-direction, \(|k| = \omega/c = 2\pi/\lambda\) is the magnitude of the wavevector, whose direction is the same as the propagation direction \(\hat{z}\), and the wave has a complex phase given by \(\phi_0\). The electric field is directly measurable and therefor must be described by a real function, requiring the complex conjugate of the complex exponential. For simplicity we will set aside the spatial components of the light wave to focus on the time-frequency dependence of the field. We will also be dropping the complex conjugate, but it must be remembered that the field is a real function.

A laser pulse is represented by a coherent superposition of a large number (\(N\)) of the plane waves described by equation 2.1 with a range of frequencies \(\omega_m\) each having an amplitude \(A_m\) and a fixed phase relationship between all frequency components \(\phi_m\). This
is expressed by:

\[ e(t) = \sum_{m=-N/2}^{N/2} A_m e^{i(\omega_0 + m\Delta \omega)t + \phi_m} \]  

(2.2)

where the frequencies of the individual modes are equally spaced by \( \Delta \omega \) and are defined with respect to a central carrier frequency, \( \omega_0 \). The spectral phases \( \phi_m \) need not all be equal to generate a field pulse, but they must remain constant between pulses. We will see later that when the all frequency components have the same phase the pulse duration is minimized. Converting the sum over discrete modes to a continuous integral equation becomes:

\[ e(t) = \int A(\omega)e^{i\phi(\omega)}e^{i\omega t}d\omega \]  

(2.3)

Recognizing this as the Fourier transform integral, we can see that the electric field in the time domain is the Fourier transform of the complex spectral distribution function, \( \tilde{E}(\omega) \), and vice versa:

\[ e(t) = \mathfrak{F}[\tilde{E}(\omega)] = \mathfrak{F}[A(\omega)e^{i\phi(\omega)}] \]

\[ E(\omega) = \mathfrak{F}^{-1}[e(t)] \]  

(2.4)

The Fourier transform relationship between \( e(t) \) and \( E(\omega) \) links the functional width of the pulse in the time and frequency domains. It is a general property of Fourier transforms that a more localized function \( f(x) \) must have a more spread-out transform \( F(\xi) \). The uncertainty relation of the second moments (variances) of any complementary Fourier variables is:

\[ \langle \sigma_t^2 \rangle \cdot \langle \sigma_\omega^2 \rangle \geq \frac{1}{4} \]  

(2.5a)

\[ \Delta t_{FWHM} \cdot \Delta \omega_{FWHM} \geq TBP \]  

(2.5b)
where the second equation 2.5b gives the time-bandwidth product \((TBP)\) in terms of the more commonly measured full-width at half-maximum (FWHM) of the pulse duration and bandwidth, the value of the TBP depends on the pulse shape. The uncertainty product is minimized for a Gaussian function, with the product of the variance in time and frequency being 1/4. The FWHM time-bandwidth product for a Gaussian pulse shape, \(TBP_{\text{Gauss}} = 0.441\), is actually not the smallest TBP. Another common pulse shape, the \(Sech^2\) pulse (associated with the temporal profile of a pulse with a square spectral amplitude distribution) has a smaller \(TBP_{\text{Sech}^2} = 0.315\). Regardless of pulse shape, it is a defining characteristic that a broader spectrum generates a shorter temporal pulse.

It is also clear from the Fourier relationship in Equation 2.4 that the shortest laser pulse is associated with a constant or linear spectral phase, i.e. when all the spectral modes of the pulse overlap at the same time: \(t=0\) for the constant phase, or \(t=\tau\) for linear spectral phase \(\phi(\omega) = \omega \tau\). This can be seen, because any higher order phase terms in an expansion of \(\phi(\omega)\) would constitute a frequency dependent delay, stretching the pulse out as different spectral components arrive at different times. This flat spectral phase condition is called a transform-limited pulse, and is the shortest duration laser pulse attainable for a given spectral distribution function.

### 2.1.2 Short-Pulse/Broad Bandwidth Light Sources

Consistent, sub-100 femtosecond laser pulses were first produced in 1981 with the development of the colliding pulse mode-locked (CPM) ring dye laser [8]. While compressed dye lasers were the first to reach the femtosecond regime, they have been nearly completely supplanted by diode-pumped solid-state lasers by virtue of their higher intensity thresholds, shorter pulses, and their ease of use compared to the dye systems. In spectral regions where solid-state lasers are not available Optical Parametric Oscillators are employed to shift the frequency of a pump laser by optical mixing in a non-linear medium.

**Titanium:Sapphire Laser**
The earliest self mode-locking Titanium:Sapphire laser, developed in 1991 [9] generated pulses with 60-45 fs pulse durations, and this system was rapidly improved achieving pulse durations as low as \( \sim 5.5 \) fs within a few years [10,11]. The Ti:Sapphire laser has a number of desirable properties that make it an ideal gain medium for ultrashort pulsed lasers. The source of the laser gain is the Ti\(^{3+}\) dopant ion, which yields a huge emission spectrum from 660-1100 nm due to coupling of the ion to the Al\(_2\)O\(_3\) crystal lattice. The excitation of the ion from its ground state is conveniently in the range of several potential pump lasers such as the Ar ion (514.5 nm), doubled Nd:YAG or Nd:YVO (532 nm), or Nd:YLF (523.5 nm). The crystal has a high thermal conductivity, which is vital as effective temperature stabilization is critical to low noise laser emission. The crystal has a large energy storage density approaching 1 J/cm\(^2\) [12]. This was originally thought to be an undesirable trait, as earlier passive mode locked dye lasers relied on low storage density to initiate pulsing above a threshold pumping intensity [13]. The Ti:Sapphire laser achieves mode-locking by a unique non-linear optical effect known as Kerr lens mode-locking [14]. When first discovered, this mode-locking mechanism was not well understood and was initially described as “magic mode-locking” [9] prompting an enormous rush to understand the physics behind this new capability. Keller and Cunningham were first to connect the self-starting mode-locking to the Kerr effect [15]. The Ti:Sapphire crystal has a nonlinear, intensity dependent refractive index: \( n(I) = n_0 + n_2I \). As a developing laser pulse with Gaussian spatial mode travels through the crystal, the index of refraction is greatest at the beam center, generating a positive focusing lens from the perspective of the pulse. This is illustrated in Figure 2.1, where the beam paths of two lasing conditions are outlined on the right. The cavity is designed to intentionally attenuate the continuous wave (CW) lasing condition. As fluctuations in the laser intensity develop, more intense regions of the CW output experience a larger focusing effect from the Kerr lensing in the crystal. This reduces the attenuation from the aperture further increasing the developing pulse’s intensity. This feedback amplification leads to a dominant pulse train emerging from the
Figure 2.1: Kerr lens effect responsible for self mode-locking in Ti:Sapphire lasers. Due to the intensity dependent index of refraction and the Gaussian beam mode of the laser cavity, the pulse sees the crystal as a positive lens which focuses more intense pulses through a restricting aperture within the cavity. This favors the first intense pulse train to begin to develop, causing all of the energy stored in the gain material to dump into that mode.

CW beam and extracting all the energy stored in the gain medium. In this way, self-starting mode-locking is achieved with no active components and without restricting the energy storage density of the lasing medium, enabling the powerful and robust Ti:Sapphire laser to become the gold-standard ultrashort pulsed laser source for over a decade.

**Optical Parametric Amplifier and Non-collinear OPA**

Two Optical Parametric Amplifiers (OPAs) are utilized in the experiments in this dissertation to shift the output of the Ti:Sapphire laser, centered at $\lambda_0 = 800$ nm, to other spectral ranges. The broad bandwidth of the Ti:Sapphire laser is useful for generating ultra-short pulses, but the near-IR spectrum is outside the range of many high-value applications. The 800 nm spectrum can be doubled using a $\beta - BBO$ non-linear crystal to generate UV pulses centered at 400 nm, but the spectral domain between these two extremes is the most valuable region on the electromagnetic spectrum, especially in the growing field of bio-chemistry where many processes have naturally developed to take advantage of the solar spectrum. To reach these wavelengths we utilize one of two parametric amplifiers, a linear Optical Parametric Amplifier (OPA) and a Non-collinear OPA (NOPA). Both OPAs are commercial systems: the Palitra broadly tuneable OPA made by Quantronix generates narrow-band spectra (~5-10 nm) that are tuneable from 300 nm to 2000 nm. The TOPAS-White NOPA from Laser Quantum has a broader output...
bandwidth that can exceed 200 nm (nearly 4850 cm$^{-1}$) and is also tunable from the UV to the Mid-IR.

The theory of the optical parametric amplification process is based on difference-frequency generation (DFG) in a non-symmetric crystal via the $\chi^{(2)}$ nonlinear optical polarizability. For a more thorough description of the theory of optical parametric amplification refer to the featured review articles [16,17], or a more complete text on Non-linear optics [1,2]. In the NOPA, a high intensity pump laser beam (400 nm pulse generated by doubling the 800 nm Ti:Sapphire amplifier output) and a low power, lower frequency seed beam are mixed in the non-linear crystal, which transfers power from the pump laser into the seed beam is down-converted into two lower frequency fields, a shorter wavelength signal beam at $\omega_s$ and a longer wavelength idler beam at $\omega_i$, such that $\omega_s + \omega_i = \omega_{pump}$ to satisfy the energy conservation relation. The signal generation efficiency is dictated by the phase-matching condition of the crystal in which the mixing takes place: $\hbar \vec{k}_P = \hbar \vec{k}_S + \hbar \vec{k}_i$. This condition satisfies the conservation of photon momentum. This phase-matching condition cannot be satisfied in homogeneous crystals, which is why we use a non-linear, birefringent amplifying crystal like $\beta - BBO$. Within the birefringent crystal, efficient phase matching occurs when the pulses propagate at a specific angle to the ordinary and extra-ordinary crystal axis, set by the cut angle of the crystal as displayed in Figure 2.2. When the pump, signal, and idler beams propagate at the specified crossing angles with respect to each other and the crystal axis there will be effective energy transfer from the pump field into the signal and idler fields.

The phase-matching and frequency relations dictate when amplification is allowed to occur based on the rules of conservation of phase and energy, respectively; but the pump, signal, and idler beams still are required to be synchronized and spatially overlapped in the crystal for amplification to occur. This is where the difference between a Non-collinear OPA and a collinear OPA is manifest. We know that the light waves traveling through the crystal have a wavelength dependent group velocity: $v_g = d\omega / dk$. This spectrally varying
Figure 2.2: Crystal cut angles for effective phase matching (x-axis) at output signal wavelengths (y-axis) for a series of internal crystal crossing angles ($\alpha$) and a pump wavelength of 400 nm. The flattest vertical section of the curves will yield the greatest phase matching bandwidth. This occurs with a BBO crystal angle $\theta \simeq 31.1$ deg, at an internal crossing angle $\alpha \sim 3.7$ deg matching signal output from $\lambda_{\text{sig}} = 525 - 725$ nm. [16]

speed means that as the pump, signal, and idler beams traverse the crystal the pulses gradually separate, limiting the effective amplification length. By introducing an angle between pump, signal, and idler (as shown in Figure 2.3) it is possible to keep the pulses spatially and temporally overlapped for a longer propagation length in the amplification crystal, increasing bandwidth and power.

In the NOPA, the amplification process begins by generating a white light supercontinuum in a thick Sapphire optical window to act as a seed from which the final NOPA output grows. The white light is then stretched or compressed in time as part of the control designating the desired output characteristics. The goal is to overlap the portion of the seed with the pump beams for their entire duration. For narrower pulses that are tuned across the accessible spectral range the seed is stretched so that only portions of the continuum spectrum overlap with the pump, while for the broadest bandwidth pulse the supercontinuum seed duration should match that of the pump pulse. After the seed
\[ \hbar k_P = \hbar k_S + \hbar k_i \]

Figure 2.3: top: phsae matching conditions for Non-Collinear OPA. By introducing an angle between signal and idler the angle of the idler beam varies with wavelength to match wavevectors with pump and signal beams. bottom: compensating from group velocity mis-match in NOPA. When signal and idler beams are collinear the faster idler pulse no longer spatially overlaps with signal pulse and parametric amplification halts. When an angle is introduced the signal and idler pulses partially overlap over a longer propagation distance. Adapted from [1].

stretcher the seed is overlapped with the first fraction of the pump beam in the non-linear crystal at the proper crossing angle to achieve amplification. After this first pass amplification, the signal beam is collimated, synchronized with remaining high intensity pump beam, and beams are crossed in the non-linear crystal again for a second, higher power amplification stage. Typical broad-bandwidth spectrum of the NOPA output are given in Figure 2.4. The final output power is typically \(~ 20 \) mW when driven by 1 W from the Ti:Sapphire amplifier, generating a 400 nm pump beam of 400 mW. The spectrum shown in the figure is the broadest stable spectrum attainable with the instrument used in these experiments, though broader spectra are possible with thinner non-linear crystals. This additional bandwidth comes at the expense of power and stability, as will be discussed in later sections.
Figure 2.4: Representative spectra of the NOPA output when aligned for ultra-broad bandwidth signal output. The first pass is weaker than the second pass, but broader and more stable. The second pass amplification generally has an overall conversion efficiency of only $\sim 5\%$, but the frequency range of the light is not only greatly expanded, but tuneable to nearly the entire visible and NIR spectrum. The output shown here has a spectrum ranging from 600-710 nm, over 2500 $cm^{-1}$.

2.2 Pulse Shaping

The previous sections have focused on creating ultrashort pulses by coherently synchronizing the spectral modes of a broad bandwidth laser, producing a ‘transform-limited’ pulse with the maximum temporal resolution and peak intensity. In this section we will focus on spreading these spectral modes back out and arranging them to form tailored electromagnetic pulses with structure and features carefully designed to enhance, direct, or observe coherent molecular dynamics. The ability to tailor the temporal field of a laser pulse to forms of nearly arbitrary complexity is not merely a novelty and a trick of the light. Ultrashort laser pulse shaping has become an important field yielding knowledge of laser matter interactions and methods for improved pulse compression and tailoring; pulse shaping has impacted fields as diverse as ultrafast spectroscopy, microscopy, fiber communication, and metrology.
2.2.1 Mathematical Description of Pulse Shaping

As we have seen, the electric field of a laser pulse can be described as the sum of a set of complex exponential waves expressed in the time or frequency domain with the two descriptions related by a Fourier transform. To modify an input pulse $e_{in}(t)$ into an arbitrary waveform $e_{out}(t)$ we could act upon it by applying time based modulation or frequency domain modulation. To shape a pulse in the time domain we take the convolution of the initial waveform with a modulation function $m(t)$:

$$e_{out}(t) = e_{in}(t) \otimes m(t) = \int e_{in}(t')m(t-t')dt'$$  \hspace{1cm} (2.6)

But because we are working with ultrashort pulses in the femtosecond regime, there is no instrument that can create a masking function $m(t)$ able to modulate the field at such a fast rate. By the characteristics of the convolution relation, the above problem is mathematically equivalent to the product of the two functions in their Fourier conjugate dimension:

$$E_{out}(\nu) = E_{in}(\nu) \cdot M(\nu)$$  \hspace{1cm} (2.7)

Where $E_{out}(\nu), E_{in}(\nu), \text{and } M(\nu)$ are the Fourier transform of their respective time domain functions: $E_{in}(\nu) = \mathfrak{F}[e_{in}(t)], \text{etc.}$ The key to Fourier transform pulse shaping is that by operating in parallel on all the spectral components of the incident pulse at once, it is possible to produce femtosecond time-scale variations on the field without needing modulators that operate at this speed [18]. To express the whole shaping process mathematically:

$$e_{out}(t) = \mathfrak{F}^{-1}[\mathfrak{F}[A_{in}(t)e^{i(2\pi \nu t + \phi_{in}(\nu))}] \cdot M(\nu)e^{i\phi_{m}(\nu)}]$$  \hspace{1cm} (2.8)

By operating on the input waveform in the frequency domain with a complex modulation function equal to the ratio of the desired and initial spectral waveforms, we should be able to create any laser pulse profile – assuming sufficient spectral bandwidth. The remainder
of this section addresses the real world attributes of the laser field and real pulse shapers that begin to constrain the accessible shaped fields.

### 2.2.2 Pulse Shaping Apparatus

Figure 2.5(a) shows the model of the most common “4-f pulse shaper” design [18]. This apparatus can be seen as the physical implementation of Equation 2.8. The first grating Fourier transforms the initial pulse by dispersing the spectral modes of the input pulse, mapping the spectral domain onto a spatial dimension. The amplitude and phase of the spectral components are then operated on by a spatial light modulator (SLM) acting as the masking function $M(\nu) \cdot e^{i\phi_m(\nu)}$. Finally, the modified spectral modes are recombined on the second grating performing an inverse transform to form the newly shaped pulse in the time domain.

For the shaper to function properly the incoming laser pulse must be perfectly reproduced in the absence of a masking operator by the SLM. The configuration in the diagram is referred to as a zero-dispersal 4-f configuration because each optical component is placed one focal length apart. This geometrical symmetry ensures that pulses entering either side of the shaper will undergo identical interactions in the two halves of the shaper before the SLM, and therefore must be identical on exiting. In practice, this shaper design is typically constructed in a slightly different arrangement and with different components. The concept of a pair of dispersing and focusing elements, symmetrically arranged on with a modulator in the middle is the same, but the changes must be made to accommodate non-ideal features of real optical elements. While these alterations do not alter the function of the system, they can complicate the schematic representation and obscure the function of the elements of the shaper system. A schematic of one of the pulse shapers used in the experiments in this work is shown in Figure 2.5(b). The differences between this shaper and the ideal cartoon on the left are described below.
Figure 2.5: (a) Diagram of 4-f geometry pulse shaper. The input pulse is a compressed broad bandwidth pulse, its spectral components are dispersed by the grating (GR) and mapped onto the Spatial Light Modulator (SLM) at the focal plane of the focusing element (FL). The SLM modulates the phases and/or amplitudes of the frequencies of the pulse before they are recombined on the second arm of the shaper into a complex structured shaped pulse. (b) Schematic of the pulse shaper used in the following experiments. The second arm has been replaced with a mirror at the back of the SLM and the lenses are replaced by cylindrical mirrors (CM). The reasons for these alterations are listed in the text.

In the construction of most pulse shapers operating on pulses with more than 10 nm of bandwidth the lenses must be replaced by cylindrical mirrors. This is partially to limit dispersion, as it is generally desirable to avoid passing femtosecond laser beams through any more glass than absolutely necessary, but this is actually not the main reason for using mirrors. Chromatic aberration makes it impossible to focus the broad spectra of the pulse onto a single focal plane where the SLM can be placed. Crafting adequately corrected achromatic doublet or triplet cylindrical lenses of sufficient size to cover the spatial extent of the SLM is simply prohibitively difficult and costly. Replacing the transmissive lens with a reflecting mirror folds the beam path back on itself. This necessitates placing
another mirror between the grating and curved mirror to redirect one of the beams to prevent the SLM and grating from being on top of each other.

Another common alteration is to place a mirror as close as possible to the SLM element to reflect the modulated beam back through the same optical path as the incoming beam with a small vertical angular tilt to allow the outgoing beam to be picked off and separated from the incoming un-shaped beam. There are many advantages to this design: it simplifies the construction and optical alignment by cutting the number of optical elements (and the potential misalignments that accompany each element) in half and guarantees that the two arms of the shaper will be symmetrical. This also doubles the optical path through the SLM, increasing the maximum phase shift. The reflected, double pass configuration does introduce two geometrical aberrations that could be reduced by using a full 4-f configuration. To properly align the system the mirror must be placed at the focal plane of the lenses. This means the SLM must be slightly in front of the focal plain and the spot size of the focusing spectral modes will be slightly larger. We have seen in the last section that this limits the shaping capabilities of the device, decreasing the size of the Gauss-Sinc window of accessible pulse shapes. This effect is generally negligible if the reflecting mirror can be placed within a few millimeters of the SLM: at these distances the difference in beam spot size and divergence is quite small. SLM manufacturers are aware of this and design the systems to allow the close placement of the back-reflecting mirror, some models even come with this mirror pre-installed as part of the liquid crystal stack. Further, this aberration would still be present in a non-reflecting shaper geometry. The second aberration would not be present in the non-reflecting shaper geometry, and can impact very sensitive measurements, but it is generally acceptable to ignore. The tilting of the beam as it passes over and under itself introduces a pulse front tilt in the vertical beam cross-section [17]. This effectively places a diameter dependent delay on the pulse, a spatial-temporal coupling that makes the pulse appear longer than it is and can reduce
the field intensity when the beam is focused. This is generally a small aberration, and
the tilt of all mirrors are kept to a minimum to limit the effect.

Depending on the spectral range and central wavelength of the laser being shaped, it
can sometimes be difficult to find acceptable gratings. In some cases it can be difficult to
disperse the spectrum across the width of the SLM within the desired grating-to-mirror
distance. In some cases the grating efficiency characteristics are unsatisfactory and too
much initial intensity is lost to reflections or higher order diffraction modes. In ultra-
broad bandwidth pulses the different diffraction orders can overlap. In these situations the
gratings can be replaced by prisms, though these carry their own problems of non-linear
dispersal of the spectrum and dispersion from propagating through significant lengths of
glass [19].

The masking device that provides the modulation function can be anything that will
act on the transmission or the optical path length of a portion of the dispersed pulse. In
the earliest ultrashort pulse shaping experiments this mask was a fixed, etched plate [20].
While providing excellent fidelity waveforms, these plates were difficult to work with and
obviously not programmable: requiring replacement, re-etching, and re-alignment with
each new waveform design. Since that time, dynamically reconfigurable masks have been
developed that allow real time alterations to the pulse profile. The most commonly used
SLM devices are liquid crystal arrays [21–23] and acousto-optic modulators that can both
modulate the spectral phase and amplitude [24].

**Special Considerations for Ultra-Broadband Pulse Shaping**

Pulse shaping tools and techniques develop alongside the ultrashort pulse laser sources
that they are used with. To this end, much of the standard body of knowledge and
many of the instruments used in pulse shaping are optimized for the spectral range of the
Ti:Sapphire laser. When designing and using a pulse shaper for a different source, such as
the ultra-broad bandwidth NOPA discussed above, there are a few details that must be
taken into consideration that do not arise when building a pulse shaper for a Ti:Sapphire laser. The most prevalent difficulty is the rarity of dielectric mirrors and other optics with optical coatings supporting good quality over the ultra-broad bandwidths. This results in most optics being replaced with silver where possible, but in cases of anti-reflection coatings in lenses or windows there is currently not many suppliers of quality optics because there are so few users making the demand. Surely this will improve as these sources become more widely used.

Depending on the spectral range and central wavelength of the laser being shaped, it can sometimes be difficult to find acceptable gratings. In some cases it can be difficult to disperse the spectrum across the width of the SLM within the desired grating-to-mirror distance. In some cases the grating efficiency characteristics are unsatisfactory: too much intensity is lost to reflections or higher order diffraction modes. Effective pulse shaping of ultra-broad bandwidth pulses with octave-spanning spectra using diffraction grating based shapers is not feasible, as the different diffraction orders of the ultra-broad spectrum will overlap. In these situations the gratings can be replaced by prisms, though these carry their own problems of non-linear dispersal of the spectrum and dispersion from propagating through significant lengths of glass [19].

The voltage dependent phase retardation of the liquid crystal SLMs is strongly wavelength dependent, with the maximum per pass phase offset for light at 500 nm being nearly a full $\pi$ radians greater than for light at 800 nm. This is shown in Figure 2.6, where the measured phase offset as a function of liquid crystal voltage is plotted at four wavelengths. Looking at the effect this has on transmitted intensity as a function of applied pixel voltage shows that at large offsets the differences in the two calibration curves can cause the shaper to fully attenuate a pulse when it is meant to be fully transmitting it. Typically one can use a global conversion table between the desired (phase, amplitude) settings of each pixel address and the pixels’ assigned voltages. With ultra-broad
Figure 2.6: Wavelength dependence of the liquid crystal SLM voltage driven phase offset, measured at 4 wavelengths. While the differences do not seem particularly striking in the phase offset graph, looking at the effect on the transmission as a function of set voltage for the highest and lowest wavelengths reveals the effect of the wavelength dependent phase retardation. Improperly indexed pixels using the wrong calibration can end up fully attenuating a spectral element when it is meant to be fully transmitted.

bandwidth sources the wavelength associated with each pixel must be known to correctly assign phase and amplitude values.

2.3 Light Matter Interaction

Spectroscopy is the use of light to passively interrogate a system, revealing the composition, structure, and dynamics of molecules within a sample through their interaction with the optical field. In photo-chemistry photons of light participate in chemical reactions to produce a product state, somewhat like any other chemical reagent, typically initiating reactions by providing sufficient energy to overcome an activation barrier. In these reactions the light is not much different from an environmental control variable like temperature or pressure, with more control over the application and specificity of the energy delivery. Quantum Control (QC) encompasses both these classes of light matter interaction and goes beyond: both observing and manipulating systems of atoms or molecules with optical control fields, and at all points exploiting the wave-like nature of quantum states.
The aim of QC is to drive a system to a desired product state by actively manipulating the coherent interferences between quantum states excited within a wave packet.

Quantum control is facilitated by the coherent interaction of a quantum system with an ultra-short pulsed laser. There is a critical, fundamental difference between excitation of a molecule/quantum system by an ultrashort pulsed, coherent laser field and a conventional light source, such as a broad spectrum incoherent white light lamp. A single molecule sees a white-light lamp as a population of photons with a distribution of frequencies. Any absorption/interaction between a single molecule and a single photon occurs at a single, well defined energy/optical frequency. When a large number of molecules interact with a large number of photons an absorption spectrum can be observed with a statistical distribution of all the transitions taking place in the sample, but every unique transition was from one defined quantum eigenstate to another. After this excitation there may be a degree of coherence during the relaxation of the molecule brought on by an-harmonic coupling of vibrational modes, but the relaxation process is generally a stochastic, incoherent process facilitated by coupling to a random (thermal) reservoir of bath states in the surrounding environment. By contrast, a single molecule sees the ultrashort coherent pulse not as a collection of photons with well defined energy, but as a wave composed of all frequencies within the bandwidth at once. This excites a superposition of several quantum states in a single molecule, rather than a distribution of those quantum states across many separate molecules. This superposition wavepacket undergoes unique dynamics specified by the energy structure of its composite states and the phase relationship between the wavefunctions. When an ultrashort pulse interacts with a large number of molecules, each molecule is excited to such a superposition, creating a distribution of superposition wavepackets. During excitation of a quantum system by a ultrashort pulsed laser field, the pulse transfers its coherence to the molecule in the generation of the wave packet.
2.3.1 Non-linear Polarizability

The following material is summarized primarily from reference [7]. Where the radiation field propagates through a material with non-zero optical response there is a reciprocal interaction between the field and the medium: the field excites the system generating dipoles, and in the bulk medium an induced polarization develops, expressed as the sum of the individual dipoles. When the induced polarization develops it becomes a source or sink term in Maxwell’s Equations, associated with emission or absorption of the field, respectively. For weak fields this induced polarization is directly related to the incoming field: \( P = \chi E \), where the constant of proportionality \( \chi(\omega) \) is the Linear Susceptibility.

With the addition of this source term the wave equation derived from Maxwell’s equations:

\[
\nabla^2 E - \frac{1}{c_0^2} \frac{\delta^2 E}{\delta t^2} = \mu_0 \frac{\delta^2 P}{\delta t^2}
\] (2.9)

still have complex exponential wave-like solutions, but the relation between frequency and wavevector must be adapted:

\[
\left( \frac{k_c}{w} \right)^2 = 1 \rightarrow \left( \frac{k_c}{w} \right)^2 = 1 + \chi(\omega) = \epsilon(\omega)
\] (2.10)

The quantity \( \epsilon(\omega) \) is the dielectric constant of the material. Like the susceptibility, \( \epsilon(\omega) \) is typically complex: composed of a real part \( \eta \), the refractive index of the medium, and an imaginary part \( \kappa \), the extinction coefficient associated with absorption. \( \frac{k_c}{w} = \sqrt{\epsilon(\omega)} = (\eta + i\kappa) \). Incorporating these relations, the travelling wave solutions from equation 2.1 now take the form:

\[
e^{i(kz - \omega t)} = e^{i\omega \left( \frac{\eta z}{c} - t \right)} \cdot e^{i\left( \frac{\omega \kappa z}{c} \right)}.
\] (2.11)

The role of \( \kappa \) as the term responsible for exponential absorption as a function of propagating length \( z \) becomes clear. More subtle is the index variation to the wavevector,
\( k^{(1)} = \left( \frac{\eta \omega}{c} \right) \), which is responsible for scattering effects in materials with inhomogeneous or non-uniform refractive index.

For stronger fields the induced polarization in the medium is larger and has greater impact on the propagating field. The non-linear relationship between the polarization and the electric field can be expressed as a Taylor expansion of terms:

\[
P(\omega) = \chi^{(1)} \cdot E + \chi^{(2)} : E^2 + \chi^{(3)} : E^3 + P^{(1)} + P^{(2)} + P^{(3)} + .
\]  

(2.12)

as the order of non-linear polarizability increases, the dimensionality of \( \chi^{(n)} \) grows from a constant (with respect to \( E \)), to a matrix, to an n-dimensional tensor. The associated products \( \chi^{(n)} : E^n = \chi^{(n)}(\omega) : \{E(\omega_1)E(\omega_2)...E(\omega_n) \} \) are increasingly high dimensional tensor products between multiple frequency components of the field. In the higher order terms the frequencies of the participating fields (including negative frequencies associated with complex conjugate terms) must sum to the local frequency \( \omega \) of the total polarizability:

\[
P^{(N)}(\omega \equiv \sum_{i=1}^{N} \omega_i) = \chi^{(N)} : (E(\omega_1) \cdot E(\omega_2) \cdot ...E(\omega_N)).
\]  

(2.13)

This derivation of the non-linear polarizability interaction between the field and an optically responsive medium through which it propagates is a macroscopic relationship with a bulk material, but it is built on a microscopic origin of an induced dipole moment in individual molecules. On a microscopic, quantum mechanical level we re-connect the polarizability with the dipole moment:

\[
P = \langle \psi | \hat{\mu} | \psi \rangle = e \langle \psi | \hat{x} | \psi \rangle
\]  

(2.14)

By reconnecting the polarizability to the changes in the quantum wavefunction induced by the field we can move to discussion of laser generated wavepackets.
2.3.2 Laser Generated Wavepacket Dynamics

Returning to an isolated molecule, we can study the light driven coherent dynamics of a quantum systems by observing the creation and dynamics of wavepackets. The following presentation is summarized from \[4, 25, 26\]. The time-dependent Schrödinger equation describes the dynamics of a particle in a constant potential \( V(r) \):

\[
i\hbar \frac{\partial}{\partial t} \psi(r, t) = H \psi(r, t)
\]

\[
H = -\frac{\hbar^2}{2m} \nabla^2 + V(r)
\]

(2.15)

Because the Hamiltonian is independent of time we can generate static wavefunction eigensolutions: \( H\phi_n(r) = E_n\phi_n(r) \), where \( E_n \) is the energy of the associated eigenstate. With these time-independent eigenstates in hand we find the time-dependent Schrödinger equation (TDSE) has the solutions:

\[
\psi(r, t) = \sum_{n=1}^{\infty} c_n \phi_n(r) \exp \left[ \frac{i}{\hbar} E_n t \right]
\]

(2.16)

This solution describes a wavepacket: a coherent superposition of eigenstates \( \phi_n(r) \). The wavefunction of the system is time-dependent when multiple eigenstates are combined, with the real probability density:

\[
|\psi(r, t)|^2 = \sum_{n,m=1}^{\infty} c_n^* c_m \phi_m(r)^* \phi_n(r) \exp \left[ \frac{i}{\hbar} (E_n - E_m) t \right]
\]

(2.17)

having dynamics associated with the frequencies corresponding to the energy differences between combined states.

When a laser field interacts with this system it introduces a time-dependent perturbation into the Hamiltonian. The new Hamiltonian contains a term associated with the
dipole coupling between the system and the field: 

\[ H_{Tot} = H_0 + H_1 = \left[ -\frac{\hbar^2}{2m} \nabla^2 + V(r) \right] + [-\mu_{nm} \cdot \mathbf{E}] \].

In the case of a resonant transition between two molecular states \(|\psi_1\rangle\) and \(|\psi_2\rangle\) we can express the TDSE as [4]:

\[
\begin{align*}
    i\hbar \frac{\partial}{\partial t} \begin{pmatrix} \psi_1(t) \\ \psi_2(t) \end{pmatrix} &= \begin{pmatrix} H_1 & -\mu_{12}^* E^*(t) \\ -\mu_{21} E(t) & H_2 \end{pmatrix} \begin{pmatrix} \psi_1(t) \\ \psi_2(t) \end{pmatrix} \\
    &= \begin{pmatrix} \langle \psi_1|\mu_{1,2}|\psi_2 \rangle E \neq 0. 
\end{align*}
\]

In the frame of Born-Oppenheimer approximation, the Hamiltonians \(H_1\) and \(H_2\) contain the kinetic energy term and the potential energy term associated with the two molecular states being excited. Each could be represented by a quadratic potential with an energy difference between their lowest levels, or more complex models of molecular potentials could be used, but the two states are independent aside from the optical coupling. The off-diagonal terms in \(H\) describe the optical transition between states, with the system absorbing a photon when the transition dipole moment from state \(|\psi_1\rangle\) to \(|\psi_2\rangle\) is non-zero:

In the Born-Oppenheimer approximation, we are able to separate the molecular wavefunction into a product of independent electronic and nuclear wavefunctions \(|\psi(x_e, X_v)\rangle = |\varphi_{elec}(x_e, X_v)\rangle \cdot \phi_{Nuc}(X_v)\rangle\), defined in terms of a set of electronic coordinates \(x_e\) and the nuclear coordinates \(X_v\) [26]. We make this approximation on the basis that the electronic motions are several orders of magnitude faster than the nuclear motions, so the electron depends on the nuclear coordinates but views the nuclei as fixed while the nuclei respond to an invariant time-averaged potential from the electrons. With this approximation in hand, the dipole operator is also separated into components expressed in electronic and
nuclear coordinates:

\[ M = \langle \psi_1(x_e, X_v) | \mu_{12}(x_e, X_v) | \psi_2(x_e, X_v) \rangle \]  

(2.19a)

\[ = \langle \varphi_{e1} \cdot \phi_{e1} | \mu_{12}(x_e) | \varphi_{e2} \cdot \phi_{e2} \rangle + \langle \varphi_{e1} \cdot \phi_{e1} | (\mu_{12}(X_v)) | \varphi_{e2} \cdot \phi_{e2} \rangle \]  

(2.19b)

\[ = \langle \varphi_{e1} | \mu_{12}(x_e) | \varphi_{e2} \rangle \cdot \langle \phi_{e1} | \phi_{e2} \rangle + \langle \varphi_{e1} | (\mu_{12}(X_v)) | \phi_{e2} \rangle \]  

(2.19c)

\[ = \langle \varphi_{e1} | \mu_{12}(x_e) | \varphi_{e2} \rangle \cdot \langle \phi_{e1} | \phi_{e2} \rangle \]  

(2.19d)

where the terms containing \( \mu_{12}(x_e) \) are associated with the electronic transition and the term associated with \( \mu_{12}(X_v) \) does not contribute because the electronic eigenstates are orthogonal: \( \langle \varphi_{e1} | \varphi_{e2} \rangle = 0 \). The first term in equation 2.19d is the electronic transition dipole moment, giving the strength of the dipole coupling, while the second term is the vibrational overlap integral, the square of which is called the Franck-Condon factor. We see that the Franck-Condon principle, that electronic transitions are more likely to occur when vibrational eigenstates on the initial and final state have greater overlap, is a direct consequence of the Born-Oppenheimer approximation. When a broad bandwidth, ultrashort pulse field interacts with such a system it’s field spans many vibrational levels, generating a coherent superposition each with an amplitude associated with a transition dipole moment like equation 2.19d and a phase related to the time dependence of the spectral modes of the pulse associated with the vibrational coupling energies.

2.4 Summary

This chapter presented mathematical and technical background that will be helpful in understanding the work presented in the following chapters. The overview of the mathematical description of the ultrashort laser pulses lays a framework for descriptions throughout the remaining chapters and gives insight into the way the lasers are constructed and function. The mathematical and practical details regarding pulse shaping will be useful in
understanding the optimal pulse shaping to discriminate similar fluorescent bio-reporters in chapter 4 as well as the goal for shaped control of optogenetic switches in future experiments beyond the work presented in chapter 5. Finally the last section on Laser-Matter interaction covers a range of levels of complexity from the semi-classical to the quantum mechanical. All of these models rely heavily on approximations, and real laser-matter interactions are significantly more complex than what is represented here. However, the models are useful for building an intuition and understanding of the interactions between the coherent ultrafast laser and the molecular sample.
References


Chapter 3

Imaging of Colliding Microdroplets with Chemically Sensitive Fluorescence

Overview

We present a novel and simple optical capability for generating spatially resolved chemical concentration maps of systems of mixing fluids using a chemically sensitive dye detected by planar laser induced fluorescence (PLIF). To demonstrate an application of this capability we investigate the collision and mixing of a pair of micro-droplets in air. The two micro-droplets are composed of different fluids, methanol and water, with the dye initially in the methanol droplet. When the two droplets collide the fluorescence of the dye shifts from blue to green as the solvent environment changes. A series of spectral-temporal images of the collision and subsequent mixing are recorded, from which we extract information about the distribution of the two intermixing droplet species reflected in the spatially resolved dye spectra. Images reveal material transfer between droplets in both coalescing and non-coalescing droplet collisions.

3.1 Introduction

The intermixing of two fluids, often with each containing dissolved species, is a prevalent physical process, often accompanied by mixing enabled chemical reactions. In particular, the fluid dynamics of micro-droplets (i.e., their collision, coalescence, and material transfer) is important to many processes in nature and technological applications. At the microscopic scale fluid flow can have unusually behaving physical characteristics (i.e., surface tension and energy, surface area-to-volume ratio, etc.) which vary non-linearly,
impacting the fluid flow. At microscopic scales the resistance of fluids to mixing can become a major obstacle to chemical reactions. Micro-droplets provide a model system to carefully study fluid mixing at these scales. Diverse processes rely on the unique physical properties of microscale droplet formation, including bio-aerosol transport on ocean mist [1] and the movement of fluids in the micro-capillaries of plant or animal cellular tissues [2]. Similarly, many applications can be found throughout science and industry, including nano-liter analytical chemistry of rare or precious samples [3], or those too hazardous to work with in large quantities [4], sprayed fuel-oxidizer reactions in rocket engines [5–8], drug discovery and delivery [9, 10], and the rapidly growing field of micro-fluidic ‘lab-on-chip’ systems [11–13].

There are many detailed and thorough studies characterizing the mixing of fluid flows in general and of droplets in particular. As far back as 1881 Osborne Reynolds observed droplets persisting on the surface of similar liquids for several seconds before coalescing into the underlying pool [14]. In 1956 Linton and Sutherland studied the impact of droplet size on coalescence between pairs of droplets on a surface and observed the effect of the trapped air between surfaces [15]. Many more experiments followed studying binary collisions of free-falling identical sized droplets in air [16–19], and cases of unequal size [20, 21] and the collision and combination of droplets in turbulent flows [22]. Still more recent studies surveyed a wide variety of colliding droplets of varying sizes and composition with different fluid properties, colliding in a wide range of relative geometries to identify and interpret the effects of all these parameters on coalescence, mixing time, and intersample transport [23–27]. This long history and continuing current activity demonstrates that the study of droplet mixing remains a vibrant and important field.

All of these studies required appropriate experimental tools to accurately measure and characterize the mixing of the fluids. In this paper we present a novel, easily-implemented diagnostic technique to produce high quality imaging of the complex mixing taking place over a range of interaction scales. We image the Planar Laser Induced Fluorescence
(PLIF) from a chemically sensitive dye, Hydroxypyrene Trisulfonic acid (HPTS), inside a pair of colliding micro-droplets of different liquid solvents. PLIF, uses a laser pulse that is expanded to produce a planar sheet of light in order to illuminate a fluorescent object for imaging concentration distributions, especially in flowing fluids [28–30]. PLIF imaging has many benefits over short-exposure, high-illumination photography. In particular, the emitted fluorescence signal is detected over a dark background, enabling the detection at the earliest onset of mixing. The laser spectrum is well defined and can easily be filtered to prevent reflections and scatter of the excitation from obscuring the signal from the sample. The present work utilizes a chemically sensitive dye rather than an inert molecule such as Rhodamine-WT to extract additional information about the local, molecular scale sample make-up [31]. The HPTS dye undergoes an instantaneous deprotonation reaction (i.e., at the timescale of \( \sim 3 \) picoseconds [32]) with its solvent environment, reacting differently when solvated in either water or methanol, thereby producing a measurable shift in fluorescence. When the HPTS dye is carried in only one of a pair of droplets, with one composed of methanol and the other water, the instantaneous, local reaction gives an easily detected readout of the environment of the dye molecule, while the imaging of the colliding droplet pair reveals larger scale inertial or convective transport from one droplet to the other. Characterizing the coalescence and mixing of the micro-droplets over these vastly different length scales offers a broad view of the rich dynamics of mixing and provides a simple and effective tool for spatio-temporal analysis of fluid dynamics under a variety of conditions.

Section 3.2 presents a brief overview of the important aspects of fluid dynamics pertinent to the mixing of micro-droplets as well as background on laser induced fluorescence measurements for quantitative sample characterization. Section 3.3 describes the apparatus for producing and colliding the micro-droplets and the method of imaging fluorescence emission from these collisions. The resultant images of the colliding and mixing droplets
are presented in Section 3.4, along with their analysis to generate quantitative mapping of local species relative concentration. Finally, concluding remarks are given in Section 3.5.

3.2 Background

Micro-scale Fluid Dynamics:

We present a brief discussion of the characteristics of fluidic behavior at the microscale and its implications in mixing. For a more comprehensive review the reader is referred to [33–35]. Moving fluids may be categorized by two extreme limits of behavior, with flow conditions being either laminar or turbulent. Laminar flow corresponds to smooth motion with characteristic stream lines. In contrast, turbulent flow is described by fluctuating local flow vector fields possibly including features such as spatial vortices. The two regimes differ in terms of the relative magnitude of internal viscous and bulk inertial forces. The relative strength of these two types of forces for a given flow condition is quantified by the dimensionless Reynolds number (Re): \( Re = \frac{\rho u D_h}{\mu} \), where \( \rho \) and \( \mu \) are the fluid density and viscosity, \( u \) is the net velocity of fluid and \( D_h \) is the hydraulic diameter of the fluid system, specifically defined for different flow geometries. At low Re viscous effects overwhelm inertial effects and the fluid flow is laminar (i.e., local domains within the fluid tend to move in concert, forming well defined streamlines [34]). At higher Re, turbulence dominates, the net flow is broken into many small local domains with divergent velocity vectors, creating a large number of interfaces between inhomogeneous regions on multiple length scales [34]. The latter circumstance is ideal for mixing, but turbulent flow is difficult to achieve in micro-scale systems due to the small size of \( D_h \). The transition between laminar and turbulent flow conditions is often accepted as \( Re \sim 2,000 \) [36]. In the case of colliding droplets the Reynolds number is calculated for each droplet independently using the relative collision velocity as the fluid velocity and the droplet diameter as the hydraulic diameter. For the methanol and water micro-droplets utilized in the experiments of this
paper, the Reynolds numbers are estimated to range from 500-650, which is comfortably within the laminar flow regime. As a result, the droplet mixing is primarily achieved by local diffusion across the collision interface and across the interfaces between any streamline flows generated by bulk advective flow between the droplets.

As suggested above, there are two mechanisms for mixing of the droplets: diffusion and advection. Diffusion is defined as the transfer of fluid particles (differentially small volumes or molecules) from a region of high concentration to one of lower concentration by random Brownian motion. Advection is the transfer of a sub-domain within the larger volume by bulk transport, conserving physical properties of the domain (i.e., composition, momentum, or energy) while exchanging its position within a flowing current. The diffusion of a material with concentration field $\varphi(r,t)$ can be described by Fick’s first law:

$$\mathbf{J} = -D\nabla \varphi$$

(3.1)

where $\mathbf{J}$ is the material flux vector (i.e., the amount of material that flows through a unit area during a unit time) and $D$ is the diffusion coefficient [33], taken as a constant here. Combining Fick’s law with the continuity equation, $\partial \varphi / \partial t + \nabla \cdot \mathbf{J} = 0$, gives the diffusion equation:

$$\frac{\partial \varphi}{\partial t} = D\nabla^2 \varphi$$

(3.2)

For the diffusion of spherical particles within a viscous medium, the diffusion coefficient can be derived by the Einstein-Stokes equation:

$$D = \frac{kT}{6\pi \mu R}$$

(3.3)

where $kT = 4.11 \times 10^{-21}$ J at room temperature, $R$ is the radius of the particle (or molecules) and $\mu$ is the viscosity of the medium [37]. For a small molecular dye like HPTS, using the longest axis of the molecule as the sphere diameter ($2R_{HPTS} = 1.5nm$)
the diffusion coefficient in water at room temperature is \( D_{HPTS,H20} = 1.4 \times 10^{-10} \text{m}^2/\text{s} \) and the diffusion coefficient in methanol is \( D_{HPTS,H20} = 2.5 \times 10^{-10} \text{m}^2/\text{s} \).

To demonstrate what the magnitudes of these coefficients mean we can solve the diffusion equation for the uniaxial spread of dye molecules in one dimension and find that the average time (\( \tau \)) for particles to diffuse a distance \( x \) is given by the relation:

\[
\tau = \frac{x^2}{2D} \tag{3.4}
\]

For a diffusion coefficient \( D = 2 \times 10^{-10} \text{m}^2/\text{s} \), over a period of \( \tau = 1 \text{ ms} \) the diffusion length \( x=0.6 \ \mu \text{m} \); thus, to travel 100 \( \mu \text{m} \) (the scale of the diameter of micro-droplets in our experiments) the diffusion time will be \( \tau \approx 25 \text{ seconds} \). Because of the slow diffusion rate at room temperature, diffusion alone is typically not sufficient for effective mixing. Rather, in micro-scale systems effective mixing is achieved by combining advection and diffusion, where advection can break the bulk volume into small domains such that the distance between dissimilar domains is small compared to the diffusive length scales.

**Pulsed Planar Laser Induced Fluorescence Imaging:**

To visualize the mixing of the droplets, a small concentration (\(< 1 \mu \text{M}\)) of the chemically sensitive dye HPTS was added to methanol and PLIF images were collected of the methanol solution mixing with the water droplet (initially free of dye). The intensity of the fluorescent signal collected from the droplets is a measure of the local dye concentration, while the spectral character reflects the solvent environment of the dye. The fluorescence emission from from a differentially small volume located at \( \vec{r} \), a position vector in the plane of the collision, in a solution carrying a spatially varying concentration of dye, \( C(\vec{r}) \), is given by \( F(\lambda; \vec{r}) \):

\[
F(\lambda; \vec{r}) = \left[ \int I_0(\lambda_{Ex}; \vec{r}) \cdot (1 - \exp\left[-\varepsilon(\lambda_{Ex})C(\vec{r})L\right]) \ d\lambda_{Ex} \right] \cdot \Phi \cdot S_{emit}(\lambda) \tag{3.5}
\]
where \( I_0(\lambda; \vec{r}) \) is the excitation laser intensity at the point \( \vec{r} \), \( \varepsilon(\lambda) \) is the absorption coefficient of the fluorescent molecule (units of \((M \text{ cm})^{-1}\)), \( C(\vec{r}) \) is its local Molar concentration, and \( L \) is the optical path length through the differential volume (in cm). The integrand involving these terms gives the amount of light absorbed by the sample. \( \Phi \) is the quantum efficiency of the dye molecule, given as the fractional amount of emitted fluorescence compared to the absorbed light and \( S_{\text{emit}}(\lambda) \) is the fluorescence emission spectrum. The measured integrated fluorescence from this point in the solution, \( F(\vec{r}) \), is given by:

\[
F(\vec{r}) = \left( \frac{\Omega}{4\pi} \right) \cdot \int R_{\text{Det}}(\lambda) \cdot F(\lambda; \vec{r}) d\lambda
\]

\[
F(\vec{r}) = \left( \frac{\Omega}{4\pi} \right) \cdot I_0(\vec{r}) \cdot C(\vec{r}) \cdot \int R_{\text{Det}}(\lambda) \cdot \tilde{S}_{\text{emit}}(\lambda) d\lambda
\]

where the ratio \((\Omega/4\pi)\) defines the collection geometry (i.e., the the solid angle of emission captured by the imaging system, \( \Omega \), divided by the full angular area of a unit sphere) and the spectral response function of the imaging system, \( R_{\text{Det}}(\lambda) \), includes the spectral efficiency curve of the detector as well as the transmission spectra of lenses and filters in the imaging system. In the second equation we collect a number of terms characteristic of the dye molecule that will not vary with mixing to define the value \( \tilde{S}_{\text{emit}}(\lambda) = \left[ \int I_0(\lambda_{Ex}; \vec{r}) \cdot \varepsilon(\lambda_{Ex}) d\lambda_{Ex} \right] \cdot \Phi \cdot S_{\text{emit}}(\lambda) \). To further simplify the expression we make two assumptions about the nature of the laser excitation field and the sample. By assuming the sample is optically thin \((\varepsilon(\lambda)C(\vec{r})L \ll 1)\) and that the excitation laser intensity spectrum and spatial profile are independent we can separate all spatially varying functions from the spectral integral, this will be useful below.

In order to utilize fluorescence as a quantitative measure it is necessary to perform calibrations of the experimental system to relate the collected fluorescent signal intensity to the local absolute concentration. The fluorescence signal is related to the local intensity of the excitation laser (which can vary as it is absorbed by the sample being probed) and the collection geometry of the imaging system (which may not be well characterized).
However, we can bypass these complexities by taking the ratio of the emission of two fluorescent species under the same excitation and collection geometries. The ratio of the fluorescence signals at two wavelengths from a mixture of two dyes with distinct, non-overlapping emission spectra becomes:

\[
\frac{F_{\lambda A}(\vec{r})}{F_{\lambda B}(\vec{r})} = \frac{C_A(\vec{r})}{C_B(\vec{r})} \left[ \frac{R_{\text{Det}}(\lambda_A) \cdot \tilde{S}_{\text{emit},A}(\lambda_A)}{R_{\text{Det}}(\lambda_B) \cdot \tilde{S}_{\text{emit},B}(\lambda_B)} \right]
\]  

The ratio removes the spatial dependence of the laser excitation source and the collection geometry, and we are left with the measurable properties of the fluorescent species. For simplification, in the equation above the fluorescence from dye A at \(\lambda_B\) is assumed to be zero and \textit{vice versa}. When the emission spectra of the two species being detected overlap the relationship between the fluorescence ratio and concentration ratio becomes more complex, but it is still separable from the experimental laser and imaging spatial dependencies.

In this experiment the two emitters are the single chemically sensitive HPTS dye in two solvents: water and methanol. Here, the local concentration of the dye, \(C_{\text{Dye}}(\vec{r})\), is the same for both parts of the fluorescence ratio and can be removed as well. The total emission becomes a sum of the contribution of the dye in the methanol environment and the contribution from the water environment. The integrated fluorescence from the dye in a mixed sample of methanol and water with spatially dependent volume fraction of methanol and water of \(f_M(\vec{r})\) and \(f_W(\vec{r})\), respectively, would be given by:

\[
F_{\text{Tot}}(\vec{r}) = I_0(\vec{r}) C_{\text{Dye}}(\vec{r}) \cdot \int R_{\text{Det}}(\lambda) \cdot \left[ f_M(\vec{r}) \cdot \tilde{S}_{D,M}(\lambda) + f_W(\vec{r}) \cdot \tilde{S}_{D,W}(\lambda) \right] d\lambda
\]  

where the absorption spectra, quantum efficiency, and emission spectra of the dye vary in the methanol (M) or water (W) environment. The ratio of the integrated fluorescence over two spectral regions can now be expressed as a function of the spatially dependent...
volume fraction in either of the two solvents (for droplets composed of only two solvents $f_M(\vec{r}) + f_W(\vec{r}) = 1$ at all positions within both droplets before and after collision). For the present experiments the spectrum is integrated over two broad spectral regions defined by the blue (B) and green (G) color filters of a digital color camera and the mixture of the system is measured in terms of the methanol volume fraction $f_M(\vec{r})$:

$$
\frac{F_B(\vec{r})}{F_G(\vec{r})} \int R_{Det,B}(\lambda) \left[ f_M(\vec{r}) \cdot \tilde{S}_{D,M}(\lambda) + (1 - f_M(\vec{r})) \cdot \tilde{S}_{D,W}(\lambda) \right] d\lambda
\int R_{Det,G}(\lambda) \left[ f_M(\vec{r}) \cdot \tilde{S}_{D,M}(\lambda) + (1 - f_M(\vec{r})) \cdot \tilde{S}_{D,W}(\lambda) \right] d\lambda
$$

(3.9)

The fluorescence ratio is solely a function of the spatially varying methanol volume fraction $f_M(\vec{r})$, with no dependence on the geometric characteristics of the laser illumination or the collection optics, or the local dye concentration. This relationship is inverted to give the local volume fraction of the two solvent species from the fluorescence emission integrated over two spectral regions. The fluorescence ratio, $F_B/F_G$ is experimentally measured over a range of volume fractions, $f_M$, and fit to a polynomial, as shown later in Figure 3.3, to arrive at the calibrated conversion function for the local volume fraction of methanol that is the inverse function of Equation 3.9.

### 3.3 Experimental Setup

A schematic of the droplet collision apparatus and imaging system is shown in Figure 3.1. The apparatus employs a pair of piezoelectric droplet generators (PDGs, described below) [23, 25] affixed in a rigid positioning and alignment mechanism with large range of motion for investigating a variety of collision geometries. The two PDGs create a stream of micro-droplets with consistent, reproducible size, velocity, and point of collision. The streams are photographed from an plane orthogonal to the collision plane using a calibrated digital color camera, with the color filters of the image sensor pixels acting as emission filters for the dye, and illuminated by a planar laser sheet to excite fluorescence from the HPTS dye.
Figure 3.1: Schematic of the colliding droplet apparatus (a) and the PLIF imaging system (b). The schematic in (a) shows the droplets colliding in a symmetric geometry and highlights how the dye (initially in the methanol droplet and dark blue) shifts emission wavelength to green as it mixes with the initially dye free water droplet. (b) The colliding droplets are photographed perpendicular to the plane defined by the collision geometry. The illuminating laser is expanded into a sheet by a pair of cylindrical lenses to cover a broad area (shaded light blue in schematic (a)), exciting fluorescence from the droplets at their collision point and over the long falling and mixing path. Laser sheet is brought in at a slight angle (exaggerated in the figure) to reduce reflection.

Initially carried in the methanol droplets and transferred to the water during post-collision mixing. To produce the pulsed laser illumination, the output from a Ti:Sapphire laser was frequency-doubled by passing the beam through a thin crystal of $\beta$-BBO. The primary 800 nm beam was blocked by a glass absorptive filter while the doubled 400 nm beam was expanded by a pair of cylindrical concave lenses of focal length -5 cm and -40 cm to give a planar sheet of height $\sim 30$ mm and width $\sim 10$ mm at the droplet collision plane, enveloping the droplet streams and illuminating the initial free fall before collision, the point of collision, and the subsequent droplet dynamics of either coalescence or break-up.

The Ti:Sapph laser generates ultra-short pulsed illumination, which completely eliminates motion blur (alternate sources of pulsed UV light could be utilized, as discussed later) and operates at a variable repetition rate of up to 1 KHz. Importantly, we can only collect fluorescent emission representative of water from the volume in the coalesced droplet that the dye has mixed into, as the portion of the droplet that is still 100% water contains no dye and can not contribute to the intensity. Once the droplets collide and mixing occurs
the dye is carried with the methanol into the water environment, and from this point
the rise of the green fluorescent signal indicates the degree of mixing on the molecular
scale. This is done to more clearly observe the initial onset of green emission from the
first contact of the dye with the water environment and avoid the faint initial signal being
overwhelmed by emission from an initially dyed water droplet.

From images of a calibrated 500 µm etched grid placed in the image plane we can
measure the length scale of the pixels and the spatial resolution of the imaging system.
With this calibration the size and speeds of the droplets can be measured. The camera
system resolution is 10.95 µm/pixel and there is no measured geometrical aberration over
the field of view used to image the droplets. For the images presented here the focus
was on long term mixing, and images were collected in a wide angle imaging geometry to
fit the field of view to the entire laser exposure area. Higher image resolution could be
achieved with greater magnification imaging optics, but at the expense of imaged area.
By virtue of the pulsed laser exposure, motion blur is not a factor in image resolution as
the droplets are essentially stationary over the timescale of laser exposure and subsequent
fluorescence.

Piezoelectric Droplet Generators:

The PDGs used in this experiment were fabricated from a design by Yang et. al. [38].
The PDG, illustrated in Figure 3.2, produces a consistent, reproducible stream of droplets
whose diameter is specified by the cross-section of the glass capillary nozzle. With different
nozzle diameters, droplets of diameters from 25 µm to 1.5 mm can be generated. Although
nominally spherical, the droplets retain internal energy after discharge from the PDGs,
causing them to be slightly ellipsoidal and vibrate and tumble as they fall. The device
operates on the piezoelectric effect: when a current is passed through a piezoelectric
material it translates that current into mechanical vibrations. In the PDG these vibrations
at the back of a fluid volume produce a pressure wave that forces a small droplet out of
Figure 3.2: Piezoelectric micro-Droplet Generator (PDG): a ceramic piezoelectric plate is deformed by a current pulse from a high voltage driver to generate a pressure wave in the PDG, forcing a droplet of fluid out of the glass capillary nozzle. The nozzle size dictates the droplet diameter. Experiments were performed on 100 µm diameter droplets. The device can deliver single droplets on-demand or can be operated in a periodic pulsed mode.

The piezoelectric plate can be driven by individual pulses, generating single droplets on demand (a capability not shared by similar droplet generating devices like vibrating orifice aerosol generators [39]), but the most effective operation is achieved at a resonant frequency associated with the thickness of the ceramic plate, the density of the fluid, and environmental conditions including room temperature and air pressure. In our experiment this frequency was typically 20-35 Hz.

A pair of these PDGs are mounted on rigid posts and aligned by 5-degree (3 linear, 2 angular) micro-positioning translation stages to cross the two streams at a consistent, stable collision point. The timing of the pulsed voltage source that activates the PDGs is synchronized to the pulsed laser system that excites fluorescence: maintaining a fixed timing between the generation, collision, laser exposure, and imaging of the droplets. Adjusting a relative phase between the laser trigger and PDG trigger scans the relative timing of the laser illumination and the collision, allowing nanosecond control of the
synchronization of the collision and flash illumination of the laser. The 1 KHz repetition rate of the laser allows multiple images to be taken of the collision and coalescence of a particular droplet pair.

An attractive feature of this technique is its simplicity and ease of implementation/integration into existing mixing and flow experiments, therefore if a pulsed UV laser is not available then similar measurements could be made with a Mercury or Xenon arc lamp or continuous UV illumination and fast exposure photography, depending on the desired time resolution of measurements. However, these sources offer lower intensity and lower temporal resolution, such that motion blur could adversely impact data collection and interpretation.

*The Chemically Sensitive HPTS Dye:*

The chemically sensitive dye HPTS is commonly employed as an acid-base titration indicator [40], and in measurements of cellular pH [41] as it is highly water-soluble and exhibits a strong Stokes shift that varies with environmental acidity. The shift in the fluorescence band, illustrated in Figure 3.3(a), is caused the loss of a hydrogen atom from the HPTS molecule to the solvent. In neutral pH water, the hydrogen is stripped from the molecule and the band shifts to 510 nm peak emission in the green; while in methanol HPTS retains the hydrogen atom and fluoresces at 440 nm peak emission, in the blue band [42]. The hydrogen abstraction is nearly instantaneous (occurring in ∼ 3 ps) making it an effective probe of sample environment and mixing [32].

The fluorescence shift in Figure 3.3(a) is significant not only for its magnitude, making the emission from the dye in the different environments easily separable, but also due to its particular wavelength location. The emission from the two states of HPTS conveniently fall within the standard blue and green color channels of Bayer RGB filter arrays used in most commercial color digital imaging sensors [43]. This feature greatly simplifies photographing the dyed samples using a single camera with built in spectral filtering rather than separately filtering, overlapping, and synchronizing the images from multiple
Figure 3.3: (a) Shift of the HPTS dye fluorescence peak caused by deprotonation in water. The fluorescence peaks at 440 nm in methanol and 510 nm in water. (b) Calibration curve to determine the volume fraction of methanol ($f_M$) by measuring the ratio of HPTS fluorescence in the green pixel channel of the camera divided by the blue pixel channel. The volume fraction of water is the complementary curve ($f_W = 1 - f_M$). The calibration curve is fit to a sixth order polynomial used to convert image pixel intensities to relative concentrations.

Monitoring the ratio of fluorescence in each pixel’s green channel associated with water compared to the pixel’s blue channel associated with methanol gives a measure of the relative local concentration of each, as shown in the calibration curve in Figure 3.3(b). The spectral response of the image sensor of the camera used in this experiment was measured by photographing a calibrated tungsten-halogen white light lamp passed through a grating and slit monochromator and fit to Gaussian distribution functions as shown in Figure 3.4. The spectral response curves of the blue and green channels of the camera pixels were used to calculate the measured pixel response to the fluorescence as a function of the volume fraction of Methanol. The red pixel response was used as an indicator of background light, scatter, or saturation. The measured calibration curve is inverted to provide a measurement of the local volume fraction of methanol or water as a function of the measured emission from the droplets in the camera images.
Figure 3.4: The images of the droplets were collected on a commercial color digital camera. The spectral response of the camera pixels’ three color channels (Red, Green, and Blue) were measured using a calibrated tungsten-halogen source and fit to Gaussian distribution functions. The response of the pixels beyond 700 nm does not affect the fluorescence measurement.

3.4 Measurements and Interpretation

Mixing experiments were carried out by colliding two streams of droplets of equal size and velocity with one droplet composed of methanol doped with HPTS and the other droplet composed of pure water. The streams were aligned to collide in a planar, ‘head-on’ geometry, symmetric about the point of impact with each droplet having equal and opposite horizontal momentum and minimum off-set from the plane of collision. After collision, the droplets would either coalesce and fall straight down while continuing to mix or break apart depending on the collision parameters.

Figure 3.5 shows a typical multi-exposure image of a pair of droplets that coalesce into a single droplet after collision. For this image the laser repetition rate is 500 Hz, the camera was set to expose for 50 ms, and the PDG frequency was 22 Hz, allowing the individual snapshots of the droplets to be spatially separated (without double exposure) and analyzed. We are able to make out several details about the collision and subsequent coalescence dynamics from studying the series of exposures. The droplets are initially
Figure 3.5: Multi-exposure image of a single pair of droplets colliding, coalescing, and mixing (evidenced by onset of green dye emission). Droplet collision is imaged at 500 Hz laser repetition rate (each snapshot is separated by 2 ms). Figure (a) shows full color image of droplets. For clarity, the dark background has been masked out and for schematic purposes a circle indicates the initial water droplet position (invisible without the dye) and path before collision. Figure (b) shows the processed 2-D spatial map of methanol relative volume fraction, with the presence of water indicated by the lighter (white) portions of the droplets. Enlarged volume fraction maps of the coalesced droplet 22 ms and 30 ms after collision (indicated in the figure with stars) are shown in detail in Figure 3.6.
approximately spherical, with diameters $\sim 750 \mu m$. Residual internal energy due to ejection from the PDGs causes the droplets to be slightly ellipsoidal, with vibrational and rotational modes of motion. The droplets are travelling at a velocity of $730 \mu m/ms$ with a vertical component of $650 \mu m/ms$ and a horizontal component of $330 \mu m/ms$. We see significant distortion of the droplets at the point of collision and in the first few milliseconds after contact until the two droplets merge into a single new dumbbell shaped droplet that rotates about its center as it falls. After collision the newly coalesced droplet falls at the same vertical velocity and the horizontal momentum of each drop is cancelled out. This absorbed kinetic energy, along with the surface energy released when the droplets merge, is converted to internal energy of the combining fluid flows, fueling advective mixing of the two liquids. The newly formed droplet remains strongly segregated between the formerly pure methanol and water domains for several milliseconds, but by the final exposure of Figure 3.5 (30 ms after collision) the droplet is more evenly mixed.

To more clearly illustrate the analysis of the mixing, two of the snapshot exposures from the collision shown in Figure 3.5 (indicated by stars in the figure) are presented in greater detail in Figure 3.6. By 22 ms after collision (Figure 3.6(a-b)) significant diffusive mixing has occurred, as indicated by the green dye emission, however the droplet is still strongly segregated into two distinct sub-domains composed of the initial droplets’ liquids. In the later exposure collected at 30 ms after the collision (Figure 3.6(c-d)) the samples are more evenly distributed, but the mixing is still dominated by diffusive transfer across the interface boundary between the original droplets at $X \approx 1400 \mu m$. Superimposed on the two-dimensional maps is an integrated trace of the volume fraction of methanol (blue lines) and water (green lines) for a band running through the central axis of the droplet along the X-direction, with a height in the Y-direction of $\sim 110 \mu m$ (10 pixels), indicated by the red outline. The dimensions of the coalesced droplet at 22 ms are $770 \mu m \times 1310 \mu m$ (in the X and Y directions, respectively), and $850 \mu m \times 1300 \mu m$ at 30 ms. These integrated stripes show the complementarity of the methanol and water.
Figure 3.6: Detailed images of a coalesced droplet at two extended delay times after collision. Original full color images of the droplets are on the left (a,c), while on the right (b,d) the two-dimensional mapping of the volume fraction of the methanol solvent is calculated from the ratio of the integrated fluorescence intensity in the blue and green color channels based on the pre-measured calibration. Integrated traces of the volume fraction of methanol (blue line) and water (green line) through the center of the droplet (indicated by red outline) are plotted super-imposed on the concentration maps.

In Figure 3.6 we characterize the violent collision and subsequent break-up of two non-coalescing droplets. This image series is exposed at 1 KHz and the initial droplets diameters are approximately $\sim 650 \mu m$. In this collision the water droplet pierces through the methanol droplet, causing large amounts of deformation and shear flow, before breaking free on the other side and splitting up into three smaller droplets. This non-coalescing collision was created by increasing the relative velocities of the two droplets (i.e., by increasing the driving voltages of the PDGs). This image series demonstrates the value of...
Figure 3.7: Image series of a non-coalescing collision of two droplets with high impact velocity. The droplets push through each other and break up into smaller droplets. Importantly, we see evidence of material transfer and mixing even when droplets do not coalesce, indicated by the green fluorescence from the dye transferred into the final droplets comprised primarily of the initial water droplet. This material transfer and the subsequent mixing occurs much more rapidly than in the coalescing streams, indicating that while the droplets interact for a shorter time (2-3 ms before break-up) the more turbulent character of the flow facilitates better mixing.

continuously monitoring a single collision event with multiple exposures over a long time rather than repeated images of perfectly reproducible collisions over a series of varied collision-exposure delays. Such a complex fragmenting collision could not be studied by the latter technique, as the exact dynamics and geometry of the break-up would not be repeatable. It is clear from the strong green emission and the volume fraction map that a significant degree of mass transfer occurs during the 2-3 ms interaction time before the breakup. This mass transfer and subsequent rapid mixing of the dye into the final water droplets is much more rapid and effective than the mixing observed in the coalescing
streams shown in earlier figures. This emphasizes that subtle and likely non-reproducible conditions can lead to effective mixing (turbulent flow and chaotic advection) calling for rapid imaging of the dynamics of each unique collision event to gain a full understanding of the processes involved. Further study of the rapid mixing and complex dynamics of such non-coalescing, non-repeatable droplet collisions would yield valuable information about micro-droplet collision reactions.

3.5 Conclusion

We have demonstrated a novel and simple technique for monitoring and characterizing the localized mixing of chemically distinct microdroplets using PLIF of a chemically sensitive dye. The ability to image a single pair of droplets at several exposures removes the necessity to image stable, repeatable collisions and enables us to analyze a larger range of collision geometries and non-coalescing streams. We observe a significantly higher degree of mixing and sample transfer in non-coalescing collisions compared to coalescing collisions.

Further experiments can be envisioned using the embedded chemically sensitive dye to analyze the effect of chemical reactions on the coalescence and mixing of reactive species. Measuring titration of strong Acid-Base neutralization (an exothermic reaction with a near instantaneous reaction rate) would be feasible with the HPTS dye molecule utilizing the same chemical mechanism for the fluorescence shift. Furthermore, comparisons between the collision of non-reactive and reactive droplets could reveal how the energy release of interfacial reactions affects coalescence and mixing.

In future experiments, a number of simple improvements could be made to improve data collection. Currently, the collision of a single droplet pair is observed in a single long camera acquisition under multiple fast laser exposures, generating a number of freeze-frames of the collision on a single image. We have shown that this is valuable for observing
non-repeatable collision events, however this places a limit on the exposure rate, set by the terminal velocity of the droplets, to avoid image overlap and double-exposure. This could be improved in two ways. Droplets sheathed in a flowing carrier gas can be made to travel much more rapidly and imaging of the colliding streams could expose much finer details of the early coalescence dynamics. This situation would also be more representative of the physical environment of micro-droplets in certain applications, such as rocket nozzles [8]. The use of a flowing carrier gas would need to be suited to the application being studied. Rather than increasing the speed of the droplet, one could increase the speed of the image collection. More advanced cameras are available for observing the droplet collision point. These cameras can acquire images at a much faster rate, up to 30,000 frames per second at restricted resolution, and have active intensifiers that amplify the small signals from short acquisitions [44, 45]. These advanced cameras remove the requirement that the droplets are displaced between exposures and increase the temporal resolution by several orders of magnitude. In the present experiment we had a single dye in only one of the pair of colliding droplets, making the other droplet invisible except where mixing occurred. This is valuable for visualizing the earliest onset of mixing, but has the drawback of losing information about the initially invisible second droplet. A suitable, color distinct dye could be placed in the second droplet to gain further insight in the collision induced mixing. Whatever alterations are made to the details of the experimental execution, the core of the technique, the LIF imaging of chemically sensitive dyes, is a very simple and flexible optical diagnostic technique that can easily be implemented in a variety of configurations (i.e., beyond colliding droplets) or integrated into existing setups to yield useful information about fluid field mixing across a broad range of length scales, from the microscopic to the macroscopic.
References


Chapter 4

Characterizing Mixtures of Two Similar Fluorescent Proteins by Optimal Dynamic Discrimination

The experiment presented in this chapter employs Optimal Dynamic Discrimination (ODD) as a novel means for quantitatively characterizing mixtures of fluorescent proteins with a large spectral overlap. To illustrate ODD, we simultaneously measured concentrations of in vitro mixtures of Enhanced Blue Fluorescent Protein (EBFP) and Enhanced Cyan Fluorescent Protein (ECFP). Building on this foundational study, the ultimate goal is to exploit the capabilities of ODD for parallel monitoring of cellular functions and genetic circuits by suppressing the spectral cross-talk among multiple fluorescent reporters. The work in this chapter has been published in Scientific Reports in 2016 by A. Goun, D.I. Bondar, A.O. Er, A.O., Z. Quine, and H. Rabitz [1]

4.1 Introduction

The development of fluorescent proteins (FPs) has revolutionized the life sciences. These proteins, used as fluorescent reporters of gene expression and biological function, have enabled qualitative as well as quantitative in vivo studies of genetic and protein network dynamics [2]. As a result, fluorescent proteins are extensively used in most modern biological laboratories. The standard practice for interrogating biological systems with FP reporters utilizes filtered excitation/emission spectroscopy to detect their expression. While this technique has proven extremely effective and produced a wealth of new results and applications, it is not without shortcomings. Most importantly, spectral crosstalk
limits the number of FPs that can be simultaneously monitored in a single experiment, due to the broad overlap of emission and absorption spectra of different reporters [3–7]. In addition, while there are hundreds of types of FPs with unique characteristics suited to specific applications, the low photon yield of many of these reporters forces overexpression, producing concentrations that interfere with biological activity. Furthermore, the absolute measurement of concentration is difficult, especially in biologically relevant conditions with low concentrations of FPs and high scattering through biological media, making these measurements primarily qualitative in nature. The ability to simultaneously, reliably, and quantitatively measure concentrations of several FPs could significantly advance a number of areas in the biological sciences, including synthetic biology [7], neuroscience [8], and cytometry [9–12]. In the current work, we take a first step towards this goal by employing Optimal Dynamic Discrimination (ODD) [13–20]: a scalable optical technique that operates on different physical principles from conventional linear excitation/emission spectroscopy. We demonstrate the capability of ODD to make reliable measurements of sub-micromolar concentrations of FPs with overlapping spectra. The experimental utility of ODD is illustrated by quantitatively characterizing mixtures of Enhanced Blue Fluorescent Protein (EBFP) and Enhanced Cyan Fluorescent Protein (ECFP) in cell extract.

ODD was originally inspired by theoretical analysis showing that nearly identical molecules may be distinguished by means of their quantum dynamics induced by properly shaped laser pulses [13]. This theory has been confirmed experimentally for a number of systems [14–20]. ODD differentiates near-identical molecules actively driving the unique excited state dynamics of each species of molecules within the mixture using the coherent nonlinear interaction between the laser field and the molecule. This operation is in contrast to conventional linear fluorescence spectroscopy, which passively excites all molecules in a mixture and monitors the fluorescence emitted by the molecules as they randomly decay back to their ground states. In ODD, femtosecond laser pulses with tailored temporal
structure interact with molecules on the time scale of their natural motion: by controlling the shape of the laser pulse we excite the molecules within the mixture being interrogated to a specific superposition of states for each different molecular species. The optimal pulse will drive the dynamic evolution of the wave packet of one species far from the others in the mixture to a particular detection state, where it will be measured. These interactions can be complex, even for the simplest molecules, and the laser pulse shapes that uniquely excite the molecules generally can not be determined from first principle calculations.

For this reason, we use closed-loop, adaptive feedback optimization in the laboratory to discover the specially shaped laser pulses that produce distinct responses from each FP within the mixture [13, 16, 20–22]. These optimally shaped pulses, or Photonic Reagents, allow us to draw apart the dynamic spectral signatures of species that would be very difficult to distinguish by means of linear spectroscopy [15, 23, 24]. Moreover, we may choose the feedback to the optimization such that the procedure not only increases the intensity of signals collected from the molecule of interest, but also enhances the accuracy of the concentration determination; thereby yielding more reliable characterization of sample composition. With adequate optical resources (i.e., broad bandwidth, stable, coherent radiation) the ODD technique may be extended to a much larger number of optical reporters than accessible by current techniques. These characteristics hold out the prospect of making ODD a valuable technique for enhancing biological characterization.

4.2 The ODD Algorithm for concentration determination

Optimal Dynamic Discrimination is implemented in two stages. In the learning stage, a stochastic algorithm discovers an optimal series of photonic reagents where each yields distinguishable signals from pure samples of individual FPs. In the next stage, the dis-
covered set of optimal photonic reagents can be applied to any mixture of the FPs to measure the component concentrations. The algorithm is described below for two FPs, and presented more generally for an arbitrary number of species in the Appendix A.

4.2.1 The Learning Stage: Photonic reagent optimization.

The total fluorescence from a mixture of FPs is simply the sum of the fluorescence signals of each component, weighted by their respective relative concentrations. For a mixture of two FPs we have

$$F_{total} = n_1 F_1 + n_2 F_2,$$

where $F_1$ and $F_2$ are the integrated fluorescence signals from reference samples of known concentration, and $n_1$ and $n_2$ are the unknown concentrations (relative to the reference samples) in the mixture. To determine these unknown concentrations the samples are excited by (at least) two different photonic reagents ($PR_1$ and $PR_2$), and the measured signals form a system of linear equations which can be arranged into the matrix below:

$$\begin{bmatrix} F_{total}(PR_1) \\ F_{total}(PR_2) \end{bmatrix} = \begin{bmatrix} F_1(PR_1) & F_2(PR_1) \\ F_1(PR_2) & F_2(PR_2) \end{bmatrix} \begin{bmatrix} n_1 \\ n_2 \end{bmatrix},$$

where the fluorescence intensities are a function of the photonic reagent used to excite the samples. The values of the known reference concentrations normalize the measured fluorescence signals; in this way they are incorporated in the final determination of the unknown concentrations.
The system of linear equations in Eq. (4.2) has a unique solution when the determinant of the matrix of measured reference fluorescence intensities (F) is non-zero:

\[ D = \det(F) = \begin{vmatrix} F_1(PR_1) & F_2(PR_1) \\ F_1(PR_2) & F_2(PR_2) \end{vmatrix}. \]  

(4.3)

Furthermore, based on the Cramér-Rao inequality [25], the error in the concentration determination is inversely proportional to the magnitude of |D|. Therefore, we perform stochastic optimization to find an optimal pair of photonic reagents which maximize |D|. Such an objective function has a degree of robustness to additive noise (e.g. shifting all fluorescence signals by a constant leaves D unchanged). Moreover, |D| = abs(det(F)) is a convex function of the matrix argument F, suggesting that the optimization procedure should be robust [22]. In the engineering literature such problems are related to what is called D-optimal experimental design [25]. Even though the unknown concentrations enter linearly in Eq. (4.2), the optimization problem is a non-linear function of the control field because the fluorescence depends non-linearly on the interrogating photonic reagents.

The details of the experimental implementation are given below.

In order to experimentally discover an optimal pair of photonic reagents, we have developed the following revision of the conventional closed-loop adaptive algorithm [21]. We begin by generating 2N random photonic reagent pulses grouped into N pairs (i.e., iterations:)

\[
\begin{pmatrix} PR_1^{(1)} \\ PR_2^{(1)} \end{pmatrix}; \begin{pmatrix} PR_1^{(2)} \\ PR_2^{(2)} \end{pmatrix}; \begin{pmatrix} PR_1^{(3)} \\ PR_2^{(3)} \end{pmatrix}; \cdots; \begin{pmatrix} PR_1^{(N)} \\ PR_2^{(N)} \end{pmatrix}
\]  

(4.4)

The upper index labels the photonic reagent iteration. In the current experiment, we employed N = 30. For each photonic reagent, PR\textsubscript{k}^{(n)}, we record the fluorescence from the reference samples of known concentration: \( F_j \left( PR_k^{(n)} \right) \) for \( k, j = 1, 2 \), and \( n = 1, \ldots, N \).
This yields fluorescence intensities from $2N$ pulse shapes, while the objective function in Eq. (4.3) depends on only 2 pulses. Thus, we form all possible pairwise combinations from the recorded $2N$ measurements (1770 combinations) and then calculate and sort the objective functions, $|D|$, for these combinations to be used as feedback to the genetic algorithm (GA). This bundling of the individual photonic reagents into pairs and the subsequent unbundling and resorting of the measured fluorescences is a consequence of the nature of the GA used to discover the optimal combinations of photonic reagents. This procedure becomes more significant at higher dimensions, and is discussed further in Appendix A. Based on the fitness of the previous generation, the GA determines new pulse shapes for each subsequent iteration, where the “genes” are the pulse shaper settings that modulate the complex temporal field of the laser pulse [21]. This procedure is iterated to discover the combination of photonic reagents that maximizes the objective function $|D|$. In principle, this algorithm may be repeated until full convergence is reached; however, in practice the FPs photo-degrade if exposed to laser radiation for extended periods of time, even when circulated, yielding an increasingly less informative signal at long optimization times. In the present experiments, we found that the algorithm returns reliable results even after a few iterations before significant photo-degradation occurred. The degree to which photo-damage occurs was measured and corrected by comparing the reference solution fluorescence intensities at the beginning of the experiment to the intensities during and after optimization under equivalent excitation conditions. All results provided below have been corrected for photo-degradation.

4.2.2 The Application Stage: Measuring concentration with optimized photonic reagents.

At the end of the learning stage, we have a final $N$ pairs of photonic reagents ranked in descending order by the value of the objective function $|D|$, characterizing the accuracy of
the concentration measurement. At this point, the top ranking pair of photonic reagents is the optimum solution, which is nominally sufficient to determine the sample concentrations within a mixture of FPs for analysis. However, to further increase the accuracy of the measured concentrations, we pick the $P$ highest performing pairs (in our experiment, $P = 10$) and use them to interrogate the mixture, thereby collecting $2P$ measurements of the left hand side on the following system of equations:

$$
\begin{bmatrix}
F_{\text{total}}(PR_1^{(p)}) \\
F_{\text{total}}(PR_2^{(p)})
\end{bmatrix} =
\begin{bmatrix}
F_1(PR_1^{(p)}) & F_2(PR_1^{(p)}) \\
F_1(PR_2^{(p)}) & F_2(PR_2^{(p)})
\end{bmatrix}
\begin{bmatrix}
n_1 \\
n_2
\end{bmatrix},
\quad p = 1, \ldots, P.
$$

This is an overdetermined system of $2P$ measurements with just two unknowns, which we solved using the unconstrained least squares method with no assumption on values of $n_j$. In cases where faster recording or lower light exposure is preferred, concentrations could be determined using as few as a single optimized pair of photonic reagents. The number of photonic reagent pairs used to characterize the mixture would depend on the degree of accuracy required of the concentration measurements and the experimental noise conditions. This rather straightforward approach worked well in our experiments; however, if the absolute concentrations of FPs are very low, thereby decreasing the signal-to-noise ratio, other more advanced techniques may be needed to compensate for measurement noise. For example, constrained least squares fitting, D-MORPH regression [26], Bayesian inference, etc. could be used to incorporate knowledge about the system, such as forbidding negative concentrations: $n_j \geq 0$. 

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4.3 Experimental Demonstration of ODD with fluorescent proteins

In any experimental implementation of ODD, the measured feedback signal must probe the coherent dynamics of the vibronic wave packet excited by the tailored photonic reagent. In the work presented here, we employ ultrafast coherent depletion of the excited state of the FP by stimulated emission, as represented in Figure 4.1. An FP molecule is interrogated by one photonic reagent, made up of a dual sub-pulse excitation-sub-pulse and an unshaped depletion sub-pulse. The vibronic wave packet created by the shaped excitation sub-pulse undergoes unique dynamics (Paths 1,2,3), which interact differently with the later depletion sub-pulse - the second path is coherently transferred to the ground state and does not complete its dynamical evolution and fluoresce like the other paths. The three paths above could either represent three different species excited by the same photonic reagent or three dynamic pathways taken by the same molecule after excitation by different shaped excitations.

Figure 4.1: Physical mechanism of ODD: The photonic reagent is a combination of a shaped excitation sub-pulse and an unshaped depletion sub-pulse. The vibronic wave packet created by the shaped excitation undergoes unique dynamics (Paths 1,2,3), which interact differently with the later depletion sub-pulse - the second path is coherently transferred to the ground state and does not complete its dynamical evolution and fluoresce like the other paths. The three paths above could either represent three different species excited by the same photonic reagent or three dynamic pathways taken by the same molecule after excitation by different shaped excitations.
depletion sequence. The excitation portion of the photonic reagent is an optimally shaped ultrashort laser pulse, which coherently transfers the ground state population of each FP into a particular superposition of vibrational states in the first excited electronic level. This creates a coherent wave packet with tailored dynamics that influence its interaction with the later portion of the photonic reagent. This second portion is a longer wavelength, unshaped “depletion” pulse, which is tuned to overlap the emission spectra of the FPs (see Figure 4.2). The interaction of the excited wave packet with the depletion portion of the photonic reagent dictates whether a particular FP will be preferentially driven back down to the ground state coherently, or will remain in the excited state to decay at a later time by fluorescing. In this way, the final fluorescence signal of the FPs is altered after interacting with photonic reagents of equivalent spectral intensity but distinct tailored temporal structure. By collecting the response of a set of reference FP samples to several pairs of photonic reagents in the learning stage the shaped excitation pulse may be algorithmically optimized to maximally distinguish the individual components in the mixture [19,24].

From an examination of the absorption spectra of EBFP and ECFP in Figure 4.2, it is evident that these two specific FPs could be effectively distinguished using standard UV excitation/emission measurements; however their significant spectral overlap still makes them good candidates to test and develop the novel, scalable nonlinear optical capability of ODD. The ultimate utility of ODD lies in the multiplexing of several photonic reagents to expand the application of the technique to several FPs, beyond the limitations of conventional linear excitation/emission measurements. This multiplexed scaling rests on operating in the ultrafast coherent regime, where we can exploit the rich dynamics of the excited molecular states by drawing on the ability to create an arbitrary number of distinctly shaped interrogating photonic reagents. It is notable that ODD will likely be most valuable in complementary applications to linear spectroscopy.
The application of stimulated depletion of fluorescent emission as a contrast mechanism is commonly used in microscopy as a super-resolution imaging technique (STED microscopy) [27, 28]. However, the utilization of coherent stimulated emission in ODD is distinct from its application in STED microscopy. In STED the goal is to maximize the contrast between the undepleted sub-diffraction-limit sized spot and the depleted surrounding region through complete suppression of the fluorescence. To accomplish this, the depletion pulse arrives after vibrational decoherence has transferred all excited molecules into the lowest vibrational state of the excited electronic level; and it is typically tens to hundreds of picoseconds long, allowing it to interact with the molecule until the excited state is completely depleted. By contrast, ODD depletes the excited state before...
decoherence of the vibrational wave packet, forcing the depletion pulse to be very short and nearly simultaneous with the excitation pulse. This reduces the maximum achievable depletion, but grants access to the highly species-specific information carried in the rich motion of the coherent excited vibronic wave packet. Because ODD only requires that each FP in a mixture responds in a unique way to a particular photonic reagent, total excited state depletion is not necessary for successful discrimination.

![Depletion curves](image)

Figure 4.3: Depletion curves (using unshaped excitation) for EBFP and ECFP in cell extract. The shaded region around \( t = 0 \) indicates the domain where the excitation and depletion pulses overlap, allowing effective manipulation of fluorescence by shaping of the complex phase of the excitation pulse.

Because of the extremely short coherence lifetime of the excited vibrational wave packet (typically on the order of \( \sim 1 \) ps) the relative timing between the excitation and depletion pulses is a critically sensitive parameter in ODD. The dependence of the fluorescence on this time delay with no shaping of the excitation pulse is shown in Figure 4.3 for EBFP and ECFP in cell extract. Both curves are normalized to the total fluorescent emission intensity without depletion, and \( t = 0 \) on the figure corresponds to synchroniza-
tion of the centers of the excitation and depletion pulses. As shown in the figure, the depletion curve can be split into three time domains. When the depletion pulse follows the excitation pulse by longer than the coherence lifetime of the vibrational wavepacket (positive delays \(\gtrsim 0.5\) ps) a steady state portion of the excited state population is stimulated down to the ground state. This circumstance will not exploit the species-specific sensitive information in the short-time dynamics created by the shaped excitation pulse. At much longer delays than shown in Figure 4.3 (~nanoseconds) the molecules naturally decay to the ground state and the fluorescence returns to its undepleted intensity. At negative delays (beyond \(\sim -0.5\) ps in Figure 4.3) the sample fluoresces as if solely exposed to the excitation, because the depletion pulse is outside the absorption spectrum of the ground state of both FPs. It is only at very short delay times, in the highlighted region between approximately \(-0.5\) and \(0.5\) ps in Figure 4.3, that there exists the unique dynamical window for effective coherent manipulation of fluorescence, as in that domain the photo-dynamics become sensitive to the phase structure of the laser pulses. This region is where the ODD algorithm can effectively exploit those dynamics. There is flexibility in setting the precise depletion delay, with each set of FPs exhibiting its own characteristics. For this experiment the initial delay was set to zero (excitation and depletion pulses synchronized) allowing the algorithmically guided pulse shaper to seek out the best delay as part of the optimization procedure.

4.3.1 Experimental Apparatus.

The schematic of the experimental apparatus is shown in Figure 4.4. The primary 800 nm laser pulses were generated by a Titanium:Sapphire ultrafast regenerative amplifier system (Coherent Legend) with a repetition rate of 1 kHz (in contrast to the MHz laser typically employed for microscopy), average power of 2 W, and pulse duration \(\sim 40\) fs. The source beam was split in two parts to make the excitation and depletion beams. The excitation
beam was created by doubling the primary beam to 400 nm in a BBO crystal and the complex temporal structure of the pulse was constructed in a 4-F configuration pulse shaper with a computer controlled acousto-optic modulator [29], which independently adjusts the phases and amplitudes of each spectral component of the pump beam. To generate the depletion beam, a portion of the primary amplifier output was used to drive a Quantronix variable wavelength Optical Parametric Amplifier (OPA) with an output at 1.25 μm. This beam was mixed with the remainder of the primary 800 nm beam by sum frequency generation in a BBO crystal to obtain the desired 488 nm depletion pulse. A band pass filter (FL488-10, Thorlabs) was used to spectrally isolate this depletion beam from its parents.

The characteristics of the excitation and depletion pulses are given in Table 4.1. The pulse energies were chosen to maximize the stimulated depletion signal while avoiding undesirable power effects, such as self-focusing, extensive sample bleaching, or absorption
Table 4.1: Laser pulse parameters. The typical excitation pulse width depends on the phase-amplitude mask of the pulse shaper, and ranges from a transform limited width of 30 fs to the maximum shaper range of ∼1 ps.

from the excited state. The two beams are synchronized with a computer controlled delay stage and recombined on a dichroic mirror before passing through routing and focusing optics into the sample cuvettes. The excitation and depletion beams must overlap both spatially, at their shared focal point, and temporally, within the dynamic coherence window, to produce ODD coherent fluorescence depletion, necessary for achieving reliable concentration measurements. The beam waist of the depletion beam is spatially wider than the excitation beam, to ensure the entire population of excited FPs are exposed to the depletion pulse.

A special design feature of our experiment is an automated sample switcher configuration. Four quartz flow cells were fixed onto a computer controlled translation stage. The total integrated fluorescence from each sample was measured on a single photomultiplier tube while each sample was moved into the beam path. Two cells contain reference samples of the FPs with known concentration measured during the learning stage to discover optimal photonic reagents. The other two cells contain different mixtures, whose concentrations are to be characterized. All cells are connected to piezo pumps for circulation to minimize photo-bleaching. This sample setup has many advantages over a configuration where the laser beam is divided among multiple stationary cells. The setup utilized here not only exploits the full beam energy in each sample, but also ensures that the pulse is identical and not distorted by beam dividing optics. Additionally, this configuration
occupies less space and is readily scalable to a larger number of sample cells. In order to obtain reliable concentration measurements, it is necessary that all samples are maintained in identical condition during laser field interrogation: the cells must be made of the same material and carefully aligned with respect to each other.

Overcoming laser beam scattering from the cell extract was a major obstacle in measuring fluorescence intensity since the depletion beam spectrally overlaps with fluorescence. Figure 4.5 shows the setup to disentangle the (comparably) weak fluorescent signal from the bright background of scattered dump. Even though the depletion beam and fluorescence emission are spectrally alike, they have very different spatial characteristics. A high numerical aperture lens is seen on the left of Figure 4.5. At the center of this lens, a small mirror is glued to guide the control beam towards the sample and to block its reflection from the sample cell. The large numerical aperture lens collects the broadly emitted fluorescence along with the remaining scattered light. To improve the isolation of the fluorescence, a notch filter (NF488-15, Thorlabs) combined with a lowpass filter (FEL0450, Thorlabs) removes a substantial portion of the remaining scattered dump pulse without completely blocking the fluorescence. The final portion of the scattered laser is suppressed by spatial filtering with a pinhole, similar to the technique used in confocal microscopy, ensuring that the signal is collected only from the spatial point of the sample where the excitation and depletion beams meet. In this way, a clean depleted fluorescence signal is obtained.

We found that the signal-to-noise ratio of the fluorescence measurement in the application stage should be at least as high as during the learning stage of ODD to ensure accuracy. This was readily achieved by increasing the fluorescence acquisition time to 2 sec per measurement when interrogating the unknown mixtures, while 1 sec of acquisition was sufficient during the learning stage. Extending the acquisition time ensured that adequate signal would be collected from the unknown samples, whose component concentrations could be lower than the reference samples.
4.3.2 Fluorescent Protein Samples.

Lyophilized EBFP and ECFP were purchased from Biovision in 100 µg quantities and reconstituted in phosphate buffer. Quantum yields of EBFP and ECFP are 0.15 and 0.68, while the extinction coefficients are $1.9 \times 10^5$ and $2.6 \times 10^6$ (M$^{-1}$cm$^{-1}$), respectively. Each sample was diluted to 1mL with 10 mM phosphate buffer, 140 mM NaCl, and 2.7 mM KCl at pH 7.4. The absorption and emission spectra of ECFP and EBFP are shown in Figure 2.

Proteins absorbing in blue region were selected not only because they were convenient for our laser sources, but also because this regime is known to be challenging for biological imaging due to low quantum yields of the FPs. The success of the experimental implementation of ODD in this regime strongly suggests that such an approach will function well for other FPs operating at longer wavelengths.

The protein samples were studied in cell extract, to simulate a biologically relevant sample environment, and were prepared according to the following protocol: *E. Coli*
<table>
<thead>
<tr>
<th>Mixtures of EBFP/ECFP</th>
<th>Mixing Ratio Measured by ODD</th>
<th>Concentration (µM) Measured by ODD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% - 50%</td>
<td>(47±2)% - (53±2)%</td>
<td>1.50±0.06 / 1.80±0.07</td>
</tr>
<tr>
<td>75% - 25%</td>
<td>(74±1)% - (26±1)%</td>
<td>2.37±0.03 / 0.88±0.03</td>
</tr>
<tr>
<td>0% - 100%</td>
<td>(4±8)% - (96±6)%</td>
<td>0.1±0.3 / 3.3±0.2</td>
</tr>
<tr>
<td>100% - 0%</td>
<td>(96±1)% - (4±1)%</td>
<td>3.07±0.03 / 0.14±0.03</td>
</tr>
</tbody>
</table>

Table 4.2: Results of ODD. The first column contains the prepared mixing ratios of the FPs. The second and third columns contain results of concentration determination with ODD, for the mixing ratio and absolute concentrations, respectively.

BL21 DE3 cells were incubated with PCA24N plasmid, containing a control gene that expresses a small four-helix bundle protein, and transformed by electroporation. The transformed cells were plated on Luria-Bertani (LB) agar plates supplemented with 30 µg/ml chloramphenicol (CAM) and grown overnight at 37°C. A single fresh colony was used to inoculate a 1L flask of LB+CAM. The culture was grown to OD600=0.6 and induced with IPTG at a final concentration of 100 µM. The culture was grown over night, and then centrifuged and frozen. Cell lysates/extracts were prepared by resuspending frozen cell pellets in 50 mls of 1X phosphate buffered saline at pH 7.4. Cells were then lysed on ice using an ultrasonic homogenizer set to 40% power with 5 seconds of sonication followed by 5 seconds of dwell time for a total of 10 minutes. The whole cell lysate was centrifuged at 8000 g’s for 30 mins. The supernatant cell extract was decanted and frozen in small aliquots for subsequent use in the experiments.

4.3.3 Results of concentration measurements.

Utilizing ODD, we analyzed a series of two-component mixtures of EBFP and ECFP dissolved in cell extract at biologically relevant concentrations. The results of these measurements are collected in Table 4.2.

A typical pair of photonic reagents $PR_1$ and $PR_2$ utilized to characterize the protein mixture [see equations (4.2) and (4.3)] are shown in Figure 4.6. The instantaneous pulse
intensity [Figure 4.6(b)] are obtained as the Fourier transform of the recorded masks [Figure 4.6(a)]. Extracting the physical mechanism for discrimination is a subject of our current investigation.

Figure 4.6: An example of shaped excitation pulses pairs used to characterize a mixture of EBFP and ECFP. (a) The normalized amplitude and phase masks for the photonic reagents, and (b) the corresponding pulses in time domain.

The experimental errors presented in in Table 4.2 were calculated as the standard deviation of five consecutive concentration measurements. Knowing the absolute concentrations of the reference solutions allowed us to translate the measured relative mixing ratios into absolute concentrations. The reference solutions were prepared by diluting the stock cell extract to micromolar concentration and characterized by UV excitation/emission spectroscopy. The EBFP and ECFP reference solutions were, respectively, prepared at a concentration of 3.20 µM and 3.40 µM. Four mixed samples were prepared from these reference solutions and characterised by ODD. In all cases, ODD reliably measured the absolute concentrations of the fluorescent proteins in the mixtures to high accuracy in spite of their overlapping spectra.

4.4 Conclusions and Outlook

Using EBFP and ECFP as examples, we have demonstrated that ODD successfully discriminates and accurately measures concentrations for mixtures of FPs with significantly
overlapping absorption/emission spectra. This operation was accomplished in a solution of *E. Coli* cell extract, establishing the suitability of ODD for interrogation of environments typically encountered in biological samples. The true utility of ODD does not lie in discriminating two FPs (e.g., as here, where linear spectroscopy could function), but rather in its scalability to draw on unique coherent FP excitation by utilizing a nearly endless set of distinct photonic reagents. To further advance ODD, we are currently reducing the complexity of the experimental system investigating alternate ultrashort pulse laser resources to replace the multi-laser system, including a single Non-colicinear Optical Parametric Amplifier (NOPA) and an Optical Frequency Comb (OFC) source. Both sources are ultra-broadband coherent light sources with emission covering the entire visible to Near-IR spectrum. These flexible radiation sources can create both the excitation and depletion pulses in unison, with the full absorption and emission bands of several FPs within their spectral range. The unparalleled Future studies should also be able to extend the capabilities of ODD by fully shaping the photonic reagents, building optical structure into both the excitation and depletion pulses, thereby allowing the photonic reagents to exploit more complex FP dynamics. These capabilities should enable more accurate, reliable, simultaneous detection of a larger number of FPs while greatly simplifying the technique.
References


Chapter 5

Enhanced Coherent Control of Optogenetic Switching by Stimulated Depletion Quenching

Overview

Optogenetics is a revolutionary new field of biotechnology, achieving optical control over biological functions in living cells by genetically inserting light sensitive proteins into cellular signaling pathways. Applications of optogenetic switches are expanding rapidly, but the technique is hampered by spectral cross-talk: the broad absorption spectra of compatible biochemical chromophores limits the number of switches that can be independently controlled and restricts the dynamic range of each switch. In the present work we develop and implement a non-linear optical photoswitching capability, Stimulated Depletion Quenching (SDQ), to overcome spectral cross-talk by exploiting the molecules’ unique dynamic response to ultrashort laser pulses. SDQ is used to enhance the control of Cph8, a photo-reversible phytochrome based optogenetic switch designed to control gene expression in E. Coli bacteria. The Cph8 switch can not be fully converted to it’s biologically inactive state ($P_{FR}$) by linear photoswitching, as spectral cross-talk causes a reverse photoswitching reaction to revert to it’s active state ($P_{R}$). SDQ selectively halts this reverse reaction while allowing the forward reaction to proceed. The results of this proof of concept experiment lay the foundation for future experiments that will use optimal pulse shaping to further enhance control Cph8 and enable simultaneous, multiplexed control of multiple optogenetic switches.
5.1 Introduction and Background

5.1.1 Introduction

The variety and complexity of tasks performed by living cells is astonishing: they harvest and refine chemicals from their environment [1], synthesize complex chemicals in response to demand [2], respond to changes in their environments in ways that mirror logic and memory [3], self-replicate and multiply under globally limited constraints [4,5], and work together - communicating and combining to form multi-cellular tissues [6]. A cell accomplishes all these tasks by measuring an array of internal and external environmental conditions using various sensor protein complexes and performing a specific biological function in response, as dictated by a set of instructions in its genetic code. In this way, cells behave as functional machines, performing actions in direct response to stimuli.

Recently, biologists have developed the capability to genetically engineer cells, supplanting the natural sensors with light sensitive proteins. These engineered proteins enable the activation or suppression of specific cellular functions with optical signals, turning a piece of the naturally developed sensing-response system into an externally accessible “control panel”. This breakthrough, called optogenetics [7,8], enables the precise and reversible control of specific biological processes in living cells by external optical stimuli to systematically perturb the system and observe its response [9].

Currently available optogenetic tools can control a range of biological processes including ion channel activation (enabling high fidelity neuromodulation [10–12]), organism mobilization [13–15] and behavior [16–18], protein synthesis in cells [19,20], gene expression or inhibition [21–24], protein localization at specific cell sites [25–29], or sequestration of active proteins away from their site of reaction [30,31]. Optical techniques can be very appealing for the control and monitoring of biological systems, with many advantages over physical or chemical/pharmacological activation. Light is a noninvasive tool that interacts strongly with specific biologically relevant molecules and can be applied with high spatial
and temporal resolution. Precise activation signals can be encoded into many controllable parameters of the optical signal, including the light’s intensity, frequency spectrum, phase structure, and the duration or temporal pattern of the exposure. Moreover, optical stimuli can be rapidly halted, in contrast to diffusonally controlled chemicals.

In spite of these favorable aspects, the practical implementation of optogenetics is prevented from reaching its fullest potential by spectral cross-talk: the overlapping optical responses of the switches. Most chromophores, the molecules within the optogenetic switch that absorb light, have broad, featureless absorption spectra, which limits the number of switches that can be independently addressed by linear photo-excitation. More problematic is that for many switches even the absorption spectrum of the activated and de-activated states overlap, preventing the switch from being completely converted from one state to the other. This inability to distinguish the two states of the switch limits the accessible dynamic range of the system and fidelity of control.

The capability now exists to overcome the spectral cross-talk: it has been shown that even molecules with nearly identical static absorption spectra can exhibit unique dynamical responses to ultrashort laser pulses [32]. These distinguishable dynamics can be accentuated by a set of optimal optical control pulses to distinguish similar molecular species, this technique is known as Optimal Dynamic Discrimination (ODD) [33–41]. By interacting with the time-dependent non-linear optical response of a molecule, rather than just the static absorption spectrum, we increase the number of control parameters that can be varied to search for orthogonal optical controls that are not restricted by the same spectral cross-talk as the linear optical responses. In principle, a suitable set of orthogonal optical controls should be capable of separating the optical responses of several similar optogenetic switches that would be extremely difficult to independently address by conventional linear photo-excitation. The ultimate goal of this research is the simultaneous (multiplexed) control of multiple optogenetic switches over their maximum dynamic range, thereby independently and simultaneously controlling a large number of
cellular functions. In the present work we take the first step toward the goal of multiplexed control of multiple optogenetic switches by achieving enhanced control over a single optogenetic switch, increasing it’s dynamic range beyond the photoequilibrium limit imposed by spectral cross-talk.

This study utilizes non-linear optical interactions to control the photoswitching reaction of Cph8, a phytochrome based red/far-red sensing optogenetic switch developed for controlling gene expression in E. Coli bacteria [20,42,43]. More detailed information about the Cph8 switch will be provided in Sections 5.1.2 and 5.1.3. To summarize briefly, Cph8 is an artificial “fusion” protein, a synthesis of the light sensing domains of the Cph1 protein originally found in Cyanobacteria with the Histidine-Kinase Two-Component Switch (TCS) signalling domains of EnvZ, a protein native to E. Coli that is associated with gene transcription [44]. The chromophore in the Cph8 switch, phycocyanobilin (PCB), converts between two states by photo-isomerization, as shown in Figure 5.1(a). The gene-expressing “On” state absorbs red light and is called $P_R$, while the biologically inactive “Off” state is called $P_{FR}$ because its absorption spectrum is shifted to longer wavelengths in the far-red. The absorption spectra of these two states is shown in Figure 5.1(b). The Cph8 switch can be fully converted from the $P_{FR}$ state to the $P_R$ state by continuous illumination with far-red light ($\lambda \geq 720$ nm), this is described as the “reverse” photo-isomerization reaction. Deactivation of the switch, converting from the $P_R$ state to the $P_{FR}$ state, is described as the “forward” photo-isomerization reaction, and due to the overlapping absorption spectra of the two states there is no wavelength of light that will initiate the forward photo-isomerization reaction without also initiating the reverse reaction. The simultaneous photo-reaction between the two states under red-illumination comes to photoequilibrium with a maximum $P_{FR}$ population of $\sim 65\%$ ($P_R \sim 35\%$). The absorption spectrum of a sample in this equilibrium mixed state is also shown in Figure 5.1(b). Using ultrashort laser light pulses we will exploit the unique dynamic optical responses of the $P_R$ and $P_{FR}$ states to selectively “quench” the reverse photoswitching
Figure 5.1: Overview of the Controlled Optogenetics experimental concept: (a) Phot switching of Cph8 is initiated by photo-isomerization of the PCB chromophore between an active, red-light absorbing state ($P_R$) and an in-active, far-red-light absorbing state ($P_{FR}$). The $P_{FR}$ state can be completely removed with far-red light, however spectral cross-talk prevents complete removal of the $P_R$ state. (b) Absorption spectra of a sample of Cph8 in the pure 100% $P_R$ state, the photoequilibrium mixed state of 65% $P_{FR}$/35% $P_R$, and the pure 100% $P_{FR}$ state that we aim to reach by Stimulated Depletion Quenching (SDQ) enhancement. (c) SDQ Concept: The Cph8 sample in a mix of $P_R$ and $P_{FR}$ states is exposed to ultrashort Excitation and Depletion pulses, separated by a delay $\tau$. Both states are excited and undergo dissimilar coherent ultrafast excited state dynamics along a chromophore isomerization coordinate to either the transient product states (LUMI-R and LUMI-F) or back to their initial ground states (grey arrows). The transient products more slowly transform the remaining protein domains downstream of the chromophore, completing the photoswitching reaction. Precision timing of the depletion pulse permits the selective transfer of a larger portion of the $P_{FR}$ molecules back to the $P_{FR}$ ground state (i.e., quenched molecules no longer follow grey dashed arrows) compared to the same process for $P_R$, thereby quenching the reverse reaction more than the forward. This process shifts the equilibrium of the photoswitching reaction toward $P_{FR}$, increasing the dynamic range of the Cph8 switch.
reaction, $P_{FR} \rightarrow P_R$, by stimulated depletion, shifting the photoequilibrium to increase the dynamic range of the switch.

A schematic of the proposed *Stimulated Depletion Quenching (SDQ)* mechanism is shown in Figure 5.1(c), and will be more fully explained in Section 5.1.4. To summarize, the actuation of the Cph8 switch is initiated when the PCB chromophore absorbs a red or far-red photon. This excitation weakens the bond order of the methine bridge joining the outer C and D rings of PCB, resulting in molecular isomerization by rotation of the D-ring, producing an irreversible cascade of down-stream protein domain reorganizations converting that single electronic excitation into the actuation of a cellular signaling pathway. Due to spectral cross-talk of the $P_R$ and $P_{FR}$ states both are excited by photons of red light, it is not possible to prevent these unwanted electronic excitations of the $P_{FR}$ state of the PCB chromophore. However, it *is* possible to prevent the switching of the remainder of the Cph8 protein complex and subsequent activation of the cellular signaling pathways by de-exciting the chromophore before the larger protein “notices” that it has been triggered. After the excitation to the $P_R^*$ or $P_{FR}^*$ state there is a brief interval of time before the chromophore begins isomerization. During this interval the molecule can be returned to its initial state by stimulated depletion, making it appear as though no absorption had occurred and quenching the photoswitching reaction. Further, to facilitate this capability, there should be sufficient differences in the excited state dynamics and stimulated emission spectra of the two forms of the chromophore to preferentially de-populate the $P_{FR}^*$ excited state over that of the $P_R^*$ excited state, allowing the forward reaction to proceed while quenching the reverse reaction. This selective quenching shifts the photoequilibrium of the simultaneous forward and reverse photoswitching reactions, thereby increasing the maximum attainable $P_{FR}$ population. The total photoswitching reaction can be represented by a simple linear transformation map for an incremental $P_R \rightarrow P_{FR}$ step per laser interaction; upon repeated iteration of the mapping transform (i.e., repeated laser exposure) the full transition of large populations of the Cph8 switch
from the $P_R$ to the $P_{FR}$ state can be accomplished. The components of the transformation map can be calculated from a small number of measurements of the system, enabling us to rapidly calculate the photoequilibrium population and the enhancement of the dynamic range accessible by the Cph8 switch brought on by the SDQ mechanism.

The experimental research described here is a feasibility study of this proposed SDQ mechanism, aiming to demonstrate control of the photo-isomerization reaction in the Cph8 optogenetic switch by achieving greater stimulated depletion quenching of the reverse photoswitching reaction compared to the forward reaction by harnessing the unique dynamics of the two photo-isomerization pathways. This work has a limited goal, but represents an important first step toward the ultimate goals of achieving maximal dynamic range control of a single optogenetic switch and simultaneous independent control of multiple optogenetic switches in living cells by multiplexed, optimal non-linear photoswitching.

The remainder of this introduction section contains additional background information on the Cph8 phytochrome based switch (Section 5.1.2), the photo-isomerization of the PCB chromophore associated with Cph8 (Section 5.1.3), and a more detailed description of the proposed SDQ technique (Section 5.1.4). Section 5.2 describes a model for simulations assessing the feasibility of the experiment and exploring the significant reduced parameter space to determine an effective range of experimental parameters. Section 5.3 presents the experimental controlled photoswitching of the Cph8 system and the procedure for data processing and analysis of the resulting measurements. These processed data are further analyzed and results are presented in Section 5.3.4, followed by a discussion of the current results and recommended future experimental directions in Section 5.4.

5.1.2 Background: The Cph8 Optogenetic Switch

Cph8 is a recently developed phytochrome based optogenetic switch designed to control gene expression in E. Coli bacteria [42]. Naturally occurring phytochromes are light
sensing proteins in plants, algae, cyanobacteria, and other micro-organisms that harness light as an energy source \cite{45,46}. These organisms rely on phytochromes to respond to daily and seasonal changes to the light environment. In plants phytochromes control seed development and growth \cite{47,48}, while in algae and cyanobacteria they act as primitive vision sensors to steer toward or away from light during phototaxis \cite{49,50}. Phytochromes use the photo-isomerization of a covalently attached open-chain tetrapyrrole (bilin) chromophore to reversibly convert between two states in response to light. The chromophore cycles between a dark-stable, red-absorbing $P_R$ state and a meta-stable, far-red-absorbing $P_{FR}$ state, functioning as a molecular switch to regulate numerous responses to light intensity, color, duration, and direction \cite{51}.

Structurally, phytochromes all share a conserved, modular architecture consisting of an N-terminal photo-sensory module and a C-terminal signal-output module, shown in Figure 5.2. The photo-sensory module is made up of three domains: PAS (Per/ARNT/Sim), GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA), and PHY (phytochrome-specific) \cite{52}. The bilin chromophore is covalently anchored to a conserved cysteine residue in the GAF domain; and the PAS and GAF domains surround the chromophore, forming the protective binding pocket that isolates the molecule from the surrounding environment, enabling coherent optical interactions within the “hot, wet, and noisy conditions inside a living cell” \cite{53}. The PHY domain detects the isomerization of the chromophore and mediates the translation of the nanometer scale isomerization of the chromophore into the large scale re-orientation of the remaining phytochrome that can be registered by the cellular signaling architecture and affect biological functions. The phytochrome photo-sensory module has a simple modular architecture that shows up naturally in a wide range of species and can be artificially implemented in a number of cell types: including mammalian and bacterial cells \cite{55}. Phytochrome based optogenetic controls seem to be possible in any organism containing the appropriate chromophore (either natively generated, as in many plant and mammalian cells, or exogenously created.
Figure 5.2: The Cph1 protein complex, divided into the conserved architecture of the canonical phytochrome, which makes up the Photo-Sensory Module of Cph8. The PAS and GAF domains anchor and form the binding pocket around the chromophore phyco-cyanobilin (PCB). The PHY domain detects the state of the chromophore and passes the information to the Signal Output Module on the other side of the cell membrane, which transforms the information into a cellular signal pathway initiating biological function. (Adapted from [54])

in bacteria and other cell types) [56]. This adaptability and natural wide utility across many cell types will be important for the expansion of phytochrome based switches and the possible multiplexed optical control of multiple switches, which will be expanded upon in Section 5.4.

Cph8 is an artificial fusion protein that combines a phytochrome light-sensing domain with a Two Component Signaling system (TCS) native to E. coli. The phytochrome light sensor is Cph1, originally from the cyanobacterium *Synechocystis PCC6803*, which utilizes phycocyanobilin (PCB) as a chromophore [57]. The E. Coli TCS is EnvZ, whose normal function is sensing changes in the osmolarity of the surrounding environment [58]. Under red light illumination the light-sensing domain is excited from the dark stable $P_R$ state to the $P_{FR}$ state, sending a stimulus to the downstream signal output module of the EnvZ half of the Cph8 switch. When activated by stimuli from the sensor domain, EnvZ selectively phosphorylates the OmpR response regulator, a small mobile enzyme in the cell cytoplasm.
that promotes gene transcription when phosphorylated [59]. When the switch returns to the $P_R$ state, either by far-red light illuminated or by slow, automatic dark reversion, the EnvZ output module de-phosphorylates the P-OmpR response regulator, inhibiting transcription. The specific gene sequence that OmpR associates with can be engineered into the system. For example, cells have been programmed to generate pigments [20] or fluorescent proteins [44] in response to optical switching under exposure to light, so as to provide measurable evidence of the switching function. A schematic of this TCS system is shown in Figure 5.3(a), along with a representative measurement of the light activated production of GFP protein in Cph8 expressing bacteria (b). In Figure 5.3(b) the non-zero signal under 650 nm illumination shows the effect of spectral cross-talk (i.e., the system is incapable of being turned off under normal illumination).

Figure 5.4 (an expansion of Figure 5.1(b)) shows the absorption spectra of the Cph8 sample after exposure to red and far-red light until saturation of the photoswitching transition. After far-red exposure the switch has fully converted to the $P_R$ state, as this state does not appreciably absorb at wavelengths longer than 720 nm and there is no
Figure 5.4: Absorption spectra of the Cph8 sample. The red line shows the spectrum of the pure $P_R$ state, obtained by maintaining the sample under saturated far-red illumination ($\lambda = 725$ nm). The purple line is associated with a sample in the photoequilibrium mixed state of 65% $P_R$ and 35% $P_{FR}$, obtained by maintaining the sample under saturated red illumination ($\lambda = 650$ nm). From the difference of these spectra we can extract the pure $P_{FR}$ spectrum, shown by the dashed blue line. The ultimate goal of this work is clean switching to this projected pure $P_{FR}$ state.

forward ($P_R \rightarrow P_{FR}$) photoswitching reaction. However, due to overlap of the $P_R$ and $P_{FR}$ absorption spectra at shorter wavelengths, the red light exposure activates both the forward and reverse ($P_{FR} \rightarrow P_R$) photoswitching reactions, and the sample does not fully convert to the $P_{FR}$ state. This photoequilibrium is defined by the ratio of the forward and reverse reaction rates at the excitation wavelength. At 650 nm this equilibrium is 65% $P_{FR}$, 35% $P_R$ [60]. This photoequilibrium greatly limits the functional application of these switches (as with the majority of optically sensitive biological molecules) due to the broad overlapping absorption spectra of naturally evolved chromophores. Stated another way, this spectral cross-talk limits the dynamic range of the switch: the ratio of the final cellular functional output under activating and de-activating light inputs is restricted by preventing the switch from being fully converted to the $P_{FR}$ state.
The source of the spectral cross-talk is the overlap in the absorption spectra of the two chromophore states in the light-sensing module. To increase the dynamic range of the switch we must uncouple the optical interaction with the \( P_R \) state (which activates the forward photoswitching reaction) from the optical interaction with the \( P_{FR} \) state (which activates the reverse reaction). We have demonstrated theoretically [33–35] and experimentally [36–40] that even molecules with spectra that are nearly functionally identical can be differentially excited by exploiting their unique, coherent excited state dynamics using ultrashort tailored pulsed light sources. To independently optically address the two states of the Cph8 protein complex we must investigate the photo-isomerization dynamics of the PCB chromophore molecule.

5.1.3 Background: Chromophore Dynamics

The chromophore in the photo-sensory module of Cph8, phycocyanobilin (PCB), is made up of four pyrrole rings labeled A-D in Figure 5.5(b) (an expansion of Figure 5.1(a)) in an open configuration joined by conjugated methine bridges. The A-ring of the chromophore is covalently bound to the GAF domain at the Cys259 residue [54,61](as shown in Figure 5.5(a)). This domain surrounds the chromophore, forming the binding pocket, which constrains the structure of the chromophore and influences its spectral response and dynamics, while isolating it from environmental disturbances that would destroy the effectiveness of the photo-isomerization reaction. The structure of the chromophore and the surrounding binding pocket shown in Figure 5.5(a) is based on x-ray crystallographic analysis of the Cph1 phytochrome in the \( P_R \) state [54]. The D-ring of the chromophore is less constrained than the rest of the molecule, giving it the freedom of movement to flip between two conformational states. The planar \( ZZZ_{ssa} \) isomer conformation corresponds to the red-light absorbing \( P_R \) state. A rotation of the D-ring about the \( C_{15}=C_{16} \) carbon bridge flips it out of the plane of the other three pyrroles converting the chromophore...
to the $ZZE_{ssa}$ conformation associated with the far-red-light absorbing $P_{FR}$ state. Here $Z(\text{usammen})\&E(\text{ntgegen})$ and $s(\text{yn})\&a(\text{nti})$ refer to the symmetry of the double and single bonds, respectively, of the $C5$, $C10$, and $C15$ carbons in the methine bridges joining the four pyrrole rings of the bilin chromophore. In the $P_{FR}$ state, interaction with the Tyr176 residue of the GAF domain (also labeled in Figure 5.5(a)) affects down stream changes in the rest of the phytochrome, converting the nanometer scale isomerization of the chromophore into a large-scale re-distribution of neighboring domains producing a signaling pathway that can influence cellular function. The optical characteristics of the chromophore are very sensitive to the binding pocket: mutation of alleles in the PAS and GAF domains of the phytochrome can prevent photoswitching or shift the absorption spectrum by up to 30 nm [62]. In spite of these shifts, the change in the central wavelength is always smaller than the broad, overlapping absorption spectrum characteristic of the bilin chromophores, meaning it is still susceptible to spectral cross-talk. However, it should be noted that while not sufficient to distinguish species by linear absorption, these subtle shifts to the chromophore binding pocket should provide an ultimate basis to exploit pulse shaping for optimal dynamic discrimination (ODD) of multiple similar switches (see Section 5.4).

As discussed above, the absorption of the $P_{FR}$ state extends to longer wavelengths than $P_{R}$, yielding near complete ($>97\%$) conversion from the $P_{FR}$ to the $P_{R}$ state (the reverse reaction) under constant far-red excitation. The opposite reaction is challenging due to spectral cross-talk: the red light excitation is absorbed by both the $P_{R}$ and $P_{FR}$ states. Since we cannot solve the problem of spectral cross-talk by altering the absorption spectra, we must turn to an alternate set of optical interactions to find suitable controls to differentiate the states. While the ground state absorption of the $P_{R}$ and $P_{FR}$ states are similar and overlapping, the excited state dynamics are very different and open a new avenue for controlling the chromophores and differentiating the two states. Thus, we seek
Figure 5.5: (a) (Adapted from [54]) Ribbon representation structure of the binding pocket in the GAF domain of the Cph1 photo-sensory module. The PCB chromophore is covalently attached to the GAF domain at C-259. The tyrosine residue Y176 is believed to help stabilize the $P_{FR}$ state and is important in the photoswitching reaction. (b) PCB Chromophore in the $P_R$ and $P_{FR}$ states. The $C_{14}−C_{15}=C_{16}$ methine bridge linking the C-ring and D-ring is highlighted. The two states partially interconvert with red light (650 nm), and concomitantly under far-red light (720 nm) or dark-reversion.

to exploit the distinct dynamics of the forward and reverse photo-isomerization reaction to halt the reverse reaction while minimizing the impact on the forward reaction.

The photo-isomerization of the PCB chromophore in both directions is initiated by photo-excitation of the molecule, immediately followed by very rapid coherent vibronic relaxation on the excited state surface [63]. This sequence is then followed by a portion of the excited molecules transitioning to a transient intermediate product state beginning the isomerization. The remaining excited state population returns to its respective initial ground state. In the forward isomerization reaction ($P_R \rightarrow P_{FR}$) this intermediate is LUMI-R; in the reverse ($P_{FR} \rightarrow P_R$) the intermediate is LUMI-F [64, 65]. These two transient intermediate species are unique to the forward and reverse reaction, respectively, and the subsequent relaxations progress along distinct, separate reaction coordinates. The LUMI-R and LUMI-F transient intermediate products persist for several nanoseconds and trigger a series of large scale conformational reorganizations of the protein residues in the downstream domains to complete the photoswitching transformation between the $P_R$ and
$P_{FR}$ states of the full Cph8 switch. The names “$P_R$” and “$P_{FR}$” are used to refer to the red-light and far-red-light absorbing states, respectively, of the PCB chromophore, the phytochrome light-sensing module, and the overall Cph8 switch depending on context.

The initial coherent steps of the ultrafast photo-isomerization are not as well understood as the slower domain reorganizations associated with the latter steps of the switching. It is generally agreed that the isomerization in both directions is initiated when the PCB chromophore absorbs a photon of light, exciting an electron from the π-conjugated system primarily localized on the C and D pyrrole rings and the methine bridge that joins them. This promotes an electron to the anti-bonding $\pi^*$ orbital, weakening the double bonding character of the $C_{15}-C_{16}$ carbon atoms in the bridge (labeled on the chromophores in Figure 5.5(b)), enabling rotation of the outer D ring and interconversion between the $P_R$ state and the $P_{FR}$ state, and vice versa, by way of non-shared transient intermediates. [63, 65, 66]. It is here that the forward ($P_R \rightarrow P_{FR}$) and reverse ($P_{FR} \rightarrow P_R$) photo-isomerization reactions start to differ and also where the disagreements about the earliest dynamics of the excited state relaxation begin. In particular, the early stage dynamics of the initial excited state relaxation and isomerization steps of the Cph1 phytochrome have been studied using numerous ultrafast electronic [63,67,68] and vibrational [69–72] transient spectroscopies, often arriving at different and sometimes even contradictory conclusions. In all reported studies, analysis of time-resolved spectra reveal complex multi-exponential excited-state relaxation dynamics, but there are different interpretations of these measurements. The extent of the isomerization reaction occurring on the excited state and the homogeneity of the ground state population are the principle areas of disagreement. There are two schools of thought with regard to ground state population: (1) there is an ensemble of ground state sub-populations that each undergo distinct single-exponential dynamics after excitation (the inhomogeneous perspective), and (2) there is a single uniform ground state population and the com-
plex, non-exponential excited state dynamics are due to multiple vibronic resonances at a conical intersection (the homogeneous perspective).

Each perspective is supported by a number of experimental studies using a variety of measurement techniques. Resonant Raman intensity analysis [73] and hybrid quantum mechanics/molecular mechanics (QM/MM) simulations [74] support a homogeneous \( P_R \) state that undergoes multi-phasic excited state dynamics. Solid-state NMR resolved two distinct ground state \( P_R \) sub-populations: one structure consistent with the known crystal structure and a second interpreted as having a modified charge distribution and hydrogen bond network [75]. Some pump-dump-probe (PDP) studies find evidence of a homogeneous ground state in \( P_R \) [67, 70], while other PDP experiments observe a heterogeneous ground state in Cph1 [68, 76, 77] and in a related phytochrome [78]. There is currently no consensus as to the exact nature of the ultrafast dynamics of the photo-isomerization. The results of the most recently published experiments characterizing the forward and reverse photo-isomerization reactions are summarized below.

The forward photo-isomerization of \( P_R \) to \( P_{FR} \) of the Cph1 phytochrome has been characterized by the Larsen group from UC Davis [79]. The study consists of a combination of temperature-dependent and excitation-wavelength-dependent static and ultrafast transient absorption measurements. Using a global analysis of the combined measurements they resolve five distinct ground state sub-populations (Pr1, ..., Pr5) co-existing at thermal equilibrium, each undergoing single-exponential dynamics through separate excited and intermediate states. The sub-populations can be grouped into two distinct categories: the fast-decaying photo-active populations (Pr1 and Pr5) and the slow-decaying fluorescent populations (Pr3-Pr5). The exponential lifetimes associated with each sub-population at room temperature are summarized in Figure 5.6. The time scale of the forward isomerization is given by the formation of LUMI-R in 61 picoseconds.

The reverse \( (P_{FR} \rightarrow P_R) \) photo-reaction has been less thoroughly studied due to the transient nature of the \( P_{FR} \) state and inability to fully remove the confounding \( P_R \)
Figure 5.6: (adapted from [79]) Measurements of forward photoswitching dynamics reveal heterogeneous ground state sub-populations. The photoswitching population ($P_{r2}$ above) forms the LUMI-R intermediate isomerization product with a time constant of 61 ps.

In a collaboration between the Heyne group at the Freie Universitat Berlin and the Mathies group at UC Berkeley, a combination of Femtosecond Stimulated Raman Spectroscopy (FSRS) and polarization-resolved transient IR spectroscopy was used to map the initial steps of the reverse photo-isomerization reaction from the $P_{FR}$ state of the Cph1 phytochrome [80]. Researchers detected the extremely rapid sub-picosecond development of the LUMI-F intermediate product. This observation is supported by direct measurement of the orientation of the chromophore D-ring by the femtosecond resolved polarization anisotropy of the $C_{19}=O$ vibrational absorption (a good indicator of the orientation of the ring) as shown in Figure 5.7(a-c). The FSRS measurement of the $C_{15}=C_{16}$ stretch in Figure 5.7(d) shows the shift to lower wavenumber of the stretching vibration, which reflects a reduction in the vibrational force constant after relaxation from the FC region. The inset of Figure 5.7(d) shows the ground-state recovery dynamics of the 1607 cm$^{-1}$ bleach feature fit to a bi-exponential decay curve with time constants of $t_1=(300\pm50)$ fs and $t_2=(6\pm3)$ ps, representing a $\approx70\%$ loss of the excited population within a picosecond of excitation; in agreement with the timescale of the ring flip given by the IR measurement.

Analysis of multiple vibrational modes on the chromophore and the direct measure of the ultrafast change in the polarization anisotropy of the $C_{19}=O$ on the D-ring support sub-picosecond isomerization of the PCB chromophore to the LUMI-F transient photo-
Figure 5.7: (adapted from [80]) (a-b) Measurements and analysis of the polarization resolved transient IR absorption and (d) FSRS measurements of the reverse photoisomerization of PCB in Cph1 phytochrome. (a) Ultrafast transient absorption of the ground state $\nu(C_{19}=O)$ and $\nu(C_{19}=O)^*$ vibrational modes on the electronic ground and excited state energy surfaces. Black dots in (a) represent IR probe polarization parallel to the visible excitation, while red circles represent perpendicular probe polarization. The vertical blue bars illustrate that the difference between the two polarized absorption signals remains fixed in the ground state, but rapidly changes in the excited state. This provides direct measurement of the rotation of the $(C_{19}=O)^*$ vibrational mode and, by extension, the D-ring of the chromophore. (b) The polarization anisotropy quantifies the difference between the two polarized absorption signals and is a measure of the angle between the transition dipole moments of the electronic excitation and the vibrational mode. The time varying anisotropy of the excited state $(C_{19}=O)^*$ mode is strong evidence of rotation of the D-ring in the excited state with a time constant of 0.7 ps. (c) Charts of the shift in peak FSRS frequency of the excited state $C_{15}=C_{16}$ stretch associated with a weakening of the bond order after excitation. The inset shows the ground-state bleach dynamics for the $C_{15}=C_{16}$ stretch, exhibiting bi-exponential recovery of the ground state over two time domains: a fast 300 fs recovery and a slower 6 ps recovery. These figures are a condensed selection of many measurements from reference [80] which support the conclusion of sub-picosecond coherent isomerization in the reverse ($P_{FR} \rightarrow P_R$) photoswitching reaction.
Figure 5.8: (adapted from [80]) (a) Sketch of the multi-dimensional potential energy surface representative of the isomerization reaction, which is shown on the right (b). Non-bonding interactions between the methyl groups on the C and D rings (indicated with green “”) in (b) place a strain on the $P_{FR}$ ground state which enhances the speed of the reverse isomerization once initiated by absorption of a photon. The important vibrations that are part of the reaction coordinate in the multi-dimensional PES are depicted on the axes in (a). The two-step mechanism of the isomerization process is depicted by the black and red trajectories. The conical intersection, denoted by a grey square, enables very fast generation of the LUMI-F photoproduct and $P_{FR}$ ground state.

The ultrafast structural change in the bilin chromophore may indicate formation of the first intermediate state on the excited state potential surface, and is accelerated by a non-bonding methyl-methyl clash of the outer methyl groups on the C-ring and D-ring in the $P_{FR}$ ground state. A representation of the excited state dynamics on a 2-D potential energy surface is shown in Figure 5.8. The relaxation from the initial Frank-Condon excitation point ($P_{fr}^*$ in Figure 5.8) occurs in 300 fs, at which point the molecule either relaxes back to the $P_{FR}$ ground state or remains on the excited surface, depending on whether its D-ring has initiated rotation. Those chromophores remaining in the excited state continue to rotate until reaching the conical intersection with the LUMI-F photo-product surface over the next 700 fs. The conical intersection mediates the rapid generation of the LUMI-F photo-product.
These studies inform our understanding of the Cph8 switch and guide the development of the experiment reported in this chapter. While the exact details of the excited state dynamics are neither fully understood nor agreed upon, this situation does not hinder our ability to conduct experiments exploring a pathway to the control of the optogenetic switches. The ability to distinguish the two states of the Cph8 switch does not require exact knowledge of the coherent excited state dynamics of the molecule, only the ability to interact with the system while the excited state coherent dynamics are ongoing. Built on the essence of the background experiments reported here, a kinetic-based model may be constructed using the generalized switching picture captured in Figure 5.1(c) (Section 5.2). We will see that, there are inherent features of the photo-physics in the latter figure (i.e., the stability of the two switch states, and the irreversibility of the switching dynamics once initiated) that are amenable to identifying the per-exposure iterative map for the transfer of population between the switch states. The existence of this iterative map is more important than a detailed quantitatively accurate model. The experiments in this work (Section 5.3) will identify the map from the laboratory data, and then use the properties of the iterative map to calculate the effective transfer for the $P_R \rightarrow P_{FR}$ photoswitching reaction. Importantly, the conclusions drawn from these initial experiments should apply to further advanced studies, including pulse-shaping assisted multiplexed optimal dynamic discrimination of multiple similar optogenetic switches.

5.1.4 Description of the SDQ Mechanism

In this experiment selective stimulated emission is employed to coherently transfer PCB chromophores initially excited to the $P_{FR}^*$ state back to the $P_{FR}$ ground state before isomerization can occur, while simultaneously allowing chromophores initially excited to the $P_R^*$ state to persist and undergo photoswitching to the $P_{FR}$ state. The concept of this SDQ mechanism is represented in the diagram in Figure 5.1(c). The sample is exposed to
two ultrashort laser pulses: an excitation pulse and a depletion pulse. The spectrum of the excitation pulse overlaps with the absorption spectra of both the $P_R$ and $P_{FR}$ states of the PCB chromophore. Exciting the molecule with the ultrashort pulse creates corresponding coherent wave packets from superpositions of vibrational levels on the electronic excited state surfaces of both forms of the chromophore. These wave packets undergo dissimilar coherent dynamics as they relax from their initial Frank-Condon excitation states toward a coherent vibronic transition through a conical intersection either back to their initial ground state or to a transient intermediate photo-product state. Before the molecules complete these dynamics, a second pulse arrives after a short, controlled delay ($\tau_{delay}$). This depletion pulse has a central wavelength set to overlap more favorably with the stimulated emission spectrum of the $P_{FR}^*$ state than the $P_R^*$ state. The combination of spectral overlap and timing permit the depletion pulse to selectively drive a larger portion of the $P_{FR}^*$ excited molecules back to the $P_{FR}$ ground state, preferentially slowing the reverse photo-isomerization reaction while allowing the forward reaction to continue transferring some portion of the $P_R$ population to the $P_{FR}$ state. Those molecules remaining in either of the excited states after the depletion pulse continue their unperturbed dynamics, either isomerizing to the transient photo-product state or returning to their initial ground state.

SDQ shares many similarities with Stimulated Emission Depletion (STED), a common super-resolution imaging technique used in microscopy. In STED microscopy the suppression of fluorescence from a ring-shaped outer spot provides contrast to an undepleted inner spot, yielding an instrument response function with sub-diffraction limited spatial resolution. In both STED and SDQ the goal is to fully de-populate an excited electronic state before the system can relax by an undesired pathway. However, there is a critical difference between conventional STED of a fluorescent molecule and our proposed SDQ of the excited state of a photoswitch. In most fluorescent species the lifetime of the excited electronic state exceeds the lifetime of the coherent vibrational states by several
orders of magnitude. In these species the molecule vibrationally relaxes to a stationary state on the electronic excited surface and a (relatively) long depletion pulse can interact with the population over an extended period, maximizing the population transferred to the ground state. As discussed in Section 5.1.3, the vibrational and electronic excited state lifetimes are of similar duration for the chromophores, and it is believed the molecule undergoes semi-coherent vibronic dynamics throughout the isomerization/relaxation (i.e., the initially coherent superposition persists while also interacting with the surrounding protein and solvent environments in ways which support the coherence or induce decoherence, the details of which are presently not well characterized [74, 81]). This impacts the potential effectiveness of the proposed SDQ mechanism in two ways. First, because the excited state lifetime is so much shorter than a fluorescent species, the depletion pulse interacts with the molecule for less time, likely reducing the maximum achievable depletion. Second, because the population is never in a stationary state on the excited surface, the depletion pulse must interact with a spectrally shifting wave packet, possibly negatively impacting the coherent transfer to the ground state. Future experiments should be able to overcome both of these obstacles by utilizing pulse shaping and feedback control to discover suitable pulse shapes for the excitation and depletion pulses that accommodate or exploit the excited state dynamics of the $P_R^*$ and $P_{FR}^*$ states in a maximally distinguishable fashion. To demonstrate that even without optimally tailored pulse shaping the extremely rapid dynamics of the photo-isomerization would not prevent the SDQ mechanism from controlling the optogenetic switching of Cph8, in Section 5.2 we run simulations with a model system exhibiting comparable optical response and dynamics upon exposure to the dual-pulse excitation-depletion sequences employed in the experiments of Section 5.3.

In practical applications, optogenetic switches are expressed in large numbers in genetically targeted cells [82], not as isolated molecules. Optogenetic control of cellular processes involves the global photoswitching of a large population of protein switches
from an arbitrary initial distribution of states to a maximally active or inactive population, restricted by the Maximal Dynamic Range (MDR) of the system and light exposure condition. This will necessarily be a gradual transfer of population between states over many laser exposure iterations. In particular, the low quantum efficiency of the photoswitching reaction (10-20% in both reaction directions [80]), as well as the fact that not all of the molecules will absorb photons in a single exposure, means that it would not be possible to transfer the entire population of a group of switches to a desired final state in one pulsed laser iteration. The iterative transfer of population is made possible by the relative stability of both active and inactive states in phytochrome-based switches, allowing the state distribution to persist between laser exposure iterations. The \( P_{FR} \) state of Cph8 is meta-stable, reverting to the \( P_R \) state in the dark, but at such a slow rate (variable in different implementations and environments, from many seconds to many hours [83]) that the state distribution can be said to be constant between laser pulses repeated at more than a few Hertz. Each laser exposure iteration transfers a fraction of the proteins from one state to the other, with a per exposure fractional yield that is independent of the states’ populations, defined exclusively by the characteristics of the light. Because of the stability of the product state of the photoswitching reactions, we are able to transfer large populations of switches to the desired product state in many small steps. This iterative transfer of population is important for a mechanism like SDQ, which acts by inhibiting an undesired reaction pathway rather than enhancing the rate of a desired pathway. The SDQ mechanism, while reducing the per-exposure fractional yield of the photoswitching reaction will none the less positively impact the final photoequilibrium after many exposures, which is the feature of interest for the practical implementation of controlled photoswitching.

In this work, the excitation and depletion pulse parameters will be varied to determine the wavelengths, powers, pulse durations, and delay timings which selectively deplete the \( P_{FR}^* \) level most effectively, quenching the reverse reaction while minimally hindering the
forward reaction. This exploration of the experimental control parameter space aims to be a feasibility study for the proposed mechanism, identifying a path through the vast, unexplored control landscape toward a region amenable to effective control of the photoswitching reaction, thereby laying the foundation for more sophisticated optimal control experiments on optogenetic switches in the future.

5.2 SDQ Feasibility Assessed by Rate Equation Model Simulations

We perform simulations using a Rate Equation Model (REM) to qualitatively assess how a system, with optical characteristics and ultrafast dynamics corresponding to the Cph8 switch, responds to ultrashort-pulsed laser excitation and depletion under experimentally reproducible conditions. The SDQ mechanism is an iterative control scheme, shifting the equilibrium condition of the simultaneous forward and reverse photoswitching reactions such that over repeated laser exposures the system gradually moves toward the desired final product distribution in many small steps. To this end, we first model how the system responds to a single excitation-depletion pulse sequence in Section 5.2.1. These single exposure simulations generate a set of Characteristic Coefficients, which describe the photoswitching reaction products independent of the initial state conditions, defined exclusively by the laser exposure condition. In Section 5.2.2, the products of the simulated single exposure photoswitching reaction are used to generate an Optical Transformation Matrix (OTM), which is a linear transformation from the initial to the final state population. The OTM can be used to easily simulate repeated exposures of a system, generating an iterative mapping from the initial state to any final state of the system within the Maximal Dynamic Range (MDR) accessible by the associated laser exposure defined by the particular control parameter set. We apply this iterative mapping to show how repeated exposures allow the $P_{FR}$ state to gradually reach a higher photoequilibrium enhanced by
SDQ. Once the mechanics of the simulation are explained, in Section 5.2.3 we present a collection of results showing the functional dependence of the photo-reaction on the laser control parameters and locate the region of the parameter space that most effectively enhances the photoswitching dynamic range. These simulations are designed to provide a basis to guide and understand the experiments of Section 5.3, and the model predictions for the SDQ enhancement will be compared to the measured results in Section 5.3.4. While the simplicity of the model precludes total quantitative agreement, the simulations and experimental results share the most common feature of an iterative map driven transfer mechanism. The Matlab code used in these simulations will be maintained on the servers of the Rabitz group and can be provided upon request.

5.2.1 Simulation of Dual-Pulsed Excitation-Depletion Photowitching

A schematic of the model system is shown in Figure 5.9. In comparison to Figure 5.1(c) or Figure 5.8(a), the multi-dimensional potential energy surfaces of the $P_R$, $P_R^*$, $P_{FR}$ and $P_{FR}^*$ states have each been collapsed to two discrete levels representing each surface, and a pair of levels represent the transient intermediate products LUMI-R=LR5 and LUMI-F=LF5, totaling ten levels. Rather than describing discrete states separated by single-valued energy differences, the levels should be viewed as representing manifolds of molecular states, and the optical responses and associated dynamics of the levels are defined by experimentally measured absorption and emission spectra and state lifetimes associated with time resolved measurements, as discussed further below. The model supports excitation to a Frank-Condon point and subsequent relaxation in the electronic excited state followed by either isomerization to the intermediate product state or transition to a hot ground state by spontaneous relaxation or stimulated emission. The populations of the intermediate states, LUMI-R and LUMI-F, do not cross or interact (i.e., the inter-
mediate transition states during the forward isomerization from $P_R$ to $P_{FR}$ are not shared with the transient states of the reverse reaction); once isomerization begins it will continue unabated. The branching ratio of the isomerization/spontaneous emission pathways is dictated by the relative transition rate constants, which are based on excited state level lifetimes from experimental measurements [79,80]. The rate constants associated with the spontaneous transitions in Figure 5.9 are collected in Table 5.1. Using these parameters, the model reproduces the expected behavior under single-pulse excitation. In particular, when exposed to continuous red illumination ($\lambda = 650$ nm) the switch reaches a mixed state equilibrium of $P_{FR}/P_R \simeq 65%/35\%$ and fully resets to $>99\% P_R$ under continuous far-red illumination ($\lambda > 725$ nm).
Because details of the structure and coherent dynamics of the chromophore molecules in the phytochrome switching systems are still not fully understood, the coherent energy-time coupling properties of the molecule and the laser pulses are not contained in this kinetic model. The high-dimensional, vibronically coupled potential energy surfaces are simplified to a reduced number of levels associated with the integrated spectral response of the molecule and linked by spontaneous incoherent transitions. The pulsed lasers are characterized under the slowly varying envelope (SVE) approximation: the temporal dynamics of the pulse serves only to scale the fixed spectral intensity distribution, leaving the coherent spectral phase and fast oscillating component of the complex-valued electric field out of the calculation. These simplifications allow us to simulate the system without making further assumptions about characteristics of the excited state dynamics that are not conclusively accepted. The next generation of improved modeling, incorporating better knowledge of the system’s structure and dynamics from future measurements would allow for a degree of coherence in the dynamics of the system using an appropriate quantum master equation (i.e., the Lindblad equation) to simulate the evolution of the sample population distribution. While details associated with the coherent dynamics of the molecule are not present, the rate equation model offers qualitative insight into how the rapid kinetics of the photo-isomerization reaction impacts the practicability of the SDQ method. In this way, the rate equation model can be thought of as a worst-case limit of the capability of the SDQ method as it does not exploit coherence.

Table 5.1: Static rate coefficients of REM. Coefficients are expressed as inverse lifetimes associated with values from the literature \cite{79, 80}.

<table>
<thead>
<tr>
<th>Electronic Relaxation</th>
<th>Fast Isomerization</th>
<th>Vibrational Relaxation</th>
<th>Slow Isomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{3,4}^R$ = 1/26ps</td>
<td>$k_{3,5}^R$ = 1/61ps</td>
<td>$k_{2,3}^R$ = 1/0.15ps</td>
<td>$k_{4,1}^R$ = 1/0.15ps</td>
</tr>
<tr>
<td>$k_{3,4}^{FR}$ = 1/0.30ps</td>
<td>$k_{3,5}^{FR}$ = 1/1.50ps</td>
<td>$k_{2,3}^{FR}$ = 1/0.05ps</td>
<td>$k_{4,1}^{FR}$ = 1/0.05ps</td>
</tr>
<tr>
<td>$k_{LR5- FR1}$ = 1/10^9ps</td>
<td>$k_{LF5- R1}$ = 1/10^9ps</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Starting with the measured reference absorbance spectra of the Cph8 protein in the $P_R$ and $P_{FR}$ states, we can simulate or approximate the remaining features of the model. The reference absorbance spectra are shown in Figure 5.10, scaled to the peak molecular absorption cross-section. The absorption spectra in the literature [60] are reported as the molar attenuation coefficient, but the calculations require the molecular cross-section; the conversion between the two is simply: $\sigma(\lambda) = \frac{10^{3\ln(10)}}{N_A} \times \varepsilon(\lambda)$. The peak molar attenuation coefficient of the $P_R$ state is $\varepsilon_{R}(650 \text{ nm}) = 85 \ (mMcm)^{-1}$ and the corresponding value for the peak of the $P_{FR}$ state is $\varepsilon_{FR}(705 \text{nm}) = 65.9 \ (mMcm)^{-1}$, resulting in a peak absorption cross-section of $\sigma_{R}(650) = 3.24 \times 10^{-16} \text{cm}^2$ and $\sigma_{FR}(705) = 2.51 \times 10^{-16} \text{cm}^2$ for the $P_R$ and $P_{FR}$ states, respectively. There is no measurement of the stimulated emission cross-section for the chromophore; however, we can represent the emission spectrum by reflecting the absorption spectrum across the zero phonon line [84]. Comparing the absorption/emission spectra of similar species, notably the iRFP fluorescent protein species developed by mutation of the Cph1 photo-sensory module [85], this symmetry is common and the assumption is reasonable. These simulated emission spectra are also plotted in Figure 5.10 using dashed lines.

We define the excitation and depletion pulses in terms of their real-valued spectral intensity, described here by a scaled Gaussian distribution. For the pulse $p = (Ex. \ (excitation) \ or \ Dp. \ (depletion))$ centered at wavelength $\lambda_p^p$ and with full width at half max of $\Delta \lambda_p$, the real spectral intensity is:

$$I_p(\lambda; \lambda_p^p, \Delta \lambda_p, \langle P_p \rangle, d_p) = \left(\frac{4\langle P_p \rangle}{\pi \cdot d_p^2}\right) \left(\frac{2\sqrt{\ln 2}}{\Delta \lambda_p \sqrt{\pi}}\right) e^{-\left(2\sqrt{\ln 2} \cdot (\lambda - \lambda_p^p)/\Delta \lambda_p\right)^2} \quad (5.1)$$

The spectral distribution function is scaled by the average power $\langle P_p \rangle$ (in units of Watts) and beam waist diameter $d_p$ (in units of cm) to give the spectral intensity in $(W/cm^2)$. The “beam diameter” is defined in these simulated pulses to describe the intensity in terms of experimentally measured and controlled parameters. The spectra of the excitation
Figure 5.10: Absorption (solid lines) and stimulated Emission (dashed lines) spectra of Cph8 in the $P_R$ state (red curves peaking at 665 nm and 725 nm, respectively) and the $P_{FR}$ state (black curves peaking at 710 nm and 770 nm, respectively). Also plotted are spectra representing an excitation pulse centered at 625 nm and two different depletion pulses centered at 775 nm and 835 nm (orange, light red, and dark red shaded areas, respectively).

and depletion pulses are plotted with the molecular absorption and emission spectra in Figure 5.10 for an excitation pulse centered at 625 nm with width 30 nm and two separate depletion pulses with width 10 nm, one centered at 775 nm and the other at 835 nm.

The temporal intensity of the pulse is defined by a normalized Gaussian function with variable delay $t_0^p$ and an adjustable pulse width $\Delta t_p$:

$$I_{pulse}(t; t_0^p, \Delta t_p) = \left( \frac{2 \sqrt{\ln 2}}{\Delta t_p \sqrt{\pi}} \right) e^{-\left(2 \sqrt{\ln 2} (t-t_0^p) / \Delta t_p \right)^2}$$  \hspace{1cm} (5.2)

As stated above, the model simulates the pulsed laser under the slowly varying envelope (SVE) approximation, the temporal intensity envelope is defined independently from the fixed spectral intensity distribution, rather than using a complex phase term in the field.
amplitude to stretch the broad bandwidth, ultrashort pulse beyond its transform-limited
duration. This approximation is acceptable where the spectral frequency bandwidth of
the pulse is much smaller than the central frequency: $\Delta \omega/\omega_0 \ll 1$. For the spectral
bandwidths and pulse durations used in these simulations we are straining the acceptable
usage of the SVE approximation (for a Gaussian spectrum 30 nm wide centered at 625 nm
the ratio of bandwidth to central frequency is 0.048). However, because the model only
incorporates the integrated spectral overlap of the lasers and the total spectral response
of the molecules (which are much broader than the laser bandwidth) a time-varying spec-
tral distribution would have little effect on the calculated rate coefficients while greatly
complicating the calculations. In subsequent models, where different laser pulse shapes
will excite distinct excited vibronic population distributions with distinct dynamics, it
will be necessary and useful to represent the laser pulse by a spectral-temporal coupled
field and incorporate coherent aspects of the molecular dynamics.

With the reference absorption and emission spectra of the simulated Cph8 molecule
and the modeled excitation and depletion pulses, we can calculate the dynamic rate
constants for the optical transitions in the molecule and simulate the time dependent
populations of each level for both the $P_R$ and $P_{FR}$ states. The initial conditions are
defined, with each state having a fraction of the total chromophore population in its
lowest ground state:

\begin{align}
[P_R]_{\text{init}} &= R_{10} \\
[P_{FR}]_{\text{init}} &= FR_{10} = (1 - R_{10})
\end{align}

where $0 \leq R_{10} \leq 1$. The time dependent populations of the levels of the $P_R$ and $P_{FR}$
states are given by the solution to a set of coupled first order differential rate equations
(see Figure 5.9 for reference to the notation). The five coupled differential equations
describing the populations of the five levels associated with the $P_R$ state (R1-LR5) are
shown below in Equations 5.5a-5.5e. The five $P_{FR}$ levels (FR1-LF5) are described by five more equations of the same form in Equations 5.6a-5.6e, exchanging appropriate rate constants and populations for the $P_{FR}$ levels. The $P_R$ and $P_{FR}$ states are coupled by the transfer from level LR5 to FR1 (eqs. 5.5e,5.6a) and LF5 to R1 (eqs. 5.6e,5.5a). The rate equations for the $P_R$ associated levels are:

\[
\frac{d(R1(t))}{dt} = [K_{1,2}^{R,Ex}(t) + K_{1,2}^{R,Dep}(t)] \cdot (R2(t) - R1(t)) \\
+ (k_{4,1}^R \cdot R4(t)) + (k_{LF5\to R1} \cdot LF5(t)) \tag{5.5a}
\]

\[
\frac{d(R2(t))}{dt} = [K_{1,2}^{R,Ex}(t) + K_{1,2}^{R,Dep}(t)] \cdot (R1(t) - R2(t)) \\
- (k_{2,3}^R \cdot R2(t)) \tag{5.5b}
\]

\[
\frac{d(R3(t))}{dt} = [K_{3,4}^{R,Ex}(t) + K_{3,4}^{R,Dep}(t)] (R4(t) - R3(t)) \\
+ (k_{2,3}^R \cdot R2(t)) - (k_{3,4}^R \cdot R3(t)) - (k_{3,5}^R \cdot R3(t)) \tag{5.5c}
\]

\[
\frac{d(R4(t))}{dt} = [K_{3,4}^{R,Ex}(t) + K_{3,4}^{R,Dep}(t)] \cdot (R3(t) - R4(t)) \\
+ (k_{3,4}^R \cdot R3(t)) - (k_{4,1}^R \cdot R4(t)) \tag{5.5d}
\]

\[
\frac{d(LR5(t))}{dt} = (k_{3,5}^R \cdot R3(t)) - (k_{LR5\to FR1} \cdot LR5(t)), \tag{5.5e}
\]
While the rate equations for the $P_{FR}$ associated levels are:

$$\frac{d(FR1(t))}{dt} = [K_{1,2}^{FR,Ex}(t) + K_{1,2}^{FR,Dep}(t)] \cdot (FR2(t) - FR1(t))$$

$$+ (k_{4,1}^{FR} \cdot FR4(t)) + (k_{LR5\rightarrow FR1} \cdot LR5(t))$$

(5.6a)

$$\frac{d(FR2(t))}{dt} = [K_{1,2}^{FR,Ex}(t) + K_{1,2}^{FR,Dep}(t)] \cdot (FR1(t) - FR2(t))$$

$$- (k_{2,3}^{FR} \cdot FR2(t))$$

(5.6b)

$$\frac{d(FR3(t))}{dt} = [K_{3,4}^{FR,Ex}(t) + K_{3,4}^{FR,Dep}(t)] \cdot (FR4(t) - FR3(t))$$

$$+ (k_{2,3}^{FR} \cdot FR2(t)) - (k_{3,4}^{FR} \cdot FR3(t)) - (k_{3,5}^{FR} \cdot FR3(t))$$

(5.6c)

$$\frac{d(FR4(t))}{dt} = [K_{3,4}^{FR,Ex}(t) + K_{3,4}^{FR,Dep}(t)] \cdot (FR3(t) - FR4(t))$$

$$+ (k_{3,4}^{FR} \cdot FR3(t)) - (k_{4,1}^{FR} \cdot FR4(t))$$

(5.6d)

$$\frac{d(LF5(t))}{dt} = (k_{3,5}^{FR} \cdot FR3(t)) - (k_{LF5\rightarrow R1} \cdot LF5(t)).$$

(5.6e)

In the rate equations above, the molecule moves from level $i$ to level $j$ via spontaneous transitions, governed by rate constants $k_{i,j}^{S}$ or by optically coupled transitions governed by coefficients $K_{i,j}^{S,pulse}(t)$ (where the $S=$(R or FR) denotes the association with the $P_{R}$ or $P_{FR}$ initial state). The transition coefficients have units $ps^{-1}$. As stated earlier, the spontaneous transition coefficients are related to measured excited state dynamic lifetimes from the literature, and are collected in Table 5.1. The vibrational relaxation rate constants of both states ($k_{2,3}^{S}$ and $k_{4,1}^{S}$) are very high, associated with the very short vibrational relaxation lifetimes. As such, levels 2 and 4 have fleeting populations serving primarily as intermediaries to levels 1 and 3.

The optically coupled transition rate coefficients ($K_{i,j}^{S,pulse}(t)$) are “dynamic”, and vary with the intensity of the pulse temporal envelope. These coefficients are calculated individually for the excitation and depletion pulses. For the chromophore population in state $S$ = (R or FR), interacting with pulse($p$) = (Excitation or Dep(Depletion)) the time
dependent rate of transition from level $i$ to $j$ is given by:

$$K_{i,j}^{S,pulse}(t) = I_{pulse}(t; t_0^p, \Delta t_p) \int \sigma_{i,j}^S(\lambda) \cdot I_{pulse}(\lambda) \cdot (\lambda/hc)d\lambda$$ (5.7)

Here, the rate coefficient has a temporally invariant spectral component given by the pulse photon flux (the pulse spectral intensity from equation 5.1 divided by the energy per photon ($hc/\lambda$)) and the absorption/emission cross-section ($\sigma_{i,j}^S(\lambda)$ is the absorption spectrum of state $P_R$ or $P_{FR}$ in $cm^2$, while $\sigma_{i,j}^S(\lambda)$ is the stimulated emission spectrum). The invariant spectral component is multiplied by the temporal intensity envelope of the pulse given in equation 5.2. Combined, the product of the two functions in Equation 5.2 gives a time dependent rate coefficient for the optically coupled transitions.

The final term in equation 5.5a ($k_{LF5 \rightarrow R1}$) is the gain term from $LF5$ to $R1$ that mediates the reverse switching from $P_{FR}$ to $P_R$ by way of the transient intermediate LUMI-F. The final term in equation 5.5e ($k_{LR5 \rightarrow FR1}$) is the loss term from $LR5$ to $FR1$ that mediates the forward switching from $P_R$ to $P_{FR}$ by way of the transient intermediate LUMI-R. These terms are present in the corresponding equations for the dynamics of the $P_{FR}$ levels $LF5$ (eq. 5.6e) and $FR1$ (eq. 5.6a), acting as loss and gain terms, respectively. The $k_{LR5 \rightarrow FR1}$ and $k_{LF5 \rightarrow R1}$ rates are much smaller than the other rate constants (more than eight orders of magnitude). This distinction arises because the large scale reorientation of the protein domains associated with the transformation from the transient intermediate products to the final product states occurs on a completely different time scale than the initial photo-isomerization (hundreds of microseconds compared to a few picoseconds). When calculating the time dependent level populations the $k_{LR5 \rightarrow FR1}$ and $k_{LF5 \rightarrow R1}$ rates are effectively zero, and the final transient product populations at the end of the simulated time must be handled by a separate calculation to give the final product state populations. The inclusion of the latter rates here serve to link the ODEs that describe the $P_R$ levels (equations 5.5a-5.5e) to the ODEs that describe the $P_{FR}$ levels.
As stated earlier, the transfer from the intermediate states (LR5 and LF5) are 100% efficient, with the entire accumulated population of LR5 and LF5 at the end of the simulated time being added to FR1 and R1, respectively, to complete the simulated photoswitching reaction with all switches in one of the two ground states.

Solving the set of rate equations above gives the dynamic populations of the indicated levels. The two sets of five dynamically coupled ODEs were solved using Matlab’s ODE45 solver, based on the explicit Runge-Kutta (4,5) integrator from references [86,87]. To avoid the differential equations becoming “stiff” the time domain of the calculation was broken into intervals, preventing rapid jumps in the magnitude of the time-varying optically coupled rate coefficients between steps of the algorithm. The level populations are calculated over a sufficiently long time interval to allow the laser pulses to pass and the dynamic optically coupled rate coefficients to go to zero, taken as five pulse widths before the center of the first pulse and after the last pulse. After this time the equations are reduced to a simple exponential relaxation of the population excited during the optically driven dynamics back to the ground or transient levels. These relaxations can be analytically determined and the final product populations calculated directly once the results of the optically coupled transitions are computed.

With laser intensities of zero the dynamic rate equations for the PR levels (equations 5.5a-5.5e) and the PFR levels (equations 5.6a-5.6e) simplify to the following analyt-
an intermediate level population at time $T$ the dynamic rate equation solutions at the end of the optically coupled time interval. For solution. The initial conditions of these reduced equations are set by the populations of can be directly added to level 3, and level 4 can be bypassed without altering the final Here the transient levels 2 and 4 are removed, as any residual population of level 2 can be directly added to level 3, and level 4 can be bypassed without altering the final solution. The initial conditions of these reduced equations are set by the populations of the dynamic rate equation solutions at the end of the optically coupled time interval. For an intermediate level population at time $T_1$ of $[R_{11}, R_{31}, LR_{51}, F_{11}, F_{31}, LF_{51}]$ the post-exposure exponential dynamics and steady-state populations at long time are calculated to be:

\[
\begin{align*}
R_3(t) &= R_{31} \cdot e^{-(k_{3,1}^R + k_{3,5}^R)t} & \Rightarrow R_{3S.S.} = 0 \\
R_1(t) &= R_{11} + \frac{k_{3,1}^R}{k_{3,1}^R + k_{3,5}^R} R_{31} \left(1 - e^{-(k_{3,1}^R + k_{3,5}^R)t}\right) & \Rightarrow R_{1S.S.} = R_{11} + \frac{k_{3,1}^R}{k_{3,1}^R + k_{3,5}^R} R_{31} \\
LR_5(t) &= R_{51} + \frac{k_{3,5}^R}{k_{3,1}^R + k_{3,5}^R} R_{31} \left(1 - e^{-(k_{3,1}^R + k_{3,5}^R)t}\right) & \Rightarrow LR_{5S.S.} = R_{51} + \frac{k_{3,5}^R}{k_{3,1}^R + k_{3,5}^R} R_{31} \\
FR_3(t) &= F_{31} \cdot e^{-(k_{3,1}^F + k_{3,5}^F)t} & \Rightarrow F_{3S.S.} = 0 \\
FR_1(t) &= F_{11} + \frac{k_{3,1}^{FR}}{k_{3,1}^{FR} + k_{3,5}^{FR}} F_{31} \left(1 - e^{-(k_{3,1}^{FR} + k_{3,5}^{FR})t}\right) & \Rightarrow F_{1S.S.} = F_{11} + \frac{k_{3,1}^{FR}}{k_{3,1}^{FR} + k_{3,5}^{FR}} F_{31} \\
LF_5(t) &= F_{51} + \frac{k_{3,5}^{FR}}{k_{3,1}^{FR} + k_{3,5}^{FR}} F_{31} \left(1 - e^{-(k_{3,1}^{FR} + k_{3,5}^{FR})t}\right) & \Rightarrow LF_{5S.S.} = F_{51} + \frac{k_{3,5}^{FR}}{k_{3,1}^{FR} + k_{3,5}^{FR}} F_{31}
\end{align*}
\]

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The “long time” at which the system reaches these steady state intermediate populations is $\sim$-nanoseconds after excitation, which is orders of magnitude longer than the $\sim$ picosecond excited state relaxation/isomerization but still orders of magnitude shorter than the final LUMI-R/LUMI-F relaxation occurring over $\sim$ microseconds. Calculating the ground and transition state populations in this direct manner is much more rapid and reliable than running the ODE solver over the vastly different time-scales of the optically driven transitions and the subsequent post-exposure relaxation. In the final step of the simulated photoswitching reaction, the accumulated transition state population is transferred to the switch’s alternate ground state ($[P_R]_{\text{fin}} = R1_{S.S.} + LF5_{S.S.}$ and $[P_{FR}]_{\text{fin}} = FR_{S.S.} + LR5_{S.S.}$). This completes a single photoswitching reaction from a single dual-pulse excitation-depletion exposure iteration. Section 5.2.2 describes how this final product state population distribution can subsequently be used as a new initial population condition, allowing the simulated photo-reaction to be repeated, replicating the gradual transfer of larger populations over multiple exposures. This is representative of the experiments performed in Section 5.3, where the sample is exposed to many Excitation-Depletion pulse pairs in a single measurement, specified by the measurement time and the repetition rate of the laser, to iteratively accumulate a larger final product population.

For characterizing the effect of the depletion pulse in the selective quenching of the forward and reverse photoswitching reactions, it is useful to monitor not just the final product populations, but also the fraction of the population taking part in the forward and reverse reactions. To this end, we define the transformation of the initial state population distribution to the final product state populations in terms of a set of “characteristic coefficients”: the forward and reverse Yield Coefficients ($Y_{R,FR}$ & $Y_{FR,R}$) and the forward
and reverse Quenching Coefficients \((Q_R & Q_{\text{FR}})\).

\[
P_{R,f} = (1 - (1 - Q_R)Y_{R,FR}) P_{R,f} + ((1 - Q_{\text{FR}})Y_{\text{FR},R}) P_{\text{FR},i} \tag{5.10a}
\]

\[
P_{\text{FR},f} = ((1 - Q_R)Y_{R,FR}) P_{R,i} + (1 - (1 - Q_{\text{FR}})Y_{\text{FR},R}) P_{\text{FR},i} \tag{5.10b}
\]

Here, the per exposure yield in the forward direction, \(Y_{R,FR}\), is defined as the fraction of molecules transferred from \(P_R\) to \(P_{FR}\) by the excitation pulse alone and the forward quenching coefficient, \(Q_R\), is the relative change in forward yield brought on by the interaction with the depletion pulse; the corresponding reverse coefficients \(Y_{\text{FR},R}\) and \(Q_{\text{FR}}\) are defined for the reverse reaction in the same way. Importantly, these **characteristic coefficients** are independent of the population distribution of the sample, being exclusively related to the variable experimental control parameters used to define and describe the excitation and depletion laser pulse pair. In this way the **characteristic coefficients** serve as a link between the control parameters and the optogenetic switch product output. It is straightforward to see that the ratios of the steady-state isomerization product populations to the initial state populations are equal to the product of the yield and quench coefficients for the forward and reverse reactions, respectively:

\[
LR5_{S.S.}/R_{10} = (1 - (1 - Q_R)Y_{R,FR}) \tag{5.11a}
\]

\[
LF5_{S.S.}/FR_{10} = (1 - (1 - Q_{\text{FR}})Y_{\text{FR},R}) \tag{5.11b}
\]

This parallel will serve as a useful means to extract information about the transient intermediate level populations without requiring ultrafast absorption measurements during the experiments presented in Section 5.3. The Net Gain of \(P_{FR}\) for a single photoswitching event is the difference between the forward and reverse reaction products:

\[
\Delta P_{FR} = (1 - Q_R)Y_{R,FR} \cdot P_R - (1 - Q_{\text{FR}})Y_{\text{FR},R} \cdot P_{FR} \tag{5.12}
\]
To illustrate the output of the simulation, Figure 5.11 shows the un-depleted dynamic level populations of a system exposed to only the short-pulsed excitation. The system in Figure 5.11 is composed of an initial equal mixture of states, $[P_R]_{init.} = [P_{FR}]_{init.} = 0.5$ (this initial condition is arbitrary and chosen for these figures to show the dynamics of both states on the same scale). These dynamic level populations are plotted in the short, optically coupled time interval as well as the long, post-exposure relaxation time domain. At $t=0$ ps the $P_R$ and $P_{FR}$ populations of the system are exposed to the excitation pulse with 100 fs duration and 30 nm spectral bandwidth centered at 625 nm, the average power is 0.50 mW corresponding to a peak intensity of $18.69 \text{GW/cm}^2$. The temporal envelope of the excitation pulse is shown in the figure as the shaded Gaussian profile. It must be noted here that, for illustration purposes within this figure, the power of this excitation pulse is exceptionally high. To make the features of the level dynamics more distinguishable and to highlight the alteration of those dynamics achieved by the stimulated depletion quenching, the powers of the excitation and depletion pulses in Figures 5.11 and 5.12 are at the upper range of powers examined in this study. In Figure 5.11 the total excited state populations ($P_R^*=R_2+R_3$, $P_{FR}^*=FR_2+FR_3$) are plotted as the thicker, darker green trace to illustrate how the excited state mirrors the ground state bleach initially, as well as to illustrate how the excited population moves from the initial upper excited level 2 (red trace) to the vibrationally relaxed level 3 (thin green trace).

Looking at the optically coupled time domain in Figure 5.11 it is clear that, as measured in studies of the actual chromophore, the reverse isomerization reaction is much more rapid than the forward isomerization. The $P_{FR}$ excited state (FR3) has already peaked by $\sim 0.1$ ps after the excitation begins and by 1 ps has nearly completely converted to the LUMI-F transient product (LF5) or back to the ground state (FR1). In contrast, around 1 ps the $P_R$ excited state R3 is still growing from population stored in R2, and very little population has switched to the transient state LR5. The switch in the $P_R^*$ state does not fully isomerize to LR5 or return to the ground state R1 for over 100
Figure 5.11: Un-depleted dynamic populations of the five levels of each switch state ((a) $P_R$ and (b) $P_{FR}$) with the excitation pulse envelope (yellow area). In both (a) and (b) the laser excitation pulse has zero intensity by 0.5 ps (five pulse widths) and the remaining dynamics are described by exponential relaxation from the lower level of the excited state (level 3, green trace) to the transient product state (level 5, red trace) or back to the initial ground state (level 1, blue trace, by way of level 4, teal trace). The distinctions between figures (a) and (b) highlight the much faster dynamics of $P_{FR}$ compared to $P_R$. Excitation laser parameters associated with the figure are listed in the text and Table 5.2. For this excitation pulse, the single exposure Net Gain of $\Delta P_{FR} = +0.046$ or 9.1% of the initial $P_{FR}$ population.
Table 5.2: Collective laser parameter settings for simulating laser excitation and depletion pulses in Figures 5.11, 5.12, and 5.13. Average power \((\langle P \rangle)\), central wavelength \((\lambda_0)\) and spectral width \((\Delta \lambda)\), temporal duration \((\Delta t_{\text{width}})\) and excitation-depletion delay \((t_{\text{Ex,Dep}})\) used in the calculation of pulse intensities by Eq. 5.1 and Eq. 5.2. These laser pulse intensities are exceptionally high, and used here only for illustration purposes. It is shown later that similar results can be achieved with lower pulse intensities.

<table>
<thead>
<tr>
<th>Laser Pulse</th>
<th>(&lt;P&gt;(\text{mW}))</th>
<th>(\lambda_0) (nm)</th>
<th>(\Delta \lambda) (nm)</th>
<th>(\Delta t_{\text{width}}) (ps)</th>
<th>(t_{\text{Ex,Dep}}) (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation</td>
<td>0.50</td>
<td>625</td>
<td>30</td>
<td>0.10</td>
<td>n/a</td>
</tr>
<tr>
<td>Deplet. 775nm</td>
<td>10.0</td>
<td>775</td>
<td>10</td>
<td>0.140</td>
<td>0.080</td>
</tr>
<tr>
<td>Deplet. 835nm</td>
<td>10.0</td>
<td>835</td>
<td>10</td>
<td>0.140</td>
<td>0.080</td>
</tr>
</tbody>
</table>

ps. The steady state level populations, as well as the final product state populations and forward and reverse Yield coefficients are shown on the figure beside each graph. For the excitation only exposure in Figure 5.11 the Quenching coefficients are zero and the Net Gain is \(\Delta P_{FR} = 0.0456\) or 9.126%.

The ultrafast dynamics of the \(P_{FR}\) photo-isomerization forces us to keep the depletion pulse short and very close to the excitation pulse to quench the reaction before it forms the transient LUMI-F product. Rather than being a preventative factor, these distinct dynamics allow us to entirely deplete the \(P_{FR^*}\) excited state before the excited \(P_{R^*}\) population has fully relaxed from the initial excitation point. This gives a window to halt the reverse reaction without fully stopping the forward reaction. The shorter interaction time of the depletion pulse limits the amount of stimulated depletion possible per exposure, restricting the maximum Quenching coefficient; but by cycling many exposures this advantage can drive the equilibrium in favor of the \(P_{FR}\) photo-product.

Figure 5.12 shows the altered dynamics after excited state quenching at two different depletion wavelengths. To simplify the graphs, the figure shows the summed populations of the levels to represent the populations of the ground states \((R1 + R4 = P_R, FR1 + FR4 = P_{FR})\), excited states \((R2 + R3 = P_{R^*}, FR2 + FR3 = P_{FR^*})\), and intermediate product states \((LR5=LR, LF5=LF)\). The excitation pulse and initial \(P_R\) and \(P_{FR}\) populations are the same as used in Figure 5.11, and the un-depleted level dynamics
Figure 5.12: Comparing depleted dynamics of the $P_R$ (a) and $P_{FR}$ (b) states at two depletion wavelengths. The ground-state bleach ($-\Delta P_R, -\Delta P_{FR}$), excited state ($P_R^*, P_{FR}^*$), and transient product (LR, LF) populations of each state are plotted along with the excitation and depletion pulse envelopes (yellow and pink, respectively). The forward and reverse Yield and Quenching coefficients, and the final net Gain of $P_{FR}$ are listed below the key for each exposure condition. For the depletion at 835 nm with $Q_{FR} \sim (2 \times Q_R)$, effectively halting the reverse reaction while allowing the forward reaction to proceed. This produces a lower Gain per exposure, but will shift the photoequilibrium. The depletion at 775 nm quenches forward and reverse reactions equally. Laser parameter settings are listed in the text and Table 5.2.
Table 5.3: Collective results from exposure of an initial 50/50 mixed state to a single excitation-depletion pulse pair. The final populations are listed in the first two columns, followed by the Net Gain of the $P_{FR}$ state. The intermediate steady-state populations of the transient isomerization products are next; followed by the Quenching coefficients, which give the percent change in the transient population with and without depletion. The forward/reverse Yield coefficients from the excitation pulse for all conditions are $Y_{R,FR} = 14.83\%$ and $Y_{FR,R} = 5.703\%$, respectively.

<table>
<thead>
<tr>
<th>Exposure Condition</th>
<th>Final $P_R$</th>
<th>Final $P_{FR}$</th>
<th>Net Gain $\Delta P_{FR}$</th>
<th>LUMI-R ($LR5_{S.S.}$)</th>
<th>LUMI-F ($LF5_{S.S.}$)</th>
<th>Forward Quench $Q_R$</th>
<th>Reverse Quench $Q_{FR}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-Depleted</td>
<td>0.454</td>
<td>0.546</td>
<td>9.1%</td>
<td>.0742</td>
<td>.0285</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Deplet.775nm</td>
<td>0.481</td>
<td>0.519</td>
<td>3.7%</td>
<td>.0306</td>
<td>.019</td>
<td>58.7%</td>
<td>58.2%</td>
</tr>
<tr>
<td>Deplet.835nm</td>
<td>0.461</td>
<td>0.539</td>
<td>7.7%</td>
<td>.0438</td>
<td>.0052</td>
<td>41.0%</td>
<td>81.9%</td>
</tr>
</tbody>
</table>

The forward and reverse Yield and Quench coefficients and the $P_{FR}$ Net Gain are shown on Figure 5.12 by the key for each exposure condition. These values, as well as the final populations of the $P_R$ and $P_{FR}$ states, and the steady-state populations of the LUMI-R and LUMI-F transient states are collected in Table 5.3. The final $P_R$ and $P_{FR}$ populations include the transfer of the accumulated population of the transient states to their final product ground states. The laser parameters associated with these results are collected in Table 5.2 for reference.
Quenching of the photo-isomerization is achieved at both depletion wavelengths for both states, as demonstrated by the drop in transient product states (levels LR5 and LF5, red in both graphs in Figure 5.12) and recovery of the initial ground states (levels R1 and FR1, blue in both graphs). Looking at the values in Table 5.3, we see that SDQ lowers the Net Gain ($\Delta P_{FR}$) per exposure for both depletion pulses, which would seem to run counter to our objective. However, we will see that the $\Delta P_{FR}$ per exposure is less consequential than generating a significant difference between the Forward and Reverse Quenching Coefficients, $Q_{FR} \gg Q_R$. The shorter wavelength depletion pulse ($\lambda_{0,D} = 780$ nm) quenches both $P_R^*$ and $P_{FR}^*$ excited states equally, $Q_{FR} \simeq Q_R$. This only slows the overall reaction, giving no selective control between the forward and reverse reaction directions. The longer wavelength depletion pulse ($\lambda_{0,D} = 835$ nm) nearly completely depletes the $P_{FR}^*$ excited state before much transient product can form, while the $P_R^*$ excited state retains some population that continues the forward reaction. This results in a lower Net Gain per exposure than the un-depleted excitation, $\Delta P_{FR} = 7.72\% < 9.13\%$, but nearly no loss of $P_{FR}$ to the reverse reaction. It will be shown next in Section 5.2.2 that this shift in the balance of the forward and reverse reactions enhances the maximum $P_{FR}$ population achieved at photoequilibrium over multiple exposures.

These single exposure simulations show that with moderate laser parameters attainable experimentally it is reasonable to expect depletion of the excited state of the PCB chromophore in Cph8 before photo-isomerization occurs, thereby quenching the ($P_{FR} \rightarrow P_R$) reverse photoswitching reaction. The simulations further show that the extremely rapid dynamics of the $P_{FR} \rightarrow P_R$ photo-isomerization, rather than being a hindrance to control, allows selective depletion of the $P_{FR}^*$ excited state without complete depletion of the $P_R^*$ excited state, enabling favorable quenching of the reverse $P_{FR} \rightarrow P_R$ photoswitching and relative enhancement of the forward $P_R \rightarrow P_{FR}$ reaction. Next, simulations of the system exposed to multiple repeating laser exposures will show that the SDQ enhancement shifts the photoequilibrium, greatly increasing the dynamic range of the switch.
5.2.2 Simulating Multiple Exposures with the Optical Transformation Matrix

The simulations of the last section determine the final population of a system \( (P_{R,f}, P_{FR,f}) \) after exposing an initial population \( (P_{R,i}, P_{FR,i}) \) exposed to a single Excitation-Depletion pulse sequence, defined by a set of laser control parameters, by solving a set of differential rate equations 5.5a-5.6e. To determine the results of multiple exposures, rather than repeatedly solving the ODEs with new initial conditions at each iteration (which is not only slow, but also prone to calculation errors due to round-off approximations and inaccuracies of the ODE solver) it is more convenient to represent the transfer of the population of the system from an initial state to a final state as a linear transformation of a vector of state populations by an \emph{Optical Transformation Matrix} (OTM) \( T \):

\[
\mathbf{P}_f = \begin{bmatrix} P_{R,f} \\ P_{FR,f} \end{bmatrix} = \begin{bmatrix} T_{1,1} & T_{1,2} \\ T_{2,1} & T_{2,2} \end{bmatrix} \cdot \begin{bmatrix} P_{R,i} \\ P_{FR,i} \end{bmatrix} = T \cdot \mathbf{P}_i.
\] (5.13)

The introduction of the OTM and its identification in the laboratory (reported in Section 5.3.4) is a key feature of the experiments in this work. The elements of the OTM are independent of the initial population distribution, as they are defined solely by the laser control parameters. Because of the complexity of the interactions, it is prohibitively difficult to directly calculate the matrix elements from the optical response functions of the molecule and laser parameters. To compute the values of the matrix elements we simulate a single iteration of the transformation using the rate equation ODEs, as described in Section 5.2.1, for two particular initial conditions and then solve for the matrix elements from the relationship between the initial and final populations. This reduces the number of times we must solve the rate equation system of ODEs from hundreds to just two. Similarly, in the experiments of Section 5.3 the OTM formalism allows us to extrapolate to the final equilibrium population distribution after measuring a smaller number of laser exposures. In this way we can measure the SDQ enhancement of a larger laser control
space, without having to wait for each permutation of the control parameters to reach photoequilibrium. In the simulations, we are able to solve the rate equations using the two initial conditions \[ \begin{bmatrix} 1 \\ 0 \end{bmatrix} \] and \[ \begin{bmatrix} 0 \\ 1 \end{bmatrix} \], which provides the matrix elements directly:

\[
T \cdot \begin{bmatrix} 1 \\ 0 \end{bmatrix} = \begin{bmatrix} T_{1,1} \\ T_{2,1} \end{bmatrix}, \quad \text{and} \quad T \cdot \begin{bmatrix} 0 \\ 1 \end{bmatrix} = \begin{bmatrix} T_{1,2} \\ T_{2,2} \end{bmatrix}
\]

highlighting the practical definition of the OTM elements: the off-diagonal elements \( T_{1,2} \) and \( T_{2,1} \) are the fractional gain per exposure between the two states. We could also refer to the definition of the characteristic coefficients in Equation 5.10 and write the OTM relation as:

\[
\begin{bmatrix} P_{R,f} \\ P_{FR,f} \end{bmatrix} = \begin{bmatrix} (1 - (1 - Q_R)Y_{R,FR}) & (1 - Q_{FR})Y_{FR,R} \\ (1 - Q_R)Y_{R,FR} & (1 - (1 - Q_{FR})Y_{FR,R}) \end{bmatrix} \begin{bmatrix} P_{R,i} \\ P_{FR,i} \end{bmatrix}
\]

(5.15)

To calculate the transfer of population from an arbitrary state distribution over a series of exposures the initial population vector is simply multiplied by, \( T^N \), the OTM raised to the number of exposures. This iterative transfer of population over many laser exposures is possible because the population distribution of the system does not change between iterations when the period between laser pulses is less than the dark-relaxation time of the switch (minutes to hours for Cph8 [52]). This gradual population transfer is plotted in Figure 5.13. The system begins in the pure \( P_R \) state \( \begin{bmatrix} 1 \\ 0 \end{bmatrix} \) and is exposed to three different exposure conditions: un-depleted single-pulse excitation, and two sets of sequential excitation-depletion exposures with a depletion pulse centered at 775 nm and 830 nm. The laser parameters for Figure 5.13, collected in Table 5.2, are the same as those used to plot Figures 5.12 and 5.11. The photoequilibrium threshold (i.e., the maximum \( P_{FR} \) or minimum \( P_R \) population) under each exposure condition is annotated on the figure. Also noted are the forward and reverse Yield associated with the excitation pulse and the forward and reverse Quenching coefficients for each depletion pulse wavelength. When the sample is exposed to the excitation-depletion pulse pair, with the depletion pulse cen-
Figure 5.13: Multi-exposure photo-isomerization yield with an un-depleted linear exposure, and an excitation-depletion pulse sequence exposure at two separate depletion wavelengths. In the first few exposures the faster reaction rate of the un-depleted exposure enables greater $P_{FR}$ generation, but after several exposures the quenching enhancement allows the sample exposed to the depletion at 830 nm to overcome the linear equilibrium limit and eventually reach a new equilibrium at $\sim90.2\%$. Laser parameters are listed in the text.

tered at 775 nm, the depletion cross-section is similar for both states of the chromophore, and the quenching parameters of the forward and reverse reaction are nearly identical ($Q_{R,FR} = 58.7\%, Q_{FR,R} = 58.2\%$). As a result, the depletion produces a small increase in the final equilibrium ($74.63\% P_{FR} 25.37\% P_{R}$); but mainly serves to slow the reaction, increasing the number of exposures to reach 90% of the equilibrium threshold ($N_{DEC}$) from 11 to 28 exposures. When the central wavelength of the depletion pulse is 835 nm the photoswitching reaction continues rising past the linear threshold to a new enhanced equilibrium. Initially, the slower reaction rate of the selectively quenched photoswitching produces less $P_{FR}$ than the standard linear excitation; however, after $\sim20$ exposures the quenched photoswitching reaction surpasses the cross-talk limited linear excitation threshold, achieving the final product population of 90.25% $P_{FR}$ and 9.75% $P_{R}$. This
selective depletion significantly enhances the final yield of the desired \( P_{FR} \) product and decreases the undesired \( P_R \) product by a factor of 4 compared to the linear exposure limit.

Another feature of the iterative mapping formulation of the photoswitching process is that it is possible to solve for the associated eigenvectors and eigenvalues of the OTM:

\[
\mathbf{T} \cdot \mathbf{v}_n = \lambda_n \mathbf{v}_n
\]  

(5.16)

which hold valuable information. We know from the physics of the photoswitching reaction that one of the eigensolutions will have an eigenvalue of 1 and be associated with the equilibrium state population, because once equilibrium is reached subsequent exposures no longer alter the population, so \( \mathbf{T}^N \cdot \mathbf{v}_{eq} = 1 \cdot \mathbf{v}_{eq} \). The set of eigenvectors of a matrix are linearly independent and span the associated space, meaning that any initial population vector can be expressed in this eigenbasis:

\[
\mathbf{P} = \begin{bmatrix} P_R \\ P_{FR} \end{bmatrix} = c_{eq} \begin{bmatrix} R_{eq} \\ F_{eq} \end{bmatrix} + c_{tr} \begin{bmatrix} R_{tr} \\ F_{tr} \end{bmatrix}
\]

(5.17)

and application of \( \mathbf{T} \) to the population vector becomes simply:

\[
\mathbf{T} \cdot \mathbf{P} = 1 \cdot c_{eq} \begin{bmatrix} R_{eq} \\ F_{eq} \end{bmatrix} + \lambda_{tr} \cdot c_{tr} \begin{bmatrix} R_{tr} \\ F_{tr} \end{bmatrix}
\]

(5.18)

In this form it is clear that the second eigensolution is associated with the population that is transferred between states to take the initial population vector closer to the equilibrium eigenvector with each iteration of the transform. The second eigenvalue \( \lambda_{tr} \) is related to the number of exposures it takes to reach the equilibrium state. From this eigenvalue we define the Decimation Count, \( N_{Dec} \), as the number of exposures necessary to reduce the transitional population to less than 10% of its initial value:

\[
N_{Dec} = \frac{-1}{\log_{10}(\lambda_{tr})}
\]

(5.19)
The decimation count is a useful metric of the effectiveness of a set of laser exposure parameters: a pulse pair that achieves a final equilibrium threshold of $100\% P_{FR}$ and $0\% P_R$ but requires infinitely many pulses to reach this equilibrium is not practically useful.

Knowledge of the eigensolutions of the OTM provide a direct, simple means to calculate the photoequilibrium threshold and efficacy of a set of laser control parameters without having to calculate or measure the photoswitching product populations over many iterations until the system actually reaches the photoequilibrium.

5.2.3 Parameter Space Survey Results

With the methods of calculating the photoswitching reaction product populations and final photoequilibrium threshold established in the previous sections, we survey the reduced space of laser control parameters that can be experimentally varied without complex pulse shaping to determine the dependence of the SDQ enhancement of the photoswitching reaction on these parameters independently and in concert. Except where otherwise noted, the following surveys are conducted around a fixed starting point in the parameter space specified in Table 5.4. The excitation and depletion pulse pair described in the table produces the highest SDQ enhancement accessible by varying the chosen control parameters, reaching an enhanced equilibrium population of $> 90\% P_{FR}$ and $< 10\% P_R$ with powers, spectra, and pulse durations accessible with the laser sources in our experimental facilities. Increasing the pulse peak intensities can enable higher equilibrium dynamic range, however this is achieved at the expense of slower reactions taking much longer to reach equilibrium, as shown later in Figure 5.17. Above an excitation power of 1 mW or a depletion power of 10 mW increasing the pulse powers gives diminishing returns and enters an intensity range where the rate equation model used here will become increasingly inaccurate. While this location in the control space produces the best SDQ enhancement from a survey within this restricted parameter basis, it is emphasized that reaching full
Table 5.4: Best laser parameters for SDQ enhancement of Cph8 dynamic range based on a few-parameter survey of the control space. For this parameter set, SDQ enhanced the photoequilibrium to 90.25% $P_{FR}$ / 9.75% $P_R$. Increasing the pulse powers can always increase the dynamic ranges, though at the expense of slower reactions and higher decimation count, $N_{DEC}$. Fully optimized control of the switches to their maximum dynamic range will require more elaborate quantum models and proper shaping of the laser pulse pairs in SDQ.

Performance optimality will require more sophisticated quantum dynamics modelling and coherent pulse shaping guided by optimal feedback control algorithms in the laboratory to address and exploit the complex coherent dynamics of the system. The present results should be viewed as illustrative of the effectiveness of the SDQ mechanism for enhancing the dynamic range of the switch.

We first scan over a range of excitation pulse parameters with no Depletion pulse to confirm that the model behaves as expected in the single pulse, linear regime. In Figure 5.14 the central wavelength of the excitation pulse is varied to confirm that the photo-reaction fully resets the switch to the $P_R$ state under far-red illumination and reaches a photoequilibrium between $P_R$ and $P_{FR}$ under red illumination. For the measurements in the figure the excitation power was 0.20 mW, the pulse duration was 100 fs, and the spectral width was 30 nm while the central wavelength of the excitation spectrum was varied from 550 nm to 800 nm. For wavelengths longer than 725 nm the equilibrium population of the $P_R$ state is $[P_R]_{EQ} > 99\%$, and for wavelengths in the red (600 nm < $\lambda$ < 670 nm) the final photoequilibrium ranges from 60-75% $P_{FR}$. The photostatic equilibrium at shorter wavelengths is higher than measured for the Cph1 molecule (65-67%). Looking at the decimation count $N_{DEC}$ of the system (inset in the figure) we see that for $\lambda$ < 610 nm the exposures necessary to reach equilibrium begins rising rapidly, due to the diminished absorption cross-section at these shorter wavelengths. If we cap the number of exposures...
at $N=20$, the value where the decimation count starts to rise divergently, we see that the photoequilibrium rises to $\sim 65\% \ P_{FR}$ in the red spectral region before falling again as the molecule stops absorbing the shorter wavelength light. Because the absorption spectra of the chromophores is so much broader than the width of the excitation or depletion spectra varying the spectral width of the excitation pulse has little effect on the photoswitching behavior. The molecule still fully resets to $P_R$ under far-red illumination and reaches the same photoequilibrium under red illumination, increasing the spectral width only smears out the transition between these two regions of the molecular response, as shown in Figure 5.15. Varying the pulse temporal duration similarly has essentially no effect on the single-pulse photoswitching reaction. With the excitation pulse power fixed at 0.20 mW and the spectrum centered at 625 nm with a bandwidth of 30 nm, stretching the pulse from 20 fs to 2 ps changes the equilibrium population by $\sim 2\%$. Having confirmed that the model returns the expected results in the linear, single-pulse exposure conditions we can begin to test the dependence on the depletion pulse parameters.

The depletion pulse central wavelength was scanned over a series of values to find the optimal depletion spectrum to maximize the final $P_{FR}$ product yield and minimize the unwanted $P_R$ population. Figure 5.16 shows the dependence of the population as the depletion wavelength is scanned from 700-950 nm. The stimulated emission cross-section of both states is higher at shorter wavelengths, increasing the depletion quenching of the excited state, but reducing the selectivity. In addition, at $\lambda_{Dep} < 750$ nm the $P_{FR}$ ground state is also absorbing the depletion pulse at a non-trivial rate, initiating the reverse reaction we are attempting to halt. At longer wavelength there is an increased difference in the response of the states but lower overall depletion due to the falling cross-section. The maximum effective enhancement of the $P_{FR}$ state is found between 830 - 840 nm, with the enhanced photoequilibrium of $[P_{FR}]_{EQ} = 90.25\%$. Varying the spectral width of the depletion pulse has slightly more effect than varying the excitation spectral width, but again is the same as averaging over the features of the wavelength dependence.
Figure 5.14: Excitation wavelength dependence of photoswitching under the condition of single pulse, linear excitation. Far-red light (\( \lambda > 725 \text{nm} \)) resets the system to the pure \( P_R \) state, while red light (600 nm < \( \lambda \) < 670 nm) reaches a photoequilibrium between 60-75% \( P_{FR}/P_R \). At wavelengths with low absorption cross-section the final equilibrium population (dashed lines) becomes numerically unreliable as the excitation pulse interacts less with the molecule and the decimation count \( N_{DEC} \) (inset) rises rapidly. For this reason we cap the number of exposures at 20 and look as the behavior of the system (solid lines), which reach the expected photoequilibrium of 65% \( P_{FR} \).

Without the use of complex pulse shaping, the depletion spectrum should be kept as narrow as possible while still being broad enough to support the desired temporal pulse characteristics. This behavior will likely be different with optimally tailored, shaped pulse excitation-depletion. A spectrally broad depletion pulse with a spectral phase designed such that its instantaneous frequency was relatively narrow and followed the dynamically varying energy difference between an excited wavepacket and the ground state should be more effective, but can not be represented by the present model.

Next we measure the power dependence of the SDQ. The depletion pulse power is varied while the spectrum is fixed at the optimal wavelength of 833 nm with a spectral width of 10 nm, the excitation pulse is 0.20 mW, 30 nm bandwidth centered at 625
Figure 5.15: Varying the spectral width of the excitation pulse has little effect on the photoswitching reaction. The switch still resets under far-red illumination and comes to the same photoequilibrium under red illumination, but the transition between these two regions is less distinct as the excitation spectrum is broadened.

Figure 5.16: Dependence of the SDQ enhanced equilibrium population of the $P_R$ and $P_{FR}$ states on the depletion wavelength, under fixed excitation power, depletion power, and pulse durations and delays. The optimum depletion wavelength is approximately 833 nm, yielding an enhanced final $P_{FR}$ population of 90.25%.
nm, with a pulse duration of 100 fs. The depletion pulse duration is 140 fs and the excitation-depletion delay is 0.08 ps. Figure 5.17 shows the power dependence of the SDQ enhanced populations. Even at low depletion power there is an increase in $P_{FR}$ over the linear equilibrium of 70%. With these exposure parameters the SDQ enhanced $P_{FR}$ population passes 90% with a depletion pulse power of 6.5 mW. Within this model, the enhancement of the $P_{FR}$ state is likely not to reach 100% $P_{FR}$, as there will always be a residual reverse reaction to produce a trace of $P_R$. As the depletion power increases the final photoequilibrium is shifted to higher $P_{FR}$ population; however, the higher depletion powers also slow the photoswitching reaction, thereby increasing the number of exposures necessary to reach this higher equilibrium state, as shown in the chart of the $N_{DEC}$ inset in Figure 5.17. The maximum $P_{FR}$ population achievable with a depletion power of 10 mW is 92.5%; increasing the power to 50 mW this can be increased to 92.7% (this power is far above the acceptable power range for this model, this would not just cause damage to the molecule but filamentation and dielectric breakdown of the solution). This remark does not necessarily mean that more sophisticated models or experimental operating conditions which address the ultrafast coherent dynamics of the excited state will not be able to break through this new higher photoequilibrium threshold to come even closer to the 100% $P_{FR}$ state.

Finally we vary the temporal parameters of the depletion pulse. In general, increasing the depletion pulse duration (while maintaining the same instantaneous intensity) should increase the depletion of an excited state; the longer the depletion pulse interacts with the system the higher the probability it will trigger the transition to the ground state by stimulated emission. However, maximizing the depletion of the $P_{FR}^*$ state by lengthening the pulse will also lead to increased depletion of the slower to develop $P_R^*$ state, eliminating the selectivity of the quenching enhancement. The optimal pulse duration and delay will balance these opposing demands, interacting with the system as long as possible while still maintaining a selective enhancement. Beyond that, the gains of quenching the reverse
Figure 5.17: Dependence of the SDQ enhanced equilibrium population on the depletion pulse power; under fixed excitation power, spectrum, duration, and excitation-depletion delay. With these parameters the enhanced $P_{FR}$ population surpasses 90% with a depletion power of 6.5 mW. The inset shows that while increasing the power enhances the final equilibrium population, it also increases the decimation count $N_{DEC}$ by slowing the photoswitching reactions in both directions.

reaction are outweighed by the losses caused by preventing the forward reaction. To find the optimal depletion delay and pulse duration, these two parameters are scanned in concert at the optimal central wavelength of 833 nm with a depletion power of 6.5 mW. This 2-dimensional scan is shown in Figure 5.18. The peak of the surface occurs at a delay of 0.08 ps and pulse duration of 0.14 ps with an equilibrium $P_{FR}$ population of 90.25%.

It is interesting to note that significant quenching enhancement can be achieved with the depletion pulse arriving before the excitation pulse if the width of the depletion pulse is greater than the negative delay. This allows the depletion pulse to interact at high intensity with the excited $P_{FR}$ state as early as possible, without waiting for the leading edge of the depletion pulse to ramp up. This also helps to have the depletion pulse drop
Figure 5.18: 2-D scan of depletion pulse temporal parameters: Dependence of the $P_{FR}$ equilibrium population on the excitation-depletion pulse delay and depletion pulse duration. Depletion spectrum is set at the previously determined optimal value of 10 nm at 830 nm central frequency. The peak value of the enhanced $P_{FR}$ photoequilibrium is 90.35% at a delay of 0.08 ps and pulse duration of 0.14 ps.

away before the excited $P_{R}$ state fully develops. At higher power, the pulse can be moved to shorter or more negative delays, as the power on the trailing edge is high enough to overcome wasting the higher peak intensity before the population arrives at the lower level of the electronic excited state. Raising the power to 10 mW increases the equilibrium $P_{FR}$ population to 92.5%, but also shifts the optimal depletion pulse to 10 femtoseconds shorter delay and 25 fs longer duration to compensate. At a power of 10 mW it is possible to achieve higher than 90% $P_{FR}$ with zero delay pulses.
5.2.4 Summary of Rate Equation Model Simulations

The rate equation model simulations reveal that, using laser control fields achievable with the sources available to us, and in many laboratories, the SDQ technique should effectively quench the reverse photoswitching reaction and increase the dynamic range of the Cph8 optogenetic switch. Further, we developed the iterative mapping framework using the Optical Transformation Matrix (OTM). The OTM enables projecting forward to an arbitrary number of exposure iterations to compute the photoequilibrium population based on the determination of a small set of Characteristic Coefficients which can be calculated after simulating (or measuring) the response of a sample in a small number of initial state distributions.

The dependence of the SDQ photoequilibrium enhancement was simulated over a domain of laser control parameters, surveying the dependence on the power, temporal, and spectral control characteristics for the excitation and depletion pulses. These simulated results will later be compared to experimental measurements in Section 5.3.4. The parameter space survey, within the simplified model, revealed the optimal exposure conditions to maximize the dynamic range of the switch within a fixed number of exposures. These optimal parameters, collected in Table 5.4, increase the dynamic range to 90.25% $P_{FR}$ and 9.75% $P_R$. The kinetic model, which does not incorporate the coherence of the laser fields and only approximates the true chromophore excited-state dynamics, should likely be taken as a limiting worst-case approximation, as it leaves out the potential to generate coherent excited state wave-packets with favorable dynamics using optimally shaped ultrashort pulse excitation and depletion. The next section describes the design and construction of the experimental apparatus used to carry out the SDQ technique, guided by the predictions of the model.

The iterative mapping framework established in Section 5.2.2 is a powerful tool that greatly accelerates the survey of the high-dimensional control parameter space. The capa-
bility to calculate the photoequilibrium population from a small number of measurements gives the same information that would have taken hundreds of laser exposures to reach by waiting for the sample state population distribution to equilibrate. In the simulated parameter space surveys of this last section this speed enhancement is a matter of convenience, but the direct calculation of the photoequilibrium from the OTM makes the experimental parameter space surveys of the next section and future optimization experiments realistically feasible.

5.3 Experimental Control of photoswitching by Stimulated Depletion Quenching (SDQ)

The simulations presented in Section 5.2 predict that the SDQ mechanism effectively halts the reverse photoswitching reaction of the Cph8 optogenetic switch while allowing the forward photoswitching reaction to continue, shifting the photoequilibrium and increasing the maximal dynamic range accessible by the switch after many laser exposure iterations. In this section we experimentally utilize the SDQ technique to enhance the control of the Cph8 photoswitching reaction. These experiments determine the Optical Transformation Matrix (OTM) iterative map associated with a particular laser control parameter set by measuring a group of characteristic coefficients, the analog of the same process in the simulations, to calculate the final photoequilibrium populations after many of exposures to the associated laser control pulses. Further, we characterize the dependence of the SDQ enhancement on the experimentally variable laser control parameters, comparing the measured dependence to the results of the simulated parameter space survey of Section 5.2.3 and determining the most effective exposure conditions for maximizing the dynamic range of the switch. The results of these experiments, presented in Section 5.3.4, are in qualitative agreement with the results of the rate equation model (REM) simulations of the last section, and confirm the feasibility of the optimal control of the photoswitching reaction.
in Cph8 by the SDQ mechanism, laying the ground work for further experiments incorporating pulse shaping and closed-loop optimization to achieve higher fidelity control of optogenetic switches.

5.3.1 Experimental Apparatus

A diagram of the laser sources and optics for the generation, conditioning, and routing of the excitation and depletion pulses to the Sample Flow Circuit (SFC), where the Cph8 sample is stored and circulated for exposure and measurement, are presented in Figure 5.19. A more detailed diagram of the SFC is shown later in Figure 5.22. The primary laser source for this experiment is a Ti:Sapphire regenerative amplifier (Coherent Legend) generating high intensity, ultrashort-pulse 800 nm laser pulses of duration $\sim 35$ fs (50 nm spectral bandwidth) with average pulse energy 2.1 mJ/pulse and 1KHz repetition rate. This source beam is split evenly by a high damage threshold, low GVD 50:50 beam splitter to generate the excitation and depletion beams.
The excitation beam is generated in a Topaz-White Non-collinear Optical Parametric Amplifier, which is configured to generate pulses with energy of \(\sim 20 \mu J/\text{pulse}\) and spectral range from 550-780 nm, peaking at 580 nm. The NOPA beam is filtered with a 610 nm long-pass glass absorption filter and a 650nm short-pass interference filter angle tuned to give a maximum wavelength of 640 nm, producing a nearly constant intensity spectrum from 610 nm to 640 nm. This filtered beam is temporally compressed at the sample exposure site (after passing through all routing optics) by maximizing SHG bandwidth and intensity in a thin 100 \(\mu\text{m}\) thick \(\beta\)-BBO crystal. The pulse is compressed by adjusting an internal Bi-Prism compressor in the TOPASWhite NOPA.

The depletion beam is generated by spectrally filtering a portion of the Ti:Sapphire amplifier beam using short-pass and long-pass interference filters. The depletion quenching was analyzed at two wavelengths: 835 nm, corresponding to the peak depletion wavelength predicted by the REM simulations in Section 5.2.3, and 775 nm, corresponding to the shortest useable wavelength of the Ti:Sapphire amplifier. We compare the measured depletion quenching at these two wavelengths to the REM simulations. The longer wavelength depletion beam was generated using a 860 nm 40 nm wide band-pass interference filter angle-tuned to pick out the red edge of the Ti:Sapphire amplifier spectrum, producing a pulse with spectral width of 10 nm centered at 835 nm. The shorter wavelength depletion beam was similarly generated with with a 780 nm, 10 nm wide band-pass interference filter angle-tuned to be centered at 775 nm. The filtered depletion beam was then sent through a dual prism stretcher to adjust the pulse length. The depletion pulse is stretched to enhance the stimulated depletion transition efficiency.

The TOPAS-White NOPA is designed to maximize the possible bandwidth of the output and thereby produce the shortest pulsed output. To accomplish this, the device incorporates a grating-based pulse shaper on the supercontinuum seed beam that conditions the seed for amplification, uses cylindrical optics throughout to maximize beam energies without burning the nonlinear crystal, and employs “tilted-pulse beam mixing” [88] in the
amplification crystal. Tilted-pulse beam mixing compensates a degree of angular dispersion across the beams that are accumulated when the beams enter the mixing crystal and allows greater phase matching bandwidth (similar to the way in which a non-collinear OPA has greater phase matching bandwidth than a collinear OPA). These options increase the spectral range, minimum pulse width, and total power of the NOPA pulse; but, they do so at the expense of the spatial mode of the beam, which can be decidedly distorted at the output, as indicated in Figure 5.20. Because the non-linear optical effects exploited in the following experiments are very sensitive to the intensity distribution and beam quality we apply a spatial filter with a 100 \( \mu \)m pinhole. This cleans up the NOPA beam to a smooth Gaussian spot at the expense of laser power. The throughput of the spatial filter is typically \(~35\%\).

To ensure that the depletion spot size at the sample is much larger than the excitation spot (ensuring that the entire excited sample is uniformly exposed to the depletion pulse) there is an adjustable beam reducing telescope near the beam combiner in the depletion beam line. This telescope can be adjusted while monitoring the overlapped spots at the sample on a camera imaging the exposure spots to confirm proper coverage.
Both beams pass through adjustable delay lines to synchronize the pulses. A computer controlled micrometer driven delay stage is placed in the excitation beam line to introduce a precise delay between the excitation and depletion pulses for time-resolved studies. The computer controlled delay stage can vary the delay over 150 ps in 3 fs steps (25 mm travel in 0.5 μm steps), though most measurements described in the following sections are made between ±2 picoseconds of excitation/depletion synchronization.

The two beams are independently attenuated with neutral density filters to set the exposure intensity before being spatially combined on an 800 nm long-pass interference filter at 45deg to transmit the depletion beam and reflect the excitation beam, using close positioned steering mirrors to accurately align the overlap and direction of each beam. After the beams are combined they can be focused into the microchannel exposure cell, or to check their alignment and synchronization they can be redirected through alternate beam lines by a series of removable flip-up mirrors. A spatial overlap beam line sends the beams through multiple irises over a path length of more than 2 meters to ensure their collimation and collinearity in the near and far field. A temporal overlap beam line sends the beams through a thin β-BBO crystal (100 μm thick) for synchronization of the pulses by Sum Frequency Generation (SFG) as well as compression of each beam by maximization of Second Harmonic Generation (SHG) intensity and bandwidth. The β-BBO crystal must be very thin to maximize phase matching across the (considerable) spectral range of the two pulses. Once synchronized by SFG, the excitation-depletion delay is set by the computer controlled delay stage.

At this stage, once excitation and depletion pulses overlap in time and space, the beams are focused into the exposure cell by an achromatic doublet with focal length 150 mm (the lens and mirrors preceding the exposure cell are replicated in the synchronization beam line to compensate for dispersion effects). The focusing beams are reflected off a small silver coated turning prism mounted at the center of a 2 inch diameter high numerical aperture (NA) lens before the exposure cell. This lens images the microchannel cell and
Figure 5.21: Images of the microchannel exposure cell with the excitation and depletion beam spots imaged at the channel depth of the cell. The camera is a monochromatic CMOS chip, so the laser spot images were collected separately, then colored and layered on top of each other with photo-editing software to form the composite image above. The black lines through the spots show the edges of the 100 $\mu$m wide channel.

laser spots onto a video imaging system which monitors the sample and can be used for fine alignment of the focal spots. This high NA lens is also used to collect fluorescence from the microchannel cell in measurements of Stimulated Emission Depletion of Fluorescent Proteins used to align and characterize the instrument response function of the apparatus. The combined beams are focused onto the exposure cell and the cell is brought forward so that the excitation beam diameter is approximately 50% larger than the microchannel flow cell width and the depletion beam diameter is approximately 50% larger than the excitation spot. Images of the microchannel and focused beams from the video monitor are shown in Figure 5.21. The spot sizes of the beams are set such that all material flowing through the channel width is exposed to the laser excitation and depletion pulses with similar intensity and no portion at the sides of the flow cell is left unexposed.

A more detailed diagram of the Sample Flow Circuit (SFC) is presented in Figure 5.22. The protein sample circulates in a minimal volume continuously flowing fluid circuit to ensure each measurement is made on a fresh sample and to mitigate sample photo-damage. The SFC is driven by a piezoelectric micro-pump (Takasago fluidics) and consists of a
Figure 5.22: Diagram of SFC which circulates the solution containing the Cph8 protein. Laser exposure initiating the photo-isomerization reaction takes place in the microchannel exposure cell, a 100 µm wide microfluidic channel that flows the sample through the laser focus. Measurement of photo-reaction product yield is done in the absorption cell, a fluid filled optical waveguide allowing for long path (5 cm) measurements of low volume (12 µL) samples. Further description of system provided in the text.

The reservoir is under continuous far-red illumination by a ‘reset diode’ emitting 730 nm light (M730D2, Thorlabs) which maintains the Cph8 protein in the sample solution in an initial $P_R$ state. There is a white light source that shines on a transparent section of tubing connecting the micro-pump to the exposure cell. This actinic light source can be turned on to pre-switch a portion of the molecules before they reach the exposure cell, initializing the sample in a mixture of the $P_R$ and $P_{FR}$ states. This initial $P_{FR}$ population is related to the flow rate (exposure time) and lamp intensity, and can be variably set to
give an initial \((P_R, P_{FR})\) population up to \(\sim (55, 45)\%\). The initial state of the sample is maintained below the linear photoequilibrium of 65\% \(P_{FR}\) because the goal of the experiment is to determine the OTM map, which requires accurate measurement of the per-exposure yield and quenching coefficients. Near photoequilibrium the per-exposure yield is very small, and difficult to accurately measure.

The exposure cell is a 100 \(\mu\text{m}\) wide 300 \(\mu\text{m}\) deep microchannel flow cell (Translume Inc.). This narrow channel concentrates the sample into the small area of the laser focal spot, ensuring that the excitation beam completely covers the channel cross-section while still being focused tightly enough to drive the necessary optical transitions. This exposes the entire flowing volume to a consistent exposure dose set by the laser intensity and fluid flow rate. This experimental configuration also has similarities to lab-on-chip bio-circuits used in synthetic biology, which is a field making practical application of optogenetic switching to manipulate and harness cellular function.

The long path absorption cell is a micro-fluidic waveguide capillary cell (LWCC-M-50, WPI inc) that allows highly precise measurements of absorption spectra in low volumes of sample by using the analyzed liquid sample as the internal media in a liquid core optical fiber, keeping the light in contact with the sample by total internal reflection at the liquid-core interface and enabling long optical path lengths. The total internal volume of the absorption cell is 12 \(\mu\text{L}\). The sample is probed with a stabilized Tungsten white light lamp (Thorlabs SLS202) that provides a smooth, continuous spectrum well-suited to absorption measurements. The light source is stabilized by an electronic feedback circuit to provide \(<0.1\%\) optical power drift per hour for long term stability. The light source is coupled to the LWCC by a fiber optic cable, and the transmitted probe is coupled to a compact spectrometer (Ocean Optics MAYA4000) by another fiber optic cable. This system gives a simple, accurate, and rapid measurement of the sample absorption spectrum.
5.3.2 Data Collection and Initial Processing

Using the apparatus described in the last section, the sample is (i) circulated as absorption spectra (like those shown in Figure 5.23) are continuously measured, (ii) analyzed to extract the absolute concentration of the two conformational states of Cph8, (iii) saved by the computer, and (iv) indexed by their time of acquisition. As the laser exposure conditions are varied, the collected absorption spectra and relative photoswitching reaction product populations change in response, as shown later in Figure 5.24. Despite not having knowledge of the time dependent excited-state level populations, as we did in the simulations of Section 5.2.1, we are still able to extract useful information about the ultrafast interactions between the molecular switch and the excitation and depletion pulses. By varying the experimental laser control parameters and measuring the final photoswitching reaction product states under a series of six Exposure Conditions (described in Table 5.5) from the final product state populations we may calculate a set of six characteristic coefficients (described in Table 5.6). Just as in the simulations of Section 5.2.1 these characteristic coefficients are a series of reaction coefficients that quantify the effects of the excitation and depletion pulses in ways that facilitate analysis and allow us to build the OTM map to calculate the enhanced photoequilibrium populations.

Representative absorption measurements from two samples are shown in Figure 5.23(a), while Figure 5.23(b) shows the fitting of one of these absorption measurements to known reference spectra to extract the concentrations of the component states present in the sample. The two samples shown in Figure 5.23(a) include a sample in the pure $P_R$ state (red trace) under continuous illumination at 730 nm as well as a sample with a mixed population containing Cph8 in both $P_R$ and $P_{FR}$ states (orange trace) produced by white light illumination. Reset samples in the ‘pure’ $P_R$ state typically retain a small residual population of $1 - 3\% P_{FR}$. Backgrounds have been subtracted to place these two spectra on the same baseline.
Figure 5.23: (a) Representative absorption spectra of Cph8 taken in the absorption cell under typical experimental concentrations and conditions. This graph contains spectra from Cph8 in a ‘pure’ $P_R$ state and a mixed state of 71.5% $P_R$ and 28.5% $P_{FR}$. (b) The mixed state spectrum is fit to a linear combination of the reference spectra and a nonlinear background (BG). Dashed traces show the relative contribution of the $P_R$ and $P_{FR}$ states to the total absorption fit curve (black trace).

The measured absorption spectra are fit to a linear combination of absorption peaks and a third order polynomial background:

$$S(\lambda, c_i) = c_RS_R(\lambda) + c_{FR}S_{FR}(\lambda) + c_{R'}S_{R'}(\lambda) + c_WS_W(\lambda) + \sum_{n=0}^{3} c_n \cdot (\lambda - \lambda_0)^n \quad (5.20)$$

The four absorption peaks are the known reference spectra of Cph8 in the $P_R$ and $P_{FR}$ states ($S_R(\lambda)$ and $S_{FR}(\lambda)$) (measured independently and verified by comparison to ref [61]), a small water overtone peak at 970 nm ($S_W(\lambda)$), and a blue-shifted sub-population of $P_R$-Cph8 with a spectrum peaking at 655 nm that develops over the course of experiments ($S_{R'}(\lambda)$). This sub-population is discussed further below. This analytical function is fit to the data by an unconstrained least squared residual fitting algorithm. The reference spectra in Equation 5.20 are scaled to the known extinction coefficients found in the literature [61] and multiplied by the optical path length of the absorption cell (5 cm), so the fit coefficients $c_R$, $c_{R'}$, and $c_{FR}$ correspond to absolute concentrations.
of the component states of Cph8 in Molar units. The analytical fit to the spectrum of the mixed state sample is shown in Figure 5.23(b), the fit curve (thin black trace) is in good agreement with the measured absorption data (thicker orange trace). The spectra of the individual components of the mixture are also shown in the figure, superimposed on the calculated background curve (dashed curves). The calculated total Cph8 concentration is 2.21 µM, and the white light illumination has converted 28.5% of the sample to the $P_{FR}$ state, leaving 71.5% in the $P_R$ state.

When excited by the ultrashort laser pulses, the dynamics of the chromophore molecule – and subsequently the photoswitching reaction product distribution of the sample – are intimately coupled to the complex spectral phase and temporal field shape of the excitation and depletion pulses interacting with the system. The high-dimensional parameter space that characterizes these pulses is immense (especially when unconstrained shaping of the spectral/temporal phase of the pulses is employed to tailor the pulsed laser field profile, which is not employed at this stage of experimentation). In this initial feasibility study we limit our exploration of this vast control space to perform a reduced survey of the key control parameters that are expected to be most relevant to the photo-isomerization dynamics of the PCB molecule and the Cph8 system, paralleling the survey of simulated results performed in Section 5.2.3. This procedure lays the ground work for future experiments using optimally tailored pulse shaping to exploit the coherent ultrafast excited state dynamics of the switches.

The set of experimentally controlled parameters investigated include: the average power of the excitation and depletion beams ($\langle P_{Ex} \rangle$ and $\langle P_{Dp} \rangle$), the central wavelength and bandwidth of the excitation pulse spectrum ($\lambda_{Ex0}, \Delta \lambda_{Ex}$) and depletion pulse spectrum ($\lambda_{Dp0}, \Delta \lambda_{Dp}$), the laser pulses’ temporal widths ($\Delta t_{Ex}$ and $\Delta t_{Dp}$)\(^a\), the time delay

\(^a\)(as described in Section 5.3.1 pulse widths are set by an internal bi-prism pair inside the NOPA for the excitation pulse and the dual prism stretcher shown in Figure 5.19 for the depletion pulse, though there are plans to use coherent pulse shaping in future experiments to generate more elaborate complex tailored pulse shapes)
between the two pulses \( (\tau_{(Ex,Dp)}) \), and the sample flow rate \( (q) \), which (in conjunction with the laser repetition rate) defines the number of laser pulses the sample is exposed to as it passes through the laser focal spot in the microchannel exposure cell. Apart from the flow rate, these parameters are the same set used to define the simulated excitation and depletion pulses in Section 5.2.1. This set of experimental control parameters,

\[
[\langle P_{Ex} \rangle, \lambda_0^{Ex}, \Delta \lambda_{Ex}, \Delta t_{Ex}, \langle P_{Dp} \rangle, \lambda_0^{Dp}, \Delta \lambda_{Dp}, \Delta t_{Dp}, \tau_{(Ex,Dp)}, q],
\]

(5.21)
defines a single point in the high-dimensional parameter space to manipulate the molecular switch. The molecular response is related to all of these parameters collectively through coupled, complex relationships. In this experiment we begin to survey a portion of this parameter space, guided by the results of the rate equation model simulations of Section 5.2, which provided an initial starting point for these experimental measurements.

As in Section 5.2.1, we define the final product populations of the photoswitching reaction as a linear transformation from the initial state population distribution using a set of characteristic coefficients:

\[
P_{R,f} = (1 - D_R - (1 - Q_R)Y_{R,FR}) P_{R,i} + ((1 - Q_{FR})Y_{FR,R}) P_{FR,i} \tag{5.22a}
\]

\[
P_{FR,f} = ((1 - Q_R)Y_{R,FR}) P_{R,i} + (1 - D_F - (1 - Q_{FR})Y_{FR,R}) P_{FR,i} \tag{5.22b}
\]

The forward and reverse Yield and Quenching coefficients are defined as they were before in Equation 5.10 of Section 5.2.1. The per exposure yield in the forward direction, \( Y_{R,FR} \), is the fraction of molecules transferred from \( P_R \) to \( P_{FR} \) by the excitation pulse alone with no depletion and the forward quenching coefficient, \( Q_R \), is the relative change in forward yield brought on by the dual pulsed excitation-depletion sequence. The corresponding coefficients \( Y_{FR,R} \) and \( Q_{FR} \) are defined for the reverse reaction in the same way. There is an additional pair of experimental characteristic coefficients not present in the simulations.
of Section 5.2, $D_R$ and $D_{FR}$, associated with the loss of protein within the sample due to photo-damage by the high intensity laser pulses. Care was taken to minimize photo-damage by limiting peak laser intensities, but it is a loss channel that must be accounted for in the experimental measurements.

Because we do not have knowledge of the transient populations of the LUMI-R/LUMI-F intermediate product states as we did in the simulations of Section 5.2 we can not use these values to define the characteristic coefficients as we did there. Rather, to calculate these six unknown coefficients we measure the final product populations under series of six Exposure Conditions (described and collected in Table 5.5). The sample can be left to circulate with no laser exposure, measuring the initial population distribution in either the pure-$P_R$ (EC1, $(R_0/F_0)$) or mixed (EC4, $(R_{0M}/F_{0M})$) initial conditions. From either of these initial conditions the sample can be exposed to the excitation laser alone (EC2, $(R_{Ex}/F_{Ex})$;EC5, $(R_{MEx}/F_{MEx})$), providing the linear photoswitching reaction products, or the excitation-depletion pair (EC3, $(R_{ED}/F_{ED})$;EC6, $(R_{MED}/F_{MED})$), producing the non-linear SDQ-enhanced photoswitching reaction products. These six Exposure Conditions make up a “Full Data Set”, thereby producing sufficient measurements to calculate the full set of characteristic coefficients for a specified laser control parameter set. From the photoswitching reaction products starting in the pure $P_R$ condition we calculate the yield, quenching, and damage coefficients of the forward reaction. Using the final product states collected starting in the mixed condition and the calculated forward characteristic coefficients we can calculate the reverse yield, quenching, and damage coefficients. The set of six characteristic coefficients is collected in Table 5.6 along with their relation to the measured final product populations under the six Exposure Conditions.

Once these characteristic coefficients are calculated, we can form an Optical Transformation Matrix (OTM), just as we did in Equation 5.15 in Section 5.2.2:
<table>
<thead>
<tr>
<th>EC#</th>
<th>Initial State (µM)</th>
<th>Final State (µM)</th>
<th>Description of Exposure Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$[R_0 \ F_0]$</td>
<td></td>
<td>Pure $P_R$ state: Reservoir under continuous 730 nm diode illumination to hold switch in $P_R$ state. (typically 1 – 3% $P_{FR}$ remains).</td>
</tr>
<tr>
<td>2</td>
<td>$[R_0 \ F_0]$</td>
<td>$[R_{Ex} \ F_{Ex}]$</td>
<td>Linear Excitation: Sample exposed to excitation laser alone. Change in $P_{FR}$ gives forward yield ($Y_{R,FR}$), while difference in $P_{FR}$ produced and $P_{R}$ lost gives forward damage coefficient ($D_R$).</td>
</tr>
<tr>
<td>3</td>
<td>$[R_{ED} \ F_{ED}]$</td>
<td></td>
<td>Excitation+Depletion: Sample exposed to Excitation and Depletion pulse pair to excite and then quench forward reaction ($Q_R$). Power, spectrum, and timing of pulses are varied to map dependence of $Q_R$.</td>
</tr>
<tr>
<td>4</td>
<td>$[R_{M0} \ F_{M0}]$</td>
<td>$[R_{MEx} \ F_{MEx}]$</td>
<td>Mixed Initial State: Dual pre-illumination (730 nm diode+white lamp) prepares sample in mixed initial state. $[P_{FR}]_{init}$ dependent on lamp intensity and sample flow rate.</td>
</tr>
<tr>
<td>5</td>
<td>$[R_{M0} \ F_{M0}]$</td>
<td>$[R_{MEx} \ F_{MEx}]$</td>
<td>Mixed Linear Excitation: Sample with initial $P_{FR}$ population undergoes forward and reverse reaction. Using the calculated $Y_{R,FR}$ the reverse yield ($Y_{FR,R}$) can be extracted.</td>
</tr>
<tr>
<td>6</td>
<td>$[R_{MED} \ F_{MED}]$</td>
<td></td>
<td>Mixed Excitation+Depletion: Sample undergoing forward and reverse reaction is exposed to depletion pulse. Using the calculated $Y_{R,FR}$, $Y_{FR,R}$, &amp; $Q_R$, the Reverse Quenching Coeff. ($Q_{FR}$) can be extracted.</td>
</tr>
</tbody>
</table>

Table 5.5: Exposure conditions to calculate Characteristic Coefficients from measured final product states. The six exposure conditions defines a Full Data Series for a single control parameter set. The Full Data Series is required to calculate the full set of Characteristic Coefficients for that set of control parameters; though, useful information about individual characteristic coefficients can be extracted from partial data series.

\[
\begin{bmatrix}
  P_{R,f} \\
  P_{FR,f}
\end{bmatrix}
= \begin{bmatrix}
  (1 - (1 - Q_R)Y_{R,FR}) & (1 - Q_{FR})Y_{FR,R} \\
  (1 - Q_R)Y_{R,FR} & (1 - (1 - Q_{FR})Y_{FR,R})
\end{bmatrix}
\begin{bmatrix}
  P_{R,i} \\
  P_{FR,i}
\end{bmatrix}
\]

(5.23)

The damage coefficients are omitted from the OTM, as the inclusion of the loss term prevents calculation of the steady-state equilibrium solution from the eigensolutions (as no steady-state exists and the sample continuously loses population). Rather, accounting for the damage coefficients in the calculation of the other characteristic coefficients ensures their accuracy. With the experimentally measured OTM we can calculate the eigensolutions of the matrix, as we did in Section 5.2.2, to determine the projected photoequilibrium from the eigenvector with eigenvalue $\lambda_{eq} = 1$. Comparing the decimation
The Characteristic Coefficients relate the measured final photoswitching reaction products to the ultrafast interactions being used to control the process. The first and second columns give the name and symbols for each coefficient, while the last column describes the method of calculating the coefficient from the final product state populations measured under the exposure conditions in Table 5.5.

<table>
<thead>
<tr>
<th>Characteristic Coefficients</th>
<th>Symbol</th>
<th>Calculation from Measured Final Product Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Yield</td>
<td>$Y_{R,FR}$</td>
<td>$(F_{Ex} - F_0)/R_0$</td>
</tr>
<tr>
<td>Forward Damage</td>
<td>$D_R$</td>
<td>$1 - Y_{R,FR} - R_{Ex}/R_0$</td>
</tr>
<tr>
<td>Forward Quenching</td>
<td>$Q_R$</td>
<td>$1 - ((F_{ED} - F_0)/R_0)/Y_{R,FR}$</td>
</tr>
<tr>
<td>Reverse Yield</td>
<td>$Y_{FR,R}$</td>
<td>$R_{MEx} - R_{M0}(1 - Y_{R,FR} - D_R)/F_{M0}$</td>
</tr>
<tr>
<td>Reverse Damage</td>
<td>$D_{FR}$</td>
<td>$1 - F_{MEx} + R_{MEx} - (1 - D_R)R_{M0}/F_{M0}$</td>
</tr>
<tr>
<td>Reverse Quenching</td>
<td>$Q_{FR}$</td>
<td>$1 - R_{MED} - R_{M0}(1 - (1 - Q_R)Y_{R,FR} - D_R)/Y_{FR,R} \cdot F_{M0}$</td>
</tr>
</tbody>
</table>

Table 5.6: The Characteristic Coefficients relate the measured final photoswitching reaction products to the ultrafast interactions being used to control the process. The first and second columns give the name and symbols for each coefficient, while the last column describes the method of calculating the coefficient from the final product state populations measured under the exposure conditions in Table 5.5.

count, $N_{DEC}$, associated with the second eigenvalue of the OTM to the sample loss associated with the damage coefficient indicates the amount of sample lost to damage in the number of iterations it would take to reach the projected photoequilibrium. It is important to note that the calculation of the steady-state equilibrium population distribution from the OTM is independent of the rate equation model used in Section 5.2, and not limited by its approximations. The use of the transformation matrix is a fundamental consequence of the linear transformation of the sample from an initial distribution to a final distribution by an iterated forward and reverse transfer pathway on a conserved total population.

A Full Data Set is defined by measuring the final product concentrations under all six of the exposure conditions in Table 5.5 for a given control parameter set (see Figure 5.25). Each time one of the control parameters in the set is varied a Full Data Set is measured to extract the characteristic coefficients at that point in the parameter space landscape.
before moving on to the next point in the survey. The characteristic coefficients build on each other, with each requiring the value of the previous determined coefficient, thus making it critical that experimental conditions remain consistent throughout the measurement of each full data series. In the current construction of the apparatus it is still difficult to maintain constant and fully reproducible experimental conditions, limiting the quantitative precision and reproducibility of calculated results measured at different times and on different samples. However, the experimental conditions are sufficiently stable to measure several Full Data Sets under consistent conditions, and the relationships between the characteristic coefficients calculated from these measurements show clear conserved trends across all the measurements.

5.3.3 Representative Data Series

In Figure 5.24 we present data collected from a portion of a typical measurement series, taken over the course of one hour. The absolute concentration of the states of Cph8 in the sample are continuously calculated from the measured absorption spectra as the sample circulates under varied laser illumination conditions, designated in the figure by the Exposure Condition number corresponding to Table 5.5. The sample is initially in a pure $P_R$ configuration, with total Cph8 concentration of 1.62 µM. Between 28-43 minutes in the figure the sample is in a mixed initial state ($R_{M0}$, $F_{M0}$) with a non-zero $P_{FR}$ population. Over the course of the measurement series the sample is exposed to the laser sources to initiate the photoswitching reaction by either a linear, single-pulse excitation or a non-linear, dual-pulse excitation-depletion pair. The periods under excitation-only exposure are highlighted in yellow, while the periods exposed to the excitation-depletion pair are highlighted blue.

In the figure we mark the concentrations of two spectrally distinct sub-populations of the $P_R$ state, $R_{670}$ and $R_{655}$, named by the peak of their absorption spectra. In all
Figure 5.24: Charting of the measured concentration of the $P_R$ and $P_{FR}$ states of Cph8 protein over the course of several measurement series under varied exposure conditions, labeled by number corresponding to Table 5.5. Regions highlighted yellow are exposed to the excitation pulse alone, regions highlighted blue are exposed to the excitation-depletion pulse combination, and non-highlighted regions have no laser exposure. Two spectrally distinct sub-populations of $P_R$ are charted in the figure, $R_{655}$ and $R_{670}$, discussed in the text.

measurements the Cph8 sample is observed to be initially free of the $R_{655}$ sup-population, which gradually develops after extended periods circulating in the experimental apparatus. The conversion of the sample is observed under complete dark circulation with only intermittent observation by the probe to minimize exposure to light, indicating that the effect is not due to photo-damage of the protein. A similar blue-shifted ground state sub-population is observed by Larsen et.al. [79], as discussed in Section 5.1.3. The $R_{655}$ sub-population participates in the photoswitching with the same yield as the initial $R_{670}$ population. Typically, attempts are made to complete the measurement series before significant populations of $R_{655}$ develop.
The first feature of note in the figure is the steady decrease of the total concentration of Cph8 protein in the sample (orange trace in Figure 5.24). Over the course of the measurement series the total protein concentration falls 14%. This decline in total protein concentration is seen under all conditions of circulation, including complete darkness, and is unrelated to the laser exposure. The source and nature of the sample degradation are not investigated in this work, though there are two suspected causes. The first is aggregation of the protein complexes, which then fall out of solution and are trapped in the sample reservoir, reducing the overall concentration. The second is denaturation of the protein due to age, handling, or environmental conditions. The development of the \( R_{655} \) sub-species does not appear to be associated with the protein loss, as the two effects develop with a different time dependence and are believed to be unrelated processes. Regardless of the source of the non-optical protein loss, the trend is linear and consistent across measurement series, and can be fit and removed from the measurements without adversely affecting the data. The removal of this gradual non-optical loss trend is the first step in the processing of a measurement series.

Next, the populations of the \( P_R \) and \( P_{FR} \) states are averaged over a fixed time period under each exposure condition after the measured concentration levels have stabilized. When there is a change in exposure condition it takes time for the newly exposed sample to flow through the SFC and fill the absorption cell. The final product concentrations associated with that exposure condition can not be measured until the new sample fully replaces the old. When the system stabilizes and the concentrations of the \( P_R \) and \( P_{FR} \) states are measured for one exposure condition, the condition can be switched to another in the series and a new measurement commences. Once a Full Data Set has been measured under a given set of experimental control parameters, the characteristic coefficients of that parameter set can be calculated and the experimental control parameters can be varied to the next point in the survey. In practice, the collection of a Full Data Set for a given laser parameter setting need not be measured consecutively or in any particular order, so
Figure 5.25: Processed measurement series: The relative decrease of $P_R$, $(1 - R/C_{Tot})$, overlaps with the relative increase of $P_{FR}$, $(F/C_{Tot})$, where $C_{Tot}$ is the total Cph8 concentration, demonstrating ‘clean’ switching between the two states with little loss to photo-damage. As in Figure 5.24, Exposure Conditions are labeled corresponding to Table 5.5: yellow regions are exposed to the excitation pulse, blue regions are exposed to the excitation-depletion pulse combination, and non-highlighted regions have no laser exposure. The bracketed region from 27-60 minutes defines one Full Data Series collected at $\langle P_{Ex} \rangle = 600\mu W$, $\langle P_{Dep} \rangle = 4.5 mW$, and $\tau_{Ex,Dep} = 0.5 ps$ (full control parameters listed in the text).

Processed data from the same measurement series shown in Figure 5.24 are presented in Figure 5.25, where the non-optical loss trend has been fit and removed and the $P_R$ and $P_{FR}$ state populations plotted as ratios of the total Cph8 concentration. The markers
indicate the average state population at each specific exposure condition indexed by the experiment time at which the exposed sample travelled from the exposure cell to the absorption cell and the measured state population had stabilized. The standard deviation of the measured concentration over the averaged interval in each exposure condition is measured and used to calculate the error of each characteristic coefficient by propagation of uncertainty. The errors in the measured population are smaller than the marker boxes, but grow as they are propagated through the multiple calculations necessary to determine the characteristic coefficients, the OTM, and the projected photoequilibrium population. In the processed measurement series, the overlap of the $P_{FR}$ population increase with the $P_R$ population decrease clearly illustrates the low damage coefficients. The larger net production of $P_{FR}$ per laser exposure at the same power when starting from zero initial concentration (Exposure Condition 2, EC2), compared to when the initial concentration of $P_{FR}$ is higher (EC5) illustrates that in EC5 the forward and reverse photo-reactions are taking place simultaneously and the sample is near photoequilibrium. The regions in Figure 5.25 when the sample was exposed to the excitation-depletion pulse combination are highlighted blue (EC3 & EC6). Because the number of laser pulse iterations during the exposure is kept low to more rapidly scan a larger domain of laser control parameters, the Excitation+Depletion pulse combination causes the $P_{FR}$ population to decrease, rather than increase. Referring back to Figure 5.13 in Section 5.2.2, this is the expected result of the SDQ mechanism when the number of laser pulse iterations per exposure is less than the number required to surpass the linear excitation. This occurs because the SDQ mechanism functions by slowing down both the forward and reverse reactions, but slowing the reverse reaction more, enabling a greater final photoequilibrium while requiring more laser pulse iterations to reach it. Because of the information we extract from the iterative mapping using the Optical Transformation Matrix we are able to calculate the photoequilibrium of the laser exposure without waiting for this extended period.
The bracketed region between 27-60 minutes encloses a single Full Data Series of measurements taken at the following experimental control parameter settings:

\[
\langle P_{Ex} \rangle, \langle P_{Dep} \rangle, (\lambda_0^{Ex}, \Delta \lambda_{Ex}), (\lambda_0^{Dep}, \Delta \lambda_{Dep}), \tau_{Ex,Dep} = [600 \ \mu W, 4.5 \ mW, (625 \ nm, 40 \ nm), (835 \ nm, 10 \ nm), 0.50 \ ps].
\]

The two partial data series at earlier times are taken at different excitation and depletion powers. The first partial data series is taken at \( \langle P_{Ex} \rangle = 400 \mu W, \langle P_{Dep} \rangle = 1.5mW, \) and \( \tau_{Ex,Dep} = 0.33ps, \) and the second partial data series at \( \langle P_{Ex} \rangle = 600\mu W, \langle P_{Dep} \rangle = 1.5mW, \) and \( \tau_{Ex,Dep} = 0.5ps. \) Though these measurements can not be used to calculate the full set of characteristic coefficients, they are useful for showing trends in the power and delay dependence of the forward Yield and forward Quenching coefficients. From the Full Data Series we can calculate the full set of characteristic coefficients: 

\[
[Y_{R,FR}, Q_{R}, D_{R}] = [0.1160 \pm 0.0035, 0.29 \pm 0.02, 0.0013 \pm 0.0006],
\]

and 

\[
[Y_{FR,R}, Q_{F}, D_{F}] = [0.1010 \pm 0.003, 0.493 \pm 0.055, 0.0009 \pm 0.0009].
\]

The errors in the calculated characteristic coefficients derive from propagation of the standard deviation of the measured sample concentrations through the equations for each coefficient (see Table 5.6).

We see that the forward and reverse reaction Yield coefficients, \( Y_{R,FR} \) and \( Y_{FR,R} \), have similar magnitude with an advantage to the forward reaction, as expected at this excitation wavelength. The damage coefficients are very low, which was common throughout the experiments. As stated earlier, efforts were taken to limit photo-damage and significant levels of damage (i.e., defined as a damage coefficient higher than \( \sim 1.5\% \)) were only seen in samples compromised by age or environment (stored for several weeks at 4°C or overnight at room temperature). In extended measurement series lasting >10 hours the photo-damage parameter would increase over the course of the experiment, but typically remained under a few percent. The Quenching coefficient of the reverse reaction, \( Q_{FR} \), is 1.7 times greater than the forward Quenching coefficient, \( Q_{R} \), a significant enhancement factor. Using the calculated characteristic coefficients, we construct the Optical Transformation Matrix and calculate the equilibrium product population from its eigen-
solutions. Analytically calculating the errors of the eigensolutions of a matrix from the errors of its components is prohibitively complicated, so the eigensolution errors were numerically computed by statistics. We calculated the eigensolutions of 1000 matrixes with components defined by the measured characteristic coefficients with gaussian variations of magnitude given by the associated coefficient errors. The standard deviation of these eigensolutions gives the error on the photoequilibrium. The projected photoequilibrium threshold of the linear excitation is $[P_{FR}\text{lin}]_{EQ} = 0.54 \pm 0.09$. Based on the Quenching coefficients calculated from the measurements of the full data series in Figure 5.25 the projected SDQ-enhanced equilibrium threshold is $[P_{FR}\text{SDQ}]_{EQ} = 0.62 \pm 0.10$. This is an increase of the maximum $P_{FR}$ population by 13% or a decrease of the minimum residual $P_R$ population by 21%, a sizeable enhancement.

In the following section we present the calculated characteristic coefficients measured over a range of laser parameter settings and discuss the functional dependence of the photoswitching reaction on the experimental control parameters.

### 5.3.4 Results and Analysis

The forward Yield ($Y_{R,FR}$) is the most straight-forward of the characteristic coefficients to calculate from the experimental measurements. It can be computed after measuring just two exposure conditions, requiring only the change in $P_{FR}$ concentration after exposure of the initially pure $P_R$ state to the excitation pulse. Measurements show that the forward Yield is linearly related to the average power of the excitation beam as well as the sample flow rate over the range of values investigated in these experiments. Figure 5.26(a) shows the linear power dependence of the forward Yield taken at a constant flow rate, while increasing the average power of the excitation beam with a variable neutral density filter. These measurements were taken at high flow rate (flow rate setting q=150) to limit photodamage at higher excitation powers and to collect the data quickly, ensuring consistent experimental conditions over the series. Figure 5.26(b) shows the decrease in the forward
Figure 5.26: Power and flow rate dependence of the per-exposure yield of the forward ($P_R \rightarrow P_{FR}$) photoswitching reaction: (a) $Y_{R,FR}$ measured as a function of Excitation power. Measurements were taken at high flow rate ($q=150$) to ensure consistent sample characteristics and prevent photo-damage at high powers. (b) $Y_{R,FR}$ measured at 200 $\mu$W Excitation power while varying the piezoelectric pump driving voltage (directly related to flow rate). Difference between $\Delta R/R_0$ and $Y_{R,FR}$ gives the damage coefficient $D_R$, which increases as laser power increases or flow rate decreases and sample is subjected to more laser intensity per exposure. Error bars (~size of markers) are calculated by propagation of the standard deviation of measured concentrations through the calculation of the Yield coefficients.

Yield as the number of laser pulses per exposure iteration is decreased: the driving voltage of the piezoelectric circulation pump sets the flow rate through the circuit, as the sample flows through the exposure cell more quickly it is exposed to fewer laser pulses. As discussed in Section 5.3.1, the flow rate is measured to be directly related to the driving voltage of the piezoelectric pump over this range of pump voltages. Figure 5.26 also shows the relative decrease of the $P_R$ state ($-\Delta R/R_0$). The difference between this measure and the forward Yield gives the damage coefficient, $D_R$. At higher power and slower flow rates the increased laser intensity and number of pulses per exposure causes $D_R$ to rise. Most measurements were taken with lower excitation power, ranging from 100 - 250 $\mu$W, and the flow rate is kept as slow as possible without leading to significant photodamage or impacting the ability to complete a measurement series under consistent sample composition.
Figure 5.27: Gradual decline of forward Yield coefficient over long measurement series (≈4 hours). Laser power, spectrum, and alignment are constant over course of measurements, therefore the decrease in yield is attributed to change in sample makeup over the course of the measurements.

In Figure 5.27 we plot the calculated forward Yield from a long series of measurements lasting over four hours and at two excitation powers. The figure shows the degree of stability of the experimental conditions over the duration of this extended measurement series. The Yield coefficients in this series of measurements are higher than those shown in Figure 5.26(a) because the flow rate is lower (q=100). There is a general, systematic decrease in the $Y_{R,FR}$ value, but the trend is gradual and consistent across the measurement series rather than erratic noise. The power and beam overlap were checked repeatedly during this time to confirm that they remained fixed, therefore the drop in yield must be attributed to a variation in sample composition or flow rate. The trend is constant in time and can be compensated for in the analysis of other characteristic coefficients calculated from this measurement series by fitting the measured $Y_{R,FR}$ values to a linear trend and using the estimated forward yield at any measurement time to calculate the other characteristic coefficients.

The excitation spectrum, generated by spectrally filtering the broadband NOPA beam between 600 nm to 640 nm, was chosen to excite both the $P_R$ and $P_{FR}$ forms of the Cph8
protein, initiating forward and reverse photoswitching reactions. Measurements comparing the forward and reverse Yield coefficient are presented in Figure 5.28, hollow squares mark the values of forward Yield used to calculate the reverse Yield. The excitation power is 250 $\mu$W and the flow rate setting is $q=125$ on the pump driver. The average forward Yield is $Y_{R,FR}=0.093\pm0.002$ while the average reverse Yield is $Y_{FR,R}=0.077\pm0.0015$ (82% of $Y_{R,FR}$). Measurements across multiple days show a consistent ratio between the forward and reverse product Yield coefficient, the reverse Yield $Y_{FR,R}$ is typically 80-85% of the forward Yield $Y_{R,FR}$. This ratio of forward and reverse Yield coefficients produces a photoequilibrium $P_{FR}$ population of $[P_{FR}^{eq}] = 0.55 \pm 0.033$. This equilibrium population is lower than the maximum photoequilibrium threshold of $P_{FR} \simeq 0.65$ achieved by linear excitation by narrow-bandwidth filtered light at 655 nm because the excitation pulse is spectrally broader and centered at a higher frequency than the optimal linear exposure. This is intentional, to produce a wavepacket of vibrational states with extra energy on the excited state potential energy surface, rather than exciting at the (lower energy) peak of the $P_R$ absorption spectrum.
Knowing the un-depleted, linear Yield coefficients for the forward and reverse reactions, we move to characterizing the Quenching coefficient. The dependence of the forward Quenching coefficient on the delay between the excitation and depletion pulses is shown Figure 5.29 for a depletion wavelength of $\lambda_{Dep}=775$ nm (a) and $\lambda_{Dep} = 835$ nm (b). We observe the sub-picosecond rise time to peak depletion, corresponding to the expected ultrafast excited state dynamics of the Cph8 switch. The depletion curves for two depletion pulse powers are shown in Figure 5.29(a), and we see that the Quenching coefficient doubles when the average power of the Depletion pulse is doubled, as expected.

The calculation of the reverse Quenching coefficient requires knowledge of all five other characteristic coefficients (see Table 5.6), making it the most difficult to accurately measure. Because of the numerous measurements needed to calculate this final reaction coefficient, it was common to only be able to measure a Full Data Series at a few control parameter settings before the sample degradation made the measurements quantitatively inconsistent. However, there are clear repeatable trends in the data that can be consistently measured over many experiments and compared to the simulations of Section 5.2.
Figure 5.30: Comparing Quenching coefficients of the forward ($Q_R$) and reverse ($Q_{FR}$) photoswitching reaction at long and short depletion wavelengths. Measurements are plotted with respect to pulse delay times, all measurements taken at excitation power $P_{Ex} = 200 \mu W$ and depletion power $P_{Dep} = 2.0 mW$. For $\lambda_{Dep} = 775$ nm there is little selectivity (ratio $Q_{FR}/Q_R \simeq 1$), while the $\lambda_{Dep} = 835$ nm depletion pulse produces consistently higher quenching coefficient in the reverse direction compared to the forward, with the ratio increasing at short delay times. Both these results are in qualitative agreement with the simulations of Section 5.2.

In Figure 5.30 we compare the depletion Quenching coefficient in the forward and reverse photoswitching reactions depleted by pulses centered at $\lambda_{Dep} = 775$ nm (a) and 835 nm (b). Measurements for both graphs were taken with an excitation pulse power of 200 $\mu W$, a depletion pulse power of 2.0 mW, an excitation spectrum of 30 nm centered at 625 nm and a depletion pulse spectral width of 10 nm. When the depletion pulse spectrum is centered at 775 nm, the forward and reverse Quenching coefficients are nearly identical for all delays, giving an average ratio of $Q_{FR}/Q_R = 1.02 \pm 0.03$. As predicted by the simulations in Section 5.2, depletion at this wavelength grants no selective control over the reaction. When the depletion pulse spectrum is centered at 835 nm, there is significant enhancement of the Quenching coefficient of the reverse reaction, with an average $Q_{FR}/Q_R$ ratio of 2.1$\pm$0.4 for Excitation-Depletion delays between 0.2-0.8 ps, with a much higher ratio of 10.0 at $t_{Ex,Dp} = 0.00$ ps. The value of the reverse Quenching coefficient being at least double the forward Quenching coefficient and $Q_{FR}$ remaining high while
$Q_R$ falls off at short excitation-depletion delays is in good agreement with the predictions of the model in Section 5.2, however the quenching effect persists to much longer times than predicted by the simulations. The spectral dependence and power dependence of the measured data are in better agreement with the model than the temporal dependence. This is reasonable, considering the model is a simplistic approximation of the molecule’s true ultrafast coherent dynamics.

The enhanced depletion quenching of the reverse, $P_{FR} \rightarrow P_R$, photoswitching reaction produces a shift in the photoequilibrium: building up a greater final product population of $P_{FR}$ and removing the undesired $P_R$ population over many laser pulse iterations. To estimate this enhanced equilibrium we calculate the eigensolutions of the Optical Transformation Matrix constructed from the measured forward and reverse Yield and Quenching coefficients (Eq 5.23 in Section 5.3.2). The errors on the photoequilibrium are obtained by statistical analysis of the calculated eigensolutions of 1000 matrixes constructed from the measured characteristic coefficients varied by a gaussian noise term scaled by the magnitude of the associated coefficient’s error. The SDQ enhanced $P_{FR}$ equilibrium populations associated with the measurements in Figure 5.30 are plotted in Figure 5.31 for the depletion pulses centered at 835 nm and 775 nm. The linear exposure photoequilibrium is calculated by omitting the Quenching coefficients from the OTM, which gives $[P_{FR}]_{EQ}^{Lin} = 0.55 \pm 0.033$. This is indicated in Figure 5.31 by the blue highlighted region. The depletion at $\lambda_{Dep} = 775$ produces no shift away from this limit, however, all exposures to $\lambda_{Dep} = 835$ produce significant enhancement above the linear threshold. The highest ratio of depletion quenching coefficients at $t_{Ex,Dep} = 0$ ps does not actually produce the highest enhanced equilibrium population, the greater reverse quenching coefficients at longer times having more impact than the forward quenching coefficient going to zero at short delays. The maximum enhanced equilibrium threshold $[P_{FR}]_{EQ}^{SDQ} = 0.81$ is achieved for parameter set $\left[\langle P_{Ex} \rangle, \lambda_0^{Ex}, \Delta \lambda_{Ex}, \langle P_{Dp} \rangle, \lambda_0^{Dp}, \Delta \lambda_{Dp}, t_{Ex,Dp}\right] =$ 168
Figure 5.31: Final $P_{FR}$ product state populations at the SDQ enhanced photoequilibrium. Data from two depletion wavelengths are shown plotted with respect to the Excitation-Depletion pulse delay. Longer depletion wavelength and sub-picosecond delays consistently produce significant enhancement of the final $P_{FR}$ population over the linear photoswitching equilibrium threshold (shown by the blue highlighted region at 0.55), while shorter depletion wavelength produces no change in the final photoequilibrium population. The maximum measured result is $[P_R, P_{FR}]^{SDQ}_{EQ} = [19\%, 81\%]$ at $\lambda_{Dep} = 835$ nm, $P_{Ex} = 200 \mu W$, $P_{Dep} = 2.2$ mW, and $t_{Ex,Dep} = 0.66$ ps.

$[200\mu W, 625nm, 30nm, 2.2mW, 835nm, 10nm, 0.667ps]$, maximizing the functional dynamic range of the Cph8 switch using SDQ of the photoswitching reaction.

### 5.4 Conclusions and Outlook

#### Conclusions

The results presented above demonstrate the capability to exploit stimulated depletion of the chromophore excited state to quench the photo-isomerization reaction before it takes place, diminishing the unwanted reverse photoswitching of the Cph8 optogenetic switch. Further, by choosing optimal spectral and temporal characteristics of the excita-
tion and depletion pulses, it is possible to selectively deplete the different chromophore states by exploiting their unique excited state dynamics and spectral response. The SDQ mechanism is proven feasible without pulse shaping, though the added capability of pulse shaping should enable even greater control of the Cph8 switch (especially at shorter Excitation-Depletion delays) and enable discrimination and orthogonal control of multiple optogenetic switches in future experiments. Selective stimulated depletion of the chromophore excited state granted control over the quenching of different photo-reaction pathways, altering the final product state distributions of the photoswitching reaction. Depletion at longer wavelengths (peaking at 835 nm) and short excitation-depletion delay times (peaking at \( \sim 0.6 \) ps) results in reverse photo-reaction quenching coefficient that is more than twice as strong as the forward quenching coefficient and a final enhanced equilibrium threshold population of \([P_R, P_{FR}]_{SDQ}^{\text{EQ}} = [0.19, 0.81]\). This is a 58% reduction of the unwanted \( P_R \) state compared to the excitation pulse alone and 46% reduction compared to the best linear photoequilibrium of \([P_R, P_{FR}]_{\text{Linear}}^{\text{EQ}} = [0.35, 0.65]\).

We have shown that photo-damage is not a significant impediment to nonlinear control of photoswitching. There is a large window of laser intensities where it is possible to drive nonlinear interactions without observing significant photo-damage of fresh samples. We have also shown that the sub-picosecond dynamics of the \( P_{FR} \) isomerization, rather than inhibiting our ability to stop the isomerization, was instrumental in enabling selective quenching of the forward and reverse photo-reaction.

We developed a simple, flexible model of the Cph8 photoswitching system that can be adapted to the simulation of a number of alternate optogenetic switching systems or simultaneous control of multiple optogenetic switches. The model is based on incoherent rate equation kinetics of the two switch states, and incorporates experimentally measured optical and dynamical characteristics of the molecular switch to simulate it’s optically driven dynamics. The model reproduces the expected steady-state photoequilibrium population distribution under saturated linear illumination with red or far-red
light. The predictions of this model are found to be in qualitative agreement with the conducted measurements, and the simulations predict a maximum enhancement of the steady-state equilibrium concentration of 93% $P_{FR}$ and 7% $P_{R}$.

Our measurements reproduce the general trends of the rate equation model, with the largest discrepancies associated with differences in the excitation-depletion time dependence of the SDQ photoequilibrium enhancement predicted by the model and measured in the laboratory. This is likely due to the omission of coherence effects from the REM simulations and the exclusion of pulse shaping from the experiments, as the coherent excited state wave packet dynamics of the molecule are sure to play a large role in the effectiveness of the SDQ mechanism, especially at short delay times. The REM model and experiments agree well in those aspects of the SDQ mechanism more reliant on the spectral information, which was more accurately represented in the model. The un-depleted reaction yields in the forward and reverse directions are linearly related to power of the excitation pulse, and the reverse yield is approximately 70% of the forward yield when excited by a pulse spectrum 40 nm wide centered at 625 nm, in agreement with the simulations and the literature [61]. Depletion at shorter wavelengths ($\lambda_{Dep} = 775$ nm) quenches both photoswitching reaction directions with comparable strength and has little effect on the equilibrium concentrations but greatly slows the reaction. This was confirmed at multiple excitation and depletion laser powers and delays. Depletion at 835 nm and sub-picosecond excitation-depletion delays increases the relative enhancement of the reverse quenching in comparison to the forward, with the ratio increasing as the excitation-depletion pulse delay was reduced. Our measurements showed that for $\lambda_{Dep} = 835$ nm and an excitation-depletion delay between 0.2-0.8 ps the ratio of Reverse Quenching coefficient to Forward Quench had an average of $\langle Q_{FR}/Q_{R} \rangle = 2.1 \pm 0.4$, increasing as the delay is decreased to $Q_{FR}/Q_{R} = 4.7$ at $\tau_{Ex,Dep} = 0.066$ ps and $Q_{FR}/Q_{R} = 10.0$ at $\tau_{Ex,Dep} = 0.0$ ps.

With the current experimental setup we were unable to expose the sample to sufficient laser exposure iterations to enable the SDQ-enhanced photoequilibrium to
overcome the slower reaction rate. However, with the iterative mapping methods developed in Section 5.2.2, we were able to calculate the SDQ-enhanced photoequilibrium threshold by computing the eigensolutions to the Optical Transformation Matrix composed of the experimentally measured Characteristic Coefficients. The maximum projected photoequilibrium population is $[P_R, P_{FR}]_{EQ}^{(SDQ)} = [0.19, 0.81]$, achieved for laser control parameter set $\left[\langle P_{Ex} \rangle, \lambda_0^{Ex}, \Delta \lambda_{Ex}, \langle P_{Dp} \rangle, \lambda_0^{Dp}, \Delta \lambda_{Dp}, t_{Ex,Dp}\right] = [100\mu W, 625nm, 30nm, 3.5mW, 835nm, 10nm, 0.667ps]$. The REM model predicts greater photoequilibrium thresholds at shorter delay times, peaking at $[P_R, P_{FR}]_{EQ}^{(model)} = [0.07, 0.93]$ at an excitation-depletion delay of $\tau = 0.08$ ps. Given all of the uncertainties and the simplicity of the REM simulations, the experimental and model results are in remarkable agreement. It is also possible that, when not properly compensated and optimally shaped, the coherent interactions of the laser pulses and the molecular wave packet observed in the experimental measurements are destructively interfering, and that proper analysis of this short time region will require shaped pulses to achieve better control over the laser exposure. The demonstrated capability to selectively quench the photo-isomerization of the chromophore in the Cph8 switch is a significant step toward complete coherent control of the optogenetic switches, enabling full dynamic range activation/deactivation of cellular signaling pathways and discriminating control of multiple switches with overlapping action spectra allowing multiplexed control of several switches simultaneously.

**Outlook**

To improve the SDQ technique for ultimate practical application we must expose the sample to hundreds of Excitation-Depletion pulse exposure cycles. To accomplish this, stable high repetition rate sources will be more valuable than sources with higher energy per pulse. Improvements to the laser sources will be discussed in greater detail in the final chapter of this work, as they impact all quantum control experiments, but it
must be stated that future experiments will be greatly enhanced by more stable, broader bandwidth, higher repetition rate laser sources.

In addition, to accurately measure the number of laser pulse pairs the sample is exposed to in a single iteration, improved control over the sample circulation and a means of continuously measuring the sample flow rate must be included in future experiments. This is an important addition to the experimental apparatus, not only to accurately calculate accumulated dose, but also to spot changes in the sample characteristics. The most immediate addition could be flow measurement by Laser Doppler Velocimetry, which can be implemented in the microchannel just upstream of the excitation laser spot, ensuring accurate measurement of the flow rate at the site of the exposure. Microfluidic circuits are being widely adopted in biochemistry and synthetic biology research laboratories as they allow for the analysis, characterization, and manipulation of smaller (nano-liter) volumes and concentrations of sample [89,90]. In response to this experimental, there are now a number of commercial solutions for flow control, regulation, and measurement in microfluidic chips and circuits. A commercial, sealed, pressure driven flow controller would not only enable precise variability and measurement of the flow rate, it would also remove the exposure to atmospheric oxygen that was a suspected cause of sample damage in this experiment. Similarly, advances in the design and fabrication of microfluidic circuits allow for elaborate custom flow geometries to be fabricated, including direct optical coupling into the flow cell with optical fibers embedded directly in the microchannel chip. A single-unit sample circulation microcircuit would greatly simplify the experimental apparatus and measurement protocol, and minimize the active sample volume – removing the need to synthesize large batches of protein samples. Direct optical coupling into the sample cell would allow more controlled coupling of the coherent ultrafast exposure beams and the absorption probe. These improvements to the sample handling system will be especially important as the transition is made from \textit{in vitro} samples of purified protein in solution to \textit{in vivo} applications within living cells.
We have demonstrated a simple form of few parameter optimization by exposing the Cph8 optogenetic switch with a series of excitation and depletion pulses at various combinations of excitation and depletion pulse power, depletion wavelengths, and excitation-depletion pulse delay times to map out the relationship between each of these parameters and the stimulated depletion quenching of the photoswitching reaction. Having demonstrated that quenching enhancement by stimulated depletion of the excited state is experimentally feasible, the next phase of experimentation will employ coherent pulse shaping to vary the amplitude and complex spectral phase of the lasers, adding temporal structure to the excitation and depletion pulses or, using a single ultra-broad bandwidth pulse from the NOPA or an Optical Frequency Comb source (see Chapter 6) combining the two pulses into a single “photonic reagent” with sub-pulse structure acting as both excitation and depletion simultaneously, as demonstrated in Chapter 4. Utilizing the full 100 nm bandwidth of the broad-band NOPA in its current configuration we can generate 15 fs ultrashort pulses directly out of the NOPA (slightly stretched from the transform-limited pulse length due to limitations of the internal prism compressor). Using a pulse shaper with a Liquid Crystal Spatial Light Modulator (LC-SLM) composed of 640 pixels we can tailor that ultrashort pulse into a highly structured control field with features as short as 6 fs (calculated by the time-bandwidth product of the full spectral bandwidth and a sech² profile) stretched over a duration of over 2 ps (based on the minimum spectral width of one pixel). An incremental advance in capability would be to use this shaped excitation to further survey the parameter space of the excitation-depletion interaction by varying the complex spectral phase of the pulse along a series of reduced basis sets, adding polynomial phases of increasing degree and scalar magnitude to map the effect of chirped excitation, chirped depletion, and combined matching or colliding chirped excitation and depletion. If an adequately rapid and reliable measurement of the photoswitching reaction yields could be made, the process could be automated and optimized by a closed-loop feedback algorithm. Unconstrained shaping of both the excitation and depletion pulses in unison
would enable the excitation of highly structured and unique excited state wave-packets that would undergo tailored unique dynamics, which the matching structure of the depletion pulse could follow in real time. This would allow for the most elaborate control over the molecules and yield true Optimal Dynamic Discrimination (ODD) of the two states of the chromophore in the optogenetic switches and full independent control of the optogenetic switching system. Once a single switch can be reliably and independently controlled over its maximum dynamic range by non-linear photoswitching the next step would be simultaneous (multiplexed) optimal selective non-linear photoswitching of multiple optogenetic switches. The same unique excited state dynamics that enable ODD to differentially excite and deplete the two states of a single chromophore will be exploited to differentiate two similar optogenetic switches.

A promising path to multiplexed optogenetic switching lies in the rugged adaptability and wide acceptance of switches based on the phytochrome photo-sensory module. Several absorption spectra are presented in Figure 5.32 from a series of mutated variants of the plant phytochrome photo-sensory module (PSM) PhyB, found in Arabidopsis thaliana, assembled with its native chromophore phytochromobilin, measured in an initial dark stable state and after saturated illumination by 650 nm red light [91]. This is a different phytochrome than Syn-Cph1 from Synechocystis (the photosensory module of Cph8), but is from the same phytochrome family of switches and illustrates a key point about the potential for enhanced control of these molecules. Mutations of key amino acids in the PHY and GAF domain change the binding pocket and alter the photo response of the phytochrome. The alterations of the binding pockets have different effects. In some cases photoswitching is completely disabled. For others photoswitching is impacted but there is no effect on the optical response. However, there is a certain sub-set of variants in which there is little effect on photoswitching yield and small but measurable changes to the spectrum of the chromophore. Five such variants from a set of analyzed mutants have been highlighted with blue in the Figure 5.32. This type of subtle change to the chromophore
structure and dynamics brought on by the altered binding pocket environment is exactly what ODD is well-suited to amplifying and distinguishing. Once suitable control fields are found that independently photoswitch these five mutant photo-sensory modules, they could each be fused to different down-stream signaling output modules to independently control five separate signaling pathways, addressing over $5 \cdot 2^5 = 160$ unique combinations of cell states within what was formerly a single channel with only partial control over the dynamic range of a single phytochrome switch. The significance of such an improvement in capability is difficult to overstate!

Close collaboration with a synthetic bio-chemistry research group specializing in directed protein synthesis would be extremely beneficial for pushing forward the field of optogenetics. Advanced protein synthesis techniques guided by insight into the dynamics of the photo-isomerization could be used to tailor chromophore binding domains in optogenetic switches to make them more effectively controlled by nonlinear photo-activation. Similarly, ultrafast spectroscopic analysis could be used to more thoroughly characterize the dynamics and structure of the exotic variants generated by mutations. The right
partnership, one that is capable of attacking the problem from both fronts, should be able to accomplish something truly revolutionary.
References


[52] Georgios Psakis, Jo Mailliet, Christina Lang, Lotte Teufel, Lars-Oliver Essen, and Jon Hughes.


Chapter 6

Outlook and Conclusions

The primary component of this dissertation demonstrated the experimental feasibility of the novel non-linear optical capabilities of Optimal Dynamic Discrimination (ODD) of Fluorescent Protein (FP) sensors and the use of Stimulated Depletion Quenching (SDQ) for the control of optogenetic switches. This work lays a solid foundation for future experiments that will build upon these results to assemble a powerful toolkit for biomolecular control and interrogation. The immediate outlook for the individual projects has already been given in Sections 4.4 and 5.4. No discussion is included here on the colliding droplet work, which is fully self contained in Chapter 3. We will begin this final chapter by presenting how these capabilities will be combined to achieve multiplexed ODD control of complex biological machines composed of numerous independently controlled biochemical reactions and processes actuated and monitored by orthogonal optogenetic switches and bio-molecular sensors in Section 6.1. We will continue by discussing the recent technological advances that will make these new experiments possible in Section 6.2. Finally, in Section 6.3 we discuss how the broader Optimal Quantum Control (OQC) field could develop in the coming years and propose possible novel applications of OQC.
6.1 Multiplexed Control of Biological Machines by ODD of Bio-molecular Sensors and Switches

The long term goal based on the present work is the flexible, independent, simultaneous control of multiple biochemical pathways, activated or inhibited by several optogenetic switches and quantitatively monitored by numerous genetically encoded and internally expressed bio-molecular sensors. The work presented in this thesis was a first step toward this capability, and there are many steps before it can be fully realized. The means by which these separate projects may grow into the desired full capability is discussed in the following sections and outlined by the map in Figure 6.1.

Figure 6.1: Map of the development of the Multiplexed Biological Control System building on the current demonstrated experimental capabilities.
6.1.1 Multiplexed Control of Paired Switch and Reporter with Overlapping Spectra

There is a set of optogenetic switches that is even more hampered by spectral cross-talk than the phytochrome switches, and the ability to use coherent control to selectively quench the photo-switching reaction in these switches would yield immediate value by enabling their use with multiple FP reporters.

The blue light-responsive optogenetic switch \textit{asLOV2} is gaining wide adoption in the optogenetic community \cite{1–3}. There are a number of reasons for the popularity of \textit{asLOV2}, including the compact genetic code leading to its expression, relatively high photo-switching quantum yield, and wide adoption across cell families \cite{4}. It is also popular because the light responsive chromophore of the switch, flavin mononucleotide (FMN), is endogenous to most cell types and expressed in abundance \cite{5}. The LOV switch is functional in a number of different cellular control modalities, including light induced protein association, gene expression, and the release or capture of associated signalling domains by conformational reorientation (the last example is schematically represented in Figure 6.2) \cite{6}.

However, unlike the phytochrome based switches explored in this work, LOV domain switches are not photo-reversible. When excited by blue light the FMN forms a covalent bond with a conserved cysteine residue within the binding pocket. This causes a down-stream $\alpha$ helical domain to undock and unfold from the rest of the switch, mediating activation of a kinase and further downstream signaling. The helix unbinding can not be reversed by interacting with the chromophore; breaking the cysteine bond does not accelerate the rewinding of the $\alpha$ helix, and the dark dependent relaxation occurs spontaneously over the course of many seconds to minutes (or even hours depending on the particular switch) \cite{7}.
The latter irreversible behavior, along with the exceptionally broad absorption/emission spectra shown in Figure 6.2 prevent the use of most FP sensors with LOV domain switches. Spectral cross-talk limits compatible imaging wavelengths to $\lambda \geq 600$ nm, effectively allowing only one channel of detection by RFP sensors to monitor the system. There are other tags with excitation spectra in the longer wavelength IR, such as Cy7 or IR800 [8], but these are dyes which can not be fabricated by the cell and must be added externally, not expressed internally as an indicator of cellular function.

We propose to use SDQ to prevent the switching of the LOV domain switch in the same way demonstrated for the Cph8 switch in this work, while allowing the FP sensor to continue on its unperturbed dynamics and fluoresce. We would first experiment with the LOV domain switch and a GFP and/or YFP sensor, as the control should be possible without optimized pulse shaping. Once the multiplexed control of the LOV switch and GFP/YFP sensors is accomplished, other FP sensors can be tested. We have demonstrated the effectiveness of ODD in discriminating signals from FPs with overlapping absorption spectra, so the use of Blue, Cyan, Green, and Yellow FPs could all be investigated, in addition to the current Red FP capability. In the first implementation of the multiplexed control the only goal would be to prevent the photo-switching of the LOV domain, enabling the imaging of the system by a broader palette of FPs without setting off the switch. The signals from each of the FPs would be detected using filters, either sequentially on a single detector or by splitting the signal to enter multiple filtered detectors. In subsequent experiments a family of more elaborate photonic reagents could also discriminate the signals of each of the FPs while inhibiting the LOV domain switch as in Chapter 4, enabling simultaneous detection of all species and control of the switch state.

The ability to quench the LOV domain, adding optical capabilities to a switch that is conventionally not photo-reversible, would be an immediate benefit to the optogenetic community. Work on this experiment has already begun in our group and promises to yield results directly. Control of the LOV domain offers an excellent example of multi-
Figure 6.2: (a) LOV domain switch function: Blue light causes Jα helix to unravel and release the effector domain, activating signaling. The reverse process is not optically controllable and occurs over many seconds or minutes. (b) Absorption and emission spectra of the LOV switch in its active (Light) and inactive (Dark) states and GFP, as well as the laser spectra of the 400 nm excitation and NOPA depletion pulses that would be used in the SDQ experiment. The 400 nm Excitation pulse would excite both GFP and LOV domain switches, but the NOPA Depletion pulse would selectively quench the LOV switch while allowing GFP to fluoresce.

plexed non-linear optical control of sensors and switches that does not necessarily require optimization guided pulse shaping and uses accessible laser resources. The ability to accomplish a sub-optimal implementation of the coherent control capability with simpler laser resources potentially opens the technique for broader implementation in more facilities.

6.1.2 Optimal Multiplexed Discrimination of Many Sensors and Switches

To create a capability of handling more switches and sensors, feedback driven closed loop algorithms will be required. While these experiments can be attempted with the current sources, experience has shown that more stable sources would likely be necessary. Fortunately there has been rapid recent progress in the field of ultrafast laser sources and equipment and a convergence of technologies will likely make future ultrafast pulse shaping and optimization more readily achievable.
6.2 New Tools and Measurement Capabilities:
Fruits of Advances in Ultra-short Pulsed Laser Technology

In recent years there has been significant advancement in commercially available femto-second pulsed laser sources. Carrier Envelope Phase (CEP) stabilization has become common, giving most ultrashort pulse laser systems the stability characteristic of optical frequency combs. Manufacturers are branching out beyond the Ti:Sapphire chirped pulse amplifier that has been the standard for ultra-short pulsed lasers for decades and are now producing more stable sources with broader bandwidths, higher pulse energy, higher repetition rate, and smaller footprint. These new sources are not only higher performance, but are simpler designs with fewer moving parts, requiring less maintenance and re-alignment.

6.2.1 Improved Ultra-broad bandwidth sources with favorable noise characteristics

Our experimental setups for ODD of FPs and SDQ of optogenetic switches have a number of sources of noise, but the principle instability is the NOPA excitation source. These noise variations rise from small but significant power, mode, and phase jitter in the Ti:Sapphire amplifier that drives the NOPA. The Ti:Sapphire regenerative amplifier is sufficiently stable to act as a primary source, but due to the highly non-linear processes taking place during the parametric amplification process in the NOPA any instability is magnified and spreads in unpredictable ways in the NOPA output (i.e., a small phase instability in the Ti:Sapphire output could lead to instabilities in the NOPA seed, poor phase matching in the non-linear amplification crystal, shifting of the spectrum, or a combination of many of these problems).

In choosing a potential light source, there is a tradeoff in attributes with current technology. One must find the best balance of source pulse intensity and average power, spectral bandwidth and frequency range, phase stability, pulse train repetition rate, and
stability of all these features, in addition to other factors not directly impacting the use of the source such as size, power consumption, device complexity, and cost. At the start of this project when our group was considering sources, there were no commercially viable light source which performed well in all of the desired attributes, and so compromises were necessary. In choosing the NOPA light source we favored pulse energy and bandwidth over other attributes, but this light source is inherently less stable than some other (newer and more expensive) light sources. However, improvements in signal detection and analysis have lowered the necessary energy requirements of the light source. With this in mind we have identified stability of output power, spectral intensity, and phase as the principle features required for the light sources used in optimal control experiments. At present we consider the following optical sources that can provide next level of performance in optical control and detection experiments.

**Optical Frequency Comb Sources**

One of the most remarkable achievements in laser technology in recent years is the development of commercially available frequency comb sources. The output of such optical sources bridges the gap between ultrafast laser science and ultrastable optical metrology. From one side frequency combs provide ultrashort (up to single cycle) optical pulses at high repetition rates. From the other side the spectrum of such a source consists of millions of extremely narrow lines with spectral stability that can be transferred from a frequency standard. High peak power of ultrashort pulses, high repetition rate, and supreme degree of the phase stabilization combine to make optical frequency combs one of the most attractive sources for precise measurements of internal composition of the living cell. These OFC have a number of unique characteristics which enable new types of measurements that would not be feasible with other sources, these novel measurement techniques are discussed below in Section 6.2.3.
Optical Parametric Chirped Pulse Amplifier

Within the last year a new type of ultra broadband amplifier has been developed that matches the NOPA for spectral range, has sufficient pulse energy to perform nonlinear optical measurements, and has significantly better noise characteristics in terms of spectral energy density/average power and spectral phase stability. This new source is the Optical Parametric Chirped Pulse Amplifier (OPCPA), and there are currently two companies producing commercial sources (Laser Quantum Inc. - CA, USA and Class Five Photonics GmbH - Hamburg, DE). The excellent stability of the spectral energy density and phase is displayed in Figure 6.3, reproduced from [9], which presents two measurements of the stability of the Venteon OPCPA from Laser Quantum Inc. The plot on the left tracks the normalized output power over more than 25 hours, with less than 1% RMS variation in power. The plot on the right is an f-to-2f interferogram, which is a measure of spectral phase and intensity stability; the stable interferogram shows that neither the pulses spectrum nor phase is fluctuating in time. The system features an rms phase error smaller than 100 mrad, which is accomplished by active stabilization with an electronic feedback loop. This stability does not come at the expense of spectral range or coherence, the OPCPA is an octave spanning ultra-broadband source which is compressible to few cycle pulse duration. The NOPA is able to produce higher pulse energy, but the OPCPA can generate several $\mu J$ per pulse, sufficient for exciting nonlinear optical interactions. Furthermore, the OPCPA operates at a higher repetition frequency, producing higher average power and enabling faster signal collection or signal modulation for lock-in detection.

6.2.2 Improved Optical Pulse Shapers

The 4-f optical pulse shaper design [10] based on physically Fourier transforming the ultrashort pulse to spatially disperse the individual frequency components so they can be modulated in amplitude or phase has been the fundamental tool of pulse shaping for
decades, and promises to still be indispensable in future applications. But as ultra-short pulse sources evolve, their capabilities and characteristics push the boundaries of what these old classes of pulse shapers can handle.

Ultra-broadband pulse shaping of octave spanning pulses introduces significant difficulties compared to broadband shaping of Ti:Sapphire lasers. The standard liquid-crystal spatial light modulators (LC-SLM) being used throughout the field were designed specifically for the Ti:Sapphire source, and as such their optical properties are tuned to the associated spectral window around 800 nm. Standard LC-SLMs do not perform as well at shorter wavelengths (where they suffer from poor transmission characteristics) or longer wavelengths (where the LC dispersion function begins to flatten out and the phase retardation drops below $2\pi$). New designed SLMs will have a broader range of sources in mind. Micro-mirror arrays are attractive SLMs as they do not suffer from many of the faults of LC-SLMs such as wavelength varying retardation response and low damage threshold. The technology is nearly at a level where they can be effectively used as SLMs, however these devices are still slightly less functional than their LC-SLM counterparts. The fact that this technology has parallel applications in the high-value field of Adaptive Optics means that we can expect the technology to continue to improve. The micro-mirror array may still hold promise as the preferred SLM in the future.
Monolithic Solid-State Arbitrary Waveform Generation and Measurement

Dynamic Optical Arbitrary Waveform Generation (OAWG) is an extremely exciting and promising avenue for advanced pulse shapers that potentially provides the most flexible and powerful means to manipulate waveforms for optimal pulse shaping. Rather than using a grating or prism to disperse the spectrum of the laser in space across an SLM, the OAWG uses a solid-state de-multiplexer and multiplexer to separate, modulate, and recombine the frequencies of the pulse on a single chip. These multiplexers are composed of an Arrayed Waveguide Filter (AWF), illustrated in Figure 6.4, in which the pulse is coupled into a waveguide which is split into numerous paths, each with a different optical path length. By carefully crafting the optical distance, each pathway has transmission characteristics to drive the different frequencies of the pulse through specified paths by interferometric recombination at the output. Each waveguide branch has a electro-optic modulator to add phase and amplitude modulation to the associated spectral component. When every comb line is independently modulated in an independent channel the result is a tailored pulse whose temporal envelope can extend as wide as the repetition period of the laser source, enabling continuous waveform generation of truly arbitrary structure. The experimental concept of the OAWG process is shown in Figure 6.5. Further, by replacing the electro-optic modulators in the OAWG shaper with phase-quadriture detectors it is possible to read out the full spectral complex phase of an arbitrary pulse with respect to a OFC reference field, enabling direct measure of the complex electric field.

Even without modulation of every line of the OFC source, such a chip-based pulse shaper would be extraordinarily valuable. The change in capability is analogous to the difference between discrete transistor computers and Integrated Circuit microchips. This device reduces the complex and difficult alignment and maintenance required by conventional 4-f pulse-shaper design; encasing the entire set of components in a single solid-state,
monolithic unit with no moving parts, optics to align, or free-space propagation to distort the beam. The electro-optic modulators on the chip operate at much higher repetition rate than the liquid crystals or micro-mirror arrays of current SLMs. This feature enables much more rapid pulse variation for a faster survey of the control utility of numerous pulse shapes as well as high frequency modulation for noise management. Work is currently underway on construction of a 64 channel OAWG device compatible with the OFC sources described above in the spectral range of 530-900 nm [14]. If such a device can be constructed and eventually scaled up to a larger number of channels it would be a truly transformative tool for the practical application of pulse shaping and pulse characterization.
6.2.3 Novel Coherent Optical Techniques
Enabled by OFC Source

The unique qualities of the optical frequency comb (OFC) source open up the opportunity for a number of new measurement techniques. Several promising techniques that could serve as better feedback signals for OQC experiments are described below. These detection schemes have many advantages over the currently utilized sample characterization measurements (i.e. fluorescence, absorption, electron/ion/fragment detection) such as improved signal to noise or avoidance of primary beam scatter (detection on zero background). But the most valuable characteristic of these measurements is the direct coherent measurement of the complex electric field rather than absolute squared intensity detection.

High Frequency Modulation Optical Heterodyne Detection

Heterodyne detection is based on the measurement of the field generated by the non-linear mixing of two coherent electric fields: a signal field carrying information to be de-convoluted and a Local Oscillator (LO) field that acts as a reference and amplifier. By mixing the signal and the LO fields two features are accomplished: the intensity of the signal field is boosted by the power in the LO, and the modulation of the complex amplitude, phase, and frequency of the mixed field can be directly measured at frequencies accessible by electronics. This transfer of the complex phase of the optical signal field into a readily measurable domain allows for direct observation of the complex polarizability of the medium that the signal field has propagated through. This direct measurement of the optical polarizability is a sensitive probe of the system being interrogated, but it can also be very sensitive to noise; thus, only the un-rivaled stability of the OFC source makes such measurements feasible. Even with the stability of the OFC laser source, other sources of noise must be eliminated from the measurement. The ability to introduce high frequency modulation on the signal and LO fields allows for shifting the detected
combination frequency to higher frequencies, away from the common sources of noise from vibrational, thermal, or turbulent fluctuations in the medium and physical apparatus.

A possible fast modulated optical heterodyne measurement experiment is described below and in Figure 6.6. A single OFC source is divided into three beams: Pump, Probe, and Heterodyne. Each beam is passed through a High Frequency Optical Modulator (HFOM), which introduces amplitude modulation on each pulse train at much higher frequency than the detection rate and lower than the repetition rate. This modulation induces side-bands on each narrow line of the OFC at $\pm$ the modulation frequency. These modulated beams are then each passed through their own independently controlled pulse shaper to add a unique complex spectral phase to the pulses. The Pump beam passes through the sample first, inducing a redistribution of the population of ground and excited states thereby changing its optical polarizability response. This alteration of the sample’s properties will have a non-trivial time dependence, determined by the quantum evolution of underlying system. As a result, the probe beam propagates through the varying media its spectrum will be enriched by the new components. Due to the high phase and frequency stability of the optical comb these newly generated frequency components can be detected after mixing with the heterodyne beam which bypassed the sample and is used as a local oscillator. In the experiment proposed in the figure, the three fields are shaped by both the HFOMs and the phase based pulse shapers to enhance the dynamic signals within the sample. The three pulse shapers will tailor and compensate the fields to maximize the distinction between the signals arising from the sets of samples to optimally discriminate the species.

**Coherence Locking Multiple Laser Sources**

Microwave or longer wave heterodyne measurements can be conducted using multiple independent field sources as the Signal and Local Oscillator at the site of the measurement because the complex electric fields of all three fields (i.e., the signal, LO, and heterodyne
Figure 6.6: Block Diagram of potential OFC based heterodyne detection apparatus for direct measurement of the complex nonlinear optical response of samples

mixed field) are measured directly and the phase difference between the LO and the signal can be measured and subtracted in real time, coherently locking the two fields. With conventional optical sources this phase measurement and coherence matching is not generally feasible for independent laser sources, and the only way to coherently mix a signal and a local oscillator is to have them originate from the same laser or from a system of lasers slaved to a mutually shared seed laser that acts as the foundation of both. Unlike conventional lasers, OFC sources can be coherently locked by remote signals, allowing multiple lasers to be coherently locked without requiring them to be pumped by the same laser. This capability can even be extended to using atomic clock signals propagated by satellite to maintain a fixed phase relationship between OFC sources separated by large geographic distances. This coherence lock between multiple separated OFC sources enables heterodyne measurement of the variation of a signal beam after propagating over long distances, enabling highly sensitive and accurate measurement of trace atmospheric gases, hazardous species, or pollutants.
Extending this coherence locking capability, comb sources can be used to establish a fixed phase relationship between laser sources of different spectral ranges and lasing material types. The different lasers can be linked by the OFC as long as there can be a nonlinear mixing signal between them and a feedback driven control on the source output (i.e., a piezoelectric motor adjusted cavity mirror to modulate the laser cavity length). With this capability, multiple laser sources, even those lying in different spectral domains, can be coherently combined, enabling multi-dimensional spectroscopy over vastly different energy domains, with the potential to eventually provide coherence locking any necessary portions of the electromagnetic spectrum. Great strides have been made in the field of 2-D spectroscopy using ultrashort pulse lasers and broad bandwidth sources to measure coherent vibrational-vibrational or electronic vibrational couplings in molecules. Having OFC locked lasers spanning several octaves of the electromagnetic spectrum could enable the observation of coherent rotational-vibrational-electronic couplings.

**New Tools and Techniques Summary**

The new sources described above have the characteristics to successfully implement ODD and future OQC experiments. The rapid development of new technologies and their ease of use should ultimately lead to implementation of ODD as a practical tool for use by the broader scientific community, and not just Optimal Quantum Control specialists. These high performance sources will be enhanced by the continuing development of more robust algorithms for optimization of the optical controls and better measurement techniques enabled by the new tools. With improved sources, detectors, and algorithms the implementation of ODD characterization of complex bio-chemical environments and reactions using several bio-chemical sensors and the independently controlled programming of cellular function with high fidelity by multiplexed optogenetic switches has the potential to produce truly ground-breaking results in understanding of biological systems and bio-engineering.
6.3 Future Applications of Optimal Quantum Control

Seeds of Future Endeavors

Looking beyond the projected applications of the current work in ODD of bio-molecules for the control of biological systems, here we will explore other possible applications of Optimal Quantum Control

6.3.1 Optimal Quantum Control Spectroscopy

Since the foundation of OQC, optimal feedback driven pulse-shaping has been employed to improve signals and reduce unwanted features of conventional spectroscopies and it will continue to grow and develop in this direction. The spread of ultra-short pulse laser sources and the simplification of pulse shaping techniques will enable more laboratories to take advantage of these tools.

Broader Applications of ODD

While it appears that ODD should be of high value in bio-chemical experiments, it was not designed to be a specialized tool for that purpose alone. The accurate identification of particular molecules in a mixture of spectrally similar species is a common challenge that is found in many branches of science. ODD provides an enhanced measurement capability for sample characterization in analytical and synthetic chemistry, drawing on details in the molecules’ dynamics that are not evident in static measurements (i.e., CW linear spectroscopy). The sensitive detection and highly species specific nature of the ODD signal could find use in the identification of explosives, bio-aerosols, and other hazardous materials, and the discrimination of these species from strong background scatter that is broad, featureless, and possibly varying in time.

Decoding Optimal Quantum Control Spectroscopy
OQC manipulates and selectively populates coherent interferences between quantum states to drive the dynamics of a quantum system toward a desired state. Once discovered, typically by closed-loop feedback optimization algorithms, these optimal control fields carry valuable information about the system being controlled and the laser-molecule interactions being utilized to achieve that control. In the same way that the tailored laser pulse transfers its coherence to the quantum system being interrogated, the system imprints itself on the optimal control field, like a key and a lock. However, it is not typically the goal of the OQC experiment to collect and analyze this information, only to achieve the target state, and so this information is typically discarded. This is not due to negligence, but because the extraction of this information from the control field is typically exceedingly difficult unless constraints are specifically defined in the optimization procedure to produce user-readable pulses, but these constraints impede search algorithms and are not preferred. Work is currently being done to make the optimization process less opaque, to reveal the mechanisms employed to optimally guide the quantum evolution of the controlled systems.

When some information about the system (i.e., the molecule’s absorption or vibrational spectrum, or excited state lifetimes and stimulated emission spectra) is known it can be incorporated into the search algorithm to improve its performance and make the final control fields easier to decipher. A system specific set of basis vectors (used to construct the control fields) can be specified which will most effectively navigate a particular landscape [15, 16]. Information can also be used to remove ineffective control fields from the family of control fields being searched over. In particular, ODD reduces to solution of a system of equations described by \( Ax = y \) where \( x \) is a vector of un-known species concentrations in a sample, \( y \) is a vector of experimental measurements to a set of control fields, and \( A \) is the response matrix of the system. To facilitate optimization, a new class of algorithms, D-MORPH [17], exist to search for non-negative sparse solutions to the above system of equations, specialized for the situation when both \( A \) and \( y \) are contam-
inated by noise. This approach will not only have a necessary robustness to laser noise, but also allow incorporating *a priori* knowledge (e.g., that the unknown species concentrations can only have non-negative values or that the mixtures is usually composed of fewer species than the total library of potential species).

Once the OQC algorithms discover an optimal pulse shape it can be measured using a variety of pulse characterization measurements or reconstructed from the applied phase and amplitude profile. For complicated control goals, the features of this temporal electric field pulse are generally not easily interpreted. An attempt to extract the information contained in the optimal control field is *Control Pulse Slicing* [18, 19]. Once the complex spectral phase shape associated with the optimal control pulse is characterized and the temporal electric field profile is calculated it may then be divided into “sliced” pieces to reveal the physical function of the different features of the optimal laser control field.

All of these techniques aim to crack open a treasure trove of information that is locked inside of the optimal control field to reveal the mechanism of control in the system and unknown information about the systems structure and dynamics.

### 6.3.2 Landscape Exploration and Survey

**Mapping the peak instead of climbing it**

The *optimal control landscape* defines the functional relationship between a measured control objective and the parameters that define the control field [20]. The topology and structure of the control landscape is fundamental to the practical feasibility of OQC experiments, but there are surprisingly few experiments studying these features [16, 21–24]; and those that do typically focus only on the local landscape at the optimal extremum. The study of the structure of the landscape as control parameters are constrained is of particular importance, as all real experimental laser resources are inherently limited [21, 25]. Understanding the restrictions such constraints place on the ability to control a system and the ease of searching on the control landscape is critical. It is important to
experimentally observe how access to various regions of the control landscape is restricted as control parameters such as laser power and intensity, or bandwidth and shaping fidelity, are varied over the experimentally accessible extremes.

These issues are generally not explored because they are extremely expensive to experimentally measure: costly in measurement time and requiring as close to an ideal laser source as can be realistically achieved. A successful OQC experiment takes a search trajectory from some starting point on the control landscape to the optimal extremum, ideally taking as few steps as possible to converge to the maximal fitness control field quickly. This performance behavior is desirable when the goal of the experiment is only to reach the optimal control objective, but if the goal is to understand the landscape itself then we must systematically measure and record the fitness at a vastly larger number of control fields. Technically, to accommodate the third canonical assumption of OQC (See Chapter 2) that the control field is not constrained, the field must be infinite dimensional! While for most practical experimental control objectives this requirement can be relaxed [26–28] to discretize the control field in some fashion, current experimental facilities are simply not capable of measuring data fast enough to survey a meaningful domain of this high dimensional space without making assumptions about the landscape structure from available knowledge of the system.

It can be advantageous to explore and characterize the local topology of the control landscape in the vicinity of this critical point. An algorithm to accomplish this critical point characterization, FOCAL [16, 29], has been developed by our group. Once at the optimal extremum of a landscape, FOCAL continues to collect information and search the space while maintaining the optimal fidelity. The additional information improves calculation of the covariance matrix, associated with the Hessian (the nonlinear curvature) at the peak of the landscape. This can unveil a broad level set of multiple equally effective control fields, as well as reveal directions in the control parameter space that can be higher or lower curvature. Higher curvature vectors in the landscape peak are sensitive: they
optimize more quickly but can also be more susceptible to noise. This information is critically valuable for maintaining or reproducing the optimal control interaction. This type of limited landscape analysis at the critical level set is an important step for understanding and taking advantage of the topology of the optimal control landscape. Work should continue on developing more algorithms focused on landscape analysis and sub-optimal level set traversal.

The dimensionality of the control space is specified by the number of control parameters used to define the control field. In experimental OQC using shaped ultra-short pulsed lasers this is set by the number of pixels on the SLM shaping the pulse (or twice this number if amplitude and phase shaping are being employed). This is typically several hundred dimensions, a daunting search space to fully explore. With some fore-knowledge of the molecular system being controlled, it can be possible to assign a physically meaningful reduced basis to construct the control pulses. For example, we can limit control vectors to being coefficients specifying the linear combination of polynomial phase functions to compensate/add dispersion to a pulse, or we can use a series of periodic phase functions to generate a multi-pulse with tailored spacing and sub-pulse intensity structure. However, these reduced bases assume specific fore-knowledge of the nature of the field-matter interaction and do not fully utilize the closed-loop feedback algorithm, potentially falling into traps due to the constrained search spaces.

It has been shown in theoretical calculations that for a number of real experimental control objectives there should be a finite, relatively small number of characteristic basis vectors to describe the landscape at the optimum [30]. In particular, for a quantum system composed of N levels, the landscape for the population transfer from state $|i\rangle$ to state $|f\rangle$ has an optimal level set of at most dimension $2N - 2$ [20]. This means that the vast majority of our control vectors accessible by our pulse shaper are degenerate and unimportant, provided that N is not large, and can be reduced to a relatively small number characteristic of the system being controlled and the control objective.
These basis vectors are not just convenient for expressing the optimal control pulse shape in fewer parameters, they contain within them the important physics of the interaction between the control field and the quantum levels of the system that enabled the propagation of the population toward the target objective state. Further, they are the eigenvectors of the Hessian matrix at the optimal extremum: these vectors and their associated eigenvalues describe the 'cardinal directions' for navigating the optimal level set and the sensitivity of the system to modulation of these control vectors. The eigenvectors associated with higher eigenvalues are more sensitive, and they can be useful for quickly ascending the landscape. However, this sensitivity also makes them susceptible to control field noise. Eigenvectors with lower eigenvalues are more robust to noise and optimal control fields made up of these lower eigenvalue basis vectors should prove more stable. Knowledge of the eigensolutions of the system Hessian help us to design better control fields: fields which not only optimally propagate the population to the control objective with high fidelity, but also do so with high robustness to control field noise.

We must explore the landscape not just at the optimal extremum, but over as much of the search space as possible to hunt for traps and saddle points (sub-optimal critical points). It is important to study these landscape features if they exist in experimental control problems, as they can cause search algorithms to become stuck at sub-optimal levels. The region that may be most important but also most difficult to experimentally study are domains far from the optimum with minimal fitness values, where most optimization experiments will start. Exploring this region is inherently difficult, as it is characterized by its low signal intensity and is also by far the largest region of the search space for most landscapes. Determining the characteristics of the minimal level sets may aid in finding ways to more rapidly navigate out of this region and reach the landscape peak.

Turning the focus of the experiments away from climbing to the peak and toward mapping the landscape is a fundamental shift in concept which will require all new al-
gorithms and possibly new experimental measurement techniques like Nuclear Magnetic Resonance spectroscopy [31]; but the information gleaned from a better understanding of the control landscape could be invaluable, leading to revelations about conserved trends and interaction mechanisms that could begin to organize and codify the library of ‘pho-tonic reagents’. There is a way to design optimization and search algorithms which do not necessarily require detailed information about the system being interrogated, but can accept and integrate that information if it is available at the start [32]. Further, the smart optimization algorithm should be able to collect information about the local landscape as it is traversed; recording the local topology and using this information along with the memory of the path it has taken to guide its ascent, rather than just robotically following the local path of steepest ascent or relying on blind random evolution. The system need not operate locally either, rather than traversing a single continuous curve on the landscape the algorithm could survey many sites in parallel and combine this non-local information to provide a more full picture of the landscape. The useful algorithm would also be able to relay all of this information back to the experimenter rather than just throwing it away once it has reached the peak.

Fundamentally, all of this is achievable with current algorithms. The impediment to accomplishing the tasks laid out above is the iteration rate of the algorithm, limited by slow data acquisition and slow pulse shaping. The promise of new, high repetition rate lasers and fast, solid-state SLMs for rapid pulse shapers could allow the algorithm sampling rate to be increased by orders of magnitude. This type of enormous leap in data acquisition would enable collection of much more information about the landscape and finally enable surveying of real complex control landscapes in realistic time scales. Speeding up the measurement process allows us to slow down and look around at the landscape!
6.4 Conclusions

In this work we have laid the groundwork for very promising new capabilities. We have opened Optimal Quantum Control to a whole new field in biology that would have seemed completely unreachable just a few years ago. The work here expands the optical capabilities for the monitoring and control of bio-chemical reactions with the promise of much greater prospects just over the horizon. There is much work remaining to develop the current projects to reach the goal of multiplexed independent control and observation of many switches and sensors, but the way ahead seems clear. The improved tools and new experimental techniques promise to enable the experiments to reach their predicted capabilities and open up whole new domains of science to explore.
References


Appendix A

General ODD Algorithm for detecting an arbitrary number of species

The following material is a generalized description of the algorithm presented in Sec. II of the main text. The ODD algorithm is scalable, drawing on an essentially endless number of distinct interrogating photonic reagents. This enables the discrimination and quantification of the individual components of a mixture by variations of their optical response to a series of photonic reagents. This optical response is not limited to the fluorescence depletion measurement used in this work, and only requires that the measured observable respond uniquely to the photonic reagent control parameters: the spectral phase and amplitude of the interrogating laser pulse. The complex pulse shapes of the tailored photonic reagents are obtained by iterative optimization of the solution to the inversion problem of concentration determination. The objective function of this optimization is defined as follows.

The total optical response from an $M$-component mixture is a linear combination of the independent optical responses $F$ of each species $j$ to a photonic reagent $PR_k$, weighted
by the relative concentrations $n_j$ of the species in the mixture:

$$F_{\text{total}}(PR_k) = \sum_{j=1}^{M} n_j F_j(PR_k), \quad k = 1, \ldots, M.$$  \hfill (A.1)

Because the signals of the individual species are independant they add linearly, but the measured optical responses are nonlinear functions of the control variables of the photonic reagent pulse.

The identities in equation (A.1) form a system of $M$ linear equations for $M$ unknown concentrations $n_j$. Such a system has a unique solution when the responses to the sequence of photonic reagents are distinct, making the following determinant non-zero:

$$D = \det(F) = \begin{vmatrix} F_1(PR_1) & F_2(PR_1) & \cdots & F_M(PR_1) \\ F_1(PR_2) & F_2(PR_2) & \cdots & F_M(PR_2) \\ \vdots & \vdots & \ddots & \vdots \\ F_1(PR_M) & F_2(PR_M) & \cdots & F_M(PR_M) \end{vmatrix}. \hfill (A.2)$$

where $F$ is a matrix of measured fluorescence intensities: each element $F_{jk}$ is the optical response from a reference sample of species $j$ with known concentration $C_j$ exposed to $PR_k$.

Based on the Cramér-Rao inequality (see, e.g., Ref. [25]), the error in the concentration determination is inversely proportional to the magnitude of this determinant: $|D|$. The larger the magnitude of $D$, the higher we expect the accuracy of the determined concentrations; therefore, this measurement is an effective objective function, or fitness score, to an iterative stochastic optimization algorithm to maximize $\text{abs}(D)$. Such an objective function has a degree of robustness to additive noise (e.g. shifting all fluorescence signals $F_j$ by a constant leaves $D$ unchanged). Moreover, $\text{abs} (\det(F))$ is a convex function of the matrix argument $F$, suggesting that the optimization procedure should be robust.
In the engineering literature such problems are related to what is called D-optimal experimental design [25].

In order to discover an optimal $M$-tuple of photonic reagents in our experiments, we developed a custom closed-loop adaptive algorithm [21]. We begin by generating $N$ random samples of $M$-tuples of photonic reagents ($PR_m^{(n)}$):

$$\left( PR_1^{(1)}, PR_2^{(1)}, \ldots, PR_M^{(1)} \right);$$
$$\left( PR_1^{(2)}, PR_2^{(2)}, \ldots, PR_M^{(2)} \right);$$
$$\vdots$$
$$\left( PR_1^{(N)}, PR_2^{(N)}, \ldots, PR_M^{(N)} \right). \tag{A.3}$$

The upper index labels the photonic reagent iteration. In the current experiment, we employed $N = 30$ and $M = 2$; however the ODD procedure is designed to be scalable and we are extending the method to a larger number of fluorescent proteins ($M \sim 10$).

For each photonic reagent, $PR_k^{(n)}$, we record the fluorescence from the reference samples: $F_j \left( PR_k^{(n)} \right)$ for $k, j = 1, \ldots, M$, and $n = 1, \ldots, N$. Since the information about the fluorescence from $NM$ pulse shapes is available while the objective function in Eq.(A.2) depends only on $M$ pulses, we form all possible combinations of $M$ out of the recorded $NM$ pulses, calculate the objective functions, and sort the results by magnitude:

$$|D \left( PR_{k_1}^{(n_1)}, PR_{k_2}^{(n_2)}, \ldots, PR_{k_M}^{(n_N)} \right)|, \tag{A.4}$$

where each $k_i$ takes on the value $k_i = 1, \ldots, M$ and each $n_j$ takes on the value $n_j = 1, \ldots, N$. The multiplicity of this set of calculations is

$$\binom{NM}{M} = \frac{(NM)!}{[M!(NM - M)!]} \tag{A.5}$$
Out of this sorted set of pulse sequences, we pick new $M$-tuples based on the largest values of the objective function.

The optimization algorithm is a stochastic genetic algorithm modeled on biological evolution, where a single genome string made up of the pulse shaper settings [21] is assigned a fitness score, the objective function in Eq.(A.2). This fitness score dictates how the controls advance and combine in the subsequent iterations to improve the response of the photonic reagents, maximize $\text{abs}(D)$, and improve the concentration determination.

The $M$ pulse shapes of each photonic reagent in the $M$-tuple of a given iteration is concatenated into a single string to be processed by the GA, then re-separated and sent to the pulse shaper to tailor the laser pulses that interrogate the sample. However, it is possible that combinations of photonic reagents from different $M$-tuples would yield higher fitness interactions than those within a group, for example:

$$\left| D \left( PR_1^{(D)}, PR_2^{(A)}, PR_3^{(B)}, \ldots, PR_M^{(A)} \right) \right| > \left| D \left( PR_1^{(A)}, PR_2^{(A)}, PR_3^{(A)}, \ldots, PR_M^{(A)} \right) \right|.$$  

(A.6)

This cross breeding across different iterations within a generation allows for higher fidelity solutions and even faster optimization in fewer generations at the expense of increased computational cost per generation.

After a number of iterations the optimization may be halted when an acceptable value of the objective function is reached. The final generation of the optimization produces $N$ samples of $M$-tuples of photonic reagents ranked in descending order by the value of the objective function, characterizing the accuracy of the concentration measurement of each $M$-tuple group.

At this point, the top ranking $M$-tuple of photonic reagents is the optimum solution and nominally sufficient to determine the sample concentrations of any combination of the characterized species. However, to further increase accuracy of the measured concen-
trations, one may pick the $P$ highest performing $M$-tuples and use them to interrogate the mixture. Beyond the statistical enhancement of repeated sample interrogation, each photonic reagent $M$-tuple has evolved to manipulate a different set of coherent dynamics in the species. We collect $P$ measurements of the left hand side of the following system of equations:

$$F_{\text{total}} \left( PR_k^{(n)} \right) = \sum_{j=1}^{M} n_j F_j \left( PR_k^{(n)} \right), \quad k = 1, \ldots, M, \quad n = 1, \ldots, P. \quad (A.7)$$

This yields an overdetermined system of $PM$ linear equations to be solved for the $M$ unknown $n_j$’s. The number of $M$-tuples ($P$) used to characterize the mixture will be determined by experimental signal to noise ratios, number of species being characterized, and desired concentration accuracy.