A time-gated fluorescence spectroscopy system capable of nanosecond gating and picosecond control of gate delays is presented. Used in conjunction with pulsed excitation, the system is capable of tracking the temporal evolution of the fluorescence spectrum from solution samples. The system uses a nitrogen discharge laser as the excitation source and a time-gated intensified CCD detector coupled to a spectrograph. Precise synchronization between the laser pulse and ICCD gate is achieved using a constant-fraction optical discriminator. In addition a high-pressure perfusion system is constructed with an optically compatible high pressure chamber for use in conjunction with fluorescence spectroscopy. System characterizations for both systems are presented, for example, the ability to both spectrally and temporally resolve the content of fluorophore mixtures is confirmed. Time resolved and high pressure perfusion data is collected using *Saccharomyces cerevisiae*. Biotechnological applications are highlighted, including gated spectroscopy for the real-time monitoring of metabolic activity via measurement of endogenous cellular fluorescence.
TOWARDS A SYSTEM FOR NANOSECOND-GATED, FLUORESCENCE BASED MONITORING OF CELLULAR RESPONSES TO HIGH HYDROSTATIC PRESSURES

A Thesis
Submitted to the
Faculty of Miami University
in partial fulfillment of
the requirements for the degree of
Master of Science
Department of Physics
by
Zac Long
Miami University
Oxford, Ohio
2013

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**Abbreviations**

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NADH:</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>KCN:</td>
<td>Potassium Cyanide</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>ICCD:</td>
<td>Intensified Charge-Coupled Device</td>
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<tr>
<td>OD:</td>
<td>Constant Fraction Optical Discriminator</td>
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<tr>
<td>POPOP:</td>
<td>1,4-Bis(5-phenyl-2-oxazolyl)benzene</td>
</tr>
<tr>
<td>9-CA:</td>
<td>9-Cyanoanthracene</td>
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Dedication

This thesis is dedicated to my family and friends helping me through difficult times and supporting me in my many endeavors.
Acknowledgments

I thank my professor, Dr. Paul Urayama, for allowing me to participate in this research project and guide me during the course of my time here at Miami University.

I thank Allison Huff for her preliminary work on the high perfusion system.

I thank my labmates who, despite their best efforts, have been a great help to me by providing company, ideas, and insight into my projects.

I thank my committee members, Dr. Herbert Jaeger and Dr. Stephen Alexander, for asking thought provoking questions and for their endurance in reading this thesis in its entirety.
Chapter 1: Introduction and Motivation

1.1 Overview

This thesis focuses primarily on the development and characterization of two techniques which will eventually be used for metabolic monitoring under high hydrostatic pressure. The first, nanosecond-gated spectroscopy, is a time resolved method of monitoring changes in a solution containing fluorophores. The second, a high-pressure perfusion system, is used in conjunction with spectroscopic techniques to monitor biological responses of cells as the pressure of their environment changes.

1.2 Motivation

The biosphere has an average pressure of 400 atm [1], ranging from 1100 atm at the bottom of the Mariana Trench to the -20 atm, used in the transport of water in trees [1, 2]. It is evident, therefore, that metabolic processes could be different at different pressures yet still functional [3]. Better understanding of the effects of pressure on life forms can help us to better understand the environmental parameters in which life can exist. This kind of knowledge could then have applications in the discussions of the origins of life both here on Earth and elsewhere.

Changes that occur as a result of pressure have potential applications in the field of biotechnology. For example pressure has been shown to be important in the utilization of enzymes and extremophiles, in addition to applications in both pharmaceutical and medical sciences [1, 4-8]. Pressure is a key factor in food processing and safety [9, 10]. Physiological pressures can also have medical or biological effects such as on the growth of load-bearing tissue [11], proper tissue growth [12], or the strength of endothelial monolayers [13].

In order to understand how pressure can affect biological systems, however, we require some way to probe the inner workings of cells. One way of doing this is to observe different molecules and compounds that control a cell’s biological processes. For example, molecules will often have different fluorescence properties depending on their conformational state. By determining how these properties change in response to various stimuli we can begin to better understand the inner workings of the cell. One such molecule of particular interest to our research group is NADH (nicotinamide adenine dinucleotide). NADH is a molecule found in
cells which is integral to the cells’ metabolic processes [14, 15]. Specifically it is used in the electron transport chain which takes place in the mitochondria of a cell. As the mitochondria are essentially made of membranes, and membranes have been shown to be sensitive to pressure changes, the study of NADH under high pressures is a topic of particular interest in this project.

NADH exists in two states within a cell: either bound to a protein or unbound [16]. By studying the change in the concentrations of the two forms of NADH in cells under high pressures, we can learn how different metabolic pathways are affected by changes in pressure. There are several properties of the molecules which differ between the bound and unbound states. The two which are important in this study are the peak emission wavelength and the excited state lifetime of the molecule [16]. Studying the ratio of the two states of the molecule within a cell and how that ratio changes can be measured using spectroscopic techniques. One can excite the molecules with a laser source and the resulting excited state spectrum can be analyzed in order to glean information concerning the state of NADH in a cellular environment. Spectral shifts in cells can be due to various outside influences such as oxygen levels, addition of chemicals such as KCN (potassium cyanide) or pressure.

Pressure effects have been studied without the use of perfusion. This is done via the examination of various single-celled organisms such as bacteria, yeast, or mammalian tissue cells in optically compatible high pressure chambers [17]. From studies such as these, we have gained significant information on the effects of high hydrostatic pressures on metabolic processes, gene-expression, and the assembly of proteins. Previously there has been work done by groups with a system, similar to ours, for high-pressure perfusion. In this case, however, the system conducted experiments for the study of morphological effects of pressure on the growth of *E. coli* using microscopic observation techniques [18]. As of yet, studies of this kind have yet to be conducted using a high-pressure perfusion system coupled with fluorescence-based spectroscopic techniques.

### 1.3 Goals

In order to study the effects of pressure on cells, we will be using nanosecond-gated spectroscopy to understand the relationship between the concentrations of bound and unbound NADH in cells and time. This sort of information will allow us to see how pressure can affect different metabolic pathways, which may allow for better understanding of the roles that
different areas of the cell play in metabolism. For example, membranes are especially pressure sensitive [19-21]. Therefore, this study could reveal more information on when the metabolic processes in the membrane of mitochondria are affected by pressure.

Information, such as the relative concentrations of different fluorophores in a solution (discussed later), can be obtained using time-resolved spectroscopic techniques. Nanosecond-gated spectroscopy is a method of measurement which involves taking nanosecond long exposures of emission spectra in such a way that allows for control over which section, in time, of the emission spectra is studied. This method allows for real time monitoring of fluorophores over time. As a result of our interest in these topics the two primary goals of this thesis were:

1. To implement a time-gated method of spectroscopy which allows for time-resolved measurements of fluorescence properties, such as intensity-weighted average wavelength and relative excited-state lifetimes, of fluorophores in solution.

   In traditional LIF (laser-induced fluorescence) spectroscopy, a sample, often a fluorophore in solution, is excited with a laser and a spectrometer is used to examine the resulting exited state or fluorescence spectrum. When a fluorophore is struck with a photon it excites an electron in the fluorophore. This electron will then decay back down to its ground state by emitting photons at specific wavelengths. These emitted photons are what we observed in a fluorescence spectrum.

   The next technique is nanosecond-gated spectroscopy. As mentioned before, nanosecond-gated spectroscopy is a method of fluorescence spectroscopy in which nanosecond exposure times are used and temporally controlled. This allows for different fluorophores to not only be distinguished between by examining their spectral shape wavelength but also by their relative excited-state lifetimes. By examining how long a certain fluorophore emits light after it has received its excitation pulse we can detect very small differences in decay lifetimes.

2. To implement a high-pressure perfusion system that allows for the monitoring of metabolic changes in cells, due to pressure or chemical stimuli, using LIF spectroscopic techniques.

   The high pressure perfusion system has the ability to trap cells, suspended in a medium, in an optically compatible pressure chamber and allow for the flow of nutrients to the cells.
Perfusion of the medium allows for *in vivo* study of the cells while the medium is pressurized. Once the cells are trapped in the pressure chamber, the environmental effects on the cells can be studied using the previously mentioned spectroscopic techniques.
Chapter 2: Nanosecond Gated Spectroscopy

2.1 Overview

This chapter describes the instrumentation and characterization of the nanosecond-gating system as it is used during the course of this thesis. The instrumentation section details the equipment used in these experiments as well as how they are used in conjunction with each other. The characterization section describes several experiments performed in order to confirm the ability of the system to detect changes in spectra in a time-resolved way. This includes synchronizing the overall system timing, confirming the manufacturer’s measured gate width, and observing the system’s ability to collect spectra for the monitoring of fluorophores in solution with respect to time.

2.2 Instrumentation

The optical side of this project is primarily concerned with the nanosecond-gated spectroscopic techniques developed for the real-time monitoring of fluorophores in solution. In essence we are taking spectra of small time windows, referred to here as detector gates, of the emission-decay curve of fluorophores in solution similar to systems developed for microscopic imaging [22]. The emission-decay curve here refers to the decrease in the fluorescence intensity of a fluorophore which has been put into an excited state. The concept behind this method is shown in Figure 2.1, end of the chapter, and detailed in the section 2.3. Figure 2.2 is a schematic of the experimental apparatus used during this portion of the thesis. This setup makes use of an ICCD (intensified charge coupled device) (Andor iStar 334T), a schematic of which is shown below in Figure 2.3, coupled with a spectrograph (Oriel MS125 #77400).

An ICCD, Figure 2.3, is a device which amplifies incoming light and takes an image. It does this by first allowing photons to enter the ICCD through a window and strike a photocathode. The photocathode emits an electron when a photon is incident on it and these emitted electrons continue through the ICCD until they strike a MCP (microchannel plate). A MCP is a thin disc comprised of a honeycomb of glass channels each of which have a resistive coating. When a large voltage is applied across the MCP, any electron which enters one of the channels will accelerate until it gains enough energy to knock off secondary electrons from the
MCP. These new electrons then strike a phosphor which in turn emits photons. The output image produced by these photons is then transported to the CCD via a fiber optic bundle coupling the intensifier to the CCD.

The CCD then converts the image into an electronic signal which we can then manipulate using software such as Andor Solis, a program which allows for control of the ICCD. This program, coupled with the ICCD hardware, allows for nanosecond control of the detector gate, similar to the exposure time of a camera. Because this control is electronic, there is delay associated with the electronics in the system which must be compensated for. As a result of this delay we introduced the optical delay shown below in the form of a 25m fiber optic (Edmund Optics #57-075). This delays the light from the laser pulse long enough to allow the ICCD to be triggered and power up before the exposure.

In order to conduct the sort of measurement for this project however, we require that the ICCD receives a trigger to turn on at specific times after the laser pulse hits a sample. In this case a constant fraction optical discriminator, OD, (Becker & Hickl GmbH #OCF-401) provides the necessary trigger. An OD is a device which senses light pulse and sends out an electronic pulse at a point that is a certain fraction of the intensity of the incoming light pulse. This has the advantage of making the timing of the electronic pulse independent of the intensity of the trigger pulse as well as reducing temporal jitter.

The introduction of the OD and a beam splitter to send some small fraction of the laser pulse to the OD allowed for the laser pulse itself to trigger the ICCD for measurement. It also introduced a small amount of electronic delay.

### 2.3 Spectral Acquisition Protocol

During the course of this research spectral acquisition protocols are needed to accomplish the goals of this thesis. The first of these, the profile protocol, was to acquire a spectrum using the ICCD at a specified gate delay and with a specified gate width. A spectrum gathered this way will contain only a small amount of the emitted light from a larger emission curve as shown by the detector gate in Figure 2.4. Once a spectrum has been acquired the detector gate is then stepped forward in time by increasing the gate delay via a setting in the ICCDs controller. This process is then iterated several times and the resulting integrated intensities of each spectra are calculated and are then plotted vs. gate delay. The interface which controls the ICCD in this case
is Andor Solis. This interface uses its own version of basic, Andor Basic, which contains specific commands to control different settings in the Andor Solis interface and through it the ICCD. Several programs were written during the course of this project in order to control the ICCD in specific ways in order to assure repeatability and speed in the data collection process. These are included along with annotations in Appendix A. The intensity graph which can be constructed as described above we refer to as an intensity profile.

Another protocol using the same system is the **two-gate scheme**. In this scheme two spectra are taken at different gate delays. These can be any number and are chosen for each sample independently. The annotated script controlling this process is located in Appendix B. Because these measurements can be taken, approximately four seconds per round of measurement, this method has the additional advantage of being able to be conducted in a way which allows for real time measurement of a sample.

For the two-gate scheme it is necessary for there to be an external trigger pulse independent of the ICCD as it is unable to send a pulse to the laser before the OD triggers the ICCD. The OD here defines zero time so the ICCD would have to produce a triggering pulse before that in order to work in a time-gated mode.

As a result of this need for external triggering an Arduino microcontroller (*hacktronics [sic] Arduino Starter Kit*), described in Appendix F, was added to the system. This Arduino provides an external trigger for the laser which can be customized to fire an arbitrary number of triggering pulses at different rates and time delays. Because the ICCD is set in an accumulate mode which accumulates five spectra before saving the file, the Arduino triggers the laser ten times and then waits for twelve seconds before repeating. As there are two gates being used in this gating scheme the laser is required to fire ten times for each round of data acquisition. Allowing for short delays between data acquisition also allows us to change the cells’ environment without disrupting the data acquisition itself. Before the two-gated scheme was developed triggering of the OD was achieved by simply allowing the laser to repeatedly trigger itself at a fixed rate.

As a result of using two different gate delays the spectra acquired will contain the emission spectra of different fluorophores in a sample in different ratios, provided the fluorophores have different decay lifetimes. For example if a sample contains both a long lifetime fluorophore and a short lifetime fluorophore then a spectra taken at a shorter gate delay
will contain the emission spectra of both of the fluorophores at a certain ratio while a spectra taken at a longer gate delay will contain the emission spectra of the longer lifetime fluorophore in a higher ratio as shown below in Figure 2.5. This is a result of the shorter lifetime fluorophore having emitted a higher fraction of its absorbed light. An effect of using this method is that shifts in relative amounts of multiple fluorophores will be apparent due to changes in the ratio of the measured intensities in the separate detector gates.

2.4 Time Gated System Characterization

In order to confirm the temporal control of the system several characterization measurements were conducted. These included synchronizing the overall system timing, confirming the gate width for the ICCD, confirming the ability to distinguish the temporal response of fluorophores, and confirming the ability to monitor spectral evolution with respect to time.

2.4.1 Overall System Timing

Before we could confirm the gate width for the ICCD, we first had to determine the appropriate gate delay that would synchronize the system and allow for detection of the laser pulse. If the ICCD gate occurred too soon or too late after the laser fires, the pulse would not be within the gate width. Initially, as a “ballpark” estimate, the arrival time for the pulse at the ICCD was calculated using the specifications provided for the ICCD, OD, fiber optic, and additional electrical wires in the system. These amounted to a total electronic delay of ~46ns (ICCD delay of 24ns, OD delay of 10ns, electrical wire delay of ~12ns due to ~4ft of wire) and a total optical delay of ~121ns (25m of fiber optic with an index of refraction of 1.458). This left us with a difference of delay time of ~75ns between the earliest time the ICCD could begin an exposure and the time when the laser pulse would reach the ICCD. Once this was determined, we located the laser pulse via the use of a binary search. The gate width was initially set to be quite long, beginning at a time slightly before the expected arrival time of the laser pulse. This gate width was then cut in half and the gate delay adjusted in order to determine whether the pulse was located in the first or second half of the exposure time. This method, shown in Figure 2.6,
was repeated until the gate width was 1ns, the lower limit of the ICCDs exposure time. This ranges from 70-80ns depending on the gate width used.

2.4.2 Confirmation of Manufacturer’s Gate Width Specifications

In order to confirm the manufacturer’s specifications of the ICCD gate, we simply collected an intensity profile of the excitation pulse using its reflection off of a 0.3 neutral density filter using the setup below shown in Figure 2.7. The reason a filter was used instead of a mirror was to reduce the amount of light incident on the ICCD. In addition to safety, the reflection had the added benefit of causing negligible widening of the pulse width profile of the laser the way a fluorophore would as reflection is an instantaneous process. Using the reflection therefore allows for a more accurate measurement of the ICCDs properties. It was expected that when directly taking the profile of the laser we would observe a convolution of the system response and the laser pulse. If we assume Gaussian temporal responses, the width we observe should follow the relation:

\[
(\Delta t_{\text{obs}})^2 = (\Delta t_{\text{gate}})^2 + (\Delta t_{\text{laser and other}})^2
\]  

(Equation 2.1)

where \( \Delta t \) is the full width half maximum of the profiles. In this case the laser pulse is 800ps (manufacturer’s specifications) and the system response is a combination of the gate width setting on the ICCD and other broadening effects.

Figure 2.8 shows example laser profile graphs. As one can see these profiles are not symmetrical. On the right hand side of the graph there is a higher peak intensity present in all of gate widths past 2ns but does not become pronounced until past 5ns and seems to be a feature of the ICCD. For this reason we found it necessary to acquire the observed gate widths in two different ways depending on the shape of the intensity profile.

The shorter gate width profiles resemble a Gaussian which allows us to fit a function to the profile and obtain its FWHM (full width half max). This FWHM of the fitted Gaussian is what we used to determine the observed gate width of these profiles. For the longer gate width settings we simply measured the width of the profile at an intensity which was half of the peak intensity. Using this method we were able to measure several different gate widths and determine how accurate the ICCDs controls were when compared to the measured gate width. This was done by finding the convolution of the manufacturer’s observed gate width at a particular setting.
for the ICCD input and the laser pulse coupled with other external factors. If the observed width of the pulse profile was similar to the calculated convolution then this suggests the ICCD is working within its specifications. After analyzing different gate widths it was found that the controls were consistent to within 0.3ns on average to the manufacturer’s value. This is smaller than the gate width resolution we associated with the ICCD, 1ns. A chart of the observed and manufacturer’s measured gate widths is shown below in Table 2.1.

2.4.3 Ability of Distinguishing Between Excited-State Lifetimes of Standard Fluorophores

Next we confirmed the profile protocol could distinguish between well-resolved fluorophores in solution using the differences in their excited state lifetimes. When taking an intensity profile we incrementally increase the gate delay after each acquisition. As the gate delay is increased we would expect the intensity to decrease exponentially for each gate delay greater than the one which is associated with the peak intensity of the profile. As different fluorophores have different excited-state lifetimes the rate of intensity decay with increasing gate delay we observe should be related to the fluorophores we have in solution.

In order to test this hypothesis we used two fluorophores with well known spectral properties. In this case the data was taken primarily using the fluorophores 9-CA (9-cyanoanthracene) and POPOP (1,4-Bis(5-phenyl-2-oxazolyl)benzene) in solutions of spectroscopic grade ethanol (SIGMA-ALDRICH, #459828-2L). These fluorophores were chosen for the large differences in their excited state lifetimes, 9-CA has an excited state lifetime of 12.12ns and POPOP 1.32ns [23], and their similarity in their peak wavelengths, both on the blue side of the visible spectrum.

Measurements were taken first of the fluorophores in separate solutions and then in solutions containing different amounts of each fluorophore. Using the setup in Figure 2.2 the fluorophores were prepared at a 5µM concentration in ethanol and placed in a quartz cuvette (NSG Precision Cells 10mm Quartz 1ES10). In each case spectra were taken over a 50ns range of gate delays. The graphs of the profiles are shown below in Figure 2.9. As one can see there is a definite difference in the decay rate of the intensity after the peak intensity in the profiles. In this case it is evident that 9-CA has a longer excited state lifetime than POPOP. In Figure 2.10, one can see the difference in the rate of decay at two different gate delays. At the 87ns gate delay
we see that both of the fluorophores are present however at the 94ns gate delay 9-CA still is present at approximately 50% of its peak intensity while the signal from POPOP is nearly gone.

2.4.4 Confirmation of Spectral Shift Detection with Respect to Gate Delay

Using the same spectra used in the construction of an intensity profile we can also calculate the intensity-weighted average wavelength and plot it vs. gate delay. The intensity-weighted average wavelength is calculated via the following relationship:

\[
\lambda_A = \frac{\sum I_i \lambda_i}{\sum I_i} \quad \text{(Equation 2.2)}
\]

where \(\lambda_A\) is the intensity-weighted average wavelength, \(I\) is the intensity of a certain wavelength in the spectrum, and \(\lambda\) is the wavelength associated with a certain pixel in the spectrum.

As a result of the differences in excited state lifetimes of the two fluorophores, we expected that a shift in the intensity-weighted average wavelength towards that of the longer lifetime fluorophore would be observed. In the case of the fluorophores 9-CA and POPOP for example we would expect to observe a red shift in the intensity-weighted average wavelength of the spectra because 9-CA's peak has a redder intensity-weighted average wavelength, \(~466\text{nm}\) for 9-CA and \(~443\text{nm}\) for POPOP, and a longer excited state lifetime than POPOP.

Next we prepared solutions containing 1 part 9-CA and 9 parts POPOP and vice versa with a total final concentration of 5µM for the fluorophores in solution. Using these we collected spectra via the same method described above. Once the spectra were collected we plotted the intensity-weighted average wavelength vs. the gate delay in order to confirm that the shifts described above would occur, these are shown below in Figure 2.11.

In the case where there is more 9-CA than POPOP the shift is more pronounced due to the initial fluorescence intensity of the POPOP. This larger concentration of POPOP should result in a larger intensity relative to the 9-CA and, therefore, a bluer center of mass wavelength. In both cases however the intensity-weighted average wavelength is observed to be attaining the same final value. The final value in this case corresponds to the same intensity-weighted average wavelength as that of a 9-CA in solution. Several individual spectra at different gate delays are shown below in Figure 2.12 illustrating this shift.
Figure 2.1: The gray shaded area above represents a detector gate, defined in 2.2, from which a spectrum can be obtained using nanosecond-gated spectroscopy.
Figure 2.2: Schematic of nanosecond gated spectroscopy experimental apparatus. The dotted lines show optical pathways while the solid lines show electronic pathways.
Figure 2.3: Schematic of an ICCD.
Figure 2.4: Emission curve and excitation pulse curve showing the detector gate used in nanosecond gated spectroscopy. The detector gate results from both the gate delay and gate width specified during a measurement.
Figure 2.5: Two different fluorophores’ profiles. In the first early gate delay both are emitting light at a similar level while in the second gate delay the longer lifetime fluorophore is emitting a much higher proportion of light.
Figure 2.6: Graphical representation of the binary search method for locating the excitation pulse.

Temporal location of excitation pulse.
Figure 2.7: Schematic of pulse characterization setup.
Figure 2.8: Example profiles for various gate widths.
Figure 2.8 continued.
Table 2.1: Table showing manufacturer’s measured gate width, observed system response, and calculated gate widths at different settings. Here we assume a constant value of $\Delta t_{\text{laser}}$ of 800ps.

<table>
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<tr>
<th>Gate Width Setting (ns)</th>
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Figure 2.9: Two different fluorophore profiles scaled to the same intensity. This demonstrates the distinction between the two fluorophores excited state lifetime.
Figure 2.10: Example spectra of 9-CA and POPOP. This shows the difference in the excited state lifetime by comparing the intensities both at their peak profile intensity and a short time after.

Figure 2.11: Graph showing the changes in the intensity-weighted average wavelength of two different fluorophores in solution with respect to gate delay.
Figure 2.12: Spectra illustrating the shift in the intensity-weighted average wavelength as a result of the difference in the two fluorophores excited-state lifetimes.
Chapter 3: High Pressure Perfusion System

3.1 Overview

This chapter describes the instrumentation, procedures, and characterization of the high pressure perfusion system as it is used during the course of this thesis. The instrumentation section details the equipment in the system as well as how they are used in conjunction with each other. The procedure section details the use of the high pressure perfusion system. The characterization section describes several experiments performed in order to confirm the functionality of the system.

3.2 Instrumentation

The high pressure side of this project is concerned primarily with the development of a high-pressure perfusion system for use in detecting changes in the metabolism of living cells using spectroscopic techniques. This system makes use of high pressure tubing, high pressure valves, three pressure generators, an optically compatible pressure chamber, and two stepper motors. A schematic and photos of the system are shown below in Figure 3.1.

Initially the perfusion system was constructed by Allison Huff using Tygon tubing. Her work included the initial proof of concept, design, and characterization of the perfusion system. She exhibited the ability to trap samples, regulate pressure in the system, and maintain consistent flow rates within the system. Since then the system has been modified in order to improve it and adapt it to high pressure. These modifications included

- replacing the Tygon tubing with high pressure lines (High Pressure Equipment Company).

Tygon tubing cannot withstand pressures in the range in which we are interested. Therefore, in order to increase our usable pressure range we were required to replace the Tygon tubing. This was done using a variety of 60,000psi rated high pressure tubing, fittings, and valves. This allows for much higher pressures to be reached and increases the overall robustness of the system.
• installing kill switches to the stepper motors.

In order to prevent damage to the pressure generators we connected kill switches to the stepper motors. When one of the pressure generators reaches the end of its track it will press a small button which cuts power to the stepper motor it is coupled with.

• adding a hand-cranked pressure generator to the system.

Before the addition of the hand-cranked pressure generator, the time required to pressurize the system was ~30 minutes depending on the desired pressure. The addition of the hand-cranked generator reduced this pressurization time to ~1 minute. This both improves the rate at which data can be collected and minimizes the time the samples are idle.

The pressure chamber, shown below in Figure 3.2, is made up of two pieces of aluminum enclosing a quartz capillary attached to high pressure tubing with epoxy (Epoxies Etc. 10-3004C15 and 10-3004RCL25). Inside of the capillary is a small glass tube which allows passage of the transport medium but not cells. This process included the construction process of this and the high pressure chambers is described in Huff’s thesis [24] barring minor changes. These included

• sanding down a small area on the aluminum supports of the pressure chamber.

We did this primarily to excite the sample at a lower angle as shown in Figure 3.3. At higher excitation angles we noticed several artifacts in the spectra collected which interfered with the collection of fluorescence signals from our samples.

• the removal of a quartz fiber plug in the capillary, shown in Appendix H.

Initially the plug was used to confine the cells to a certain volume within the capillary while still allowing flow of liquids past the plug. The quartz fiber plugs had two issues which made data collection very difficult. The first of these is the plug would often become clogged with cells eventually leading to a buildup of pressure inside of the pressure chamber which resulted in their destruction. The second, and perhaps much more difficult to fix, issue is the fluorescence qualities of the quartz fiber. Quartz fiber fluoresces in a wavelength range which overlaps significantly with that of the NADH fluorescence spectrum, Figure 3.4. This overlap of spectra can significantly reduce our ability to distinguish between any changes that may occur in the
spectrum of NADH as the quartz fiber fluorescence tends to wash out the signal of the NADH. Because of this we tried removing the quartz fiber from the pressure chamber to see if cells could be trapped despite the lack of a physical barrier. The reasoning behind this decision was that the cells may become trapped in the small area between the glass block and the capillary wall. Once this hypothesis was tested it was apparent that the cells were being trapped in the capillary.

3.3  Procedure for Sample Loading and Pressurization

Cells are loaded into the pressure system via a loading point, in this case a high pressure T-connection, on Reservoir One in Figure 3.1. Another solution such as phosphate buffered saline, PBS, or potassium cyanide, KCN, dissolved in PBS is loaded into Reservoir Two. Once the system is sealed an optical excitation fiber is placed at a low angle above the pressure chamber.

The system is then pressurized using the hand-cranked pressure generator. Once the desired pressure has been reached Generator One begins contracting while Generator Two retracts in order to keep the pressure static and the medium flowing. Control of this process is done via a Labview program written by Huff and is shown below in Appendix G [24]. The sample is then excited using an excitation fiber and the resulting emission is collected and the spectrum is analyzed. Collection of the spectra is done via a microscope coupled with an optical fiber. This transfers light to the ICCD which is controlled using an Andor script included below in Appendix C.

The reservoirs, as shown below in Figure 3.1, can be blocked at the driving end of the system using a high pressure valve. While a valve is closed, the medium in the associated reservoir is isolated from the cell while still allowing for the medium to be pressurized via the opposite end of the reservoir. By changing which of these valves is open, the medium the cells are exposed to can be changed. This system also allows for the introduction of media containing different chemicals to trapped cells while still maintaining static pressure and perfusion.

3.4  High Pressure Perfusion System Characterization

In order to confirm control of samples, reservoirs, perfusion, and pressure it was necessary to conduct several preliminary characterization experiments. These included
confirming that cells can be trapped and remain trapped in the pressure chamber during reservoir changes, determining the flow rate of the perfusion system with respect to jog speed observing the ability to switch between reservoirs, and confirming that all of the above can be done under high pressure.

3.4.1 Confirmation of Ability to Trap Cells

We began by showing that we could indeed trap cells, as in Figure 3.5, without the use of a quartz fiber plug in the pressure chamber. The graph shows two main aspects of trapping cells without the use of the quartz fiber. The first of these is that there is a delay between the reservoir switching process, the closing of one reservoir and opening of the other, and the arrival of the second medium. The second is that once a different medium arrives at the excitation point the intensity begins to drop off to a constant value. This is most likely a result of some cells in the not becoming trapped and moving through the pressure chamber uninhibited. Upon closer inspection of the chamber via use of a microscope we observed some of the cells adhering to the walls of the capillary while the rest of the trapped cells appear to be suspended within the capillary. The reason for this has yet to be determined.

3.4.2 Determination of Relationship Between Jog Speed and Flow Rate

Once we determined that we could trap cells while the system was undergoing perfusion we needed to determine the relationship between the jog speed, the speed at which the stepper motors rotate, and the flow of liquid through the system. This was done by filling up the pressure generator with a 50/50 mixture of ethanol and water and then measuring the amount of liquid expelled from the generator during a certain amount of time at various jog speeds. The flow rate was then average over several trials to find that a jog speed of 1 on the manufacturer’s control software corresponds to a flow rate of 0.00132mL/s. The relationship we found was linear though it differed slightly from that previously calculated by Huff, 0.0016ml/s [24]. This allowed for more accurate control flow rates for the perfusion of the system and opened the door for future examination of the effects of flow rates on metabolism.
3.4.3 Determining Level of Control Over Switching Between Reservoirs

Next we moved on to determining the level of control we had over switching between the separate reservoirs by filling them with 5µM solutions of the standard fluorophores 9-CA and POPOP. Both of these fluorophore solutions were loaded into separate reservoirs and the pressure generator was set to a flow rate of 0.005mL/s. In order to monitor transition between samples under flow we used an Andor script which took a spectrum every 15 seconds, shown in Appendix C, producing the graphs below in Figure 3.6.

It took ~21 minutes for the 9-CA to arrive at the chamber. At the flow rate stated above, this is several minutes longer than the time determined using the inner diameter, length, and flow rate of the perfusion system. Initially the discrepancy seemed to be a calibration issue with the flow rate however this seems to occur only if the system is not pressurized before monitoring occurs. This leads us to believe that during the pressurization process, the time during which the system is brought from ambient pressure to the desired pressure, there is little to no flow of the fluids in the perfusion system. This is most likely due to the differences in compressibility of air and the pressurizing medium. Before pressurization the bubbles in the system take up a lot of space. During pressurization however, the bubbles compress much more than the pressurizing medium. As a result the pressurizing medium will not begin to flow until the volume change of the bubbles is very small.

3.4.4 Examination of Diffusion Rates

Before pressurization occurs the liquids in the system are static and will diffuse at the boundaries between them as in Figure 3.7. Ideally the interface between the pressurizing media and the contents of the reservoir would be a step function with no mixing. This is important because if the diffusion rate is too high then the pressurizing media, containing ethanol and water, or the contents of the other reservoirs could change the metabolic properties of the cells prematurely. Once it was established that we had control over switching between the reservoirs we needed to confirm that the diffusion rates between the media were small.

In order to get an idea of what diffusion rates we could expect we used a 1D diffusion model. The equation in question was [25]:

\[
C(x, t) = \frac{c_0}{2} \text{erfc} \left( \frac{x}{2\sqrt{Dt}} \right)
\]

Equation 3.1
Where \( C_0 \) is the initial concentration of the liquid at a certain position \( x \), \( \text{erfc} \) is the complementary error function, \( D \) is the diffusion constant of the liquid, and \( t \) is time. Using this, Fick’s First Law, diffusion constants of fluids similar to those used, and the number of moles per unit volume of sample in the perfusion system we calculate diffusion rates on the order of \( 10^{-6} \text{m/s} \). This is small when compared the rate of flow results which in a translational speed on the order of \( 10^{-2} \text{m/s} \) in through the perfusion system; therefore, assuming the model is accurate there is more mixing of the liquids at their interfaces due to mechanical mixing than diffusion.

In order to confirm this we conducted several trials using the standard fluorophores 9-CA and POPOP. In these trials the reservoirs were filled with solutions containing the standard fluorophores at 10µM concentrations and flowed through the system while we monitored a fixed point in the pressure chamber. Once one fluorophore arrived at the monitoring point we switched between the reservoirs in order to allow the second fluorophore to flow through the system.

Figure 3.8 is an example of the difference in the level of mixing at different pressures and times. In this case the same procedure was followed as above which produced Figure 3.6 except the perfusion system was set to pressures ranging from 0-400 atm. When the trials were completed we found that the level of mixing in the perfusion system appears to depend primarily on how much the system was agitated during the data collection process. However, it does not seem to be dependent on the pressure level inside of the perfusion system or the time the fluorophores remained in the system.

In Figure 3.6, 9-CA arrived at the pressure chamber first and the POPOP arrived at the chamber second after ~20 min after the 9-CA intensity began to sharply decline. In Figure 3.8 however we notice that the fluorophore at 100 atm takes significantly longer to reach its maximum intensity than that of either the 0 atm or 400 atm trials. There are several differences between these trials aside from pressure. The first of these is that the intensity increase in the 0 atm trial was observed at a much earlier time than that of the other two trials. This is primarily due to the amount of time it takes to pressurize the system. However, although the 100 atm and 400 atm trial took place at similar times after the start of the perfusion system, the 400 atm intensity increase occurring several minutes later than the 100 atm trial, the 100 atm trial seems to exhibit more mixing at the interface. This suggests that the level of mixing is not as strongly dependent on time or pressure as it is to other factors. In this case we believe that most of the
mixing is a result of agitation of the pressurizing medium due to the opening and closing of the valves. This occurred more in the 100 atm trial than that of either of the other two trials.
Figure 3.1: Schematic and photos of the high pressure perfusion system.

Figure 3.1a
Figure 3.1b

Figure 3.1c
Figure 3.2: Schematic and photos of the pressure chamber.

Figure 3.2a
Figure 3.3: Photo showing the low angle the excitation fiber must obtain in order to remove artifacts.
Figure 3.4: Graph showing the overlap of the spectra of quartz fiber and NADH.

Figure 3.5: Graph showing the intensity of cells before and after the arrival of PBS in the pressure chamber of the perfusion system.
Figure 3.6: Graph of two different fluorophores in solution as they travel through the perfusion system. This shows the control we have over the different reservoirs within the perfusion system.
Figure 3.7: This Figure shows the mixing region between two media. The mixing region corresponds to the interface between the two media where we see an increase in intensity in Figure 3.8.

Figure 3.8: Graph showing the difference in diffusion over time at several different pressures. It seems that the mixing of the interfaces depends more on the amount of mixing done by the opening and closing than by time or pressure. During the 100 atm trial, the fluid was agitated more than in the other two and was observed at an earlier time than the 400 atm trial.
Chapter 4: Application of Techniques to Metabolic Monitoring

4.1 Overview

This chapter discusses techniques as they are used in the monitoring of biological metabolism. Section two shows the control experiments conducted prior to implementing the protocols developed for this thesis. In sections two and three we implement those protocols for the monitoring of biological monitoring. Each section includes a brief description of how the data was collected and examples of representative data for each type of collection method.

4.2 Control

Upon completion of characterization of the systems described above we began applying techniques to the study of yeast. The first step of this process was to confirm the response of yeast to different stimuli. As mentioned previously, NADH, the source of the fluorescence we are monitoring in cells, exists in two states, protein-bound and free. The protein-bound form has a bluer emission peak, as shown below in Figure 4.1, and a longer excited state lifetime than the free form [16].

*S. cerevisiae*, baker’s yeast, was grown at 25°C for 48 hour on a solid YPD agar medium (1.0% yeast extract, 2.0% peptone, 2.0% agar, 2.0% glucose). The cells were triple-washed in PBS (phosphate buffered saline) (pH 7.2) in order to remove the growth medium from the cells. Cells were then resuspended and 1mL of the cell suspension was pipetted into a quartz cuvette containing 1mL of PBS. Previously we have observed cells’, prepared via this method, reaction to potassium cyanide (KCN) at an 8µM concentration as shown below in Figure 4.2.

As one can see, there seems to be an overall redshift in the intensity-weighted average wavelength of the solution and an increase in intensity which may be caused by a shift in the relative concentrations of the bound and free forms of NADH.
4.3 Nanosecond Gated Spectroscopy

4.3.1 Metabolic Sensing with Intensity Profiles at Atmospheric Pressure

Once the cellular response was confirmed we began conducting trials of the nanosecond gated technique on yeast cells suspended in PBS in a quartz cuvette. Unfortunately the data which has been gathered using yeast suspended in PBS has a much lower signal to noise ratio, Figure 4.3, than that of the standard fluorophores POPOP and 9-CA. As a result the spectra gathered have more uncertainty associated with them than that of the standard fluorophores. In Figure 4.4 we see example intensity profiles of NADH response to KCN.

The before and after label in the key of this graph refers to spectra taken before and after adding potassium cyanide (KCN), at a final concentration of 8µM, to yeast suspended in PBS. Here we see a slight difference in the excited state lifetime before and after the addition of the KCN which matches what we expected if there is indeed a shift in the relative concentrations of the two forms of NADH toward the unbound from. This sort of measurement is promising because it shows that we can detect small shifts in the spectra even with a much weaker signal and poor spectral resolution. In Figure 4.5 we see an example of the intensity-weighted average wavelength associated with the profiles in Figure 4.4. As one can see, the addition of KCN to the yeast resulted in an overall redshift in the intensity-weighted average wavelength of the yeast which may be due to a higher concentration of unbound NADH in relation to the bound NADH.

4.3.2 Metabolic Sensing Using the Two-Gating Scheme at Atmospheric Pressure

Another protocol used during the course of this project is conducted using a two-gated scheme for real time measurements of sample. Instead of taking profiles of the sample in question we can take spectra at two separate gate delays as in Figure 2.5 which correspond to two different pools of fluorophores. The earlier gate here would be sensitive to both conformation of NADH as both are present in the early gate. Because of its longer excited state lifetime however, the bound form of NADH is the only form present in the later gate. This makes the later gate valuable in the sensing of changes in only the bound pool of NADH.

Unlike the shifts seen in section 4.3.1, when using the two-gating scheme we observed a blueshift after the addition of KCN, the reason for this is beyond the scope of this thesis. When
we observe a shift in the intensity-weighted average wavelength of any kind we expect there to be a difference in the ratio of intensities of the two gate delays when comparing them before and after the addition of KCN to the yeast solution. In the case of a blueshift the difference in the ratio of intensities is due to the higher concentration of the bound NADH with respect to the unbound NADH after the addition of KCN.

As mentioned before the bound form of NADH has a bluer intensity-weighted average wavelength and a longer excited state lifetime than that of the unbound form of NADH. As a result, if the ratio of the bound to the unbound forms of NADH were to increase, as they do when KCN is added to a solution containing yeast, the amount of the bound form still emitting light at a longer gate delay will also increase. This implies that if we take spectra at two different gate delays, one just after the profiles peak intensity and one at a gate delay several nanoseconds later, we should find that after the addition of KCN the later gate delay will have a higher intensity relative to the early gate delay than before the addition of KCN. Figure 4.6 shows this sort of gating scheme using previously collected data.

Notice that although both of the intensities increased after the addition of cyanide the difference between the two intensities changed. The ratio of the intensities, early gate to late gate, before the addition of KCN was $\approx 1.25$ and after the addition of KCN was $\approx 1.17$. This would correspond to a shift of relative concentrations for the two forms of NADH to the form with the longer excited state lifetime, the bound form, as we expected. Once it was determined that the information we wanted had potential we implemented this two-gated data collection scheme in a way which would allow for the real time monitoring of fluorophores in solution. In light of this we wrote an Andor script, shown in Appendix B, in order to easily collect this data when it is coupled with the Arduino controller. It allows for the two gate times to be changed independently of one another in order to maximize usable signal. Some example trials are shown below in Figure 4.7.

Note for the intensity graph the first portion of the graph was scaled to the same intensity in order to better detect any changes in the relative ratios of the different gate delays. As one can see, after the addition of KCN we see an approximately 20% increase in the relative intensity of the later gate. This again implies a shift to the bound form of NADH which is reinforced by the blueshift observed in the center of mass wavelength.
4.4 Metabolic Monitoring Using the High Pressure Perfusion System

4.4.1 Control: Monitoring Switch Between Fluids with No Cell Response.

Once we demonstrated the efficacy of nanosecond-gated spectroscopy we began to study the yeast in the perfusion system. The cells were first prepared using the same method as in the nanosecond-gated spectroscopy project and then loaded into the perfusion system in reservoir 1. In reservoir 2 we loaded a 5µM solution of Rhodamine B suspended in PBS. Rhodamine B was chosen as a marker in this case for 2 reasons. The first is be sure that the PBS had arrived from the reservoir. As the PBS does not fluoresce under our lasers excitation is crucial to confirming that PBS had arrived at the sampling point. Secondly Rhodamine B fluoresces on the red side of the visible spectrum thus minimizing any overlap with the NADH spectrum. Some representative data of this kind is shown below in Figure 4.8.

In region A the flow is stopped for ~27 min. During this time we see a slight blueshift in the intensity-weighted average wavelength. In previous experiments conducted in the lab we have observed a change in the NADH fluorescence as a result of oxygen concentration [26]. This suggests that the shift observed here is most likely due to the yeast using up the available oxygen in the environment. Once the shift had stopped the flow was rate was set to 0.001mL/s in order to flow in fresh PBS. In region B we see an increase in intensity most likely to an influx of new cells to the monitoring point within the pressure chamber. Finally in region C, we see the arrival of PBS to the monitoring point. This is confirmed by observation of the Rhodamine B signal shown in Figure 4.8. As one can see, there may be slight shift in the intensity-weighted average wavelength which could be due to the oxygen concentration changing. There is also a decrease in the intensity in region C which is to be expected as some of the cells move out of the monitoring area.

4.4.2 Metabolic Sensing in the High Pressure Perfusion System

Once the control experiments had been completed we began work on the detection of metabolic changes in the cells. This was done in the same way as the method described previously with the slight change of 8µM KCN being added to the PBS/Rhodamine B solution. An example trial of this is shown below in Figure 4.9. Here we see similar results to those done
previously in the cuvette trials. There is indeed a blueshift in the intensity-weighted average wavelength of the sample which corresponds with an increase in the intensity in the second 512 pixels, signaling the arrival of the Rhodamine B and therefore the KCN. There is an additional secondary blueshift, however, which we do not know the cause of. Though, because the flow of the perfusion system was stopped upon the arrival of the KCN, this could be due to the depletion of nutrients within the pressure chamber.
Figure 4.1: This shows example spectra of both the free and protein bound forms of NADH. NADH concentration of 25µM in PBS for free NADH and in excess malate dehydrogenase for protein bound NADH.

Figure 4.2: Graph showing the response of yeast cells suspended in PBS to KCN.
Figure 4.3: Example of yeast spectra obtained at 2 min and 15 min during the collection of data the KCN trial shown in Figure 4.2.

Figure 4.4: Graph showing the difference in excited-state lifetimes before and after the addition of KCN to yeast. Here, after the addition of KCN we observe a shorter excited-state lifetime.
Figure 4.5: Intensity-weighted average wavelength of yeast in solution before and after the addition of KCN gathered via the nanosecond gated spectroscopic technique.

Figure 4.6: Simulation of overall intensity gathered at two separate detector gates both before and after the addition of KCN.
Figure 4.7: This is representative data of the two-gated scheme obtained in real time. The intensity in the top graph has been scaled to 1 for the time before the addition of KCN to the sample. Here (arb) refers to equal time steps of approximately 15s each.
Figure 4.8: Graphs showing the monitoring of cells within the perfusion system while the flow of the system is manipulated. Example spectra of region B and C are included.
Figure 4.8 continued
Figure 4.9: Graphs showing the metabolic response of cells as they are introduced to cyanide within the perfusion system.
Chapter 5: Conclusions and Future Work

5.1 Conclusions

This thesis details the construction and application of several methods of biological monitoring for use under high hydrostatic pressures. The primary goal was to show the usefulness and potential of these techniques in order to allow for future study of biological metabolism as well as chemicals of various kinds in solution. The results of this thesis and future work which could be done using these techniques are discussed below.

The primary conclusions of this thesis are two-fold. Using these techniques and systems we are able to detect changes within cells due to chemical stimuli inside our high pressure perfusion system, and we are able to monitor the solutions in a time resolve way. We have demonstrated the ability to distinguish between fluorophores based solely on their excited state lifetimes, chapter 2.4.4. We have shown that we can detect differences in ratios of different detector gates in real time and detect difference in the excited state lifetimes of fluorophores in solution, chapter 4.3. We can also gain some information as to the relative concentrations of these fluorophores in solution when comparing multiple samples, chapter 2.4.4. In addition we have demonstrated our ability to monitor metabolic changes in cells through the addition of chemical stimuli in our perfusion system while maintaining flow, chapter 4.4.

5.2 Future Work

The techniques developed during the course of this project have many more potential applications than those discussed at length in this thesis. Fluorescence spectroscopy is an incredibly useful noninvasive probe for the monitoring of biological processes among other things. The ability of our system to gather data in a time resolved way opens up another level of information which could potentially be used to distinguish between fluorophores and their states using their excited state lifetime. This will allow for a more robust data collection scheme which measures changes in a sample in multiple ways and therefore gives more evidence to the validity of certain theoretical models of what could be causing those changes. More work will need to be done in order to improve the high pressure perfusion system to allow for the collection of cleaner spectra and make the data collection itself more efficient. The ability of this system has been
shown however and will allow for future study of biological metabolism as it is affected by high hydrostatic pressures.
References


Appendix A: Andor Profile Script

LoadConfig("\CUL038780-1\Lab Member Documents\Lab Member Documents\Zac Long\Research\Configuration Files\100726-(3HBIN)(400-650nm)(1-800-microm)(50um-slit).cfg")

cooler(1) : rem sets cooler to on

SetTemperature(-5) : rem sets cooler to -5(C)

SetAcquisitionMode(2) : rem single scan

SetGateMode(4) : rem Turn off the gate when taking the background

SetTriggerMode(1) : rem Sets Trigger mode to External

SetGain(50) : rem Sets gain to number 0-255

SetAcquisitionMode(2) : rem Sets to Acumulate

SetAccumulateNumber(5) : rem sets Accumulate to 5

SetAcquisitionType(1) : rem take background

run()

SetGateMode(5) : rem DDG on;

SetDataType(2) : rem Sets data to counts(background corrected)

SetAcquisitionType(0) : rem Take signal;

dset=0
i=0
inittm=75000 : rem Initial gate delay (ps)
init=75 : rem Initial gate delay (ns)
deltm=1000 : rem Initial gate width (ps)
del=1 : rem Initial gate width (ns)
zero$="0"
zeros$="00"
path2$ = "C:\Users\longzc\Desktop\NEW DATA\Profile\"
path1$ = "C:\Users\longzc\Desktop\NEW DATA\Profile\ascii\"
tm$ = "time_"
asc$ = ".asc"
sif$ = ".sif"
dot$ = "."

while i<100

    print(inittm)
    SetDDG(2,0,inittm,deltm,0)
    run()
    Q=del
    Q$=str$(Q)
    R=init
    R$=str$(R)
    fname$=str$(init)
    R1=floor(R)

    if (R==R1) then
        fname1$=path1$ + tm$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + asc$
        fname2$=path2$ + tm$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + sif$
    else
        if(R-R1==.5) then
            fname1$=path1$ + tm$ + R$ + zero$ + dot$ + zero$ + Q$ + asc$
            fname2$=path2$ + tm$ + R$ + zero$ + dot$ + zero$ + Q$ + sif$
        else
            fname1$=path1$ + tm$ + R$ + dot$ + zero$ + Q$ + asc$
            fname2$=path2$ + tm$ + R$ + dot$ + zero$ + Q$ + sif$
        endif
    endif

    print(fname1$)
    SaveAsciiXY(#dset,fname1$,2) :rem set the delimilator as tab;
    Save(#dset,fname2$)
    CloseWindow(#1)
    print(GetGatePulseDelay())
    i=i+1
    inittm=inittm+250
init=init+.25

wend
print("end")
Appendix B: Andor Two-Gate Script

LoadConfig("CUL038780-1\Lab Member Documents\Lab Member Documents\Zac Long\Research\Configuration Files\100726-(3HBIN)(400-650nm)(1-800-microm)(50um-slit).cfg")

cooler(1) : rem sets cooler to on
SetTemperature(-5) : rem sets cooler to -5(C)
SetAcquisitionMode(2) : rem single scan
SetGateMode(4) : rem Turn off the gate when taking the background
SetTriggerMode(1) : rem Sets Trigger mode to External
SetGain(50) : rem Sets gain to number 0-255
SetAcquisitionMode(2) : rem Sets to Acumulate
SetAccumulateNumber(5) : rem sets Accumulate to 5
SetAcquisitionType(1) : rem take background

run()

SetGateMode(5) : rem DDG on;
SetDataType(2) : rem Sets data to counts(background corrected)
SetAcquisitionType(0) : rem Take signal;

dset=0
i=0
inittm=0 : rem Initial gate delay (ps)
init=0 : rem Initial gate delay (ns)
deltm=600000 : rem Initial gate width (ps)
del=600 : rem Initial gate width (ns)
zeros$="0"
zero$="00"
path2$ = "C:\Users\longzc\Desktop\NEW DATA\Profile"
path1$ = "C:\Users\longzc\Desktop\NEW DATA\Profile\ascii"
inittm1=88000 : rem Initial gate delay (ps)
deltm1=5000 : rem Initial gate width (ps)
inittm2=92000 : rem Initial gate delay (ps)
deltm2=20000 : rem Initial gate width (ps)
stepsizetm=1000 : rem Step size (ps)
stepsize=1 : rem Step size (ns)
path6$ = "C:\Users\longzc\Desktop\NEW DATA\Early Gate\"
path5$ = "C:\Users\longzc\Desktop\NEW DATA\Early Gate\ascii\"
path4$ = "C:\Users\longzc\Desktop\NEW DATA\Late Gate\"
path3$ = "C:\Users\longzc\Desktop\NEW DATA\Late Gate\ascii\"

tm$ = "time_"
asc$ = ".asc"
sif$ = ".sif"
dot$ = "."

while i<1000

SetDDG(2,0,initm,deltm,0)

print(initm)
run()

Q=del
Q$=str$(Q)

R=init
RS=str$(R)
fname$=str$(init)
R1=floor(R)

if (i<10) then

    fname1$=path1$ + tm$ + zero$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + asc$
fname2$=path2$ + tm$ + zero$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + sif$
else

    if (R==R1) then

        fname1$=path1$ + tm$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + asc$
        fname2$=path2$ + tm$ + R$ + dot$ + zeros$ + dot$ + zero$ + Q$ + sif$
    else

        + asc$
        + sif$
    else

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if(R-R1==.5) then
fname1$=path1$ + tm$ + R$ + zero$ + dot$ + zero$ + Q$ + asc$
fname2$=path2$ + tm$ + R$ + zero$ + dot$ + zero$ + Q$ + sif$
else
fname1$=path1$ + tm$ + R$ + dot$ + zero$ + Q$ + asc$
fname2$=path2$ + tm$ + R$ + dot$ + zero$ + Q$ + sif$
endif
endif
print (i)
print(fname1$)
SaveAsciiXY(#dset,fname1$,2) :rem set the delimiter as tab;
Save(#dset,fname2$)
CloseWindow(#1)
SetDDG(2,0,inittm1,deltm1,0)
run()
P=i
P$=str$(P)
if i<10 then
fname1$=path5$ + tm$ + zero$ + P$ + asc$
fname2$=path6$ + tm$ + zero$ + P$ + sif$
else
fname1$=path5$ + tm$ + P$ + asc$
fname2$=path6$ + tm$ + P$ + sif$
endif
print(fname1$)
SaveAsciiXY(#dset,fname1$,2) :rem set the delimiter as tab;
Save(#dset,fname2$)
CloseWindow(#1)
print(GetGatePulseDelay())

print(inittm2)  :rem gate 2 start

SetDDG(2,0,inittm2,deltm2,0)

run()

if i<10 then
    fname1$=path3$ + tm$ + zero$ + P$ + asc$
    fname2$=path4$ + tm$ + zero$ + P$ + sif$
else
    fname1$=path3$ + tm$ + P$ + asc$
    fname2$=path4$ + tm$ + P$ + sif$
endif

print(fname1$)

SaveAsciiXY(#dset,fname1$,2)  :rem set the delimilator as tab;

Save(#dset,fname2$)

CloseWindow(#1)

i=i+1

init=init+1
wend
print("end")
Appendix C: Andor Real-Time Monitoring of Intensity-weighted average wavelength and Intensity

LoadConfig("C:\Users\Public\Desktop\ICCD-CalibrationSpectra\100726-(400-650nm)(1-800-microm)(50um-slit).cfg")

SetAcquisitionMode(2) : rem Accumulate Mode
SetAccumulateNumber(5): rem AccumulateNumber

SetGateMode(4) : rem Turn off the gate when taking the background

SetAcquisitionType(1) :rem background

run()

delay(1000)
beep()
dset=0
i=0
temp=0
path2$ = "C:\Users\longzc\Desktop\NEW DATA\"
path1$ = "C:\Users\longzc\Desktop\NEW DATA ascii"
tm$ = "time_"
asc$ = ".asc"
sif$ = ".sif"
dot$ = "."
SetGateMode(5) : rem Gating is controlled from FIRE signal only

while i<5000
    t=0
    SetDataType(2)
    SetAcquisitionType(0)
    :rem Take signal

    run()
    delay(500)
    :rem fname$=str$(time())
    fname$=time$()
    hh$ = left$(fname$,2)
    mm$ = mid$(fname$,4,2)
    ss$ = mid$(fname$,7,2)

    hh=val(hh$) :rem Converting a text string to floating number

    if(hh<temp) then
        hh=hh+24
    endif

    hh$=str$(hh)
    fname1$ = path1$ + tm$ + hh$ + dot$ + mm$ + dot$ + ss$ + asc$
    :rem define the file path and name;
    fname2$ = path2$ + tm$ + hh$ + dot$ + mm$ + dot$ + ss$ + sif$

    print(fname1$)
SaveAsciiXY(#dset,fname1$,2) :rem set the deliminator as tab;

Save(#dset,fname2$)
CloseWindow(#1)

while t<15
    delay(1000)
    tu=-t+29
    print("Time Remaining: ",tu," sec")
    t=t+1
wend

i=i+1

temp=hh
wend
print("end")
Appendix D: Labview Center of Mass Wavelength/Profile Calculator

Instructions
1) Set 'Refresh interval' and 'Pixel range.' Default values should work in most situations.
2) Set 'Folder Path.' Folder should contain files with names 'time_HH.MM.SS.asc.'
3) Click 'Run.'
4) Click 'Stop and Save' to stop the vi and save graph output to a file.

Version history
v1 - 06/11/12, J. Cheng.
v2 - 06/12/12, P. Urayama. Changed GUI streamline code to be more flexibility in Matlab coding.
v3 - 06/14/12, P. Urayama. Corrected display of time axes. Changed intensity units to be average detector counts.
Appendix E: Labview Two-Gate and Profile Calculator
const int GledPin = 9;
const int RledPin = 10;
const int FirePin = 13;

int GledState = LOW;
int RledState = LOW;
int FireState = LOW;

long previousMillis = 0;
int i = 1;
int j = 1;

long interval = 333;
long GdelayInterval = 10000;
long RdelayInterval = 2000;

void setup() {
  pinMode(FirePin, OUTPUT);
  pinMode(GledPin, OUTPUT);
  pinMode(RledPin, OUTPUT);
void loop()
{
    do
    {
        do
        {
            unsigned long currentMillis = millis();

            if((currentMillis - previousMillis > interval)) {
                // save the last time you blinked the LED
                previousMillis = currentMillis;

                // if the LED is off turn it on and vice-versa:
                if (FireState == LOW)
                    FireState = HIGH;
                else
                    FireState = LOW;

                // set the LED with the ledState of the variable:
                digitalWrite(FirePin, FireState);
                i++;
            }while (i < 21);

            digitalWrite(GledPin, HIGH);   // sets the LED on
delay(10000);                  // waits for 10 seconds
digitalWrite(GledPin, LOW);    // sets the LED off
digitalWrite(RledPin, HIGH);   // sets the LED on
delay(2000);                   // waits for 2 seconds
digitalWrite(RledPin, LOW);    // sets the LED off

            j++;
            i = 1;
        }while (j < 1000);
    }while (j < 1000);
    digitalWrite(FireState, LOW);
    delay(10000000);
}
Appendix G: Lab-View Perfusion System Control
Appendix H: How to Make a High Pressure Chamber

1. Cut a 12” long 1/8” diameter nipple in half
2. Drill holes using tungsten drill bits to 1”
3. Attach to pressure system and flow through ethanol/water mixture to clean and confirm that the nipple is not blocked
4. Allow to dry
5. Stretch glass tubes using propane torch to until approximately 4” of thin glass tube is present
6. Break in half using diamond stylus
7. Mix epoxy at 50/50 in weigh boat
8. Apply epoxy to area approximately 2/3 from sharp end of glass rod
9. Insert into the 1.5 OD 0.5 ID capillary and twist for even coating
10. Break off glass rod leaving a very short, approximately 2mm piece, sticking out of capillary
11. Apply epoxy to the outside of the capillary approximately 1/4” from the end
12. Insert both sides into nipples and twist for even coating
13. Generously apply epoxy to inside of aluminum supports
14. Place capillary/nipples in the thick aluminum support
15. Place the thin aluminum support on top with the glass insert on the right and the ground area facing you
16. Insert 4 support screws
17. Allow to dry for 24 hours
18. Attach to pressure system to test for pressure and flow