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Intra-cardiac Side Firing Light Catheter for Monitoring Cellular Metabolism using Transmural Absorbance Spectroscopy of Perfused Mammalian Hearts --Manuscript Draft--

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Dear Dr. Steindel,

Attached please find our second revision of manuscript JoVE58992.

We have tried to make all of the suggestions made by the Editorial staff. We will have added a supplemental file containing the executable LABVIEW program to collect and analyze spectral data. This program will require a LabView license as we have stated without referring to LabView specifically. Following your earlier instructions we have downloaded this at the DropBox location <https://www.dropbox.com/request/zyMZ7gB45AcBKrUE6OYa> as requested. We have decided not to include the source code since compiling the program with appropriate drivers is not trivial and would require some expertise. If authors wish source code we will provide along with detailed instructions on compilation. We have confirmed that all drivers are open source with a LabView license.

We look forward to any further comments.

Sincerely for the authors,

Robert S. Balaban.

TITLE:

Intra-cardiac Side-Firing Light Catheter for Monitoring Cellular Metabolism using Transmural Absorbance Spectroscopy of Perfused Mammalian Hearts.

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KEYWORDS

Heart function, heart metabolism, mitochondria, myoglobin, cytochrome oxidase, cytochrome c, cytochrome b, FAD, mitochondria membrane potential, tissue oxygenation, optical spectroscopy, spectral fitting

SUMMARY:

Here we introduce a method for using an intra-ventricle optical catheter in perfused hearts to perform absorbance spectroscopy across the heart wall. The data obtained provides robust information on tissue oxygen tension as well as substrate utilization and membrane potential simultaneously with cardiac performance measures in this ubiquitous preparation.

ABSTRACT:

Absorbance spectroscopy of cardiac muscle provides non-destructive assessment of cytosolic and mitochondrial oxygenation via myoglobin and cytochrome absorbance respectively. In addition, numerous aspects of the mitochondrial metabolic status such as membrane potential and substrate entry can also be estimated. To perform cardiac wall transmission optical spectroscopy, a commercially available side-firing optical fiber catheter is placed in the left ventricle of the isolated perfused heart as a light source. Light passing through the heart wall is collected with an external optical fiber to perform optical spectroscopy of the heart in near real-time. The transmission approach avoids numerous surface scattering interference occurring in widely used reflection approaches. Changes in transmural absorbance spectra were deconvolved using a library of chromophore reference spectra, providing quantitative measures of all known the cardiac chromophores simultaneously. This spectral deconvolution approach eliminated intrinsic errors that may result from using common dual wavelength methods applied to overlapping absorbance spectra, as well as provided a quantitative evaluation of the goodness of

fit. A custom program was designed for data acquisition and analysis, which permitted the investigator to monitor the metabolic state of the preparation during the experiment. These relatively simple additions to the standard heart perfusion system provide a unique insight into the metabolic state of the heart wall in addition to conventional measures of contraction, perfusion, and substrate/oxygen extraction.

INTRODUCTION:

Optical absorbance spectroscopy for monitoring intact organ biochemistry is a widely-used approach due to its intrinsic, non-destructive nature¹⁻⁹. Myoglobin absorbance provides a measure of the average cytosolic oxygen tension¹⁰⁻¹². Mitochondrial cytochromes provide information regarding substrate entry at the level of flavins, membrane potential from cytochrome $b_L:b_H$ ¹³, and oxygen delivery to the mitochondria in the cell from cytochrome oxidase (COX) redox state¹⁴. Glancy et al. demonstrated that the activities of each complexes can be determined by measuring the mitochondrial membrane potential and the metabolic rate¹⁵. Hence, using optical spectroscopy, a wealth of information can be obtained without the need of exogenous probes or major modifications of current study systems. The goal of this paper is to present a robust method for collecting transmission optical spectra in conventional perfused heart preparations with the only major modification being performing studies in a darkened environment.

Reflectance absorption spectroscopy has been successfully used to perform optical spectroscopy of the perfused heart^{3,6,16-19} as well as the heart in vivo¹. Reflectance spectroscopy consists of impinging light on the heart surface and collecting the light scattered through the heart as well as diffuse and specular reflected light. Thus, the collected light in this approach is a composite of multiple scattering mechanisms as well as the tissue chromophore absorbances of interest. Due to the motion and complex surface of the heart, the light reflection off the surface of the heart is particularly problematic, altering the depth of penetration and amount of purely reflected light.

The limitations of reflectance absorption spectroscopy presented above were resolved by introducing an optical catheter into the left ventricle cavity, permitting the collection of transmitted light across the left ventricle free wall²⁰. The benefits of transmission spectroscopy for this type of study was appreciated in early invasive studies by Tamura et al.⁹ The current implementation provides a very robust transmission absorption spectroscopy analysis of the intact heart with regards to cytosolic oxygenation and mitochondria redox state under a variety of conditions²¹. These initial studies used a specially fabricated catheter with a powered LED on the tip oriented to generate a side-firing pattern of white light through the myocardium. However, the relatively large LED tipped catheter is only appropriate for use on medium size hearts (rabbit, guinea pig, etc.) and required custom fabrication. In the current study, a method of using a commercially available 200-micron core side-firing optical fiber as a light guide is presented. Instead of a wired LED at the tip, the catheter with the 500-micro tip redirects light from an external source increasing the versatility of the system. This approach allows the use of a wide variety of external light sources including lasers for applications such as Raman scattering spectroscopy. To quantify this data, an online full multicomponent spectral analysis using known reference spectra to improve the accuracy of the spectroscopic determination of cardiac

chromophores is presented as previously described^{20,22}. The source code for this analysis will be provided by the authors upon request. Using this approach, information on cardiac biochemistry and mitochondrial function can be obtained simultaneously with the conventional cardiac functional parameters with little or no impact on the heart preparation. As the heart is critically dependent on mitochondrial function and oxygen delivery, this technical addition to the classical perfused heart system will greatly improve the interpretation and utility of this important model of cardiac performance.

PROTOCOL

All animal protocols were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee and performed in accordance with the guidelines described in the Animal Care and Welfare Act (7 USC 2142 § 13).

1. Isolated Perfused Heart System and Perfusate

NOTE: This preparation is very similar to previous publications²³.

1.1. Make 4 liters of modified Krebs-Henseleit perfusate composed of (in mmol/L) 137.0 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 1.0 Na₂HPO₄, 10.0 glucose, 1.0 lactate, and 10.0 HEPES.

1.2. pH the perfusate to 7.4 at 37 °C with NaOH and HCl.

1.3. Filter the perfusate through a 1 µm pore membrane.

1.4. Rinse all tubes and chambers of the perfused heart system by running and draining purified water through the system.

1.5. Transfer the perfusate into the tank and oxygenate with 100% O₂ with the bubbler while maintaining the temperature at 37 °C using a heated circulating water bath.

1.6. Add 2 of 12 µm pore membrane filters and prime the system with the perfusate while recirculating in the Langendorff mode.

1.7. Attach a tubing clamp on the tube right above the aortic cannula and adjust the screw so that the aortic flow drops to about 10 mL/min.

2. Rabbit Heart Excision and Perfusion

2.1. Heart excision

2.1.1. Anesthetize male New Zealand white rabbits (about 3 kg) via a 1.5 mL intramuscular injection of ketamine/acepromazine mixture (10:1).

2.1.2. Approximately 10–15 minutes later, administer 3% isoflurane via inhalation for a complete anesthetic effect.

2.1.3. Confirm proper depth of anesthesia by toe pinching and then place a line in the marginal ear vein for administration of subsequent drugs.

2.1.4. Inject 1,500 units (or 1.5 mL of 1,000 units/mL) of Heparin and let circulate for 3 minutes.

2.1.5. Double check proper depth of anesthesia and then euthanize with 6 mEq (or 3 mL of 2 mEq/mL) of KCl.

2.1.6. Rapidly open the chest, locate the heart's apex and the aorta. Remove the heart by cutting the aorta as far from the heart as possible and cutting the pulmonary veins as close to the lungs as possible.

NOTE: Removal of lungs at this early stage is different than previous publication²³ but has not impact on preparation.

2.1.7. Place the heart in a small beaker of perfusate (same perfusate as step 1.3) sitting in a bucket of ice for transportation from surgery to perfusion.

2.2. Heart cannulation

2.2.1. Cannulate and tie the aorta securely, making sure not to include any bubbles in the aortic line.

2.2.2. Initiate flow at 70 mmHg perfusion pressure by removing the tubing clamp on the aortic line and maintain this pressure during the remainder of the surgery and vessel cannulation.

2.2.3. Separate the pulmonary artery from the aorta and other vessels and ligate the vena cavae and pulmonary veins. Remove the fat and connective tissue still present.

2.2.4. Cannulate the pulmonary artery to provide a measure of coronary sinus flow rate and oxygen tension.

2.2.5. Discard initial flow out of the heart (for about 10 minutes) during the preparation to eliminate blood and surgical debris. After this period, recirculate the perfusate.

3. Side-Firing Fiber Optic Placement

3.1. Connect the fiber optic catheter to a high-power fiber-coupled LED white light source to help visualization as well as provide the light for spectroscopy once in the heart.

3.2. Cut a small appendage of the left atrium, insert the catheter into the left ventricle via the mitral valve, then rotate it to achieve an illuminated left ventricle free wall.

3.3. Position the pickup fiber optic directly opposite the region of maximum illumination of the left ventricle at about 1 cm from the heart.

3.4. Connect the other end of the pickup fiber to a rapid scanning spectrometer.

4. Optical Spectroscopy

4.1. Turn off the lights in the experimental area to obtain complete darkness.

4.2. Start the custom program, incorporating spectrometer drivers to perform data acquisition and real-time analysis of the transmitted light.

NOTE: An executable version of the consolidated version of the spectral acquisition and analysis program is provided as a Supplemental Coding File. Source code is available by request to the authors.

4.3. Navigate through all prompts, selecting options for perfused heart spectroscopy acquisition mode. On the next page, indicate whether auxiliary data collection is occurring. Finally, enter acquisition parameters, including location of both chromophores reference spectra and data to be saved.

4.4. Enter a bandwidth of 490–630 nm.

4.5. Enter a sampling rate of 2 Hz (i.e. 2 samples/sec).

4.6. Collect a dark current, or zero light, spectrum to correct for background signal levels by turning the light source off.

4.7. Click to select the chromophore references desired to be used in the fitting routine.

4.8. In the **Acquire Data** page, adjust the position of both the catheter and the pickup fiber to maximize the transmitted light displayed on the software with specific attention to the signal amplitude in the 500 nm region, where the oxygenated myoglobin absorbances should be observed.

4.9. Make sure the transmitted light is not saturating the detector in the 600 nm region.

4.10. Ensure no external light sources contribute to the collected spectrum by turning off catheter illumination and confirming no light is now detected.

4.11. Initiate the data collection by clicking on the **Save Spectra** button.

4.12. Click on **Set as Control** to view the difference absorbance spectrum from future spectra to the current “control” spectrum.

4.13. Perform any physiological perturbation as desired.

4.13.1. Protocol 1: Effect of Cyanide on Cardiac Performance and Chromophore Absorption

4.13.1.1. Stop recirculating fluid from the heart perfusion.

4.13.1.2. Using a syringe pump, inject cyanide (2.5 to 75 mM at pH 7) at different rates into perfusate just before the aortic cannula to achieve desired concentrations of cyanide (0.025 to 1 mM, calculated from aortic flow rate) in the flowing perfusate into the heart while monitoring cardiac function and optical properties.

4.13.1.3. Stop the cyanide syringe pump when the effects on coronary flow and heart rate along with the optical transmission through the heart wall are at steady state.

4.13.2. Protocol 2: Ischemia/Hypoxia

4.13.2.1. Stop cyanide infusion.

4.13.2.2. After 5 minutes switch the bubbling gas from 100% oxygen to 100% nitrogen to remove oxygen from the system.

4.13.2.3. After about 10 minutes, stop the flow to simulate a total ischemic/hypoxic condition.

5. Spectral data analysis

5.1. Run the program in the perfused heart analysis mode.

5.2. Select appropriate spectrometer.

5.3. Enter the data file path and reference spectra file and select catheter light source, which loads the pre-saved spectrum of the catheter light source.

5.4. Select **Read Bin Data**.

5.5. Select **Set Min** and **Max Wavelength**.

5.6. Enter the bandwidth for the data analysis as 490–630 nm.

5.7. Select **Return to Main Menu**.

264 5.8. Select **Read References**.

265
266 5.9. Confirm the reference spectra to use in the analysis.

267
268 5.10. Select **Return to Main Menu**.

269
270 5.11. Select **Time Points** in the main menu.

271
272 5.12. Select a T0 time point as control and set the range to 100 points.

273
274 5.13. Select a T1 time point as the experimental period at a range of 100 points.

275
276 5.14. Observe the raw difference spectrum in the **Averaged Abs. Spectrum** tab.

277
278 5.15. Select **Calculate Fit Coefficients** and then click on the **Fit Coefficients** tab to observe the
279 time course of the reference spectra fit.

280
281 5.16. Return to the **Main Menu** and select **Calculate Difference Abs**.

282
283 5.17. Select T0 and $\Delta T1$ at all positions. Observe the fitted spectrum in the **Difference Spectrum**
284 window and fitting elements in the **Reference Weight** window.

285
286 5.18. Repeat this procedure to compare other time points in the experiment.

287
288 5.19. Return to the **Main Menu**.

289
290 5.20. Save data and analysis in a spreadsheet report by typing in a desired name and selecting
291 **Save Data** for further analysis with other programs.

292
293 NOTE: if no name is typed, the report is saved with the same name as the input file. The report
294 is saved in a folder named **Excel Analysis Files**, located in the same folder as the original input
295 file.

296 297 **REPRESENTATIVE RESULTS:**

298
299 The system used is an off the shelf small animal perfusion heart system but was heavily modified
300 for use with a rabbit heart. The modifications were primarily to increase the bore size of all the
301 tubing to assure adequate flow delivery to the rabbit heart. Great care was made to assure, at
302 the perfusion pressures used, the flow rate of the native perfusion system exceeded the flow
303 with heart attached by at least 5-fold. 2–12 μm pore membrane filters were placed in parallel
304 between the fluid pump and the aortic preload bubble trap chamber to remove any debris from
305 the heart.

306 307 **Transmitted Light from the Rabbit Heart**

Figure 1 presents the spectrum of the catheter (**Figure 1A**) and the raw spectrum of the transmitted light from the rabbit heart free wall (**Figure 1B**). These data reveal a very large attenuation of light in the blue region of the spectrum, however the bands of absorbance from myoglobin and the mitochondrial cytochromes can be directly observed between 490 and 580 nm in the insert. It is important in these studies to assure enough transmitted light is detected in the region from 490 to 630 nm to obtain information on the metabolically responsive cardiac chromophores. The positioning of the external and internal fibers is adjusted prior to saving data to maximize light intensity but not saturate the detector in the 625 nm region.

Reference Reduced minus Oxidized Spectra of Reference Chromophores in the heart.

Figure 2 presents the reference spectra used to fit the difference spectra collected in these studies. These references include myoglobin, cytochrome aa₃ (alternatively cytochrome a₆₀₅ and cytochrome a₆₀₇, depending on the type of perturbation²²), cytochrome a₅₈₀, cytochrome b_L, cytochrome b_H, cytochrome c, cytochrome c₁, FAD, an absorbance representation of the incident light (denoted I₀, which is used to account for sieved light, that is, photons that went through the tissue without being absorbed), and a line (with varying slope and intercept to account for scattering, not shown in **Figure 2**). Some spectra are noisy, as the concentration of the pure reference material was very low²².

Time course of reference spectra fits during total experiment

Figure 3 represents the time course of a typical experiment calculated in step 5.15 of the protocol. This consists of a control phase, followed by a cyanide injection phase, followed by a cyanide washout, followed by a deoxygenation phase, and finally ischemia. The changes in the individual chromophores (myoglobin, cytochrome aa₃, and cytochrome c) over time are plotted over time along with coronary flow rate. The optical density change of each chromophore is estimated by multiplying the fit coefficient obtained from the linear least squares routine and the representative peak of the chromophore (or the maximum absorbance of said chromophore). For example, for myoglobin, the fit coefficient of the myoglobin reference is multiplied by the value of the myoglobin reference spectrum at 580 nm. Note the rapid oxygenation of myoglobin to the addition of cyanide is matched by the increase in flow but is before the significant reduction of cytochromes. This effect is partially recovered with the washout of cyanide. Finally, full reduction of cytochromes and deoxygenation of myoglobin is obtained with ischemia. These data demonstrate that dynamic data concerning the metabolic status of the heart can easily be obtained with this methodology. The position of the spectra used for the difference spectra are marked on this time course as: C baseline, CN cyanide injection, CNW cyanide Washout, H N₂ Hypoxia (nitrogen being bubbled in the perfusate instead of oxygen), and HI No flow ischemia (no perfusate flowing through the heart).

Difference Spectrum of Cyanide Treatment versus Control and Fit of Cyanide Difference Spectrum from Rabbit Heart.

To obtain a difference spectrum, two absolute spectra are subtracted. Each absolute spectrum is obtained by taking an average of many (typically 100) spectra to optimize signal to noise ratio.

Figure 4A represents the difference spectrum of the control (C) and cyanide (CN) treated heart. Using the reference spectra outlined in **Figure 2**, the fit spectrum is calculated. The residual

spectrum is the subtraction of the fit from the raw data. The same scheme is used for all the subsequent spectral presentations. **Figure 4B** presents the spectra amplitudes of the reference spectra (shown in **Figure 2**) used to fit **Figure 4A**. Strong increases in absorbance of most of the cytochromes are observed as the flow of electrons down the cytochrome chain was blocked by cyanide in the steady state. In contrast, the absorbance of deoxygenated myoglobin decreased as the consumption of oxygen was eliminated by cyanide. **Figure 4C** presents the difference spectra and fit of the difference spectrum from CNW and CN, revealing the partial reversal of the cyanide effect. This was accomplished by selecting time points in protocol step 5.18, moving T0 to the CNW and T1 to CN region of the time course. **Figure 4D** presents the difference spectrum of HI and C, which represents the fully deoxygenated and reduced state of the cytosol and mitochondria versus the control condition. Again, this was performed at protocol step 5.18, moving T0 to C and T1 to HI.

Initial time course of cyanide effects on coronary flow and chromophores

Figure 5A shows an example of the initiation of the cyanide effect on the tissue. The fits for myoglobin, cytochrome a₆₀₅ and cytochrome c along with the coronary flow are presented for a single heart. These time courses were created at protocol step 5.15 for the cyanide experiment. The individual difference versus the baseline (position 1) are shown in **Figure 5B**. The spectra were generated from the corresponding position number (1-4) on the time course. This was accomplished at protocol step 5.18, where T0 was always in position 1, and subsequently different spectra (2-4) were created by moving T1 to position 2, 3, and 4 respectively. Somewhat surprising was the observation that flow and myoglobin oxygenation increased before significant changes in cytochrome redox state. The initiation of the changes in flow and chromophore absorbance were estimated by linear extrapolation of the initial rate of change from the baseline. Using this approach, and setting the change in coronary flow as time zero, the increase in myoglobin oxygenation initiated $1.71 \text{ min} \pm 0.39 \text{ min}$ after the change in flow, while cytochrome a₆₀₅ and cytochrome c absorbance were nearly identical but much slower at $4.24 \text{ min} \pm 0.76 \text{ min}$ and $4.34 \text{ min} \pm 0.77 \text{ min}$, respectively ($n = 8$). These data suggest that cyanide relaxes vascular tone²⁴ before a major change in cardiac muscle metabolic state occurs. This effect is likely caused by cyanide encountering the vascular smooth muscle prior to reaching effective dose around the cardiac myocytes.

Estimates of Myoglobin oxygenation in control hearts

Using the cyanide data as an estimate of total myoglobin oxygenation and the ischemia data for fully deoxygenated myoglobin, we estimate that under control conditions myoglobin was only $88.2\% \pm 1.0\%$ ($n = 10$) oxygenated, consistent with prior studies^{20,21,25}.

FIGURE LEGENDS:

Figure 1: Spectra of the side-firing optical catheter. (A) This is a spectrum of the emitted light from the remote light source through the catheter detected with the pickup fiber at about 1 cm from the catheter. In this geometry, the heart is absent and the intensity of the light source is tuned so that the detector does not saturate. **(B)** The side-firing catheter is inserted in the left

ventricle and the transmitted light from the heart is collected and shown. The insert shows the 400 to 580 nm region expanded, revealing the complex transmission of light from this region.

Figure 2: Reference spectra of cardiac chromophores used for spectral fitting. Spectra were collected via a variety of methods²² and are of reduced – oxidized (for the cytochromes) and deoxygenated – oxygenated (for myoglobin). For I_0 , the spectrum in **Figure 1A** is simply converted to an absorbance term to make the reference.

Figure 3: Flow and optical changes over time. The optical density change (ΔOD) of each chromophore is simply the fitted individual chromophore's spectrum at its maximum absorbance. The maximum absorbance frequencies were as previously described^{20,26}. The presented time course is for one experiment, showing a baseline, followed by cyanide injection (0.10 mM at maximum perfusate flow), cyanide washout, nitrogen hypoxia performed by replacing oxygen with nitrogen, and then complete ischemia.

Figure 4: Fitted difference spectra of various conditions. (A) Spectrum of the cyanide injection minus the baseline. The fit spectrum obtained from the least square routine is also plotted. The residual spectrum is the difference between the raw and fit spectra. **(B)** The reference spectra used to create the fit presented in **Figure 4A**. The program scales the references in figure 2 to their relative contribution in the current difference spectrum. **(C)** Same as in **A**, but showing the difference spectrum of washout versus cyanide injection. **(D)** Same as in **A**, but showing the difference spectrum of ischemia versus baseline.

Figure 5: High temporal resolution of cyanide infusion effect on selected cytochromes, myoglobin and cardiac flow. (A) Time course of cardiac flow, deoxymyoglobin, reduced cytochrome a_{605} , and reduced cytochrome c. Numbers refer to the position of the spectra taken relative to baseline in **Figure 5B**. **(B)** Difference spectra for the 4 positions labeled in **Figure 5A** versus control (position 1).

DISCUSSION

The isolated retrograde or working perfused heart preparation is a mainstay in the study of cardiac physiology as well as the preclinical investigation of techniques and drugs on the heart. Key to its use has been the ease of preparation, robust functional characteristics and control of experimental parameters as well as the ability to measure many functional parameters of the beating heart. Optical absorbance spectroscopy provides insight into tissue oxygenation as well as mitochondria metabolic activities. Optical spectroscopy has primarily been conducted in the isolated perfused heart studies in the reflectance mode that is difficult to interpret due to motion and light scattering complications.

We have introduced ventricle wall transmission optical spectroscopy (VWTOS) to provide a robust method of monitoring cardiac tissue metabolic chromophores. In a previous publication, we demonstrated that an LED hardwired to the tip of coaxial cable²⁰ makes a unique intracardiac side-firing light source that can be used for VWTOS perfused hearts. The side-firing refers to the projection of light perpendicular to the long axis of the catheter, ideal for illuminating the

ventricle free wall. The LED catheter was small enough to not impact cardiac function but required specialized fabrication in the laboratory. The current study presents the use of a 500 micron commercial side-firing catheter that can be coupled to any light source compatible with fiber optics. These side-firing optical catheters were commercially developed for laser ablation perpendicular to the long axis of the fiber. Naturally, we are using light power much lower than required for photoablation. Smaller fibers are available for use on smaller preparations such as the perfused mouse heart²⁷. This fiber optic system provided adequate illumination through the heart wall in the wavelength range where cardiac chromophores absorb (450–630 nm). Using a pickup fiber optic on the outside of the heart, the absorbance of myoglobin and mitochondria cytochromes can be monitored with excellent temporal and spectral resolution (see **Figure 5**). The side-firing fiber optic approach has several advantages over the LED catheter for VWTOS, including a much smaller cross-sectional profile of the catheter that minimizes the impact of the catheter on the heart, more flexible reducing impact on cardiac valve and ventricle performance, no electrical connections that can short out in the saline perfusate, and finally a catheter that uses an external light source that increases the flexibility of light source selection for VWTOS.

Due to the strong absorbance of the heart below 490 nm, it is difficult to generate much information on the Soret band of the cytochromes in the region of 410–445 nm or NADH at 340 nm. Thus, the broad absorbance of FAD at 450 nm is the lowest frequency absorbance that is observed, though the entire absorption peak of this chromophores is not sampled. Using VWTOS the signal to noise ratio is very high as the entire wall is sampled in contrast to surface reflection spectroscopy, commonly used²⁰, which only samples the surface of the heart with numerous scattering issues. VWTOS sampling the entire heart wall is more analogous to Nuclear Magnetic Resonance Spectroscopy (NMRS) measures of many cardiac metabolites such as ³¹P detected adenosine triphosphate and creatine phosphate²⁸, ¹³C detected labeled metabolites^{29,30} including hyperpolarized labels^{31,32}, and ¹H detected metabolites³³. As the VWTOS can be conducted using non-magnetic devices, it is completely feasible that NMR and VWTOS could be conducted simultaneously. VWTOS is not limited to endogenous chromophores and could be used to monitor absorption from optical probes for pH, Ca²⁺, and plasma membrane potential.

We use 2 Hz (i.e. 2 samples/sec) which provides excellent single spectrum signal to noise. Though higher sampling rates can be achieved that permit cardiac cycle analysis, previous studies have demonstrated that there's no beat to beat variation in the chromophore absorbance, so no effort to selectively collect light as a function of cardiac cycle was made³⁴. Due to the VWTOS geometry, the detection of light is less dependent on tissue motion than reflection methods, since the complex surface scattering events are eliminated. We find that severe motion can disrupt these measures, but the real time spectral analysis quickly reveals spectral transitions inconsistent with tissue chromophore transitions. Again, this only occurs when the heart moves grossly away from the collecting fiber dramatically reducing the amount of collected transmitted light.

The VWTOS data is analyzed using full spectral fitting routine based on a reference library of spectra of cardiac chromophores and the spectrum of the light source as previously described^{20,22,27,35} with a simple linear least squares approach. This spectral fitting procedure compensated for overlapping absorbance spectrum and does not relying on "isobestic"

wavelengths. This full spectrum analysis eliminates artifacts associated with common dual beam (i.e. two wavelength) analysis^{1,3,6} which has been shown to be problematic²⁰. The added advantage of full spectral analysis is the generation of a goodness of fit from the residuals, not available in dual beam protocols.

In this study, we focused on the effect of cyanide on the optical properties of the heart. As cyanide blocks cytochrome oxidase, it inhibits oxygen consumption and essentially results in a net reduction of all of the cytochromes as the electrons back up in the cytochrome chain. However, the membrane potential apparently remains high, as the redox changes in b_L and b_H are very small when compared to cytochrome c^{13} . With the cessation of oxygen consumption, the oxygen tension in the tissue should approach the perfusate and we noted an early increase in oxygenated myoglobin with cyanide consistent with the notion that the saline perfused heart, even in retrograde perfusion modes, is not fully oxygenating myoglobin in the cytosol^{19-21,36}. Comparing the maximum effect of cyanide on oxygenated myoglobin with the fully deoxygenated spectrum obtained with ischemia reveals a myoglobin oxygenation of only about 88%, consistent with previous studies.

It is important to note in this study that the cyanide effects on myoglobin oxygenation and cytochrome reduction were temporally resolved. It is surprising that the effects of cyanide were first observed on coronary flow and myoglobin before large changes in the heart's cytochromes redox state was observed. The early marked increase in flow suggests that an effect on arterial smooth muscle^{24,37} may occur before gross metabolic effects in the heart cells are observed. The increase in flow, potentially with a modest cyanide induced decrease in respiration, likely results in the immediate increase in oxygenated myoglobin caused by the increase in oxygen delivery. With the spread of the cyanide inhibition to the myocytes, a further increase in coronary flow is observed (see the region marked 3 in **Figure 5a**), likely driven by numerous metabolic factors³⁸. The large early impact of cyanide on flow suggests that the metabolism of the vascular smooth muscle may be more potent in altering vascular tone than the metabolism of the myocytes. These data support the well-established notion that myoglobin has a much lower affinity for oxygen than COX, even in the intact heart, as myoglobin oxygenation occurred well before changes in mitochondria redox state (**Figure 5**). This high level of deoxygenated myoglobin under control conditions is consistent with prior studies suggesting that the isolated saline perfused heart may be partially hypoxic even under control conditions^{9,19-21,27,36}, underscoring the importance of monitoring cardiac tissue oxygenation when using this important model in cardiac physiology.

We present here the experimental details for conducting transmission absorption spectroscopy on the isolated perfused heart. We have successfully adapted this technique for use on the hearts from the rabbit to the mouse by using a thin side-firing intracardiac optical fiber. Utilizing state of the art full spectral fitting routines, the complex optical interaction of the cardiac chromophores can be easily extracted providing, a near real-time measure of critical elements of myocardial metabolism simultaneously with conventional functional measures.

DISCLOSURES:

No conflicts of interest declared.

ACKNOWLEDGEMENTS:

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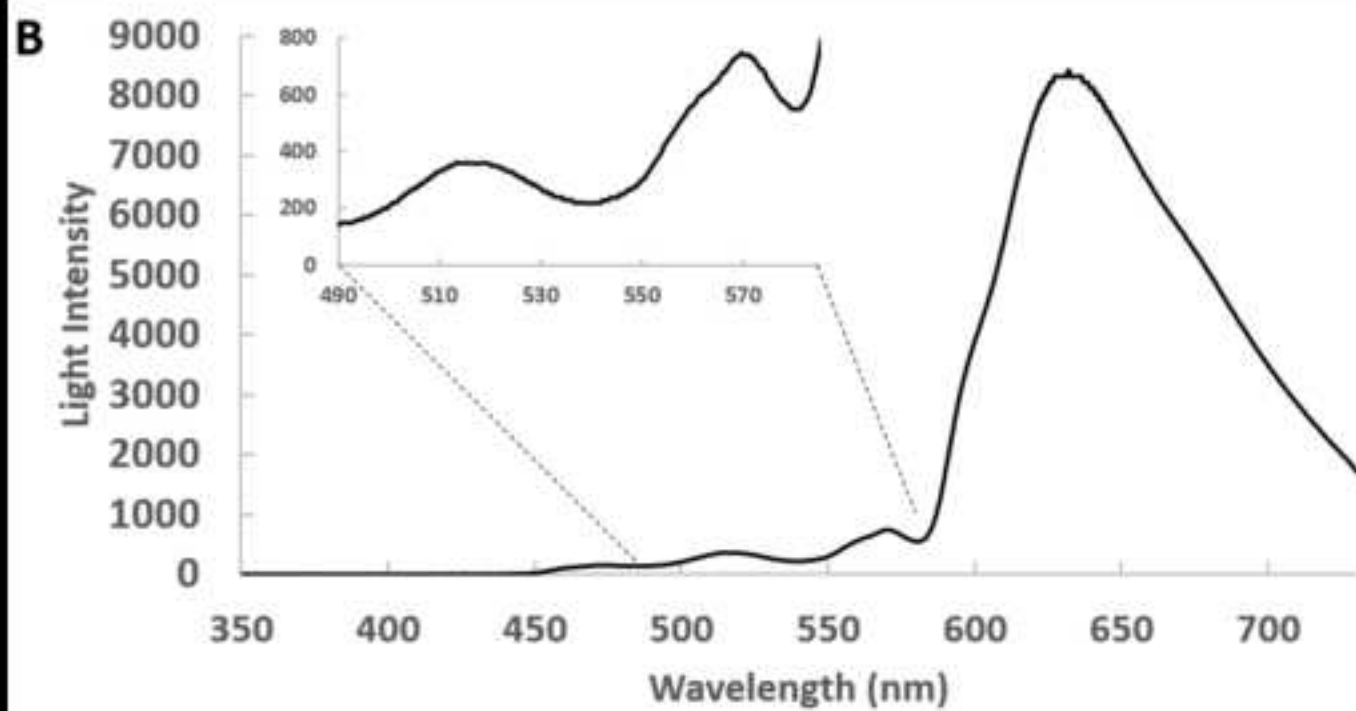
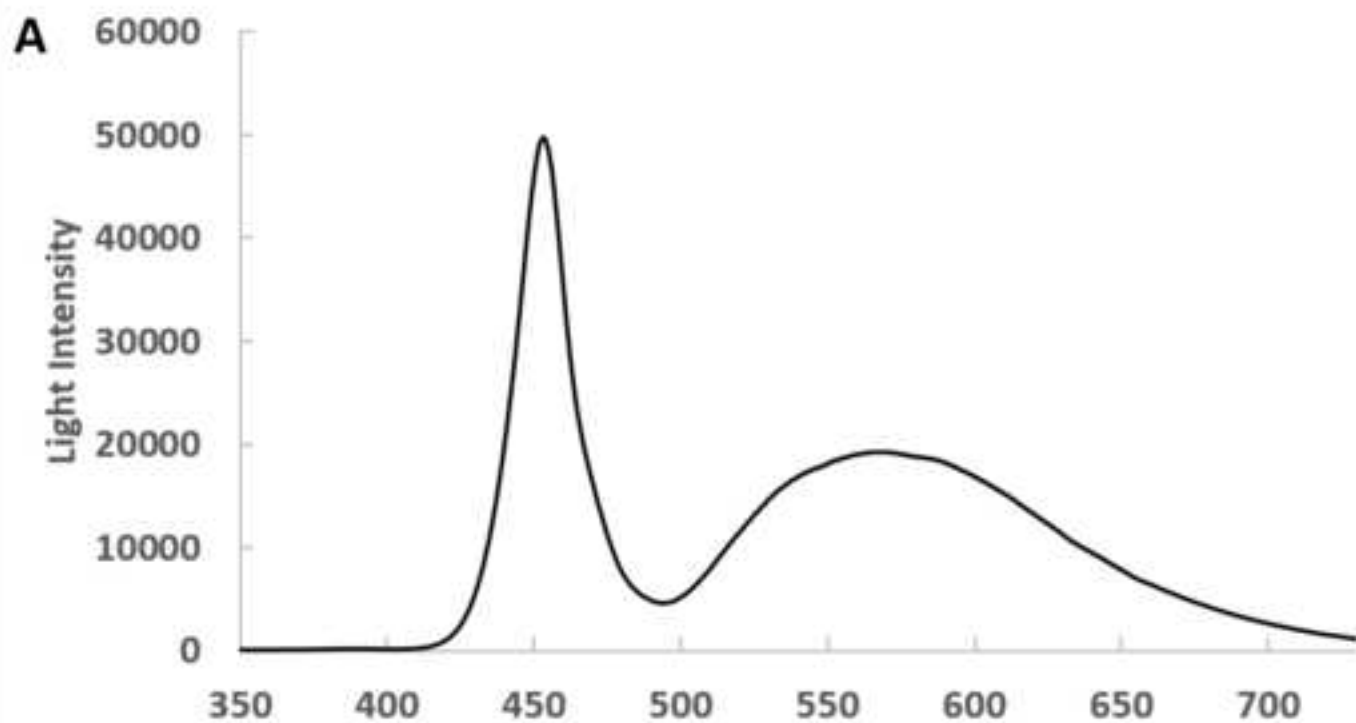
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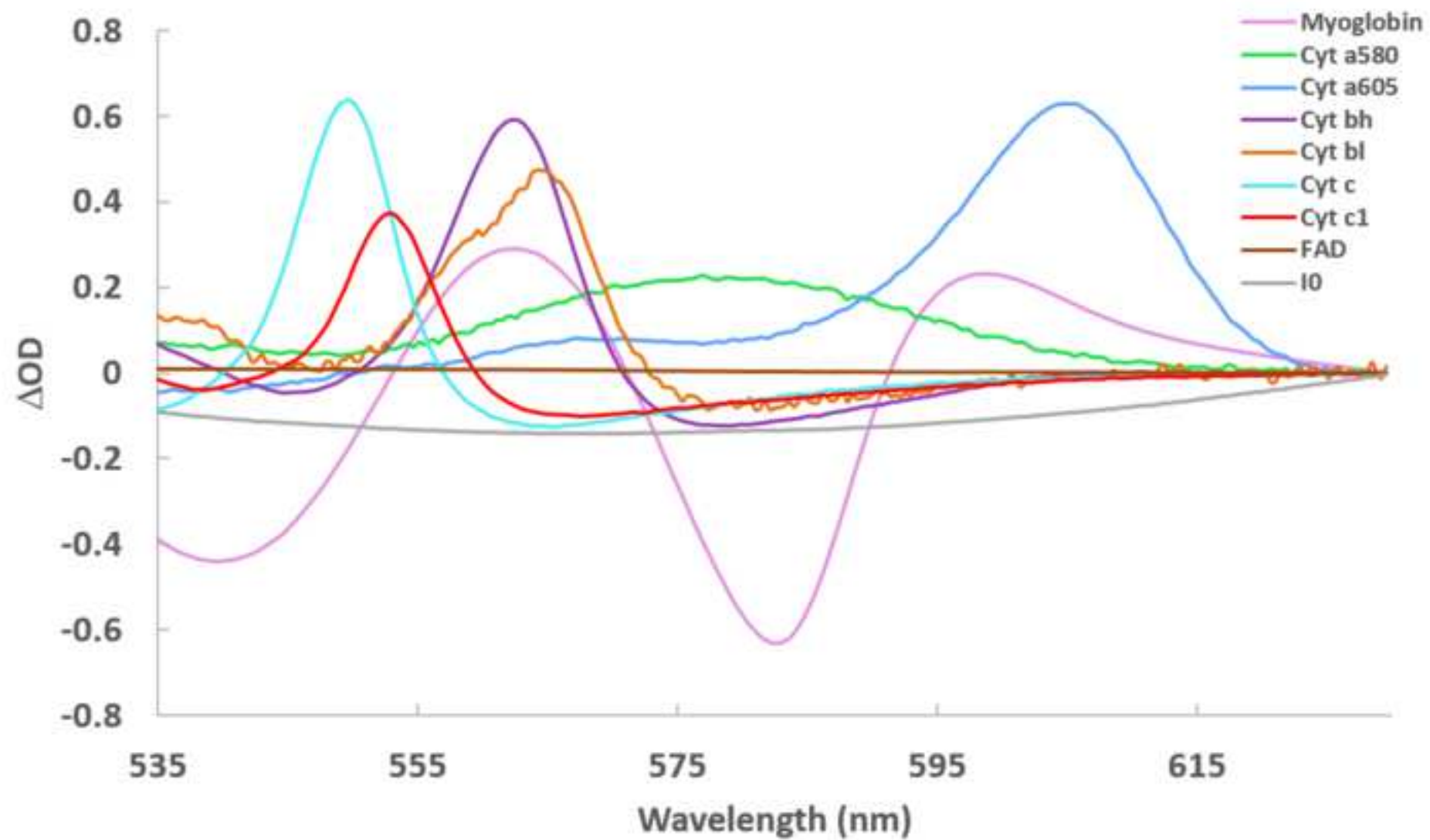
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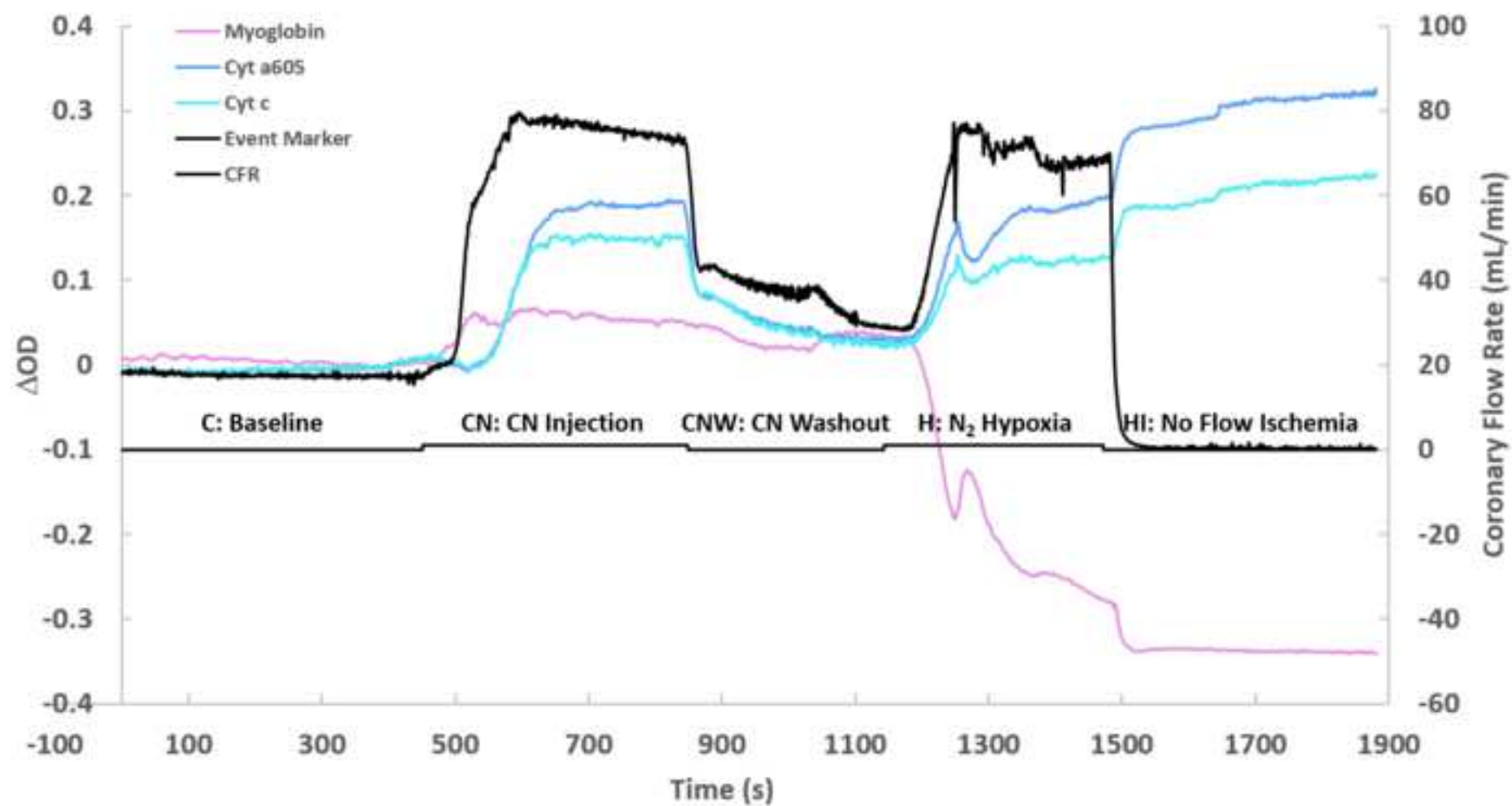
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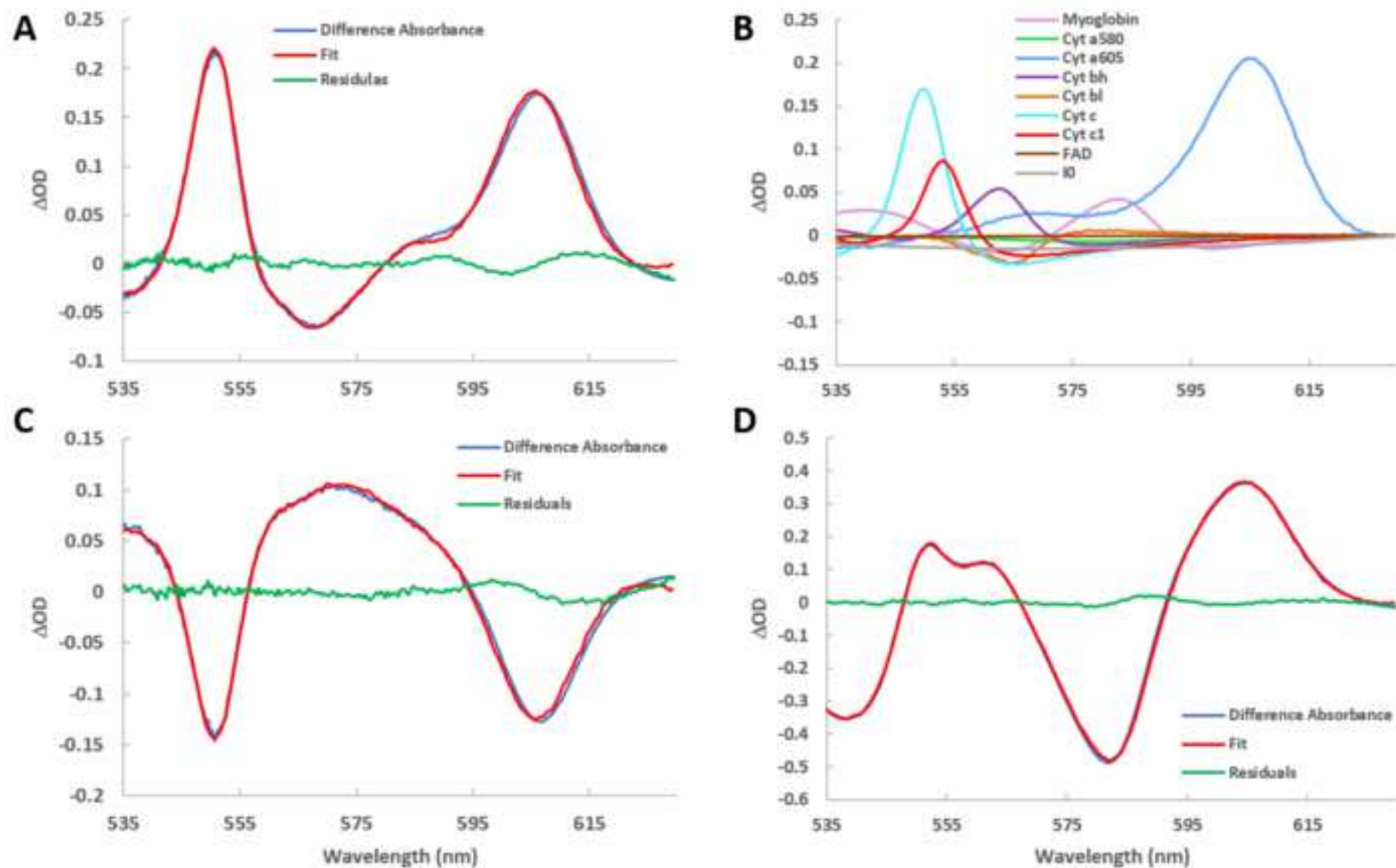
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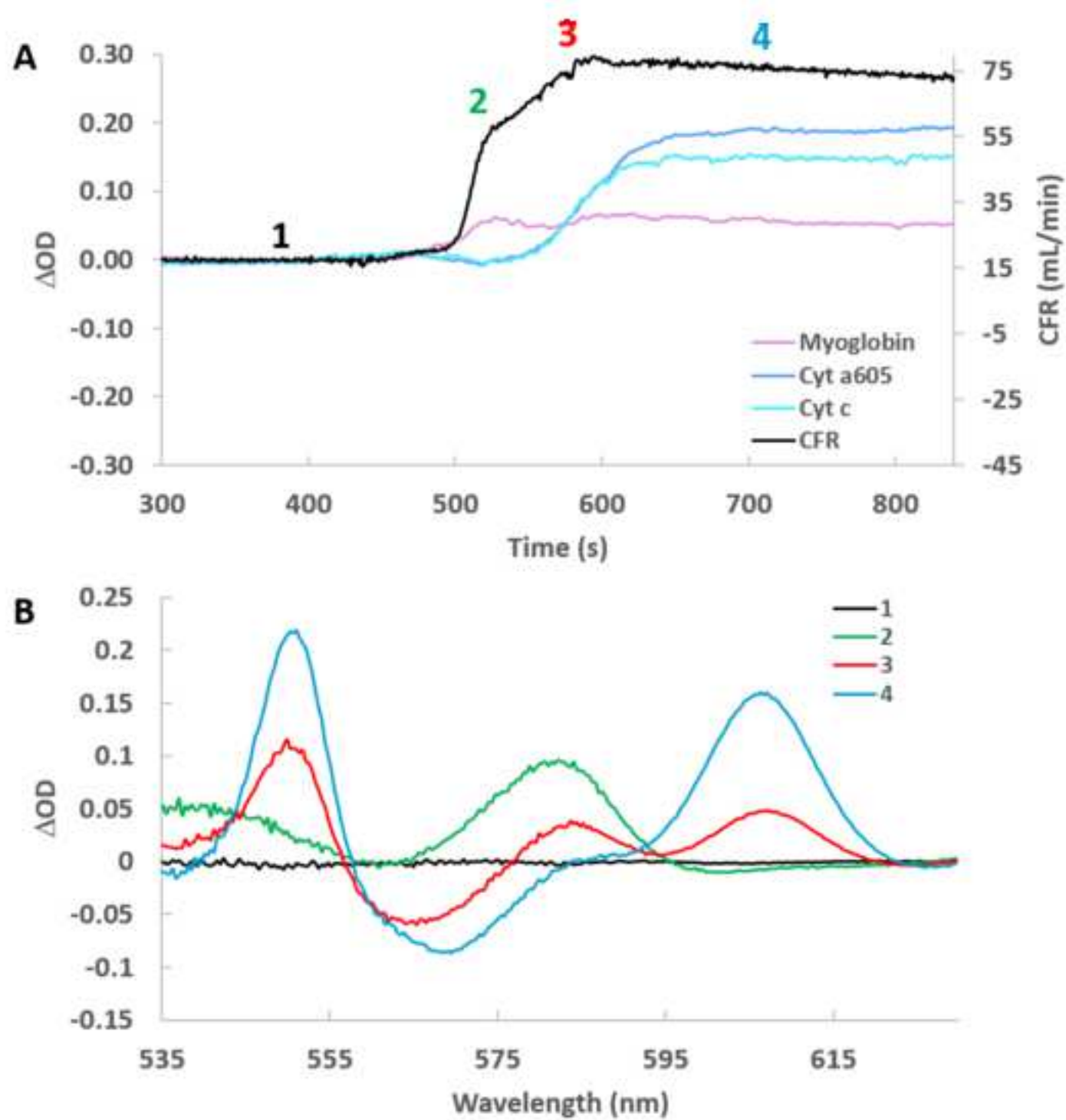
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BIOPAC data acquisition system	BIOPAC	MP150	Analog to digital conversion
BIOPAC general purpose transducer amplifiers	BIOPAC	DA100C	Pressure monitoring
BIOPAC System skin temperature amplifiers	BIOPAC	SKT100B	temperature monitoring
Compact Universal 1- and 2- Channel LED Controllers	Mightex	SLC-MA02-U	External light source power supply
Disposable pressure sensors	BIOPAC	RX104A KDS 200 / 200P LEGACY SYRINGE	Pressure monitoring
Dual Syringe, Infusion Pump	KdScientific	PUMP	drug injection
Flow-through probes	Transonic FORTUNA	4PXN	perusate flow monitoring
Glass Syringe	Optima	30 CC	Air tight fluid injection
High power fiber-coupled LED white light source	Mightex	Type-A FCS-0000	External light source This system was heavily modified to provide adequate flow (see manuscript)
Perfused heart system	Radnoti	120101BEZ	oxygen concentration
Phase fluorimeter	Ocean Optics	NeoFox-GT	Fiber for collecting transmitted light (pick up fiber)
Pickup fiber optic	Thor labs	BF20HSMA01	Analog to digital conversion
PowerLab unit	AD Instruments	PowerLab 8/35	pressure monitoring
Pressure transducers	BIOPAC	TSD104A	Software for driving spectrophotometer
Programming environment	LABVIEW	N/A	Software for driving spectrophotometer
Rapid scanning spectrophotometer	Ocean Optics	QE65PRO	Rapid scanning spectrometer for spectral analysis
	Polymicro Technologies		
	Molex, LLC		
	18019 North		
	25th Av, Phoenix	JTFLH200230500/	
Side firing fiber optic	AZ 85023-1200	1.5M	side firing fiber optic 200 microns core

Sodium cyanide	Sigma-Aldrich	380970	Metabolic inhibitor
Temperature probe	BIOPAC	TSD102A	temperature monitoring
Tubing flow modules	Transonic	TS410	perusate flow monitoring

Software 1 Software 1 Software 1 Software for driving spectrometer, digitizing data and analysis. Code available on request



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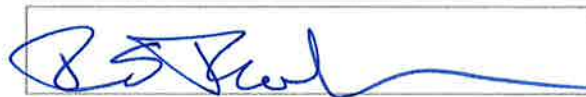
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Editorial comments:

1. Section 2 of the protocol is somewhat confusing. For one, the references cited at the beginning of the protocol both mention excising the heart and lungs, while the protocol only mentions cutting out the heart-how exactly is that done?

Reference 23 is nearly an identical procedure. When the removal of the lungs occurs is a detail of no importance in the operation of the retrograde perfused heart. Some like to use the lungs as reference points others do not. We remove the lungs at surgery. We have added a clarifying “Note” at 2.1.6. The original reference 24 was a dual chamber working rabbit heart and likely would be confusing however this approach is applicable to most heart perfusion protocols.

Also, it seems like there’s a step missing-how exactly do you ‘lower the rate of the fluid’ in 2.2.1?

This is now section 1.7 in the manuscript. It is now made clear that the rate of the fluid is adjusted with a tubing clamp.

2. Sections 4 and 5: Can you provide the custom program you used in these sections (e.g., as a Supplemental Coding File)? **Yes we are downloading a compiled version of the acquisition and analysis programs (both are contained in a single program). We have constructed this with National Instruments Drivers that can be released in this format according to the National Instruments Labview license. Users will have to have a LabView license to run. Since the source code is very large we are only providing that if asked directly.**

3. Results, “Transmitted Light from the Rabbit Heart”: You mention a note in section 4.3, but there doesn’t seem to be a note there.

That section was previously removed as the caption of figure 1A described the protocol used to acquire this spectrum. The mention of section 4.3 note is now removed from the results.

4. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

This is a intramural program with no grants. We have added this to an Acknowledgement section along with a project number.

5. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

A statement of no conflicts of interest is now included.