

Video Article

# NADH Fluorescence Imaging of Isolated Biventricular Working Rabbit Hearts

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## Abstract

Since its inception by Langendorff<sup>1</sup>, the isolated perfused heart remains a prominent tool for studying cardiac physiology<sup>2</sup>. However, it is not well-suited for studies of cardiac metabolism, which require the heart to perform work within the context of physiologic preload and afterload pressures. Newly introduced modifications to the Langendorff technique to establish appropriate left ventricular (LV) preload and afterload pressures<sup>3</sup>. The model is known as the isolated LV working heart model and has been used extensively to study LV performance and metabolism<sup>4-6</sup>. This model, however, does not provide a properly loaded right ventricle (RV). Demmy *et al.* first reported a biventricular model as a modification of the LV working heart model<sup>7, 8</sup>. They found that stroke volume, cardiac output, and pressure development improved in hearts converted from working LV mode to biventricular working mode<sup>8</sup>. A properly loaded RV also diminishes abnormal pressure gradients across the septum to improve septal function. Biventricular working hearts have been shown to maintain aortic output, pulmonary flow, mean aortic pressure, heart rate, and myocardial ATP levels for up to 3 hours<sup>8</sup>.

When studying the metabolic effects of myocardial injury, such as ischemia, it is often necessary to identify the location of the affected tissue. This can be done by imaging the fluorescence of NADH (the reduced form of nicotinamide adenine dinucleotide)<sup>9-11</sup>, a coenzyme found in large quantities in the mitochondria. NADH fluorescence (fNADH) displays a near linearly inverse relationship with local oxygen concentration<sup>12</sup> and provides a measure of mitochondrial redox state<sup>13</sup>. fNADH imaging during hypoxic and ischemic conditions has been used as a dye-free method to identify hypoxic regions<sup>14, 15</sup> and to monitor the progression of hypoxic conditions over time<sup>10</sup>.

The objective of the method is to monitor the mitochondrial redox state of biventricular working hearts during protocols that alter the rate of myocyte metabolism or induce hypoxia or create a combination of the two. Hearts from New Zealand white rabbits were connected to a biventricular working heart system (Hugo Sachs Elektronik) and perfused with modified Krebs-Henseleit solution<sup>16</sup> at 37 °C. Aortic, LV, pulmonary artery, and left & right atrial pressures were recorded. Electrical activity was measured using a monophasic action potential electrode. To image fNADH, light from a mercury lamp was filtered (350±25 nm) and used to illuminate the epicardium. Emitted light was filtered (460±20 nm) and imaged using a CCD camera. Changes in the epicardial fNADH of biventricular working hearts during different pacing rates are presented. The combination of the heart model and fNADH imaging provides a new and valuable experimental tool for studying acute cardiac pathologies within the context of realistic physiological conditions.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/4115/>

## Protocol

### 1. Setting Up for the Study

1. Prepare four liters of modified Krebs-Henseleit solution<sup>16</sup> (in mM: 118 NaCl, 3.30 KCl, 2.00 CaCl<sub>2</sub>, 1.20 MgSO<sub>4</sub>, 24.0 NaHCO<sub>3</sub>, 1.20 KH<sub>2</sub>PO<sub>4</sub>, 10.0 glucose, 2.00 NaPyruvate, and 20.0 mg/L albumin). The solution should be prepared as close to the start of the experiment as possible. The pH should be adjusted to 7.4 after sterile filtering (pore size: 22 µm, Corning). Solution osmolality should be between 275 and 295 mOsm/kg.
2. Rinse all tubes and chambers of the working heart system with purified water. Run pumps until all water has been removed from the system.
3. Add cellulose membrane filters (pore size: 5 µm, Advantec) in line with each of the perfusion pumps (Langendorff perfusion pump, left heart perfusion pump, and right heart perfusion pump).
4. Perform a two-point calibration (0 and 60 mmHg) for each pressure sensor.
5. Turn on the water baths. A heated circulating water bath (Cole Palmer) is used to warm the water-jacketed tubes and heat exchangers. Perfusate is pre-warmed in a separate water bath (Oakton Instruments). Both baths are set to maintain a solution temperature of 37 °C.

6. Turn on the pumps to circulate the perfusate in a closed loop. Perfusate passes through microfiber oxygenators (hemofilters) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 80 kPa. Oxygenated perfusate then flows through heat exchangers to maintain it at a temperature of 37 °C before entering the heart cannulae.

## 2. Heart Excision

1. Begin by setting the working heart system to operate in constant pressure Langendorff mode. Set the pressure of the aortic block within the range of 50 to 60 mmHg.
2. Anesthetize the rabbit with an intramuscular injection of ketamine (44 mg/Kg) and xylazine (10 mg/Kg). After the rabbit is sedated, pentobarbital (50 mg/Kg) and heparin (2000 U) is intravenously injected via the marginal ear vein or the lateral saphenous vein on the inside of the hind limb.
3. When the rabbit is completely non-responsive, as determined by a lack of pain reflex, the thoracic cavity is quickly opened, the pericardium is sliced, the aorta is clamped, and the heart and lungs are excised. At this point the lungs should be left attached to the heart to help with isolating the pulmonary veins.
4. Isolate and cannulate the aorta with a 5 mm diameter cannula that is attached to a syringe filled with 60 mL perfusate and 200 units of heparin. Secure the aorta to the cannula with size zero silk suture and slowly depress the syringe to flush the heart of blood.

## 3. Biventricular Cannulation

1. Connect the heart to the aortic block of the working heart system. Prevent air from entering the aorta, which can cause coronary emboli. It is best to attach the cannula to the aortic block by approaching the aortic connector at an oblique angle and allowing perfusate to gently drip from the connector into the cannula while it is attached.
2. While the heart is perfused in constant pressure Langendorff mode, remove the fat and connective tissue and locate the following vessels: inferior and superior vena cava, azygos vein, pulmonary artery, pulmonary veins.
3. Ligate the superior vena cava. Cut the pulmonary artery just below where it branches to the right and left pulmonary arteries.
4. Group all the remaining vessels (the pulmonary veins) between the heart and lungs and ligate them all using one suture. Remove the lungs.
5. Cut a small hole in the corner of the left atrial appendage. Ensure that the LA is filled with perfusate. Cannulate the LA while ensuring that the cannula is completely filled with perfusate while it is inserted. Suture the cannula to the LA appendage.
6. Turn on the left side pump (pump #2) to provide flow to the left atrium. Set preload pressure between 2 - 6 mmHg and adjust  $\pm 2$  mmHg, as determined by atrial dilation.
7. Switch the heart to the working heart mode by turning off the Langendorff pump (pump #1).
8. Momentarily decrease the aortic pressure to 10 mmHg and then slowly increase it to within the range of 80 to 100 mmHg. This will allow the aortic valve to open and function as it would during normal physiologic conditions. The final afterload pressure will depend upon the contractility of the LV. It should be set to a value that is approximately 20 mmHg less than peak LV pressure.
9. LV cardiac output can be determined by measuring the flow rate of perfusate exiting the aortic block (mL/min). Normal cardiac output is between 14.77 and 16.43 mL/min per 100 g of body weight<sup>17</sup> and averages 340 mL/min for a 2.2 kg rabbit. Aortic pressure should resemble the pressure signal shown in **Figure 1**.
10. Cannulate the RA through the inferior vena cava. Ensure that both the RA and the cannula are completely filled with perfusate and insert the cannula while preventing the formation of air bubbles. Suture the cannula to the vein.
11. Turn on the right side pump (pump #3) to provide flow to the right atrium. Set the pressure to approximately 3 mmHg.
12. Ensure that the RV is filled with perfusate and cannulate the pulmonary artery. Ensure that the cannula is completely filled with perfusate while it is inserted to prevent air bubbles. Suture the cannula to the pulmonary artery.

## 4. Signal Acquisition: Pressures, Monophasic Action Potentials, and fNADH

1. Once biventricular cannulation is complete, carefully insert the pressure transducer catheter (Millar) into the aorta via the aorta cannula. Gently navigate it past the aortic valve and into the LV. Monitor the LV pressure signal to ensure proper positioning of the catheter tip. An example of LV pressure is shown in **Figure 1**.
2. Gently press the monophasic action potential electrode against ventricular epicardium. Monitor the signal to achieve appropriate action potential measurements. Slight motion artifact in the signal is normal.
3. Place a bipolar stimulus electrode on the right atrium to pace the heart. In our protocol, hearts were paced at cycle lengths between 300 and 150 msec, corresponding to 200 and 400 bpm, respectively.
4. Measure the temperature of the LV epicardial surface. If the study requires that the temperature be maintained at 37 °C then position the heart inside a water-jacketed heart chamber or submerge the heart in a warmed superfusate bath to maintain a constant temperature throughout the heart.
5. Position the CCD camera (Andor iXon DV860, 128x128 pixels) and focus the lens such that an appropriate field of view is observed. The camera is connected to a workstation and images are acquired at 2 fps using Andor SOLIS software.
6. Turn on the mercury lamp light prior to the start of imaging. Light is directed through an excitation filter (350 $\pm$ 25 nm, Chroma Technology) and into a fiber optic light guide (Horiba Jobin Yvon model 1950-1M) to illuminate the surface of the heart. The attenuation of UV light through the light guide is small. UV illumination could also be provided using a high power LED system consisting of LED spotlights (Mightex PLS-0365-030-S) and a control unit (Mightex SLC-SA04-US).
7. Turn off the room light and minimize any ambient lighting. Aim the ferrules of the light guide (or LED spotlights) at the heart to achieve uniform epicardial illumination. Emitted NADH fluorescence (fNADH) passes through an emission filter (460 $\pm$ 20 nm Chroma Technology) and is imaged by the CCD camera.
8. Monitor fNADH changes over time by selecting a region of interest using the imaging software. Select live-update mode to monitor the mean pixel intensity within the region of interest.

9. The heart should be functioning in biventricular working mode to generate appropriate pressures. fNADH levels should be low and stable over the epicardial surface to confirm adequate coronary perfusion. At this point in the study a specific experimental protocol should be implemented to test a hypothesis.
10. When the study is completed, remove the heart from the system and drain all perfusate. Rinse the system tubing and chambers with purified water. For routine maintenance, the system should be periodically rinsed with Mucosal solution or a diluted hydrogen peroxide solution, as needed.

## 5. Off-line Processing of fNADH Images

1. One way to compare NADH datasets ( $fNADH(i,j,t)$ ) between experiments is to normalize each fluorescence image using a reference image ( $fNADH(i,j,t_0)$ ) from the dataset<sup>9</sup>, as shown in the equation below. Another way to normalize NADH fluorescence is to place a small piece of uranyl glass in the field of view before the experiment<sup>9, 18, 19</sup>. Uranyl glass will fluoresce (450 - 550 nm) when illuminated with UV light to provide a signal that can be used as a stable reference.

$$\eta fNADH(i,j,t) = \frac{fNADH(i,j,t) - fNADH(i,j,t_0)}{fNADH(i,j,t_0)}$$

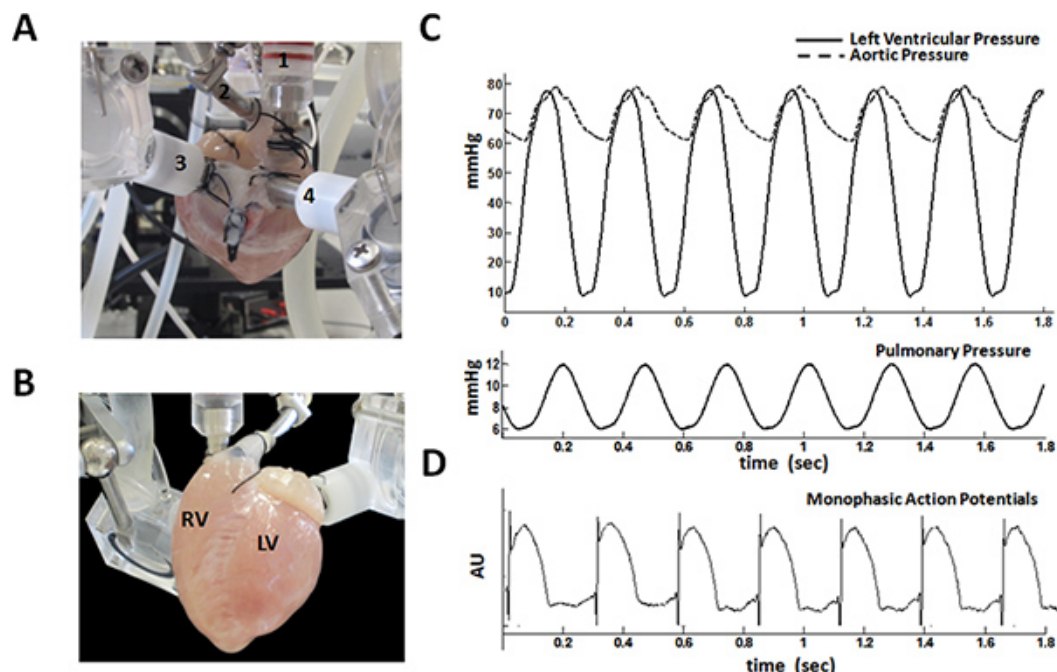
## 6. Representative Results

Anterior and basal views of a biventricular working rabbit heart preparation are shown in **Figure 1**. Left ventricular pressure was measured by navigating a pressure transducer catheter (Millar SPR-407) past the aortic valve and into the left ventricle. Aortic, pulmonary artery, and left ventricular pressures (LVP) are shown in **Figure 1C**. Diastolic LVP is usually between 0 and 10 mmHg. The minimum diastolic aortic pressure is approximately 60 mmHg. Peak systolic LVP is dependent upon filling pressure (the preload or LA pressure) and contractility and, optimally, should be between 80 and 100 mmHg. The maximum aortic pressure and maximum LVP should closely match, as shown in **Figure 1C**.

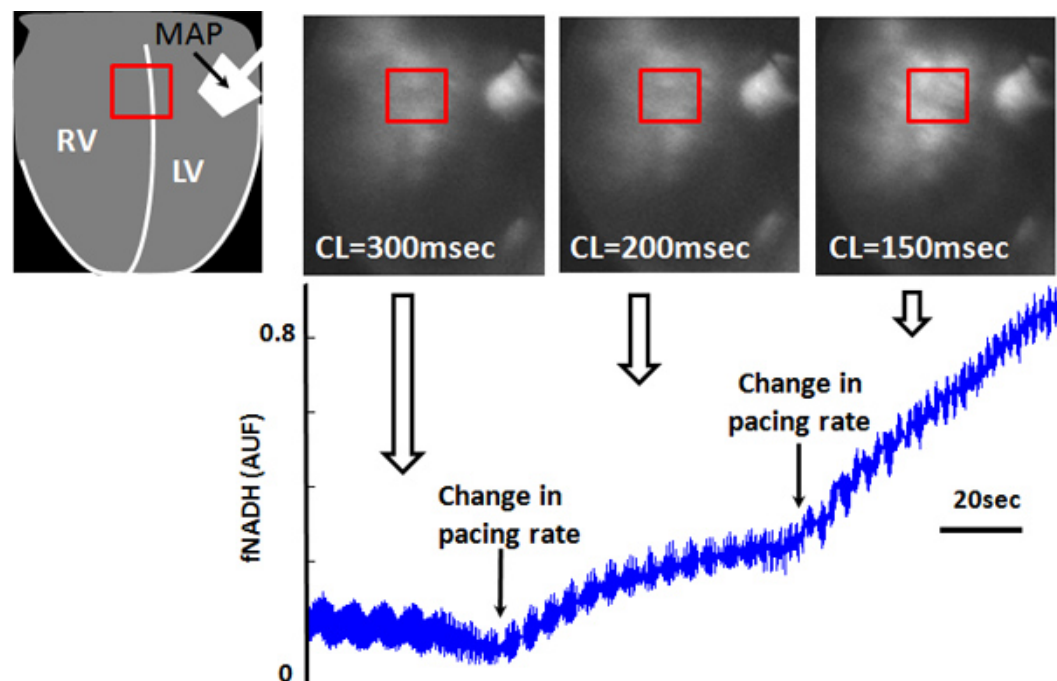
Monophasic action potentials (MAPs) with a fast depolarization phase and a repolarization phase that are typical for rabbit hearts are shown in **Figure 1D**. MAPs can be recorded relatively easily from a contracting heart but will usually have small motion artifact during diastole, as shown in **Figure 1D**. MAPs are useful for confirming successful entrainment of the heart (capture) during pacing and can also be used to measure local electrophysiological changes due to ischemia or other acute perturbations. An ECG could also be measured by submerging the heart in a bath of warm superfusate and placing an electrode in the bath on the left and right sides of the heart. A third indifferent electrode is either placed in the bath, away from the heart, or is attached to the aorta. An ECG will provide information regarding the global excitation and repolarization process, which is useful for evaluating overall electrical function and for revealing the presence of ischemia.

fNADH imaging reveals changes in the mitochondrial redox state of the heart, which can be used to measure the spatiotemporal progression of ischemic or hypoxic regions. For this study, epicardial fNADH was measured to monitor changes in redox state during three pacing rates at cycle lengths (CLs) of 300, 200, and 150 msec. Average fNADH values from a region of interest (red box, **Figure 2**) show that baseline fNADH levels increase as the cycle length is shortened. When pacing rate is close to sinus rhythm (CL=300 msec) baseline fNADH level is relatively constant. As cycle length is shortened below 300 msec, baseline fNADH levels increase, with the largest increase at the shortest CL (150 msec). High resolution fNADH imaging of the full anterior surface at 200 and 400 bpm is shown in **Figure 3**. fNADH levels at 200 bpm were constant and spatially homogeneous. At 400 bpm, fNADH levels increased substantially throughout the epicardium. Significant spatial heterogeneity was observed with the largest increases occurring within the septal regions of the RV and LV.

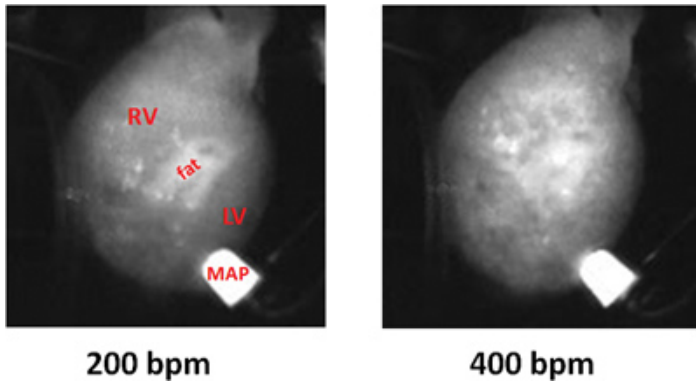
The fNADH signal oscillates with contraction (motion artifact) and the frequency of oscillation corresponds to heart rate (**Figure 2**). In biventricular cannulation, the base of the heart is held by 4 cannulae, which helps to prevent the heart from swinging during contraction. Therefore, oscillation amplitude is always less than any longer time scale (5-10 sec) trends in fNADH that are caused by ischemia or hypoxia.



**Figure 1. Typical pressures and monophasic action potentials from an isolated biventricular working rabbit heart.** **A.** Basal view of the heart showing the four cannulae: 1, aortic; 2, pulmonary artery; 3, left atrial; and 4, right atrial. **B.** Anterior view of the heart showing the left ventricle (LV) and the right ventricle (RV). **C.** Representative pressures. Top: left ventricular pressure (solid line) and the aortic pressure (dotted line). Bottom: pulmonary pressure. **D.** Representative monophasic action potentials. The signal is aligned with the pressures shown in panel C. [Click here to view larger figure.](#)



**Figure 2. fNADH imaging of an isolated biventricular working rabbit heart.** **Top:** A cartoon of the field of view (left) and three fNADH images are shown. The corresponding pacing cycle length (CL) is indicated on each image. The region of interest for the fNADH signal in the bottom panel is indicated by the red box. The tip of the monophasic action potential electrode is seen to the right of the region of interest. The epicardium was illuminated using the mercury lamp and light guide, as shown in **Figure 5**. Only the epicardial surface surrounding the region of interest was illuminated. **Bottom:** Average fNADH for the region of interest indicated by the red box in the top panel. Average fNADH increases with reduced cycle length.



**Figure 3. fNADH images of the full anterior surface of an isolated biventricular working rabbit heart.** The heart was paced from the RA at 200 bpm and 400 bpm. fNADH was imaged (2 fps, 128x128 pixels at a resolution of 0.4 mm) while illuminating the entire anterior epicardium using two high power LEDs (Mightex PLS-0365-030-S, 365 nm, 4% intensity, 50 mW max).

## Discussion

The isolated Langendorff perfused heart remains a prominent tool for studying cardiac physiology<sup>2</sup>. It is especially useful in studies of cardiac arrhythmias, particularly those that use fluorescence imaging of transmembrane potential<sup>20</sup>. An advantage is that the entire epicardium of the isolated heart can be observed<sup>21,22</sup>. Another advantage is that, in contrast to blood, perfusion with a clear crystalloid buffer solution does not interfere with fluorescence signals. A limitation is that the Langendorff technique is not well-suited for studies of cardiac metabolism, which often require the heart to perform work within the context of physiologic preload and afterload pressures.

To elevate the relevance of isolated heart preparations for metabolic studies, Neely introduced modifications to the Langendorff technique to establish appropriate left ventricular (LV) preload and afterload pressures<sup>3</sup>. The model is known as the isolated LV working heart model and has been used extensively to study LV performance and metabolism<sup>4-6</sup>. The LV working heart model is superior to the Langendorff model for functional evaluations, yet it does not provide a properly loaded right ventricle (RV). Demmy *et al.* first reported a biventricular model (LV & RV) as a modification of the LV working heart model<sup>7,8</sup>. They found that stroke volume, cardiac output, and pressure development improved in hearts converted from working LV mode to biventricular working mode<sup>8</sup>. A properly loaded RV also improves septal function by diminishing abnormal pressure gradients across the septum. Biventricular working hearts have been shown to maintain aortic output, pulmonary flow, mean aortic pressure, mean pulmonary pressure, heart rate and myocardial ATP, and creatine phosphate levels for up to 3 hours<sup>8</sup>. Biventricular working heart studies typically use hearts from small animals, such as rats and rabbits, because the cardiac output and the required volume of perfusate are much less than that for hearts of larger animals. However, biventricular working heart studies have been conducted using hearts from swine, canines, and even humans<sup>23,24</sup>.

The metabolic demand of isolated hearts in biventricular working mode is considerably higher than that of Langendorff perfusion. It is important that the perfusate solution provide enough oxygen and metabolic substrate to support biventricular heart function. Standard crystalloid buffer solutions, such as Krebs-Henseleit<sup>16,17,25</sup> or Tyrodes<sup>26,27</sup>, have oxygen solubilities as high as 5.6 mg/L. When these solutions are gassed with carbogen (a gas blend of 95% O<sub>2</sub> and 5% CO<sub>2</sub>) and contain suitable metabolic substrate (glucose, dextrose, and/or sodium pyruvate), they are appropriate for biventricular working hearts beating at normal sinus rates (approximately 180 bpm for a rabbit).

Metabolic demand increases for fast rhythms and the amount of oxygen dissolved in standard perfusates might not be enough to fully support a biventricular working heart that is contracting at high rates. Crystalloid buffer solutions containing erythrocytes or mixed with whole blood have been used in working heart preparations to ensure adequate oxygen availability. Previous studies have shown that adding erythrocytes to a Krebs-Henseleit solution improved working heart function during rigorous pacing protocols and also reduced the incidence of ventricular fibrillation<sup>16</sup>. A limitation of using erythrocytes or mixtures of whole blood is that hemoglobin interferes with light wavelengths that are used for fluorescence imaging<sup>13</sup>. Other substrates, such as albumin, may also be added to perfusate solutions to prolong heart viability and reduce edema<sup>28</sup>.

During fluorescence imaging the intensity of excitation light should be high and the light distribution should be uniform. Achieving uniform illumination is not always easy due to the curvature of the epicardial surface. In our studies, we image fNADH by filtering light (350±25 nm) from a mercury lamp. A bifurcated fiber optic light guide is used to direct the UV light onto the epicardial surface. Uniform lighting can be achieved by appropriate positioning the two output ferrules. UV LED light sources could also be used, as we have demonstrated in **Figure 3**. LED sources are relatively inexpensive so multiple sources could be incorporated into an imaging system. LEDs can also be cycled on and off at high rates to synchronize excitation light with image acquisition.

Photobleaching of NADH should be minimized<sup>29</sup> by reducing the time of tissue illumination. This can be done by cycling the illumination on and off using an electronic shutter and a lamp or with an LED lighting system and a controller. If illumination is synchronized with the cardiac cycle, then fNADH image acquisition could be confined to diastole, which would reduce motion artifact in the fluorescence signals. Triggering illumination and image acquisition using a pressure signal, such as LV pressure, would be one way to do this.

In our studies we have observed that changes in fNADH per unit time can be more than 5X higher at 400 bpm than at 200 bpm. This indicates that fast rhythms elevate the redox state of the heart. Whether or not this is caused by hypoxia or the inability of myocytes to oxidize NADH to NAD<sup>+</sup> quickly enough to avoid the accumulation of NADH is still an unanswered question.



The performance of a biventricular working heart preparation is contingent upon multiple factors. One of the most important is to set appropriate preload and afterload pressures to mimic the physiological conditions that are under investigation. In particular, the LV afterload (aortic pressure) must be adjusted to represent systemic pressure. If it is too high, the LV will not be able to overcome the pressure, resulting in regurgitation. Pressure that is too low will adversely affect coronary perfusion. The LV preload pressure (left atrial pressure) should also be adjusted to provide an end diastolic volume that is appropriate for the experimental protocol.

fNADH imaging of living tissue is an established mode of fluorescence imaging<sup>13</sup>. Its application to cardiac tissue was illustrated by Barlow and Chance when they reported striking elevations of fNADH within regionally ischemic tissue after ligation of a coronary vessel<sup>14</sup>. Their fNADH images were recorded on film using a Fairchild oscilloscope camera and UV flash photography. Coremans *et al.* expanded upon this concept using the NADH fluorescence/UV reflectance ratio to measure the metabolic state of the epicardium of Langendorff blood-perfused rat hearts<sup>30</sup>. A videofluorimeter was used for imaging and data was recorded using a video recorder. Later, Scholz *et al.* used a spectrograph and photodiode array to measure average fNADH from a large area of the LV. This approach reduced the effects of epicardial fluorescence heterogeneities and local variations in circulation while revealing macroscopic work-related variations of fNADH<sup>31</sup>. This approach is similar to computing average fNADH levels for a region of interest across all frames of an fNADH imaging dataset, as illustrated in **Figure 2**. As we have presented in this article, today's technology provides high-speed CCD cameras and digitally controlled high-power UV spotlights. These technologies enable the spatiotemporal dynamics of fNADH and cardiac metabolism to be studied from many new perspectives. The relatively low-cost of the optics and light source makes fNADH imaging a useful accessory for conventional cardiac optical mapping systems.<sup>9, 32</sup>

## Disclosures

No conflicts of interest declared.

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