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Abstract

Fluorometry is used to detect intrinsic flavoprotein (FP) and nicotinamide adenine dinucleotide NADH signals in an open-chest rabbit model of myocardial ischemia-reperfusion injury. Myocyte apoptosis has been shown clinically to contribute to infarct size following reperfusion of ischemic myocardium. A noninvasive means of assessing apoptosis in this setting would aid in the treatment of subsequent ventricular remodeling. We show that in vivo fluorometry can be useful in apoptosis detection in open-chest surgeries. Specific changes in myocardial redox states have been shown to indicate the presence of apoptosis. Two main mitochondrial intrinsic fluorophores, NADH and FP signals, were measured during normoxia, ischemia, and reperfusion experimental protocol. Ischemia was induced by occlusion of the largest branch of the circumflex coronary artery and fluorescence signals are collected by applying two different fluorescence techniques: *in vivo* fluorometry and postmortem cryoimaging. The first technique was employed to detect FP and NADH signals *in vivo* and the latter technique uses freeze trapping and lowtemperature fluorescence imaging. The heart is snap frozen while still in the chest cavity to make a "snapshot" of the metabolic state of the tissue. After freezing, the ischemic area and its surrounding border zone were excised and the sample was embedded in a frozen buffer for cryoscanning. These two data sets, *in vivo* fluorometry and low temperature redox scanning, show consistent extreme oxidation of the mitochondrial redox states (higher redox ratio) suggesting the initiation of apoptosis following reperfusion. This represents the first attempt to assess myocyte apoptosis in the beating heart.

Keywords

fluorescence imaging, fluorometery, mitochondrial redox state, ventricular remodeling, myocardium, apoptosis

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Fluorescence spectroscopy and imaging of myocardial apoptosis

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Abstract. Fluorometry is used to detect intrinsic FP (Flavoprotein) and NADH (Nicotinamide Adenine Dinucleotide) signals in an open chest rabbit model of myocardial ischemia-reperfusion injury. Myocyte apoptosis has been shown clinically to contribute to infarct size following reperfusion of ischemic myocardium. A non invasive means of assessing apoptosis in this setting would aid in the treatment of subsequent ventricular remodeling.

We show that *in vivo* fluorometry can be useful in apoptosis detection in open-chest surgeries. Specific changes in myocardial redox state have been shown to indicate the presence of apoptosis. Two main mitochondrial intrinsic fluorophores, NADH and FP signals were measured during normoxia, ischemia, and reperfusion experimental protocol. Ischemia was induced by occlusion of the largest branch of the circumflex coronary artery and fluorescence signals are collected by applying two different fluorescence techniques: *in vivo* fluorometry, and post mortem cryoimaging. The first technique was employed to detect FP and NADH signals *in vivo* and the latter technique uses freeze trapping and low temperature fluorescence imaging. The heart is snap frozen while still in the chest cavity to make a “snapshot” of the metabolic state of the tissue. After freezing, the ischemic area and its surroundings border zone were excised and the sample was embedded in a frozen buffer for cryo-scanning. These two data sets, *in vivo* fluorometry and low temperature redox scanning, show consistent extreme oxidation of the mitochondrial redox states (higher redox ratio) suggesting the initiation of apoptosis following reperfusion. This represents the first attempt to assess myocyte apoptosis in the beating heart.

Keywords: fluorescence imaging; fluorometery; mitochondrial redox state; ventricular remodeling; myocardium; apoptosis;

1 Introduction

Optical techniques in *in vivo* small animals models have been shown to be useful in determining mitochondrial dysfunction.[1-5] Myocyte apoptoisis has been shown to influence infarct size following reperfusion of ischemic myocardium.[6] It has also been shown to play and important part in the progression of post infarction ventricular remodeling leading to heart failure after a myocardial infarction.[7] Currently the only way to quantitatively assess myocyte apoptosis is by molecular assays applied to biopsy tissue specimens. A catheter based non-invasive technique to assess apoptosis in the beating heart would aid in the diagnosis and treatment of both diseases.

Apoptosis is an orderly regulated process which results in mitochondrial dysfunction and disruption. To study the ability of optical techniques to assess apoptotic mitochondrial dysfunction we used a clinically relevant rabbit model of reperfusion injury. This model has been shown to be a strong stimulus for apoptosis as measured by standard molecular assays. In order to study this apoptosis progression in cellular level, fluorescence spectroscopy and imaging of rabbit heart infarction is studied.

We monitor myocardial fluorescence signals by a fiber optic coupling to a spectrofluorometer, which reads the mitochondrial redox state and the oxygenation state of hemoglobin/myoglobin in normoxia and ischemia. Spectroscopic measurements of FP and NADH surface fluorometry as well as high resolution imaging of redox state of frozen rabbit heart are obtained. Metabolic state of myocardium in this experiment is measured *in vivo* and in cryo images.

2 Materials and Methods

Fluorescence spectroscopy of rabbit myocardium is conducted with a fluorometer, which is a mobile optoelectronic apparatus that collects fluorescence signals of any type of tissue through a 3mm tip lightguide. The incident light is a broadband mercury arc lamp can be filtered at four different wavelengths by an air turbine filter wheel rotating at 50 Hz. Consequently, up to four signals could be multiplexed to a photodetector in order to make four-wavelength channel optical measurements of tissue metabolism as shown in figure 1 but in this study just two of these channels are used. The light intensity that is incident on tissue at the fiber tip is $\sim 3\mu w$. In cardiac fluorometry experiments, the excitation wavelengths of FP and NADH are obtained by filtering the mercury arc lamp at 436 nm and 366 nm resonance lines (interference filters: 440DF20, 365HT25). The fluorescence signals are then detected by a PMT (photomultiplier tube, R928 Hamamatsu), converted to a digital signal with an A/D converter, and displayed. Hence, NADH and FP signals could be monitored using myocardial surface fluorometry in an open chest rabbit heart under different perfusion experimental conditions such as normoxia, ischemia, and reperfusion.

Male New Zealand white rabbits (3-4 Kg) were anaesthetized with Ketamin (100mg/kg), Glycopyrolate (0.01 mg/kg) and Buprenorphine (.05 mg/kg.) The surgery was conducted in compliance with Institutional Animal Care and Usage Committee (IACUC) standards. Left thoracotomy was performed in the fourth intercostals space and the heart was exposed.

The experimental protocol begins under normal conditions with the ventilator on and the optical fiber placed on the rabbit heart. During the experiment the fiber is positioned at two locations, the first- on the basal anterolateral wall (non-infarct zone) and the secondclose to apex in the anteroapical apical myocardial wall distal to the largest branch of the circumflex coronary artery (later called infarct zone) as shown in Figure 2. A baseline for the absolute heart signal in normal conditions is obtained by recording the fluorescence signals of FP and NADH simultaneously for three minutes at each of the two positions. These sequential data from these two points are later time-averaged to provide control (baseline) signals. Myocardial ischemia is induced thereafter by tightening the coronary artery snare and confirmed by ECG and distinct color change. Right after occlusion, data are collected at the same positions. After 30 minutes of ischemia another set of data is acquired. The ischemic area is then reperfused by loosening the coronary snare. To avoid arrhythmias pretreatment with lidocaine and magnesium is conducted. Repeat fluorescence signals 1, 2, and 3 hours after reperfusion are then collected. Following this, the heart is freeze-clamped while still in the chest cavity, utilizing Wollenberger tongs and subsequently cooled with liquid N2, to preserve the instantaneous metabolic redox state of the tissue at the freezing moment.

The low-temperature 3D Fluorescence Scanner (Redox Scanner or Cryo-imager) provides high resolution fluorescence images of the frozen heart. The scans of the surface of the frozen sample are provided via a microlightguide to obtain the fluorescence. The high resolution scanner consists of a light source, synchronized optical filter wheels with bandpass filters, a bifurcated light guide, two PMTs, a sample chamber, and stepper motors to drive the fibers in different directions. The scanner has a mercury arc lamp as the source to provide wavelengths from 300-600 nm. In order to obtain maximum fluorescence, the excitation wavelength for NADH should be in the range of 330-370 nm, and within 440-470 nm for FP. The peak energy of the excitation spectrum of NADH is at 365 nm and that of FP at 436 nm. These wavelengths can be selected by using bandpass filters to exclude the excitation wavelength. Filter wheels rotate at 50 Hz and the emitted signals are detected by a PMT (photomultiplier tube R928, Hamamatsu). Figure 3 shows the instrumentation of the low temperature fluorometer. Filtered light from a mercury arc lamp is coupled into one branch of a bifurcated fiber lightguide (1 emission fiber in the middle and 6 excitation fibers around the emission fiber, each with a 70 μm diameter core and a numerical aperture of 0.34) and illuminated on the tissue surface. The fluorescence signals of the fluorophores are collected through the other branch of the fiber and detected by the PMT after passing through emission filters.

The detected signals are converted to digital data with an A/D converter to allow the computer to obtain images of FP and NADH fluorescence signals via software programming. Two stepper motors drive the optical fiber bundles to scan the tissue surface in X and Y planes with a distance of 60 μm in Z direction from the surface of the sample. The planar resolution is approximately as small as 40 x 40 μm^2. In the Z direction, tissue can be shaved with a resolution of 10 μm via a miller head. The sample is fixed in a round sample holder cup inside the chamber filled with LN2, the depth of which is monitored by a thermal sensor.[8]

The low temperature scan provides a snapshot of the metabolism state of the myocardium and increases the fluorescence quantum yield, typically 10-fold, to give a better S/N ratio of fluorescence signal compared to room temperature. To observe deeper tissue metabolic state, the metal cutter shaves the sample surface with a desired depth (couple of hundred microns). The ischemic area and its surroundings myocardium is excised and embedded in the frozen buffer for redox scanning. The operations of the redox scanner such as milling, stepper motors movements in X, Y, Z directions and measurements are automatically controlled by computer. The number of pixels in each scan can be varied from 64 x 64 to 128 x 128 or 256 x 256 depending on sample size. The resolution of the step size at each X and Y direction can also be specified for a desired resolution. This technique allows a high level of details and signal averaging as well as 3D imaging by shaving the surface of frozen sample and scanning at different depths.

3 Experimental Results

Coronary arterial occlusion in *in vivo* heart experiments causes regional hypoperfusion and local tissue hypoxia. This is a model for a possible human heart myocardial ischemia. The shift from aerobic to anaerobic metabolism and local tissue hypoxia can be assessed by metabolic collection of mitochondrial intrinsic fluorescence of NADH (Nicotinamide Adenine Dinucleotide) and FP (Flavoprotein). The normalized ratio of these fluorophores (FP/FP+NADH), called the redox ratio, is an indicator of the metabolic state of tissue.

Figure 4 displays the fluorescence intensity of the FP redox ratio in the infarct zone versus the non-infarct zone (two regions of interest observed from the beginning of the experiment) after 180 minutes of reperfusion. The data reveals the redox ratio of infract zone has become more oxidized with respect to the non-infarct zone. It is quite anomalous that a previously hypoxic region has become more oxidized. Previous data suggest this anomaly represents ongoing apoptosis.[9] The non-infarct zone shows a drop in FP redox ratio at ischemia due to reduction of fluorophores and a gradual increase during reperfusion due to oxidation. The variation of redox ratio in non-infarct region is smaller (~10% from reperfusion start to end) than infarct region during reperfusion (~48% variation). In the surface fluorometery data, each heart serves as its own control (infarct zone vs. non-infarct zone). Thus, in this experiment, the FP redox ratio for normal myocardium versus Ischemic/Reperfused (IR) zone shows a distinct rise due to oxidation in the I/R zone during the reperfusion period (180 min), suggesting that we are able to detect apoptosis during reperfusion.

The mitochondrial redox state obtained by redox scanning is suitable for identifying heart metabolic states since the redox state is sensitive to apoptosis detection, workloads, oxygen and substrate supplies. The *in vivo* fluorescence signals at 180 min of reperfusion were found to correlate with post mortem frozen heart signals obtained by cryogenic redox scanning. The frozen sample scans shown in Figure 5 indicate an increased FP redox ratio after 3 hours of reperfusion at the ligation I/R zone and reduction of NADH redox ratio in that area. The histograms of FP redox ratio as well as NADH redox ratio show a bimodal distribution that can be due to a much higher intensity of oxidized fluorophores in the infarction zone. This is clear by a higher spectrum mean in FP redox ratio and a smaller spectrum mean in NADH redox ratio. Based upon previous studies,[10,11] this is indicative of on going apoptosis is occurring in this region.

This high resolution scan suggests a clearly defined apoptotic zone surrounding the rather small infarct zone. Thus, we are able not only to identify apoptosis but also, and more importantly to show the borders of the apoptotic region.

4 Discussion

Noninvasive optical techniques are useful in assessing tissue oxygenation on site during open-heart surgery. We demonstrate the technique of direct fluorometry of mitochondrial signals as non-tissue destructive indicators of myocardial damage due to ischemia along with the identification of activation of apoptosis. Further studies are in progress to distinguish the apoptosis signal from necrosis by using infarcted but non-reperfused hearts. A noninvasive/non-tissue destructive optical technique to assess tissue oxygenation, redox states and apoptosis in human hearts would have tremendous clinical potential in a variety of clinical settings. This study suggests the feasibility of such an approach. It is hypothesized that the main reason for heart failure months after a heart attack is the initiation and progression of apoptosis.

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Figure captions:

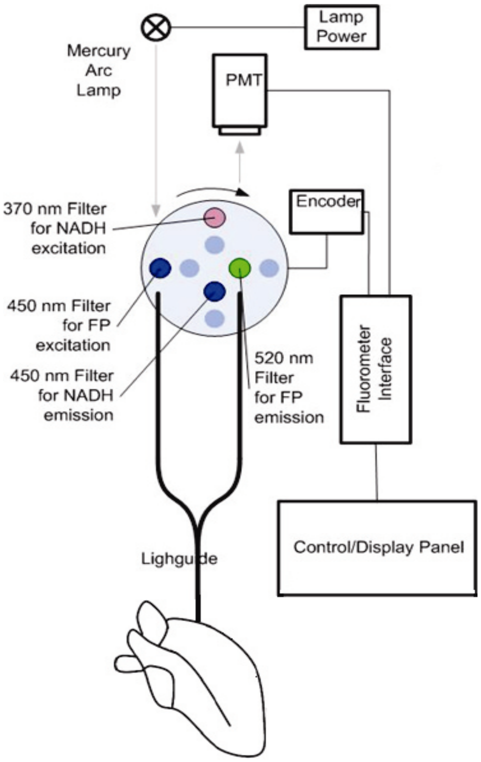
Fig.1 Rotating Disk Fluorometer

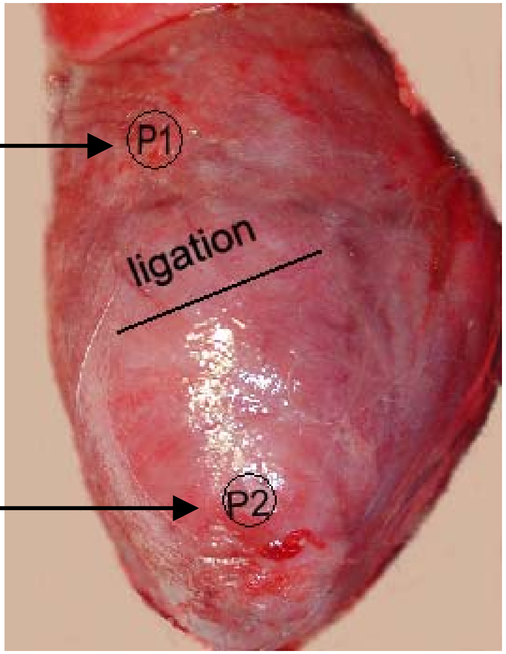
Fig.2 Position of ligation area and data acquisitions on the heart

Fig.3 Diagram of the low temperature fluorometer (redox scanner)

Fig.4 FP redox ratio of non infarct zone and infarct zone during experiment

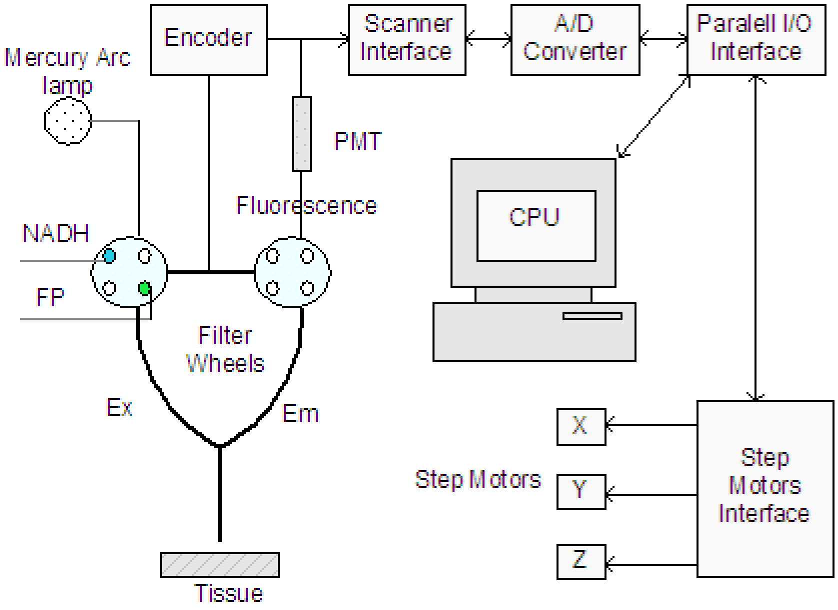
Fig.5 Redox ratio fluorescence signals of frozen heart (Depth from surface =1100 μm)


Lamp Power
Mercury Arc Lamp
PMT
Encoder
370 nm Filter for NADH excitation
450 nm Filter for FP excitation
450 nm Filter for NADH emission
520 nm Filter for FP emission
Fluorometer Interface
Lighguide
Control/Display Panel



Basal anterolateral wall

Anteroapical myocardial wall distal to the largest branch of circumflex coronary artery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Scanner Interface
A/D Converter
Paralell I/O Interface
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FP
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Ex
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Step Motors
X
Y
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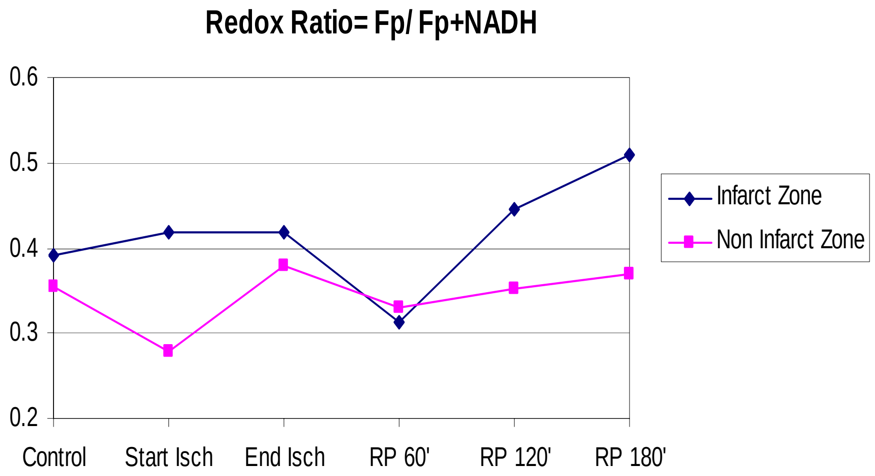
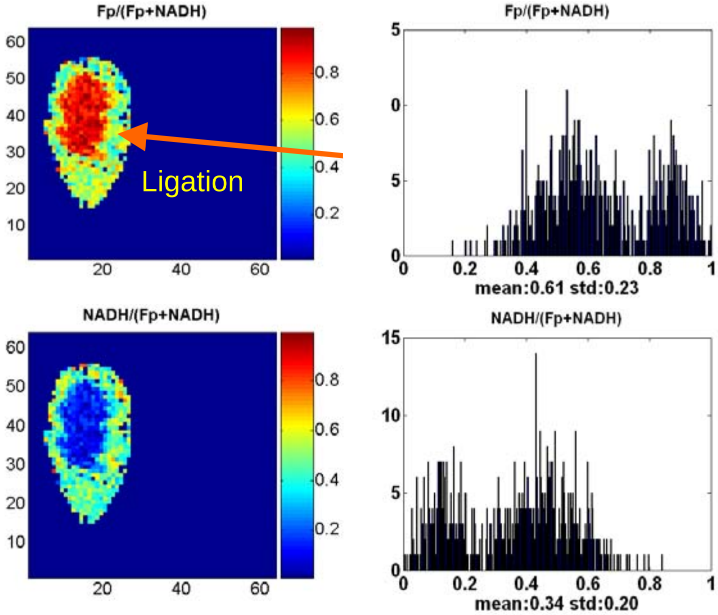
Step Motors Interface


Redox Ratio= Fp/ Fp+NADH
0.6
0.5
0.4
0.3
0.2
Control
Start Isch
End Isch
RP 60'
RP 120'
RP 180'
Infarct Zone
Non Infarct Zone


Fp/(Fp+NADH)
60
50
40
30
20
10
20
40
60
0.8
0.6
0.4
0.2
Ligation
Fp/(Fp+NADH)
5
0
5
0
0
0.2
0.4
0.6
0.8
1
mean:0.61 std:0.23
NADH/(Fp+NADH)
60
50
40
30
20
10
20
40
60
0.8
0.6
0.4
0.2
NADH/(Fp+NADH)
15
10
5
0
0
0.2
0.4
0.6
0.8
1
mean:0.34 std:0.20