

Monitoring changes in the redox state of myoglobin in cardiomyocytes by Raman spectroscopy enables the protective effect of NO donors to be evaluated

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Abstract

Raman microspectroscopy has been used to monitor changes in the redox and ligand-coordination states of the heme complex in myoglobin during the pre-conditioning of *ex vivo* cardiomyocytes with pharmacological drugs that release nitric oxide (NO). These chemical agents are known to confer protection on heart tissue against ischemia-reperfusion injury. Subsequent changes in the redox and ligand-coordination states during experimental simulations of ischemia and reperfusion have also been monitored. We found that these measurements, in real time, could be used to evaluate the pre-conditioning treatment of cardiomyocytes, and predict the likelihood of cell survival following a potentially-lethal period of ischemia. Evaluation of the pre-conditioning treatment was done at the single-cell level. The binding of NO to myoglobin, giving a 6-coordinate ferrous-heme complex, was inferred from the measured Raman bands of a cardiomyocyte by comparison to pure solution of the protein in the presence of NO. A key change in the Raman spectrum was observed after perfusion of the NO-donor was completed, where if the pre-conditioning treatment was successful then the bands corresponding to the nitrosyl complex were replaced by bands corresponding to metmyoglobin, Mb^{III}. An observation of Mb^{III} bands in the Raman spectrum was made for all the cardiomyocytes that recovered contractile function, whilst the absence of Mb^{III} bands always indicated that the cardiomyocyte would be unable to recover contractile function, following the simulated conditions of ischemia and reperfusion in these experiments.

Introduction

Cardiovascular disease is the leading cause of mortality worldwide, with coronary artery disease responsible for approximately 42% of these deaths (7.4 million in 2012).¹ Many of the deleterious effects of coronary artery disease are caused by ischemia-reperfusion injury. Tissue damage occurs when the cardiac muscle is starved of oxygen and nutrients due to blocked coronary vessels but yet further damage occurs when the tissue is reperfused with blood in an attempt to restore circulation.² The molecular mechanisms of reperfusion injury are not fully understood. Strategies for pre-conditioning cardiac muscle to confer protection against ischemia/reperfusion injury have been known since the mid-1980s. For instance Murry et al. observed that a few brief cycles of sub-lethal ischemia and reperfusion could protect the heart.³ Other studies have identified a diverse range of pharmacological agents that are also cardioprotective.⁴

Minners et al. demonstrated that pre-conditioning of isolated rat hearts with the mitochondrial uncoupler, 2,4-dinitrophenol (DNP), reduced infarct size following ischemia and reperfusion.⁵ The effects of this type of chemical agent can also be demonstrated in laboratory experiments that monitor, among other things, the contractile function of *ex vivo* cardiomyocytes from heart muscle. Cardiomyocytes are the individual rod-shaped cells found in cardiac muscle which contain an abundance of the contractile proteins actin and myosin. Rodrigo et al. showed that DNP depolarises the mitochondrial membrane and decreases nicotinamide adenine dinucleotide (NADH) in isolated cardiomyocyte models whilst conferring protection from metabolic inhibition and reperfusion.⁶

Another notable class of cardioprotective agents are chemical drugs that release nitric oxide (NO).⁷⁻⁹ Endogenous production of NO is already known to play a role in cardioprotection.¹⁰ Respiration and cellular viability are regulated under conditions of hypoxia by the reduction of nitrite ions to NO mediated by the heme protein, myoglobin.¹¹ The oxygenated state of myoglobin also acts as a scavenger of cellular NO and, hence, the protein plays a vital role in NO homeostasis, which is particularly important in cardiomyocytes as elevated levels inhibit mitochondrial respiration.^{12,13}

Non-resonance^{14,15} and resonance¹⁶⁻¹⁹ Raman spectroscopy have been used before in structural imaging of heart tissue¹⁶ and to monitor dynamic changes in both isolated hearts¹⁸, cardiomyocytes¹⁴⁻¹⁷ and exposed mitochondria.¹⁹ In our recent work, the cellular mechanisms in *ex vivo* cardiomyocytes during hypoxia and hyperoxic reoxygenation were studied by Raman spectroscopy using a (non-resonant) excitation wavelength of 488 nm.¹⁵ This excitation wavelength, which lies between the Soret and Q bands for heme proteins, was found to give a strong Raman signal for the pyrrole half-ring symmetric stretch (ν_4) of the heme prosthetic

group; the notation for the assignment of heme vibrational modes has been described by Spiro and co-workers.²⁰ The measured scattering cross-section of the ν_4 band obtained using a non-resonant wavelength of 488 nm was comparable to that obtained using a resonant wavelength of 405 nm, but the cardiomyocytes could be exposed to higher total energies of irradiation without causing photoinduced damage of cells.¹⁵ The centre wavenumber of the ν_4 band is distinct for deoxygenated myoglobin (Mb^{II} ; 1356 cm^{-1}) oxygenated myoglobin or metmyoglobin ($\text{Mb}^{\text{II}}\text{-O}_2$, Mb^{III} ; 1372 cm^{-1}), ferrous cytochrome *c* (Cyt^{II} ; 1363 cm^{-1}) and ferric cytochrome *c* (Cyt^{III} ; 1374 cm^{-1}). Identification of these species is also assisted by the centre wavenumber of the bands measured in the region 1500-1650 cm^{-1} ; where the high-spin heme proteins, Mb^{II} and Mb^{III} , exhibit a similar pattern of bands, which differs from that exhibited by low-spin heme proteins, such as $\text{Mb}^{\text{II}}\text{-O}_2$. Although the individual cytochromes (*a*, *b* and *c*) cannot be resolved separately, the dynamics of myoglobin in cardiomyocytes can be distinguished from cytochromes in time-lapse sequences of Raman spectra.

In the present study, we have employed confocal-Raman spectroscopy to monitor changes in the redox and ligand coordination states of the heme complex in myoglobin during the pre-conditioning of cardiomyocytes with NO-donor compounds. We have also monitored the subsequent changes that take place during experimental simulations of ischemia and reperfusion. Due to the important role played by myoglobin in NO-homeostasis, it was anticipated that the determination of redox and ligand coordination states of myoglobin could be used to evaluate the pre-conditioning treatment of cardiomyocytes and the likelihood of cell survival following a potentially-lethal period of ischemia. Established methodologies to evaluate pre-conditioning with pharmacological agents have involved the post-treatment determination of infarct size in isolated hearts⁵ or cell-survival statistics in a population of cardiomyocytes.⁶ The novelty of the present method is that information on the impact of treatment can be obtained at the single-cell level and in real time during the different stages of pre-conditioning, ischemia and reperfusion.

Experimental Methods

The isolation of single ventricular cardiomyocytes from adult male Wistar rats (250-300 g) was described previously.¹⁵ The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institute of Health.²¹ The procedure yielded ~80% quiescent rod-shaped ventricular myocytes with approximate dimensions of 100 μm -length and 20 μm -width. Cardiomyocytes were stored at room temperature in Tyrode solution, which is approximately isotonic with interstitial fluid, in a density of approximately 0.4 million per ml. [Tyrode solution: 6 mM KCl, 135 mM NaCl, 0.33 mM NaH_2PO_4 , 5 mM Na pyruvate, 10 mM glucose, 10 mM HEPES, 2 mM CaCl_2 and 1 mM MgCl_2 .]

The isolated cardiomyocytes were placed in a rhombus-shaped chamber and allowed to settle on the lower-imaging surface, after which the cells were perfused with Tyrode solution. The flow rate was maintained at approximately 4.5 ml/minute using a peristaltic pump. Full details of the perfusion apparatus were given in the earlier publication,¹⁵ with the exception that, in the present experiments, (i) the chamber was regulated at a temperature of $\sim 34^\circ\text{C}$ and (ii) a pair of platinum electrodes was positioned in the chamber aligned parallel to the direction of flow. The cardiomyocytes were stimulated by an electric field at 1 Hz with a magnitude of 25 V and duration of 5 ms using a physiological stimulator (Digitimer).

Two different apparatus were employed in the experiments: either (i) a population of cardiomyocytes was monitored by brightfield imaging of a large area of the perfusion chamber using a commercial-inverted microscope, or (ii) a single cardiomyocyte was monitored by the combination of confocal Raman spectroscopy and brightfield imaging using a homebuilt-inverted microscope (described previously¹⁵).

After at least 10 minutes perfusion with Tyrode solution, the cardiomyocytes were perfused with the pre-conditioning agents for either 5 or 10 minutes. Two different NO-donor compounds were used in separate experiments: diethylammonium (Z)-1-(N, N-diethylamino)diazen-1-ium-1, 2-diolate (diethylamine NONOate; DEA) and sodium nitroferricyanide (III) dihydrate (sodium nitroprusside; SNP). A comparison of the mechanisms of NO release by these compounds is given in (22). Both compounds were purchased from Sigma Aldrich (D5431 and 228710, respectively) and used without further purification. Concentrations of 100 μM DEA and 100 μM SNP were prepared in substrate-free Tyrode solution. A control experiment was also performed using 2,4-dinitrophenol (DNP), 50 μM , in substrate-free Tyrode solution. The control experiment replicated the results reported previously.⁶ Perfusion of the pre-conditioning agent was followed by perfusion of a substrate-free Tyrode solution for 5 minutes. Control experiments were also performed on cardiomyocytes without any pre-conditioning treatment.

[Substrate-free Tyrode solution: 6 mM KCl, 140 mM NaCl, 0.33 mM NaH₂PO₄, 10 mM sucrose, 10 mM HEPES, 2 mM CaCl₂ and 1 mM MgCl₂.]

Ischemic conditions were simulated by perfusion of a metabolic inhibitor consisting of 2 mM sodium cyanide, with 1 mM iodoacetic acid, in substrate-free Tyrode solution, for 7 minutes. Iodoacetic acid inhibits glyceraldehyde 3-phosphate dehydrogenase and the cyanide ion blocks electron transfer from complex IV, cytochrome *c* oxidase, of the electron transport chain.⁶ The solution mimics ischemia chemically as both components inhibit ATP synthesis, which also occurs in low oxygen conditions; the substrate-free Tyrode solution lacks the metabolic substrates glucose and pyruvate which are necessary for ATP generation.

Reperfusion of normal Tyrode solution can promote ATP synthesis again and result in the re-energisation of cells. Contractile recovery of the cardiomyocytes was determined after 10 to 25 minutes. Myocytes that did not respond to electric-field stimulation or contracted asynchronously were deemed not to have recovered contractile function.

In one set of experiments, the percentage of cells that recovered contractile function, following metabolic inhibition and reperfusion, was determined by brightfield imaging of a population of cardiomyocytes occupying a large area of the perfusion chamber. Electrical stimulation at the start of the experiments was used to identify those cells in the field-of-view that exhibit normal contractile function. At the conclusion of the experiment, the contractile responses of the same cells were recorded. Data is reported as the mean number of cells that exhibit contractile recovery in a series of replicate experiments; the standard error of the mean from the replicate experiments is also reported. The full details of the total number of cells recorded, the number of experiments performed and number of hearts involved in the study are reported in parenthesis. The statistical significance of measured differences in the data sets was considered using an unpaired t-test (Prism 5, GraphPad), in which appropriate and significant difference was accepted where the calculated *p*-value was < 0.01 (**), < 0.001 (***) or < 0.001 (****).

The study of populations of cardiomyocytes in the aforementioned experiments contrasts with the strategy adopted in the other part of this research work. A further set of experiments were performed in which a single cardiomyocyte was selected to be studied each time. In the first instance, it was confirmed by brightfield imaging that the single cardiomyocyte exhibited normal contractile function in response to electric-field stimulation. Raman spectra were then recorded from a diffraction-limited volume inside the cardiomyocyte during pre-conditioning, metabolic inhibition and reperfusion. An excitation wavelength of 488 nm was used, with a power of 0.9 mW incident on the cell for a spectral-acquisition time of 30 s. There was a minimum-time lapse of 5 minutes between the recording of sequential Raman spectra, and the laser was shuttered during this interval of time. Fluorescence background was subtracted using a

polynomial-fitting algorithm in ORIGIN 9.1 software (OriginLab Corp.). Electric-field stimulation was used again to determine the contractile function of the single cardiomyocyte following metabolic inhibition and reperfusion.

Reference Raman spectra were recorded for solutions containing nitrosyl ferro- and ferri-myoglobin. The preparation of ferro and ferric- myoglobin solutions has been described before.¹⁵ Addition of 100 μM SNP to these solutions resulted in the nitrosylation of the myoglobin species.

Results and discussion

Recovery of contractile function after simulated ischemia reperfusion injury

A brightfield image of isolated ventricular cardiomyocytes perfused with Tyrode solution is shown in figure 1 **(a)**. Cells were stimulated to contract in response to electric-field stimulation (1 Hz). Subsequent images of the same group of isolated cardiomyocytes during simulated ischemia with metabolic-inhibitor solution, and during reperfusion with Tyrode solution is shown in figure 1 **(b)** and **(c)**, respectively. Many of the cells show hypercontracture after 10 minutes of reperfusion, and the number of cardiomyocytes that recovered contractile function in response to electric-field stimulation was quantified. In the absence of pre-conditioning, the percentage of control cells that recovered contractile function was determined to be $27 \pm 2\%$ ($n = 547, 25, 20$) and $28 \pm 3\%$ ($n = 84, 4, 4$) in two separate experiments; see figure 1 **(d)** and **(e)**. [$(n =$ total number of cells studied, number of experiments performed, number of hearts used).]

Two different compounds that release NO were tested as pre-conditioning agents for cardiomyocytes. Pre-conditioning treatment with sodium nitroprusside (SNP) for either 5 or 10 minutes showed a significant increase in the percentage of cells recovering contractile function: 5 minutes, $50 \pm 2\%$ ($n = 138, 8, 8$); 10 minutes, $66 \pm 2\%$ ($n = 238, 11, 11$); see **(d)**. A similar increase in contractile recovery was observed following pre-conditioning with diethylamine NONOate (DEA): 5 minutes, $48 \pm 3\%$ ($n = 99, 4, 4$); 10 minutes $66 \pm 2\%$ ($n = 86, 4, 4$); see **(e)**. The response of the cardiomyocytes to pre-conditioning with the NO donors is comparable with that observed by the known cardioprotective agent, 2, 4-dinitrophenol (DNP). The percentage of cells that recovered contractile function, following 10 minutes pre-conditioning with DNP, was determined to be $63 \pm 3\%$ ($n = 217, 10, 8$) and $62 \pm 5\%$ ($n = 107, 5, 4$) in two separate experiments; see **(d)** and **(e)**. In order to account for any effects of SNP and DEA that were independent of NO release, the chemical agents were left overnight (o/n) in solution at room temperature to allow the entire NO content to evolve. These solutions were then tested as pre-conditioning agents. The percentage of cells that recovered contractile function following the use of the NO-depleted solutions was similar to the data measured in the control experiments without pre-conditioning treatment: 5 minutes SNP o/n, $26 \pm 3\%$ ($n = 96, 4, 4$); 10 minutes SNP o/n, $28 \pm 2\%$ ($n = 101, 5, 4$); 5 minutes DEA o/n, $24 \pm 3\%$ ($n = 86, 4, 4$); 10 minutes DEA o/n, $25 \pm 4\%$ ($n = 82, 4, 4$); see **(d)** and **(e)**.

Raman spectroscopic measurement of nitrosyl myoglobin in cardiomyocytes

The Raman spectrum measured from single ventricular cardiomyocytes (*ex vivo*), using a laser excitation wavelength of 488 nm, was shown in earlier work¹⁵ to be dominated by bands from deoxygenated myoglobin, Mb^{II}, with smaller contributions made from oxygenated myoglobin, Mb^{II}-O₂, and cytochromes. A typical spectrum of an isolated ventricular cardiomyocyte, perfused with Tyrode buffer, is shown in figure 2 (**a**). The assignment of the most intense bands to vibrational modes of Mb^{II} is consistent with previously reported work by Spiro and co-workers.^{20,23} A Raman spectrum of the pure protein Mb^{II} in aqueous solution is shown for comparison in figure 3. Raman spectra are also shown in figure 2 for an isolated cardiomyocyte measured in Tyrode buffer containing a NO-donor compound: (**b**) DEA (100 μ M) or (**c**) SNP (100 μ M). Addition of NO results in a shift of the ν_4 band for the cellular myoglobin from the value for Mb^{II} at 1356 cm⁻¹ to a new value of 1375 cm⁻¹. The spin-marker region of the spectrum for the cardiomyocyte in the presence of NO is dominated by the ν_2 & ν_{19} bands at 1585 cm⁻¹ and the ν_{10} band at 1637 cm⁻¹.

The identity of the myoglobin species which dominates the spectra of cardiomyocytes in the presence of the NO-donor compounds, in figure 2 (**b**) and (**c**), is nitrosyl ferrous myoglobin, Mb^{II}-NO. Both spectra resemble the Raman spectrum recorded for an aqueous solution of the pure protein, Mb^{II} following the addition of a NO donor compound; see figure 3. The ν_4 band, at 1375 cm⁻¹, the ν_2/ν_{19} bands, at 1585 cm⁻¹, and the ν_{10} band, at 1637 cm⁻¹, for Mb^{II}-NO are located at approximately the same positions as the corresponding bands for Mb^{II}-O₂.¹⁵ The pattern of the ν_2 , ν_{19} and ν_{10} bands for Mb^{II}-NO is characteristic of a low-spin state of the 6-coordinate metal ion.²⁰ The position of the ν_4 band is often used to deduce the oxidation state of iron in heme proteins,²⁰ and the appearance of the ν_4 band for Mb^{II}-NO at a relatively high wavenumber position would usually lead to a conclusion that the character of the metal centre is ferric. Approximately the same wavenumber position for the ν_4 band of Mb^{II}-NO has been reported by others.^{24,25} The electron paramagnetic resonance (EPR) spectrum of Mb^{II}-NO (which has a bent Fe-N-O geometry) shows that the ferrous metal is not sufficiently reduced by charge transfer to justify the description of the complex as possessing ferric character,²⁶ therefore, the wavenumber position of the ν_4 band is unusual for the nitrosyl ferrous complex, Mb^{II}-NO. A similar wavenumber for ν_4 is observed for the weakly-bound complex formed between the ferric state of myoglobin, Mb^{III}, and NO.^{24,27} In this example, EPR studies indicate that charge transfer, in the linear Fe-N-O geometry, gives a high spin complex possessing ferrous character, *i.e.* Mb^{III}-NO \leftrightarrow Mb^{II}-NO⁺,²⁸ however the higher electron density on the metal ion in Mb^{II}-NO⁺ must not lead to increased π -backbonding to the porphyrin as the anticipated shift in ν_4 to lower frequencies is not observed (see figure 3). Thus, both the nitrosyl

ferrous and ferric complexes of myoglobin have a centre frequency for ν_4 which is characteristic typically of ferric complexes despite structural studies indicating that both complexes have predominantly ferrous character. The high spin electron configuration of the $\text{Mb}^{\text{III}}\text{-NO}$ complex is reflected in the similarity in the position of the bands between 1500 and 1650 cm^{-1} to other high spin forms of myoglobin, Mb^{II} and Mb^{III} ; see figure 3.

The comparison between the spectrum of the cardiomyocyte, figure 2 **(b)** and **(c)**, and the spectrum of the pure protein, Mb^{II} , in solution with NO (figure 3) is convincing evidence that NO is bound to myoglobin in the cardiomyocyte. The amount of myoglobin in the heart is substantial (approximately 200 $\mu\text{mol/kg}$) and nitrosyl-myoglobin complexes have previously been measured in an ischemic heart by EPR.²⁹ Nitric oxide is known to bind to other heme proteins¹² and the complex formed between myoglobin and NO has also been characterised by infra-red spectroscopy,³⁰ NMR³¹ and theoretical modelling,³² in addition to the present study using Raman spectroscopy and the aforementioned work using EPR.²⁹

Raman spectroscopic monitoring of metabolic inhibition and reperfusion of cardiomyocytes

In figure 4, the change in the Raman spectrum of a single cardiomyocyte is shown following the sequential perfusion of **(a)** Tyrode solution, **(b)** substrate-free Tyrode solution and **(c)** a metabolic inhibitor, and **(d)** the subsequent reperfusion of the cardiomyocyte with Tyrode solution for 25 minutes. In this experiment, the cardiomyocytes were not subjected to a pre-conditioning treatment. Measured data obtained from a number of different examples of single cells can be subdivided into two categories corresponding to cardiomyocytes that either recovered contractile function, or failed to recover contractile function in response to electrical stimuli after metabolic inhibition and reperfusion. Representative examples of data recorded on a single cell that fell into each of these categories are illustrated in figure 4: Left – a cardiomyocyte that failed to recover contractile function; Right – a cardiomyocyte that recovered contractile function. Sequences of Raman spectra were recorded for 6 replicates of this experiment, where 3 cells recovered and 3 cells failed to recover contractile function, respectively. The spectra shown in figure 4 are representative of all datasets obtained for cells that fell into these two different categories.

When the solution is changed from normal Tyrode solution to a substrate-free Tyrode solution **(a)** to **(b)**, there is no discernible change in the Raman spectrum of cardiomyocytes that were observed to either recover, or not to recover, contractile function at the conclusion of the experiment. During the perfusion of a metabolic inhibitor in **(c)** and the subsequent reperfusion of Tyrode solution in **(d)**, there is also no change in the Raman spectrum for examples of single

cells that were observed to recover contractile function (Right panel in figure 4), however, the Raman bands for myoglobin were seen to disappear for single cells that failed to recover contractile function (Left panel in figure 4). In the latter case, the most prominent feature remaining in the spectrum is the amide I band (c.1650 cm^{-1}), which could originate from contractile (actin and myosin) proteins. It is possible that myoglobin has been released through ruptured membranes in cells that fail to recover contractile function. There is evidence reported in the literature that this takes place following ischemia but it might not be a process that is specific to myoglobin.^{33,34,35,36}

Monitoring cardioprotection by NO-donor compounds during simulated reperfusion injury

It was shown in figure 2 that the pre-conditioning of cardiomyocytes with a solution containing either DEA or SNP causes a change in the dominant state of myoglobin from the ferrous 5-coordinate complex, Mb^{II} , to the 6-coordinate nitrosyl complex, $\text{Mb}^{\text{II}}\text{-NO}$. The same change is observed in sequences of Raman spectra, shown in figures 5, 6, 7 and 8 (recorded from different single cells), between the perfusion of Tyrode solution in (**a**), and either DEA solution (figures 5 (**b**) and 6 (**b**)) or SNP solution (figures 7 (**b**) and 8 (**b**)). The formation of $\text{Mb}^{\text{II}}\text{-NO}$ is apparent from the increase in wavenumber of ν_4 at 1375 cm^{-1} and the intensity increase of the overlapping ν_2 and ν_{19} bands at 1585 cm^{-1} and the ν_{10} band at 1637 cm^{-1} . Either one of two things could happen to single cells in the next stage of the experiment during the perfusion of a substrate-free Tyrode solution in (**c**). The different responses of single cells appear to directly correlate with the final outcome of the experiment when it was observed whether or not the individual cell recovered contractile function; *i.e.* after metabolic inhibition in (**d**), and reperfusion in (**e**).

In figures 5 (**c**) and 7 (**c**), there is no clear shift in ν_4 , and the overlapping ν_2 and ν_{19} bands are still observed at 1585 cm^{-1} , following perfusion of the substrate-free buffer subsequent to pre-conditioning of cardiomyocytes with DEA and SNP, respectively; *i.e.* the position of the ν_4 , ν_2 , ν_{19} and ν_{10} bands remain characteristic of the nitrosyl myoglobin complex, $\text{Mb}^{\text{II}}\text{-NO}$. For the experiments illustrated in figures 5 and 7, these examples of single cells failed to recover contractile function after metabolic inhibition and reperfusion of Tyrode solution. As in the control experiment (figure 4 left), the Raman bands for myoglobin were also observed to disappear following perfusion of the metabolic inhibitor, figure 5 (**d**) and 7 (**d**), and reperfusion with normal Tyrode solution, figure 5 (**e**) and 7 (**e**).

In figures 6 (**c**) and 8 (**c**), there are significant changes in the Raman spectrum following perfusion of the substrate-free buffer subsequent to pre-conditioning of cardiomyocytes with

DEA and SNP, respectively: (i) there is a small shift in ν_4 from 1375 cm^{-1} to 1372 cm^{-1} ; (ii) the overlapping ν_2 and ν_{19} bands at 1585 cm^{-1} are much weaker and the ν_{10} band at 1637 cm^{-1} is not resolved (these disappearing bands originate from low-spin heme complexes); (iii) a ν_2 band appears at 1565 cm^{-1} , which indicates the formation of a high-spin heme complex; and (iv) a $\nu(\text{C}_a=\text{C}_b)$ band appears at 1615 cm^{-1} . In summary, the spectra in figure 6 (c) and 8 (c) are characteristic of a ferric, high spin, complex which exhibits a strong $\nu(\text{C}_a=\text{C}_b)$ band. All of these features are consistent with metmyoglobin, Mb^{III} , becoming the dominant species in substrate-free Tyrode solution after pre-conditioning treatments. A Raman spectrum of Mb^{III} recorded in aqueous solution is shown in figure 3. Mb^{III} is a 5-coordinate complex in which a water molecule can bind reversibly to the 6th coordination position. The position of the ν_4 band for Mb^{III} , at 1372 cm^{-1} differs significantly from the position of the same band for Mb^{II} (at 1356 cm^{-1} ; see figure 3). The ν_4 band for Mb^{III} is at a slightly lower wavenumber than for $\text{Mb}^{\text{II}}\text{-NO}$ (at 1375 cm^{-1} ; see figure 3) and at a similar wavenumber to that observed for $\text{Mb}^{\text{II}}\text{-O}_2$.¹⁵ In contrast, the pattern of vibrational bands in the spin-marker region of the metmyoglobin spectrum resembles that observed for Mb^{II} ; ν_{11} (1545 cm^{-1} shoulder), ν_2 (1565 cm^{-1}) and ν_{10} (1608 cm^{-1}), however, the ν_{10} band is obscured in Mb^{III} by the strong C=C stretching band. Both the ferrous and ferric metal ion in Mb^{II} and Mb^{III} , respectively, have a high spin electron configuration in contrast to the low spin electron configuration of the metal ion in $\text{Mb}^{\text{II}}\text{-O}_2$ (ν_2 , ν_{19} and ν_{10} are found at higher wavenumbers in $\text{Mb}^{\text{II}}\text{-O}_2$).

Significantly, in both experiments shown in figures 6 and 8, these examples of single cells recovered contractile function following metabolic inhibition in (d), and reperfusion in (e). The Raman spectrum following metabolic inhibition in (d) is similar to that observed at the previous stage in (c), however, the Raman spectrum changes to that characteristic of Mb^{II} after reperfusion with normal Tyrode solution in (e). The final spectrum at the conclusion of the experiments shown in figures 6 (e) and 8 (e) resembles the initial spectrum of the single cell recorded in perfused Tyrode solution in (a). This could indicate the return of cellular homeostasis with resumed production of NADH which is necessary for the function of metmyoglobin reductase, the enzyme necessary for the conversion of metmyoglobin to deoxymyoglobin.³⁷

The example data shown in figures 5 to 8 are illustrative of a larger number of replicate experiments on single cells. A total of nine experiments were performed on single cells from different hearts; in six of the experiments, the single cells were observed to recover contractile function following pre-conditioning with either DEA or SNP solution and, in each example, the appearance of Mb^{III} bands in the Raman spectrum during the perfusion of substrate-free Tyrode solution was observed (4 examples with SNP, and 2 examples with DEA); in three of the

experiments, the single cells failed to recover contractile function following pre-conditioning with either DEA or SNP and, in each example, there was no significant change in the Raman spectrum during the perfusion of substrate-free Tyrode solution (2 examples with SNP, and 1 example with DEA).

Conclusion

The pre-conditioning of cardiomyocytes with NO donors (*i.e.* DEA and SNP) results in the nitrosyl complex becoming the dominant state of cellular myoglobin. We expect that the NO released from the donor compounds will also interact with other heme proteins in mitochondria as NO is known to suppress mitochondrial respiration, however, the Raman measurements were not able to detect these interactions in cardiomyocytes. A change in the Raman spectrum after the perfusion of the solution of the NO donor is observed in individual cardiomyocytes that have been pre-conditioned successfully with the pharmacological agent. In this example, the nitrosyl heme complex reacts with oxygen dissolved in substrate-free Tyrode solution resulting in the formation of metmyoglobin (Mb^{III}), *i.e.* according to $\text{Mb}^{\text{II}}\text{-NO} + \text{O}_2 \rightarrow \text{Mb}^{\text{III}} + \text{NO}_3^-$; this reaction has been followed previously by $^1\text{H-NMR}$.³¹ This reaction will ensure that NO is maintained at a low concentration, which is essential as the radical NO would otherwise be cytotoxic, blocking the mitochondrial chain and leading to production of reactive oxygen species. Although a relatively small number of cardiomyocytes were tested, if the oxidised state of myoglobin was detected in the Raman spectrum directly after the pre-conditioning treatment, then the individual cardiomyocyte was observed always to recover contractile function following the simulated conditions of ischemia and reperfusion in these experiments. During the period of reperfusion, the ferric state of myoglobin is reduced back to the ferrous state. This reduction reaction will be catalysed internally in cardiomyocytes by metmyoglobin reductase.

If the Raman bands remain at the positions identified with the nitrosyl myoglobin state ($\text{Mb}^{\text{II}}\text{-NO}$) after the pre-conditioning treatment, then the individual cardiomyocyte was observed not to recover contractile function following the simulated conditions of ischemia and reperfusion. In this example, the formation Mb^{III} is not significant and the reaction between $\text{Mb}^{\text{II}}\text{-NO}$ and O_2 must not have taken place. We can speculate that the nitrosyl-myoglobin complexes must release large quantities of NO during metabolic inhibition, which will subsequent block the production of ATP in mitochondria during reperfusion of the cardiomyocyte with oxygenated buffer, and hence the cell will not recover contractile function. This is accompanied by a decrease in the intensity of the heme bands and only broad features remain in the Raman spectrum which are derived from amino acid side-chains.

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Figure captions

Figure 1: Contractile recovery of cardiomyocytes following simulated ischemia-reperfusion injury. Images of cardiomyocytes **(a)** prior to metabolic inhibition (MI), **(b)** after 7 minutes of MI and **(c)** after 10 minutes of reperfusion. **(d)** and **(e)** The percentage of cardiomyocytes recovering a contractile response to electrical-field stimulation was measured after reperfusion. Results are presented for untreated cells, positive-control experiments where the cells were pre-conditioned with DNP, pre-conditioned cells with SNP and DEA for 5 minutes and 10 minutes, and pre-conditioned cells with SNP and DEA solution that were left overnight (o/n).

Figure 2: Raman spectra of a single ventricular cardiomyocyte **(a)** in Tyrode solution **(b)** in substrate-free Tyrode solution containing DEA, and **(c)** in substrate-free Tyrode solution containing SNP. The data were recorded from different cells.

Figure 3: Raman spectra of solutions containing nitrosyl ferrous and ferric myoglobin complexes, $\text{Mb}^{\text{II}}\text{-NO}^{\bullet}$ and $\text{Mb}^{\text{III}}\text{-NO}^{\bullet}$, deoxymyoglobin, Mb^{II} , and metmyoglobin, Mb^{III} .

Figure 4: Raman spectra of single ventricular cardiomyocytes. The data were recorded from two different cells. In Example A, the cell did not recover contractile function (Left) and, in Example B, the cell recovered contractile function (Right) after reperfusion of normal Tyrode solution. Sequential perfusion of **(a)** Tyrode solution, 5 minutes, **(b)** substrate-free Tyrode solution, 5 minutes, and **(c)** a metabolic inhibitor, 7 minutes, and **(d)** reperfusion of Tyrode solution, 25 minutes.

Figure 5: Raman spectra of a single ventricular cardiomyocyte that did not recover contractile function after reperfusion of normal Tyrode solution. Sequential perfusion of **(a)** normal Tyrode solution, 5 minutes, **(b)** substrate-free Tyrode solution containing DEA, 10 minutes, **(c)** substrate-free Tyrode solution, 5 minutes, and **(d)** a metabolic inhibitor, 7 minutes, and **(e)** reperfusion of normal Tyrode solution, 25 minutes. All data shown were recorded from the same cell.

Figure 6: Raman spectra of a single ventricular cardiomyocyte that recovered contractile function after reperfusion of normal Tyrode solution. Sequential perfusion of **(a)** normal Tyrode solution, 5 minutes, **(b)** substrate-free Tyrode solution containing DEA, 10 minutes, **(c)** substrate-free Tyrode solution, 5 minutes, and **(d)** a metabolic inhibitor, 7 minutes, and **(e)** reperfusion of normal Tyrode solution, with spectra recorded in 5 minute intervals for a duration of 25 minutes. All data shown were recorded from the same cell.

Figure 7: Raman spectra of a single ventricular cardiomyocyte that did not recover contractile function after reperfusion of normal Tyrode solution. Sequential perfusion of **(a)** normal Tyrode solution, 5 minutes, **(b)** substrate-free Tyrode solution containing SNP, 10 minutes, **(c)**

substrate-free Tyrode solution, 5 minutes, and **(d)** a metabolic inhibitor, 7 minutes, and **(e)** reperfusion of normal Tyrode solution, 25 minutes. All data shown were recorded from the same cell.

Figure 8: Raman spectra of a single ventricular cardiomyocyte that recovered contractile function after reperfusion of normal Tyrode solution. Sequential perfusion of **(a)** normal Tyrode solution, 5 minutes, **(b)** substrate-free Tyrode solution containing SNP, 10 minutes, **(c)** substrate-free Tyrode solution, 5 minutes, and **(d)** a metabolic inhibitor, 7 minutes, and **(e)** reperfusion of normal Tyrode solution, with spectra recorded in 5 minute intervals for a duration of 25 minutes. All data shown were recorded from the same cell.

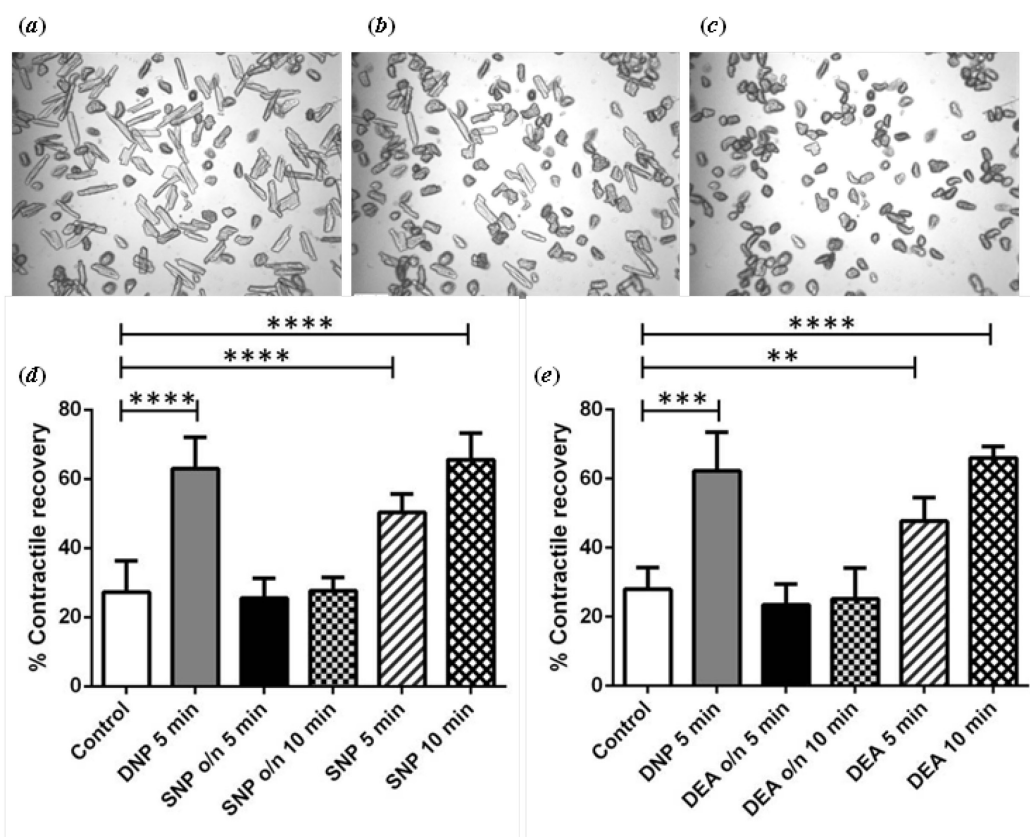


Figure 1

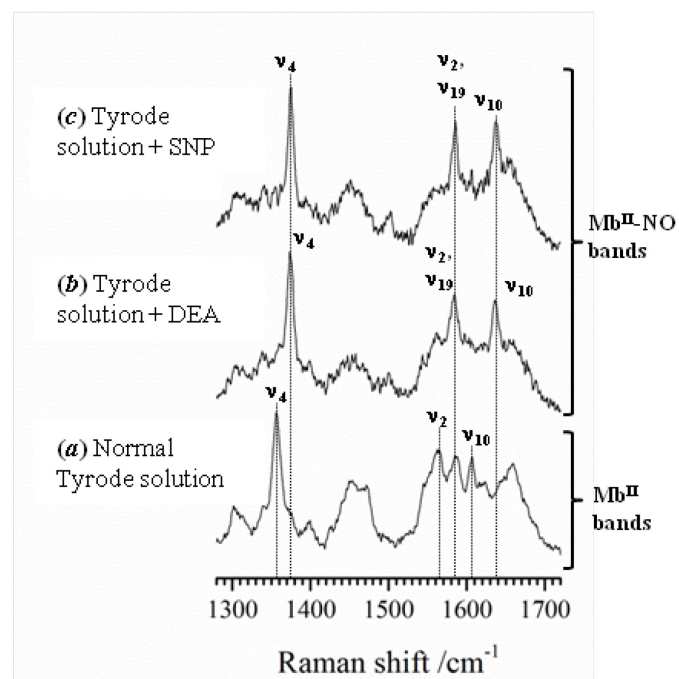


Figure 2

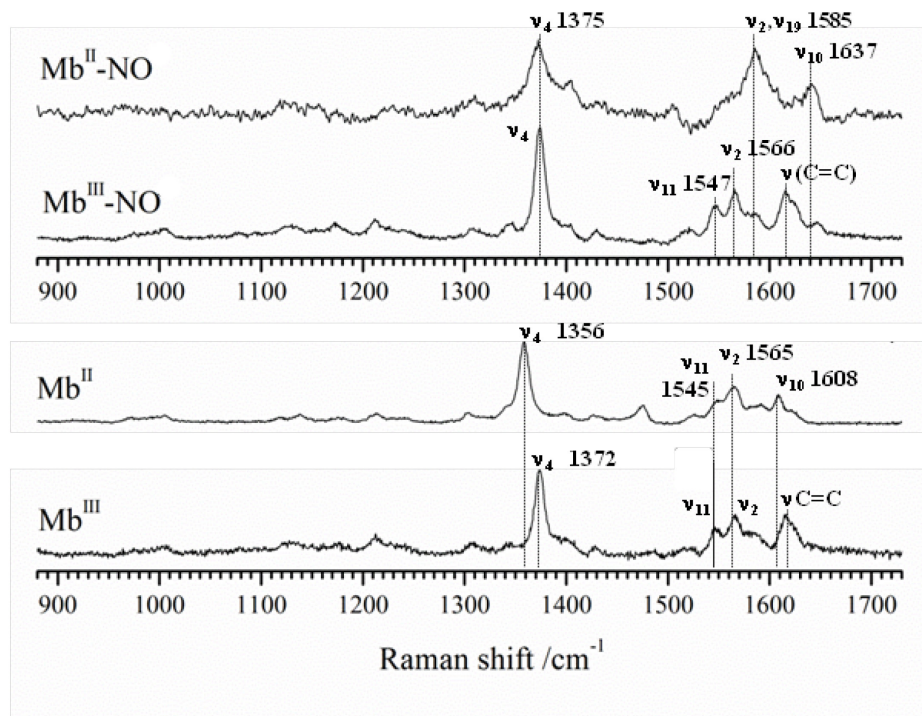


Figure 3

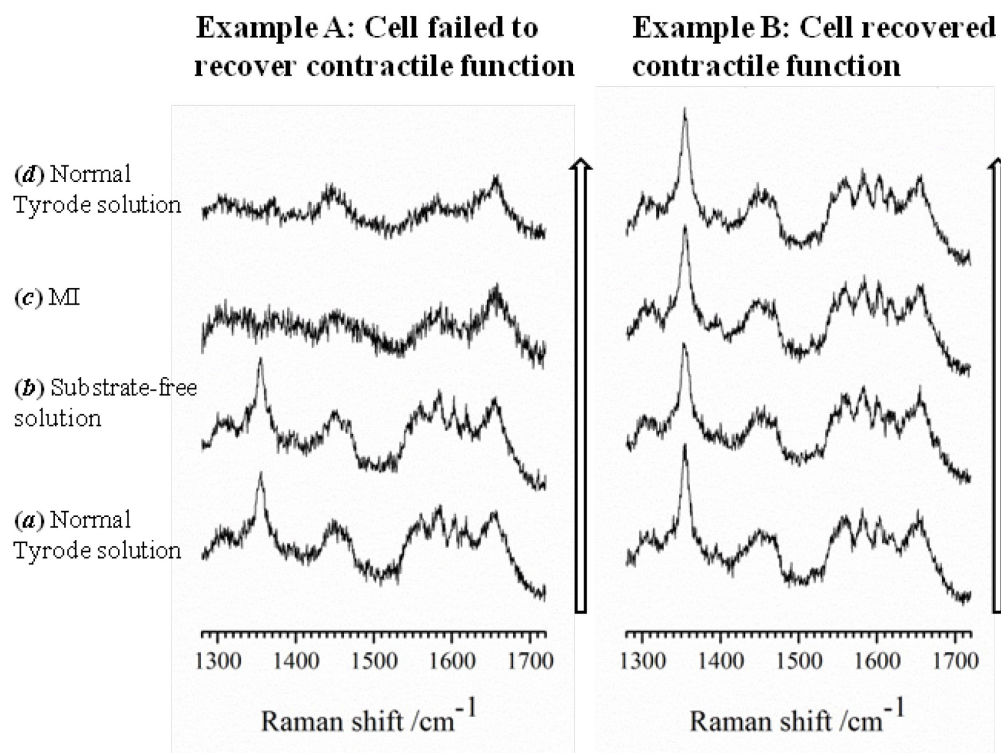


Figure 4

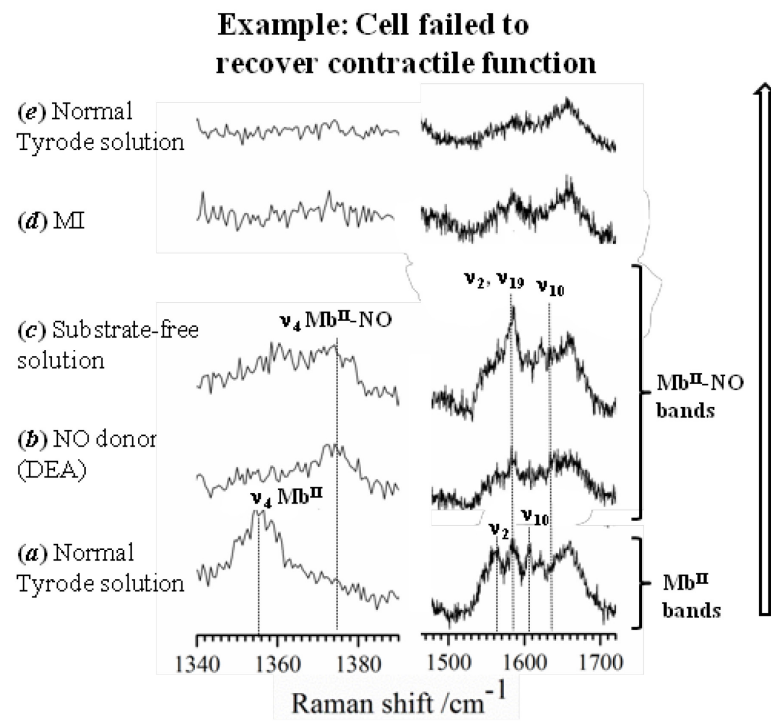


Figure 5

**Example: Cell recovered
contractile function**

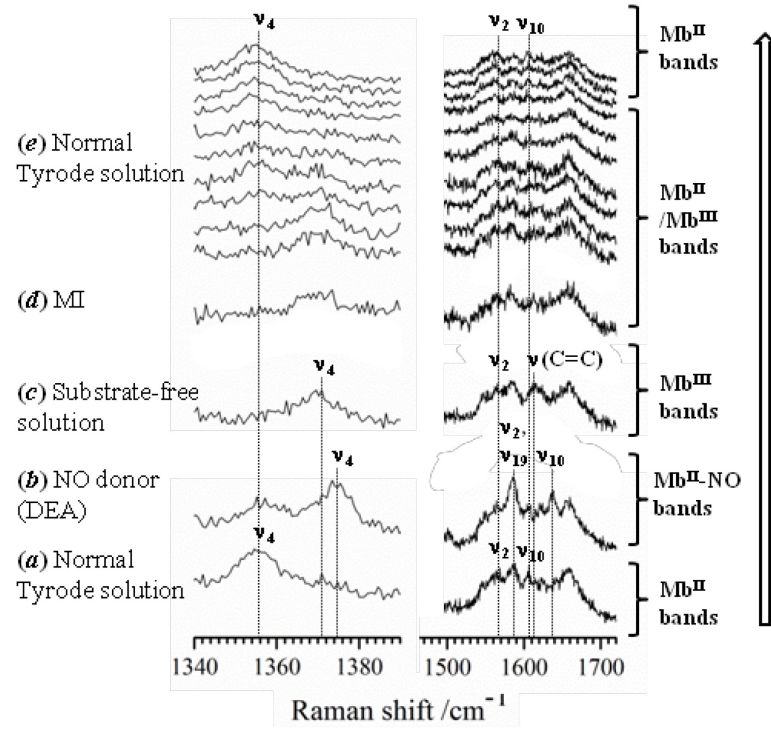


Figure 6

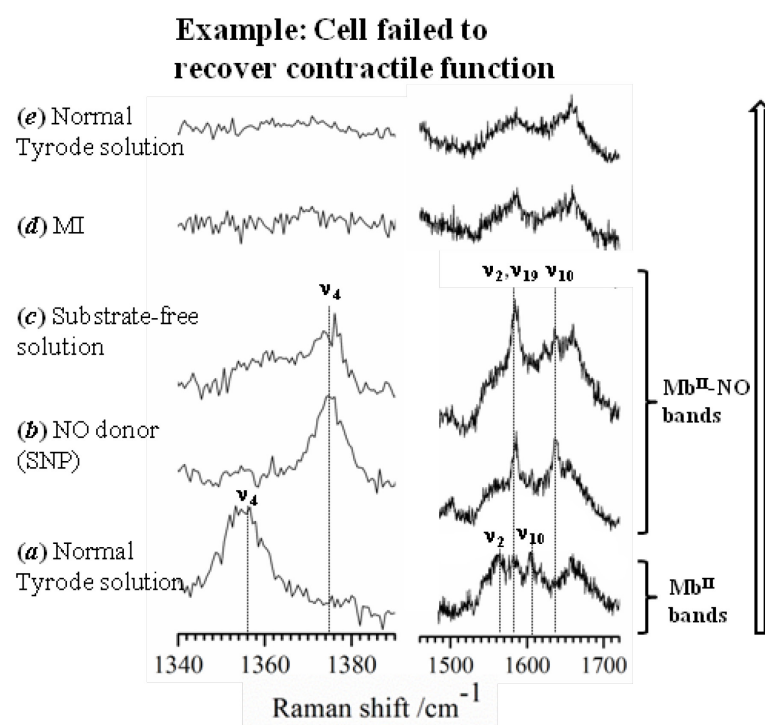


Figure 7

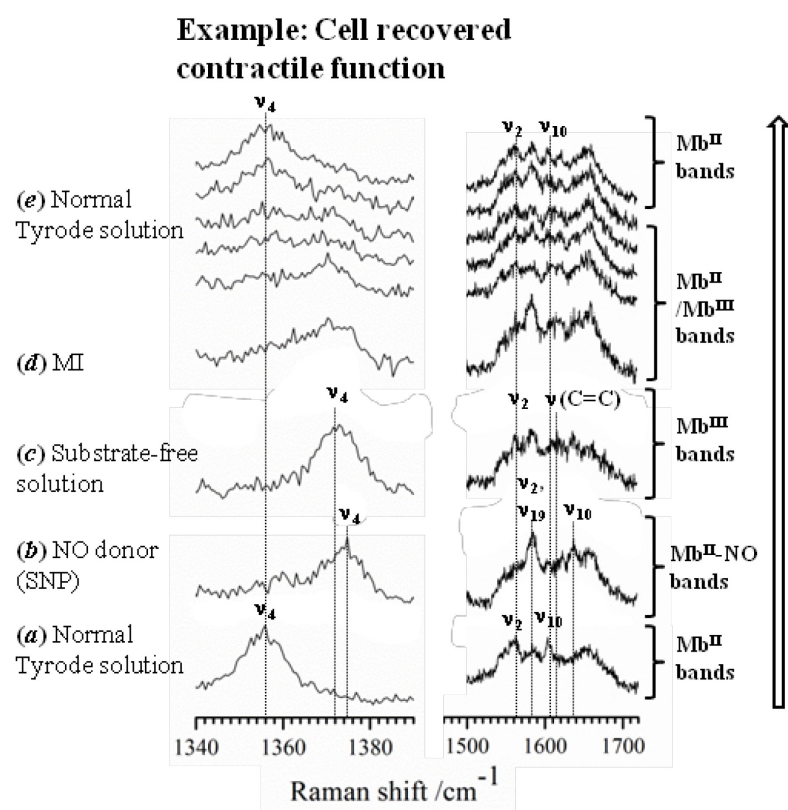


Figure 8

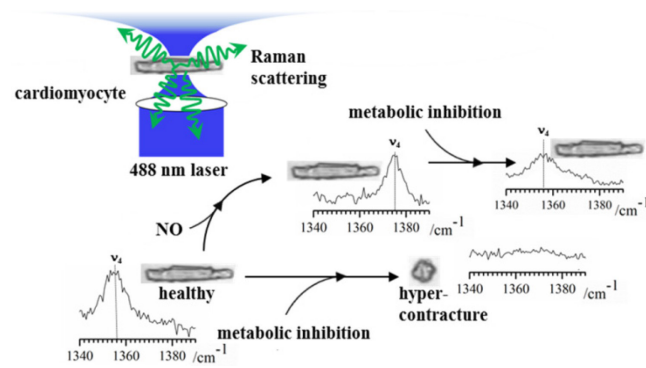


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