Monitoring cytochrome redox changes in the mitochondria of intact cells using multi-wavelength visible light spectroscopy

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Abstract

We have developed an optical system based on visible light spectroscopy for the continuous study of changes in the redox states of mitochondrial cytochromes in intact mammalian cells. Cells are suspended in a closed incubation chamber in which oxygen and nitric oxide (NO) concentrations can be monitored during respiration. Simultaneously the cells are illuminated with a broad-band tungsten–halogen light source. Emergent light in the visible region (from 490–650 nm) is detected using a spectrophotometer and charge-coupled device camera system. Intensity spectra are then converted into changes in optical attenuation from a ‘steady-state’ baseline. The oxidised-minus-reduced absorption spectra of the mitochondrial cytochromes are fitted to the attenuation spectra using a multi-wavelength least-squares algorithm. Thus, the system can measure changes in the redox states of the cytochromes during cellular respiration. Here we describe this novel methodology and demonstrate its validity by monitoring the action of known respiratory chain inhibitors, including the endogenous signalling molecule NO, on cytochrome redox states in human leukocytes.

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1. Introduction

Optical spectroscopy has for many years been employed as a research tool for studying the composition and function of biological tissues both in vitro and in vivo (for review see Ref. [1]). The majority of in vivo studies have exploited the oxygen-dependence of light absorption by chromophores such as haemoglobin [2], myoglobin [3] and cytochrome \textit{c} oxidase [4,5] to monitor oxygen (O\textsubscript{2}) delivery and consumption in tissue. Spectroscopic studies of in vitro systems have focused on events involving O\textsubscript{2} utilisation in isolated cytochrome \textit{c} oxidase [6,7], via the electron transport chain (ETC) in mitochondria [8–10] and, to a lesser extent, in whole cells [11,12].

The capacity of the signalling molecule nitric oxide (NO) to modulate respiration through the reversible inhibition of cytochrome \textit{c} oxidase (complex IV) has previously been demonstrated in mammalian mitochondria using visible spectroscopy [13]. The extent of inhibition is known to be dependent on O\textsubscript{2} concentration due to a competition between NO and O\textsubscript{2} for the haem \textit{a}\textsubscript{3}/Cu\textsubscript{B} binding site of cytochrome \textit{c} oxidase, with a half-inhibition for respiration of 60 and 250 nM of NO at O\textsubscript{2} concentrations of 30 and 145 \textmu M, respectively [14]. Under physiological conditions, O\textsubscript{2} tensions of 34.8 \textpm 6.9 mm Hg (43.5 \textpm 8.9 \textmu M) for terminal arterioles and 23.5 \textpm 5.3 mm Hg (29.4 \textpm 6.6 \textmu M) for tissue have been observed [15]. Hence, in vivo concentrations of O\textsubscript{2} are such that the nanomolar concentrations of NO present in tissues [16] could potentially modulate the affinity of cytochrome \textit{c} oxidase for O\textsubscript{2} under physiological conditions. Studies have in fact shown that NO from vascular endothelial cells, under basal and stimulated conditions, modulates the respiration of these cells in an O\textsubscript{2}-dependent manner [17]. However, these effects of NO have not previously been studied by following the redox states of the ETC cytochromes in an intact cell system.
Here we describe an optical system based on visible light spectroscopy (VLS) that can monitor changes in the redox states of mitochondrial cytochromes of mammalian cells during respiration and hence, the effects of endogenous NO on cellular respiratory parameters. Various limitations of measuring cellular respiratory parameters in a closed system such as the one described here have been pointed out [18], including errors due to the time response of the O2 electrode and back-diffusion of O2 into the system at low partial pressures of O2. In our system, corrections for both the time response of the O2 electrode as well as back-diffusion have been systematically applied. Changes in cytochrome redox states are determined by multi-wavelength least-squares fitting of pure absorption spectra, measured previously on the isolated enzymes, to attenuation changes measured in the cells in the range 530–615 nm. In order to validate the response of the system we have treated the Jurkat cell line of human leukocytes with a variety of known mitochondrial inhibitors, namely rotenone (an inhibitor of complex I), antimycin A and myxothiazol (complex III) and potassium cyanide (complex IV). Furthermore, we have monitored the effects of the inhibitor NO, generated by an NO donor, on the cytochrome redox states. These studies demonstrate the ability of the technique to separate out redox changes in individual cytochromes due to inhibition at different stages of the ETC during cellular respiration.

2. Materials and methods

2.1. Instrumentation

A diagrammatic representation of the VLS system is shown in Fig. 1. Cells are placed into a glass incubation chamber (Rank Bros., Cambridge, UK) maintained at 37 °C. O2 concentration ([O2]) within the chamber is monitored by a Clark-type O2 electrode (Rank Bros.), the calibration and measurement corrections of which are described in Section 2.2. As shown in Fig. 1, a plunger is inserted into the top of the chamber during respiration studies in order to seal the sample from the atmosphere. The plunger supports two 1-mm-diameter optical fibres (aligned parallel to one another and separated by 3.2 mm) and an additional electrode for monitoring NO (ISO-NOP World Precision Instruments, Stevenage, UK). The NO electrode is calibrated based on a reaction in which a known amount of nitrite is reduced to produce a known amount of NO. The response of the NO electrode, i.e. the current produced for a given NO concentration, was on average 2 pA.nM−1 calculated from four to six additions of 1 nmol of nitrite per calibration.

The cells are illuminated with a broad-band tungsten–halogen light source (77501 LOT Oriel, UK), filtered by a blue-green bandpass filter (038FG11-25 LOT-Oriel, 50% short wavelength cut-off 350 nm) and an infrared blocker to reduce stray light and remove unwanted heating effects (100FL07-25 LOT-Oriel, 50% long wavelength cut-off 1000 nm). The light back-scattered from the cell suspension is delivered to a spectrophotometer (270M Instruments SA, USA) by the detecting fibre. All experiments were performed in a dark room to minimise the amount of ambient light reaching the spectrophotometer. Light entering the spectrophotometer is dispersed by a 600 lines-mm−1 diffraction grating such that wavelengths in the visible region of 490–650 nm (calibrated against mercury emission lines) are incident upon a charge-coupled device (CCD) camera (Wright Instruments, Enfield UK). The spectral resolution of the spectrophotometer, with the entrance slit width set to 200 μm, is 1.25 nm. The spectrophotometer and CCD camera are connected to an IBM-compatible computer and controlled by custom-written software developed at UCL.

Spectra are acquired at a rate of 50 Hz (every 20 ms) and averaged over a period of 500 ms (to improve signal-to-noise ratio) such that data are recorded at a sampling rate of 2 Hz. The output of the NO electrode is input to an analogue-to-digital converter (World Precision Instruments) and sampled simultaneously with the spectral data by the acquisition software.

2.2. [O2] calibration and measurement corrections

The Clark-type O2 electrode is capable of measuring a current that is proportional to the partial pressure of O2 (P O2) in the sample. The current is converted to a voltage and input to the analogue-to-digital converter, which is again sampled at a rate of 2 Hz by the acquisition software. The software converts the measured voltage to [O2] using values determined from the calibration procedure described below. The rate of O2 consumption (VO2) in the chamber is also calculated on-line from the differential of [O2] with respect to time, determined from a 20-data-point fourth-order polynomial (i.e. fitting over a period of 10 s).
The conversion of the O₂ electrode output to [O₂] involves a two-point calibration procedure, performed daily in 1 ml of the medium the cells are suspended in during their subsequent measurement in the chamber. This comprises phenol-red-free RPMI-1640 medium (Life Technologies, Paisley, UK), with 1% foetal bovine serum (FBS), 20 mM glucose, 2 mM glutamate, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 25 mM HEPES buffer. With the chamber open to the environment, O₂ in the medium is allowed to equilibrate with the surroundings until it reaches ambient Pₐₒ, approximately 159 mm Hg. The zero point is achieved by adding an excess of sodium dithionite (Na₂S₂O₄) to reduce O₂ completely and then isolating the system by insertion of the plunger. The calibration takes into account sample temperature and salinity, ambient barometric pressure and water vapour pressure, all of which will affect the solubility and hence concentration of O₂ dissolved in a given sample [19]. The salinity correction is based on the concentration of NaCl, the dominant salt by an order of magnitude, in the medium described above. At a barometric pressure of 760 mm Hg, for example, [O₂] in the medium is calculated to be 201.9 μM at 37 °C. [O₂] is assumed to vary linearly with voltage between the ambient and zero points.

Due to the finite time taken for O₂ to diffuse across the membrane, the signal response of the Clark electrode to a step change in Pₐₒ (or [O₂]) is not immediate. The response can be approximated by a mono-exponential function with time [20], characterised by the time constant τ of the electrode response. From a step change in [O₂] from ambient, induced by the addition of Na₂S₂O₄, the time constant of the electrode was determined to be 7.7 ± 0.8 s (n = 5) at 37 °C. The time constant is then used to make a first-order correction of the measured O₂ signal for the finite response time of the electrode [20]

$$[O₂]_{\text{corr}} = [O₂]_{\text{meas}} + \frac{1}{\tau} \frac{d[O₂]_{\text{meas}}}{dt}$$  \hspace{1cm} (1)

where [O₂]corr is the time response-corrected O₂ concentration, [O₂]meas is the measured O₂ concentration and d[O₂]meas/dt is the differential of the measured concentration with respect to time. This correction procedure has been extensively described by Gnaiger and colleagues [18,22]. In our system the regression parameters for a linear fit of O₂ concentration against O₂ flux were found to be 0.010 ± 0.004 min⁻¹ and −0.63 ± 0.78 μM-min⁻¹ for the slope and offset, respectively. The O₂ consumption due to the cells is then determined by subtracting the background O₂ flux from the consumption measured in the presence of cells

$$\langle V_{O₂} \rangle_{\text{corr}} = - \frac{d[O₂]_{\text{corr}}}{dt} - (a₀ + a₁[O₂]_{\text{corr}})$$  \hspace{1cm} (2)

where $$\langle V_{O₂} \rangle_{\text{corr}}$$ is the corrected O₂ consumption by the cells, [O₂]corr is the time response-corrected O₂ concentration, defined in Eq. (1), d[O₂]corr/dt is its derivative with respect to time, and a₀ and a₁ are the slope and offset, respectively, found from the linear regression of background O₂ flux against concentration. All [O₂] and VO₂ values given in the results refer to the corrected values [O₂]corr and $$\langle V_{O₂} \rangle_{\text{corr}}$$, respectively.

2.3. VLS algorithm

The attenuation A of a sample is defined as the log ratio of incident to emergent light

$$A = \log_{10} \left( \frac{I₀}{Iₙ} \right)$$  \hspace{1cm} (3)

where I₀ is the intensity of the light incident on the sample and Iₙ is the intensity of the emergent light. In a non-scattering sample, attenuation is a linear function of absorption and hence chromophore concentration, as described by the Beer–Lambert Law. In a scattering sample such as cells, however, multiple pathlengths render application of the Beer–Lambert relation invalid and attenuation is a nonlinear function of both light scattering and absorption. To account for the nonlinearity between attenuation and absorption, the following approximation may be made: if changes in absorption and scattering give rise to a small change in attenuation, then that change in attenuation at any wavelength λ can be given by

$$\Delta A(\lambda) = \frac{\partial A}{\partial \mu_a} \Delta \mu_a(\lambda) + \frac{\partial A}{\partial \mu_s} \Delta \mu_s(\lambda)$$  \hspace{1cm} (4)

where μₐ is the absorption coefficient, μₐ is the scattering coefficient and Δμₐ(λ) and Δμₐ(λ) are changes in the absorption and scattering coefficients, respectively. This is well approximated by our experimental situation, since changes in the redox states of the cytochromes induce relatively small changes in absorption (and hence attenuation) compared to the degree of nonlinearity between attenuation and absorption [23]. Moreover, if scattering changes are zero or negligible, the second term on the right-hand side of Eq. (4) can be ignored and a linear relationship between changes in attenuation and absorption exists where the constant of proportionality, the term $\frac{\partial A}{\partial \mu_a}$, is known as the differential
pathlength, given the symbol $\beta$. The differential pathlength $\beta(\lambda)$ represents the (weighted) mean path the light travels through the scattering sample at a given wavelength $\lambda$ [24]. In this regime, changes in attenuation can be related to changes in chromophore concentration by

$$\Delta A(\lambda) = \beta(\lambda) \sum_i \epsilon_i(\lambda) \Delta c_i$$

where $\epsilon_i$ is the specific absorption coefficient and $\Delta c_i$ is the change in the concentration of the $i$th chromophore, respectively. Our experiments indicate negligible changes in light scattering during cellular respiration (see further discussion in Section 3.2), thus validating the use of Eq. (5) for calculating changes in chromophore concentrations. The algorithm described here for determining cytochrome redox changes is thus based on changes in attenuation from an initial reference, i.e. the first measurement in time. The change in attenuation $\Delta A$ at any subsequent time $t$ is determined from experimental measurements by

$$\Delta A(t) = \log_{10} \left( \frac{I_g(0)}{I_g(t)} \right)$$

where $I_g(0)$ is the initial intensity emerging from the sample at time $t=0$ and $I_g(t)$ is the intensity recorded at time $t$.

The specific absorption spectra for cytochromes $b_{Ht}$ and $aa_3$ are displayed in Fig. 2. The spectra in fact represent the absorption spectrum to the difference attenuation data $\Delta A$. The differential pathlength parameter by the pathlength. Fig. 3 shows the two fits to the region 530–580 nm, whereas for cytochromes $aa_3$ the fit is in the range 590–615 nm. The two fits also include a constant background, i.e. a flat spectrum, to account for potential changes in attenuation that are independent of the cytochrome redox states. For each chromophore the regression parameter of the fit is the product of the change in concentration $\Delta c$ recovered by dividing the regression parameter by the pathlength. Fig. 3 shows the two fits described above to a difference attenuation spectrum measured at anoxia, when the cytochromes have become reduced. The cytochrome difference absorption peaks can be seen at approximately 550, 560 and 605 nm, due to an increase in the reduced form of cytochromes $c$ and $aa_3$. The redox state of the other $b$-type haem belonging to cytochrome $b$, $b_{Ht}$ ($b_{S660}$), is assumed unchanged under $O_2$-limiting conditions, due to its low redox midpoint potential in relation to $b_{Ht}$ [25]. The cytochrome $c$ difference spectrum was measured previously at UCL using bovine cytochrome $c$, oxidised with a small amount of cytochrome $c$ oxidase then reduced with Na$_2$S$_2$O$_4$. Changes in the redox state of cytochrome $c$ will also contain contributions from the $c_1$ cytochrome of the $bc_1$ complex, since their absorption spectra are very similar in this region and cannot easily be resolved. However, the two cytochromes exist in rapid equilibrium, their redox potential mid-points separated by only 5 mV [26]. The ratio of cytochromes $c$ to $c_1$ is reported to be approximately 2.8:1 in rat liver mitochondria [27]. The $aa_3$ (complex IV) spectrum was measured using bovine mitochondria [28] and represents the difference spectrum of a combination of the $a$ and $aa_3$ cytochromes which comprise the cytochrome $c$ oxidase enzyme. In this wavelength range the spectrum contains a much greater contribution from cytochrome $c$ oxidase $a$ (80–90%) than from cytochrome $aa_3$ [10]. Thus, redox changes determined from the difference spectrum used here give an indirect measure of redox events at the cytochrome $c$ oxidase oxygen binding site, the haem $aa_3/CuB$ binuclear centre.

Fitting with the difference spectra shown in Fig. 2 yields changes in concentration of the difference species, as opposed to changes in absolute concentrations of the oxidised and reduced forms of the cytochromes. The cytochrome $b_{Ht}$ difference absorption spectrum was measured using bovine $bc_1$ complex (complex III) [25] and represents the high potential haem $b_{Ht}$ ($b_{S660}$) of cytochrome $b$. Although $b_{Ht}$ is only one of the $b$-type haems that constitute cytochrome $b$ of the $bc_1$ complex, we refer to it as cytochrome $b_{Ht}$ to be consistent with the references to measurement of cytochromes $c$ and $aa_3$. The redox state of the other $b$-type haem belonging to cytochrome $b$, $b_{Ht}$ ($b_{S660}$), is assumed unchanged under $O_2$-limiting conditions, due to its low redox midpoint potential in relation to $b_{Ht}$ [25]. The cytochrome $c$ difference spectrum was measured previously at UCL using bovine cytochrome $c$, oxidised with a small amount of cytochrome $c$ oxidase then reduced with Na$_2$S$_2$O$_4$. Changes in the redox state of cytochrome $c$ will also contain contributions from the $c_1$ cytochrome of the $bc_1$ complex, since their absorption spectra are very similar in this region and cannot easily be resolved. However, the two cytochromes exist in rapid equilibrium, their redox potential mid-points separated by only 5 mV [26]. The ratio of cytochromes $c$ to $c_1$ is reported to be approximately 2.8:1 in rat liver mitochondria [27]. The $aa_3$ (complex IV) spectrum was measured using bovine mitochondria [28] and represents the difference spectrum of a combination of the $a$ and $aa_3$ cytochromes which comprise the cytochrome $c$ oxidase enzyme. In this wavelength range the spectrum contains a much greater contribution from cytochrome $c$ oxidase $a$ (80–90%) than from cytochrome $aa_3$ [10]. Thus, redox changes determined from the difference spectrum used here give an indirect measure of redox events at the cytochrome $c$ oxidase oxygen binding site, the haem $aa_3/CuB$ binuclear centre.

Fitting with the difference spectra shown in Fig. 2 yields changes in concentration of the difference species, as opposed to changes in absolute concentrations of the oxidised and reduced forms of the cytochromes. An increase in the oxidised-minus-reduced absorption indicates that the cytochrome has become more oxidised (assuming that the total concentration remains the same and oxidation occurs at the expense of the reduction) and a decrease means it has been reduced. The acquisition software uses a linear least-squares routine to fit the absorption spectra to the difference attenuation data using Eq. (5). For cytochromes $b_{Ht}$ and $c$, a fit is performed to the region 530–580 nm, whereas for cytochromes $aa_3$ the fit is in the range 590–615 nm. The two fits also include a constant background, i.e. a flat spectrum, to account for potential changes in attenuation that are independent of the cytochrome redox states. For each chromophore the regression parameter of the fit is the product of the change in concentration $\Delta c$ recovered by dividing the regression parameter by the pathlength. Fig. 3 shows the two fits described above to a difference attenuation spectrum measured at anoxia, when the cytochromes have become reduced. The cytochrome difference absorption peaks can be seen at approximately 550, 560 and 605 nm, due to an increase in the reduced form of cytochromes $c$, $b_{Ht}$ and $aa_3$, respectively. Since the redox changes are occurring from a steady-state baseline of zero, the reduction in the cytochromes means the peaks are reversed in sign relative to the oxidised-minus-reduced spectra displayed in Fig. 2.

![Graph showing specific difference absorption spectra of cytochromes](image-url)


2.4. Cell culture

Jurkat cells (human leukocytes) were purchased from American Type Culture Collection (TIB 71; Manassas, VA). Cells were cultured in suspension in a glass stirrer bottle in RPMI-1640 with 25 mM HEPES, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg·ml⁻¹ streptomycin. The cells were maintained as a stirred culture at 37 °C in a humidified incubator containing 5% CO₂ in air. Cells were harvested by centrifugation at 400 × g at room temperature, washed once with the complete medium and resuspended in medium without phenol red dye (to avoid interference with the optical technique) containing 1% FBS (and other components mentioned in Section 2.2) to a final concentration of 2–3 × 10⁷ cells·ml⁻¹. Cell counting and determination of viability at this point was carried out using the Trypan blue exclusion method and only batches of cells with a viability above 95% were used.

2.5. Protocol

After harvesting, cells were placed into a 50-ml Falcon tube and incubated at 37 °C in a water bath for 40–60 min prior to measurement, gently resuspending every 15–20 min. At the time of measurement 1 ml of cells was placed into the respiration chamber and kept in suspension by stirring with a magnetic glass bar at 750 rpm, sealing the cells from the atmosphere with the plunger. At the end of each experiment, the cells were collected from the chamber and placed on ice for further determination of cell number.

2.6. Cell counting

In order to quantify the cell density of each individual sample taken from the pool, a further procedure was performed. After each experimental measurement in the incubation chamber, six aliquots of 100 μl from the 1-ml sample were centrifuged and the pellets kept at −20 °C. Cell pellets were later dissociated by 100 μl of 10% sodium dodecylsulfate (SDS) for 20 min and diluted to a final concentration of 2% SDS. A protein assay kit (Pierce, USA) based on the reaction with bichromolin acid (BCA) was developed in a 96-well plate, using cell concentrations from 0.2 to 2 × 10⁵ cells per well (2–20 μg protein·ml⁻¹) to obtain a standard linear regression curve and a sample volume of 20 μl per well [29].

2.7. Cell viability

In several independent tests it was observed that the viability of cells incubated at 37 °C remained above 95% over a post-harvest period of 3–4.5 h, as determined by the Trypan blue exclusion method. Cell viability was also examined by flow cytometry at 4 h post-harvest. Aliquots of approximately 10⁶ cells in 1 ml of PBS, with 1% FBS and 0.02 μM propidium iodide, were analysed by flow cytometry on a FACScan instrument (Becton Dickinson). Dead cells were distinguished from live cells by measuring forward-angle light scattering versus fluorescence intensity. For each sample, 10⁵ cells were counted by the software (CELLQUEST) to express viability, which again remained above 95%.

3. Results

3.1. Pathlength measurement

To obtain changes in chromophore concentrations using Eq. (5), the pathlength b(λ) in the scattering sample must first be determined. Furthermore, because the fitting of the absorption spectra extends over a range of wavelengths, the wavelength-dependence of the pathlength (WDPL) should also be considered. The WDPL was initially measured in the range 490–650 nm, the range within which the fits are performed, by adding a small amount of absorbing dye to the cell sample. The consequent change in attenuation ΔA was then divided by the specific absorption spectrum of the dye at each wavelength, yielding the pathlength β scaled by the change in concentration Δc. The WDPL was then found by normalising to unity at a given wavelength. This procedure was performed for cell concentrations in the range 1.6–4.9 × 10⁷ cells·ml⁻¹ (n = 10), which encompasses the range used in subsequent experiments. The spectrophotometer was then re-calibrated to the range 620–775 nm in order to obtain the absolute pathlength β by the second derivative method, using the spectral features of water absorption at 740 nm [23]. The WDPL measurement was repeated as described above for the new range, for cell concentrations between 1.7 and 4.3 × 10⁷ cells·ml⁻¹ (n = 10). Between the wavelengths of 740 nm (the value...
around which the absolute pathlength was determined) and 530 nm (where the spectral fitting begins), the WDPL, and hence the absolute pathlength, varies by less than 4% (3.3 ± 2.9%). Thus, the pathlength determined at 740 nm can be used to represent the pathlength over the entire wavelength range, without correcting for the WDPL.

The absolute pathlength was determined by fitting the second derivative of the water absorption spectrum to that of the absolute attenuation spectra of the cells (referenced to air) in the range 700–770 nm [23]. The second derivative method approximates a linear relationship between the second differential of attenuation (with respect to wavelength) and the second differential of absorption via the differential pathlength, then determines the pathlength by assuming a water concentration of 100% for the cell suspension. The pathlength was measured over a wide range of cell concentrations from 1.3 to 10.5 × 10⁷ cells·ml⁻¹. Within the range 1.5–4.5 × 10⁷ cells·ml⁻¹ the pathlength was found to be very similar, with a mean of 2.3 ± 0.1 cm (n = 11). This value was then used in the fitting routine to determine the redox-dependent changes in cytochrome concentrations, as described below. Above 4.5 × 10⁷ cells·ml⁻¹ the pathlength decreased with cell concentration. This is most likely attributable to a decrease in the component of the pathlength due to reflections from the base of the chamber as cell density increases.

3.2. Control experiments

Control experiments were performed in order to characterise cellular respiration under ‘normal’ conditions, i.e. in the absence of mitochondrial inhibitors. Fig. 4 shows typical traces of cytochromes \(b_\text{H}, \ c\) and \(a\alpha_3\) against time and \([\text{O}_2]\) (Fig. 4A and B, respectively) and the corresponding \(V_{\text{O}_2}\) (Fig. 4C and D, respectively), during respiration to anoxia. The term ‘anoxia’ as opposed to ‘hypoxia’ is used to describe the minimum \([\text{O}_2]\) reached, although it should be noted that \([\text{O}_2]\) will never truly reach zero due to the
presence of O2 back-diffusion. As discussed earlier, the ability of the VLS method to determine changes in cytochrome concentrations relies on the validity of the assumption that attenuation changes are due to changes in absorption only. It has been noted that light scattering changes can occur due to osmotic swelling of mitochondria on cessation of respiration [30]. In response to a 20-min period of hypoxia (\( \approx 25 \mu M O_2 \)), Kreisman and LaManna [31] reported a decrease in light intensity of approximately 26\% (a change in attenuation of \( \approx 130 \text{ mOD} \)) in rat brain tissues. They attributed this change to cell swelling upon hypoxia and determined its time constant to be approximately 6 min. Changes in the baseline of our measured attenuation spectra, as indicated by the flat offsets included in the spectral fitting, were of the order of 1 mOD and did not occur as a direct result of reduction in the cytochromes. We therefore conclude that scattering changes, if present, are negligible during the time frame of our experiments (\( \approx 5 \text{ min total}, < 1 \text{ min at anoxia} \)).

In the intact cell the \( P_{50} \) for cellular respiration has been defined as the \( P_O \) at which \( V_{O_2} \) reaches 50\% of its maximal rate [9,18]. This parameter has been used to estimate an apparent O2 affinity by analogy with the Michaelis–Menton constant (\( K_m \)) for \( O_2 \), and has been determined using a hyperbolic function to describe the relationship between \( [O_2] \) and \( V_{O_2} \) [9]. However, it has been noted (as was our experience) that the value of \( P_{50} \) depends strongly on the \( [O_2] \) range used in the hyperbolic fitting [18]. Hence we determine the \( P_{50} \) by simple bi-linear interpolation between data points at half-maximal \( V_{O_2} \). We have also used the term \( P_{50} \) to define the \( [O_2] \) at which the reduction of each cytochrome is half-maximal. Note that whilst \( P_{50} \) refers to a partial pressure of \( O_2 \), we have expressed it in terms of concentration for consistency.

The parameters reported here for cellular respiration under normal conditions (see Table 1) have been determined for a cell concentration of \( 2.6 \pm 0.2 \times 10^7 \text{ cells} \cdot \text{ml}^{-1} \) \((n = 16)\), measured over 5 (non-consecutive) days. The average maximal \( V_{O_2} \) was determined to be 30.0 \pm 2.7 \( \mu M \cdot \text{min}^{-1} \), i.e. a value of 11.5 \pm 1.0 \( \mu M \cdot \text{min}^{-1} \) per 10^7 cells. For comparison, Beltran et al. [32] observed an average (uncorrected) \( V_{O_2} \) for Jurkat cells of 12.8 \pm 1.4 \( \mu M \cdot \text{min}^{-1} \) per 10^7 cells under similar cell culture and experimental conditions. We determined the \( P_{50} \) for \( V_{O_2} \) to be 2.3 \pm 0.4 \( \mu M O_2 \) \((n = 16)\), similar to the value obtained by Rumsey et al. [33] of 2.2 \pm 0.1 \( \mu M \) for a \( V_{O_2} \) of 16.6 \pm 1.2 \( \mu M \cdot \text{min}^{-1} \) with cardiac myocytes. Further discussion of the results of these experiments is given in Section 4.

### 3.3. Mitochondrial inhibitors

In order to verify the response of the system to changes in mitochondrial respiration via the cytochrome redox states, various known inhibitors were added to cells in the closed chamber, keeping all other experimental conditions as previously described. In each case the concentration of inhibitor used was the minimum required to produce the maximum response in terms of cytochrome redox changes. The implications of these findings are discussed in further detail in Section 4.

#### 3.3.1. Rotenone

On addition of 0.25 \( \mu M \) rotenone (Sigma), all of the cytochromes undergo an immediate oxidation, as shown in Fig. 5A (the arrow indicates the addition of rotenone at 50 \( \mu M O_2 \)), with a corresponding decrease in \( V_{O_2} \). Rotenone has been shown to be specific for inhibition of complex I only at stoichiometric concentrations [34]. The observed oxidation is thus due to a shift in the redox steady-state caused by a decrease in electron flux downstream of complex I. Table 2 gives the concentration changes for each cytochrome after addition of rotenone, for a cell concentration of 2.9 \( \pm 0.1 \times 10^7 \) cells \( \cdot \text{ml}^{-1} \) \((n = 3)\). Subsequent to the addition of the inhibitor, 250 \( \mu M \) of potassium cyanide (KCN) (Sigma) was added to the chamber to inhibit respiration completely. The remaining non-mitochondrial consumption of \( O_2 \) was used to calculate the percentage inhibition of mitochondrial \( V_{O_2} \), due to the addition of rotenone, found to be 95.2 \pm 2.9\%. The residual \( O_2 \) consumption was 1.7 \pm 0.1 \( \mu M \cdot \text{min}^{-1} \).

#### 3.3.2. Myxothiazol

Myxothiazol inhibits at the level of the bc1 complex at the proximal Qo site, the site of ubiquinone oxidation [35]. The effect of adding 1 \( \mu M \) of myxothiazol (Sigma) to the cells (at 62 \( \mu M O_2 \)) is shown in Fig. 5B. According to the binding site of myxothiazol, one would expect an oxidation of cytochrome \( b_{11} \), but instead a slow reduction was observed. This effect is suggested to be due to a leak of electrons from semiquinone at the Qo site [36]. The concentration changes are given in Table 2 for a cell density of 2.6 \( \pm 0.2 \times 10^7 \text{ cells} \cdot \text{ml}^{-1} \) \((n = 4)\). Mitochondrial respiration was inhibited by 95.5 \pm 1.4\%, as determined by the further addition of 250 \( \mu M \) of KCN. In this case the residual \( O_2 \) consumption was 2.1 \pm 0.8 \( \mu M \cdot \text{min}^{-1} \).

#### 3.3.3. Antimycin A

Antimycin A is a complex III inhibitor, blocking electron flow at the Qi site [37], the site of ubiquinone reduction, downstream of the b cytochromes. Fig. 5C demonstrates how the addition (at 48 \( \mu M O_2 \)) of 0.5 \( \mu M \) of antimycin A

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Changes in concentration (nM)</th>
<th>( P_{50} ) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( h_{11} )</td>
<td>(-16.7 \pm 2.3)</td>
<td>1.3 \pm 0.5</td>
</tr>
<tr>
<td>( c^- )</td>
<td>(-40.2 \pm 3.6)</td>
<td>2.5 \pm 0.6</td>
</tr>
<tr>
<td>( a_{1a} )</td>
<td>(-9.8 \pm 1.2)</td>
<td>4.6 \pm 1.0</td>
</tr>
</tbody>
</table>
(Sigma) caused an immediate reduction in cytochrome \( b_H \) and an oxidation in both cytochromes \( c \) and \( aa_3 \). Table 2 gives the changes in cytochrome concentrations on addition of the inhibitor for a cell concentration of \( 2.8 \pm 0.6 \times 10^7 \) cells\( \cdot \)ml\(^{-1} \) (\( n=3 \)). The percentage inhibition of respiration was \( 98.9 \pm 0.8\% \), determined by adding 250 \( \mu \)M of KCN as described above, with a residual \( O_2 \) consumption of \( 2.9 \pm 0.4 \) \( \mu \)M\( \cdot \)min\(^{-1} \).

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[Cells] ( \times 10^7 ) cells( \cdot )ml(^{-1} )</th>
<th>( \Delta [b_H] ) (nM)</th>
<th>( \Delta [c] ) (nM)</th>
<th>( \Delta [aa_3] ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>2.9 ± 0.1</td>
<td>2.8 ± 0.7</td>
<td>16.8 ± 0.7</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>2.6 ± 0.2</td>
<td>−6.7 ± 0.7</td>
<td>20.7 ± 1.3</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>2.8 ± 0.6</td>
<td>−8.4 ± 0.9</td>
<td>19.5 ± 0.8</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>KCN</td>
<td>3.2 ± 0.3</td>
<td>−6.6 ± 0.7</td>
<td>34.4 ± 1.3</td>
<td>−7.7 ± 1.3</td>
</tr>
</tbody>
</table>

For the given cell concentration the changes in cytochrome concentrations on addition of rotenone (0.25 \( \mu \)M), myxothiazol (1 \( \mu \)M), antimycin A (0.5 \( \mu \)M) and KCN (250 \( \mu \)M) are shown.

### 3.3.4. Cyanide

Fig. 5D shows the effects on the cytochrome redox states of the addition of 250 \( \mu \)M of KCN (at 62 \( \mu \)M \( O_2 \)), which inhibits at the level of complex IV. All three cytochromes became reduced on addition of KCN, concomitant with a block of electron flow at the \( O_2 \) binding site. The changes in concentration are given in Table 2, for a cell concentration of \( 3.2 \pm 0.3 \times 10^7 \) cells\( \cdot \)ml\(^{-1} \) (\( n=3 \)). The inhibition of respiration was in this case assumed to be maximal, since further addition of KCN did not decrease \( V_{O_2} \), giving a residual \( O_2 \) consumption of \( 2.0 \pm 0.3 \) \( \mu \)M\( \cdot \)min\(^{-1} \).

### 3.3.5. NO donor

To demonstrate the ability of our system to study the effects of NO on the parameters of cellular respiration DEA-NO (Alexis Chemicals), a short-lived NO donor with a half-life of 2 min at pH 7.4 and 37 °C [38] was used. A concentration of 5 \( \mu \)M of the donor was added to the closed chamber during cellular respiration at an \( O_2 \) of 100 \( \mu \)M. Fig. 6A shows the typical reduction of the cytochromes on...
addition of 5 μM DEA-NO, with the concomitant changes in \(V_{O_2}\) and the release of NO displayed in Fig. 6B and C, respectively. The peak concentration of NO measured in the cells was 4.4 ± 0.2 μM (n = 3). It is known that NO released from NO donors is degraded by the autoxidation of NO to nitrite, the extent of which is proportional to \(O_2\) concentration [39]. In characterising the release of NO from DEA-NO in the absence of cells, therefore, the \([O_2]\) was first reduced to 100 μM by bubbling with nitrogen prior to addition of the donor. Since the NO release by DEA-NO is also pH-dependent [38], the measurement was carried out in the supernatant of control cells, collected subsequent to their measurement in the chamber, in which the pH was 7.2. The peak NO release by 5 μM DEA-NO was found to be 6.6 ± 0.3 μM (n = 3), approximately 40% higher than that measured in fresh medium at pH 7.4.

Fig. 6A and C shows that the sudden release of NO on addition of DEA-NO is accompanied by a sharp inhibition of the cytochromes, and the slower degradation of NO is matched by the gradual recovery of their redox states towards baseline. For a cell concentration of \(2.0 \pm 0.1 \times 10^7\) cells·ml\(^{-1}\) the inhibition of \(V_{O_2}\) by 5 μM DEA-NO was 95.2 ± 1.3%, whereas for cytochromes c and aa\(_3\) it was 87.4 ± 2.0% and 76.1 ± 2.6%, respectively (n = 3). Unlike cytochromes c and aa\(_3\), the redox state of cytochrome b\(_4\) does not fully recover from inhibition, remaining partially reduced until anoxia whereupon its final reduction is submaximal compared to controls. The reason for this observation has yet to be elucidated.

4. Discussion

We have developed a novel optical system for studying the redox behaviour of respiratory complexes in the ETC which, to our knowledge, has not previously been applied to intact cells. The determination of redox-dependent changes in cytochrome concentrations is based upon an algorithm that assumes changes in light attenuation are dominated by changes in absorption, and that scattering changes are negligible. With the optical arrangement selected, we saw no evidence for the scattering changes upon anoxia that have been reported elsewhere. This confirms the validity of the algorithm. Furthermore, since we have measured the optical pathlength through the cells for the chosen range of cell concentration, we are able to quantify the changes in cytochrome redox states during respiration as real (absolute) concentration changes. To improve the accuracy of our measurements, we have rigorously applied corrections to account for such factors as the finite time response of the Clark electrode and the background \(O_2\) flux in the absence of cells.

Using the VLS system described here, we first characterised cellular respiration in the Jurkat cell line of human leukocytes in the absence of respiratory inhibitors. As shown in Fig. 4B, it is clear that cytochrome c starts to

![Fig. 6. Effect of inhibition with DEA-NO on cytochrome redox states. Typical changes in cytochromes b\(_4\) (closed circles), c (open squares) and aa\(_3\) (closed triangles) (A), the corresponding \(V_{O_2}\) (B) and the release of NO measured by the ISO-NOP electrode (C) following the addition of 5 μM of DEA-NO at an \([O_2]\) of 100 μM are all displayed against \([O_2]\). In each panel the arrow and the dashed line indicate the addition of DEA-NO.](image-url)
reduce from around 50 μM O₂. This early reduction of cytochrome c, whilst \( V_{\text{O}_2} \) is still maximal, has been noted by others both in cells [40] and mitochondria, the latter of which was associated with changes in pH of the suspension medium [9]. Chance [41] saw no early reduction in cytochrome c in yeast, and explained this observation by others as due to O₂ gradients in cells that have aggregated, or a spectroscopic interference from the deoxygenation of haemoglobin.

Cellular respiration was characterised by the changes in cytochrome concentrations at anoxia and the \( P_{50} \)s for \( V_{\text{O}_2} \) and the cytochromes. Values of \( P_{50} \) for \( V_{\text{O}_2} \) below 1 μM O₂ have been obtained for cell concentrations \(<10^6\text{cells·ml}^{-1}\), the increase in \( P_{50} \) with cell concentration being explained, in part, by the intracellular O₂ gradient [42]. In a study using isolated mitochondria, Gnaiger et al. [43] observed erroneously high \( P_{50} \)s for \( V_{\text{O}_2} \) when the maximal \( V_{\text{O}_2} \) was greater than 30 μM·min⁻¹. We found that cell concentrations below \( 2.0 \times 10^6 \text{cells·ml}^{-1} \) did not give a sufficient signal-to-noise ratio to determine cytochrome concentrations accurately. In our experiments, therefore, the average cell concentration was \( 2.8 \pm 0.3 \times 10^6 \text{cells·ml}^{-1} (n = 29) \), with a maximal \( V_{\text{O}_2} \) of \( 28.4 \pm 2.4 \mu\text{M·min}^{-1} \). Moreover, in the range \( 2–3 \times 10^7 \text{cells·ml}^{-1} \), we observed no correlation between cell concentration and \( P_{50} \).

Experiments with mitochondrial inhibitors have demonstrated that the system is able to monitor cytochrome redox changes according to the site of mitochondrial inhibition, indicating that the measured absorption changes are sufficiently specific to allow decomposition of the three cytochrome spectra by our algorithm. Experiments with the short-lived NO donor DEA-NO established the ability of the system to observe the reversibility of inhibition of cytochrome c oxidase, and hence cellular respiration, whilst simultaneously monitoring NO release from the donor.

The addition of rotenone should enable us to estimate the ratio of oxidised to reduced cytochromes during the steady-state of electron transport, assuming total inhibition of complex I and hence complete oxidation of the downstream cytochromes. Under our experimental conditions, we found that cytochromes \( aa_3 \) are approximately 31% reduced during steady-state respiration, in agreement with the findings of others in mammalian mitochondria [10]. However, cytochrome \( b_{11} \) was only 14% reduced, an observation that appears to conflict with the concept that cytochromes closer to the oxidising (O₂) end of the ETC will be more oxidised than those at the reducing end. Whether this unexpected finding could be attributed, at least in part, to the formation of reactive oxygen species, observed following treatment with rotenone [34], has yet to be determined.

We noted that with the addition of the complex III inhibitor myxothiazol there was a gradual reduction in \( b_{11} \) continuing beyond 30 s after addition (Fig. 5B). Antimycin A, however, produced a rapid reduction in cytochrome \( b_{11} \) that reached a stable level within 5–10 s (Fig. 5C). Whilst this difference could be related to the time taken for the inhibitors to bind to their respective sites, this observation could also reflect the gradual reduction of cytochrome \( b_{11} \) due to a leak of electrons from the Qo site with myxothiazol, compared to the immediate downstream inhibition, and hence reduction of \( b_{11} \) by antimycin A. Both inhibitors produced a sub-maximal reduction of cytochrome \( b_{11} \) compared to controls, which could be a consequence of an equilibration of electron transfer between \( b_{11} \) and the ubiquinone pool [36]. This bypass reaction of electrons has been associated with superoxide production after inhibition of mitochondria with antimycin [44] and in purified \( bc_1 \) complex [45].

Inhibition of the terminal enzyme in the ETC should result in the maximal reduction of upstream cytochromes. On addition of KCN, however, the reduction of cytochromes \( aa_3 \) was only 79% of that at anoxia. This has also been observed in mitochondria [10] and is due to the fact that KCN binds to cytochrome \( a_3 \) mainly in its oxidised form. Hence, only the subsequent reduction in cytochrome \( a \), which is known to contribute 80–90% of the \( aa_3 \) signal [10], will account for the change in the measured spectrum. It should be noted, however, that spectral changes in the region over which the \( aa_3 \) fitting is performed will contain a small contribution due to KCN binding to oxidised cytochrome \( a_3 \) that will not be accounted for by the current fitting. These changes include a slight shift (≈ 5 nm) in the difference peak to shorter wavelengths and the appearance of a shoulder on the short wavelength side of the peak [28].

In summary, our system based on VLS is capable of monitoring cytochrome redox states in mammalian cells and the effects of NO on parameters of cellular respiration. It is important to emphasise the value of monitoring \( V_{\text{O}_2} \) and the cytochrome redox states simultaneously, as we have done in intact cells, when studying the modulation by NO of the apparent affinity of cytochrome c oxidase for O₂. This novel combination of methodologies that we have now described will doubtless allow us to answer many physiologically relevant questions relating to the role of endogenous NO in the control of cell respiration, particularly at the low concentrations produced by the constitutive NO synthases.

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References


