

MEASUREMENT OF CYTOCHROME OXIDASE REDOX STATE BY NEAR INFRARED SPECTROSCOPY

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INTRODUCTION

Although near infrared spectroscopy (NIRS) is primarily used to probe changes in oxyhaemoglobin (HbO₂) and deoxyhaemoglobin (dHb) concentrations, it has long been realised that there is a significant oxygen-concentration dependent near infrared signal from the mitochondrial enzyme cytochrome *c* oxidase. In this paper we discuss the origins of this near infrared (NIR) signal, the possible factors affecting its intensity and its likely physiological and clinical significance. This paper complements our recent review on this subject¹.

ORIGIN OF THE CYTOCHROME OXIDASE NEAR INFRARED SIGNAL

Cytochrome oxidase is the terminal electron acceptor of the mitochondrial electron transport chain and responsible for over 90% of cellular oxygen consumption. The energy generated in this process is utilised to synthesise ATP. There are reviews pertaining to the mechanism² and control^{3,4} of the enzyme. The three-dimensional structures of both the bacterial⁵ and the mammalian enzymes⁶ have been solved.

The evidence demonstrating that cytochrome oxidase is detectable *in vivo* by near infrared spectroscopy (NIRS) is primarily from experiments of the type illustrated in Figure 1. The blood is removed from an animal and replaced with an oxygen-carrying perfluorocarbon emulsion. The inspired oxygen fraction (F_{iO_2}) is then reduced and the NIR difference spectrum (hypoxic minus normoxia) plotted. A decrease in absorbance is observed, with a trough centred at 820 - 840 nm. As can be seen this is very similar to the reduced minus oxidised absorbance spectrum of the isolated cytochrome *c* oxidase enzyme. A reduction of the metal centres in the enzyme would be expected as oxygen tension dropped to levels that began to limit tissue oxygen consumption. In the wavelength range used primarily in commercial NIR spectrometers (770 - 900 nm) most of the observed *in vivo* signal can be attributed to cytochrome oxidase, although improved results are obtained if the *in vitro* cytochrome *b* and cytochrome *c* spectrum are included in the fit. Below 750 nm the cytochrome oxidase spectrum alone becomes an increasingly poor fit to the data as the near infrared spectrum of cytochrome *c* becomes significant¹.

What is the nature of the species that is responsible for the NIR spectrum of cytochrome oxidase? Before the advent of tissue NIRS it had long been known that this enzyme had a unique reduced minus oxidised NIR difference spectrum⁷. Characterising the

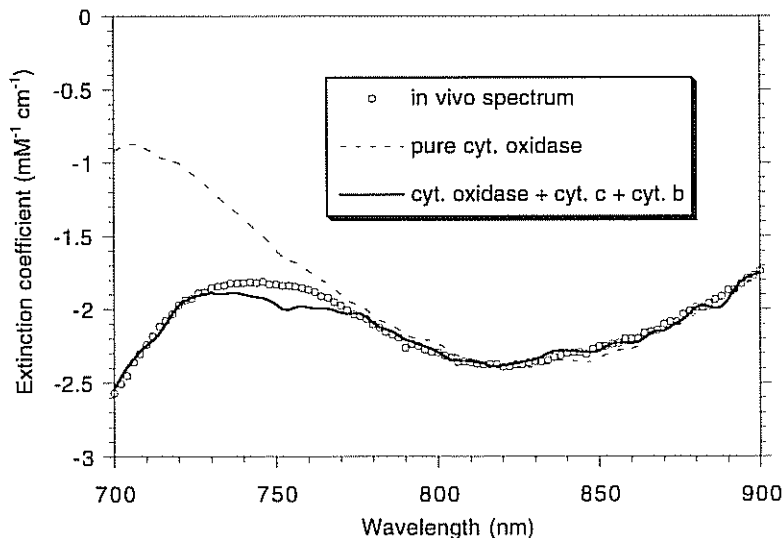


Figure 1. Effect of reducing F_{iO_2} to zero on the *in vivo* near infrared spectrum of a perfluorocarbon-perfused rat brain. The *in vivo* deoxygenated minus oxygenated spectrum is fitted to the *in vitro* reduced minus oxidised spectra of the mitochondrial cytochromes. Fitted compounds are either pure cytochrome oxidase (from bovine heart) or the same ratio of cytochrome oxidase, cytochrome *b* and cytochrome *c* present in rat brain cyt. oxidase:cyt. *b*: cyt. *c*; 1:2.6:1.15).

precise nature of this signal has proved problematic, as the enzyme contains four redox active metal centres, all of which are unique in the body and could contribute to the signal. The two redox-active iron atoms are present in prosthetic haem *a* groups - containing hydroxyethylfarnesyl and propionate side-chains which clearly perturb the visible spectrum compared to the more common haem *b* moiety usually found in haem enzymes. However, after much study⁷⁻¹¹ it has been shown that it is another centre in the enzyme, Cu_A , that dominates the NIR spectrum. The best evidence for this is that cytochrome oxidases containing Cu_A , but no haem *a*, have very similar NIR spectra to the native enzyme^{12, 13} and that bacterial enzymes lacking Cu_A , but containing haem *a*, have no strong NIR spectra¹⁴.

To our knowledge there has been no reported spectrum of human cytochrome oxidase in the NIR region. However, it is unlikely to differ significantly from that of other mammals. Figure 2 compares the deoxygenated minus oxygenated spectrum of a perfluorocarbon-treated adult rat with that of a neonatal pig. Similar spectra have also been reported from the cat brain^{15, 16}. The similarity in these spectra suggests that age and species differences will not affect the shape of the cytochrome oxidase spectrum unduly - although they will relate to the total cytochrome oxidase content of the tissue under investigation.

It is important to note that the spectra displayed in Figure 1 and 2 are *difference* spectra. They relate solely to the fraction of tissue chromophores whose NIR signal is affected by reducing the oxygen tension in the tissue. There may exist a large variety of chromophores whose spectra are not oxygen-dependent, but which would still contribute to the tissue absorbance. For example one would expect all metal enzymes (especially haem iron enzymes) to have weak absorbances in the NIR due to metal-ligand charge

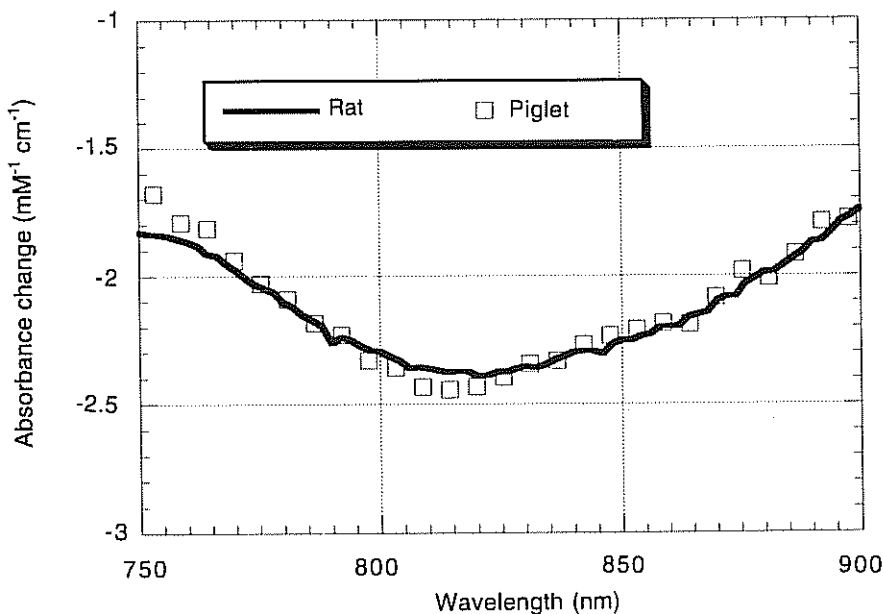


Figure 2. Comparison of NIR spectra of oxygen-dependent chromophores in perfluorocarbon-perfused rat brain, compared to that of a 1-day old perfluorocarbon-perfused pig. NIR difference spectrum (deoxygenated minus oxygenated) obtained by reducing FiO_2 to zero. Rat spectrum from Figure 1, piglet spectrum normalised to that of the rat at 780nm.

PROBLEMS OF DETECTING CYTOCHROME OXIDASE SIGNALS IN THE PRESENCE OF HAEMOGLOBIN

Whilst detecting cytochrome oxidase redox state changes in blood-free animals may be of some interest to the physiologist/biochemist, the key question for the clinician is whether these signals can be detected in the presence of the greater (approximately 10-fold) oxyhaemoglobin (HbO₂) and deoxyhaemoglobin (dHb) concentrations. These problems have been discussed and debated at some length by ourselves^{1, 17-19} and others^{16, 20-23} - the key problems being tissue heterogeneity and the non-linear absorption and scattering dependent relationship between wavelength and the optical pathlength.

We compared all the published (at that time) algorithms for determining cytochrome oxidase redox state changes in the presence of haemoglobin concentration changes¹⁹ and found that, with one exception²³, they all showed similar qualitative trends in the cytochrome signal, although there were significant quantitative differences. However, this in itself is not evidence that the algorithms are valid, as many were based on similar assumptions. In an attempt to "challenge" the efficacy of the algorithms further we have developed an animal model where blood volume and oxygenation are varied dramatically, but in which cytochrome oxidase redox state should be fixed. This involves the addition of a dose of cyanide intravenously to an anaesthetised rat. The brain is especially sensitive to this treatment as it contains only small amounts of rhodanese, the enzyme that metabolises cyanide. Cyanide binds to haem a₃ of cytochrome oxidase, preventing oxygen reduction by electrons leaving Cu_A and haem a²⁴. Therefore Cu_A should become completely reduced²⁵. However, [HbO₂] will rise and [dHb] fall as oxygen consumption is inhibited; vasodila-

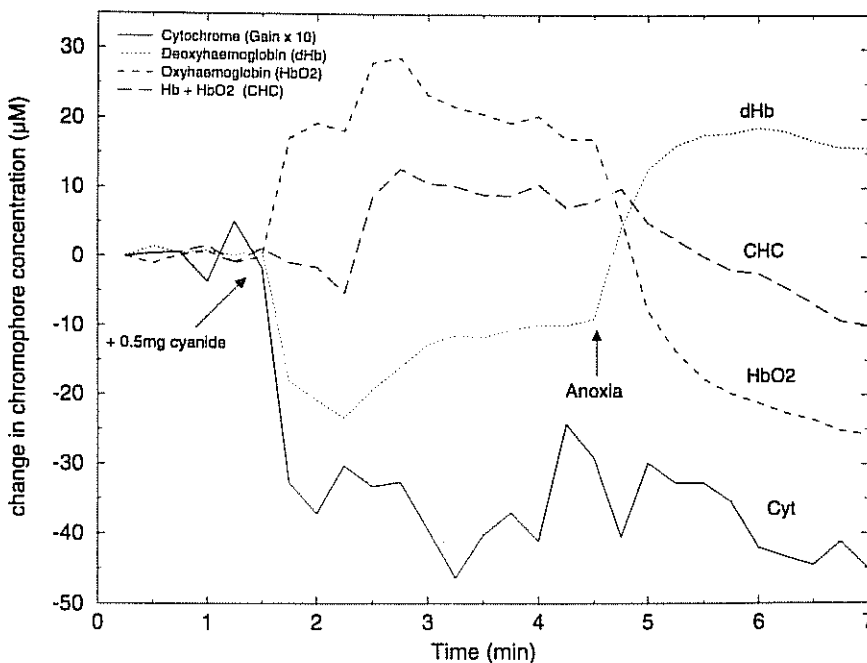


Figure 3. Effect of cyanide and anoxia on NIR chromophore changes. Adult rat with additions as shown on Figure; optodes placed directly on skull following removal of surface muscle as described in Wray *et al.* (1988)²⁶.

this treatment as it contains only small amounts of rhodanese, the enzyme that metabolises cyanide. Cyanide binds to haem a_3 of cytochrome oxidase, preventing oxygen reduction by electrons leaving Cu_A and haem a ²⁴. Therefore Cu_A should become completely reduced²⁵. However, $[\text{HbO}_2]$ will rise and $[\text{dHb}]$ fall as oxygen consumption is inhibited; vasodilation will also occur, increasing the blood volume and thus the total haemoglobin concentration (CHC). The FiO_2 is then reduced to zero. Following anoxia cytochrome oxidase Cu_A should remain reduced, whereas $[\text{HbO}_2]$ should now fall and $[\text{dHb}]$ rise. Any cross-talk in the algorithms will be revealed if the cytochrome signal changes following anoxia. Figure 3 shows that the algorithm used (the multiwavelength, 780–900nm, UCL algorithm described in detail by Matcher et al.¹⁹) is robust to these extreme changes in $[\text{dHb}]$, $[\text{HbO}_2]$ and CHC.

Two problems still remain, however. Firstly the studies in Figure 3 were performed in transillumination mode on an adult rat with the surface muscles removed and the optodes placed directly on the skull. It is not straightforward to transfer this “internal validation” to other systems where the optical properties and tissue heterogeneity may be different and/or more complex^{16, 27, 28}. In similar studies with the neonatal pig (with no skull surgery) we have found that the change in the cytochrome signal is critically dependent on the wavelength range over which the fit is performed.

In a clinical/human setting this direct “internal validation” technique is clearly not possible and one has to fall back on measuring correlations i.e. cytochrome oxidase changes that correlate with those of another chromophore under a wide variety of conditions might be treated with a degree of scepticism.

FACTORS AFFECTING THE IN VIVO CYTOCHROME OXIDASE REDOX STATE

Electron Flow to Cytochrome Oxidase

In a recent review we discussed the factors affecting the redox state of Cu_A *in vitro*¹. Our conclusion was that *in vivo* Cu_A was likely to be highly oxidised and that *large* changes were only likely to be seen as oxygen tension fell and started to affect the electron transfer from Cu_A to the other metal centres in the enzyme, resulting in significant reduction of Cu_A . Theoretically inhibitors acting upstream of cytochrome oxidase might be expected to oxidise cytochrome oxidase Cu_A , either directly by reducing the flow of electrons from cytochrome c to Cu_A or indirectly by reducing the proton motive force and hence facilitating electron flow between Cu_A and haem a . However, in halothane-anaesthetised rats we have been unable to significantly oxidise cytochrome oxidase by the addition of the barbiturate amytal, a well-characterised inhibitor of mitochondrial NADH dehydrogenase^{29–31}.

The NADH/NAD⁺ ratio can be lowered (and hence the rate of electron entry to cytochrome oxidase decreased) by reducing the rate of glycolysis. However, we have also been unable to detect any significant oxidation of cerebral cytochrome oxidase following a reduction in blood glucose levels to <1mM in either the neonatal pig or the adult rat. Small oxidations have been observed though following perfusion of the haemoglobin-free isolated rat head with a glucose-free medium²¹.

Although it is difficult to further *oxidise* Cu_A *in vivo* by the addition of inhibitors of mitochondrial respiration it is possible, however, to *reduce* the enzyme by adding compounds that increase oxygen consumption whilst maintaining a high tissue oxygen tension

the concentration of HbO_2 ¹⁸. The most likely explanation for an increased reduction state in the mitochondrial respiratory chain is increases in the NADH/NAD^+ ratio. This could be achieved by, for example, increases in intramitochondrial calcium which is known to activate the mitochondrial dehydrogenases³³.

Oxygen Tension

By far the greatest changes seen in the *in vivo* Cu_A redox state are when oxygen tension is changed. When oxygen tension is reduced the reaction of reduced haem a_3 with oxygen starts to limit the steady state rate of electron transfer in the enzyme, resulting in an increased reduction in all the metal centres including Cu_A . Thus the cytochrome oxidase NIR signal might be expected to be an effective monitor of tissue, as opposed to vascular, oxygenation. The oxygen tension at which redox state changes occur has been a matter of ongoing debate amongst those who state that mitochondrial electron transfer centres start to become reduced at relatively high oxygen tensions³⁴⁻³⁷ and those who view that changes occur only when haemoglobin is essentially fully deoxygenated³⁸⁻⁴¹.

The problem of the oxygen dependence of redox state changes may be linked to the observation that the K_m for oxygen consumption by cytochrome oxidase is higher *in vivo* than *in vitro*. Various theories have been put forward to explain this phenomenon^{4, 42}; most recently attention has focused on the idea that the intercellular messenger nitric oxide may compete for oxygen at the active site of the enzyme^{43, 44}. Thus the oxygen dependence of cellular respiration and the Cu_A redox state would vary with the nitric oxide concentration.

In the brain there is a well-characterised⁴⁵ biphasic response of oxygen consumption (CMRO_2) to changes in the rate of oxygen delivery (DO_2). As DO_2 declines there is no change in CMRO_2 until a critical rate of O_2 delivery is reached. It might be expected that the Cu_A redox state would display a similar biphasic response. This has been clearly shown in a variety of studies in the blood-free, perfluorocarbon perfused, brain in both rats^{16, 46} and cats^{15, 16, 47}; in the latter case Cu_A redox state, CMRO_2 , and somatosensory evoked potential were all unchanged until DO_2 dropped below $5\text{ml}/100\text{g}/\text{min}$ ⁴⁷.

A priori one would expect the physiological oxygen carrier (haemoglobin) to be at least as efficient as perfluorocarbon emulsions and therefore that under normal levels of oxygen delivery in the blood-perfused brain the Cu_A redox state would be relatively insensitive to small changes in DO_2 . To address this question we have been using NIR on the blood-perfused rat brain, varying DO_2 by different methods, and observing the relationship between the haemoglobin and the cytochrome oxidase redox states.

In the adult rat brain we have dropped mean arterial pressure by exsanguination and measured the NIR spectra at defined and measured DO_2 ¹. $[\text{HbO}_2]$ drops linearly as DO_2 declines, but, as in the bloodless rats⁴⁶, the Cu_A redox state shows a biphasic response. Thus a plot of $[\text{HbO}_2]$ versus Cu_A redox state shows that the Cu_A redox state does not start to fall until the HbO_2 concentration falls below $10\mu\text{M}$ (Figure 4).

The rapid time resolution NIR affords allows us to perform rapid changes in FiO_2 and observe the effects on $[\text{HbO}_2]$ and the Cu_A redox state. Again we see no change in the Cu_A redox state until significant haemoglobin desaturation is observed (Figure 5). A full recovery of the Cu_A NIR signal is observed upon reoxygenation and the FiO_2 swing can be repeated. Following complete removal of O_2 from the brain, $5\mu\text{M}$ of oxidised Cu_A was observed to undergo reduction. This is further evidence that, under normoxic conditions, Cu_A is highly oxidised as the total cerebral cytochrome oxidase concentration in the adult rat brain is between 5 and $5.5\mu\text{M}$ ⁴⁸.

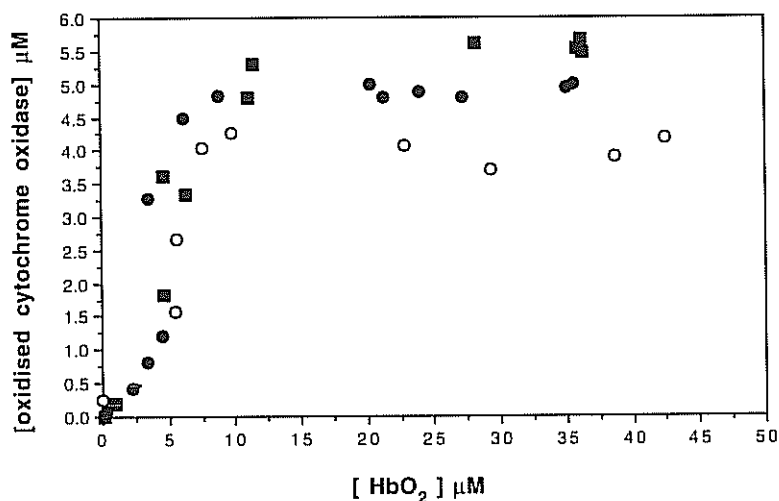


Figure 4. Effect of reducing oxygen delivery on cerebral $[\text{HbO}_2]$ and cytochrome oxidase NIR signals. Different symbols represent different rats. Optodes placed directly on skull following removal of surface muscle. Zero values for oxyhaemoglobin and oxidised cytochrome oxidase were obtained following extended ventilation with $\text{FiO}_2 = 0$. DO_2 was lowered by reducing the blood pressure.

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Plotting the $[\text{HbO}_2]$ against the Cu_A signal again shows that no significant change is seen in the Cu_A redox state until $[\text{HbO}_2]$ drops below $10\mu\text{M}$. (Figure 6). We thus agree with other authors^{41, 47} in stating that, at least in animal models with normal cerebral perfusion, large scale changes in the cytochrome oxidase NIR signal will not be observed until cerebral rates of oxygen delivery are very compromised. Changes in the cytochrome oxidase redox state therefore reflect a condition where oxygen supply is already inadequate to meet oxygen demand (i.e. tissue dysoxia), not an early warning of impending hypoxia.

CONCLUSION

In the 770–900nm region the only significant (non-haemoglobin) oxygen-dependant chromophore is cytochrome oxidase. The tissue NIR spectrum of cytochrome oxidase is dominated by the Cu_A centre in the enzyme. The distinctive reduced minus oxidised centre of this centre is unchanged throughout a variety of mammalian species and developmental ages. Small changes in the redox state of Cu_A are readily detected in the blood-free brain.

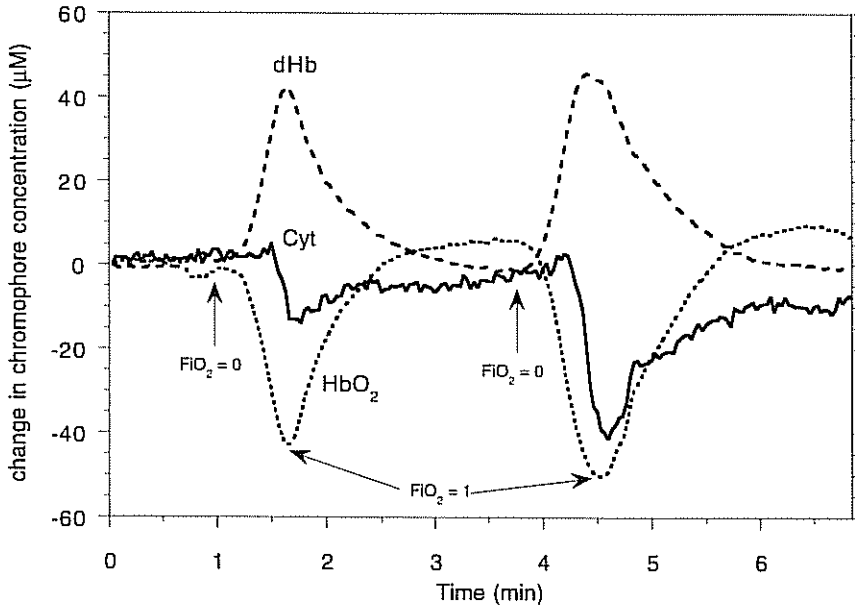


Figure 5. Kinetics of cerebral haemoglobin and cytochrome oxidase NIR signals in the halothane-anaesthetised rat brain following a rapid reduction in F_{iO_2} . Other conditions as per Figure 3.

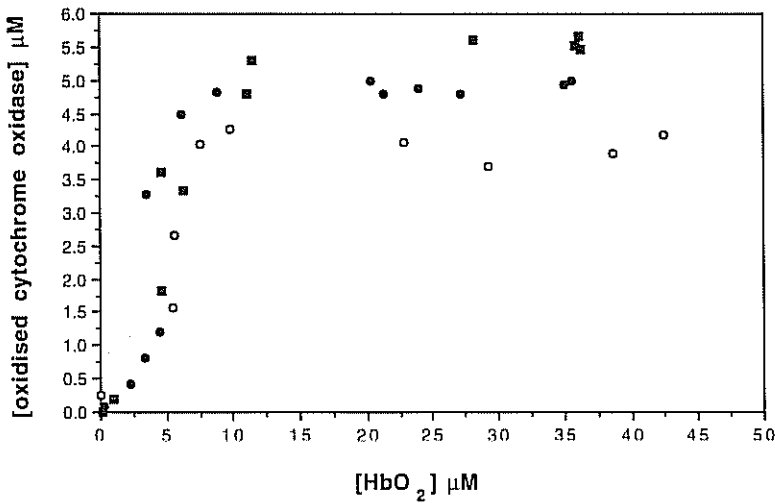


Figure 6. Variation of cytochrome oxidase and $[HbO_2]$ changes during hypoxia. Data derived during decrease in F_{iO_2} shown in Figure 5.

oxygen. Large reductions are seen in the Cu_A redox state when oxygen delivery drops significantly. However, the Cu_A changes are only observed once extensive haemoglobin desaturation has occurred. We conclude that the cytochrome oxidase near infrared signal is an important measure of actual tissue dysoxia - haemoglobin desaturations that do not induce cytochrome oxidase reduction are unlikely to cause long-term tissue damage.

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