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1 Introduction

A patient’s health is in great danger when there is a prolonged lack of oxygen delivery to meet the metabolic demand of the tissue; even brief periods of hypoxia can cause permanent damage to brain tissue. This is a huge problem in patients with acute brain injury, for example, where derangements of cerebral oxygen delivery and utilization often occur, rendering the brain susceptible to secondary, or additional, injury processes. Secondary injury is also common in neonatal brain injury after perinatal hypoxia-ischemia (HI), affecting neurodevelopmental outcome. A number of hemoglobin-based oxygenation monitors are available; however, clinicians currently cannot monitor the biochemical status of the injured brain continuously and noninvasively at the bedside. There is therefore an urgent need for real-time, in vivo measurements of brain tissue oxygenation and biochemistry in the clinic; a need for a bedside sensor that can improve diagnostic information and indicate when to alter and/or redirect therapy to improve clinical outcome.

Since 1977, when Franz Jöbsis reported a new optical method in a seminal Science article,1 near-infrared spectroscopy (NIRS) has been seen as the technique, which could deliver a solution to this clinical need. NIRS has become an established research and clinical tool for measuring changes in cerebral oxygenation, in particular, changes in oxygenated and deoxygenated hemoglobin (HbO2 and HHb) concentration. This groundbreaking paper has been cited close to 3000 times. However, Jöbsis’s intention was to develop an optical technique to measure in vivo changes in cytochrome-c-oxidase (CCO), an enzyme in the mitochondria, and hence monitor tissue metabolism (see his candid account of his discovery2). Jöbsis discovered that near-infrared (NIR) light penetrates deep into living tissue and this optical window could be used to monitor changes in the concentrations of absorbing compounds inside the tissue. The tissue light attenuation measurements are related to changes in the concentrations of HbO2 and HHb, and the redox state of CCO. The legacy of Jöbsis’s work lies in the measurement of changes in hemoglobin oxygenation with NIRS, as it is much simpler to measure the hemoglobin chromophores, which are present in high concentrations compared to CCO; but his goal of an in vivo monitor of tissue metabolism is as important today as it was almost four decades ago.

There has been a significant amount of work on measuring hemoglobin concentration changes with NIRS since 1977 (see the recent reviews by Wolf et al.3 and Scholkmann et al.4). A typical commercially available NIRS system is a noninvasive, inexpensive, portable, bedside monitor that can measure, at multiple sites, the changes in HbO2 and HHb via the modified Beer-Lambert law (described in Ref. 4). From those, it is possible to derive estimations of changes in oxygen delivery (via hemoglobin difference, HbD = HbO2 − HHb) and changes in total blood volume (total hemoglobin, HbT = HbO2 + HHb). In addition, from technical developments in the late 1990s, we now have clinical NIRS cerebral oximeters that measure absolute tissue saturation [known as cerebral oxygen saturation (ScO2)], tissue oxygen saturation (StO2), or tissue oxygenation index (TOI)], which is the ratio of absolute HbO2 and absolute HbT, using techniques, such as spatially resolved spectroscopy.5,6 However, these measurements do not monitor metabolism at a cellular level unlike a measurement of the redox state of CCO, which has the potential to yield an in vivo indication of cellular energy metabolism. Simultaneous measurement of the CCO and hemoglobin signals can therefore provide complimentary information on hemodynamics, oxygenation, and metabolism. Monitoring of CCO with NIRS has been shown to be consistent with other measures of metabolism; including those from more invasive techniques.

Abstract. Near-infrared spectroscopy (NIRS) measurements of cytochrome-c-oxidase (CCO) have the potential to yield crucial information about cerebral metabolism at the patient bedside. Developments in instrumentation and the analytical methods used to resolve changes in CCO have led to many clinical applications of the measurement since its first demonstration in 1977 by Jöbsis. There is a substantial literature of work on measures of CCO in animal and in vitro studies; however, this review focuses on translational studies. Almost 40 years from the advent of the first measurement of CCO using NIRS, this signal continues to hold significant interest in our understanding of the human brain in health and disease. We discuss methodologies for obtaining NIRS measurements of CCO in the clinic and review studies in neonates and adults. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.JBO.21.9.091307]

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such as microdialysis in humans, or more expensive and less portable techniques, such as magnetic resonance spectroscopy (MRS) in animal studies. Furthermore, the concentration of CCO is much higher in the brain than in extracerebral tissues, so it is a more brain-specific signal that is less prone to scalp and skull contamination than the hemoglobin signals. This has potentially huge importance for functional NIRS (fNIRS) studies in which the hemoglobin signals suffer from contamination from the extracerebral tissues.

Preclinical and in vitro studies have contributed enormously to our understanding of the optical issues surrounding the NIRS measurement of CCO, its role as marker of cellular metabolism, and its potential as a clinically relevant measure. This was a focus for several research groups in the 1980s and 1990s. More recently, a study in a piglet model of HI brain injury has demonstrated the specificity of the CCO signal as a marker of tissue metabolism. In this study, the percentage recovery of CCO after HI to baseline levels was highly correlated with percentage recovery of phosphorus ($^{31}$P) MRS measures of cellular metabolism (see Fig. 1). Furthermore, this study demonstrates the potential for CCO measures to identify outcome after injury. However, in this review, we focus on the translational clinical and human cerebral studies that have been ongoing since 1985 and we aim to identify and summarize this work from then until the present day.

Figure 2 shows how the number of publications on and the populations studied by NIRS-measured CCO has varied over the years. Between 1977 and 1997, there was a gradual increase in the number of papers published, varying in subject and research group [Duke University, University College London (UCL), Hokkaido University, Sapporo, and Keele University were home to the main groups involved]. After 1997, the interest in the CCO measurement gradually declined due to both controversies over the optical instrumentation, methods and algorithms used to resolve it, and a shift of clinical interest due to the emergence of the absolute tissue saturation measurement with NIRS. In the 2000s, efforts were made to optimize NIRS instrumentation and methods specifically for the measurement of CCO and studies were performed in healthy volunteers and patients to characterize the behavior of the CCO signal as a marker of cerebral cellular oxygen metabolism. A resurgence in the number of papers published over the last few years reflects the continued clinical desire for in vivo measures of metabolism and a renewed interest in the CCO signal following the demonstration of new technological and analytical methods.

Our focus in this review article is on measurements in patients and healthy volunteers, however, in order to fully understand the chronology and advances made in the methodology, some important preclinical work is also discussed. While there is extensive work on the measurement of CCO changes in the visual part of the light spectrum (see the review by LaManna for a historical perspective), here, we have focused on the measurements with NIR light (650 to 1000 nm). We aim to summarize work on the instrumentation, algorithms, and clinical applications of the CCO signal and assess the current state of the measurement to look forward to the future of the technique. We provide a simplified description of the biochemistry of the enzyme to understand the origins of the optical signal; the methods, algorithms, and instrumentation used to study it; a discussion of the issues with the methodology; a summary of the applications of cerebral NIRS measurements of CCO in neonatal and adult studies; and a forward perspective on the future of the technique. Almost 40 years from the advent of the first measurement of CCO using NIRS, this signal continues to hold significant interest and hope in providing the ultimate noninvasive, in vivo, bedside, and real-time sensor of brain tissue well-being; a sensor with the capacity to indicate when to alter and/or redirect therapy to improve long-term clinical outcome.

We are interested in an indicator of the redox state change of CCO. Previous NIRS studies, both preclinical and clinical, have referred to this signal as CytOx, aa3, CuA, CCO, and oxCCO. For clarity, in this review, the term oxCCO is used to describe the NIRS measurement of the oxidation state of cytochrome-c-oxidase, which arises from its redox state-dependent changes in the NIR spectrum. Although the dominant chromophore in the NIR is the CuA center, other cytochrome redox centers and oxygen intermediates can contribute to this signal.

2 Biochemistry and Spectral Features of Cytochrome-C-Oxidase

In order to fully understand the CCO NIRS signal, one must understand the biochemistry from which the signal stems. CCO is a small but important part of the aerobic metabolism of glucose. Glycolysis converts glucose into pyruvate and in the process generates ATP and NADH. After glycolysis, pyruvate is transported into the mitochondria and converted to acetyl CoA, which then enters the tricarboxylic acid (TCA) cycle. During the TCA cycle, additional ATP and NADH are produced. NADH is an electron donor; electrons from NADH can be used to, ultimately, convert oxygen to water, releasing useful chemical-free energy in the process. The mitochondrial electron transfer chain (ETC) mediates this transfer, in the process converting ADP to ATP. The consequent change in ATP/ADP ratio provides usable energy to drive a wide variety of cellular processes.
Figure 3 shows the mitochondrial protein complexes involved in this oxidative phosphorylation. Oxidative phosphorylation is the process where energy is harnessed through a series of protein complexes (known as complexes I to V) embedded in the inner membrane of mitochondria to create ATP. Oxidative phosphorylation can be broken down into two parts: oxidation of NADH (and other electron donors) in the ETC via complexes I to IV and phosphorylation, the production of ATP, at Complex V. Complex I accepts electrons from NADH and transfers them to the soluble membrane-bound electron carrier ubiquinol (Q), producing ubiquinone (QH2). QH2 is oxidized by complex III, in the process reducing the soluble electron transport protein, cytochrome c. Cytochrome c is then oxidized by the terminal electron acceptor cytochrome-c-oxidase (also known as complex IV), with the electrons ultimately being used to reduce oxygen to water. The redox potential drop in the electron transfer processes in complex I, III, and IV is converted to a proton electrochemical potential, which ultimately drives mitochondrial ATP synthesis via the mitochondrial ATP synthase (complex V).

The transfer of electrons within the mitochondrial ETC complexes occurs via a number of protein bound redox factors. Of particular interest in this review, CCO contains four redox centers: two hems (known collectively as aa3) and two copper sites (see Fig. 3). The electrons pass between these centers in a series of redox reactions. All of these redox changes have associated optical transitions. In the NIR region, one of the copper sites, the Cu-Cu dimer copper A (CuA), dominates the absorption spectrum, with a strong peak in the oxidized form centered, in mammalian enzymes, around 830 to 840 nm (see Fig. 4). For a recent detailed discussion of the relative contributions of CCO chromophores to the NIR spectrum, see Ref. 30 and references therein.

The difference between these absorption spectra (oxidized-reduced CCO) can be used to monitor changes in the redox states of the mitochondrial proteins. Note that the total CCO concentration does not change over a short-time period (in the order of hours); therefore, for analytical purposes, it is only necessary to use the difference spectrum between the oxidized and reduced species to obtain an indicator of the changes in the CCO redox state.

The UCL group has shown that using the oxidized-reduced difference spectrum resolves changes in oxidized CCO (and conversely, using the reduced-oxidized difference spectrum resolves changes in reduced CCO). The proof uses the modified Beer–Lambert law and involves two scenarios. In scenario 1 [Eqs. (1)–(5)], CCO is fully oxidized (ox) at the initial time \( t_0 \) and so the Beer–Lambert law states that the attenuation (A) due to CCO is related only to the concentration (c) of oxidized CCO, its extinction coefficient (\( \varepsilon \)), and the optical path-length of light (l):

\[
A(t_0) = l\varepsilon_{\text{oxCCO}}C_{\text{oxCCO}}(t_0). \tag{1}
\]

At a later time (\( t_1 \)), CCO is not fully oxidized and so the attenuation is related to both oxidized and reduced (red) forms of the enzyme:

\[
A(t_1) = l[\varepsilon_{\text{oxCCO}}C_{\text{oxCCO}}(t_1) + \varepsilon_{\text{redCCO}}C_{\text{redCCO}}(t_1)]. \tag{2}
\]

Assuming that the total CCO concentration does not change, the initial concentration of fully oxidized CCO is equal to the sum of the oxidized and reduced forms at the later time:

\[
C_{\text{oxCCO}}(t_0) = C_{\text{oxCCO}}(t_1) + C_{\text{redCCO}}(t_1). \tag{3}
\]

Therefore, it is possible to substitute the equations above into the difference measurement of the attenuation:
\[ \Delta A = A(t_1) - A(t_0) \]
\[ = l(\varepsilon_{\text{oxCCO}}[C_{\text{oxCCO}}(t_1) - C_{\text{oxCCO}}(t_0)] + \varepsilon_{\text{redCCO}}[C_{\text{redCCO}}(t_1) - C_{\text{redCCO}}(t_0)]) \]
\[ = l(\varepsilon_{\text{oxCCO}} - \varepsilon_{\text{redCCO}}) \Delta C_{\text{oxCCO}}. \]  

The oxidized-reduced difference (diff) extinction spectrum, \( \varepsilon_{\text{diff}} = \varepsilon_{\text{oxCCO}} - \varepsilon_{\text{redCCO}} \), gives rise to Eq. (5), which expresses the change in attenuation due to a change in the concentration of oxidized CCO from the difference extinction spectra:

\[ \Delta A = l\varepsilon_{\text{diffCCO}} \Delta C_{\text{oxCCO}}. \]  

In a scenario 2 [Eqs. (6)–(10)], where CCO is not fully oxidized initially (i.e., CCO exists in both oxidized and reduced forms), the same expression can be achieved:

\[ A(t_1) = l(\varepsilon_{\text{oxCCO}} C_{\text{oxCCO}}(t_1) + \varepsilon_{\text{redCCO}} C_{\text{redCCO}}(t_1)). \]

Assuming again that the total CCO concentration does not change:

\[ C_{\text{oxCCO}}(t_0) + C_{\text{redCCO}}(t_0) = C_{\text{oxCCO}}(t_1) + C_{\text{redCCO}}(t_1). \]

Therefore,

\[ \Delta A = A(t_1) - A(t_0) \]
\[ = l(\varepsilon_{\text{oxCCO}}[C_{\text{oxCCO}}(t_1) - C_{\text{oxCCO}}(t_0)] + \varepsilon_{\text{redCCO}}[C_{\text{redCCO}}(t_1) - C_{\text{redCCO}}(t_0)]) \]
\[ = l(\varepsilon_{\text{oxCCO}} - \varepsilon_{\text{redCCO}}) \Delta C_{\text{oxCCO}}. \]

which gives rise to the same expression as Eq. (5):

\[ \Delta A = l\varepsilon_{\text{diffCCO}} \Delta C_{\text{oxCCO}}. \]
These proofs show that the oxidized-reduced difference spectrum can be used to resolve changes in the concentration of oxidized CCO. An increase in the oxCCO signal therefore reports an increase in oxidized CCO and an equivalent decrease in reduced CCO. Note that if the sign of the measured signal is changed, it represents reduced CCO, not oxidized CCO. The oxCCO term does not imply that we are only sensitive to the oxidized species. Nevertheless, oxCCO is the term used to describe the measured changes from the difference spectra in this review article.

To understand what a change in oxCCO means physiologically we need to know what the concentration of oxidized and reduced CCO is dependent on and how it can be affected in vivo. The redox state is dependent on both the availability of oxygen in the cells and the supply of electrons entering the ETC. The review by Cooper et al.\textsuperscript{31} gives an overview of the influencing factors on the CCO redox state. In the purified isolated enzyme, these include changes in the three substrates [protons (pH), reduced cytochrome c, and oxygen tension], as well as a range of terminal inhibitors (such as nitric oxide and cyanide) and electrochemical potential. The complex interplay between these factors in the mitochondrial ETC has been investigated mathematically by Banaji.\textsuperscript{32} This informs on clinical in vivo measurements. Possible factors that might increase oxCCO in vivo include increase in oxygen tension, increased ATP turnover, decrease in proton electrochemical gradient, decrease in pH, or decrease in supply of reducing equivalents (substrates, i.e., NADH) to the ETC. These factors have been discussed by Heekeren et al.\textsuperscript{33}

The oxCCO concentration can readily be decreased in animal models, either by large drops in oxygen tension or the addition of inhibitors that act at the oxygen reduction site.\textsuperscript{16} There have, though, been questions regarding how easy it is to increase the oxCCO signal in vivo, but animal models have shown that there is the capacity to increase the oxidation state at normoxia\textsuperscript{17,34} as well as adult volunteer studies in hyperoxia and hypercapnia.\textsuperscript{13,35} We acknowledge that there are some differences in the CCO response between the preclinical and clinical work, in particular, when compared to results from nonanesthetized human adults; it is not our intention in this review to discuss those physiological differences.

### 3 Methods of Cytochrome-C-Oxidase Measurements

CCO is one of the most abundant enzymes in mammalian systems. However, its relatively low concentration compared to the hemoglobin chromophores presents some challenges for NIRS techniques. The absolute concentration of CCO in the human brain is unknown, but optical methods have obtained values of 5.5 and 4.5 μM in the adult rat.\textsuperscript{36,37} This should be considered an upper limit for the human CCO concentration as rats have a significantly higher cerebral metabolic rate of oxygen than humans.\textsuperscript{38} The contribution of CCO to overall absorption in tissue is considerably less than that of hemoglobin, because of its lower concentration, despite a higher specific extinction spectrum in this review to discuss those physiological differences.
must be chosen, along with the selection of other variables, such as the pathlength that the light has traveled, the absorption spectrum of the chromophore, and a set of measurement wavelengths. It is important that the algorithm and the other variables do not unknowingly induce artifacts. The factors that make up the algorithm and its variables have been the subject of discussion for many years. The choices to be made are:

- the algorithm
- the determination of the chromophore absorption spectra
- how the optical pathlength is estimated
- the number and choice of wavelengths.

Calculating and validating changes in hemoglobin in both of its oxygenation states is simpler because its concentration in tissue is high. It is also relatively simple to isolate the molecule to produce an absorption spectra in vitro. The absorption spectra have defined features in the NIR (see Fig. 5), such as a peak at 750 to 760 nm in HHb and the isobestic point at 800 nm. The CCO difference absorption spectrum, once identified, has a broad peak, which is significantly different to that of the hemoglobin chromophores. However, the broad nature of the three chromophore peaks throughout the NIR region confounds simple deconvolution via standard optical techniques, such as dual wavelength or derivative spectroscopy. Therefore, to successfully separate the CCO signal from the larger hemoglobin signals, careful selection of the algorithm, wavelengths, and extinction spectra is required. One of the biggest problems for the oxCCO signal is that there is no gold standard with which to compare and validate the NIRS signal experimentally. Although the same problem exists for NIRS detectable hemoglobins, in this case, there are at least invasive measurements that can be assumed to report on similar parameters (such as tissue oxygen, blood volume, and arterial/venous oxygen saturation). The lack of external comparators is a problem in assessing which criteria produce the most physiologically accurate oxCCO signal. Yet, there have been a range of in vitro and in vivo controlled studies that inform on the success of the optical separation of the cellular oxCCO signal from changes in the hemoglobin signals. Additionally, integrated mathematical models of the biochemistry and physiology can aid the interpretation of results.

3.1 Algorithm

Various algorithms have been developed by different groups, and these were assessed and described fully in 1995 by Matcher et al. The algorithms can be reduced to those developed at Duke University (Duke), University College London (UCL), Hokkaido University, Sapporo (Sapporo), and Keele University (Keele).

The UCL algorithm is a generalized algorithm based on the modified Beer–Lambert law and the concentrations are derived using multilinear regression. The modified Beer–Lambert law forms the mathematical basis of spectroscopic algorithms that relate wavelength-dependent optical attenuation signals to changes in chromophore concentrations. The generalized form of this algorithm for n wavelengths (termed UCLn) is

\[
\begin{bmatrix}
\Delta[HbO_2] \\
\Delta[HHb] \\
\Delta[oxCCO]
\end{bmatrix}
= \frac{1}{\text{pathlength}} 
\begin{bmatrix}
\epsilon_{HbO_2}(\lambda_1) & \epsilon_{HHb}(\lambda_1) & \epsilon_{oxCCO}(\lambda_1) \\
\epsilon_{HbO_2}(\lambda_2) & \epsilon_{HHb}(\lambda_2) & \epsilon_{oxCCO}(\lambda_2) \\
\vdots & \vdots & \vdots \\
\epsilon_{HbO_2}(\lambda_n) & \epsilon_{HHb}(\lambda_n) & \epsilon_{oxCCO}(\lambda_n)
\end{bmatrix}^{-1} 
\times \begin{bmatrix}
\Delta A(\lambda_1) \\
\Delta A(\lambda_2) \\
\vdots \\
\Delta A(\lambda_n)
\end{bmatrix}
\]

Fig. 5 Specific extinction coefficients measured by Mark Cope. The ox-redCCO spectra are not the same as the difference between the CCO spectra presented in Fig. 4. Data taken from the UCL BORL website in Ref. 29.

The algorithm assumes that tissue scattering and pathlength remain constant throughout the measurement period and is therefore suitable for all geometries and optical systems. It ignores, however, the multiple scattering effects including the nonlinear relationship between absorption and attenuation; this is discussed further in the Sec. 3.2. A three-wavelength version of this algorithm was also developed by researchers at Keele using different extinction spectra.

The algorithm developed in Sapporo is a four-wavelength algorithm, which uses attenuation differences between three measuring wavelengths and a fourth reference wavelength. The reference wavelength was selected in a region in which, the authors claim, has zero attenuation from CCO. The algorithm was developed specifically for the intact rat head through a vertical plane from the roof of the mouth to the top of the skull. The coefficients used (not shown here but presented in Refs. 41 and 46) contain optical instrumentation factors so are only specific to one instrument. Their algorithm does not attempt to quantify the concentration changes in hemoglobins and oxCCO, and thus measurements are expressed in relative terms. The authors assert their algorithm to be robust to changes in scattering and optical pathlength changes during the measurement period.
The Duke algorithm uses four wavelengths with specific extinction spectra determined in vivo from the intact cat brain to determine concentration changes. The algorithm is not displayed here but can be found in Ref. 41, where it is also discussed.

3.1.1 Chromophore Absorption Spectra

The difference between the oxidized and reduced form of CCO can be determined in many different environments, such as the isolated enzyme, in mitochondria, and in vivo. The first NIRS measurement used difference spectra that were taken through animal heads during anoxic challenges. These spectra showed clear differences between oxidized and reduced CCO states. Later, authors replaced blood with optically clear oxygen carrying perfluorocarbon solutions in an attempt to produce hemoglobin-free CCO spectra. In addition, later studies were able to remove this contribution completely in cats, pigs, and pigtails.

The use of perfluorocarbon perfused blood-free animals has been a key to the development of algorithms to detect oxCCO. In some cases, these spectra were themselves used to generate the algorithms; in others, they were used to validate the use of in vitro spectra. The latter was the case for the UCL algorithms. Cope found that there is no appreciable difference between the spectra measured in vivo and in vitro. Matcher et al. found similar results when using the four-wavelength UCL algorithm, which uses in vitro extinction spectra, and the three-wavelength Keele algorithm, which uses in vitro spectra but with whole blood, not purified hemoglobin, to resolve concentration changes from the same attenuation data.

3.2 Optical Pathlength

The estimation of optical pathlength is one of the most notable challenges for the calculation of changes in oxCCO, as it is thought that erroneous changes resolved in oxCCO (i.e., cross talk) could be due to pathlength changes causing insufficient chromophore separation. As mentioned in Sec. 3.1, the modified Beer–Lambert law does not account for tissue scattering and assumes that the reduced scattering coefficient \(\mu'_s\) is constant throughout a measurement period. The optical pathlength term is the only consideration of scattering in the equation, so it must be estimated sufficiently to produce physiologically accurate representations of chromophore changes in vivo.

The first attempts at optical pathlength estimation assumed a constant pathlength, equal to the product of the distance between the source and detector optodes and the differential pathlength factor (DPF), an experimentally determined factor to account for the increased pathlength of light due to all forms of scattering. The DPF can be determined using frequency-domain NIRS (FD NIRS) or time-resolved NIRS (TR NIRS), which estimate the optical pathlength. In addition, to monitor the changes in pathlength in real time with CW NIRS, Matcher et al. at UCL developed a method to resolve the pathlength using the water absorption spectrum and the measured broadband attenuation; features in the second differential of the water absorption spectra around 740 and 820 nm can be combined with an assumed tissue concentration of water to estimate pathlength.

In 1993, the modified Beer–Lambert law-based algorithm was updated to account for the wavelength-dependency of the DPF, which allowed for the decreasing scattering effects at increasing wavelengths in tissue. DPF varies with wavelength through another attenuation mechanism; high absorption of light at a specific wavelength decreases the likelihood that light at that wavelength will reach the detector.

A few experiments have been set up specifically to test whether changes in pathlength are responsible for cross talk in the oxCCO measurement; none of these studies found significant changes in the pathlength during events where changes in oxCCO were observed, such as hypoxemia or functional activation. Skov and Greisen suggest that the wavelength-dependent DPF is a solution to the cross talk problems; they observed a linear relationship between HbT and oxCCO during hypoxemia when using a constant DPF but not when using wavelength-dependent DPF.

There are physiological factors that will affect tissue scattering. For example, glucose levels affect the refractive index of extracellular fluid, which contributes to the reduced scattering coefficient; therefore, changes in the glucose concentration may affect the overall scattering of the tissue. Swelling of the brain, or edema, is likely to change cerebral scattering properties. There is therefore a need to understand the influence of changing tissue scattering properties on the calculation of chromophore concentrations or to measure scattering during the measurement period, particularly in cases such as brain injury.

3.3 Wavelength Selection

In theory, to extract information for three chromophores requires measurements at three wavelengths. Yet this approach is prone to noise and cross talk artifacts that may lead to inaccurate quantification of changes in concentrations and misinterpretation of data. When measurements at a larger number of wavelengths are used in spectroscopic algorithms, any detrimental effects of cross talk and noise are expected to diminish.

Due to the broad spectral peak in the oxidized-reduced CCO spectra and its relatively low concentration in vivo, the choice of selection of the specific wavelengths and number of wavelengths used for spectroscopy is a big factor. This is also relevant for the hemoglobin chromophores. Matcher et al. performed analysis to assess the separation of the oxCCO signal from the hemoglobin signals with different number of wavelengths (4, 6, and “n” or 112) in a range of data: rat head, piglet head, adult forearm, and a simulated dataset. The results showed that the higher number of wavelengths produced more accurate simulations and improved the in vivo measurements. Despite this, preclinical studies showed that limiting broadband measurements from 780 to 900 nm improves the resolution of oxCCO as exclusion of shorter wavelengths lessens the contribution of HHb, which has a large peak at 760 nm. Figure 6 shows graphically the selection of wavelengths used in the clinical papers mentioned in this review.

Broadband spectroscopy, offering a full range of measurement wavelengths, can be difficult to incorporate into wireless and multichannel systems. A recent study has used a genetic algorithm method to assess the minimum number of wavelengths needed to resolve oxCCO accurately (compared to the current gold standard of a broadband NIRS system across 780 to 900 nm at 1-nm wavelength resolution). The results show that the optimal combination of wavelengths is a set that almost evenly spans the spectral range (see Fig. 6). The error against the gold standard reduces as the number of wavelengths increases; increasing from 3 to 4, 5, and 8 wavelengths leads to a...
two-, three-, and sevenfold improvement in the reduction of errors. The wavelengths selected need to be spread across this range in order to resolve the shape of the ox-redCCO spectra and disentangle it from the other chromophores; Arifler et al. found that the optimum eight wavelengths were 784, 800, 818, 835, 851, 868, 881, and 894 nm (displayed in Fig. 6).

### 3.4 Optical Instrumentation

Advances in optical methods have allowed further development of the instrumentation, as well as the algorithm, which have contributed to the increased robustness of the measured oxCCO signal with NIRS. For a full review of NIRS instrumentation, see Ref. 4.

Initially, NIRS instrumentation for the measurement of oxCCO was performed using three or four narrowband lasers at specific wavelengths between 740 and 910 nm (see Fig. 6, for more examples) using a photomultiplier tube (PMT) for detection. Commercial systems were developed to measure oxCCO using four discrete wavelengths using lasers and either photodiode detectors or PMTs (NIRO 300 and NIRO 500, respectively, Hamamatsu, Japan).10

In UCL, systems have increasingly been developed in-house and focused on broadband spectroscopy in the aim of obtaining a more robust oxCCO signal (see Sec. 3.4). These systems typically use a broadband white light source (i.e., a tungsten halogen bulb) with a spectrophotograph and charge-coupled device using optical fibers to transmit light both to and from the tissue.10

The UCL broadband NIRS systems include (1) a broadband instrument developed in the 1990s by Springett et al.;10 (2) a multistand hybrid broadband and FD system developed by Tachtsidis et al. in 2010 (called hybrid optical spectrometer or pHOS);25,101 and (3) in 2014, two variations of that system without the FD component developed by Bale et al. to be used in neonates (called Cytochrome Research Instrument and application or CYRIL),25 and Phan et al. with the capacity to perform imaging. All UCL-developed systems are broadband instruments with the capacity to apply the UCLn algorithm in real time and are explicitly mentioned in the reported studies below.

Miniaturization is a trend seen in all forms of technology and NIRS is no exception. The trend toward making devices smaller, more portable and cheaper has pushed broadband spectroscopy toward miniature spectrometers, and discrete wavelength systems toward wireless NIRS. For wireless devices, in which there is a need to eliminate heavy fiber optics, LEDs and laser diodes can be applied directly to the head with wireless transmitters to send the data to a nearby computer. Versions of both of these technologies are in development with the aim of incorporating a measurement of oxCCO.10

In 2000, a combined CW and FD NIRS system was developed to use measurements of $\mu'_s$ and the absorption coefficient (\(\mu_a\)) to inform the calculation of absolute chromophore concentrations.10 This was further developed by UCL to the pHOS system that attempted to measure oxCCO using the UCL hybrid algorithm.25,101 These hybrid devices use broadband CW systems and several lasers in the FD (7 and 4 wavelengths) to achieve this. By resolving for both absorption and scattering, it may be possible to measure absolute concentrations of the chromophores.

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**Fig. 6** Chart of wavelengths used in NIRS systems from all clinical publications mentioned in this review. Systems are listed in chronological order from their first publication in this review and are separated into three- and four-wavelength combinations and broadband spectra. The suggested wavelengths determined by Arifler et al. are also shown in Ref. 62. Clinical papers using the wavelengths are Duke (3),22,63,64 UCL (4),65 Duke (4),66-68 Keele (4),69-71 NIRO 300,72-75 Critikon 2020,76-78 UCLn,79-84,86-85 NIRO 1000,86-93 Keele 3,76,77 NIRO 500,94-95 Humboldt A,96-99 Humboldt B,100 and UCL Hybrid.101,102

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It is important to ensure that the NIRS signals are sensitive to the brain, but it is not possible to measure this experimentally. Therefore, light propagation models have been used to estimate the sensitivity of NIRS measurements to the brain in the adult \(^{100,101}\) and neonate \(^{112-114}\) at different source–detector separations. Due to the different geometries and tissue densities in adults and neonates, there are differences in the propagation of light. \(^{112}\) The cerebrospinal fluid (CSF) has a large positive impact on the sensitivity of absorption change in the brain, \(^{110}\) whereas the skull thickness has a negative impact. \(^{111}\) The thin skull and CSF layers in the neonate allow for more penetration of light into the cortex. \(^{112,113}\) Brigadoi et al. \(^{112}\) found that in neonates, a source–detector separation of <2.14 mm has a brain sensitivity of 5%, which means that signals from a typical setup of ~25-mm separation will have a significant cerebral contribution; a source–detector separation of more than 8.4 mm will achieve more than 5% brain sensitivity in adults. In addition, computational modeling work from Fukui et al. \(^{112}\) suggests that while the white matter sensitivity in adults is almost negligible, even for large (50 mm) source–detector separations, the neonatal model shows high white matter sensitivity at as low as 10-mm separation. Furthermore, the CCO concentration in the brain is likely to change with maturity. Rodent studies have shown that there is an increase in the number of mitochondria per cell, as well as an increase in the number of mitochondrial proteins and respiratory enzymes per mitochondrion in the first postnatal days. \(^{115}\) Additionally, there is a difference in energy metabolism and functional activity in the rodent brain during postnatal development compared with the adult, and so this must be taken into consideration when monitoring CCO with NIRS.

4 Analysis

4.1 Analysis of Broadband Cytochrome-C-Oxidase Measurements

Here, we document clinical experiments that assess the oxCCO signal for robustness and independence from other chromophores (i.e., cross talk). We focus mainly on clinical papers that use many wavelengths (broadband NIRS) and the wavelength-dependent DPF methods, which are required to minimize cross talk. We present a strong weight of evidence against cross talk if a sufficient number of wavelengths are utilized.

The simplest way to assess cross talk is to compare the oxCCO signals with the HbO\(_2\) and HHb, and this has been done in many different experiments. In the assessment of the low frequency oscillations during visual stimuli, there was a phase shift between the hemodynamics and the oxCCO signal. \(^{116}\) Additionally, the authors noted that the oxCCO recordings were “remarkably stable . . . compared to the changes in the hemoglobins, making a simple cross talk rather improbable.” Another functional activation study also showed differences in the oxCCO directional changes despite normal hemodynamic response functions. \(^{112}\) Furthermore, the same study confirmed that there was no relationship between oxCCO and the measured systemic changes. During blood pressure changes in orthostatic hypotension patients, oxCCO dropped despite different HbT directional responses. \(^{113}\) During spontaneous desaturation events in infants with hypoxic-ischemic encephalopathy (HIE), different directional changes were seen in oxCCO despite consistent decreases in HbO\(_2\) and increases in HHb. \(^{96}\) As discussed in Sec. 3.2, no study, where oxCCO and pathlength have been measured simultaneously, has a correlation been found between their changes.

Ulundug et al. \(^{98}\) found from simulations that incorrect estimation of the wavelength dependency of partial pathlength can contribute to cross talk in chromophores with low concentration. However, when this was investigated experimentally by studies in healthy adult volunteers using the Humboldt B broadband NIRS system, they found that the majority of oxCCO changes could not be explained by this effect. \(^{114}\)

Wolf et al. \(^{116}\) investigated the changes in oxCCO in 22 preterm infants during slow oxygen saturation (SpO\(_2\)) changes caused by inspired O\(_2\) changes. \(^{116}\) Changes in the concentrations of HbO\(_2\), HHb, and HbT, induced by small and slow O\(_2\) changes, were inversely correlated to the measured changes in the oxidation state of oxCCO with a four-wavelength Critikon 2020.

The most compelling evidence of a unique metabolic signal comes from human studies in which simultaneously measured changes in metabolism have been made using other modalities. Increases in oxCCO during hyperoxia correspond with decreases in microdialysis measures of lactate/pyruvate ratio, which is a marker of anaerobic metabolism. \(^{7}\) Tisdall et al. \(^{116}\) saw a linear relationship between oxCCO and estimated oxygen delivery [measured via pulse oximetry and transcranial Doppler (TCD) ultrasound] during hypoxemia. This relationship was not seen between HbD and oxygen delivery.

Residual analysis enables an observation of the goodness of fit of the chromophore spectra to the measured attenuation. To see if the CCO difference spectrum is necessary to explain the measured attenuation, two- and three-chromophore fits can be performed with and without CCO. Heekeren et al. \(^{33}\) found that using the three-chromophore fit explains in vivo data better than the two-component fit. \(^{33}\) The residuals for two-component fit have the same shape as oxCCO, which suggests that a chromophore with a similar shape to oxCCO is needed to fully explain the spectra. Above 760 nm, the three-component fit residuals were random. This result was also found in other broadband adult studies \(^{12,58}\) and a neonatal study \(^{96}\)—an example of this process is shown in Fig. 7.

5 Neonatal Studies

In this section, we review NIRS measures of oxCCO in neonates during spontaneous changes in systemic physiology (e.g., periods of hypoxia, increases in blood pressure, and so on), during surgery, with neonatal drug administration, and during functional activation.

5.1 Hypoxia and Other Spontaneous Systemic Changes

In 1985, Jöbsis’s group published the first NIRS study on newborn infants. \(^{22}\) They observed a decrease in oxCCO during spontaneous oxygen desaturations in three preterm infants using the Duke three-wavelength system. Furthermore, the timing and magnitude of the oxCCO decrease depended on the preceding oxygenation state, the depth and length of desaturation event and presence of circulatory disorder. Another study in which eight preterm infants were monitored with the NIRO 1000 showed that small SpO\(_2\) changes did not alter oxCCO. \(^{117}\) To add to the heterogeneity of the oxCCO response, Wickramasinghe et al. \(^{118}\) saw an inconsistent (increases and decreases) and insignificant change in oxCCO in seven infants during the reduction of inspired oxygen fraction (FiO\(_2\); SpO\(_2\))
change ~10%) using a three-wavelength Keele system.118 A study of infants during repetitive apneas found a consistent trend of reduced oxCCO during the breathing cycle.80 More recently, a UCL broadband NIRS study (with CYRIL) of over 200 oxygen desaturation events in six term infants with HIE saw changes in the concentration of oxCCO in both directions but with a decrease in oxCCO on average.96 The heterogenic response to these hypoxias may be a reflection of the level of brain injury, prematurity, previous oxygenation levels or influence of systemic pathophysiology. This heterogeneity is not seen in adult broadband NIRS studies of controlled hypoxias12 (see Sec. 6.1) so is specific to the neonatal brain. Animal work during anoxic swings in piglets has shown that as the oxygen delivery rate is reduced, there is a linear decrease in HbO₂ but a biphasic elbow in oxCCO reduction.104 A reduction in oxCCO may be a signal of impending damage, and this has also been previously suggested by Cooper et al.31 The heterogeneity of the oxCCO response to hypoxia has also been seen in a piglet study of increasingly severe episodes of hypoxia.11 At lower severities of hypoxia (8 and 12% FiO₂), there were small increases in oxCCO, while at higher degrees of hypoxia (6%, 4%, and 0% FiO₂), oxCCO decreased with severity; consistent decreases in HbO₂ and corresponding increase in Hb were observed in all hypoxic episodes. In 1985, Brazy called for an investigation into the degree of CCO reduction that causes brain injury in order to protect those at risk of injury;22 30 years later, there is still no answer but there is renewed effort to find it.96

Following from the hypoxia work, the effect of respiratory changes, which cause a subsequent change in carbon dioxide tension (PaCO₂), on the cerebral metabolism was investigated in neonates. The 1985 infant study from Brazy et al.22 saw oxCCO increase during hyperventilation; this was the first human study to suggest that oxCCO is not maximally oxidized in vivo at normoxia and can be increased by hyperventilation. The ability to increase oxCCO from normoxia is supported by another investigation where an increase in oxCCO was observed during an increase in PaCO₂.71 Edwards et al.117 suggest that the strong relationship between PaCO₂ and oxCCO is either due to cerebral hemodynamic changes caused by vasodilation due to
CO₂ or CO₂ decreasing the pH in cells. However, not all studies have seen this strong correlation; a study of PaCO₂ changes in 24 ventilated preterm infants observed changes in cerebral blood flow and volume (CBF and CBV, respectively), but not in oxCCO (four-wavelength Keele NIR system). Bale, Elwell, and Tachtsidis: From Jöbsis to the present day: a review of clinical near-infrared spectroscopy.

Another early NIRS experiment examined changes in oxCCO during alterations in mean arterial blood pressure (MABP). This study showed that increases in MABP were accompanied by increases in HbT and HbH and a decrease in oxCCO as measured by a three-wavelength Duke system. Infants whose clinical condition did not improve over the study duration showed larger changes in HbT and oxCCO. This area has potential for further investigation, especially in terms of cerebral autoregulatory effects on tissue metabolism.

The effects of changing intracranial pressure (ICP) were investigated during lumbar puncture in six infants with posthemorrhagic ventricular dilation with the NIRO 1000; three infants had an increase in ICP, which was accompanied by an increase in oxCCO, the other three had no change in either ICP nor oxCCO; this is the only study to link oxCCO to ICP. In a 2004 study of 12 infants with intraventricular hemorrhage, removal of CSF, which is expected to cause a reduction of ICP, did not produce a change in oxCCO measured with the NIRO 500.

5.2 Surgery and Anesthesia

Concern for cerebral health during major surgery such as cardiopulmonary bypass (CPB) brought the introduction of NIRS to the operating room. The first use of oxCCO monitoring during neonatal surgery was to monitor cerebral metabolism throughout cardiac surgery. In 1991, 15 children were monitored during CPB at Duke with a four-wavelength system and a decrease of oxCCO was observed at onset of bypass with hypothermia and reduced further when rewarming began. In 1995, 63 infants were monitored during CPB and hypothermia, and interestingly, oxCCO decreases were observed despite HbO₂ increases measured by the NIRO 500. Recovery of oxCCO was delayed despite HbO₂ recovery to above baseline levels. Larger and more rapid decreases were seen in oxCCO in older (>14 days) infants and those infants had worse recovery. The divergence in intravascular and mitochondrial oxygenation suggests abnormal response, which the authors suggested could be due to hypothermic temperature, effects of pH on the ETC, a disturbance in oxygen delivery to the mitochondria, disturbed perfusion, or decreased HbO₂ dissociation. The delay in recovery suggests mitochondrial dysfunction. Changes in hemodynamics and oxCCO measured by a four-wavelength Keele system were seen in 5 out of 14 children, who were hypoxic during CPB for congenital heart disease. Some recent studies with the NIRO 300 during repair of coarctation of aorta (without CPB) and cardiac surgery showed no significant change in oxCCO throughout either procedure.

Other strains during surgery such as the use of therapeutic hypothermia and anesthesia will have effects on cerebral metabolism; such effects are interesting and important to understand if NIRS monitoring of metabolism is to become clinically relevant. However, as Du Plessis et al. note, it is difficult to disentangle the effects of hypothermia on oxCCO from the pathology or the stress of surgery.

5.3 Effects of Drugs on the Neonatal Brain

The effects of the anesthetic, propofol, during CPB in children (n = 11) and CPB controls without propofol administration (n = 13) were studied in 2000. A decrease in oxCCO was observed during CPB and a lower decrease was seen in those with propofol.

The oxCCO response to other neonatal drugs, such as aminophylline, has been monitored. A NIRO 1000 was used to assess aminophylline delivery to 13 preterm infants in order to wean them off ventilators. A decrease in oxCCO was seen after administration, which the authors suggest could be mediated by reduction in perfusion of CO₂. A later similar study, using a NIRO 300 on the effects of aminophylline and caffeine for treatment of apneas in preterm infants, found that neither drug made a significant change to oxCCO.

Measures of oxCCO have been used to identify cerebral metabolic changes caused by indomethacin for the treatment of patent ductus arteriosus. Despite using different NIRS systems, three separate studies recorded decreases in oxCCO, HbO₂, CBV, and CBF with increases in HbH when indomethacin was administered. This result, combined with other evidence from changes in CBF, has provided evidence against the use of the drug due to its reduction in CBF and cerebral oxygen consumption, in favor of other drugs, such as ibuprofen, which do not have such a cerebral metabolic effect.

5.4 Neonatal Brain Injury

In 1997, a review by Cooper and Springett highlighted the potential for NIRS-measured oxCCO to monitor the progression of neonatal HI brain injury. The management strategy of HIE could be improved with additional information regarding the cerebral metabolic state as the development of the injury progresses over time and can lead to a secondary energy failure (SEF). There have been a handful of clinical studies of HIE with oxCCO which have promising results. A study of 11 infants with HIE showed that increases of SpO₂ and PaCO₂ increased oxCCO (measured with the UCL4 algorithm), which is a normal response and does not suggest pathophysiology. van Bel et al. saw a CBV and oxCCO (using the Four-wavelength algorithm) decrease in the first 12 h after birth in severe HIE, but stable changes in mild HIE and controls; this could suggest the delayed neuronal cell death as predicted by the animal models. They also saw a positive relationship between PaCO₂ and oxCCO in all HIE infants.

Recently, the relationship between the UCLn NIRS-measured oxCCO and oxygen delivery (HbD) was investigated in six HIE infants and found that an increased affinity between cerebral metabolism and oxygenation indicated brain injury. Analysis of the rewarming data after therapeutic hypothermia from this cohort provides more evidence that the relationship between the multimodal signals is indicative of brain injury. Further analysis of this data with a multivariate statistical technique showed that a strong relationship between oxCCO and the systemic physiology indicated more severe brain injury (as measured by 31P MRS). This suggests that the importance of the oxCCO measurement may not lie in the signal itself, but in its relationship with oxygenation or
systemic physiology, which can inform on the delicate equilibrium in pathology. However, most of this study occurred during therapeutic hypothermia, now a routine treatment for HIE, so it is difficult to compare with other earlier studies.

Furthermore, therapeutic hypothermia may make it difficult to observe SEF and cause unusual metabolic responses, as mentioned before. As previously discussed, there need to be more studies of the oxCCO response during cooling.

Recent animal studies of HIE have shown promising results. The UCL group demonstrated a relationship between broadband NIRS-measured oxCCO recovery fraction and HIE outcome in a piglet model.

The oxCCO signal has recently been observed during neonatal stroke in a term infant for the first time. Repeated transient decreases in cerebral HbT, HbD, and oxCCO were noted on both cerebral hemispheres without significant changes in the monitored systemic physiology. A clear asymmetry was noted in the degree of change between the two sides of the brain. Cerebral oxygenation (measured with HbD) and oxCCO were only highly coupled on the injured side of the brain.

5.5 Functional Activation

There has been a huge wealth of work in fNIRS using HbO2 and HbB as indicators of the hemodynamic response to neuronal activity since it was first presented in 1993. We have an opportunity with the oxCCO signal to inform directly on cerebral metabolism during neurovascular coupling; there is no other noninvasive, in vivo modality that can assess oxygen consumption during functional tasks. Concerns have been raised regarding the likelihood of measuring false positives or negatives in the hemodynamic response using fNIRS from hemodynamic signals that do not originate from neurovascular coupling.

Given that the oxCCO signal is more brain specific than the hemodynamic signals, it could prove to be a very valuable addition to the fNIRS toolbox.

There has only been one functional activation study involving infants and oxCCO and this used the NIRO 300. Nineteen newborns were given auditory stimulus and no significant increase in oxCCO was observed despite a normal hemodynamic response. The expected response of oxCCO during functional activation is unknown, although an increase is often seen in adult studies of functional activation (see Sec. 6.2). Differences between the mature and neonatal brain are supported by the NIRS study during cardiac surgery by Du Plessis et al., where different oxCCO responses were seen at different ages. This could either reflect greater mitochondrial hypoxic tolerance in younger infants or differences in the concentration of CCO at different ages.

6 Adult Studies

In order to characterize measured changes in the oxCCO signal, an increasing number of studies on adults have been performed over the past 10 years. These studies fall into three categories: volunteer studies with controlled inspired gas challenges to investigate the oxCCO response to systemic physiology, functional activation studies on healthy volunteers, and clinical studies.

6.1 Volunteer Studies: Systemic Challenges

The first NIRS studies on adult volunteers were performed in the early 1990s at Duke University. Hypoxic challenges were used to assess the changes in oxCCO in the adult brain at normocapnia and hyper- and hypocapnia with four-wavelength Duke NIRS systems. This work demonstrated a reduction of oxCCO during hypoxia at all levels of PaCO2.

More recently, the UCL group has performed hypoxemia studies (SpO2 to 80%) during normocapnia in healthy volunteers using the UCLn algorithm to estimate oxCCO. The hypothesis was that in nonanesthetized humans, a significant decrease in oxygen saturation should reduce oxCCO; this was confirmed in the study. There was a linear correlation between oxygen delivery [estimated from the measurement of the velocity of the middle cerebral artery (Vmca), an indicator of changes in CBF as monitored with TCD ultrasound] and oxCCO. A temporal delay between the HbD drop and oxCCO of ~5 s was also observed. A later study using the pHOS and the UCLn algorithm confirmed the decrease in oxCCO during hypoxia.

Conversely, in hyperoxia, the oxCCO signal has been found to increase. Tachtsidis et al. found that the oxCCO signal as measured with the UCLn algorithm was correlated with TOI (measured with the NIRO 300), as well as Vmca. Later, Kolyva et al. repeated this study using the pHOS and also showed that the magnitude of the oxCCO decrease during hypoxia was dependent on the distance between the source and the detector.

The oxCCO response to changes in PaCO2 was investigated first in 2009. During hypercapnia, Tachtsidis et al. increased inspired CO2 and saw a significant increase in oxCCO (estimated with the UCLn algorithm) and Vmca from baseline. There was no correlation between oxCCO and Vmca, but there were correlations with oxCCO and TOI (as measured with the NIRO 300). More recently, Kolyva et al. confirmed the increase in oxCCO during hypercapnia when monitored with the UCL pHOS system. They also performed hypocapnia and saw a decrease in oxCCO. Increased CO2 in the blood has a vasodilatory effect, which can increase CBF, but it also decreases the pH, which can reduce oxCCO. The authors suggested that the increases in blood flow from hypercapnia outweigh the potential damage to the respiratory chain from acidosis. In these studies, the oxCCO signal had a different trend to changes in HbT but showed a similar trend to TOI. A hyperventilation experiment from a different group also showed minor decreases in oxCCO with the NIRO 500.

As well as answering physiological questions, the study by Kolyva et al. was also designed to address scattering issues and brain-specificity questions using the UCL pHOS system. The magnitude of the oxCCO response increased with detector distance, which was not true for the hemoglobin signals. This oxCCO depth dependence shows that the signal is more brain specific, which is due to the higher CCO concentration in the brain, so the signal is less prone to extracerebral contamination than HbO2 and HbB. Neither this study nor the previous one saw any changes in pathlength during any of these physiological challenges.

6.2 Volunteer Studies: Functional Activation

Functional activation in adults has been studied with fNIRS since 1993 (see Sec. 5.5). Visual stimulation of the occipital cortex provides a robust stimulation method. There have been a few studies in this area to evaluate transient changes in the cellular energy metabolism that occur during changes in neuronal activity. Heekeren et al. saw an oxCCO increase during functional activation with a broadband NIRS (Humboldt A system); they speculate that the cause of the increased oxCCO is not due to increase in oxygen, as it would imply that the tissue is in a low
PaO$_2$ state normally, but due to decreases in proton electrochemical gradient increasing the rate of electron flow. This phenomenon was also seen by the same group in later papers with the Humboldt B broadband system. Fourier analysis of the frequencies showed that oxCCO and HHb signals have the same frequency as the stimulation, whereas HbO$_2$ has other peaks, which can be due to other factors.

Significant heterogeneous oxCCO responses were seen in an anagram-solving working memory study using the UCL phos system. No group significant change in oxCCO was seen during the anagram-solving task, but there were significant increases and decreases seen in different subjects. In some subjects, a significant decrease in oxCCO was seen in the presence of a typical hemodynamic response (increase in HbO$_2$ and decrease in HHb). It is hard to predict or model the effect of functional activation on oxCCO; the authors suggested that the NADH oxidation and proton motive force can affect oxCCO without affecting hemodynamics, which may provide an explanation as to the heterogeneity of the oxCCO response despite a consistent homogeneous hemodynamic reaction. This would support the theory of Heereken et al., which suggests a decrease in supply of reducing equivalents (substrates, i.e., NADH) to the ETC that might alter oxCCO without affecting the hemoglobins.

Different depths give different oxCCO responses, which could be explained by spatial distribution of CCO, which is more highly concentrated in the brain than in the extracerebral tissue. A recent working-memory study with CYRIL at multiple source–detector distances showed that the increase in oxCCO observed in the longer channels was not present in the short separation channels, despite a typical hemodynamic response in all channels. The addition of the measurement of the brain-specific oxCCO signal in fNIRS studies could help identify false positives and negatives and solve the problem of surface contamination.

### 6.3 Clinical Studies

Monitoring cerebral dysfunction in a clinical setting is important both in cases where the brain is at risk of injury (e.g., cardiac surgery) or where brain injury has already occurred [e.g., traumatic brain injury (TBI)]. There have been an increasing number of clinical NIRS studies in adults for pathologies, such as obstructive sleep apnea (OSA), cardiac surgery and hypotension, as well as brain injury itself.

The first investigation of cerebral oxCCO with NIRS during surgery occurred in 1995 due to concerns regarding cerebral stress of cardiac surgery causing postoperative neuropsychological damage. A NIRO 500 study of 41 patients undergoing cardiac surgery with CPB found that patients who suffered from neurological defects after surgery had lower minimum oxCCO values during surgery compared to those without defects. The oxCCO and venous saturation were inversely correlated during CPB suggesting that an increase in oxygen saturation may not be representative of an increase in tissue oxygenation. Kikihana et al. using the Sapporo algorithm and instrument saw that their measurement of oxCCO was a good predictor of postoperative cerebral outcome in a group of 66 patients who underwent thoracic aortic surgery; the recovery fraction of broadband NIRS-measured oxCCO following the surgery was predictive of neurological outcome.

Further, in cardiac surgery investigators recorded changes in oxCCO with the NIRO 300 during implantation of subpectoral implantable cardioverter defibrillators under general anesthesia. A random change in both directions was seen in oxCCO (measured with the NIRO 300) during implantable cardioverter defibrillator testing, despite consistent decrease in HbO$_2$ and increase in HHb. This suggests that the availability of oxygen at mitochondrial level was not consistently affected by a short term lack of oxygen delivery and that mitochondrial function may be able to identify cerebral abnormalities in this group of patients that hemodynamic changes do not reveal. A very early study also came to this conclusion, in patients who were candidates for carotid endarterectomy, carotid artery compression tests were performed and a reduction of oxCCO was seen in some, but not all, patients. The authors suggest that the presence of a decrease in oxCCO is indicative of a more severe impairment of brain function.

A case study of an adult woman with a long-term neurological disorder examined the oxCCO response to seizures with the NIRO 500. Prior to seizure activity, a gradual increase in HbO$_2$ and HbT was observed with a simultaneous reduction in oxCCO. Throughout the seizure there was an increase in oxCCO, and HHb; seizures cause increased metabolic demand and the increase in oxCCO corroborates this.

To investigate the response to hyperoxia in the brain injury, eight patients with TBI undertook a hyperoxia challenge (60% and 100% FiO$_2$). The concentration of oxCCO measured with the UCLn algorithm increased during hyperoxia and correlated with brain tissue oxygen tension as measured by microdialysis; there was a negative correlation between oxCCO and lactate/pyruvate ratio which shows that the oxCCO NIRS changes are related to cellular metabolism. Another investigation of six patients with TBI showed a mean increase in oxCCO during hypcapnia, but not in all individuals (four increase, two decrease) despite increases in Vmca—this could be due to heterogeneity of TBI. This study did not find an association between oxCCO and the lactate/pyruvate ratio—the authors suggest that this may be due to more complex changes induced by CO$_2$, such as pH, nitric oxide (NO) and cerebral metabolic rate of oxygen (CMRO$_2$) changes.

Bed tilts were performed in patients with primary autonomic failure to assess cerebral oxCCO during hypotension measured with the NIRO 300. There was a range of NIRS-measured oxCCO responses during large decrease in HbD and HbT. In patients where a significant decrease in oxCCO was observed, there was a threshold in HbD and HbT reduction before it occurred. A similar threshold has been seen in piglet studies of anoxia and may indicate the point at which damage to the cells due to hypoxia and/or ischemia occurs.

Another population at risk of hypoxic injury is OSA patients and these have been studied with the NIRO 300. The first study looked at OSA patients during daytime naps with NIRS monitoring, polysomnography, and laser Doppler. Correlations were seen during spontaneous hypoxias between changes in oxCCO and changes in TOI, CBF, SpO$_2$, and MABP, there was a weak negative correlation between oxCCO and end-tidal CO$_2$. The oxCCO signal was in phase with TOI but not HbO$_2$ or HHb. These results suggest that OSA hypoxias cause anaerobic metabolism or CO$_2$ changes to reduce CCO. In another study of 62 sleep apnea patients, six had deep apneas (TOI drop of >10%) and oxCCO dropped for each apnea.

### 7 Discussion

NIRS-measured oxCCO has been studied in many clinical environments. For neonates, this has included term and preterm
infants, infants studied on neonatal intensive care units, and during cardiac surgery. The results from the neonatal cohort are not consistent, but that is largely due to the heterogeneity of the populations studied and differences between the protocols. An excellent example of the application of oxCCO in neonates is the indomethacin work, which has shown that the oxCCO signal can provide clinically important information.\textsuperscript{70,71,72} The most recent work on neonatal HIE has shown that the relationship between cerebral oxCCO and oxygenation is indicative of the level of brain injury, as measured by a \textsuperscript{31}P MRS biomarker of outcome,\textsuperscript{96} so oxCCO has potential clinical importance as a bedside marker of cerebral well-being.

The literature on adults also covers a wide range of clinical environments. The large study by Kakihana et al.\textsuperscript{130} is an excellent example of the potential of oxCCO to be a clinical tool that is predictive of neurological outcome after surgery. Further, there is a great potential for the oxCCO measurement to be used in fNIRS studies for a deeper understanding of oxygen metabolism during neurovascular coupling and for its brain specificity.\textsuperscript{12}

The work on adults also covers a portfolio of volunteer studies, which have been used to deepen our knowledge of the oxCCO signal in humans. The behavior of the oxidation state of CCO is not fully understood as there is no gold standard for the measurement in vivo. However, from the summary of the results found in this review, we are able to summarize the changes in its behavior from challenges performed on healthy adult volunteers. We are limited to the adult brain as it is not possible to obtain such controlled data on neonates. Quantification of a normal oxCCO response in a healthy brain will allow the identification of abnormal, and perhaps pathophysiological, cerebral responses. The generalized trends of the three NIRS chromophores in response to systemic changes are summarized in Fig. 8.

Interpretation of a change in the oxidation state of CCO is complex because it represents a change in equilibrium, which may reflect fluctuations in any of several factors and the balance between them including O\textsubscript{2} supply, glucose or other substrate supply, pH, temperature, and other ETC rates. Understanding the CCO signal is sometimes challenging as there are many different factors that can cause increased oxidation or reduction of the enzyme. This complexity can be dealt with by using computer modeling of the physiology.\textsuperscript{81}

The UCL group has developed a mathematical model of the physiology of brain metabolism and circulation to aid the interpretation of the measurable cerebral signals.\textsuperscript{38–40,134} The first version of the model was an elaborate representation of the adult brain\textsuperscript{134} and was extended to simulate changes in the NIRS variables, in particular, changes in oxCCO.\textsuperscript{35} The model has been used with NIRS-based adult studies,\textsuperscript{38} and after modification for the piglet brain,\textsuperscript{39} has been used in controlled preclinical studies of HIE.\textsuperscript{46}

\section{Future Directions}

Measuring a real-time, bedside marker of brain tissue metabolism is an active area of NIRS research. Many biomedical optics groups are measuring cerebral CMRO\textsubscript{2} with combined NIRS and diffuse correlation spectroscopy.\textsuperscript{135–138} Others are combining MRI and NIRS to measure CMRO\textsubscript{2},\textsuperscript{139} confirming the need for a noninvasive measurement of tissue metabolism. We see NIRS-measured oxCCO to be an alternative and/or complimentary measurement to these other monitors of cerebral metabolism.

NIR imaging or diffuse optical tomography is under continuous development.\textsuperscript{2} Recent work by Phan et al.\textsuperscript{105} demonstrates the first oxCCO images acquired using a multichannel broadband spectroscopy system during activation of the visual cortex (Fig. 9). This work has demonstrated differences between the localization of changes in oxCCO and the hemodynamic signals (HbO\textsubscript{2} and HHb). This difference could be due to the oxCCO signal originating directly from the brain tissue, while the hemoglobin signals result from changes in the surrounding vasculature. Topographical images with oxCCO in addition to HbO\textsubscript{2} and HHb will allow investigation of regional changes in cerebral oxygenation and oxygen consumption in the healthy and injured brain.

The most challenging but exciting foreseeable development for CCO would be an absolute measurement. To resolve changes in the oxidized concentration with respect to the reduced concentration of CCO would be revolutionary, as it would allow easier comparison between patients or volunteers, and give a better understanding of the state of tissue metabolism. The UCL group is working toward methods to do this, with both new hardware and algorithms. The group has recently developed a multiwavelength TR spectrometer to quantify absolute changes in absorption and scattering with a view to measuring absolute CCO redox changes.\textsuperscript{140,141}

In terms of the future of the instrumentation, we believe that the capacity to measure changes in light attenuation using many wavelengths is a key in separating the contribution of CCO from the total attenuation spectra. While our recent theoretical work suggests that measurements at a specific combination of eight wavelengths (between 780 and 900 nm) can accurately resolve oxCCO changes to 2\% difference when compared to a broadband (121 wavelengths) measurement,\textsuperscript{82} we have yet to develop and test an instrument that implements this solution. Instruments based on broadband spectrometers with the capacity to measure the changes in light attenuation at more than 100 wavelengths have been extremely successful in separating the CCO signal from the hemoglobins and are considered a key component to an optimal CCO instrument. Broadband spectrometer

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_8.png}
\caption{Physiological stimuli and predicted NIRS-measured hemoglobin oxygenation and oxCCO changes based on findings from this review for the healthy adult brain.}
\end{figure}
instrumentation has advanced significantly the last 30 years and we now have systems with enhanced light throughput and photon sensitivity in the NIR, with reduced noise and improved electronics for fast acquisition, so they are robust enough to be used in a clinical setting. In addition, through advances in laser instrumentation and the development of supercontinuum fiber laser sources, we now have the capacity to perform time-of-flight measurements of multiple wavelengths of our choice, allowing us to resolve tissue light absorption and scattering across the NIR spectra to quantify oxCCO.\textsuperscript{140–142} Ten years from now, we see NIRS instruments and cerebral oximeters enhanced with the added capacity to measure oxCCO through the utilization of an increased number of wavelengths.

9 Conclusion

In this review, we described the rationale, methods and analysis behind NIRS measures of oxCCO. We summarized the range of human brain studies (in healthy volunteers and patients) from those first performed by the Jöbsis group, as early as 1985, to the present day. We found 103 papers published on cerebral oxCCO NIRS measurements in humans, 65% of the studies are those first performed by the Jöbsis group, as early as 1985,\textsuperscript{1264–1267 (1977).} the neonatal brain observed by in vivo near-infrared spectroscopy, 20. Y. Hoshi and M. Tamura, \textit{Brain Res.} \textbf{102} (2), 173–83 (2013).


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