HIDEX

Application note
413-006

Determination of hemoglobin spectra in living red blood cells of fish

Introduction

Hemoglobin is the intracellular oxygen-carrying molecule in all vertebrates and it transports oxygen in the blood from the respiratory organs to the rest of the body. In vertebrates, hemoglobin (Hb) consists of four subunits: two α subunits and two β subunits, each with one heme group and a central iron ion. The iron is the site of oxygen binding. Oxygen binding and release induce substantial structural changes in the hemoglobin molecule which result in spectral changes of hemoglobin. The Hidex sense microplate reader together with the Hidex/Okolab gas controller unit was used to record the absorbance spectra of oxygenated and deoxygenated hemoglobin in living red blood cells of rainbow trout (*Oncorhynchus mykiss*).

Material and methods

Plate reader and gas control unit

The Hidex Sense will help your lab become more effective. The touch screen user interface makes the operation safe and comfortable. Straightforward application focused operation minimizes the time spent on instrument training, and is essential for superior results. ELISA, protein quantification and enzyme activity assays are measured with ultrafast full spectrum readout, using a high sensitivity spectrograph for absorbance detection.

The Sense environment control system consists of an external digital gas flow control unit connected with tubing to optional gas connectors at the rear panel of the Sense. The control unit is equipped with a touch screen to adjust settings and monitor the gas concentrations.

The unit controls CO_2 and O_2 concentration by mixing CO_2 and Nitrogen (N_2) (only when oxygen control is needed) continuously. The system works with a closed loop control mode. A vacuum pump takes gas from the enclosed Sense measurement chamber to regulate each gas flow. The composition is monitored inside the volume every 30 seconds.

Test procedure

Blood was collected from rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) via caudal puncture into falcon tubes containing sodium heparin (50 USP units per ml blood) and stored on ice. Red blood cells were washed three times in cold 1 x PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and finally resuspended at a hematocrit of 20%. For the measurements red blood cells were diluted 1:10 in 1 x PBS, and 1.5 ml of the dilution was pipetted into a clear 12-well plate without a lid.

Hypoxic conditions were provided by a gas controller unit (Hidex/Okolab) connected to the Hidex sense. Oxygen concentration was decreased from initially 18% down to 3% and increased again to 18%. Changes in set points were done manually.

Absorbance scans were recorded at room temperature from 350 nm to 700 nm. Furthermore spectral changes were recorded at a wavelength of 576 nm (window 5, 25 flashes), which corresponds to the absorbance of the α-band. Changes in absorbance spectra were followed hourly for a total of 7 hours, and the plate was shaken between scans with gentle orbital shaking to ensure homogenous gas conditions in the whole sample.

Results & discussion

The Hidex sense plate reader with the Hidex/Okolab gas controller was used to monitor absorbance changes of fish Hb in living red blood cells during exposure to hypoxia and during reoxygenation.

After lowering the new set point in the gas controller unit to 3% oxygen, the ambient oxygen in the chamber declined in a steep slope to 6.7% within the first half hour. Then the curve flattened until it reached its lowest level at 3.1% after 4 hours (Fig. 1).

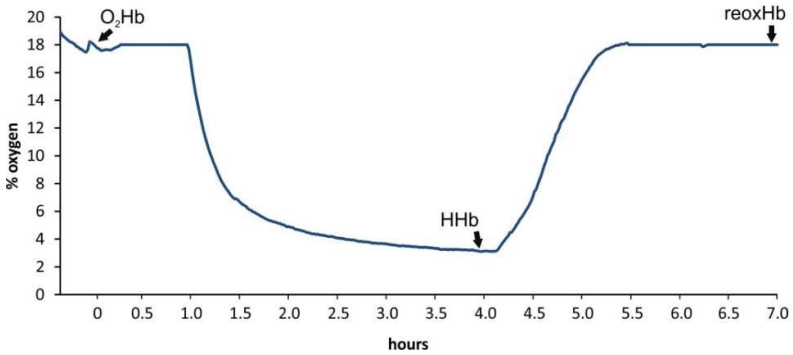

Figure 1. Oxygen profile created by the Hidex/Okolab gas controller unit during the spectrophotometric run. Arrow heads show the time points at which the representative absorbance spectra for oxyhemoglobin (O_2Hb; Figure 2), deoxyhemoglobin (HHb; Figure 2) and reoxygenated hemoglobin (reoxHb) were recorded.

The absorbance spectrum of fully oxygenated Hb in living red blood cells of rainbow trout shows three peaks in the range of 350-700 nm. The Soret band peaks at 416 nm, the β-band at 541, and the α-band at 576 nm. The Soret band peak in deoxygenated rainbow trout Hb is red shifted to 426 nm, while in reoxygenated Hb it is slightly blue shifted relative to the fully oxygenated Hb (Fig. 2A).

The α-band and β-band of the fully oxygenated Hb are replaced by one broad peak at 551 nm in the deoxygenated form, but they reappear during reoxygenation (Fig. 2B).

The shoulders present in the spectrum of the deoxygenated Hb could either indicate the presence of at least two Hb isoforms in rainbow trout red blood cells which differ in their oxygen affinity or the formation of metHb (Fig. 2).

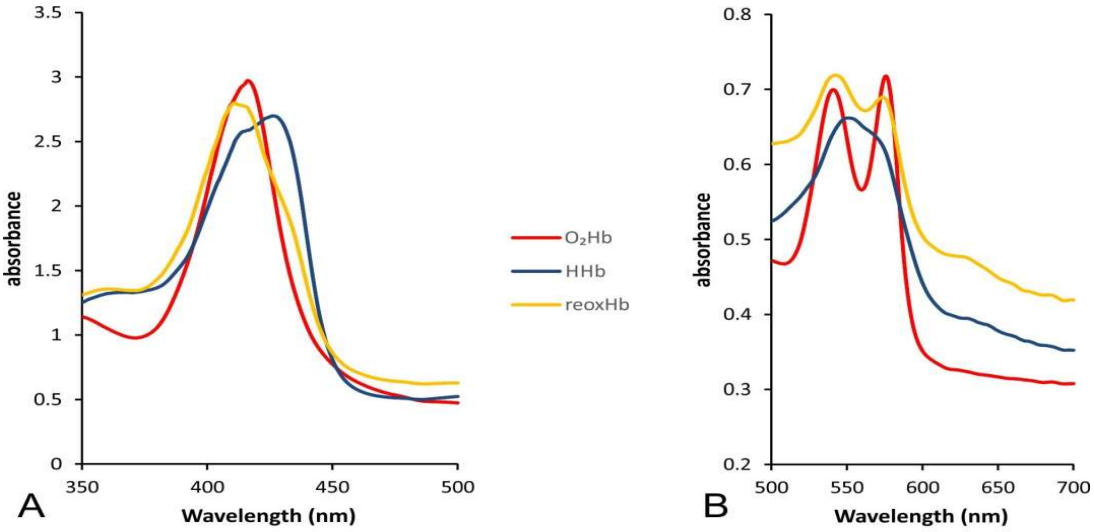

Figure 2. Absorbance spectrum of rainbow trout oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb) and reoxygenated hemoglobin (reoxHb) in living red blood cells. A, Soret band peaks. B, α-and β-band peaks.

By monitoring spectral changes at the specified wavelength of 576 nm, which represents the α-band, we were able to follow the transition from fully oxygenated Hb to deoxygenated Hb and back to oxygenated Hb more closely (Table 1).

Table 1. Changes in absorbance reads during the time course of the experiment at 576 nm representing the α-band peak.

abs. read 0 h oxyHb	abs. read 1 h	abs. read 2 h	abs. read 3 h	abs. read 4 h HHb	abs. read 5 h	abs. read 6 h	abs. read 7 h reoxHb
0.7177	0.7770	0.7379	0.6421	0.6143	0.6409	0.6716	0.6862

Conclusions

We demonstrate that it is possible to follow changes in the oxygenation status of hemoglobin in intact red blood cells. The absorbance spectra obtained are comparable to those published from lysed red blood cells or purified hemoglobin solutions from fish. By using the Hidex/Okolab gas controller unit we were able to manipulate the gas atmosphere in the chamber of the Hidex sense microplate reader to deoxygenate the hemoglobin molecule in red blood cells. The decrease and increase in O_2 occurred rapidly and was stable. The reader together with the gas controller thus has a wide range of application possibilities in different disciplines of biology.