Validation of a high-power, time-resolved, near-infrared spectroscopy system for measurement of superficial and deep muscle deoxygenation during exercise

Shunsaku Koga,1 Thomas J. Barstow,2 Dai Okushima,1 Ⓡ Harry B. Rossiter,3 Narihiko Kondo,4 Etsuko Ohmae,5 and David C. Poole2

1Applied Physiology Laboratory, Kobe Design University, Kobe, Japan; 2Departments of Anatomy and Physiology and Kinesiology, Kansas State University, Manhattan, Kansas; 3Rehabilitation Clinical Trials Center, Division of Respiratory & Critical Care Physiology & Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California; 4Kobe University, Kobe, Japan; and 5Hamamatsu Photonics K.K., Hamamatsu, Japan

Submitted 10 November 2014; accepted in final form 31 March 2015

Koga S, Barstow TJ, Okushima D, Rossiter HB, Kondo N, Ohmae E, Poole DC. Validation of a high-power, time-resolved, near-infrared spectroscopy system for measurement of superficial and deep muscle deoxygenation during exercise. J Appl Physiol 118: 1435–1442, 2015. — Near-infrared assessment of skeletal muscle is restricted to superficial tissues due to power limitations of spectroscopic systems. We reasoned that understanding of muscle deoxygenation may be improved by simultaneously interrogating deeper tissues. To achieve this, we modified a high-power (~8 mW), time-resolved, near-infrared spectroscopy system to increase depth penetration. Precision was first validated using a homogenous optical phantom over a range of inter-optode spacings (OS). Coefficients of variation from 10 measurements were minimal (0.5-1.9%) for absorption (μa), reduced scattering, simulated total hemoglobin, and simulated O2 saturation. Second, a dual-layer phantom was constructed to assess depth sensitivity, and the thickness of the superficial layer was varied. With a superficial layer thickness of 1, 2, 3, and 4 cm (μa = 0.149 cm⁻¹), the proportionnal contribution of the deep layer (μa = 0.250 cm⁻¹) to total μa was 80.1, 26.9, 3.7, and 0.0%, respectively (at 6-cm OS), validating penetration to ~3 cm. Implementation of an additional superficial phantom to simulate adipose tissue further reduced depth sensitivity. Finally, superficial and deep muscle spectroscopy was performed in six participants during heavy-intensity cycle exercise. Compared with the superficial rectus femoris, peak deoxygenation of the deep rectus femoris (including the superficial intermedius in some) was not significantly different (deoxyhemoglobin and deoxymyoglobin concentration: 81.3 ± 20.8 vs. 78.5 ± 13.6 μM, P > 0.05), but deoxygenation kinetics were significantly slower (mean response time: 37 ± 10 vs. 65 ± 9 s, P ≤ 0.05). These data validate a high-power, time-resolved, near-infrared spectroscopy system with large OS for measuring the deoxygenation of deep tissues and reveal temporal and spatial disparities in muscle deoxygenation responses to exercise.

to drive blood-to-muscle O2 flux. In this regard, investigation of near-infrared spectroscopy (NIRS)-derived muscle deoxygenation within and among muscles is crucial for resolving the mechanistic bases for vascular and metabolic control following the onset of exercise (e.g., Refs. 3, 10, 11, 15, 20, 25, 29, 35).

The dynamics of muscle microvascular deoxygenation are spatially heterogeneous within the quadriceps muscles in transient states where metabolic rate is changing rapidly (~1.5 cm depth; e.g., Refs. 4, 8, 23–25, 36). However, in humans, NIRS measurements of spatial heterogeneity have been limited to the relatively superficial regions of the quadriceps muscles (13, 23, 24). In rat muscle, the deoxygenation response kinetics (time constant) at the onset of contractions is slower in deep compared with superficial tissues, implying a better matching of the increase in QO2 to VO2 following the onset of electrically induced contractions (22). This response has been explained on the basis of deep muscle containing proportionally more slow-twitch (type I) muscle fibers with a greater sensitivity of vasodilatory control (2, 28). Goodwin et al. (14) used NIRS directly on the surface of canine hindlimb muscles to access both superficial and deep muscle deoxygenation during stimulated contractions. They showed that slowed blood flow speeded the rate of muscle deoxygenation during the transition from rest to steady state. This finding supports the expectation in humans that there will be faster deoxygenation responses in superficial muscle where the QO2/VO2 is lower than in deep muscle. However, while skeletal muscle fiber-type expression differs between superficial and deep muscles in humans (19), it remains unknown to what extent the amplitude and kinetics of deoxygenation of deeper muscle tissues differ from those of more superficial muscle tissues.

Magnetic resonance spectroscopy reveals that the activation and intramuscular phosphate metabolism of quadriceps muscles are widely heterogeneous during bipedal exercise (5, 34). Additionally, positron emission tomography suggests that perfusion in vastus intermedius (VI) muscle is greater than that of the lateralis during knee-extension exercise (17, 21, 26). Thus practical methods are needed to determine whether these regional variations in recruitment, perfusion, and metabolism are well matched in both deep and surficial tissues. Such knowledge may improve our understanding of factors contributing to development of limitations to skeletal muscle function during exercise. Reliance on deoxygenation sampling that is limited to superficial muscle, as in conventional NIRS studies, therefore stifles the ability to test mechanistic hypotheses regarding the recruitment and coupling, or otherwise, of QO2 and VO2 in humans and its role in limiting exercise performance.

http://www.jappl.org
8750-7587/15 Copyright © 2015 the American Physiological Society
1435
To address these questions, we used a high-power, time-resolved (TRS)-NIRS system to characterize the dynamic and spatial heterogeneity of superficial and deep quadriceps muscle microvascular deoxygenation during exercise. This development increases the output power delivered at the extremity of the NIRS irradiation optical fiber by ~30 times that of the previous TRS-NIRS system used in our laboratory (24), allowing deoxygenation of relatively deeper muscle tissues to be assessed. Herein we validate this system for precision (the variability between repeated measurements) and tissue depth penetration. We also demonstrate the application of high-power TRS-NIRS in the superficial and deep regions of rectus femoris (RF) and the superficial regions of the VI, to assess spatial heterogeneity of human muscle deoxygenation during high-intensity cycling exercise.

METHODS

Three separate experiments were conducted to validate the high-power TRS-NIRS developed for this study. First, TRS-NIRS measurement precision at different optical depths was assessed using a homogenous optical phantom. Second, the TRS-NIRS depth resolution was determined using dual- and triple-layer optical phantoms. Finally, the utility of the TRS-NIRS to measure superficial and deep tissue deoxygenation during high-intensity cycling exercise in humans was demonstrated.

Description of the TRS-NIRS systems. The principles of operation and algorithms utilized by the equipment have been described in detail elsewhere (31, 32). Briefly, a high-frequency light pulser emits light at three wavelengths, and a single-photon detector measures the light reflected from the material or tissues under the probe at 5 MHz. This detection-time curve is fitted to resolve the absorption coefficient ($\mu_a$), the reduced scattering coefficient ($\mu_s'$), and the mean path length (PL; calculated from the measured mean time of flight), by application of diffusion theory (31). Knowledge of these three variables allows measurement of absolute deoxygenation [tHb] and oxyhemoglobin and oxymyoglobin concentration ($\text{oxy[Hb-Mb]}$) in micromolar units, and their sum, total hemoglobin and myoglobin concentration ($\text{[Hb]}$) (16, 18). From these, tissue O$_2$ saturation (StO$_2$) may be calculated using $\text{oxy[Hb-Mb]/[tHb]}$.

Two TRS-NIRS systems were used for this study. A TRS-20 (Hamamatsu Photonics K.K.), previously used in our laboratory (24), was used to assess deoxygenation in superficial muscle tissues. The TRS-20 uses a picosecond light pulser that emits three wavelengths (759–762, 793–800, and 833–835 nm), with a repetition frequency of 5 MHz, and a full-width at half-maximum of 70–100 ps. The average power at each wavelength is ~200 $\mu$W at the light pulser and 100 $\mu$W at the extremity of the irradiation optical fiber. A time-correlated single photon counting board acquires the temporal profiles of detected photons passing through tissue. The laser diodes and photomultiplier tube are connected to lightweight plastic probes by optical fibers for single-photon detection.

A second high-power system was developed by modifying a TRS-NIRS device previously described for deep brain measurement (37) (TRS-20SD, Hamamatsu Photonics K.K.). This system emits three wavelengths of high-power light from nanosecond light pulsers at 761, 801, and 836 nm. A photomultiplier tube with a gallium arsenide type photocathode is used for single-photon counting, by a TRS circuit based on the time-correlated single-photon counting method. The instrument is attached to the material of interest by an irradiation optical fiber and a detection optical fiber with a numerical aperture of 0.29. Each nanosecond light pulser generates a light pulse with full-width at half-maximum of ~1.7–1.8 ns, at a pulse rate of 5 MHz, and an average power of ~7.5–8.0 mW at the light pulser and 3.3 mW at the extremity of the irradiation optical fiber. The irradiation bundle fiber was 3 mm in diameter to ensure that the maximum permissible exposure to the skin was not exceeded (22.4 mW, defined by the International Electrotechnical Commission). The system operator and the subjects wore laser safety glasses for eye protection.

The output frequency of both TRS systems was set to 0.5 Hz. Calibration of both instruments was performed before each test by measuring the response when the input and receiving fibers face each other through a neutral-density filter in a black tube.

High-power TRS-NIRS validation. Previous studies show that deoxygenation measured by TRS spectroscopy correlates with hemoglobin O$_2$ saturation in blood (18) and in a purified-hemoglobin phantom solution (16), which are considered to be the “gold standard.” In the present study, precision and depth sensitivity of the high-power TRS-NIRS system were validated using optical phantoms.

Solid optical phantoms (Biomimic, INO, Québec City, Quebec, Canada) were used to measure the precision and depth penetration of both TRS systems. The phantoms are made of hard polyurethane matrix, with titanium dioxide as scattering medium and carbon black as an absorbing medium. In the first experiment, a single homogenous phantom (thickness of 7 cm) was used to determine the influence of probe spacing (OS) on the precision of NIRS measurements. For this, inter-OS was set at 3, 4, 5, 6, and 7 cm in separate trials on the homogenous phantom with known optical characteristics ($\mu_a = 0.096$ cm$^{-1}$; $\mu_s' = 11.0$ cm$^{-1}$; each at 800 nm). PL, $\mu_a$, $\mu_s'$, simulated [tHb], and simulated StO$_2$ were measured over 10 different trials. Additionally, for comparison, the absorption, scattering, and PL characteristics of the homogenous phantom were measured using the low-power TRS-20 with OS at 3 cm.

In the second experiment, a series of dual- and triple-layer optical phantoms were constructed to investigate the depth-sensitivity of the TRS-20SD system. The values of $\mu_a$ and the $\mu_s'$ of the superficial layer were selected according to data of a previous study (30). The values of $\mu_a$ of the deeper layer were set to be greater than the superficial layer value, with $\mu_s'$ values targeted to be similar to the superficial layer. For the dual-layer optical phantoms, the $\mu_a$ of the superficial layer was 0.149 cm$^{-1}$, and the deep layer was 0.250 cm$^{-1}$ (each at 800 nm), while the $\mu_s'$ of the superficial layer was 7.7 cm$^{-1}$, and the deep layer was 8.0 cm$^{-1}$ (at 800 nm). The thickness of the superficial layer was varied among 1, 2, 3, and 4 cm (with the deep-layer thickness constant at 4 cm) (Fig. 1). The measured mean $\mu_a$ was used to calculate the fraction of the $\mu_a$ deriving from the deep-layer phantom at each superficial-layer thickness, i.e., depth sensitivity was calculated as follows: (measured $\mu_a - 0.149$)(0.250 – 0.149). OS of 3, 4, 5, 6, and 7 cm were used to determine the influence of OS on depth sensitivity. Subsequently, a third-layer optical phantom with low absorption characteristics was added to simulate the effect of adipose tissue on the NIRS measurement of superficial and deep muscles. The $\mu_a$ and $\mu_s'$ of this 0.5-cm adipose-layer phantom were 0.048 and 10.9 cm$^{-1}$ (each at 800 nm) (Fig. 1). Both with and without the additional adipose-layer phantom, we hypothesized that the greater the OS, the greater would be the percentage of the $\mu_a$ received from the deep layer.

Superficial and deep muscle deoxygenation during cycle ergometry. The absolute changes in muscle deoxygenation and oxygenation profiles during high-intensity cycle ergometry were measured in the quadriceps of the dominant leg of six healthy men (age 21 ± 3 yr, height 172 ± 3 cm, weight 62 ± 5 kg). Written, informed consent was obtained from each volunteer before his participation, as approved by the Institutional Review Board of Kobe Design University. All procedures complied with the latest revision of the Declaration of Helsinki and Belmont Report.
fixed to the skin with adhesive tape. This helped ensure that the position of the optodes was fixed and invariant during the exercise. For superficial muscle, the OS of 6 cm was used because the signal-to-noise ratio was better than that for 7 cm for the high temporal-frequency measurements required for kinetics analysis. The optodes were placed on the midbelly of the RF muscle.

At the end of the exercise test, pen marks were made on the skin to indicate the margins of the rubber NIRS optode holders so that adipose tissue thickness (ATT) and the depth of the RF and VI muscles could be measured subsequently. RF and VI thickness were measured at rest with the subject in an upright seated position using B-mode ultrasound (Logiq 400, GE-Yokogawa Medical Systems) (Table 1; Fig. 2). ATT was measured at the TRS-NIRS optode site of the RF muscles. To quantify the influence of ATT on dynamic changes in NIRS signals, we used the ATT correction method of Bowen et al. (4). This method was based on the NIRS signal being derived predominantly from the muscle and skin (assuming minimal signal from Hb and none from Mb in adipose tissue), such that the attenuation in the [tHb] signal should be proportional to the volume of adipose within the sample, represented by the ATT. Resting [tHb] was determined from a 2-min resting average in the upright seated position. Measured deoxy[Hb+Mb] and [tHb] values at each individual muscle site were corrected to a common ATT of 0 mm using the regression between [tHb] and ATT. This normalization process allowed absolute values of both deoxy[Hb+Mb] and [tHb] to be compared among subjects and muscle sites differing in ATT.

Participants were familiarized with exercise testing procedures. All exercise tests were performed in the upright position on an electronically braked cycle (75XL-III, Combi) with breath-by-breath alveolar gas exchange measured using a flowmeter and gas analyzer system (Aeromonitor AE-300S; Minato Medical Science, Osaka, Japan). Heart rate was measured beat by beat from a three-lead electrocardiogram. Initially, an incremental exercise test (20 W/min, constant pedal frequency at 60 rpm) was used to determine peak VO\(_2\) and the gas exchange threshold (GET) and assign a heavy-intensity work rate for the constant work rate exercise tests. A detailed description of the pulmonary gas exchange measurement in our laboratory has been published elsewhere (4, 8, 23, 24, 36). For each exercise session, subjects reported to the laboratory at least 2 h after their last meal. They were asked to avoid caffeine, alcohol, and strenuous exercise for 24 h before the test. The temperature and relative humidity of the laboratory were maintained at 22°C and 50–65%, respectively.

Constant work rates were calculated for each individual to require a VO\(_2\) equal to 50% of the difference (Δ) between the subject’s GET and peak VO\(_2\) (heavy intensity), i.e., a value of (GET + 0.5Δ) based on the VO\(_2\)/work rate relation with account taken of the lag in VO\(_2\) relative to the work rate during the ramp exercise. Actual percent difference was 47 ± 5%. Exercise was performed for 6 min and was preceded by 2 min of rest and 4 min of unloaded cycling. Subjects performed two exercise transitions with only one exercise transition performed on any single day. Additionally, to compare the optical parameters of the same interrogation sites between the two NIRS devices, separate measurements of the RF were made with an OS of 3 cm using both TRS-20 and TRS-20SD in four single exercise transitions.

**Kinetic analysis.** Individual responses of deoxy[Hb+Mb] during the baseline-to-exercise transitions were time-interpolated to 1-s intervals and averaged across each transition for each subject. Subsequently, the deoxy[Hb+Mb] data were fit from the exercise onset to 180 s with a monoeponential model (Eq. 1):

\[
deoxy[Hb+Mb](t) = \text{deoxy}[Hb+Mb]_b + A_p \cdot \left[1 - e^{-(t - \tau_p)/T_D}\right]
\]

where the subscripts b and p refer to baseline unloaded cycling and primary component, respectively; deoxy[Hb+Mb]\(_b\) is the unloaded exercise baseline value; \(A_p\) is the amplitude of the exponential term; \(\tau_p\) is the time constant; and TD represents the time delay. The TD and \(\tau_p\) of the deoxy[Hb+Mb] response were summed to provide an indication of the overall dynamics of muscle deoxygenation in the first 180 s of exercise [mean response time (MRT)]. Separately, values for deoxy[Hb+Mb], oxy[Hb+Mb], [Hb], and StO\(_2\) were measured from the mean of the last 60 s at baseline and the 20 s immediately before 180 s and 360 s during exercise.

**Statistics.** Data are presented as mean ± SD. Coefficient of variation [CV (%); 100·SD/mean of the measurement values] was used to assess measurement precision from 10 repeated phantom measurements, and intraclass correlation (ICC) was used to assess test-retest variability in the human study. A paired t-test was performed to evaluate significant differences for the superficial and deep RF muscle deoxygenation kinetics. Relationships between two variables were analyzed by Pearson product moment correlation or nonlinear regression analysis. Significance was accepted at \(P \leq 0.05\).

---

**Table 1. Adipose tissue thickness and muscle thickness of the rectus femoris and the vastus intermedius of the quadriceps**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>ATT</th>
<th>RF</th>
<th>ATT+RF</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>17.6</td>
<td>24.6</td>
<td>16.9</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
<td>22.4</td>
<td>27.5</td>
<td>16.5</td>
</tr>
<tr>
<td>3</td>
<td>6.9</td>
<td>23.5</td>
<td>30.4</td>
<td>19.1</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>24.9</td>
<td>29.9</td>
<td>14.1</td>
</tr>
<tr>
<td>5</td>
<td>7.5</td>
<td>24.6</td>
<td>32.1</td>
<td>18.4</td>
</tr>
<tr>
<td>6</td>
<td>8.3</td>
<td>17.8</td>
<td>26.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Mean</td>
<td>6.6</td>
<td>21.8</td>
<td>28.4</td>
<td>16.9</td>
</tr>
<tr>
<td>SD</td>
<td>1.3</td>
<td>3.3</td>
<td>2.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Values are in mm. ATT, adipose tissue thickness; RF, rectus femoris; VI, vastus intermedius.
Innovative Methodology

Deep and Superficial Muscle Deoxygenation during Exercise • Koga S et al.

RESULTS

High-power TRS-NIRS precision and accuracy. Using a homogeneous phantom and the high-power TRS-20SD NIRS, the mean PL became longer as OS increased at each wavelength (Table 2). There was a linear relationship between OS and PL across the optode separation distances investigated. The \( \mu_a \) and \( \mu_s' \) were relatively unaffected by OS, such that simulated muscle \([tHb]\) and \(\text{StiO}_2\) varied little around the mean (37 \(\mu\)M and 59\%, respectively) (Table 2). The \( \mu_a \) at 801 nm was essentially identical to the phantom (0.097 vs. 0.096 cm\(^{-1}\)), and \( \mu_s' \) was slightly but systematically less than the phantom (10.4 vs. 11.0 cm\(^{-1}\)), demonstrating \( \mu_a \) accuracy. The precision was also high, with the CV of 10 measurements being narrow: \( \mu_a, 0.5–1.3\%; \mu_s', 0.5–1.1\% \); simulated \([tHb]\), 0.5–0.7\%; simulated \(\text{StiO}_2\), 1.1–1.9\%.

The characteristics of the homogeneous phantom interrogated by the low-power TRS-20 and high-power TRS-20SD at 3-cm OS were also compared. Overall the two devices showed similar values for PL and absorption (Table 2). The \( \mu_s' \) measured by the TRS-20 tended to be slightly closer to the phantom value than the TRS-20SD, and simulated \(\text{StiO}_2\) measured by the TRS-20 tended to be slightly less than the TRS-20SD (by ~4\%), because the wavelengths were slightly different for the two TRS systems.

The TRS-20 showed high precision. The CV of 10 measurements were as follows: \( \mu_a, 0.5–0.6\%; \mu_s', 0.3–0.4\% \); simulated \([tHb]\), 0.3\%; simulated \(\text{StiO}_2\), 1.0\%.

Table 2. Optical parameters of a homogeneous phantom with different optode spacings measured using low- and high-power time-resolved near-infrared spectroscopy

<table>
<thead>
<tr>
<th>NIRS Power</th>
<th>NIRS Unit</th>
<th>Optode Spacing Distance</th>
<th>Path Length, cm</th>
<th>Absorption coefficient, cm(^{-1})</th>
<th>Reduced Scattering Coefficient, cm(^{-1})</th>
<th>Simulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>761 nm</td>
<td>801 nm</td>
<td>836 nm</td>
<td>761 nm</td>
</tr>
<tr>
<td>High</td>
<td>TRS-20SD</td>
<td>3 cm</td>
<td>22.3</td>
<td>22.7</td>
<td>22.7</td>
<td>0.094</td>
</tr>
<tr>
<td>Low</td>
<td>TRS-20</td>
<td>3 cm</td>
<td>(22.6)</td>
<td>(23.1)</td>
<td>(23.1)</td>
<td>(0.106)</td>
</tr>
<tr>
<td>High</td>
<td>TRS-20SD</td>
<td>4 cm</td>
<td>30.5</td>
<td>31.1</td>
<td>31.1</td>
<td>0.103</td>
</tr>
<tr>
<td>High</td>
<td>TRS-20SD</td>
<td>5 cm</td>
<td>39.2</td>
<td>40.2</td>
<td>40.1</td>
<td>0.104</td>
</tr>
<tr>
<td>High</td>
<td>TRS-20SD</td>
<td>6 cm</td>
<td>48.1</td>
<td>49.1</td>
<td>49.2</td>
<td>0.104</td>
</tr>
<tr>
<td>High</td>
<td>TRS-20SD</td>
<td>7 cm</td>
<td>56.8</td>
<td>58.0</td>
<td>58.2</td>
<td>0.104</td>
</tr>
</tbody>
</table>

NIRS, near-infrared spectroscopy; TRS, time-resolved system; total \([Hb + Mb]\), total hemoglobin and myoglobin concentration; \(\text{StiO}_2\), tissue O\(_2\) saturation. TRS-20 (759 nm, 793 nm, 834 nm) measurements are shown in parentheses for comparison with TRS-20SD at 3-cm optode spacing.

Depth sensitivity of high-power TRS-NIRS. The depth sensitivity of the high-power TRS-NIRS system using a series of dual and triple-layer phantoms is shown in Figs. 3 and 4. Figure 3A shows the influence of the deep phantom layer on the absorption over the range of OSs used (OS = 3–7 cm). Using the widest OS (7 cm), which is expected to return light from the deepest region of the phantom (i.e., PL is greatest; Table 2), the influence of the deep-layer phantom could be detected at depths up to 3 cm. With a superficial-layer thickness of 1, 2, 3, and 4 cm, the proportional contribution of the deep-layer to total \( \mu_s \) was 80.1, 26.9, 3.7, and 0.0\%, respectively (at 6-cm OS; Fig. 4A). Thus, using a superficial-layer thickness of 1 cm, the influence of the superficial layer is minimal. With 3- or 6-cm OS, the deep layer provides the dominant variable determining the absorption. At superficial-layer thicknesses of 3 and 4 cm, there is little effect of OS on the contribution of the deeper layer, because essentially all of the \( \mu_s \) derives from the superficial layer.

To simulate the effect of adipose tissue, a third layer was added on top of the dual-layer phantom (Figs. 3B and 4B). The addition of an adipose-layer phantom reduced the depth sensitivity of the high-power NIRS. With a 0.5-cm adipose phantom and a superficial muscle-layer phantom of 1, 2, 3, and 4 cm, the relative contribution of the deep-layer phantom to the measured \( \mu_s \) was 34.9, 12.2, 4.1, and 3.4\% (at 6-cm OS; Fig. 4B).

The confidence intervals of the \( \mu_s \) values were calculated as an estimate of the signal-to-noise characteristics of the mea-
Superficial and deep muscle deoxygenation kinetics during heavy-intensity exercise. The sum of the ATT and the RF thickness was 28.4 mm, on average (Table 1). An example of deoxy[Hb+Mb] response to heavy-intensity exercise in the superficial and deep RF is shown in Fig. 5. In all subjects, the MRT for deoxy[Hb+Mb] kinetics in the deep muscle tissue was significantly slower compared with that of superficial muscle tissues (MRT, 37 ± 10 s vs. 65 ± 9 s, P = 0.05) (Table 3). Deoxy[Hb+Mb], oxy[Hb+Mb], and [tHb] at the baseline, 180 s, and 360 s were not different between superficial and deep muscle regions, except StO2 values at 360 s (Table 4). The ICC of test-retest deoxy[Hb+Mb] measurements was 0.670 at baseline and 0.624 at end-exercise.

There was no significant difference in the deoxy[Hb+Mb] response when measured by TRS-20 or TRS-20SD in the same superficial RF muscle region (OS = 3 cm, N = 4): low- vs. high-power TRS; baseline, 59.3 ± 3.9 vs. 58.7 ± 8.5 μM; end-exercise amplitude, 85.8 ± 18.9 vs. 82.3 ± 12.3 μM. This supports that the increased power of emitted light does not have an effect on tissue perfusion or deoxygenation.

DISCUSSION

To our knowledge, this is the first investigation to validate a high-power TRS-NIRS (type 20SD), which, when paired with the lower power TRS-NIRS (type 20), is capable of quantifying microvascular deoxygenation simultaneously in both superficial and deeper quadriceps muscles. Overall, high-power TRS-NIRS showed high technical precision (0.5–1.9%) and was able to detect differences in optical properties up to 3 cm deep in a phantom. The depth-sensitivity of the high-power TRS-NIRS was, therefore, greater than that of most commercial NIRS systems. This system revealed profound differences in muscle deoxy[Hb+Mb] kinetics following the onset of heavy-intensity exercise in superficial and deep RF (or superficial VI in some participants). These data suggest that high-power TRS-NIRS is precise and may be of benefit in resolving the mechanistic bases of regional vascular and metabolic control in skeletal muscle during exercise.

Without adipose layer phantom

Fig. 3. Change in μa as a function of the thickness of the superficial layer of a dual layer phantom without (A) and with (B) an additional superficial 0.5-cm adipose layer phantom. The μa of the superficial layer is 0.149 cm⁻¹, and μa of the deep layer is 0.250 cm⁻¹. At 4-cm superficial layer thickness, there is essentially no influence of the greater absorption of the deeper layer. Increasing OS reveals the influence of the deeper layer is detectable at 3-cm depth penetration. Note: the 0.5-cm adipose phantom is not included in the thickness measurement of the superficial phantom in B.

With adipose layer phantom

Fig. 4. The proportional contribution of the deep layer absorption (μa) to total absorption without (A) and with (B) an additional 0.5-cm superficial adipose layer phantom at 3 and 6 cm OSs. Note: the 0.5-cm adipose phantom is not included in the thickness measurement of the superficial phantom in B.
High-power TRS-NIRS precision. TRS-NIRS instruments have the advantage of improving the depth sensitivity by exploiting the temporal information of photon migration through tissues (see Ref. 12). The depth sensitivity of the NIRS signal depends on many factors, including the inter-OS, source power, detector sensitivity, and optical properties of the skin, adipose, and muscle tissues (13). To test whether the new high-power TRS-NIRS system could provide accurate and valid estimates of superficial and deep tissues, we first investigated the system precision using a homogenous optical phantom over a wide range of OS. The mean PL became longer as OS increased at each wave length, consistent with detection of light from deeper within the optical medium at a wide OS. The relatively constant values of $\mu'_s$, $\mu_a$, simulated muscle [Hb], and StiO$_2$ among the different OS investigated, and the narrow CV, validate the technical precision of the TRS-NIRS instrument for use in biological investigation of superficial and deep tissues.

Depth sensitivity of high-power TRS-NIRS. The depth penetration of NIRS light has been estimated as approximately one-half of the distance between the emitter and the receiver optodes (7). However, it should be noted that the PL (and, therefore, depth penetration) estimated in this fashion represents an average of an infinite number of potential PL traveled by the photons as they transit from source to detector. Thus, in the present study, some of the signal at wider OS likely includes measurements from both deep and superficial regions, as photons must travel through superficial layers to reach deeper tissues (Figs. 3 and 4). Thus, using this approach, it is not possible to accurately subtract out the influence of the superficial layer, to extract information solely from the deeper tissues. Nevertheless, this technology affords the heretofore unavailable opportunity to assess the influence of deeper regions of biological tissues to the deoxygenation response during exercise.

With this in mind, the phantom experiments validate that a greater proportion of the $\mu_a$ derives from deeper tissues using an OS of 6 cm compared with 3 cm. However, we found that an OS of 7 cm reduced the signal-to-noise ratio for high-frequency measurements, i.e., such as those needed to measure kinetic responses during exercise. We assumed that the proportional depth sensitivity established in phantom studies could be reasonably extrapolated to human tissue and, therefore, selected OS values of 3 and 6 cm to interrogate superficial and deep muscles, respectively, in the physiological studies during exercise.

It is recognized that the dynamic optical profile of exercising muscles (continuously changing from baseline to end-exercise) represents different optical conditions from those in a static phantom, such that the precise depth sensitivity will likely be different in the phantom from those in vivo. Our phantom studies demonstrate that the absorption characteristics are altered when three layers are used (analogous to adipose, superficial and deep muscle tissues).

Table 3. Kinetic characteristics of the initial deoxy[Hb+Mb] response following the onset of heavy exercise in the superficial and deep RF

<table>
<thead>
<tr>
<th></th>
<th>Superficial RF</th>
<th>Deep RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above GET (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline, $\mu$M</td>
<td>54.5 ± 4.7</td>
<td>54.9 ± 8.8</td>
</tr>
<tr>
<td>Amplitude of primary component, $\mu$M</td>
<td>21.4 ± 17.6</td>
<td>22.3 ± 16.3</td>
</tr>
<tr>
<td>Time delay, s</td>
<td>8 ± 5</td>
<td>10 ± 7</td>
</tr>
<tr>
<td>Time constant, s</td>
<td>30 ± 8</td>
<td>55 ± 26*</td>
</tr>
<tr>
<td>Mean response time, s</td>
<td>37 ± 10</td>
<td>65 ± 9*</td>
</tr>
<tr>
<td>Coefficient of determination: $r^2$</td>
<td>0.907</td>
<td>0.824</td>
</tr>
</tbody>
</table>

Values are mean ±SD. Deoxy[Hb+Mb], deoxyhemoglobin and deoxymyoglobin concentration; GET, gas exchange threshold. *Superficial vs. deep, $P \leq 0.05$. 

Table 4. Absolute values of deoxy[Hb+Mb], oxy[Hb+Mb], total[Hb+Mb], and StiO$_2$ in the superficial and deep RF at baseline and 3 min and 6 min of heavy exercise

|                      | Superficial RF | Deep RF |  |
|----------------------|---------------|---------|
| Oxy[Hb+Mb]           |               |         |
| Baseline             | 137.9 ± 20.7  | 149.0 ± 11.5 |
| 180 s                | 117.6 ± 16.6  | 131.3 ± 4.2 |
| 360 s                | 120.1 ± 17.4  | 135.0 ± 10.3 |
| Deoxy[Hb+Mb]         |               |         |
| Baseline             | 54.5 ± 4.7    | 54.9 ± 8.8 |
| 180 s                | 76.9 ± 23.0   | 73.8 ± 11.7 |
| 360 s                | 81.3 ± 20.8   | 78.3 ± 13.6 |
| Total[Hb+Mb]         |               |         |
| Baseline             | 192.4 ± 24.9  | 203.9 ± 6.2 |
| 180 s                | 194.5 ± 34.1  | 205.1 ± 10.6 |
| 360 s                | 201.4 ± 30.1  | 213.3 ± 10.6 |
| StiO$_2$             |               |         |
| Baseline             | 71.5 ± 1.8    | 73.2 ± 4.3 |
| 180 s                | 61.0 ± 6.0    | 64.1 ± 3.9 |
| 360 s                | 60.0 ± 6.0    | 63.4 ± 5.3* |

Values are mean ±SD in $\mu$M. Oxy[Hb+Mb], oxyhemoglobin and oxymyoglobin concentration. *Superficial vs. deep, $P < 0.05$. 

J Appl Physiol • doi:10.1152/japplphysiol.01003.2014 • www.jappl.org
consequence of greater $\dot{Q}_{\text{O}_2}/V_{\dot{O}_2}$ in slow-twitch muscle, which

In addition, the relative absorption of the different phantom layers will influence the depth sensitivity. Were greater absorbing layers to be more proximal to the NIRS probes, then the depth penetration would be expected to diminish. For this reason, we selected the characteristics of the adipose, superficial, and deep phantoms to be a model of the human lower limb. In human quadriceps, the fiber-type population and relative blood flow varies between superficial (more type II, less perfusion) and deep muscle (more type I, greater perfusion) (17, 19, 25). Thus, using a very low absorption in the adipose phantom, greater absorption in superficial phantom, and the greatest in deeper phantom should be representative of the anatomy sampled by the NIRS in vivo and, therefore, provide physiologically relevant depth-sensitivity estimates.

**Superficial and deep muscle deoxygenation kinetics during heavy-intensity exercise.** The depth sensitivity of the high-power TRS-NIRS in the present study (up to $\sim$3 cm) enabled detection of the influence of the deep RF and in some subjects the superficial region of the VI. The ICC of test-retest variability of high-power deoxy[Hb+Mb] measurement were similar to previous reports for low-power NIRS (6). The test-retest reproducibility was moderate, emphasizing the necessity for repeated measurements in human studies to improve the signal-to-noise ratio for kinetic parameterization (38). The kinetics of muscle deoxygenation were markedly slower in deeper muscle compared with superficial (Fig. 5), despite similar steady-state responses. This suggests that the $Q_{\text{O}_2}/V_{\text{O}_2}$ of the deep muscles remained greater for a longer period following the onset of constant high-intensity exercise. The difference in kinetic control matching of $Q_{\text{O}_2}$ to $V_{\text{O}_2}$ during the transient, depending on the depth of the tissue within the RF and VI muscles, reinforces the utility and sensitivity of kinetic analysis to detect differences in control features of physiological responses, even when differences in steady state may or may not exist.

**Mechanistic bases for the temporal profile of $Q_{\text{O}_2}/V_{\text{O}_2}$ in superficial and deep muscle.** The significantly slower deoxygenation responses observed in the deeper muscle could be explained by deeper regions containing proportionally more slow- (type I) than fast-twitch (type II) muscle fibers. Behnke et al. (2) and McDonough et al. (28) demonstrated blunted responses of microvascular partial pressure of $O_2$ ($P_{\text{mvO}_2}$) in rat muscles composed of more slow-twitch fibers, i.e., slower $P_{\text{mvO}_2}$ kinetics and reduced difference in $P_{\text{mvO}_2}$. This was a consequence of greater $Q_{\text{O}_2}/V_{\text{O}_2}$ in slow-twitch muscle, which is consistent with the greater sensitivity of vasodilatory control (27) and reduced sympathetic vasoconstriction (1) in these muscles. In human quadriceps, the fiber-type population varies between superficial (more fast twitch) and deep muscle fibers (more slow twitch), although these distributions are not as distinct (with respect to containing predominantly oxidative or glycolytic fibers), as seen among different muscles in animal preparations. The consequence of this distinction between rat and human thigh muscles may be that, in humans, the difference among the kinetic responses as a function of depth may be more pronounced than any differences in the steady-state response. Confirmation of these findings must await systematic evaluation in future work. Thus high-power TRS-NIRS of superficial and deep muscle provides the opportunity to investigate how these differences in fiber type may manifest as different deoxygenation responses to exercise. While high-power deep tissue TRS-NIRS aggregates deep and superficial responses and thus provides an overall response that is intermediate between the two fiber populations, the preliminary data in this validation study suggest that deeper muscles may deoxygenate at a profoundly slower rate than previously thought, based on superficial NIRS measurements. Blood flow in humans is often greatest in the VI compared with the other three superficial muscles of *m. quadriceps femoris*, at least during moderate exercise (17). This muscle is located deep within the thigh nearest the femur and thus contributes primarily to resist gravity and assist in body posture maintenance. Moreover, even at heavy and severe exercise intensities when a far greater spectrum of fibers is recruited, blood flow remains far greater in the deeper muscle tissues compared with superficial, at least in the rat (9, 33). Thus this study emphasizes that spatial heterogeneity of responses for both surface and deep muscles should be accounted for to better understand muscle regional contributions to overall metabolic and vascular responses to exercise. High-power TRS-NIRS may provide a method to unlock these heterogeneous responses during exercise in humans.

**Conclusions.** The high-power TRS-NIRS system presented herein permits evaluation of the dynamic and spatial heterogeneity of quadriceps microvascular deoxygenation simultaneously for both superficial and deep tissues following the onset of heavy-intensity cycling exercise. Moreover, it offers a technique for investigating muscle microvascular oxygenation and deoxygenation in subjects who have a thicker overlying adipose tissue layer. Precision and depth sensitivity of the high-power TRS-NIRS system was validated using optical phantoms chosen to mimic adipose tissue and superficial and deep muscles in humans. This demonstrated high instrumental precision (0.5–1.9% CV) and an effective mean depth penetration of $\sim$3 cm. The primary component kinetics of deoxy[Hb+Mb] in deeper muscles (deep RF and superficial VI) were significantly slower compared with the superficial RF. These results suggest that deep muscle has a greater $Q_{\text{O}_2}/V_{\text{O}_2}$, which reduces the likelihood that $Q_{\text{O}_2}$ may limit $V_{\text{O}_2}$ kinetics compared with superficial muscle i.e., deep muscles may reside to the right of the $Q_{\text{O}_2}$ “tipping point” (25). These data validate a high-power TRS-NIRS system for measuring the deoxygenation of deep muscle in humans and reveal spatial disparities in muscle deoxygenation responses to exercise.

**ACKNOWLEDGMENTS**

The authors thank Motoki Oda (Hamamatsu Photonics K.K.) for construction and validation of the high-power TRS-NIRS system.

**GRANTS**

S. Koga was supported by grants from Japan Society for the Promotion of Science (KAKENH- 24247046, 265650362), H. B. Rossiter and S. Koga were supported by a UK-Japan Partnering Award from Biotechnology and Biological Sciences Research Council, UK (BB/I024798/1).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


REFERENCES