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NIR spectroscopic imaging to map hemoglobin + myoglobin oxygenation, their concentration and optical pathlength across a beating pig heart during surgery

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The purpose of this paper is to demonstrate that near-infrared (NIR) spectroscopic imaging can provide spatial distribution (maps) of the absolute concentration of hemoglobin + myoglobin, oxygen saturation parameter and optical pathlength, reporting on the biochemical-physiological status of a beating heart *in vivo*. The method is based on processing the NIR spectroscopic images employing a first-derivative approach. Blood-pressure-controlled gating compensated the effect of heart motion on the imaging. All the maps are available simultaneously and noninvasively at a spatial resolution in the submillimeter range and can be obtained in a couple of minutes. The equipment has no mechanical contact with the tissue, thereby leaving the heart unaffected during the measurement.

A map of the oxygen saturation parameter (OSP) distribution across the heart surface.

1. Introduction

The mapping of hemoglobin (Hb) + myoglobin (Mb) oxygenation in pig hearts *in vivo* has been reported using a near-infrared (NIR) spectroscopic

imaging technique [1–7]. Although the maps of the oxygen saturation parameter (OSP) have been determined on a limited scale of 50 through 100%, they have been shown to be useful in cardiac research for the *ex vivo* and *in vivo* assessment of

myocardial oxygenation or regional ischemia in the subepicardium of a beating heart.

The method did not provide absolute concentrations of (Hb + Mb) in cardiac tissue, while for cardiac surgeries its determination in the beating heart *in vivo* would be important. For example, monitoring the Hb concentration [Hb] in the blood every hour during a cardiopulmonary bypass (CPB) surgery was recommended, and a value of hematocrit connected with [Hb] and indicative of oxygen delivery is important for the estimation of blood viscosity during hypothermic CPB [8]. Arterial and venous [Hb] were used to represent critical and actual oxygen delivery parameters in cardiac surgery and to identify a "hemoglobin reserve" [9]. It was found that [Hb] inversely relates to a degree of acute anemia and can indicate a tolerance to anemia in CPB surgery [10]. Changes in [Hb] could be used to reasonably initiate a transfusion during CPB [11].

Blood biochemistry determines arterial and venous oxygenated and deoxygenated Hb (HbO₂ and deoHb, respectively). In cardiac tissue, blood oxygenation is a weighed sum of arterial and venous HbO₂, which depends on the arterial and venous capillary volumes. Total Hb content in the tissue directly depends on the blood volume.

For intraoperative estimation of [Hb], conductivity, photometric and centrifugation techniques have been compared [12]. It was recommended [13] that at the point of care during CPB the use of hematocrit based on [Hb] values is critical. All the tests report on oxygenation and [Hb] in the blood, while in the heart tissue they may differ from the whole blood values. The biochemical blood-assay results may not be informative when these parameters are estimated in the living cardiac tissue *in situ*, for example, regarding the coronary flow heterogeneity [14].

The optical pathlength is a parameter quantifying a light pathway between the entrance and the exit of the tissue. It depends on both (Hb + Mb) concentration and oxygenation and can, in general, report on structural features of the tissue (see Refs. [15–19], for example). If the light propagates inside the epicardium and subepicardium, then OSP, [Hb + Mb] and the optical pathlength correspond to these layers of the heart. The determination of optical pathlength that is currently used was developed for diffuse reflectance spectroscopy at a single point on cardiac or other tissues [20–23]. For the diffuse reflectance-based NIR spectroscopic imaging of biological tissues the optical-pathlength issue has not been developed in detail.

Recently, the method has been reported to determine maps of the absolute concentration of Mb, its oxygenation and optical pathlength in buffer-perfused arrested pig heart with a 0.4 mm spatial resolution, rapidly, noninvasively and without mechanical

contact with the object [24]. The mapping is the result of the NIR spectroscopic imaging and employing a first-derivative approach of the pseudo-optical density spectrum. Originally, this approach was developed for a single-point spectroscopy of the NIR light diffuse reflectance from cardiac tissues [20, 21].

The present paper addresses application of the method to blood-perfused beating living hearts, showing how it can be practically realized in a large animal *in vivo* during cardiac surgery. The method provides the image acquisition and mapping noninvasively, in several minutes, with high spatial resolution. The quantitative distribution of the absolute (Hb + Mb) concentration and optical pathlength in the epicardium and subepicardium of beating pig heart during cardiac surgery is presented for the first time. The OSP mapping scaled from zero to 100%. The spatial resolution allows one to distinguish features in the submillimeter range. Such mapping of the OSP and oxygenated and deoxygenated (Hb + Mb) concentrations can bring new opportunities for a cardiac surgeon to monitor these parameters across the heart surface visually and quantitatively in a minute time range.

2 Materials and methods

2.1 Theoretical

The theoretical background has been developed in detail elsewhere [20, 21, 24]. Below it is described in brief.

The diffuse reflectance spectra $I_{xy}(\lambda)$ (heart surface) and $I_{0xy}(\lambda)$ (reference) at each pixel (x, y) were converted to pseudo-optical density (POD) spectral images $P_{xy}(\lambda)$:

$$P_{xy}(\lambda) = -\log_{10} \frac{I_{xy}(\lambda)}{I_{0xy}(\lambda)}. \quad (1)$$

White polyurethane sponge was employed as a reference (see Ref. [22, 25]). Figure 1 shows five images $P_{xy}(\lambda)$ at selected wavelengths.

According to a modified Lambert–Beer law, the POD spectrum $P_{xy}(\lambda)$ at each pixel (x, y) is:

$$P_{xy}(\lambda) = [\text{HbO}_2 + \text{MbO}_2]_{xy} \epsilon_{\text{HbO}_2}(\lambda) L_{xy} + [\text{deoHb} + \text{deoMb}]_{xy} \epsilon_{\text{deoHb}}(\lambda) L_{xy} + \mu_w(\lambda)_{xy} L + s_{xy}(\lambda) + a_o, \quad (2)$$

where $\mu_w(\lambda)$ is the light absorption of water per unit of optical pathlength. $[\text{HbO}_2 + \text{MbO}_2]_{xy}$ and $[\text{deoHb} + \text{deoMb}]_{xy}$ are concentrations of the hemes of oxygenated and deoxygenated (Hb + Mb), respectively, at each pixel. Allowing that the Hb and

Mb light absorption spectra are not distinguishable, the same extinction coefficients $\epsilon_{\text{HbO}_2}(\lambda)$ and $\epsilon_{\text{deOHb}}(\lambda)$ were applied for both of them. $\epsilon_{\text{HbO}_2}(\lambda)$ and $\epsilon_{\text{deOHb}}(\lambda)$ and the water absorption coefficient spectrum $\mu_w(\lambda)$ were taken from Refs. [26–29]. The Hb spectra per heme were obtained by dividing intensities of these spectra by four (number of hemes in the tetrameric Hb molecule). The parameter L_{xy} can be considered as an averaged optical pathlength at each pixel (x, y) . Although it is, in general, wavelength dependent, in a short range of wavelengths it was assumed to be relatively wavelength independent in the NIR range as shown for muscles [30–32]. The offset a_0 reflects the sample-dependent structural inconsistency of the reference and the sample as well as a wavelength-independent contribution of the reference $\log_{10}(I_0)$. A light-scattering component $s_{xy}(\lambda)$ at each pixel (x, y) was described by a power function of wavelength approximated by a linear function as $s_{xy}(\lambda) = -\beta_{xy}\lambda$.

Differentiation of Eq. (2) results in the first-derivative spectrum of cardiac tissue at each pixel (x, y) in the NIR:

$$\begin{aligned} P'_{xy}(\lambda) = & [\text{HbO}_2 + \text{MbO}_2]_{xy} \frac{d\epsilon_{\text{HbO}_2}}{d\lambda} L_{xy} \\ & + [\text{deOHb} + \text{deOMb}]_{xy} \frac{d\epsilon_{\text{deOHb}}}{d\lambda} L_{xy} \\ & + \frac{d\mu_w}{d\lambda} L_{xy} - \beta_{xy}. \end{aligned} \quad (3)$$

Equation (3) is a system of linear equations of rank 4 with regard to four unknowns $\xi_{1xy} = [\text{HbO}_2 + \text{MbO}_2]_{xy} \times L_{xy}$, $\xi_{2xy} = [\text{deOHb} + \text{deOMb}]_{xy} \times L_{xy}$, $\xi_{3xy} = L_{xy}$ and $\xi_{4xy} = \beta_{xy}$ for each pixel (x, y) . $P'_{xy}(\lambda)$ forms an n -component vector-column $\mathbf{P}_{xy}$. The matrix:

$$\mathbf{M} = (\epsilon'_{\text{HbO}_2}; \epsilon'_{\text{deOHb}}; \mu'_w; \mathbf{E}) \quad (4)$$

consists of three vector-columns ϵ'_{HbO_2} , ϵ'_{deOHb} and μ'_w of n elements representing the first derivatives $d\epsilon_{\text{HbO}_2}/d\lambda$, $d\epsilon_{\text{deOHb}}/d\lambda$ and $d\mu_w/d\lambda$ respectively, and a vector-column $\mathbf{E}$ consisted of n ones. Once the unknowns form a vector-column $\mathbf{X}_{xy} = \{\xi_{1xy}, \xi_{2xy}, \xi_{3xy}, \xi_{4xy}\}$, Eq. (3) in a matrix form is:

$$\mathbf{P}_{xy} = \mathbf{M} \otimes \mathbf{X}_{xy}, \quad (5)$$

where $\otimes$ denotes matrix multiplication. The solution gives all unknowns, from which the absolute concentrations for each pixel can be deduced easily as: $[\text{HbO}_2 + \text{MbO}_2]_{xy} = \xi_{1xy}/\xi_{3xy}$ and $[\text{deOHb} + \text{deOMb}]_{xy} = \xi_{2xy}/\xi_{3xy}$.

Equation (5) was solved using the MatLab function *lsqnonneg*, resulting in the best least squares solution $\mathbf{X}_{xy}$ for each pixel (x, y) with a constraint that all unknowns are positive. Based on this solution, the matrices $[\text{HbO}_2 + \text{MbO}_2]_{xy}$, $[\text{deOHb} + \text{deOMb}]_{xy}$, L_{xy} and β_{xy} were the maps of the spatial distribution

of the oxygenated and deoxygenated (Hb + Mb) concentrations, optical pathlength and light-scattering factor determined independently of each other. OSP was available at each pixel (x, y) as:

$$\text{OSP}_{xy} = \frac{[\text{HbO}_2 + \text{MbO}_2]_{xy}}{[\text{HbO}_2 + \text{MbO}_2]_{xy} + [\text{deOHb} + \text{deOMb}]_{xy}}. \quad (6)$$

2.2 Diffuse reflectance imaging

Three halogen lamps of 50 W with mirror reflectors homogeneously illuminated the pig heart *in vivo* from a distance of 25–30 cm above the pig heart surface. The reflectors concentrated the light beam in a 24° stereo angle.

Diffuse reflectance imaging was performed with a NIR system consisting of a Hamamatsu C7042 back-thinned FFT-charge coupled device (CCD) camera (Japan), Nikon AF Micro 60 mm f/2.8D lens and a Cambridge Research & Instrumentation, Inc. (Woburn, MA, USA) VariSpec SNIR/NNIR liquid-crystal tunable filter (LCTF). The lens aperture was 5.6–8 providing a significant depth of focus and reducing the chromatic aberration to 0.8–0.88 and vignetting to 0.1 (www.photozone.de). The system was assembled and operating software was developed at the NRC Institute for Biodiagnostics.

The camera allowed imaging with a resolution of 532×256 pixels and frame readout time of 536.8 ms in the 200–1100 nm wavelength range. The LCTF worked as an electronically activated tunable interference filter in the range of 650–1100 nm with a bandwidth of 10 nm and response time of 150 ms. The resulting spectral image is a 3D matrix $I_{xy}(\lambda)$ (two-dimensional xy -image of light intensity taken at n wavelengths λ). NIR imaging of beating hearts was made possible by capturing an image at each wavelength at the same point in the cardiac cycle by employing the acquisition gating as described below.

For each heart, two NIR spectroscopic images were acquired as replicates.

Absolute (Hb + Mb) concentration, OSP, optical pathlength and related calculations were performed with a program written using MatLab version 7.1 software (The MathWorks, Natick, MA).

2.3 Gating of image acquisition

A video clip *nongated_pig_heart.mpg* (see Supplement online) shows an example of real pig heart motion. To compensate for the heart motion, the image capture was triggered by a signal from a blood-pres-

sure monitor. The gating hardware monitored the cardiac cycle using the first derivative of the systemic blood pressure, which triggered an image acquisition with some delay from the moment when $dLVP/dt$ reduced to a plateau (end-systole). The delay provided the capture of the NIR image at a chosen wavelength at every second diastolic cardiac cycle phase. This corresponded to an acquisition time window of 50–100 ms (approx. 10–16% of the entire cycle of 600–670 ms) during early diastole. The pig heart motion after gating can be watched in a video clip gated_pod_pig_heart.mpg (see Supplement online), in which the intensity at every pixel is the POD determined by Eq. (1) (see below). The additional heart motion, which is resulting from the respiration-induced lung motion, was not compensated for.

In addition to triggering an image acquisition, the trigger also caused a 4 nm increment in the LCTF wavelength through the range of 650–1050 nm. LCTF-based spectral imaging principles have been discussed elsewhere [2, 33].

2.4 Statistics

The raw diffuse reflectance spectra $I_{xy}(\lambda)$ at each pixel (x, y) were smoothed employing a Savitzky–Golay approach [34] for a five-point linear function. The smoothed spectra $I_{xy}(\lambda)$ (sample) and $I_{0xy}(\lambda)$ (reference) were used to find the pseudo-optical density spectra $P_{xy}(\lambda)$ calculated by Eq. (1) as described in Section 2. Before numerical differentiation of $P_{xy}(\lambda)$, no additional smoothing was done.

To quantify a goodness of the fit for the $P_{xy}(\lambda)$ spectra averaged over a region of interest (ROI), a χ^2 -criterion [35, 36] was used for estimating the statistical significance of the difference between the $P_{ROI}(\lambda)$ and fit spectra. Following Meyer [35], the χ^2 -parameter was calculated as:

$$\chi^2 = \sum_{i=1}^n \frac{[y_i - f_i]^2}{\sigma_i^2}, \quad (7)$$

where y_i is a measured spectrum intensity, f_i is its fit value, σ_i is a standard deviation for y_i , where i refers to wavelength. In the spectral imaging studies, i changed from 1 to $n = 86$, which corresponds to the wavelength range from 700 to 1040 nm with an increment of 4 nm. A null hypothesis $y_i = f_i$ was considered to be true if:

$$\chi^2 \leq \chi_{\alpha}^2 = \frac{1}{2}(\sqrt{2n-3} + z_{2\alpha})^2. \quad (8)$$

Here, a critical χ_{α}^2 was given for $n > 30$ [36, 37]. At a significance level of $\alpha = 0.05$, we have $z_{2\alpha} = 1.65$ and $\chi_{\alpha}^2 = 107.3$ at $n = 86$.

A Student's t -test was applied as described elsewhere [35].

2.5 Pig hearts

All pigs (24–26 kg, $n = 4$) received humane care in compliance with the guidelines of the Canadian Council on Animal Care [38]. The NRC-IBD institutional Animal Care Committee approved all experiments.

For details of pig heart surgery see Refs. [20 and 39]. Briefly, to perform acute experiments, preanaesthesia of the animals was induced with midazolam, ketamine, and atropine. After endotracheal intubation, the pig was ventilated mechanically (rate of 12–14 beat/min) to maintain the arterial pO_2 of 350 mm Hg (100% arterial HbO_2) and pH at 7.4–7.5. Anaesthesia was maintained with isoflurane. A temperature probe was placed in the anus to monitor core temperature. A lateral thoracotomy was performed and the pericardium was opened. A lidocaine infusion was administered to prevent the development of arrhythmias. The heart rate varied in the range of 90–100 beat/min or 0.60–0.67 s per beat.

The color photograph in Figure 2A illustrates a general view of the representative pig heart in the open chest during surgery. It was captured with a Canon PowerShot A710 IS camera at a distance and a view angle different from the distance and the view angle of the CCD camera during the NIR spectroscopic imaging.

2.6 Biochemical blood assay

The assay was done with a NOVA Biochemical Critical Care Xpress Stat Profile M7 (Waltham, MA) blood-gas analyzer, which provided hematocrit, Hb concentration in g/l, oxygen saturation, ionic composition, glucose, lactate content and other biochemical parameters.

3 Results and discussion

3.1 Epicardial/subepicardial maps

This approach provides the determination of maps of the distribution of concentrations of deoxygenated, oxygenated and total (Hb + Mb), OSP and optical pathlength L across the heart surface. Figure 2 shows three of them for a representative pig

Figure 1 Images of the pseudo-optical density of diffuse reflectance from a representative pig heart at five selected wavelengths and determined according to Eq. (3). A white arrow in each image shows a point, the coordinates of which in pixels are indicated in parentheses. The image size of 532×256 pixels is shown.

heart as well as a regular color photograph for general comparison.

The $P_{xy}(\lambda)$ images (Figure 1) show dark spots in the heart surface. The spots relate to a significant contribution of specular reflectance in addition to diffuse reflectance. Therefore, these spots cannot be used for the analysis although they demonstrate the apparent maximal oxygenation in the OSP map (Figure 2B) and the minimal heme concentration in the total (Hb + Mb) map (Figure 2C). The bottom part of the B–D panels correspond to images of the lamp fixture used for the heart illumination. The scale of the OSP from zero to 100% appeared as a result of calculations and accorded to Eq. (6). The [Hb + Mb] map scale from 0 to 3 mM was predetermined. In the map areas corresponding to the lamp fixture images of both OSP and [Hb + Mb] were found to have zero values.

In all the maps, regions of interest (ROI) were chosen to represent the most important parts of the

heart, left and right ventricles and left anterior descending artery (LAD). In addition, some of the map area represents liquid blood outside the heart, the data of which can be compared with the biochemical analysis of blood. Figure 2 shows maps for a representative pig heart and all the ROIs. In general, selection of each ROI is a matter of experimental choice.

After the ROIs were chosen, a MATLAB program calculated the across-ROI mean values of five parameters mentioned above for each ROI. For four ROIs in Figure 2, the values are combined in Table 1. The *Out of heart* ROI corresponded to the area filled out with liquid blood.

The biochemical blood assay data for OSP and [Hb] can be compared with the corresponding results deduced from the NIR imaging of a limited area with a minor bleeding (ROI *Out of heart*). The comparison demonstrated no statistical difference between them. This biochemically confirmed the correctness of the NIR imaging results. The biochemical

Figure 2 (online color at: www.biophotonics-journal.org) Color photograph (panel A) and 532×256 pixel images of a pig heart *in vivo*. Panel B, map of the oxygen saturation parameter (OSP) distribution across the heart surface. Panel C, map of total (Hb + Mb) concentration in mM of heme, which is a sum of concentrations of oxygenated and deoxygenated (Hb + Mb). Panel D, map of actual optical pathlength in cm. Positions of the heart apex and left anterior descending (LAD) artery in the color photograph are the same as in panels B–D. Four solid curves in panels B–D indicate regions of interest (ROIs) chosen as described in the text. ROI 1, a limited area out of the heart body with minor bleeding; ROI 2, left anterior descending (LAD) artery; ROI 3, left ventricle wall normal tissue; ROI 4, right ventricle wall normal tissue. These ROIs were used to determine the averaged $P_{xy}(\lambda)$ spectra and calculate the parameters shown in Table 1. See Section 2 for detail.

Table 1 Oxygenation (OSP), (Hb + Mb) concentration, and optical pathlength (L) determined in the selected areas of a representative pig heart surface using NIR spectroscopic imaging.

	Left ventricle	Right ventricle	LAD	Out of heart	Blood assay
ROI	3	4	2	1	n.a.
OSP, %	81.8 $\pm$ 2.5	80.2 $\pm$ 2.5	94.5 $\pm$ 2.4	100.0 $\pm$ 0.2	99.9 $\pm$ 0.1*
[total (Hb + Mb)]	955 $\pm$ 138	983 $\pm$ 79	1890 $\pm$ 307	6356 $\pm$ 2432	6364 $\pm$ 152*
[deoxy (Hb + Mb)]	173 $\pm$ 28	194 $\pm$ 17	101 $\pm$ 40	31.2 $\pm$ 29.2	n.d.
[oxy (Hb + Mb)]	781 $\pm$ 122	789 $\pm$ 81	1789 $\pm$ 314	6355 $\pm$ 2433	n.d.
L , mm	6.3 $\pm$ 0.3	6.9 $\pm$ 0.3	5.0 $\pm$ 0.5	1.1 $\pm$ 0.4	n.a.
Pixels in ROI	1096	1393	633	269	n.a.
χ^2	60.9	98.4	42.7	91.5	n.a.
Criterion (Eq. (8))	$\chi^2 < \chi_\alpha^2$	$\chi^2 < \chi_\alpha^2$	$\chi^2 < \chi_\alpha^2$	$\chi^2 < \chi_\alpha^2$	n.a.

Notes. LAD, left anterior descending artery; ROI, region of interest. The data and ROIs correspond to the images shown in Figure 1 panels B–D. All data are means and standard deviations (std) over all pixels in the ROI. [total (Hb + Mb)] = [HbO₂ + MbO₂] + [deo (Hb + Mb)]. The concentrations are in μ M of heme and were not corrected for water content of the tissue. The *Out of heart* region was filled with blood. For details of the blood assay, χ^2 and $\chi_\alpha^2 = 107.3$ see Section 2, the biochemical blood assay related to Hb, only. n.d., not determined; n.a., not applicable.

verification of the method's correctness has also been presented for buffer-perfused arrested pig hearts, as reported elsewhere [23].

The $P_{xy}(\lambda)$ data obtained according to Eq. (1) and then numerically differentiated (left part of Eq. (3)) for each pixel (x , y) were averaged over each of four ROIs (Figure 2). The data shown in Table 1 provided parameters for the right-hand side of

Eq. (3), which is a fit of the experimentally obtained averaged differential spectra in these three ROIs. When plotted together (Figure 3), the fits were found to be very close to the experimental spectra regarding both the band position and their intensities. The goodness-of-fit χ^2 -criterion (Eqs. (7) and (8)) confirmed this quantitatively. This is a spectroscopic verification of the NIRSI results.

Table 2 Statistical results of oxygenation (OSP), (Hb + Mb) concentration, and optical pathlength (L) determined in the selected areas of pig hearts using NIR spectroscopic imaging.

	Left ventricle (LV)	Right ventricle (RV)	LAD	Out of heart	Blood assay
OSP, %	86.2 $\pm$ 5.9	82.5 $\pm$ 6.7	91.7 $\pm$ 4.8	96.6 $\pm$ 7.2	99.9 $\pm$ 0.1
	LV	$P > 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.001$
		RV	$P < 0.001$	$P < 0.001$	$P < 0.001$
			LAD	$P < 0.001$	$P < 0.001$
				Out of heart	$P > 0.05$
[total (Hb + Mb)]	1314 $\pm$ 475	1193 $\pm$ 362	2200 $\pm$ 11620	6165 $\pm$ 358	6885 $\pm$ 895
	LV	$P > 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.01$
		RV	$P < 0.01$	$P < 0.001$	$P < 0.001$
			LAD	$P < 0.001$	$P < 0.001$
				Out of heart	$P > 0.05$
[deo (Hb + Mb)]	186 $\pm$ 92	192 $\pm$ 59	155 $\pm$ 67	268 $\pm$ 385	
[HbO ₂ +MbO ₂]	1134 $\pm$ 434	1002 $\pm$ 382	2051 $\pm$ 1616	5968 $\pm$ 698	
L , mm	6.4 $\pm$ 0.9	5.9 $\pm$ 1.0	4.8 $\pm$ 1.1	1.5 $\pm$ 0.5	
	LV	$P > 0.05$	$P < 0.001$	$P < 0.001$	
		RV	$P < 0.01$	$P < 0.001$	
			LAD	$P < 0.001$	
n	20	22	13	6	10

Notes. LAD, left anterior descending artery. [total (Hb + Mb)] = [HbO₂ + MbO₂] + [deo (Hb + Mb)]. The concentrations are in μ M of heme and were not corrected for water content of the tissue. The mean values and standard deviations were calculated over n regions of interest (ROI) chosen in normal tissue of four hearts using averaged data for each ROI as in Table 1. The *Out of heart* region was filled with blood. For each heart, two NIR spectroscopic imaging was performed. Blood was biochemically probed during each heart surgery as Section 2 describes. The probability P that the comparing values are not statistically different was determined according to the Student's t -test. When $P > 0.05$, the values are not different.

Figure 3 The first derivative of the pseudo-optical density averaged over regions of interest (ROIs) in Figure 2. The spectra related to area out of the heart body containing blood (ROI 1), left anterior descending (LAD) artery (ROI 2), left ventricle wall normal tissue (ROI 3) and right ventricle wall normal tissue (ROI 4) are shown as open circles. Their fits obtained by Eq. (3) and values from Table 1 are bold curves.

Table 2 compiles the statistical results. Similar ROIs were chosen several times for the same functional compartments of a heart (both ventricles and LAD), for each of four hearts and for each of two replicated NIR images. The statistical results were similar to the results for an individual pig heart (Table 1) and support the high reproducibility and feasibility of the NIR spectroscopic imaging approach.

3.2 Oxygenation

Arterial blood was used for the biochemical assay. OSP for both the arterial blood and blood in the *Out-of-heart* area was about 100% as expected. In both the individual heart (Table 1) and multiple ROIs of four hearts (Table 2), OSP decreased to about 92% for LAD and to 80–86% for left or right ventricle walls.

As discussed below (Section 3.4), the functional parameters OSP and [Hb + Mb] can be determined for the volume, in which NIR light propagates in the tissue. According to this, the OSP decrease for LAD may be a result of two causes. First, the LAD diameter ranges in 2–3 mm at the real special resolution of the NIR imaging of about 0.65 mm (see Section 3.5). Because the spatial resolution and the LAD size are of the same order of magnitude, the LAD ROI may partially contain the ventricle wall tissue, the OSP of which was significantly lower than for blood. Secondly, the optical pathlength was found to be close to 5 mm, which may result in about 2 mm of the NIR light penetration into the LAD. Because the LAD wall, which does not contain Hb or Mb, should significantly affect the NIR light propagation, the OSP reduction in the LAD reflected the absence of the heme chromophore in the LAD wall.

The OSP for the left and right ventricles was found to be similar. However, these values were significantly lower than that for the blood or the LAD. Similar values of 75–85% for the ventricle myocardial oxygen saturation for the beating heart have been reported [3–5, 39, 40]. Because the cardiac tissue contains both 100% oxygenated blood in arterial capillaries and 50% oxygenated blood in venous capillaries, the low OSP values for the tissue probably reflected a combination of arterial and venous OSP. Also, low OSP can be partially due to the OSP value of 91–92% for Mb contributed to the NIR light absorption in a pig heart [23, 41].

Blood-oxygen-level-dependent cardiovascular magnetic resonance (BOLD CMR) has been shown to have a potential to noninvasively measure myocardial oxygenation because the BOLD CMR signals are sensitive to deoxygenation of Hb (see recent applications in Refs. [42, 43] as examples). No data

on myocardium oxygenation in terms of OSP or related parameters was available because these signals are not sensitive to either HbO_2 or total Hb content. However, this approach remains effective for estimation of changes in the regional myocardial oxygenation, not absolute values of parameters like OSP.

3.3 (Hb + Mb) concentration

In both right and left ventricular walls, the total (Hb + Mb) concentration in the pig hearts was found to be in the range of 1.2–1.3 mM (Table 2). Estimations of Mb in a buffer-perfused pig heart [23] or from biochemical studies [41] resulted in [Mb] of approximately 0.3 mM. Obviously, the difference $[\text{Hb} + \text{Mb}] - [\text{Mb}] = 0.9\text{--}1.0$ mM should correspond to [Hb] in the tissue. Using CT imaging of porcine heart wall, the intramyocardial blood volume ρ (mainly microvascular) was determined to be 0.06–0.22 ml per gram of the left ventricular wall tissue depending on the myocardial blood flow varying in the range of 1–4 ml/g of myocardium per min [44]. Since $[\text{Hb}]_{\text{blood}} = 6.17 \pm 0.36$ mM (Out of heart from Table 2), the Hb concentration in the heart wall should be $[\text{Hb}]_{\text{myocardium}} = \rho d[\text{Hb}]_{\text{blood}} = 1.09 \pm 0.06$ mM at $\rho = 0.167 \pm 0.055$ ml/g (the mean and standard deviation, $n = 23$, deduced from (Ref. [44]) and muscle density $d = 1.06$ g/cm³ [45]. This value closely corresponds to the estimate of [Hb] of 0.9–1.0 mM mentioned above.

The (Hb + Mb) concentration determination may be affected by variation of water content in the tissue. In Eqs. (2) and (3), light absorption was taken for pure water, while the water content may range from 80–85% in muscles to 95% in blood. The lower water content leads to a decrease in the effective optical pathlength L through water in tissue by the factor $f_w = 1/0.8 = 1.25$ for myocardium and $f_w = 1/0.95 = 1.05$ for blood. Respectively, for the lower L value, the (Hb + Mb) concentration found as a ratio $(\xi_{1xy} + \xi_{2xy})/\xi_{3xy}$ will increase by factor f_w . Because the exact water content in myocardium in the heart in these experiments was unknown (in general, water content in blood and muscles may vary depending on physiological status of animal at the moment of the imaging), we did not introduce the f_w factor into calculations of the (Hb + Mb) concentration.

Obviously, if the (Hb + Mb) content is compared for different parts of the sample, the f_w factor becomes less important when (Hb + Mb) differs by more than 30%. From Tables 1 and 2, the logical difference of (Hb + Mb) between ventricle walls and LAD does not depend on whether the f_w factor is

applied or not, while the numerical difference may occur.

It is important to note that the variation of water content across the tissue does not affect the OSP values and maps because of the lack of an effect of optical pathlength on OSP.

The absolute concentration of total Hb in muscular or skin tissues can be measured by a frequency-domain photon migration technique (see Refs. [46–48] for instance). It allowed determination of the total light absorption μ_a , and then [Hb], with a single source–detector separation. This approach does not provide data on optical pathlength and is only suitable for imaging with a spatial resolution of hundreds of mm² employing fiber-optic optodes in contact with the tissue.

In another method, the Hb concentration can be calculated from the Lambert–Beer law utilizing the optical pathlength that is either predetermined or theoretically predicted (for instance, see Refs. [2, 49–51]). Our results indicate that the optical pathlength may vary significantly across the sample surface (see below). Therefore, using the predetermined or predicted optical pathlength may result in errors in the diffuse reflectance-assisted determination of the [Hb + Mb] distribution across epicardium and subepicardium.

3.4 Optical pathlength

The significance of optical-pathlength determination arises from two issues. First, the functional parameters OSP and [Hb + Mb] determined from diffuse reflectance of NIR light correspond to the volume, inside which the light propagates from the point of the entrance of the tissue to the exit of the tissue at a given point. The optical pathlength estimates the distance of the propagation. Secondly, the optical properties of the tissue strongly depend on the light scattering, which is a function of the tissue structure. Therefore, the optical pathlength can, in general, report on the tissue structure along the path of the light propagation.

Figure 2D shows an optical pathlength map. Heterogeneity of the optical-pathlength distribution across the heart surface confirms the inappropriateness of using a single optical pathlength value for the whole heart. The averaged values for the ROIs are in Tables 1 and 2.

Because the imaging was performed in the diastolic phase, the ventricular volume was maximal and the wall should have minimum thickness. In our experiments, the optical pathlength of 6–7 mm was not statistically different in both ventricles (Table 2). The pig heart left and right ventricular wall thickness can be estimated to be in the ranges of 7–10 and

5–6 mm, respectively, on the basis of *in vivo* magnetic resonance imaging, and postmortem analysis of fluorescence imaging of fluorescent microspheres deposited in the heart slices as well as triphenyltetrazolium chloride staining of heart slices [38, 52]. The depth of the light penetration, defined as the distance in which the light intensity decreases *e*-times, into the muscle (780–835 nm [53]) or cardiac (653 nm [54]) tissues has been estimated to be in the range of 3.5–3.8 mm. Obviously, the data of Tables 1 and 2 indicate that the NIR light was collected from inside the ventricular walls and, therefore, OSP and [Hb + Mb] were found as a combination of values for the epicardial, subepicardial and intramural layers.

In our experiments, the increase of [Hb + Mb] from the ventricles to the LAD and liquid blood and minor changes in their oxygenation correlated with the decrease of the optical pathlength for both individual heart (Table 1) and multiple ROI statistics (Table 2). This finding closely corresponds to a well-known effect (see Refs. [16, 17], for instance), when optical pathlength inversely depends on the total light absorption coefficient $L \sim 1/\mu_a^{0.5}$, where $\mu_a = [\text{deo. (Hb + Mb)}] \epsilon_{\text{deoHb}} + [\text{HbO}_2 + \text{MbO}_2] \epsilon_{\text{HbO}_2} + \mu_w$ in our case.

Optical pathlength in diffuse reflectance in turbid media depends on the light-scattering efficiency μ_s and the anisotropy factor *g* of the tissue $L \sim [(1 - g) \mu_s]^{0.5}$ [16] characterizing the tissue structure. Heterogeneous insertions in the tissue affect the optical pathlength [15]. The inhomogeneous distribution of optical pathlength shown in Figure 2D probably resulted from the variation of both [Hb + Mb] and OSP, as well as structural variations across the heart epicardium and subepicardium.

To avoid the effect of the geometry of the optical elements of illumination and light collection (the measurement setup), a differential pathlength factor (DPF) has been introduced as an optical pathlength per interoptode distance, $\text{DPF} = L/d$ [14] when one optode serves for illumination and the second optode for diffusely reflected light collection. In the case of the macroscopic NIR imaging no special optodes can be selected because illumination is over the entire exposed heart surface and the diffusely reflected light is collected from that surface. In fact, the light, which is collected from a certain point of the heart, may result from diffuse reflectance of the light incident to the multiple points in the surrounding area. Therefore, the distance corresponding to *d* cannot be easily determined, and an idea of DPF is inapplicable in our case.

The time-of-flight method [14] was developed to experimentally determine optical pathlength in turbid media. It employs a picosecond pulse of light travelling through the tissue, requires highly specialized equipment, is time consuming and hardly appropriate for bedside operations. Another variant of this

method employs a phase modulation of the light instead of pulses [55]. Both of the methods do not provide estimates of oxygenation or [Hb + Mb]. Therefore, the use of the optical pathlength found by the time-of-flight approach can serve as predetermined. It should be applied to any point of the heart, while its surface cannot be characterized by a single optical pathlength value. Therefore, the optical pathlength measured by the time-of-flight method is inapplicable. Only the actual optical pathlength values at different locations can be used.

3.5 Spatial resolution

The CCD camera provided imaging with a resolution of 532×256 pixels (see Section 2). Regularly, the heart short axis ranges in about 5–6 cm, which resulted in 57–68 mm for 256 pixels according to Figure 1. Therefore, the camera-limited spatial resolution was 0.244 ± 0.022 mm/pixel. In the 2D notation, this spatial resolution was $0.049\text{--}0.071$ mm²/sq. pixel.

Other limitations also contribute to the total image resolution. As shown recently [23], defocusing can significantly reduce the spatial resolution. For a beating heart, the distance between the camera lens and the sample surface changes with a rate of the beating that can lead to a partial defocusing. This defocusing is significant when the beating-dependent heart motion along the imaging axis (the camera-to-heart direction) expands over the depth of lens focus. Partial defocusing may occur because of chromatic aberration of the lens. However, in our case, chromatic aberration was probably insignificant as no color fringes were observed at the VIS-NIR imaging of heart.

The motion of lung adjacent to the heart body caused the heart motion along the imaging plane. In the video clip *gated_pod_pig_heart.mpg* (see online attachment) one can observe such heart motion. Figure 1 shows five frames of this video related to specific wavelengths. We chose a representative point on the heart surface, which moved significantly. The change of this point position in the image allowed demonstration of a maximal reduction of the spatial resolution determined by the lung motion in this particular case.

The images in Figure 1 correspond to one respiration cycle of 4.3–5 s. As the gating provided imaging every 2nd cardiac cycle of 600–670 ms, the time interval between images is 1.2–1.3 s. This resulted in about 3–4 images per respiration cycle. Therefore, among five images in Figure 1, two heart positions can be found as maximally distant each from other. The maximal difference of the representative point coordinates in the images was between 814 and 822 nm (or 806 nm). The distance between the points was calculated as $d^2 = (178 - 175)^2 + (438 - 436)^2$ for the pixel coordinates of two points

in Figure 1 was $d = 3.6$ pixels = 0.88 mm (at 0.244 mm/pixel). Then, the spatial linear resolution in each of two axis of the image is $r = d \times \sin 45^\circ = 0.62$ mm. Hence, in this particular case, the lung-dependent heart motion determined a lower resolution than the camera resolution. Therefore, the total spatial resolution was about 0.60–0.65 mm (linear) or 0.36–0.42 mm² (square).

A similar spatial resolution of 0.40 mm² has been achieved by applying spectral imaging of hand palms but without quantifying the absolute concentration of heme proteins and oxygenation [56]. Because no sample motion occurred during such imaging, only the camera determined the total resolution. Other NIR imaging systems based on the application of multiple individual optodes or probes provided significantly lower spatial resolution measured in the range of tens of hundreds of mm² [57–60].

4 Conclusions

It was demonstrated that NIR spectroscopic imaging can provide the maps of absolute concentration of oxygenated and deoxygenated hemoglobin + myoglobin, oxygen saturation parameter and optical path-length as important biochemical-physiological information on the beating heart status *in vivo*. All the maps can be obtained at high spatial resolution in a 1-mm range simultaneously, noninvasively and in a couple of minutes. When the method is used during actual cardiac surgery for intraoperative monitoring of the immediate heart status, this approach provides a great advantage.

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