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Use of derivative diffuse reflectance spectroscopy in CAM assay combined with multivariate analysis as an approach to detect features of vulnerable plaques

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Abstract: Identifying vulnerable plaques at an early stage is critical to reducing patient mortality associated with cardiovascular diseases. Diffuse reflectance spectroscopy (DRS) can directly measure of absorption and scattering properties of tissue and can detect oxy-haemoglobin inside the plaque associated with intraplaque haemorrhage, which is a feature of vulnerable plaques. This study assesses the potential of using a diffuse reflectance spectroscopy combined with a machine learning algorithm to identify oxygenated haemoglobin in a chick embryo chorioallantoic membrane (CAM) assay model. A total of 88 diffuse reflectance spectra measurements were collected from five 12 to 14-day-old embryos. The first and second derivative of the reflectance spectra were calculated, followed by the use of partial least square-linear discriminant analysis (PLS-LDA) to identify oxygenated haemoglobin in chick embryo vessels. The model achieved a sensitivity and specificity of 96% and 72%, respectively, in differentiating arteries from veins (oxy-haemoglobin) using reflectance data. The sensitivity and specificity were 92% and 88% using the first derivative of reflectance data, and 100% and 92% using the second derivative of reflectance data in the wavelength range of 500-600 nm. Initial results indicate that derivative reflectance combined with multivariate analysis has advantages for detecting tissue oxygenated haemoglobin in CAM assay model. This approach shows promise as a way to identify and study the features of vulnerable plaques.

Keywords: atherosclerosis; vulnerable plaque; diffuse reflectance spectroscopy; spectroscopic techniques; multivariate analysis

1. Introduction

Atherosclerosis is a major cause of most cardiovascular diseases. Some plaques remain quiescent and stable for years, while others are unstable and vulnerable. Patients with vulnerable plaques remain asymptomatic until a serious health event occurs, such as a heart attack or stroke. Thus, these plaques are difficult to detect and treat before they cause significant damage. Current methods used for detection include imaging tests such as CT and MRI scans, as well as blood tests to check for high levels of cholesterol and other markers of heart disease. However, these tests may not always be accurate, and it remains a challenge to reliably identify vulnerable plaques at an early stage.

Multimodal spectroscopic techniques have been investigated for detection of vulnerable plaque features by the Feld group [1,2,3]. The morphological features of vulnerable plaques include thin fibrous cap, necrotic core, superficial foam cells, and intraplaque haemorrhage. Diffuse reflectance spectroscopy (DRS) can directly measure of absorption and scattering properties of tissue and can detect oxy-haemoglobin, which is the main absorbers in atherosclerotic plaques. Intrinsic fluorescence can identify tissue fluorophores such as collagen, elastin, and lipids, and may provide information about the thin fibrous cap by detecting collagen.

Chick embryo chorioallantoic membrane (CAM) has been widely used to study angiogenesis and anti-angiogenesis due to its dense vascular structure [4]. Compared to other *in vivo* models, CAM assay offers several advantages – it has a relatively low cost and results can be obtained in a short amount of time [4,5]. Furthermore, CAM does not require ethical approval for animal experiment. Therefore, it is a very useful and cost effective model to study the features of vulnerable plaques.

The aim of this work was to conduct an initial *ex ovo* study to assess the potential of using diffuse reflectance spectroscopy to identify the veins and arteries of allantoic vessels in the chick embryo. In particular, we aim to establish a CAM assay as an *in vivo* model using spectroscopic techniques together with a machine learning algorithm to study the features of atherosclerotic vulnerable plaques.

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2. Material and methods

2.1 Instrumentation and diffuse reflectance spectral collection

A broadband light source (HL-2000, Ocean Insight, B.V. Netherlands) was used to illuminate the examined chick embryo. The diffuse reflectance spectra were collected using a compact spectrometer (QEPro, Ocean Insight, B.V. Netherlands) with a detection range of 400 - 1000 nm. A bifurcated bundle probe (BFL44HS01, THORLABS, Germany) was used to deliver the light to the tissue and collect the reflectance light from the tissue back to the spectrometer. The core diameters of the collection and illumination fibres were 400 µm with a numerical aperture (NA) of 0.39 in both cases. The centre-to-centre distance between the light illumination and collection fibres for reflectance was chosen as 865 µm.

Fertilized chicken eggs were obtained from County Cork, Ireland. The CAM assay was performed using the *ex ovo* method. After breaking the eggshell on day 4, the egg contents were transferred to a petri dish and incubated at 37 ˚C with 60% humidity.

Spectra were collected from multiple locations on the 12 to14-day-old embryo (Figure 1). The probe was held perpendicular to the arteries and veins. The room lights were turned off during spectral data acquisition. A set of standard measurements was performed on a reflectance standard (Spectralo®, Labsphere, Inc., North Sutton, US) prior to each measurement of an embryo sample. In total, DRS spectra were collected from 88 locations on five embryos. The sample size distribution from each vessel type is summarized in Table 1.

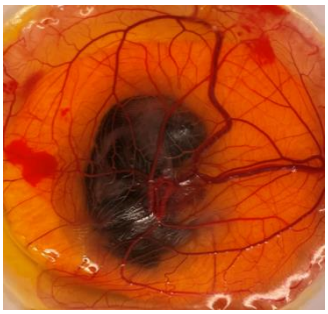


Figure 1. Distribution pattern of allantoic vessels in a 14-day embryo used in this study.

Table 1. Vessel types break down and sample size distribution

Vessel type	On egg white or yolk	Number of investigation sites
artery	white	13
	yolk	25
vein	white	25
	yolk	25

2.2 Data processing and classification analysis

Several steps were conducted to pre-process the collected spectra. First, all spectra were mean-centered. To enhance the spectral variations of the species over the background and minimize baseline variation, the first and second derivative of all spectra were calculated using discrete differences. A Savitzky-Golay filter was used to smooth the second derivative spectrum. The polynomial order and frame length used in this study were 2 and 9 respectively. Partial least square-linear discriminant analysis (PLS-LDA) was chosen for classifying arteries and veins on the yolk. PLS was first applied to the pre-processed data matrix, then a supervised classification scheme was developed using an LDA model. Due to the relatively small sample size, leave-one-out cross-validation (LOOCV) was used to evaluate the performance of the classification model built using the PLS-LDA method. The root mean squared errors of cross-validation (RMSECV) was used to optimise the models.

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All data in this paper and numerical analysis were processed using MATLAB 2019b (The MathWorks Inc., Natick, Massachusetts). All statistical analysis was conducted using the Statistics and Machine Learning Toolbox functions in MATLAB.

3. Results

3.1 Diffuse reflectance spectra of allantoic vessels in embryo

A total of 88 diffuse reflectance spectra measurements (13 arteries and 25 veins on egg white, 25 arteries and 25 veins on egg yolk) were collected from five 12 to14-day-old embryos. Figure 2 illustrates the mean reflected signal of arteries and veins in egg white (Fig. 2(a)) and yolk (Fig. 2(b)) and in addition with spectra normalization using mean-centering (Fig. 2(c) and Fig. 2(d)). The light pink or grey areas represent the standard deviation of the means for each type of vessel. Mean-centering emphasized the subtle variation in the spectra due to changing species concentrations. As can be seen, the mean-centering reduces the variance in the spectra while the spectral shape remains unchanged.

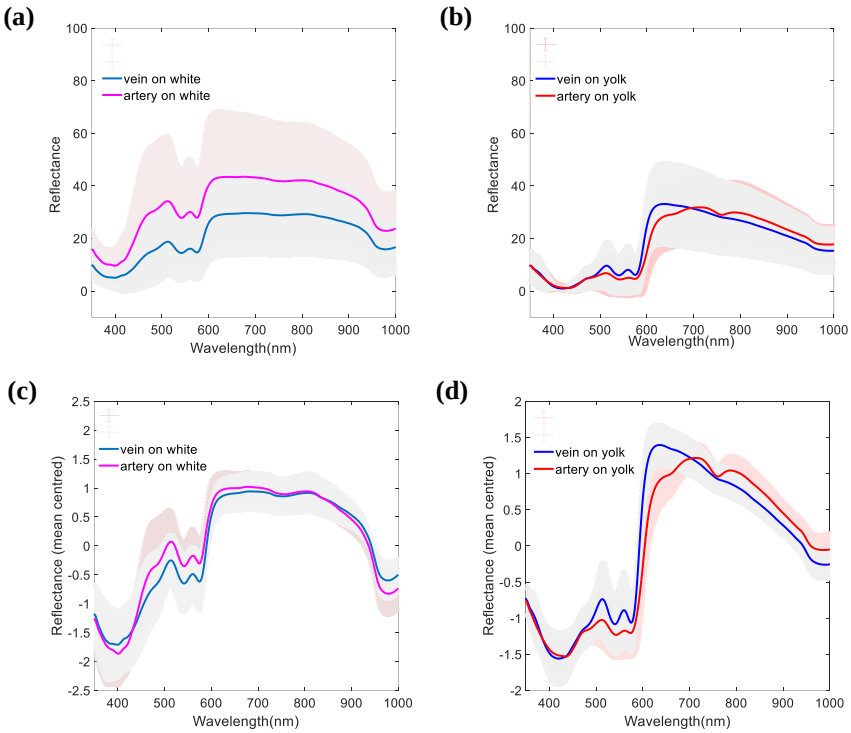


Figure 2. Mean diffuse reflectance spectra of vein (blue solid line) and artery (red solid line) on egg white (left) and yolk (right) before (top) and after (bottom) mean-centering. The grey areas indicate the standard deviation of vein (light grey) and artery (light red).

3.2 Derivative analysis and classification for oxygenated and deoxygenated haemoglobin

Figure 3(a) shows the spectral profiles of veins and arteries in the wavelength range of 500-600 nm. The first and second derivative of the DRS spectra are shown in Figure 3(b) and Figure 3(c), respectively. PLS-LDA was used for classifying arteries and veins on the yolk. The first four PLS components were selected to build the classification model based on the mean squared error of the results. Figure 4 displays the confusion matrix of the classification results using leave-one-out cross-validation. The values in blue cell reflect the percentage of correct tissue type classifications. Arteries can be differentiated from veins (oxy-haemoglobin) with a sensitivity of 96.0% and specificity of 72.0% using mean-centered reflectance data in the wavelength range of 500 - 600 nm (Figure 4(a)). To verify the derivative approach for detecting haemoglobin oxygen levels, the first and second derivatives of the reflectance spectra were calculated using discrete differences followed by PLS-LDA. Sensitivity and specificity were 92% and 88% using the first derivative of reflectance, and 100% and 92% using the second derivative of reflectance (Figure 4(b) and Figure 4(c)). It is clearly shown that the false positive rate is significantly reduced using derivative analysis.

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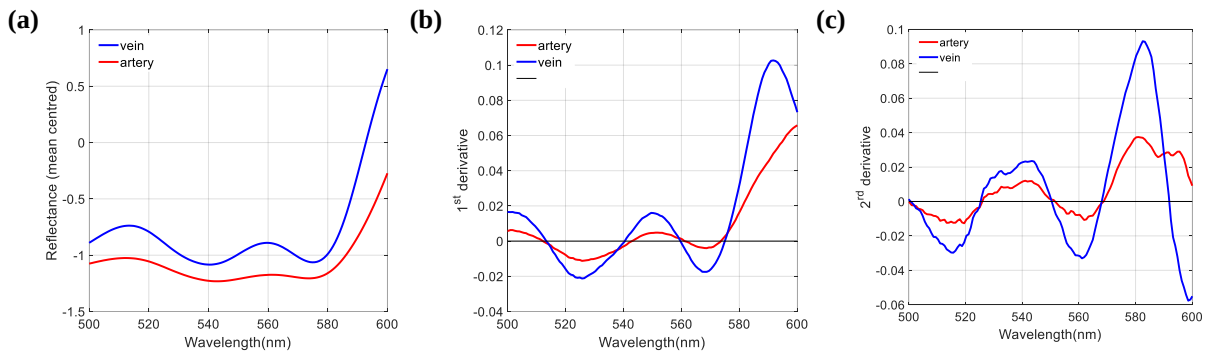


Figure 3. (a) Mean diffuse reflectance spectra; (b) First derivative; and (c) second derivative reflectance of vein (blue line) and artery (red line) in the wavelength range of 500-600 nm.

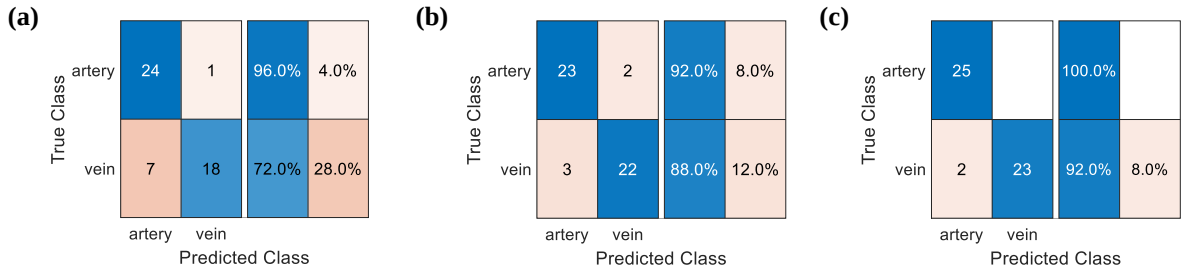


Figure 4. Confusion matrix displaying the classification of vein and artery using PLS-LDA model with (a) mean centred reflectance data; (b) first derivative reflectance; and (c) second derivative reflectance, in the wavelength range of 500 – 600 nm. The first four PLS components were selected to build the classification model. The classification results represent leave-one-out validation.

4. Discussion and future work

In this *ex ovo* study, we show that with the use of a DRS probe and a machine learning algorithm, we can identify oxygenated haemoglobin in the CAM assay model. Initial results of the study showed that arteries can be differentiated from veins (oxy-haemoglobin) with a sensitivity and specificity of 96% and 72%, respectively, using reflectance data, 92% and 88% using the first derivative of reflectance, and 100% and 92% using the second derivative of reflectance in the wavelength range of 500-600 nm.

Šćepanović *et al.* [2] demonstrated that DRS haemoglobin concentration is one of the spectroscopic parameters that shows the best correlation with morphological features of vulnerable plaques. In atherosclerotic plaques, the main absorber is haemoglobin which is associated with thrombus or acute intraplaque haemorrhage with features of vulnerable plaques. The DRS spectra is composed of tissue absorption and scattering. The absorption in vessel is primarily contributed by oxy-haemoglobin. The two dips at 544 nm and 575 nm in diffuse reflectance spectra related to oxygenated and deoxygenated haemoglobin from vein and artery are clearly seen in Figure 2. The area of these dips and humps varies with different concentrations of oxygenated haemoglobin and thus reflect the variation of tissue oxygen level. In agreement with a previous study of oxygen analyses in chicken embryos [6], our experimental results show that blood oxygen content in allantoic veins is higher than that in arteries (refer to Figure 3).

The first derivative of DRS is the rate of change of reflectance with respect to the wavelength and the values of the first derivative equals zero at the wavelength where the reflectance value is maximum. The second derivative can be described as the rate of change of the slope, and thus represents the curvature of the spectrum. Therefore, the derivative analysis can provide information on morphological variations in DRS and hence is an indirect evaluation of tissue oxygen level between artery and vein.

Though the presented algorithms and performance are considered preliminary, our results indicate that derivative combined with multivariate analysis has advantages for detecting tissue oxygenated haemoglobin. This underscores the potential for establishing a novel CAM assay as an *in vivo* model that uses spectroscopic techniques combined with a

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machine learning algorithm for studying the features of atherosclerotic vulnerable plaques. In the next step of this research, we intend to perform a combination of DRS and autofluorescence to provide more detailed chemical and morphological information to better understand other features of vulnerable plaques.

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