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Kyoto University
In vivo detection of atherosclerotic plaque using non-contact and label-free near-infrared hyperspectral imaging

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ABSTRACT

Background and aims: Detecting detailed atherosclerotic plaques is important to reduce risk factors during surgery. However, there are few methods to evaluate them during surgery. The aim of this study was to establish an in vivo, non-contact, and label-free imaging method for identifying atherosclerotic plaque lesions from outside vessels with a diffuse-reflectance near-infrared (NIR) hyperspectral imaging (HSI) system.

Methods: NIR spectra between 1000 and 2350 nm were measured using an NIR HSI imaging system outside the exposed abdominal aorta in five Watanabe Heritable Hyperlipidemic (WHHL) rabbits in vivo. Preprocessed data were input to a supervised machine learning algorithm called a support vector machine (SVM) to create pixel-based images that can predict atherosclerotic plaques within a vessel. The images were compared with histological findings.

Results: Absorbance was significantly higher in plaques than in normal arteries at 1000–1380, 1580–1810, and 1880–2320 nm. Overall predictive performance showed a sensitivity of 0.814 ± 0.017, a specificity of 0.836 ± 0.020, and an accuracy of 0.827 ± 0.008. The area under the receiver operating characteristic curve was 0.905 (95% confidence interval = 0.904–0.906).

Conclusions: The NIR HSI system combined with a machine learning algorithm enabled accurate detection of atherosclerotic plaques within an internal vessel with high spatial resolution from outside the vessel. The findings indicate that the NIR HSI system can provide non-contact, label-free, and precise localization of atherosclerotic plaques during vascular surgery.

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1. Introduction

Unstable atherosclerotic plaque is one of the major factors predicting embolism of stenotic arterial lesions. It is also a risk factor for embolization during carotid endarterectomy [1,2]. Therefore, evaluation of atherosclerotic lesions is important [3]. The properties and distribution of atherosclerotic plaques are clinically evaluated by preoperative plaque imaging, such as magnetic resonance imaging (MRI), ultrasonography (US), and computed tomography (CT). These non-invasive methods can evaluate hemorrhage, lipid core, or calcification in the atherosclerotic plaque and show the distribution of lesions. However, these methods cannot always provide accurate information about atherosclerotic lesions clinically because of their low spatial resolution. Therefore, other invasive methods, such as radiocontrast angiography and indocyanine green (ICG) video-angiography, that enable real-time and high spatial resolution imaging are often applied during vascular surgery. They can be used to diagnose the possibility of plaques as contrast defects. Radiocontrast angiography was previously considered the gold standard intraoperative method. However, it can cause embolization or arterial dissection because it needs an arterial puncture and injection of contrast agents [4]. In
addition, it only visualizes atherosclerotic lesions as defects. ICG video-angiography is a useful method with a cyanine dye with a peak spectral absorption approximately at 800 nm [5]. It suggests the plaque distribution as defects in the vascular site of the surgical field. Thus, it helps us understand the localization of plaques. However, the contrast deficits do not always correspond to atherosclerotic plaques. Furthermore, ICG hypersensitivity can occur as a complication because it requires intravenous administration of ICG [6]. Despite these methods, perioperative cerebral ischemic infarctions sometimes occur without any abnormalities seen with these methods [7,8]. Therefore, a method that can evaluate more detailed atherosclerotic lesions without any contrast agents needs to be developed.

The major atherosclerotic plaque models with spontaneous hyperlipidemia are apolipoprotein E-deficient (ApoE−/−) mice and Watanabe Heritable Hyperlipidemic (WHHL) rabbits. Whereas the main apolipoprotein of mice is composed of B48, B100 is the main apolipoprotein in rabbits and humans. Therefore, the models have different lipid metabolic pathways [9]. In addition, the atherosclerosis that occurs in ApoE−/− mice is extremely lipid-rich, and macrophage-derived foam cells stand out, with a very scarce fiber component [10–12]. Such lesions differ from the atherosclerosis seen in humans, which shows various lesions, including smooth muscle cells and collagen fibers covering the fibrous layers of lipid and foam cells [13]. In addition, the diameter of the aorta in mice is too small compared to that of the human carotid artery, which is one of the final clinical targets. The atherosclerosis of the WHHL rabbit shows a variety of lesions by age, as in humans [14]. Therefore, the WHHL rabbit seems a more suitable animal model for studying atherosclerotic plaques, especially for future applications of imaging methods and findings to humans.

Near infrared (NIR) spectroscopy with a micro-catheter has been used to identify vulnerable plaques in coronary artery regions [15,16]. NIR spectroscopy is a well-accepted method in biological science that enables chemical analyses without damaging the investigated materials. In addition, NIR light has two desirable properties for biomedical imaging. The first one is tissue permeability. The NIR spectral region, and specifically the 650–950 nm window, offers attractive characteristics for optical imaging, compared to the visible light range, due to lower light absorption by water in this wavelength band [17,18]. This enables practical photo detection even after propagation through several centimeters in tissue, for example human breast and brain [19–22]. The NIR window is also used for near infrared fluorescence molecular imaging, which offers high spatial resolution and good sensitivity of investigational targets. Macrophage-mediated inflammation [23], endothelial adhesion molecule activity [24], and thrombin activity [25,26] have been investigated by the in vivo NIR fluorescence imaging approach. However, a probe needs to be administered, and its safety in humans has not yet been verified. Therefore, a method without probes is desirable. The second one is that NIR has a unique spectral absorption pattern in each material. Several wavelengths identify characteristic components of human atherosclerotic plaques [27,28]. However, spectral interference caused by oxyhemoglobin and deoxy-hemoglobin, which cannot be avoided in the evaluation of vessels, was mainly expected in the 700–1000 nm. In addition, wavelengths over 2350 nm allow detailed information about lipids [29,30]. However, these wavelengths have low tissue penetration, and thus have no suitability for examinations through the vessel wall. On the other hand, wavelengths of 1000–2350 nm included cholesterol to 1200 and 1700 nm in the prior studies [27,28]. Furthermore, absorption peaks of vessel protein components, which are mainly collagen and other matrix proteins, have been shown in the vicinity of 2200 nm [28]. Characterization of human advanced atherosclerotic plaque using these wavelengths has already been done in clinical catheter intervention for coronary arteries [16]. However, the method has a potential risk of embolic complications and is, hence, not suitable during vascular surgery. Consequently, the distinguishing wavelengths, which have both tissue permeability for an examination from outside vessels and discriminability of components of atherosclerotic plaques, can be suitable.

Hyperspectral imaging (HSI) is a newer spectroscopic technique integrated with image information, providing both spatial and spectral data. HSI has been widely used for the quality assessment of agricultural products [31] and the evaluation of pharmaceutical materials [32]. Because HSI has more information than panchromatic or multispectral imaging, it can improve the accuracy of plaque identification. Previous reports showed the effectiveness of HSI for the identification of cholesterol crystals in plaques of ex vivo atherosclerotic mouse aorta, using coherent anti-Stokes Raman scattering HSI with wavelengths in the range of 2650–3050 nm [29,30]. In addition, the combination of a supervised machine-learning method, such as a support vector machine (SVM), which is widely used because of its remarkable performance in classification with multiple parameters [33–35], can be effective because the HSI contains considerable spectral information.

The aim of this study was to establish an in vivo, noncontact, and label-free imaging method for identifying atherosclerotic plaque lesions from outside vessels with the NIR HSI system and SVM.

## 2. Materials and methods

### 2.1. Animals

WHHL rabbits were used for the study as a suitable animal model for human familial hypercholesterolemia and atherosclerosis [36] (7–8 months of age; n = 5, male). We selected only male WHHL rabbits because a tough operation was needed in a female WHHL rabbit which has a large amount of accumulated intraportaline fat. In WHHL rabbits, aortic plaque formation is observed in all individuals more than five months old, and lesion occupancy reaches 30–40% at the age of 7–8 months [36].

Anesthesia was induced by venous injection of 12.5 mg/kg pentobarbital and continued by inhalation of 1.0–1.5% isoflurane. Local anesthesia was added in the abdominal wall by xylocaine for relief during NIR image data measurement. The abdominal aorta proximal to the renal artery branch level was exposed and measured by the NIR HSI system (Compovision, Sumitomo Electric Industries, Osaka, Japan). After measurement, WHHL rabbits were euthanized with an overdose injection of pentobarbital, and their aortas were excised for histological examination.

All experiments conformed to a protocol approved by the Standing Committee on Animals of Kyoto University.

### 2.2. Spectrum detection system

The HSI system consists of an NIR spectroscopic camera, which has a spectroscope and a two-dimensional NIR detecting chip (indium gallium arsenic detector). The NIR detecting chip has 215 wavelengths, which range from 1000 to 2350 nm (spectral resolution 6.27 nm). The camera takes 100–320 frames per second. The system is without peer with respect to the measurement speed over a wide area; it can measure the two-dimensional NIR spectra of a 15 × 20 cm² (approximately 100,000-pixel) sample in five seconds or less [32,37].

A schematic diagram of the experimental design is shown in Fig. 1. Diffuse-reflectance spectra in the 1000–2350 nm region of each pixel in a target were measured by the HSI system. Since images with high spatial resolution were needed in the study, the
Hyperspectral imaging data were calibrated by white and black reference images. The apparent absorbance at each pixel was then calculated by the modified Beer-Lambert law [39]. The preprocessed spectrum data were compared at each wavelength between vessels with and without atherosclerotic plaques by a paired t-test. The significance level threshold was \( p < 0.05 \), corrected for multiple comparisons by the Benjamini and Yekutieli false discovery rate (FDR) procedure [40]. Detailed procedures of image preprocessing are given in the Supplemental Methods.

Finally, the preprocessed data were processed by a supervised machine learning algorithm called SVM to create pixel-based images that can predict atherosclerotic plaques within a vessel. A leave-one-out cross-validation (LOOCV) strategy was used to assess the classification performance because the strategy is widely used in machine learning and allows using most of the data for training [33–35,41]. After all LOOCV repeats, the accuracy, sensitivity, and specificity for all folds are averaged together to generate the final accuracy, sensitivity, and specificity. Decision values [42] for receiver operating characteristic (ROC) curves and the area under the curve (AUC) were also evaluated. Furthermore, the LOOCV strategy was repeated 101 times to calculate the confidence intervals (CIs) of these estimates [33]. Detailed procedures of SVM-based plaque prediction are given in the Supplemental Methods.

### 2.4. Histological validation

Progression of atherosclerotic lesions is classified as inflammatory cell infiltration, lipid accumulation, and the formation of fibroptic and calcific layers. In clinical surgery, the target lesions are advanced lesion rather than atheromas that accumulate lipids. Oil red O staining (Lillie’s method) [43] was used to stain atherosclerotic lesions to assess lipid accumulation and layer structure. In order to avoid elution of lipids by dehydration and deacockelization in paraffin embedding, frozen fixing was required. Eight marks were placed on the abdominal aorta: four marks from the level of the celiac artery branch to the diaphragm and four marks from the level of the superior mesenteric artery branch to the level of the celiac artery branch. The marks were painted on the ventral vessel wall using gentian violet ink. These were also used for the identification of the ventral wall of histological samples. Dissected aortas were snap-frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and sectioned (10-μm thickness). Four sections were created per each mark. To evaluate the microscopically observed atherosclerotic plaques, histologic examination of imaged aortas was performed using 10-μm sections stained with oil red O. Locations and thicknesses of atherosclerotic plaques and thicknesses of vessels were evaluated. Each section was divided into 20 areas on the ventral vessel wall, and each area was classified as “Plaque” and “Normal artery” (Fig. 5). The “Plaque” included lipid pooling atherosclerotic lesions as intermediate lesions. atheromas, and fibro-atheromas. The “Normal artery” included histologically normal vessels. In accordance with the histologic evaluation, regions of interest (ROI) both for “Normal artery” and “Plaque” regions were manually defined on the HSI images.

### 3. Results

#### 3.1. NIR absorption spectra

Fig. 2 shows a montage of a representative hyperspectral cube (WHHL 1). More highly absorbed regions, such as at 1190 and 1720 nm, partially matched “Plaque” areas. However, there were no differences in absorption between “Plaque” and “Normal artery” areas, such as at 1410 and 1530 nm. Each spectral image had some disparity with the ROIs defined based on the histological findings throughout. The mean NIR absorption spectra of atherosclerotic plaques and normal arteries are shown in Fig. 3A. These absorption spectra both in plaques and normal arteries had marked absorption peaks at around 1200, 1750, 1450, and 1900 nm. Absorbance was significantly higher in plaques than in normal arteries at 1000–1380, 1580–1810, and 1880–2320 nm (FDR corrected \( p < 0.05 \)) (Fig. 3B). On the other hand, there were no significant differences in absorption between plaques and normal arteries at 1380–1580, 1810–1880, and 2320–2350 nm. The overall spectral patterns and differences in absorption between plaques and normal arteries were seen for all five cases (Fig. 52). WHHL 3 showed slightly higher absorption spectra at around 1600–1800 and 2100–2350 nm compared with the others.

#### 3.2. SVM-based plaque prediction

The results of plaque prediction by the SVM and LOOCV strategy in each WHHL rabbit and representative comparisons between the predictions and pathological findings are shown in Fig. 4 and 53, respectively. The plaque predictions showed good concordance with the label images defined by histological findings in all rabbits (Fig. 52). Fig. 4 shows that the distribution of predicted plaques was consistent with that of histological atherosclerotic plaques on the ventral aorta wall. Atherosclerotic plaques corresponded to the predicted images (pale yellow arrows in Fig. 4). On the other hand, thin plaques were not predicted as plaques, but as normal arteries.
In contrast, no atherosclerotic plaque lesions were found in the section predicted to be normal artery. The predictions showed high accuracies in all rabbits (WHHL 1 0.864 ± 0.012, WHHL 2 0.861 ± 0.007, WHHL 3 0.760 ± 0.028, WHHL 4 0.821 ± 0.008, WHHL 5 0.850 ± 0.005) (Fig. 5A). Overall prediction performance showed a sensitivity of 0.814 ± 0.017, a
specificity of 0.836 ± 0.020, and an accuracy of 0.827 ± 0.008. Fig. 5B shows an ROC curve with AUC and 95% CIs for differentiating plaques from normal arteries; the AUC was 0.905 (95% CI = 0.904–0.906).

4. Discussion

Diffuse-reflectance NIR spectra of in vivo arteries showed extensive significant differences at 1000–2350 nm between arteries with and without atherosclerotic plaques in the present study. Furthermore, combining with the hyperspectral spectral data and SVM, the locations of plaques were precisely visualized. These findings exemplify an imaging method for predicting precise atherosclerotic changes within an artery from outside the vessel.

Since the arteriosclerosis model used in the study has compositions closer to those of human arteriosclerosis, similar results in experiments with human subjects can be expected. In fact, histological findings of WHHL rabbit aorta in the present study included intracellular lipid accumulation, a core of extracellular lipid, and collagen fibers covering fibrous layers of lipid, which are characteristics of human atheroma. Observations of the aortic arch or thoracic aorta need to perform thoracotomy, which can deteriorate
atherosclerotic lesion of aortas and other arteries [46]. Male and female, they showed no gender differences in structure of cholesterol levels and triglyceride levels and vascular reactivity between bits used in the study revealed differences in plasma total cholesterol model [44], before clinical trials. Furthermore, gender-associated differences in human atherosclerotic plaques are important especially for our future clinical application of the system as several animal models with larger diameter vessels will be required for future applications in a human internal carotid artery or common carotid artery. Our system will need to be evaluated by more subjects including other animal plaque models with larger vessels close to humans, such as a swine model [44], before clinical trials. Furthermore, gender-associated differences in human atherosclerotic plaques are important especially for our future clinical application of the system as several human studies showed the differences [45]. Although WHHL rabbits used in the study revealed differences in plasma total cholesterol levels and triglyceride levels and vascular reactivity between male and female, they showed no gender differences in structure of atherosclerotic lesion of aortas and other arteries [46–48]. Furthermore, we need a tough operation in a female WHHL rabbit which has a large amount of accumulated intra-abdominal fat. Therefore, we selected only male WHHL rabbits in the study. It will also be needed to evaluate gender-associated differences in atherosclerotic plaques with other animal models whose plaques are similar to those in human and different between male and female in the future.

The measurement environment in the present study was quite similar to that of human vascular surgery. The target vessel with atherosclerosis was exposed in vivo, and diffuse-reflectance NIR HSI data were obtained by a non-contact type light source and a camera. The measurement conditions provide a non-invasive investigation. In addition, the measurement does not interfere with the surgical procedure because the measurement time is only 4–5 s. The limit for exposure duration in our settings is estimated to be more than 50 s for preventing thermal damage to the skin, according to the publication of IEC 62471:2006 “Photobiological Safety of Lamps and Lamp Systems”. Because the measurement time is less than five seconds in each sample, our NIR HSI system can be applied safely. Actually, thermal damage to cells by halogen lamps was not observed in the histological evaluation of vessels in this study. The NIR HSI system used in the study was also applied to quantitative analyses of biodegradable materials and showed the effectiveness of the quantitative determination of materials [49].

Overall absorption spectral patterns in all cases were similar, not only in normal arteries, but also in plaques. Absorption spectra both in plaques and normal arteries showed marked absorption peaks at around 1200, 1450, 1750, and 1900 nm in the present study. The spectral patterns were consistent with those of human aorta specimens [27]. The absorption peaks at 1450 and 1900 nm are dominated by water because of the strong OH-absorption [27]. There are no significant differences in absorbance around these peaks between plaques and normal arteries at these two wavelength regions. On the other hand, there were different spectral patterns between plaques and normal arteries in other spectral bands. The increases in absorption at wavelengths of 1200 and 1750 nm indicated the presence of cholesterol, which was the major lipid contained in atherosclerotic plaques [50]. In addition, the band of absorption increased at 1900–2350 nm, showing overlapping of the absorption peaks of collagens and chondroitin sulfate and sphingomyelin, which are known components of extracellular matrix [27,28]. In particular, increased absorption in the advanced atherosclerotic plaques at 1900–2350 nm was seen in WHHL3. Hypertrophy and disturbance of the layer structure of the tunica media were observed on histological examination of the area. The findings suggest the further possibility of evaluating different histological properties in plaques.

Plaque prediction by SVM showed good concordance with the histological findings in all rabbits (Figs. 4, 5 and S3). The results suggest that the method is useful for in vivo identification of atherosclerotic plaques from outside a vessel. However, the predicted images contained some false results (Fig. 4 and S3). The plaque areas that were predicted as “Normal artery” had thin plaques histologically (pale yellow arrow head in Fig. 4). Therefore, this mismatch between pathological findings and predictions at areas with thin plaques was one of the reasons for reduced accuracy in the present study. Moreover, the accuracy in WHHL3 was slightly

**Fig. 5.** Individual accuracy and receiver operating characteristic (ROC) curve. (A) Accuracy for differentiating normal arteries and plaques in each WHHL rabbit. The error bars are standard deviations. (B) ROC curve with area under the curve (AUC) and 95% confidence intervals (CI) to differentiate plaques from normal arteries.
lower than in others (Fig. 5A). This might be due to the difference in the nature of the atherosclerotic plaque of WHHL. Because WHHL3 was eight months of age, a month older than the others, the atherosclerotic lesions were more advanced on pathological validation, and increased absorption spectra were observed around 1750 nm and over 2100 nm (Fig. S2). The difference in spectral pattern can lead to a loss of accuracy in WHHL3 compared with the others with trained data for an SVM and LOOCV strategy. In fact, atherosclerotic lesions have a variety of compositions, thicknesses, and degrees of vessel wall degeneration. The classification performance of our method with a machine leaning algorithm can be more accurate with more data. Although this study only focused on predicting the presence and absence of atherosclerotic plaque, identification of components of atherosclerotic lesions will be better in clinical practice, such as hemorrhage, calcification, lipid core, and degeneration of a vessel wall. This method can also be extended to identify the various properties of atherosclerotic plaques with more data and their pathological information. The measurement and accumulation of human atherosclerotic lesion data will result in clinical application of the system. The properties and distribution of atherosclerotic plaques are clinically evaluated by preoperative plaque imaging, such as MRI, US, and CT. However, since those methods are absolutely preoperative examination, it is often difficult exactly to match the results to the surgical field during surgery. On the other hand, our NIR HIS system enables the evaluation of the actual surgical field during surgery. Therefore, our method has a benefit as an intraoperative support compared with the conventional plaque imaging methods. Radio-contrast angiography and ICG video angiography, which are used as intraoperative examinations of atherosclerotic plaques, are essentially techniques for assessing blood flow in the blood vessel lumen. Hence, the information of the vessel wall itself is limited in the methods. Furthermore, those methods need injection of contrast agents into the blood vessel. On the other hand, our NIR HIS system can evaluate more accurate atherosclerotic plaques within the vessel wall than those methods without any injection of contrast agent. Thus, the system can assist surgeons for decision-making, such as a selection of a recipient in bypass surgery and a decision of clamping the portion of vessels in carotid endarterectomy.

In conclusion, the NIR HIS system showed characteristic absorption spectral differences between normal arteries and plaques and enabled the prediction of atherosclerotic lesions within the internal vessel from outside the vessel with high accuracy with an SVM. The method can be applied clinically as a non-contact imaging technique. Moreover, it does not require any administration of drugs. These findings indicate that the NIR HIS system can less invasively evaluate the precise locations of atherosclerotic lesions during vascular surgery.

Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.029.

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