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**Laser-Induced Fluorescence Examination of Myocardial Biopsies in
Patients with Transplanted Hearts**

Report
by

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Laser-induced Fluorescence Examination of Myocardial Biopsies in Patients with Transplanted Hearts

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Background and Objective

Myocardial biopsies from transplanted hearts were examined by laser-induced fluorescence to investigate the possibilities of catheter-based fibre-optical "biopsy" to characterise myocardial rejection and fibrosis.

Patients and Methods

Eighty-eight myocardial biopsies from 15 patients were examined directly after resection during routine biopsy after heart transplantation. Ultra violet light was guided through an optical fibre to the biopsies. The fluorescence was collected with the same fibre and guided to the spectrum analyser. Using multivariate analysis techniques, the main characteristic spectroscopic features were extracted. The results were correlated with the histopathological characterisation.

Results

Myocardial fibrosis could be distinguished from the normal myocardium. In this study, acute rejection was not significantly different from the normal myocardium.

Conclusions

It is possible to examine myocardial biopsies using laser-induced fluorescence spectroscopy with a fibre-optic probe. Our work suggests the potential *in vivo* application of a catheter-based optical tissue characterisation for the diagnosis of myocardial fibrosis *in situ*, but not for acute rejection.

Keywords: autofluorescence, fibrosis, rejection, tissue characterisation

Introduction

Myocardial biopsy is repeatedly conducted after heart transplantation to diagnose the rejection of a transplanted heart and to monitor the myocardial response to immunosuppressive drugs. It is of great clinical interest to develop less invasive methods to monitor rejection. Different techniques have been suggested for the detection of acute rejection including electrical impedance measurements [1] and blood analysis [2]. Laser-induced fluorescence spectroscopy has previously

been proven to be useful for tissue characterisation [3-6]. It is a non-invasive method that can distinguish differences in biochemical composition in real-time. Success of distinguishing fibrous myocardial tissue from normal myocardial tissue [7-9] and of distinguishing rejected myocardial tissue from normal [10] has been reported. Laser induced fluorescence spectroscopy has also been successfully used to differentiate between nodal conduction tissue and atrial and ventricular endocardium [11]. The method has also been used to study rejection of transplanted aortic sections in rats [12] and to investigate spectral differences between atrial and ventricular endocardium in sheep [13].

The aim of this study was to investigate the possibility of using fibre-optic fluorescence spectroscopy for real-time characterisation of fibrosis and acute rejection.

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Materials and Methods

Biopsies

Measurements were performed on 88 scheduled routine biopsies taken from 15 patients with transplanted hearts at 19 different occasions (table 1). Some of the patients were newly transplanted and some of them had lived with their transplanted hearts for a few years. The average transplantation time was: mean 377 days, median 81 days. The age of the patients was ranging from 7 to 64 years. On average, five biopsies per patient were taken from the right ventricular septum. Directly after resection, the biopsies were put on wet compresses and examined with laser-induced fluorescence spectroscopy. They were then put individually in plastic tubes filled with 4% formaldehyde for fixation and sent for routine histopathological examination and classification. All specimens were stained with Haematoxylin & Eosin and van Gieson elastic stain.

Laser-induced fluorescence measurements

The laser-induced fluorescence measurements were performed with the point-monitoring optical multichannel analyser, which has been previously described in detail [8]. Laser emission from a nitrogen laser (Laser Science VSL 337) emitting 3 ns long pulses at 337 nm with a repetition rate of 10 Hz was used for excitation. The excitation light was guided to the sample by a 600 µm optical quartz fibre (Superguide, Fiberguide Industries Inc.). Due to tissue absorption and scattering, the penetration depth of the

Biopsy#	Patient#	Age	Time*	Biopsy	Rel.	Fib.	Path†	Biopsy#	Patient#	Age	Time*	Biopsy	Rel.	Fib.	Path†
1	1	53	8 days	1	0	0		48	10	61	8 days	1	0	0	
2				2	0	0		49				2	0	0	
3				3	0	0		50				3	0	0	
4				4	0	0		51				4	0	0	
5				5	0	0		52	11	56	1m 9d	1	+	+	
6	2	58	1 year 1m	1	0	+		53				2	+	+	
7				3	0	+		54				3	+	+	
8				4	0	+		55				4	+	0	
9				5	0	+		56	12	51	1m	1	0	+	
10				6	0	+		57				2	0	+	
11	3	53	3y	1	+	+		58				3	0	+	
12				3	+	+		59	9	40	2m 18d	1	+	0	
13				4	0	+		60				2	0	0	
14	4	58		1	0	+		61				3	0	+	
15			3y 11m	2	0	+		62				4	0	0	
16				4	0	+		63				5	0	0	
17				5	0	+		64			2m 3w	6	0	+	
18				6	+	+		65	1	54		1	0	0	
19				7	0	+		66				2	0	0	
20	5	14	2m 1w	1	+	+		67				3	0	+	
21				2	0	+		68			2 y 1 m	4	0	+	
22				3	0	0		69				5	0	0	
23				4	0	+		70	13	7		1	+	+	
24	6	57	2 years	1	0	0		71				2	0	0	
25				2	0	+		72			4 years	3	0	+	
26				3	0	0		73				4	+	+	
27				4	0	+		74				5	0	+	
28				5	0	+		75	14	59		1	0	+	
29	1	53	15 days	1	0	0		76			1 year	3	0	+	
30				2	0	+		77				4	0	+	
31				3	0	+		78				6	0	0	
32				4	0	+		79				5	0	0	
33	7	64	7m 2w	1	+	+		80	15	53		1	0	+	
34				2	+	+		81			15	2	+	+	
35				3	0	+		82				3	0	0	
36				4	0	+		83				4	0	+	
37				5	+	+		84				5	+	+	
38	8	52	1 year	1	0	0		85	10	61	12 days	1	0	0	
39				2	0	0		86				2	0	+	
40				3	0	0		87				3	0	0	
41				4	0	0		88				4	0	0	
42				5	0	0									
43	9	39	12 days	1	0	0									
44				2	0	0									
45				3	0	+									
46				4	0	+									
47				5	0	+									

* = Time from transplantation to biopsy
y = years m = months
w = weeks d = days
† = Pathology evaluation
0 = Not in biopsy
+ = In biopsy

excitation light is very shallow, only a few 100 μm . The distal end of the fibre was held perpendicular in light contact with the biopsies. The excitation pulse energy at the sample was approximately 5 μJ . The induced fluorescence emission was collected by the same fibre and guided back to the instrument. The fluorescence light was separated from the excitation light using a beamsplitter (BBAR 450-750 nm, CVI). It was further filtered through a cut-off filter (Schott GG 375) and focused on the entrance slit of a grating spectrometer (SpectraPro-275, Acton Research). Fluorescence emission spectra between 360 and 680 nm were captured with a cooled image-intensified CCD detector (ICCD-576G/RB, Princeton Instruments Inc.). The detector was used in gated mode to suppress ambient room light. Detector dark current and any fluorescence background originating from the optics were subtracted from the recorded spectra. Each fluorescence spectrum was also corrected for a non-uniform spectral response of the detection system with the use of recorded spectra from a white light calibration lamp (ORIEL Corp.). The fluorescence recorded from 25 sequential laser pulses was accumulated to create one spectrum used for the evaluation. Each biopsy was measured on two different locations. The whole procedure took not more than two to five minutes for each patient.

Evaluation methods

All biopsies were examined by the same pathologist and classified according to two different criteria: whether they included any fibrosis and if they showed any sign of rejection of the transplanted heart. Rejection is defined as infiltration of lymphocytes in the myocardium and is scored 0 - IV according to the criteria by International Society for Heart and Lung Transplantation. Rejection and fibrosis were classified in all biopsies independently of each other. Biopsies without fibrosis and lymphocyte infiltration were classified as normal.

The sampling volume of the fluorescence measurement is very small and occupies only a small fraction of each biopsy sample since the light penetrates only a few 100 μm into the tissue. To, in some way, resemble the pathological evaluation where the histological findings from the whole biopsy are summarised to define the classification of the tissue, one spectrum from each biopsy was chosen for evaluation. This choice was made using principal component analysis (PCA) as described below. Before evaluation, all spectra were normalised to exhibit the same total integrated intensity. All normalised spectra were put in a matrix X with 176 rows corresponding to the number of spectra and 574 columns corresponding to the wavelength points. PCA is a singular-value decomposition of the matrix X , which gives orthogonal spectral components, corresponding to the variation in X in decreasing order. Then each spectrum is correlated to these principal components and the correlation coefficients are calculated, called scores. By plotting the scores for the two first most relevant principal components in 2D graphs, any, in the spectra, inherent differences resulting in grouping can be found. Such grouping was used to choose the spectrum in every spectrum-pair that was used in the evaluation.

The selected fluorescence spectra were analysed with partial least squares (PLS), which is a mathematical tool to reveal spectral characteristics from a group of spectra. Using all spectra in the whole wavelength interval, the PLS components were calculated by correlating the variance in the spectra (X) with the variance of the classification (Y) from the pathologist. The PLS components were then calculated as the most common deviations from the mean spectra, which correlate with the histopathological classifications. The correlations between each spectrum and the components were calculated and the result is called scores. The first components are the most interesting components since they reveal the spectral characteristics, which correlate the most to the known answers. Higher terms represent noise and information not relevant to the known answer. Due to the small number of biopsies in some of the groups,

the spectra were validated with cross-validation, i.e. leaving one sample out at a time and then validating the model created by the rest with this sample. The number of components to use for validation was chosen by plotting the Root Mean Square Error of Prediction, against the number of components used for validation. RMSEP is a measure of the accuracy of validation using a certain number of components. The minimum reflects the optimal choice of components. Including more components results in including components irrelevant for prediction which just adds noise.

Results

Classification

Acute rejection was found in 17 biopsies and among these, 15 showed fibrosis. Most biopsies (n=42) were fibrous without any sign of rejection and the rest of the biopsies

were classified as normal myocardium (n=29). Examples of histologic sections from normal, fibrous and rejected myocardium are illustrated in figure 1.

PCA was performed on all spectra to select which spectra to be included in the evaluation. The first component was interpreted to correspond to increased collagen and elastin fluorescence and a decrease of the fluorescence from reduced nicotinamide adenine dinucleotide (NADH) [14]. Fibrotic samples would show a higher score of this component than the normal ones. However, these samples were very heterogeneous and the score for the first component varied much for the two measurements on each sample. In each spectrum-pair, the spectrum with the highest score of the first principal component was thus chosen for evaluation of fibrosis. It was found that among the samples histologically classified

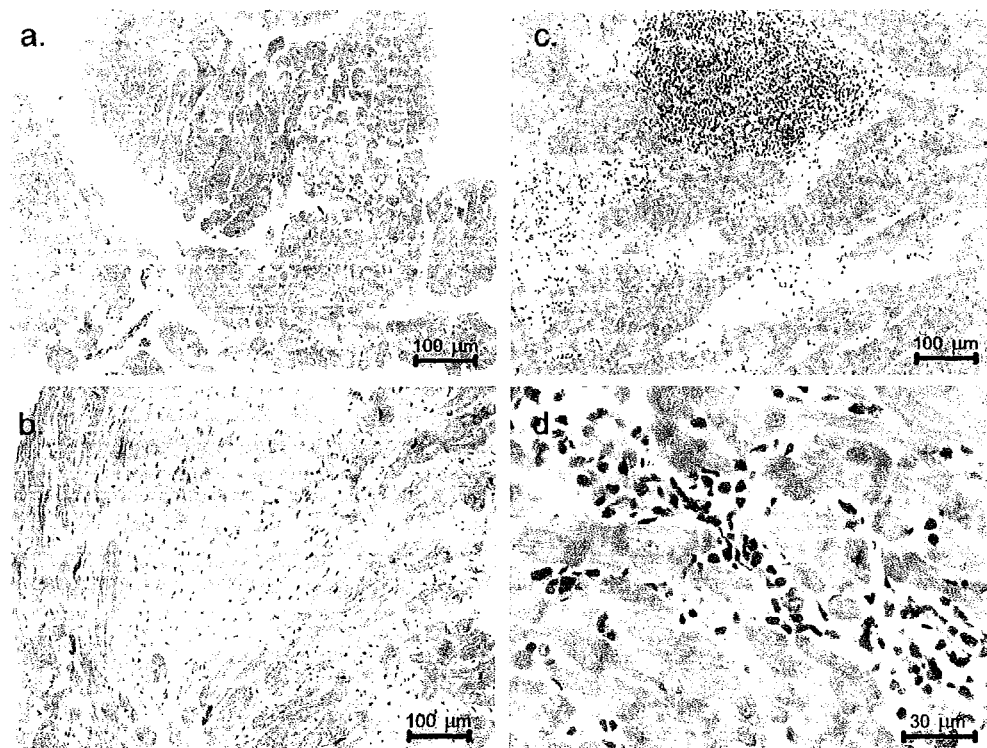


Figure 1. Myocardial biopsies with normal myocardium (a), fibrosis (b), rejection grade IIIa with invasion of lymphocytes and myocytolysis (c) and rejection grade Ib (d). All sections are stained with hematoxylin & eosin, a, b, c x100, d x400.

as fibrotic, many spectra had a very low score of the first component indicating that the spectra resemble spectra from non-fibrotic samples. Ten of the spectrum pairs looked very different (the score for the first principal component differed with more than 10) and they came all from the fibrotic samples and also, there were many (n=18) spectrum-pairs where both spectra had a very low score.

Choosing spectra for evaluation of rejection was more difficult since no information is given on which spectral characteristics can be found in rejected samples. There was no sign of any grouping when the scores of the ten first principal components were plotted. Therefore, all 176 samples were used in the evaluation of rejection.

The fluorescence spectra

Characteristic examples of the fluorescence spectra from normal and fibrous myocardium are shown in figure 2. The fluorescence between 385 nm and 410 nm

originates from elastin and collagen fluorescence. Nicotinamide adenine dinucleotide in reduced form (NADH) is the energy carrier in muscle cells with peak fluorescence around 465 nm. As could be expected, the spectrum from normal myocardium shows a higher intensity in this region. The spectra also exhibit a reduction in the fluorescence intensity around 420 nm due to reabsorption of fluorescence light at this

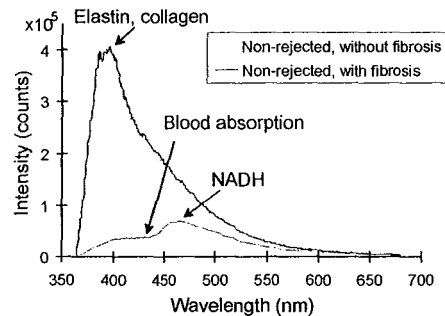


Figure 2. Typical fluorescence spectra of normal and fibrotic myocardial biopsies. Excitation wavelength 337 nm.

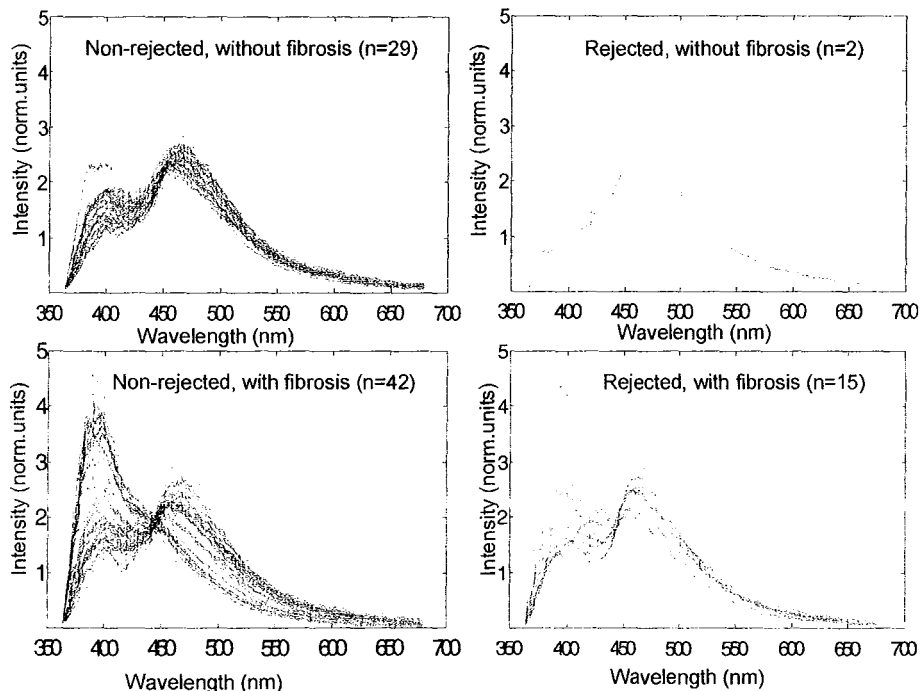


Figure 3. Fluorescence spectra from the biopsies. Excitation wavelength 337 nm.

wavelength by hemoglobin. In figure 3, the 88 spectra included in the statistical evaluation normalised to the same integrated intensity are shown. The fibrotic samples show on average a higher intensity towards lower wavelengths. There is no visible difference between the rejected and non-rejected samples.

Partial least squares analysis of fibrosis
PLS was performed on the 88 selected fluorescence spectra. In the evaluation of fibrosis, the samples histopathologically classified as fibrotic were trained to provide prediction of -1, while the non-fibrotic samples were trained to yield the prediction of +1. Whether it was rejected was not taken

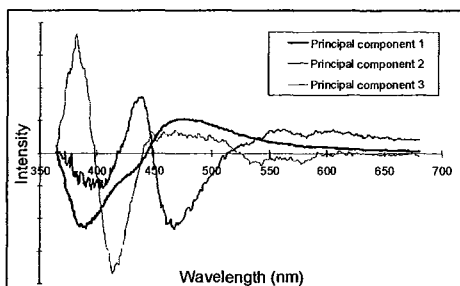


Figure 4. The first 3 PLS components representing (95%, 20%) (1%, 4%) (2%, 1%) of X (spectra) and Y (pathology answer) variations respectively.

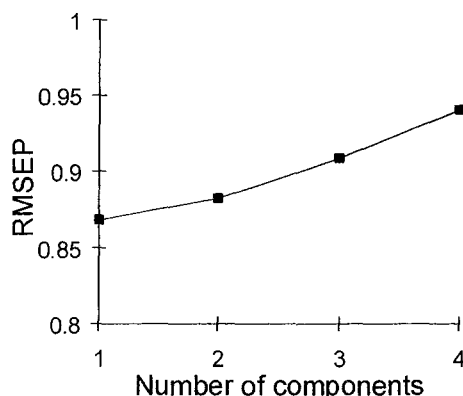


Figure 5. The figure shows the Root Mean Square Error of Prediction (RMSEP) of validating the fibrotic tissue as a function of number of used principal components for the prediction. The best prediction is found using only the first component.

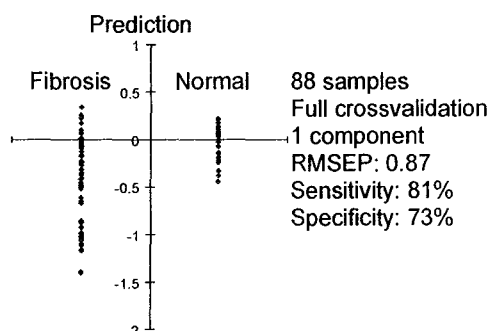


Figure 6. Results of predicting fibrosis using full crossvalidation.

into account in this analysis. The first three PLS components in this evaluation are shown in figure 4. The first component is inverted and shows an increased collagen and elastin fluorescence and a decreased NADH fluorescence.

In figure 5, the resulting RMSEP is plotted against the number of components. It shows a minimum of 0.87 at the first component and in figure 6, the validation using only the first component can be seen. The presence of fibrosis in the biopsies could in this study be classified with a sensitivity of 81% and a specificity of 73%.

Partial least squares analysis of rejection

The results of evaluating the potential of detecting rejection are more ambiguous. The number of rejected samples is small and an evaluation using the rejected/non-rejected information showed no statistical differences. The presence of a markedly increased number of lymphocytes in the tissue is the indication for acute rejection seen by the histopathologist. To investigate if there is any reason for that the fluorescence spectrum from rejected tissue is different from non-rejected tissue, measurements were performed on pure lymphocytes purified from a blood sample. The fluorescence spectrum can be seen in figure 7. The peak is around 460 nm indicating that it mainly comes from NADH fluorescence. Even though the signal/noise ratio is very small due to the small volume, it does not show any other spectral shape that could be used as a marker for lymphocyte infiltration. The

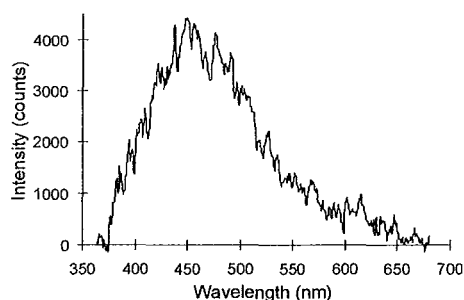


Figure 7. Fluorescence spectra from pure lymphocytes. Excitation wavelength 337 nm.

fluorescence looks very much alike the fluorescence from normal myocardial cells. The difference is only due to the absorption from hemoglobin around 420 nm in the red blood cells which alters the true fluorescence shape of the myocardial cells.

Discussion

After a heart transplantation, the new myocardial tissue is unfamiliar to the immune system and a rejection process is initiated. There are three general phases of rejection; hyperacute, acute and chronic. The hyperacute rejection is caused by the antibodies circulating in the patients blood. The new organ is attacked and the organ fails within the first few hours after transplantation. Acute rejection is most frequently seen during the first year, but could occur even several years after transplantation. The myocardial cells of a transplanted heart are attacked and even destroyed by the immune process initiated by T-lymphocytes. Thus infiltration of lymphocytes in the myocardial tissue characterises an acute phase of rejection. To monitor the rejection status of the transplanted heart, biopsies are taken once every week directly after transplantation. After the fourth week, biopsies are taken week 6, 8, 10, 12, 16, 20, 24, 32, 40 and after one and two years. Biopsies are then taken every second year. At any sign of rejection, the dose of immunosuppressive drug and/or glucocorticoid are increased to reverse the process. Acute rejection with tissue-destruction usually heals with fibrosis

(scar tissue). Fibrosis can also be due to scars from previous biopsies as well as due to other causes, e. g. hypertension.

Laser technology for cardiovascular diagnosis and therapy is an expanding field with many forms of applications [5] for instance laser angioplasty, transmyocardial revascularisation and diagnostic tissue characterisation. Laser light can be guided in optical fibres and is thus suitable for applications in conjunction with the catheter-based techniques often used in the field of cardiology. Fluorescence spectroscopy provides information about position and distribution of certain biomolecules within the tissue. This information can be used for tissue characterisation. A few substances in the tissue can give rise to detectable fluorescence signal, namely collagen, elastin, NADH, porphyrines, cholesterol and some other proteins. With the application of the fibre-optic technique, there is a possibility to perform minimally invasive *in vivo* measurements by insertion of a silica-fibre along a diagnostic catheter into the chamber of an implanted heart. Multiple measurements would be possible at different positions within the heart, including locations where biopsy is not possible due to the risk of perforation or other potential injury.

In the present study it was found that the myocardial fibrosis could be distinguished from the normal myocardium according to their main spectroscopical characteristic features. Our findings are very similar with the findings of Perk *et al.* [9]. It is not possible to compare the spectra directly from the two studies, since the spectra from the previous study [9] were not corrected for the instrumental response and spectra were recorded using different measurement geometry. However, an increased fluorescence intensity in the wavelength range between 385nm to 410 nm was observed for the fibrotic myocardium in both studies. Collagen I has its two strongest excitation/emission maxima at ($\lambda_{exc} = 340\text{nm} / \lambda_{em} = 395\text{nm}$) and ($\lambda_{exc} = 270\text{nm} / \lambda_{em} = 395\text{nm}$) [16] and since the absorption peaks are broad, collagen is excited by both wavelengths (308 nm and 337 nm) used in the two studies, respectively. Collagen fibres

are also very much present in the fibrotic tissue, as seen by the histopathology. Elastin fluoresces weaker than collagen and it is also excited by these wavelengths since it exhibits excitation/emission maxima at ($\lambda_{exc} = 360\text{nm} / \lambda_{em} = 410\text{nm}$) and ($\lambda_{exc} = 260\text{nm} / \lambda_{em} = 410\text{nm}$) [16]. Elastin fibrils are contained in mature collagen fibrosis and subendocardial tissue, especially if it is fibrotic. Thus, fluorescence from collagen I and elastin provides the basis for classification of fibrosis.

Since we have not been able to identify lymphocyte infiltration, acute tissue rejection could not be diagnosed with the current technique. This is not consistent with the findings of MacAuley *et al.* [10]. The explanations for this could be several. They used a HeCd laser for excitation ($\lambda=442\text{nm}$) and found a wavelength shift and broadening in the main fluorescence peak around 510 nm, which were used for the classification of rejected tissue. Due to the different excitation wavelength they mainly study other fluorophores in the tissue than those providing information in this investigation. In our study, the two non-fibrotic biopsies with acute rejection showed no wavelength shift or intensity increase.

It is not clear to us whether one can expect a fluorescence difference between tissues with and without lymphocyte infiltration and in that case, what causes the difference. The fluorescence spectrum from pure lymphocytes is very similar to that of normal myocardial tissue and does not seem to be an ideal fluorescence marker. In rejected tissue, there is also an increased fluid content, edema. Perhaps this change could provide an alteration in the fluorescence signal not seen in this study, which could be used for tissue classification.

A full statistical analysis of the potential of the method to identify rejected tissue was difficult to perform due to the low number of rejected samples included. However, if this method should be useful to detect acute

rejection, differences on a patient level must be present. Here, we do not find such differences. An other limitation of this study is the short penetration of the excitation light, meaning that only two small volumes of the biopsy was measured as compared with the histopathology examination, which uses the whole tissue mass. In some samples, there might have been fibrotic changes, which were missed by the fluorescence technique and this led to that many spectra from the samples histopathologically classified as fibrotic were very similar to spectra from normal biopsies. The sampling difference between pathology and fluorescence measurements could perhaps been minimised with an improved method. The biopsies could have been marked on the spots where the fluorescence measurements were performed and evaluated by the pathologists on these spots. Alternatively, if more fluorescence measurements could have been performed on each biopsy, the sensitivity and specificity of detecting fibrosis might have been increased. In addition, we did not perform quantitative histopathology of collagen content, which also could improve the statistics of fibrosis detection.

Even though our results indicate that the fluorescence spectroscopy technique at this excitation wavelength could not be used for the diagnosis of acute rejection, it can be used to identify endocardial fibrosis. The examination is minimally-invasive and could therefore be extendedly applied. Up to 30-40 measurements could be conducted in a short time. The finding of fibrosis, especially dynamic changes compared with previous examinations could provide important information for the diagnosis and/or treatment of patients with cardiomyopathy, myocardial ischemia and infarction and the tissue changes of a transplanted heart.

In conclusion, it is possible to distinguish fibrous from normal myocardial tissue using fluorescence spectroscopy with a fibre-optic probe. This suggests a potential clinical application of a catheter-based endocardial fluorescence spectroscopy technique for detecting myocardial fibrosis in situ. However, in this study we could not tell the difference

between normal and rejected myocardium, using the fluorescence spectroscopy with a fibre-optic probe. One of the reasons for this could be that we had a small amount of biopsies and further studies are therefore warranted.

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