VILNIUS UNIVERSITY

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FLUORESCENCE SPECTROSCOPY AND IMAGING STUDIES OF FUNCTIONALY DIFFERENT HUMAN HEART TISSUES

Summary of doctoral dissertation
Biomedical sciences, biophysics (02 B)

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ABBREVIATIONS

CCS – cardiac conduction system

SA – Sinoatrial node

AV – atrioventricular node

HB – Hiss bundle

MC – myocardium

CT – connective tissue

EC – endocardium

UV - ultraviolet

RCM – reflection confocal microscopy

I. INTRODUCTION

According to the Health Information Centre the diseases of hematopoietic organs and cardiovascular system still remains the most common cause of death in Lithuania. The dysfunction of these critical systems could arise from congenital or inherited disorders, or as a result of harmful lifestyle. In many cases no any preventive actions are taken and finally the only way to restore the quality of life remains the operational procedure. Additional attention requires the congenital heart diseases that usually occur in the first years of life. This is especially difficult cases, because of the pathology the cardiac structure is altered and accurate localization of its individual components can not be known, therefore it could be easily damaged during any surgical intervention.

The main function of the heart is the appropriate supply of blood to the body and it is largely dependent on an orderly and rhythmic contraction. The beating rhythm and the propagation of impulse are controlled by the cardiac conduction system (CCS). CCS is a specific muscular tissue, where a heartbeat signal originates and subsequently spreads through its branches to the different parts of the heart, initiating depolarization of the ventricles. CCS begins with the sinoatrial (SA) node, in which the depolarization of the pacemaker cells dictates the heart beat rhythm. An impulse generated in the SA node spreads through the atriums to the atrioventricular node (AV), whereat the conduction system transforms into the His bundle (HB) connecting atriums with ventricles. In the interventricular septum HB splits into two parts, which branch off in the myocardium of ventricles and end in the inner ventricular walls as thin Purkinje fibers [1,2]. In cardiac surgery is extremely important to identify the localization of CCS and to avoid the damage of this system, what may result in a dangerous obstruction of the heart functionality. However, because of muscular origin CCS is hardly distinguishable from the surrounding muscle myocardium (MC). CCS is located in almost all parts of the heart, but due to problematical identification of this system, there is still a discussion about existence of certain conduction paths and in most cases of congenital heart defects the distribution is not known at all. Insufficient knowledge about precise localization yields the possibility to harm the CCS during the surgical procedure. Complications could be avoided if the CCS could be visualized during operation. This requires real-

time analysis or imaging methods. Histological examination is the gold standard for tissue analysis, but in this case it is not suitable, since microscopic examination of tissue is performed ex vivo and is long-lasting procedure. Thus, the imaging technique should be non-invasive, simple and quick to perform, but still very informative, because the differences are sought between muscular type tissues.

Various methods of the optical spectroscopy, which provide a non-invasive acquisition of the real time data, continue to gain new application areas in biomedical research and clinical diagnostics. These methods have unique characteristics that make them attractive for diagnostics of various diseases. Most importantly, it is non-invasive, relatively simple and quick methods, however, despite the simplicity, they are extremely sensitive. Fluorescence spectroscopy is the mostly used optical diagnostics method for investigation of tissue optical properties and their changes. Biological tissue is characterized by specific (auto) fluorescence that is a result of natural tissue fluorophores. Spectral characteristics of many endogenous and exogenous fluorophores are already explored and correlation between fluorescence spectra and optical properties of biological object is also made. Autofluorescence reflecting the intrinsic features of the biological tissue has become a useful parameter for the differentiation between the tissues otherwise being visually indiscernible [3,4]. The recent development of the new compact light sources, especially in the near-UV spectral region, as well as miniaturization of the fiber-optics based registration equipment eventually creates a new boost for the fluorescence – based diagnostic techniques application during complex surgery of internal organs, such as surgery of the heart. Fluorescence spectroscopy is already used for tissue viability estimation, tumor identification and determination of tumor tissue boundaries. A variety of diagnostic techniques are created based on the specific tissue fluorescence,

Despite the extensive application of optical methods for diagnostics and biological tissue investigation, there are only a few scientific assays about application of fluorescence spectroscopy for CCS research. All experiments are carried out on ex vivo tissues, also excluding the influence of blood. Key discoveries that have been made in the investigations of experimental animal hearts, is that the fluorescence of the conduction system nodes (AV and SA) is more intense than fluorescence of ventricles and is slightly weaker than the atrial fluorescence, however no significant differences in

the AV and MC fluorescence spectra were observed. Recently has been performed a study on human heart preparations searching differences between the optical parameters of the different types of cardiac tissue. Investigations were carried out on the heart tissues that were separated from covering endocardium (EC) and regardless of its fluorescent properties. It was found that the CCS and MC have different absorption in ultraviolet (UV) region and different fluorescence intensity in 400 nm – 550 nm region. However, fluorescence intensity depends on the excitation-collection conditions and the recorded fluorescence intensity differences may be due to a different contact between the fiber and the tissue, but not because of different tissues. The direct application of intensity derived differences would not be accurate, therefore there is still a demand of the method that would be insensitive to uneven excitation-collection conditions and could be used for *in vivo* applications.

The aim of the study

To create the identification methodology for human cardiac conduction system based on the investigation of tissue optical parameters.

Objectives

1. To investigate the optical parameters of the heart tissues that are not separated from EC by fluorescence and fluorescence excitation techniques.

2. To determine the influence of blood reabsorbtion to the fluorescence spectra of heart tissue.

3. To create CCS imaging methodology based on detected spectral differences.

4. To investigate cardiac tissues by time-resolved fluorescence spectroscopy.

5. To investigate functionally different cardiac tissues by reflection confocal microscopy.

6. To evaluate the applicability of created CCS imaging methodology for *in vivo* imaging, during the surgery procedure.

Defended statements

1. Fluorescence spectra of unprepared heart tissues – CCS, MC and CT, which are not separated from EC, differs in form and intensity under the excitation of 330 nm radiation. Two fluorescence peaks at 390 nm and 430 nm are observed in the spectrum. The ratios of intensities at these wavelengths are different for CCS, MC and CT. Under excitation of 380 nm the fluorescence spectra differ in intensity, but the spectrum shape does not change significantly.

2. An intense absorption of blood is observed at ~ 420 nm and its presence in living tissues would strongly affect the registering fluorescence spectra. Studies of bloody (frozen) heart tissues allowed to evaluate the blood affect and to select the optimal fluorescence excitation and registration regions. The excitation light must be chosen from 330 nm – 380 nm region, and fluorescence must be registered at 460 nm.

3. CCS differentiation method has been created based on the determined spectral differences. The method relies on calculation of intensity ratios:

$$< R_{tiss} > (460) = \frac{I_{330}^{ex}}{I_{380}^{ex}}$$

Calculated value $<R_{tiss}>(460)$ is different for CCS, CT and MC tissues. The developed methodology is insensitive to fluorescence excitation-collection conditions and therefore may be used for unambiguous identification of CCS. Moreover the method allows the determination of CCS bundle margins and even visualization of small areas.

4. Time resolved fluorescence spectroscopy revealed, that at least three fluorophores are responsible for the fluorescence of CCS, CT and MC in the

spectral region of 430 nm – 550 nm. The lifetimes (τ) and the fractional components of fluorescence intensity (F) of muscular type tissues (CCS and MC) showed no statistically significant difference. Whereas τ_1 an τ_2 also the relative fractional components F1 – F3 of CT differed significantly from those of CCS.

5. The different reflection properties of cardiac tissue components allow to visualize collagen and elastin fibers composing different layers of EC. The method also allows to identify myocardium by distinguishing single myocytes and to detect the bundles of conduction system with the Purkinje cells composing the Purkinje fibers.

6. Spectral characteristics of cardiac tissues recorded *in vivo* during surgery procedure correspond to those, observed on *ex vivo* tissues, therefore the developed method is suitable for the CCS discrimination studies in living tissues. Nevertheless, in order to adapt this method for routine use during the surgery extremely fast and sensitive equipment is required.

Actuality and scientific novelty

Cardiac disorders still remain the leading cause of mortality and morbidity. In the numerous cases the surgery is the only way to eliminate the source of the heart malfunction, however, during the surgical intervention, especially in the case of congenital heart diseases [5], there is a possibility to harm the conduction system of the heart, which may result in a dangerous obstruction of the heart functionality. CCS is a specific tissue type where a heartbeat signal originates and then spreads through its branches to the different parts of the heart initiating the depolarization of the ventricles. Thus, it is absolutely vital for generating and synchronizing the heartbeat. Now it is known, that CCS is a muscular type tissue and its branches are surrounded by the isolating layer of connective tissue. These facts complicate the visual distinction of the conduction system from the surrounding MC and fibrous (CT-like) tissues, therefore CCS could be damaged. The exact localization of CCS in the heart is still not known

exactly and a reliable method for its visualization either *in vivo* or *ex vivo* also does not exist.

Various methods of the optical biopsy, which provide a non-invasive acquisition of the real time data, continue to gain new application areas in biomedical research and clinical diagnostics. The recent development of the new compact light sources, especially in the near-UV spectral region, as well as miniaturization of the fibre-optics based registration equipment eventually creates a new boost for the fluorescence – based diagnostic techniques application during complex surgery of internal organs, such as surgery of the heart.

The most widely used optical diagnostic method is based on the fluorescence measurements. Autofluorescence reflects the intrinsic features of the biological tissue, therefore it has become a useful parameter for the differentiation between the tissues otherwise being visually indiscernible [3,4]. CCS plays a totally different role from MC and has different electric characteristics – an impulse through HB propagates about ten times faster than in MC [6,7]. This suggests that the respective two muscular tissues should have some qualitative or quantitative differences in their structure and/or molecular composition that could be detectable by means of fluorescence spectroscopy. Spectroscopic investigations of the hearts of experimental animals revealed, that the SA and AV fluorescence is different from the surrounding tissue fluorescence. The intensity differences have been observed among nodes and ventricular EC fluorescence spectra [8-11]. Fluorescence of human CCS nodes is weaker than that of atrium, but is more intense than of ventricles [12]. However, the investigations, performed in the human heart is very limited and the heart tissues have been mainly investigated with the removed EC [13], which mostly consists of collagen and elastin fibers and would strongly affect the registered fluorescence.

More specific optical method, which provides information about the particular fluorophore in the tissue, is a time resolved fluorescence spectroscopy. The method allows to determine the number of fluorophores and to identify them by the characteristic fluorescence lifetime. This technique has been applied for investigations of tumorous and normal tissue [14,15], cardiac and vascular tissues [16]. The studies are mostly focused on the detection of atherosclerosis [17-20], while the cardiac conduction tissue has not been studied.

The differentiation between tissues could be more easily performed if the direct visualization of the tissue structure and components would be possible, however, the imaging of the tissue structures at the cellular level *in vivo* is still a challenging task. The ability to investigate biological objects with the resolution of a single cell is limited to a very few techniques, such as optical coherence tomography and confocal microscopy. Confocal microscopy is a technique that allows object imaging layer by layer, therefore high resolution and three dimensional view of an object is created. The simplest type of confocal microscopy is the reflection confocal microscopy (RCM). RCM offers non-destructive tissue sectioning to depths of up to 0.35 mm [21,22] with the capability of single cell identification. During the visualization procedure tissue is illuminated with infrared laser light and because of different reflections from the tissue structures the reflection image is obtained. Recently developed techniques enable confocal imaging even of live tissues [23-27]. The method also enables repetitive sampling without biopsy collection and does not cause any further damage to the areas under investigation. Virtual optical biopsy, based on RCM, has already been used for the assessment of benign and malignant lesions, and the findings show great potential for its applications in basic skin research and clinical dermatology [22,23,28-30]. Most reflective substances in the skin were found to be melanin, keratin and collagen, whereas contrast sources in the heart tissues are unknown, since cardiac tissues has never been investigated using RCM.

Summarizing all CCS investigations it could be concluded, that despite of the numerous studies, the visualization of the conduction system is still at the experimental level and still is not accurate. Most of the experiments have been done on the experimental animals and *ex vivo*. We could not find any study, where the human heart tissues have been investigated with optical methods *in vivo*.

II. MATERIALS AND METHODS

After a comprehensive analysis of the studies of cardiac tissues the experiments with different techniques were planned. The study with human materials has been approved by the Lithuanian Bioethics Committee. First of all a fluorescence imaging of the cardiac tissues has been performed. After that, tissue specimens have been investigated by steady state and time resolved fluorescence spectroscopy. Based on steady state fluorescence data CCS differentiation method has been established. We also carried out the first imaging experiments using RCM. Finally, the established CCS differentiation method has been tested *in vivo* during the surgery procedure.

Human heart samples from 24 subjects (of middle age, without heart pathology) were obtained at autopsy (not later that 48 h post mortem) by the pathologist in the National Centre of Pathology. Preparations were fixed in a 10 % neutral buffered formalin solution immediately after excision and kept in the dark at 4ºC. The hearts were washed with saline solution prior the excision of preparates in order to remove the remaining blood. The samples that were investigated with unwashed blood were measured immediately after the excision or kept in the dark at – 70 ^{o}C.

After the experiments, depending on the objectives, the tissue samples have been investigated histologicaly to identify/confirm the investigated tissue type.

The significance of the spectral differences has been evaluated in pairs between the fluorescence data of CCS and MC, as well as CCS and CT. The pair of MC and CT needed no evaluation due to intrinsic structural and obvious spectral differences. The testing within one specimen has been done using an independent t-Test. To test the significance of the difference between the fluorescence data from different specimens a paired t-Test has been applied.

2.1 Fluorescence imaging of cardiac tissues

Fluorescence imaging has been performed on interventricular septum. During the imaging procedure the tissue was illuminated with 365nm wavelength light. High pressure HG lamp ДРШ 250-3 (LS) was used as the light source. The group of lines around 365 nm was separated by the filter УФС8 (F1). The images were recorded by

digital camera Philips ToUcam Pro (C) and computer was used to process acquired images.

2.2 Steady state fluorescence spectroscopy of cardiac tissues

Specimens for steady state fluorescence spectroscopy were taken from 13 subjects. 18 specimens from 3 subjects were prepared from washed hearts, and 12 specimens were prepared from 2 unwashed hearts. Further studies were performed to test the established CCS differentiation method. In this stage eight human heart samples from 8 subjects were investigated.

During the method evaluation, the localization areas of the distinct types of the tissue (CCS, MC and CT) were preliminary marked by the pathologist based on anatomical features of the heart (Fig. 2.2.1).

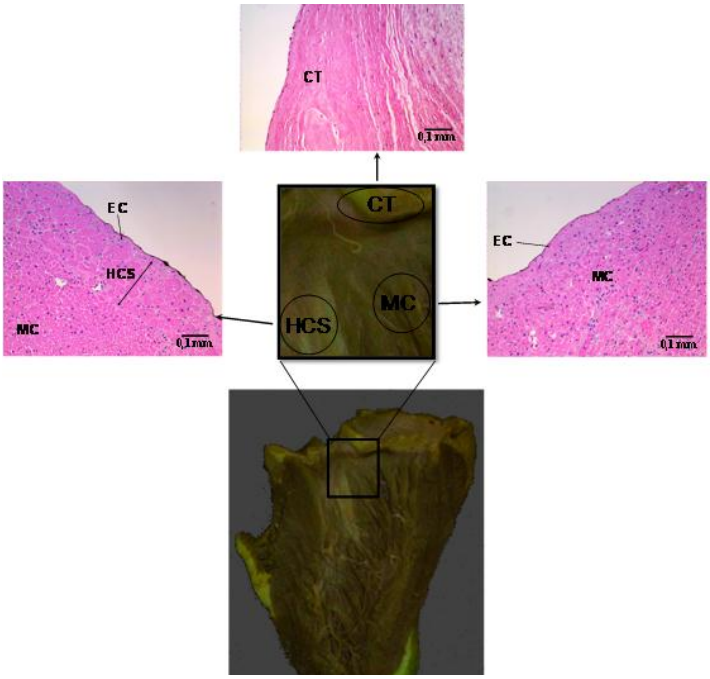

Fig. 2.2.1 The localization of distinct tissues was preliminary marked (circles) by pathologist and verified histologically after spectral measurements

After the spectral measurements the spots were investigated histologically. For that tissue samples were embedded in paraffin. Sections of 4 μm thickness were done perpendicularly to the surface with the Leica RM2145 microtome and were stained by hematoxylin and eosin according to the ordinary histology practice [31]. Histological

slides were investigated with an optical microscope Olympus BX41. The pictures were taken with an attached microscopy camera Pixelink PL-A662.

The autofluorescence spectra were measured by means of a spectrofluorimeter FLS920 (Edinburgh Instruments) Fig.2.2.2.

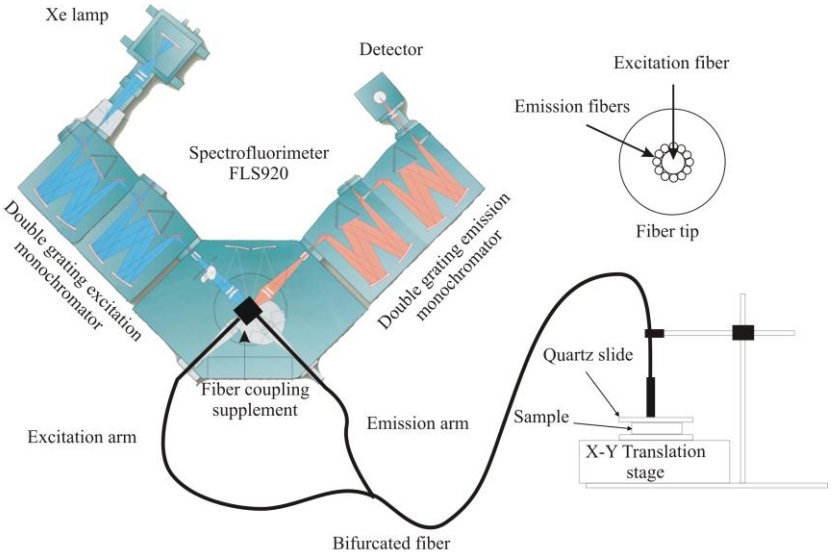

Fig. 2.2.2 The experimental setup and the fiber tip

The special fiber coupling supplement was installed in the place of a standard cuvette holder in order to measure the fluorescence through a fiber bundle probe. The fiber probe (Avantes) enabled us to measure the spectra from particular places of the specimens. It was composed of a bundle of parallel quartz fibers: a central one (600 μm in diameter) – for excitation and twelve (200 μm in diameter) being situated around the excitation fiber – for the fluorescence detection. Typical excitation light intensity at the fiber tip was ~ 50 μW.

The specimens were fixed between quartz slides and mounted on the X-Y translation stage. The thickness of the covering slide was 1 mm. A tip of the fiber was fixed in the holder and placed onto the slide surface. Different places of the specimen were selected for spectral measurements by translating the stage. It was assumed that the measurement conditions arranged in this manner had not changed for various spots of the same sample, thus allowing to compare the absolute intensities of the registered fluorescence spectra.

2.3 Spectral imaging of cardiac tissues

Spectral imaging has been performed on the specimens from 2 subjects. The experiments have been carried out using the established CCS identification method. During the CCS visualization procedure the differentiation was performed in two ways: at first – by scanning one specimen along one line with high precision, then – by performing planar scanning to test the applicability of the revealed spectral differences for the CCS visualization. During the precise linear scanning the distance between two investigated spots was 500 μm, while during the visual demarcation of CCS the planar area (11 mm x 15 mm) was scanned with the resolution of 1 mm. After the spectral measurements, four sections of the specimen were taken for histological analysis. One of the sections was taken along the scanning line and the rest were chosen randomly.

2.4 Time resolved fluorescence spectroscopy of cardiac tissues

Time resolved fluorescence spectroscopy experiments were performed on three specimens prepared from a single human heart. Spectroscopic studies were carried out on the diagnostically most important types of the heart tissue: CCS, MC and CT. The specimen that contained CCS was prepared from the left branch of His bundle. The specimen for MC was prepared from the ventricles and the CT specimen was prepared from the aorta. The location of particular tissue was marked on each specimen by the pathologist.

The spectroscopic measurements were performed by means of a spectrofluorimeter FLS920 (Edinburgh Instruments). A pulsed diode laser (λ = 405 nm, a pulse width – 66.9 ps) has been used for the excitation. The special solid sample holder (Edinburgh Instruments) was installed in the place of a standard cuvette holder in order to measure the fluorescence spectra and the fluorescence lifetimes of the heart specimens. To prevent the tissues from drying out the specimens were fixed between quartz slides and mounted on the holder. It was assumed that the measurement conditions arranged in this manner had not changed for different heart tissues, thus allowing to compare the absolute intensities of the registered fluorescence spectra. Lifetime measurements have been performed using the time correlated single photon counting (TCSPC) methodology. The fluorescence decay have been measured in 430 nm – 550 nm spectral region with a 5 nm step.

Fluorescence decay lifetimes (τ) and the fractional components of fluorescence intensity (F) were calculated using FAST software (Edinburgh Instruments). An instrument response function was measured from the polymer microspheres (Thermo Scientific) suspended in water.

2.5 Imaging of cardiac tissues using reflectance confocal microscopy

Confocal imaging experiments have been performed on 4 specimens from 4 subjects. For the investigations of the Purkinje fibers human heart tissues were selected from peripheral area of the interventricular septum. As a reference, CT and ordinary MC specimens were taken from the aorta and ventricles, respectively. Visualization of different types of the heart tissue has been performed using a reflectance confocal microscope and the specimens were then analyzed histologically. For confocal imaging, tissue samples of 2x2 cm in size were cut from the heart and no other special preparation of the tissue was required.

In confocal laser scanning microscopy, the tissue is scanned with a point-like laser beam. The laser beam is focused into the tissue and the reflected light traverses a confocal diaphragm on its way to the detector, blocking the passage of the light from levels over or under the focus level. During the scan, only the information of one point in three-dimensional volume reaches the detector. Hence, a high degree of depth discrimination is possible. The tissue is scanned in a point-by-point manner and a two-dimensional horizontal image of the confocal level (optical section) is produced. The procedure is repeated for the underlying parallel sections and a three-dimensional volume (up to a maximum depth of 0.35 mm) can be studied. The individual microstructures of the tissue possess differing reflection indices. This provides the image with its natural contrast. The main contrastive compounds in the skin are melanin, keratin and collagen, whereas only collagen is present in heart tissues. The varying reflection of the laser light by each point is measured and subsequently transformed into a digital image with different levels of gray.

To obtain confocal reflectance images, the Vivascope 1500 confocal laser scanning microscopy system (Lucid, USA) was used. In this system the tissue is illuminated with 830 nm wavelength laser light. The intensity of the laser light varied during the scan, but has not exceeded 15 mW. The lateral optical resolution of the

system was < 1.25 μm and vertical resolution was < 5 μm. The individual images were 500 μm x 500 μm in size. Respective individual images could be binned and the largest area that could be depicted with the resolution of a single cell was 8 x 8 mm. During the visualization procedure, the heart tissue specimen was fixed to the microscope imaging window. The areas of interest were scanned layer by layer from the surface to the deeper layers; therefore, comprehensive analysis of the heart tissue could be performed.

2.6 Differentiation of cardiac tissues *in vivo* during the surgery procedure

The procedure has been performed in a single subject. The study has been approved by the Lithuanian Bioethics Committee. 72 fluorescence spectra from 24 different points in the heart have been measured.

Differentiation of cardiac tissues has been performed using the developed CCS identification method. For this study, the compact optical biopsy system, suitable for the examination of biological tissues during the surgery, has been assembled. System is shown in Figure 2.6.1.

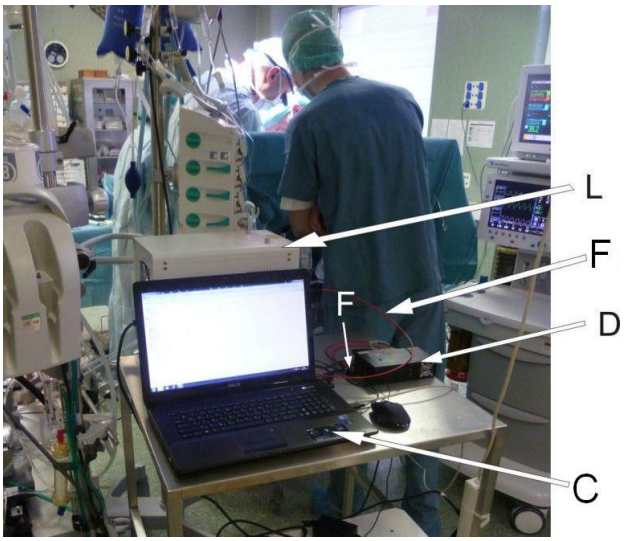

Fig. 2.6.1 Compact optical biopsy system. L – light source. F – fibers. D – detector. C – computer.

Main components of the system are the double wavelength light source (L) and the detector (D). The light from the source is focused to one arm of the bifurcated fiber (F) and through it is transmitted to the tissue. Then fluorescence is collected and transmitted to the detector. Fluorescence spectra are recordered to the computer. The calculations and evaluations have been done after the measurements.

III. RESULTS AND DISCUSSION

3.1 Fluorescence imaging of cardiac tissues

Visualization of the heart tissues were performed according to previously determined fluorescence excitation wavelength [32,33]. In that study it had been shown, that CCS fluorescence is one and a half time higher that MC fluorescence under 365 nm wavelength light excitation. The fluorescence imaging results are presented in Fig. 3.1.1.

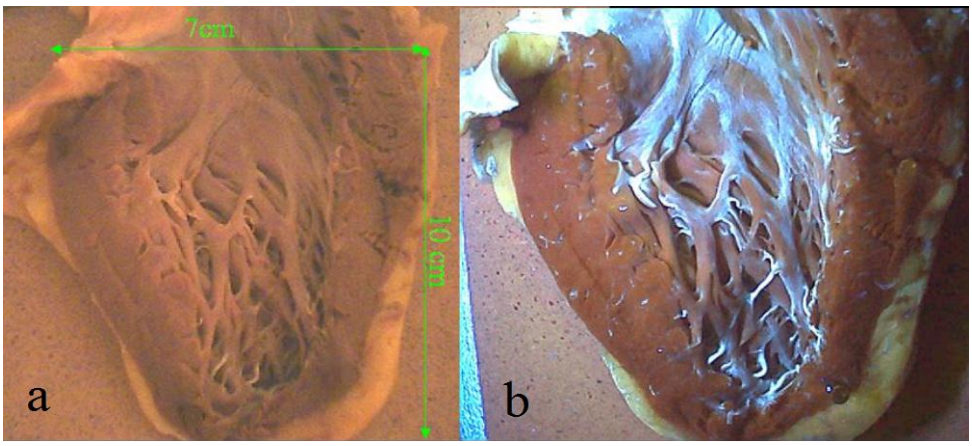

Fig. 3.1.1. The images of the interventricular septum: a) in the daylight, b) under illumination at 365 nm.

In the daylight image (Fig.3.1.1A) the cardiac internal structure and trabeculae are clearly visible; however, the localization of the conduction system could only be predicted according to the anatomy atlases. Whereas fluorescence image (Fig.3.1.1B) seems to be more informative. Obvious fluorescing bundles could be identified. The fluorescing pattern correlates with the localization of left His bundle branch. At the top it is visible a wide left branch that branches down to the bottom of the interventricular septum and ends with thin Purkinje fibers, visible as the bluish spots in MC. From these pictures it is obvious, that the determination of exact localization of CCS without any visualization method could be very complicated. However, the comprehensive analysis of the fluorescence images revealed, the not only CCS exhibit high fluorescence intensity. The structures that are rich of collagen and elastin also possess intense fluorescence; therefore CCS could not be distinguished from fibrous tissues. CT, which is the main type of fibrous tissue in the heart, is widely distributed in the heart, therefore it is essential to distinguish CCS from CT during visualization procedure. For this

purpose more sophisticated method, based on spectral properties of different tissues must be created in order to distinguish CCS from CT.

3.2 Steady state fluorescence spectroscopy of cardiac tissues

Fluorescence spectra of the heart tissues covered by the endocardium were registered on the preliminary marked places of the specimen. The apparent differences between the fluorescence spectra of the heart tissues were observed under excitation at 330 nm and at 380 nm. Typical fluorescence spectra of CCS, CT and MC registered on the same specimen of the heart through a layer of EC are presented in Fig.3.2.1.

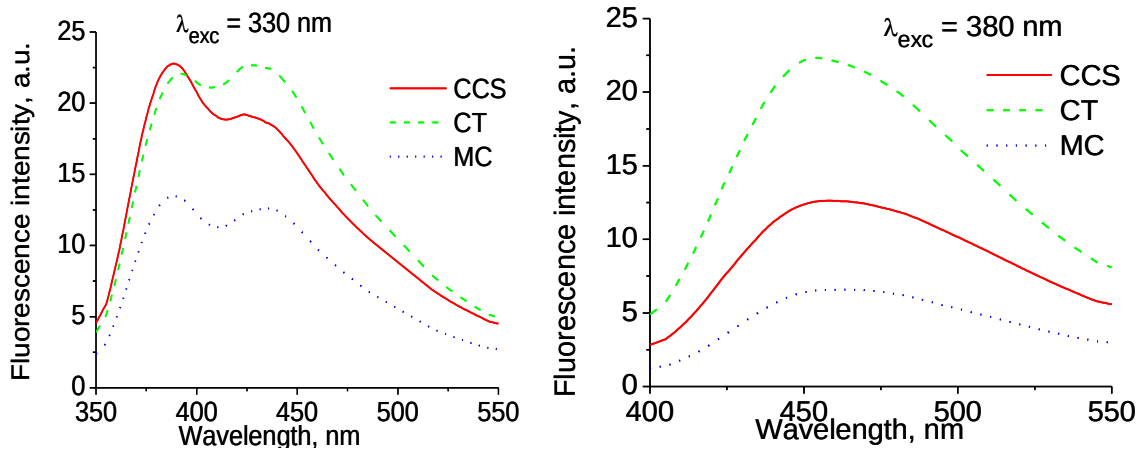

Fig.3.2.1 Fluorescence spectra of CCS, CT and MC. Spectra were measured on the same sample maintaining the same measurement conditions.

Similarly, as it was observed for the endocardium free tissues [34], the CCS covered by EC has higher fluorescence intensity than MC under 330 nm or 380 nm excitation. CT has higher fluorescence intensity than CCS when excited at 380 nm and the similar overall intensity when excited at 330 nm. It can be concluded that the excitation of the heart tissues at 380 nm results in the highest differences in fluorescence intensity, while the excitation at 330 nm results in the most apparent differences in spectral shape. As shown in Fig. 3.2.1, when excited at 330 nm all fluorescence spectra of different types of the tissue are composed of two overlapping bands with peaks around 390 nm and 430 nm, which possess different intensity distributions. However, our previous findings [34] revealed that there are no such bands in either fluorescence or fluorescence excitation spectra that would be specific for any type of the heart tissue.

This suggests that there is no specific fluorophore in CCS, CT or MC. The electrophoresis studies that have been performed on tissue extracts [35] also revealed that CCS, CT and MC contain similar proteins, but in different amounts. Consequently, the origin of the observed spectral differences is primarily the result of different amounts of collagen and elastin (330 nm is near an absorption peak of collagen, and 380 nm is mostly absorbed by elastin) [36]. These results imply that the highest contrast between different heart tissues during their spectral characterization should be obtained employing spectral properties of both collagen and elastin. The only limitation is the reabsorption of the fluorescence by blood. Peak absorption of HbO_2 and Hb is in the region of 410 nm – 430 nm, therefore, the registration should be performed around 460 nm, where the absorption of blood is minimal in the region of interest. Based on these findings the spectroscopic method is suggested for the differentiation of the CCS.

The main idea of the method is to excite autofluorescence in turn at 330 nm and at 380 nm and to register fluorescence intensity twice only at one wavelength – 460 nm. Then, calculate the characteristic ratio of the intensities $I^{ex}_{330}(460)/I^{ex}_{380}(460)$. The relative changes in fluorescence intensity are different for CCS, CT and MC (Fig.3.2.2).

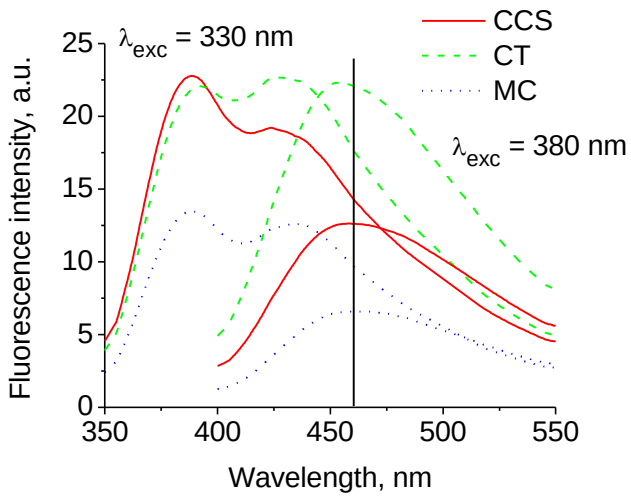

Fig.3.2.2 Fluorescence spectra of CCS, CT and MC tissues under 330 nm and 380 nm excitation. A vertical line marks 460 nm

The calculated ratios are summarized in the table 1. Table 1 also presents values of the significance levels $P(H_0 = 0)$.

Table 1. Fluorescence intensity ratios calculated at 460 nm under excitation at 380 nm and 330 nm.

Specimen	$<R^{CT}>(460)$ ±SD [n]	$P(H_0 = 0)$ $x10^{-3}$	$<R^{CCS}>(460)$ ±SD [n]	$P(H_0 = 0)$ $x10^{-3}$	$<R^{MC}>(460)$ ±SD [n]
1			1.01 ± 0.03 [3]	*6.9*	1.31 [1]
2	0.92±0.01 [2]	*49.8*	1.14 ± 0.13 [4]	*4.1*	1.60 ± 0.15 [3]
3	1.12 [1]	– *	1.64 [1]	– *	1.94 [1]
4	0.84±0.04 [2]	*17.2*	1.22± 0.09 [2]	*26.3*	1.49 ± 0.01 [2]
5			1.08 ± 0.1 [3]	*1.28*	2.16 ± 0.16 [2]
6			1.85 ± 0.02 [2]	*21.5*	2.23 [1]
7			2.75 ± 0.02 [2]	*12.7*	3.98 ± 0.28 [2]
8			1.59 ± 0.02 [2]	*5.35*	3.14 [1]
Averaged mean ratio	**0.96±0.14 [5]**	***(25.0)***	**1.54±0.58 [19]**	***(3.10)***	**2.23± 0.91 [13]**

$<R^{CT}>$, $<R^{CCS}>$and $<R^{MC}>$ – mean values of the corresponding ratios of the same sample

SD – standard deviation

[n] – represents the number of measurement points

$P(H_0 = 0)$ – values of significance levels for the null hypothesis ($<R^{CCS}> - <R^{MC}> \geq 0$, $<R^{CCS}> - <R^{CT}> \leq 0$)

* The $P(H_0 = 0)$ value could not be calculated because the difference of the standard error is 0. However at the 0.05 level, the two values are significantly different

The mean ratios $<R^{tiss}>(460)$ obtained for each type of the tissue were quite scattered comparing between different specimens (Table 1, values in columns 2, 4, 6). Such variation is caused by the quantitative differences in the inner composition of the tissues in various hearts. On the other hand, the differences between the ratios of different heart tissues in the same specimen were found to be significant. As a rule, the mean ratio of the CCS was significantly higher than the ratio of CT and smaller than the ratio of MC (Table 1, values in rows).

The $P(H_0 = 0)$ value obtained for CCS and CT (0.025, three specimens) indicates that using the proposed methodology the chance of confusing CCS with CT in a randomly chosen heart specimen is less than 3 %. The corresponding $P(H_0 = 0)$ value for CCS and MC (0.0031, eight samples) shows even a greater possibility to distinguish between these tissues on the basis of the obtained ratios.

The proposed method can be modified taking into account the variations observed between the hearts. For that all ratios in each specimen were normalized to the mean ratio of CCS for that specimen. The normalized mean values $<R^{tiss}{}_n>(460) = <R^{tiss}>(460)/<R^{CCS}>(460)$ are shown in table 2.

Table 2. Values of the fluorescence intensity ratios calculated at 460 nm under excitation at 330 nm and normalized to the ratio of CCS in the same sample.

Specimen	$<R^{CT}{}_n>(460)$	Confidence interval	$<R^{CCS}{}_n>(460)$	Confidence interval	$<R^{MC}{}_n>(460)$
1			*1 ± 0.03*		*1.3*
2	*0.81±0.01*		*1 ± 0.11*		*1.40±0.13*
3	*0.68*		*1*		*1.18*
4	*0.69±0.03*		*1 ± 0.07*		*1.22±0.01*
5			*1 ± 0.09*		*2.00±0.15*
6			*1 ± 0.01*		*1.21*
7			*1 ± 0.01*		*1.45 ±0.1*
8			*1 ± 0.01*		*1.97*
Averaged	***0.73±0.07***	**0.15**	***1 ± 0.11****	**0.24**	***1.47±0.33***

$<R^{CT}{}_n>$, $<R^{CCS}{}_n>$ and $<R^{MC}{}_n>$ – mean ratios of the corresponding tissue normalized to the $<R^{CCS}>$ value of the same sample.

* the maximal standard error value has been taken.

This normalization provides the additional possibility to calculate the confidence intervals that were calculated with significance level of 0.05. In the case of CCS and CT "0.15" indicates that $R^{tiss}{}_n(460)$ higher than 0.85 can be attributed to CCS. In the case of CCS and MC "0.24" indicates that $R^{tiss}{}_n(460)$ higher than 1.24 do not belong to CCS and could be attributed to MC. Thus, the obtained confidence interval $0.85 < R^{tiss}{}_n(460) < 1.24$ determines the heart tissue, which on the basis of the spectral properties can be attributed to CCS with 95% probability.

On the other hand, if it is not possible to find a plausible CCS location in the heart during the surgery procedure, the tissue differentiation could be started by measuring reference values from MC. In this case the averaged $<R^{MC}{}_n>$ value is also needed (Table 2). $<R^{MC}{}_n>(460)$ value of 1.47 being calculated from 8 specimens (hearts) indicates how much the ratio of the MC is expected to be higher than the ratio of the CCS in the same heart. This number can be assigned as a reference value V_{MC}. In order to ensure a higher reliability, this value obviously should be calculated from the higher number of specimens. Then, the CCS ratio could be calculated in the following way: $<R^{CCS}>(460) = <R^{MC}>(460)/V_{MC}$, where $<R^{MC}>$ is the average of the measured reference values from MC. Then, the upper and the lower limits of CCS ratio can also be evaluated from the corresponding confidence intervals (table 2).

3.3 Spectral imaging of cardiac tissues

The precise spectroscopic demarcation of CCS boundaries was demonstrated by performing the linear scanning. Like in the previous experiments, the fluorescence was excited at each spot in turn at 330 nm and at 380 nm and the fluorescence intensity was registered at 460 nm.

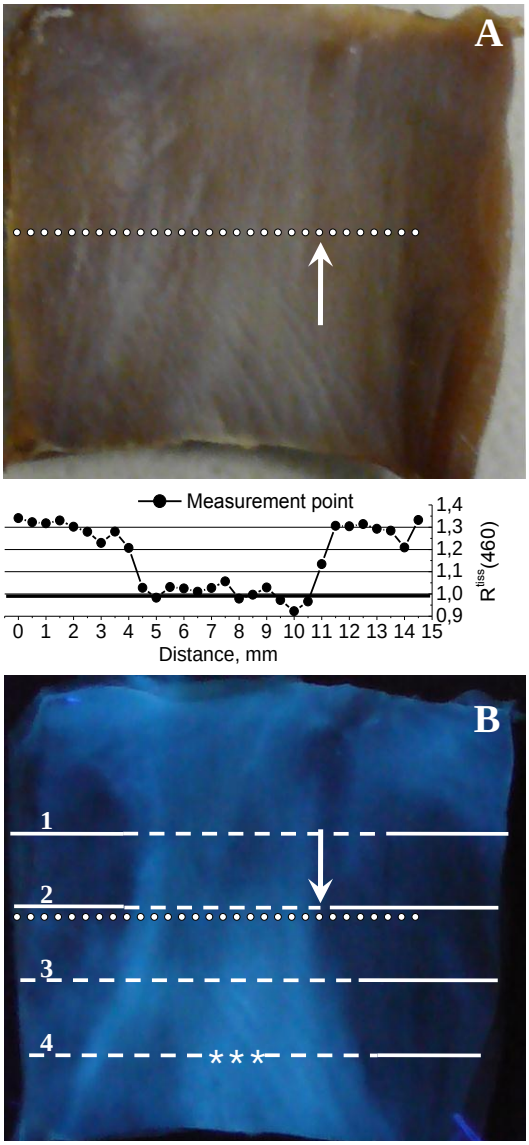

Fig.3.3.1 Linear scanning of the sample. Panel A – the image of the specimen in the daylight. Points where the ratios were measured along the line are marked by dots. Panel B – the image under 365 nm irradiation. The ratios R(460) = $I^{ex}_{330}/I^{ex}_{380}$ calculated for each spot are presented between the images.

The specimen as it looked in the daylight is presented in Fig.3.3.1A and the image of the specimen under 365 nm illumination is presented in Fig.3.3.1B. Four lines (1-4) represent the sections, where the histological analysis was carried out. The location of the CCS in each section is marked by the dashed part of the line. The solid part of the

line corresponds to the MC tissue. Stars mark the location of CT, which was found only in section 4.

The ratio $R^{tiss}(460)$ from the histologically confirmed CCS area (dashed part of the histological section No.2) is around 1. The ratio $R^{tiss}(460)$ from the histologically confirmed MC area (solid parts of the histological section No.2) is around 1.3. Also, there was a spot ($R^{tiss}(460)$ value around 1.15), which, as mentioned above, corresponded to neither type of the tissue – judging from the fluorescence image of the specimen and the histological analysis it was located on the edge between two different types of the tissue (shown by an arrow on Fig.3.3.1 picture).

The applicability of the revealed spectral differences for the visualization of the CCS tissue was demonstrated by the planar scanning of the same specimen. In this case the spectral image was obtained, each point of which corresponded to the calculated value of the ratio $R^{tiss}(460) = I^{ex}_{330}/I^{ex}_{380}$. The boundaries of the scanned area are marked on the daylight picture (Fig. 3.3.3).

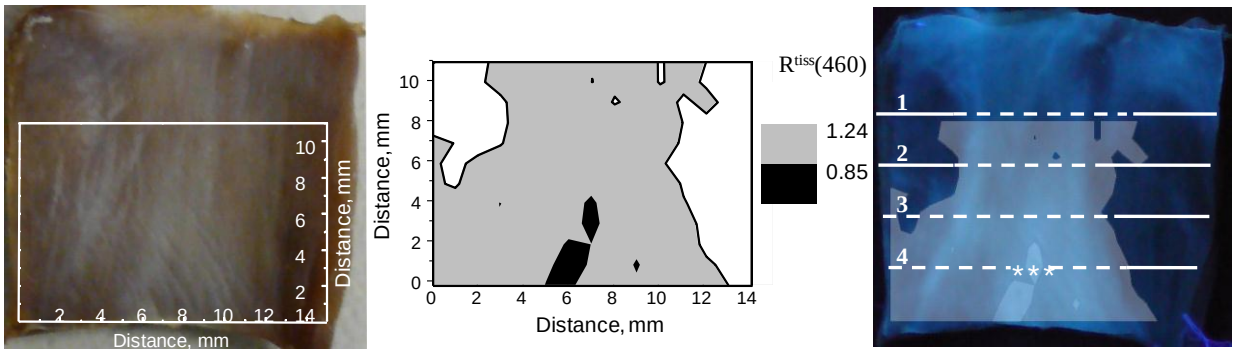

Fig.3.3.3 Planar scanning of the specimen. Picture in the daylight (left panel); spectral image (middle panel); combined picture of spectral and fluorescence image (right panel). Four lines in the right image mark sections of the sample taken for histological studies

Based on the data from the linear scanning and the confidence intervals, the area that possessed $0.85 < R^{tiss}(460) < 1.24$ was attributed to CCS with probability of 95%. The digital spectral image was then overlaid on the photograph of the sample illuminated at 365 nm (right panel, Fig. 3.3.3). As it is seen on the combined picture, the tissue area that was marked by ratio values in the range of 0.85 – 1.24 closely overlaps with the fluorescing area visible on the image. Slight variations of the boundaries could be due to the limited scanning resolution. Spectral scanning also revealed the area of the specimen where $R^{tiss}(460)$ was lower than 0.85. This area is marked black in the digital

picture (middle panel, Fig. 3.3.3). According to the spectral analysis it should be attributed to CT. The subsequent histological analysis of the tissue confirmed the presence of the CT in the central part of the section 4.

3.4 Time resolved fluorescence spectroscopy of cardiac tissues

Fluorescence lifetime is the characteristic average time that a fluorophore spends in the excited state following excitation from its ground energy level. Fluorescence lifetime varies with the molecular environment but usually does not depend on fluorophore concentration or signal attenuation caused by absorption or scattering [37-39], therefore the number of fluorophores could be determined even their fluorescence spectra overlaps.

The experimental data of the CCS fluorescence decay approximated by one and two exponentials are presented in Fig. 3.4.1.

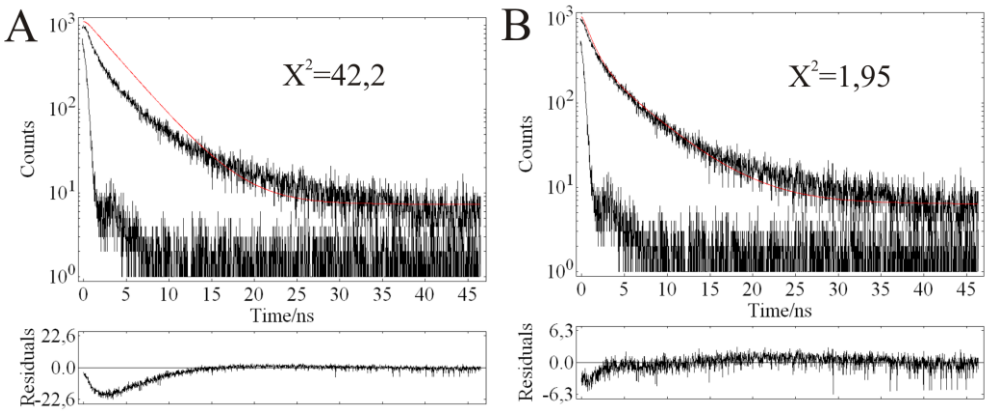

Fig 3.4.1. CCS fluorescence decay approximated by one (picture A) and two (picture B) exponentials.

The number of exponentials corresponds to the number of expected fluorophores. The fitted curves do not correspond to the measured decay that additionally could be demonstrated by the residuals and the increased χ^2 value. This indicates that more than one or two different components are forming the shape of the fluorescence decay curve.

The experimental data of MC, CCS and CT fitted by three exponentials are presented in Fig. 3.4.2.

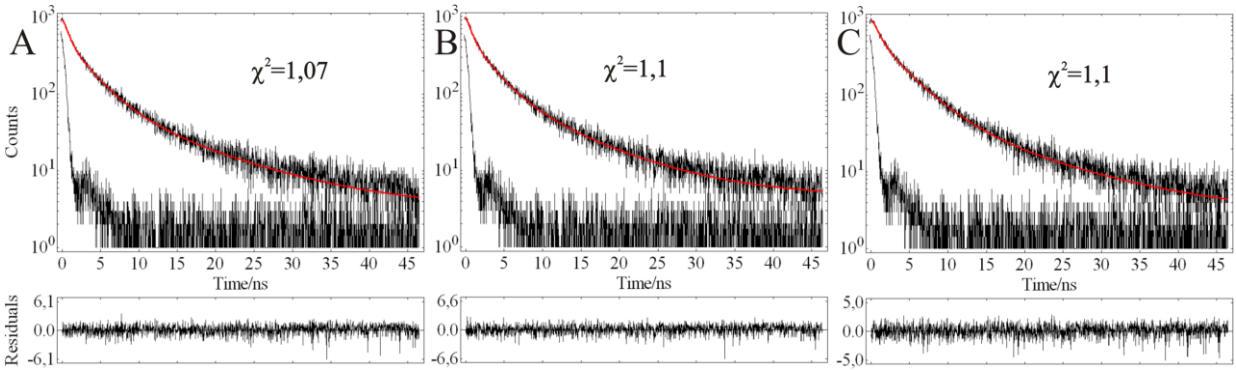

Fig.3.4.2 Fluorescence intensity decay registered at 482 nm. MC (picture A), CCS (picture B) and CT (picture C).

The fitted decay curves closely correlate with the measured values as this could be seen from residuals and χ^2. Thus, according to the mathematical analysis, at least three fluorophores are responsible for the fluorescence signal of all three cardiac tissues. The lifetimes of every tissue did not change throughout the spectral region of 430 nm – 550 nm.

The average lifetimes of the fluorophores calculated through the whole spectral region (430 nm – 550 nm, n = 25) and the standard deviations are presented in table 1.

Table 1. The average lifetimes (in ns) and their standard deviations. $P(H_0 = 0)$ – values of significance levels for the null hypothesis.

	MC	$P(H_0 = 0)$	CCS	$P(H_0 = 0)$	CT
τ_1	0.7 ± 0.1	0.07	0.7 ± 0.1	2.9×10^{-5}	0.9 ± 0.1
τ_2	3.1 ± 0.2	0.19	3.2 ± 0.3	2.2×10^{-4}	3.5 ± 0.4
τ_3	10.6 ± 1	0.94	10.7 ± 1.2	0.84	10.7 ± 1.7

The significance of the difference between averaged lifetimes of CCS and other tissues was evaluated using a paired t-Test for the null hypothesis $\tau^{CCS} - \tau^{CT} = 0$ and $\tau^{CCS} - \tau^{MC} = 0$.

The main fluorophores in the heart tissue were shown to be collagen and elastin [9,13,40,41], however, collagen in the heart tissues is found in several forms. The cardiac collagen of extracellular matrix consists of 85% type I collagen [42]. In addition to type I, other fibril forming collagen types found in the heart are type III and V [43]. The heart valve consists of collagen I (74 %), collagen III (24 %) and collagen V (2 %) [44]. Therefore, the fluorophores that mostly contribute to the registered fluorescence

decay in all three tissue types could be identified as elastin, collagen I and collagen III. The fluorescence spectrum of elastin overlaps with the fluorescence spectra of collagen III, therefore, it could be hardly distinguished by applying only techniques of steady state spectroscopy.

To determine the contribution of each fluorophore in the fluorescence spectra the fractional components of the fluorescence intensity (F) have been calculated. The average contribution values of the first, the second and the third constituent to the fluorescence spectra measured in the spectral region of 430 nm – 550 nm are presented in table 2.

Table 2. The relative contribution of different fluorophores to the fluorescence spectra. $P(H_0 = 0)$ – values of significance levels.

	MC	P(H0 = 0)	CCS	P(H0 = 0)	CT
F1	27 ± 3	0.09	28 ± 2	3.34×10^{-8}	20 ± 4
F2	46 ± 2	0.57	47 ± 2	1.51×10^{-5}	51 ± 4
F3	27 ± 3	0.08	25 ± 3	0.01	29 ± 5

The significance of the difference between averaged contribution values of CCS and other tissues was evaluated using a paired t-Test for the null hypothesis $F^{CCS} - F^{CT} = 0$ and $F^{CCS} - F^{MC} = 0$.

As it is seen from the table 2, muscular type tissues – MC and CCS – had very similar composition of fluorophores, while statistically significant differences were observed between CCS and CT (see table 2). The first constituent is present in larger amounts in an elastic tissue – CCS, whereas a stiff tissue like CT contains larger amounts of the second constituent. According to this analysis, the first constituent corresponds to elastin, the second – to collagen I and the third constituent corresponds to collagen III.

The estimation of the time-resolved autofluorescence data yielded statistically unreliable difference between these values from CCS and MC. Therefore, these two tissues could not be distinguished by fluorescence decay analysis only. On the other hand, some differences were observed between the time resolved data from CT and CCS. The decay constants τ_1 and τ_2 of CCS appeared to be significantly different from those of

CT (table 1). The relative constituents F1 – F3 of the spectral composition were also found to be significantly different between CCS and CT (table 2).

3.5 Imaging of cardiac tissues using reflectance confocal microscopy

Various types of heart tissues have been distinguished using RCM. The inner surface of the heart – EC is composed mainly of two layers: endothelial layer and CT layer composed mostly of collagen fibrils and CT cells. Endothelium – the most superficial layer of EC is composed of a single sheet of endothelial cells, CT cells and collagen fibrils. The reflectance image of endothelium is presented in Fig. 3.5.1A.

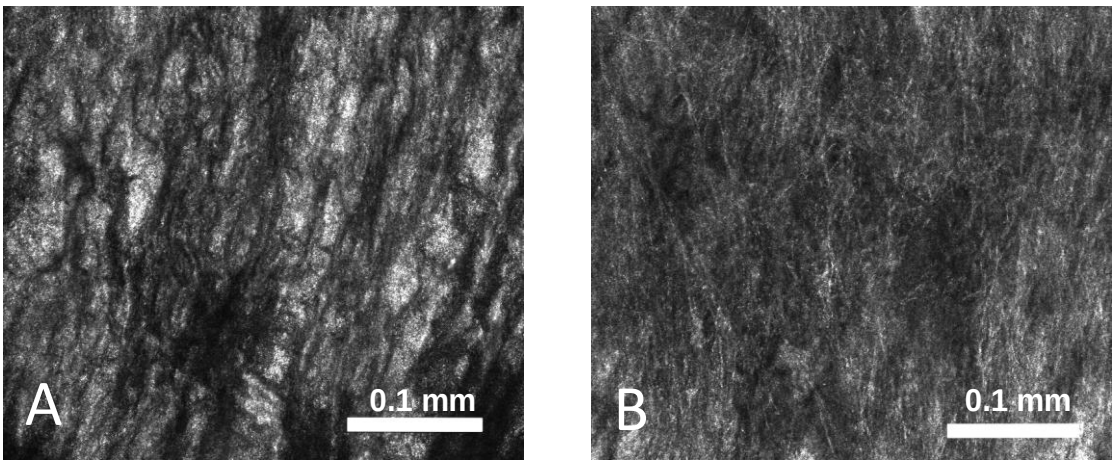

Fig.3.5.1 Reflectance images of different layers of EC. A – image of the most superficial – endothelial layer; B – image of the deeper – CT layer at 16 μm depth.

The endothelial cells are very thin (around 3 um), therefore they could not be distinguished. The bright areas visible on the surface of EC in Picture 3.5.1A is the result of the rough surface of the collagen covering the deeper layers of the EC. The bright network visible in 3.5.1B picture is the collagen fibers composing the second main layer of EC. The dark areas visible between collagen fibers represent the intercellular ground substance. The thickness of EC depends on the location and varies from 10 till 200 um [45].

Under the endocardium lying MC contains myocytes, interstitium with CT cells, elastin and collagen fibers and blood vessels. In some parts of the heart the CCS could also be detected. The MC tissue could be clearly distinguished in the confocal reflectance images (Fig.3.5.2).

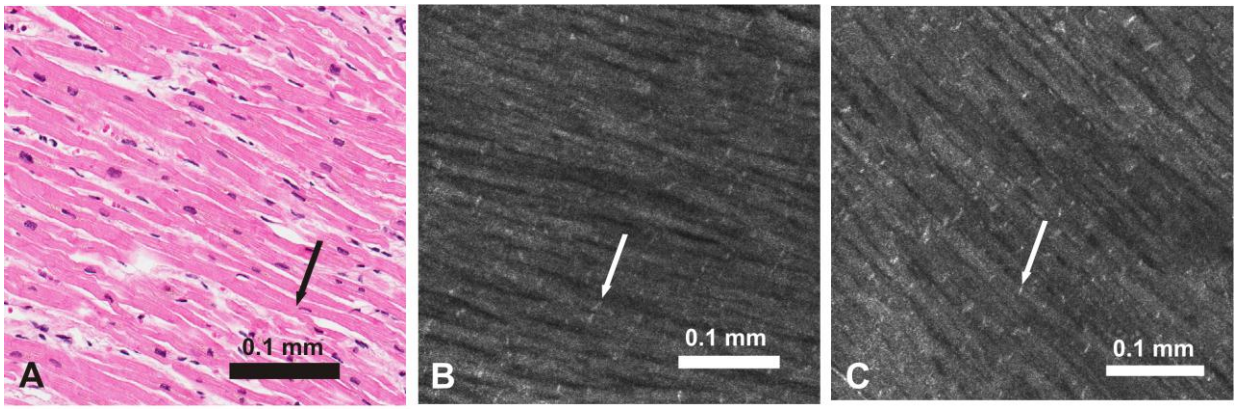

Fig.3.5.2 Ordinary myocardium. A – the view of MC slice stained with hematoxylin and eosin. B – the view of MC obtained by RCM. The depth is around 20 μm from the tissue surface. C – MC cells, the depth is around 35 μm. Intercalated discs (shown by arrows) could be more clearly distinguished in the reflectance images.

The actin and myosin fibers present in myocytes reflect the laser beam and result in grey color of myocytes, whereas interstitium is visible as dark areas. Intercalated disks between myocytes could also be differentiated (Fig.3.5.2, arrows). Intercalated discs are rich of proteins and exhibit high reflection. Therefore these junctions are visible as bright structures. The average diameter of the single myocyte was found to be around 10 μm.

Moreover, it was possible to distinguish the cells different from myocytes. These cells are much thicker, are organized as the fibers and are surrounded by ordinary myocytes arranged in the same direction (Fig. 3.5.3).

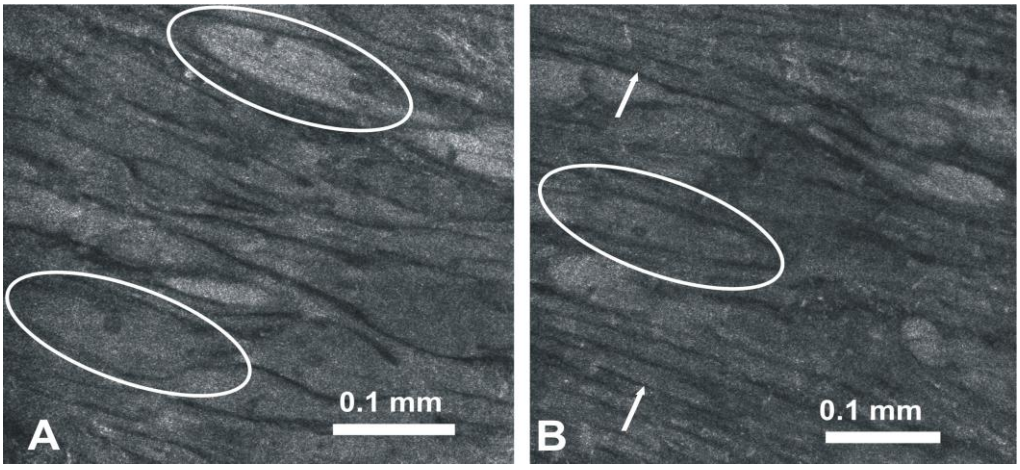

Fig.3.5.3 Reflectance images of the heart tissue at 50 μm depth. A - the image of large cells that differ from myocytes. The large cells are outlined by white contours. Nuclei are visible as the dark spots in the cells. B – the image showing that cells are organized as fibers and are surrounded by myocytes. White contour mark the large cell and arrows mark myocytes.

It is known, that Purkinje cells are responsible for signal transmission in the peripheral part of the heart [7]. In the branches of the CCS the Purkinje cells are interlaced with the myocytes and present in relatively small amounts forming Purkinje fibers. The Purkinje cells have an average diameter of ~ 46 μm and cells, which are visible in the reflectance images in Fig.3.5.3, also have the same diameter. According to the respective facts, these cells could be recognized as the Purkinje cells. Another characteristic feature of CCS is that the branches are surrounded by the isolating layer of the fibrous CT (Fig.3.5.4, arrows No.2).

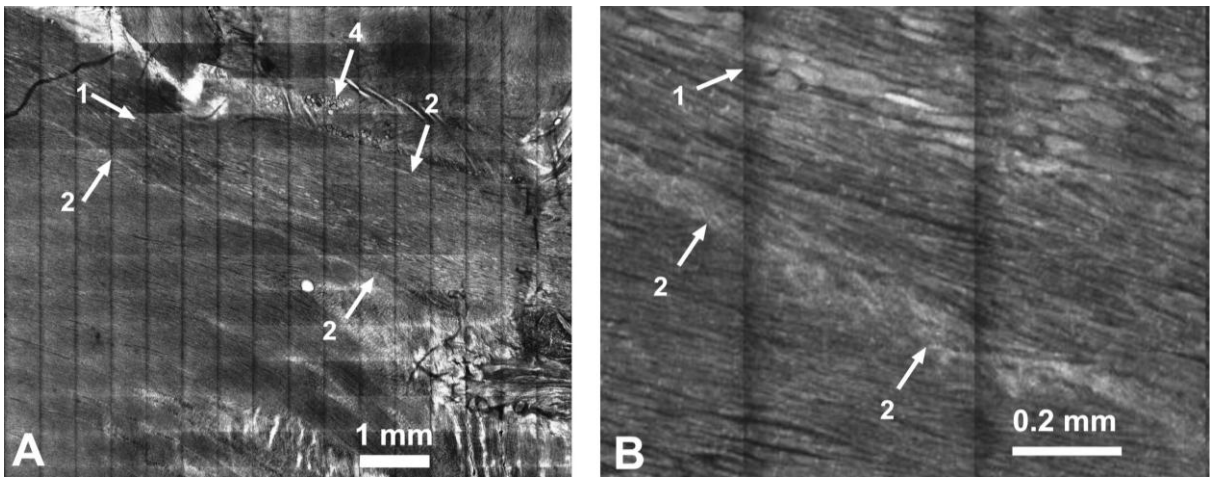

Fig.3.5.4. Reflectance images of the heart tissue at a depth of 55 μm. A – strictly confined right bundle branch of His. B – the boundary between the branch and ordinary MC. Arrow No.1 shows Purkinje cells that are situated like fibers and create a bundle-like structure with the surrounding myocytes. Arrow No.2 marks the fibrous CT that separates the bundle from the ordinary MC. Arrow No.4 marks round lypocytes.

In the background of ordinary myocardium a strictly confined bundle of CCS could be differentiated. The bundle is composed of Purkinje cells that are situated like fibers and are surrounded by myocytes (Fig.3.5.4 arrows No.1). The isolating layer of the bundle is made of CT and is visible as thin stripes of matted collagen fibers (Fig.3.5.4, arrows No.2). In the upper part of Fig.3.5.4A the aggregation of rounded lipocytes is visible outside the CCS bundle in the CT.

3.6 Differentiation of cardiac tissues *in vivo* during the surgery procedure

During the surgery 24 points in the heart have been investigated. 10 points have been measured from surface of the valve – therefore it could be clearly attributed to CT.

4 points have been measured from the ventricles – they could be attributed to MC. 10 points have been measured near the incision site where the location of CCS was sought.

Primary results of *in vivo* performed procedure revealed, that the distribution of fluorescence intensities are similar to those, observed during *ex vivo* experiments. Typical fluorescence spectra of different types of cardiac tissues are presented in Fig.3.6.1.

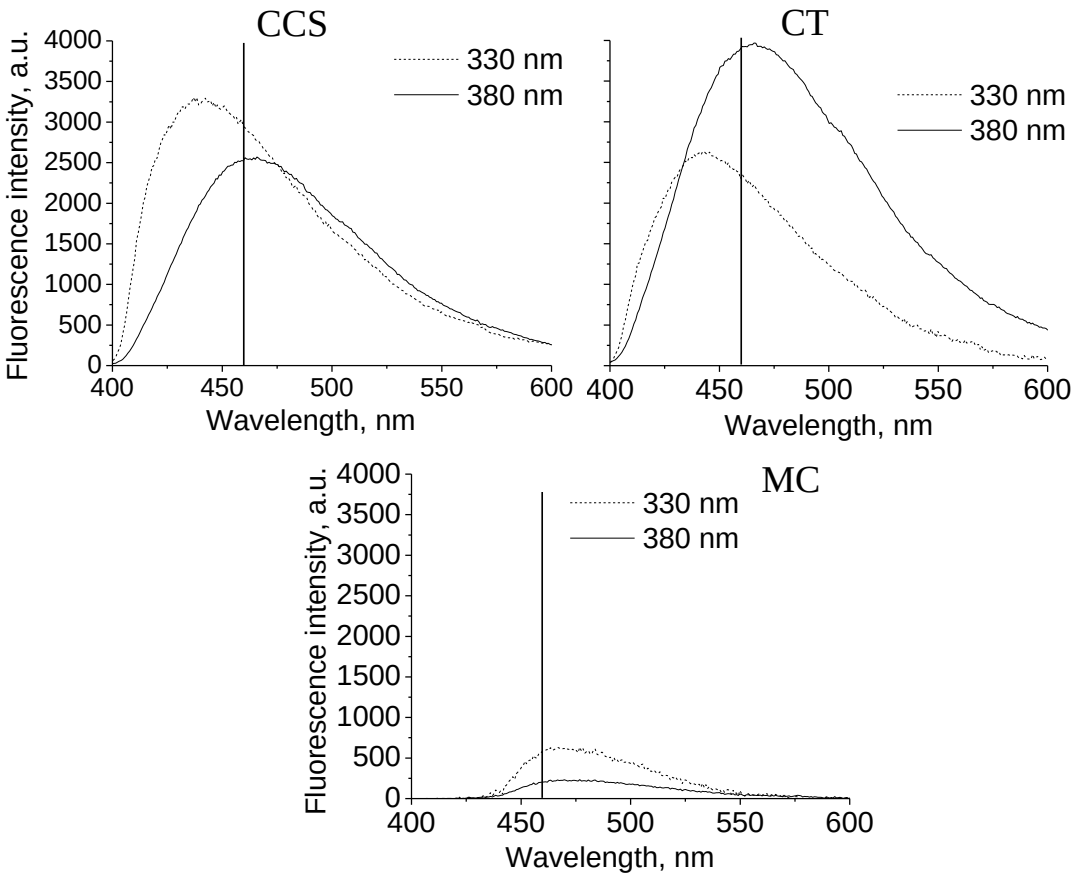

Fig.3.6.1 Typical CCS, CT and MC fluorescence spectra measured from live tissue. Fluorescence was excited in turn with 330 nm and 380 nm. The vertical line marks 460 nm, where intensity ratios have been calculated.

CT possesses the highest fluorescence intensity, while the weakest intensity was registered from MC. In those places of the heart, where CCS occurrence was expected, the fluorescence was weaker than CT. After the detailed analysis of the registered spectra some points were excluded because of obvious distortion of the spectra. During the measurements the fiber was held in one point for about 30 seconds and in some cases the position of the fiber had changed during the procedure. This resulted in alteration of fluorescence by the extrinsic light or by the blood that has

accumulated under the fiber tip. 3 of 10 measured points from CT and 3 of 10 measured points from possible CCS tissue have been excluded from calculation. The other points of CCS, CT and MC have been analyzed according to the intensity ratios method introduced in section 3.2. The characteristic $<R^{tiss}>(460)$ values have been calculated: $<R^{CT}>(460) = 0.9 \pm 0.2$; $<R^{MC}>(460) = 2.8 \pm 0.6$. Based on these values the interval for CCS tissue has been determined: $1.1 \leq CCS \leq 2.2$. According to this interval, only 2 points could be identified as CCS ($<R^{CCS}>(460)$ values 1.1 and 2.2).

The measurements were performed using far from perfect diagnostics system; however, the data so far seem encouraging in the view of the possibility to apply the fluorescence methods for the differentiation of CCS during surgery. This valuable experience also defined further trends for development of methodology and equipment.

IV. CONCLUSIONS

1. Fluorescence spectroscopy studies of cardiac tissues revealed, that most distinct spectral differences between CCS and the surrounding tissues were observed in 400 nm – 550 nm region under excitation from 330 nm – 380 nm region.
2. The evaluation of blood reabsorption revealed, that spectral region around 460 nm is the most suitable for an unambiguous differentiation of the CCS avoiding the absorption peak of blood.
3. The visualization method, based on the intensity ratios calculated for two excitation wavelengths, has been established:

$$< R_{tiss} > (460) = \frac{I^{ex}_{330}}{I^{ex}_{380}}$$

The calculated value $<R_{tiss}>(460)$ is different for CCS, CT and MC tissues, therefore the method may be used for identification of CCS. Moreover the method allows determination of CCS bundle margins and even visualization of small areas.

4. Time resolved fluorescence spectroscopy revealed no significant difference in composition and lifetimes between muscular type tissues (CCS and MC). On the other hand, the lifetimes (τ_1 and τ_2) and the relative spectral composition (constituents F1 – F3) of CT differed significantly from those of CCS. The observed differences could be explained by relatively larger amounts of elastin present in CCS and larger amounts of collagen in CT.
5. RCM allows visualizing MC, CT, Purkinje cells and CCS bundles because of different reflection properties of tissue components and their specific distribution inside the tissue. Therefore RCM method is suitable for non-destructive cardiac investigations and for CCS imaging.
6. The results of *in vivo* performed procedure revealed, that the distribution of fluorescence intensities are similar to those, observed during *ex vivo* experiments, therefore the established CCS identification method, based on intensity ratios, is suitable for cardiac investigations *in vivo*.

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VI. SANTRAUKA

Pagrindinė širdies funkcija yra tinkamas žmogaus organizmo aprūpinimas krauju, kas didžiąja dalimi priklauso nuo tvarkingo ir ritmingo širdies darbo. Širdies plakimo ritmą užduoda ir elektrinio impulso sklidimą kontroliuoja širdies laidžioji sistema (ŠLS) sudaryta iš sinusinio (SA) bei atrioventrikulinio (AV) mazgų, Hiso pluošto (HP) bei Purkinje skaidulų tinklo. Atliekant širdies operacijas yra ypatingai svarbu identifikuoti ŠLS lokalizaciją ir nepažeisti šios gyvybiškai svarbios sistemos, tačiau dėl raumeninės kilmės ji yra sunkiai vizualiai atskiriama nuo darbinio širdies raumens miokardo (MK). ŠLS yra išsidėsčiusi visose širdies srityse ir kol kas, dėl sudėtingo šios sistemos aptikimo, vis dar nėra vieningos nuomonės apie tam tikrų laidžiosios sistemos takų egzistavimą. ŠLS ir atskirų jos dalių matmenys literatūroje pateikiami labai skirtingi, o daugelio įgimtų širdies ydų atvejais, žmogaus širdies laidžiosios sistemos išsidėstymas išvis nėra žinomas, todėl operacijos metu atsiranda galimybė pažeisti ŠLS, ko pasėkoje sutrikdomas tvarkingas širdies darbas. Dalies komplikacijų galima būtų išvengti, jei operacijos metu ŠLS būtų matoma. Vaizdinimo metodika turėtų būti neinvazinė, paprastai ir greitai atliekama, tačiau vis dėlto labai informatyvi, kadangi skirtumai ieškomi tarp labai panašių – raumeninės kilmės audinių.

Sparčiai tobulėjant naujoms technologijoms bei gydymo ir diagnostikos metodams svarbią vietą tarp jų užima optinės diagnostikos technologijos, dažnai pristatomos kaip optinė biopsija. Šie metodai pasižymi išskirtinėmis savybėmis, darančiomis juos patraukliais taikyti įvairių susirgimų diagnostikoje. Svarbiausia, tai yra neinvaziniai, o taip pat palyginti nesudėtingi ir greitai atliekami metodai, tačiau, nepaisant paprastumo, jie yra itin jautrūs. Plačiausiai biologinių objektų optinėms savybėms bei jų pakitimams tyrinėti naudojamas metodas yra fluorescencinė spektroskopija. Naudojant fluorescencinės spektroskopijos metodus nustatomas audinių gyvybingumas, vaizdinama skiriamoji riba tarp sveiko ir navikinio audinio odos paviršiuje. Sukurti įvairūs diagnostikos metodai, pagrįsti savitąja audinių fluorescencija, ištirtos daugumos endogeninių ir egzogeninių fluoroforų spektrinės charakteristikos, nustatytos sąsajos tarp fluorescencijos spektrų, jų intensyvumo bei biologinio objekto

tarpląstelinės medžiagos sandaros, medžiagų apykaitos ląstelėje, morfologinių ir citologinių sveikų ir navikinių ląstelių pokyčių, jų išsigimimo.

Nepaisant plataus optinių metodų taikymo biologinių audinių tyrimams bei diagnostikai, fluorescencinės spektroskopijos metodais atliktų ŠLS tyrimų yra labai nedaug. Visi eksperimentai yra atlikti *ex vivo* audiniuose, neįskaitant kraujo įtakos. Esminiai atradimai, kurie padaryti tiriant eksperimentinių gyvūnų širdis, yra tai, kad laidžiosios sistemos mazgų (AV ir SA) fluorescencija yra intensyvesnė nei skilvelių ir šiek tiek silpnesnė nei prieširdžių, tačiau žymesnių skirtumų AV ir miokardo fluorescencijos spektruose nepastebėta. Pastaruoju metu buvo atlikti žmogaus širdies preparatų tyrimai ieškant optinių parametrų skirtumų tarp skirtingo tipo širdies audinių. Tyrimai buvo atlikti išpreparuotuose širdies audiniuose, pašalinus juos dengiantį endokardą ir neatsižvelgiant į jo fluorescencines savybes. Nustatyta, kad ŠLS ir MK pasižymi skirtinga sugertimi ultravioletinėje (UV) srityje, taip pat stebėti ir fluorescencijos intensyvumo skirtumai 400 – 550 nm srityje, tačiau intensyvumas yra tiesiogiai nuo tyrimo sąlygų priklausantis parametras ir praktinis pritaikymas nebūtų labai tikslus, todėl nepaisant visų atliktų tyrimų, fluorescencinių metodų taikymo galimybės ŠLS šakų vaizdinimui operacijos metu vis dar išlieka neaiškios.

Tyrimo tikslas:

Sukurti žmogaus širdies laidžiosios sistemos identifikavimo metodiką paremtą audinių optinių parametrų tyrimu.

Uždaviniai:

1. Ištirti nepreparuotų širdies audinių, kuriuose EK nėra atskirtas, optinius parametrus fluorescencijos bei fluorescencijos žadinimo metodais.

2. Ištirti kraujo įtaką registruojamiems širdies audinių fluorescencijos spektrams.

3. Remiantis nustatytais spektriniais skirtumais sukurti ŠLS vaizdinimo metodiką.

4. Ištirti širdies audinius fluorescencine laikinės skyros spektroskopija.

5. Ištirti funkciškai besiskiriančius širdies audinius konfokaliniu mikroskopu.

6. Atlikti ŠLS vaizdinimui sukurtos metodikos įvertinimą *in vivo,* operacijos metu.

Ginamieji teiginiai:

1. Nepreparuotų širdies audinių – ŠLS, MK ir JA, kuriuose EK nėra atskirtas, fluorescencijos spektrai žadinant 330 nm spinduliuote skiriasi tiek savo forma, tiek intensyvumu. Spektre yra stebimos dvi smailės ties 390 nm ir 430 nm, kurių intensyvumų santykis ŠLS, MK ir JA yra skirtingas. Žadinimui naudojant 380 nm spinduliuotę fluorescencijos spektrai skiriasi savo intensyvumu, tačiau esminių spektro formos pokyčių nėra.

2. Intensyviausia kraujo sugertis yra stebima ties ~ 420 nm, todėl jo buvimas gyvuose audiniuose stipriai įtakoja fluorescencijos spektrus. Tyrimai kraujingų (šaldytų) širdies audinių leido įvertinti kraujo įtaką ir atsižvelgiant į ją parinkti optimalias fluorescencijos žadinimo ir registravimo sąlygas – žadinimui tikslinga naudoti 330 – 380 nm spinduliuotę, o fluorescenciją registruoti ties 460 nm.

3. Kiekviename tyrimo taške fluorescenciją žadinant 330 nm ir 380 nm spinduliuote yra registruojami du fluorescencijos spektrai, kurių intensyvumai ŠLS, JA ir MK audinių yra skirtingi. Fluorescencijos intensyvumų santykis I(330)/I(380) ties 460 nm yra charakteringas kiekvienam audinio tipui, todėl tokia metodika, paremta audinių fluorescenciniu tyrimu gali būti naudojama taškiniam ŠLS identifikavimui bei nedidelių plotų vaizdinimui.

4. Fluorescencine laikinės skyros spektroskopija nustatyta, jog mažiausiai trys fluoroforai yra atsakingi už ŠLS, MK ir JA fluorescenciją. Raumeninių audinių (ŠLS ir MK) fluorescencijos gyvavimo trukmės bei fluoroforų santykiniai indėliai į fluorescencijos spektrus reikšmingai nesiskiria, tuo tarpu JA ir ŠLS gyvavimo trukmės skiriasi reikšmingai, taip pat skiriasi ir santykiniai fluoroforų įnašai į registruojamus fluorescencijos spektrus. Remiantis šiais skirtumais buvo identifikuoti visi trys fluoroforai, atsakingi už fluorescencijos spektrus 430 – 550 nm srityje.

5. Konfokaliniu atspindžio mikroskopu, naudojant 800 nm bangos ilgio spinduliuotę audinių apšvietimui, gali būti vaizdinami JA sudarantys kolageno ir elastino pluoštai, MK sudarantys miocitai bei Hiso pluošto šakose esančios Purkinje ląstelės ir jų skaidulos.

6. Operacijos metu *in vivo* registruojami širdies audinių spektriniai dėsningumai atitinka stebėtus *ex vivo* audiniuose, todėl sukurta metodika yra tinkama ŠLS atskyrimo tyrimams gyvuose audiniuose, tačiau, norint šį metodą pritaikyti kasdieniniam naudojimui operacijos metu, reikalinga ypatingai greita bei jautri aparatūra.

Aktualumas

Širdies ir kraujagyslių ligos vis dar yra pagrindinė mirties priežastis Lietuvoje ir visose išsivysčiusio pasaulio šalyse. Daugelio susirgimų atvejais nėra imamasi jokių prevencijos priemonių, todėl galiausiai vienintelis būdas pašalinti sveikatos sutrikdymus sukėlusias priežastis lieka chirurginė intervencija. Invazinės procedūros visada yra susijusios su tam tikra pooperacinių komplikacijų rizika.

ŠLS yra raumeninės kilmės audinys, todėl vizualiai yra sunkiai atskiriama nuo aplinkinio raumens ir operuojant lengvai gali būti pažeidžiama. Tokios situacijos galima išvengti jei būtų žinomas tikslus laidžiosios sistemos išsidėstymas, arba ŠLS būtų galima identifikuoti chirurginių procedūrų metu. Deja, bet kol kas atskirų ŠLS dalių išsidėstymas vis dar yra tikslinamas, o įvairiausių patologijų atvejais apskritai nėra aiškus.

Esama situacija rodo, jog ŠLS yra itin sudėtingas tiriamasis objektas – laidžiosios sistemos audinio kilmė yra raumeninė, tačiau jis pasižymi nerviniam audiniui būdingomis greito signalo perdavimo funkcijomis, o atskiri ŠLS pluoštai yra apsupti JA tinklu, izoliuojančiu juos nuo MK. Dėl šių priežasčių ŠLS negali būti vienareikšmiškai identifikuojama iš kito tipo širdies audinių net ir *ex vivo*. Žymenų, žyminčių tik laidžiąją sistemą, nėra, o mikroskopinis identifikavimas yra labai sudėtingas. Nors šiuolaikinių technologinių bei biocheminių įrankių pagalba buvo identifikuotos kai kurių ŠLS dalių lokalizacijos, tačiau tiksli visų ŠLS takų lokalizacija nėra žinoma net ir patologiškai sveikoje širdyje. Taigi stingant žinių apie žmogaus ŠLS anatomiją ir neesant patikimų vaizdinimo metodikų yra neabejotinas poreikis laidžiosios sistemos identifikavimo metodams tiek gyvuose audiniuose, tiek tiriant širdies audinių preparatus.

Naujumas

Gyvų biologinių audinių charakterizavimui plačiai yra naudojami neinvaziniai optiniai tyrimo metodai. Fluorescencinės spektroskopijos metodais tiriant eksperimentinių gyvūnų širdis nustatyta, kad SA bei AV mazgo fluorescencija skiriasi nuo juos supančių audinių fluorescencijos. Intensyvumų skirtumai stebimi ir tarp mazgų bei skilvelių endokardo fluorescencijos spektrų. Žmogaus ŠLS mazgų fluorescencija yra silpnesnė nei prieširdžių, tačiau intensyvesnė nei skilvelių. Iki šiol širdies audiniai daugiausia buvo tirti pašalinus dengiantį endokardą, kurio didžiąją dalį sudaro kolageno ir elastino pluoštai. Endokardas dengia visą vidinį širdies paviršių, todėl jo optinės savybės turėtų stipriai įtakoti širdies audinių fluorescencijos spektrus. Atlikti tyrimai iš esmės akcentuoja tik intensyvumų skirtumus, tačiau intensyvumas didele dalimi priklauso ne tik nuo audinio vidinių parametrų, bet ir nuo fluorescencijos žadinimo-surinkimo sąlygų, todėl siekiant vienareikšmiškai atskirti ŠLS reikalinga metodika, kuri būtų nejautri tyrimo sąlygoms.

Egzistuoja dar vienas fluorescencinis audinių optinių parametrų tyrimo metodas, kuris suteikia informaciją apie konkrečius audinyje esančius fluoroforus. Tai yra fluorescencinė laikinės skyros spektroskopija. Didžiausias dėmesys yra skiriamas aterosklerozės pažeistų kraujagyslių diagnostikai, tuo tarpu širdies laidumo audinys laikinės skyros spektroskopija kol kas nėra tirtas.

Taip pat netyrinėtos ir itin perspektyvios *in vivo* tyrimams konfokalinės mikroskopijos galimybės širdies audinių vaizdinimui. Ši metodika yra unikali tuo, kad įgalina tirti audinius ląstelių lygmenyje įvairiuose sluoksniuose nesuardant paties audinio. Konfokalinė atspindžio mikroskopija yra plačiai taikoma *in vivo* odos pigmentinių darinių diagnostikai, nustatyti kontrasto šaltiniai įvairiuose odos sluoksniuose, tačiau širdies sienelės struktūra bei sudėtis yra kitokia ir galimi kontrasto šaltiniai širdies audiniuose nėra aiškūs.

Apibendrinant ŠLS tyrimus galima teigti, kad nepaisant gausių širdies audinių tyrimų, laidžiosios sistemos vaizdinimas vis dar yra eksperimentiniame lygmenyje ir kol kas nėra tikslus. Didžioji dalis darbų yra atliekami arba tiriant eksperimentinius gyvūnus, arba audinių preparatus ex vivo, tuo tarpu gyvoje žmogaus širdyje atliktų laidumo audinių tyrimų spektroskopiniais metodais nepavyko rasti.

Pagrindiniai rezultatai

Atlikus išsamią širdies audinių tyrimų analizę buvo suplanuoti laidžiosios sistemos tyrimai net keliomis metodikomis. Pirmiausia atliktas širdies audinių fluorescencinis vaizdinimas, vėliau atlikti nuostoviosios bei laikinės skyros fluorescencinės spektroskopijos tyrimai bei spektroskopinis vaizdinimas. Taipogi atlikti pirmieji širdies audinių vaizdinimo eksperimentai atspindžio konfokaline mikroskopija, ir, galiausiai, sukurta ŠLS atskyrimo metodika buvo išbandyta širdies audinių atskyrimui *in vivo*, operacijos metu.

Atlikus tarpskilvelinės pertvaros fluorescencinį vaizdinimą nustatyta, jog tarpskilvelinės pertvaros šonuose bei viršuje išsiskiria gerokai intensyviau nei laidžioji sistema švytinčios vietos – daug kolageno ir elastino turinčios struktūros, pvz. vožtuvas, kraujagyslių sienelės, t.y. audiniai, kurių pagrindinė sudedamoji dalis yra JA. Taip yra todėl, kad JA didelę dalį sudaro audinių struktūriniai komponentai kolagenas ir elastinas, kurių fluorescencija yra smarkiai žadinama 365 nm spinduliuote, todėl papildomas 365 nm apšvietimas galėtų būti naudojamas tik apytiksliam ŠLS lokalizacijos įvertinimui, tuo tarpu tiksliam laidžiosios sistemos atskyrimui nuo kitų širdies audinių reikalinga sudėtingesnė fluorescencijos spektrų analizė.

Pirmajame tyrimų etape buvo ieškoma ŠLS atskyrimui tinkamos fluorescencijos žadinimo ir detektavimo srities bei buvo vertinama kraujo įtaka registruojamiems fluorescencijos spektrams. Nustatyta, jog žadinant 380 nm spinduliuote stebimi didžiausi širdies audinių fluorescencijos intensyvumų skirtumai, tuo tarpu žadinant 330 nm spinduliuote yra stebimi didžiausi širdies audinių spektrų formos skirtumai. Žadinant šio bangos ilgio spinduliuote fluorescencijos spektre susiformavusios smailės ties 390 nm ir 430 nm yra skirtingo intensyvumo visuose audiniuose. Vis dėlto, atlikti kraujingų audinių tyrimai leido nustatyti, kad smailės yra susiformavusios dėl kraujo reabsorbcijos ties 413 nm, todėl anksčiau nustatyti ŠLS spektriniai ypatumai 390 nm – 430 nm srityje gali būti įtakojami kraujo tiriant gyvus audinius. Atsižvelgiant į oksiduoto ir redukuoto hemoglobino sugertį bei širdies audinių fluorescencijos spektrus, tolesniems tyrimams pasirinkta fluorescencijos registravimo sritis ties 460 nm.

Remiantis šiais duomenimis buvo sukurta ŠLS atskyrimo nuo kito tipo širdies audinių metodika, paremta fluorescencijos intensyvumų, užregistruotų tame pačiame

tyrimo taške, santykio skaičiavimu. Metodikos esmė – tame pačiame tyrimo taške fluorescencija yra registruojama du kartus. Vieną kartą žadinant ties 330 nm, kita kartą žadinant ties 380 nm. Abiem atvejais fluorescencija yra registruojama ties 460 nm, t.y. neregistruojamas visas fluorescencijos spektras, o tik intensyvumas ties 460 nm. Esminis tokios metodikos privalumas – jog ji yra nejautri žadinimo-registravimo sąlygų pokyčiams.

Tyrimai šia metodika buvo atlikti *ex vivo* bandiniuose. Atlikus spektroskopinius tyrimus, bandiniai buvo išanalizuoti histologiškai ir patvirtinta tirtų vietų kilmė. Eksperimento metu buvo kelti du uždaviniai – ŠLS ribų nustatymas bei ŠLS audinių vaizdinimas. Ribų nustatymo eksperimento metu širdies audinyje buvo matuojami spektrai išilgai vienos linijos kas 500 μm, o vaizdinimo eksperimento metu spektrai buvo matuoti 11mm x 15 mm plote kas 1 mm. Tokiu būdu suformuotas vaizdas, kurio kiekvienas taškas atitiko intensyvumų žadinant 330 nm ir 380 nm santykį. Širdies audinių sritys, kurios optinės diagnostikos metodu buvo atpažintos kaip laidžioji sistema, sutapo su histologiškai nustatyta ŠLS lokalizacija, todėl toks metodas, paremtas intensyvumų santykių skaičiavimu, yra tinkamas ne tik taškiniam ŠLS atpažinimui bet ir nedidelių plotų vaizdinimui.

Optinių savybių tyrimams pasaulyje vis plačiau yra taikoma fluorescencinė laikinės skyros spektroskopija, suteikianti informaciją apie konkrečius audinyje esančius fluoroforus. Jos pagrindinis privalumas, jog laikinės spektroskopijos duomenų neįtakoja audiniuose esantis kraujas, taipogi taikant šią metodiką galima identifikuoti fluoroforus, kurie dėl persiklojančių fluorescencijos spektrų nuostoviosios spektroskopijos metodais nėra matomi. Nustatyta, jog ŠLS ir MK tiek fluorescencijos gyvavimo trukmės, tiek santykiniai fluoroforų kiekiai reikšmingai nesiskiria ir laikinės skyros metodika šie audiniai yra neatskiriami. Tuo tarpu ŠLS ir JA pasižymi ir skirtingomis fluorescencijos gyvavimo trukmėmis (τ_1 ir τ_2), ir nevienoda fluoroforų santykine sudėtimi (F_1.- F_3). Taipogi nustatyta, jog už ŠLS, MK ir JA fluorescenciją yra atsakingi mažiausiai trys fluoroforai – kolagenas I, kolagenas III ir elastinas.

Pirmą kartą atlikus širdies audinių vaizdinimo tyrimus atspindžio konfokaline mikroskopija buvo parodyta, kad horizontali ir vertikali metodo skyra įgalina išskirti skirtingus endokardo sluoksnius dėl nevienodai išsidėsčiusių kolageno ir elastino

skaidulų. Pavieniai miocitai, kurių vidutinis skersmuo yra ~ 10 μm, taip pat gali būti vaizdinami. Konfokaline atspindžio mikroskopija, panašiai kaip ir optine koherentine tomografija, vaizdai yra gaunami dėl skirtingai atspindėtos šviesos nuo tam tikrų audinio elementų. Vietos, kuriose gausu baltymų, pvz. interkaliaciniai diskai, stipriai atspindi šviesą ir išsiskiria iš aplinkinių struktūrų. Tam tikrose MK vietose buvo aptiktos didelės ląstelės, kurios remiantis anatominiais ir histologiniais kriterijais buvo atpažintos kaip Purkinje ląstelės. Taigi konfokalinė mikroskopija, įgalinanti registruoti pavienių ląstelių vaizdus gyvuose audiniuose atveria naujus kelius širdies audinių tyrimuose *in vivo* ir laidžiosios sistemos vaizdinimui.

Paskutiniame darbų etape buvo atlikta fluorescencinės metodikos, pagrįstos intensyvumų santykių skaičiavimu, testavimas *in vivo* operacijos metu. Pirminiai rezultatai patvirtino ir anksčiau, *ex vivo* bandiniuose stebėtus fluorescencijos intensyvumo ypatumus – intensyviausiai fluorescuoja JA, silpniausiai MK. Tose vietose, kur ieškota ŠLS, intensyvumas buvo šiek tiek mažesnis nei JA, tačiau gerokai didesnis nei MK. Taip pat išliko ir dėsningas fluorescencijos intensyvumų santykis – JA mažesnis už ŠLS, o MK didesnis už ŠLS intensyvumų santykio vertę.

Apibendrinant visus širdies audinių tyrimus galima padaryti išvadas, jog optiniais diagnostikos metodais registruojami širdies audinių optiniai parametrai yra nevienodi skirtingiems audiniams. ŠLS ir MK pasižymi skirtingu fluorescencijos intensyvumu, tuo tarpu pritaikius dviejų žadinimo bangos ilgių metodiką gali būti atskirti visi trys audiniai – ŠLS, JA ir MK. Fluorescencinės laikinės skyros spektroskopijos metodu taip pat aptikti tiek fluorescencijos gyvavimo trukmės, tiek kompoziciniai skirtumai tarp JA ir ŠLS. Vaizdinant širdies audinius konfokaliu atspindžio mikroskopu buvo išskirti skirtingi JA sluoksniai, taip pat MK sudarantys miocitai bei jų pluoštai ir, svarbiausia, aptikome pavienes Purkinje ląsteles, sudarančias ŠLS. Taip pat buvo užregistruotas ŠLS šakos vaizdas. Šiuo metu pasaulyje jau yra daug mažesnių ir mobilesnių konfokalinės mikroskopijos prietaisų, todėl tikėtina, kad ateityje tokia metodika galėtų būti pritaikyta ir ŠLS aptikimui gyvuose audiniuose. Galiausiai, atlikus eksperimentus operacijos metu buvo nustatyta, kad užregistruoti fluorescencijos spektrai bei suskaičiuoti intensyvumų santykiai yra labai panašūs į *ex vivo* registruojamų autofluorescencijos spektrų. Šie rezultatai sudaro prielaidas ŠLS atskyrimui nustatytomis metodikomis bei nubrėžė tolimesnes metodikos bei aparatūros tobulinimo kryptis.

Išvados

1. Fluorescenciniais metodais ištyrus nepreparuotus širdies audinius nustatyta, jog didžiausi optinių parametrų skirtumai tarp skirtingo tipo širdies audinių yra stebimi fluorescenciją žadinant 330 nm – 380 nm srityje, o registruojant 400 nm – 550 nm spektriniame ruože.
2. Ištyrus kraujo įtaką registruojamiems spektrams nustatyta, jog dėl kraujo reabsorbcijos fluorescencija turi būti registruojama ties 460 nm.
3. Remiantis nustatytais spektriniais skirtumais sukurta ŠLS atskyrimo metodika, kuri paremta intensyvumų santykių skaičiavimu:

$$< R_{aud} > (460) = \frac{I_{330}^{žad}}{I_{380}^{žad}}$$

   Suskaičiuota vertė $<R_{aud}>(460)$ yra skirtinga ŠLS, MK ir JA audiniams. Tokia metodika yra nejautri fluorescencijos žadinimo-surinkimo sąlygoms ir gali būti naudojama ne tik taškiniam širdies audinių identifikavimui, bet galimas ir tikslus ŠLS ribų nustatymas bei nedidelių plotų vaizdinimas.
4. Raumeninės kilmės audinių (ŠLS ir MK) fluorescencijos gyvavimo trukmė bei santykinė sudėtis reikšmingai nesiskiria, tuo tarpu JA ir ŠLS tiek fluorescencijos gyvavimo trukmės (τ_1 ir τ_2), tiek santykinė komponentinė sudėtis (F1 - F3 komponentai) yra skirtinga. Šie komponentiniai skirtumai yra nulemti santykinai didesnio elastino kiekio ŠLS ir didesnio kolageno kiekio JA.
5. Dėl skirtingų šviesą atspindinčių komponentų bei jų išsidėstymo širdies audiniuose konfokaliniu atspindžio mikroskopu galima identifikuoti MK, JA, Purkinje ląsteles ir ŠLS pluoštus, todėl metodas yra tinkamas neinvaziniams širdies audinių tyrimams ir ŠLS vaizdinimui.
6. Operacijos metu užregistruoti spektriniai dėsningumai atitinka stebėtus *ex vivo* tyrimų metu, todėl ŠLS identifikavimo metodika, paremta fluorescencijos intensyvumų santykių skaičiavimu, yra tinkama širdies audinių tyrimams *in vivo*.

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