

Spectroscopy of Testicular Tissues as a Tool for Azoospermia Visualization During Micro-TESE and IVF: a Feasibility Study

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Abstract

Non-obstructive azoospermia observed in 10% of infertile men and 0,6% of all men is the most severe form of male infertility. The histological structure of testicular tissue in patients with non-obstructive azoospermia is heterogeneous and is represented by various disorders of spermatogenesis. The only way to achieve pregnancy in the families of patients with non-obstructive azoospermia is the method of in vitro fertilization with intracellular sperm injection. The most effective method of detecting spermatozoa suitable for in vitro fertilization in testicles is the micro-TESE method. It is based on a subjective visual assessment of tissues and allows detecting spermatozoa in non-obstructive azoospermia only in 38-60% of cases. To increase the effectiveness of micro-TESE, the method used must be objective, high-specific, safe for reproductive cells, and also implemented during the operation in real time. The existing approaches to solving the problem of identifying seminal tubules with preserved spermatogenesis, such as multiphoton microscope, Raman spectroscopy, optical coherence tomography and etc., are either associated with the use of laser radiation with unproven safety or cannot be implemented intraoperatively and operationally. The paper considers the possibility of creating a specialized microsurgical system for intraoperative evaluation of testicular tissues histological structure using the spatial distribution of its spectral characteristics. We proposed to implement such a system based on multispectral imaging of the studied tissues with real-time data processing and displaying the results using augmented reality methods. The reflectance spectrum of testicular tissue with varying degrees of preservation of spermatogenesis in the visible and near infrared spectral ranges was studied. Spectral regions were identified that potentially provide the best differentiation of healthy tissues and tissues with impaired spermatogenesis. The described approach can be implemented intraoperatively and safely and may serve to automate and objectify the diagnosis of testicular tissues in micro-TESE.

Keywords: testicular tissue, azoospermia, micro-TESE, noninvasive diagnostics, reflection, spectroscopy, imaging.

1. Introduction

Non-obstructive azoospermia (NOA) – complete absence of sperm in the ejaculate, observed in 0.6% of all men (10% of all infertile men) is the most severe form of male infertility, difficult to correct [1,2].

The histological structure of testicular tissue in patients with NOA is heterogeneous, there may be areas with different morphologies, the quality of which is assessed on the ten-point Johnsen SG scale: from the complete absence of spermatogenic epithelium to normal spermatogenesis, however, it is most often represented by the so-called "Sertoli cells only" syndrome.

The only way to achieve pregnancy in the families of men with NOA is the use of *in vitro* fertilization (IVF) with ICSI (Intra Cytoplasmic Sperm Injection) – one of the methods of assisted reproductive technologies. Detection of spermatozoa suitable for IVF in testicular tissue is a key step of the method. For successful IVF-ICSI, it is necessary to differentiate the seminal tubules containing spermatogenesis cells from those tubules where the spermatogenic epithelium is completely absent. In a number of studies [3,4], it was shown that even with severe morphological changes in testicular tissues, in 50-60% of cases, there are areas of spermatogenesis of varying degrees of completeness in it. For the detection of such areas, the method of choice today is micro-TESE (Microdissection Testicular Sperm Extraction), based on the use of microsurgical techniques and allowing the detection of spermatozoa in NOA in 38-60% of cases (20-25% more than conventional TESE). The stages of micro-TESE are schematically depicted in the diagram (Fig. 1.): after dissecting the **tunica albuginea** of the testicle, the surgeon spreads its edges, gaining wide access to the testicular parenchyma, divided by thin partitions of connective tissue into about 200-300 lobules, each of which contains from 1 to 3 strongly convoluted **seminiferous tubules**. The total number of tubules in one testicle is about 600, their total length is 360 m. Using an optical magnification of a surgical microscope with an increase of 15-25 times, the doctor evaluates the structure of the testicular tissue and performs a biopsy from the areas with the most "mature" tubules. The resulting material is immediately transferred to the embryologist, who, using a biological microscope with a 200-400-fold magnification, studies the material in order to detect sperm in the tubules. If successful, the spermatozoa are placed in a buffer solution and used, as a rule, on the same day for IVF-ICSI. In their absence, a biopsy is performed from the next section of the testicle. The procedure continues until the spermatozoa are detected, or stops after several unsuccessful attempts.

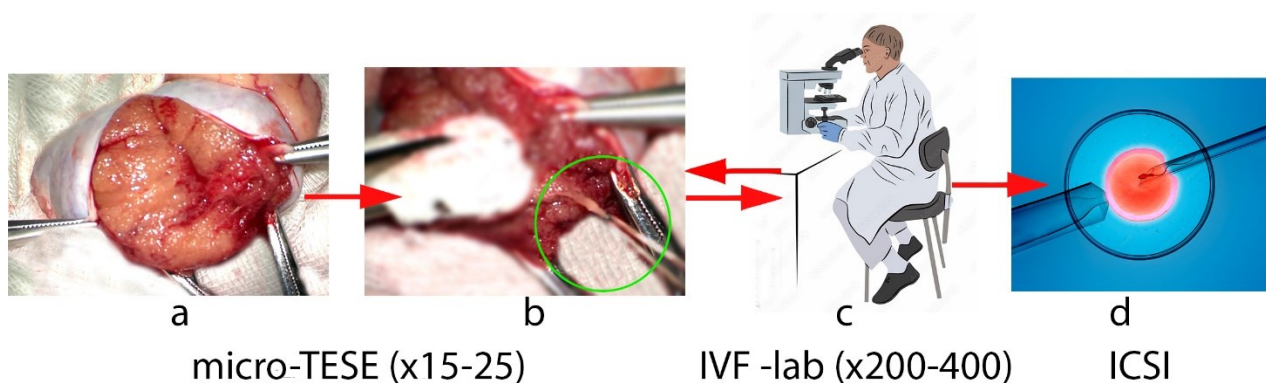


Figure 1: Pipeline of the micro-TESE – IVF-ICSI cycle: a) revision of testicular tissue using a surgical microscope, b) performing a biopsy from areas with the most expanded tubules, c) examination of the resulting tissue in the IVF laboratory, d) use of sperm for IVF-ICSI

It is important to note that the surgeon needs to examine a relatively large area of testicular tissue in a limited operating time in order to find a site suitable for biopsy. At the same time, the assessment of the degree of maturity of the seminal tubules during micro-TESE is highly dependent on the experience of the doctor, since it is based on a visual comparison of the diameters of the tubules and the consistency of their contents. The

method of evaluation of testicular tissue, characterized by objectivity, high specificity, harmlessness to reproductive cells, as well as the ability to conduct analysis in real time and intraoperatively, can increase the probability of detecting areas of spermatogenesis.

To interpret the laboratory data, it is desirable to have an idea of the differences in testicular morphology in normal and in NOA [5]. So, normally, testicular tissue consists of *tubuli seminiferi* and interstitial tissue located between them (*interstitium*) (Fig. 2).

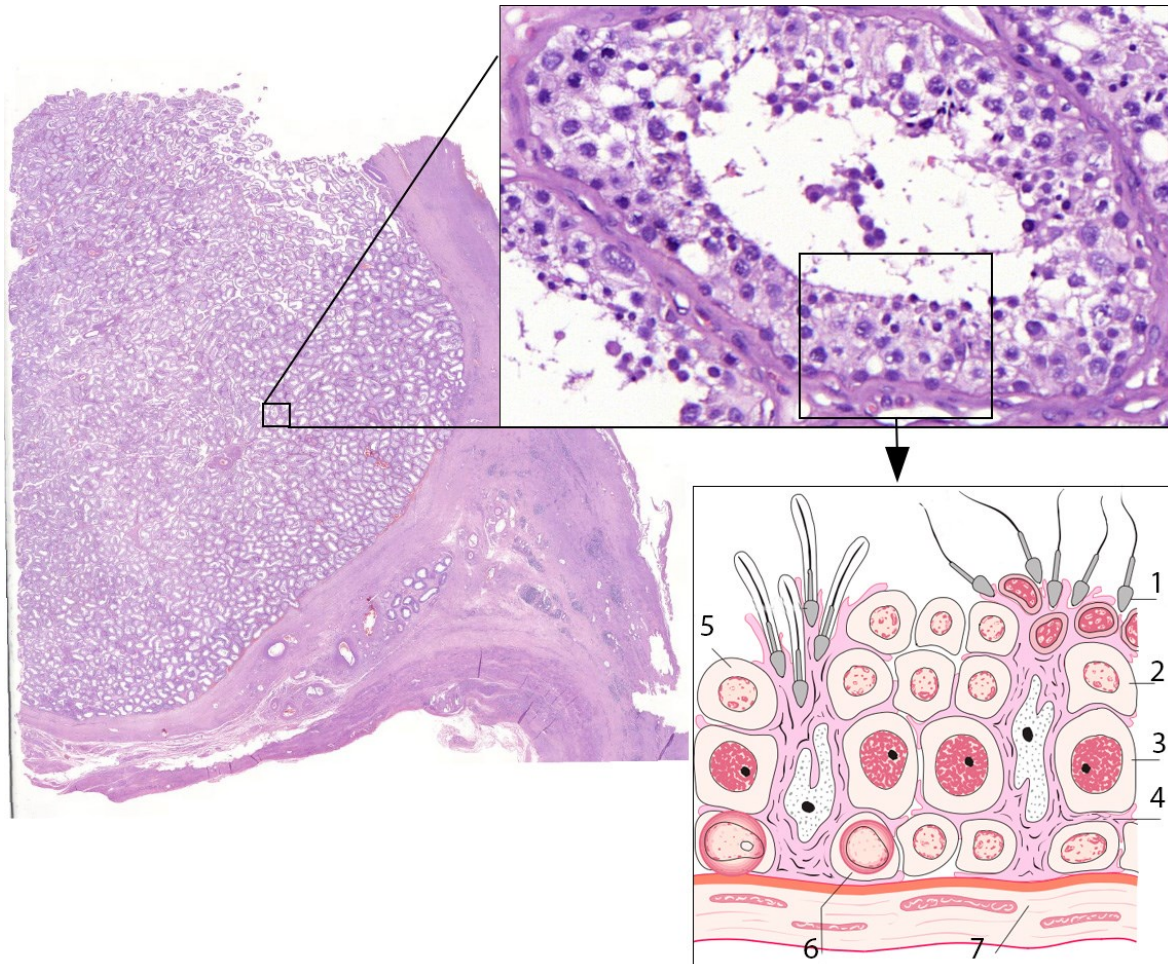


Figure 2: Testicular tissue structure: 1 – spermatozoa, 2, 5 – round-cell spermatids, 3 – spermatocytes, 4 – Sertoli cells, 6 – spermatogonia, 7 – peritubular cells

The tubular component – the vas deferens make up 60-80% of the volume of the testicle. This is the place where the production of germ cells – spermatozoa – is carried out. Each *tubulus semeniferi* has a wall consisting of a layer of collagen and peritubular cells – myofibroblasts. The lumen of the tubule is occupied by germinative cells, which differentiate into spermatozoa, and somatic cells, the main of which – Sertoli cells – have the function of maintaining and regulating sperm maturation. All phases of germ cell maturation are present in the tubule at the same time and are separated in space – as spermatogenesis cells differentiate, they move from the periphery of the vas deferens to its lumen, along the supporting Sertoli cells. Eventually, mature spermatozoa are separated into the lumen and carried further along the vas deferens. The interstitial component, which occupies 12-15% of the volume of the testicle, is represented by loose connective tissue with nerve fibers, blood and lymph vessels.

In the case of non-obstructive azoospermia, four histological types can be detected simultaneously in the testicular tissue: hypospermatogenesis – incomplete composition of the epithelium of the *tubuli seminiferi*; maturation arrest – if spermatogenesis stops at a certain stage; Sertoli cells only syndrome (SCO)- only Sertoli cells are present, germinative

epithelium is absent and tubular hyalinization - Sertoli cells and germinative epithelium is absent, the vas deferens are structurally indistinguishable.

Advances in the development of IVF- ICSI today allow not only mature sperm cells to be used for fertilization of an egg, but also their precursors – round and late spermatids [6], which ultimately reduces the task to differentiating the seminal tubules containing spermatogenesis cells from those tubules where the spermatogenic epithelium is completely absent.

In order to improve the results of micro-TESE, various teams of researchers are studying the possibility of using modern imaging methods, which in the future would allow the surgeon to assess the structure of testicular tissue with greater objectivity, increasing the probability of detecting tubules containing sperm in the testicular tissue in NOA. These methods can be divided into two groups. In the first case, physical interactions of biological tissue and radiation are used that are inaccessible to direct human observation, in the second – methods that improve the visualization of the surgical field directly by the surgeon. The world literature describes the experience of using a multiphoton microscope [7,8] to study testicular tissue. Using a near-infrared laser source to induce autofluorescence of tissues, this method creates a high-resolution image and allows for high reliability (about 92%) in real time to distinguish seminal tubules with normal spermatogenesis from pathological ones. However, the use of laser radiation is potentially dangerous by thermal damage to DNA, which can lead to further mutations. And although this laser has shown low phototoxicity in rodent experiments, its safety for human DNA needs proof.

Another promising method is Raman spectroscopy [9,10]. Based on the so-called raman scattering of photons, it is also a real-time visualization method, with the help of which it is possible to detect seminal tubules with preserved spermatogenesis with even greater accuracy (up to 96%) in animal models with induced NOA-SCO. But the relatively long scanning time at one point, which is about 2 minutes, limits the use of this method in a real operation. All of the above is also true regarding the use of a laser as a photon source.

Another method – full-field optical coherence tomography [11,12] – does not pose a danger associated with the use of a laser, since a halogen lamp can serve as a light source for it. The high speed of image acquisition - about 1 frame per second, allows scanning a relatively large surface area of the tissue in a short time, revealing the seminal tubules containing sperm by a characteristic reflected signal from the microstructures of the sperm tails, which was also demonstrated on an animal model. However, today this method has serious limitations for use during micro-TESE due to insufficient resolution, low depth of tissue scanning and difficulty in interpreting the results.

Another approach to solving the problem of improving the quality of micro-TESE is to improve the visualization of the operating field. The most modern microsurgery technology, the ORBEYE high-resolution 3D microscope [13,14], creates a three-dimensional image of the surgical field projected onto the surgeon's 3D glasses, and can improve the visualization of testicular tissue, but tissue analysis remains subjective.

There are few works where computer image processing using a neural network is used in order to improve the results of micro TESE. The algorithm is based on the detection of tubules of the largest diameter, but this approach has dubious advantages over conventional visual inspection [15].

Previous studies have established [16] that testicular tissue has different optical properties depending on the degree of completion of spermatogenesis. The use of spectral reflection characteristics as additional data in the task of differentiation of testicular tissues can increase the probability of detecting areas of spermatogenesis in NOA. The paper considers the possibility of creating a specialized microsurgical system using the spatial distribution of the spectral characteristics of testicular tissues to assess its histological structure during micro-TESE. For this purpose, studies of the reflection spectrum of testicular tissue with varying degrees of spermatogenesis obtained during testicular biopsy in the visible and near infrared ranges were carried out.

2. Materials and methods

To simulate the reflection spectrum of testicular tissue regions in NOA, testicular tissue samples were taken from patients who underwent orchiectomy for various indications: severe testicular hypoplasia / atrophy, trauma or inflammation of the testicle. The spectral characteristics of tissues with preserved spermatogenesis were collected from biopsy material obtained during autopsy in fertile men during their lifetime. A complete absence of spermatogenesis was observed in the tissues of hard hypoplastic testicles removed due to cryptorchidism. The selected tissue samples had a relatively homogeneous structure, either with intact spermatogenesis or with its complete absence, which excludes measurement errors associated with the heterogeneity of the morphological structure of the samples.

After determining the histological structure of the obtained testicular tissue (TT), its spectral characteristics were recorded by assembled setup (Fig. 3) using spectrometers (S) OceanOptics FLAME-VIS and FLAME-NIR (spectral range 350-1000 and 950-1650 nm, spectral bandwidth 1,34 and 10 nm, exposure time range 3,8 ms–20 s and 1 ms–65 s, respectively). Reflected radiation was introduced into the spectrometer using an optical fiber (OF) with collimator (CI) (field of view $5\times 5^\circ$) mounted at a fixed distance at an angle of 45° to the sample plane. A halogen lamp Dedolight DLH4 (150 W) was used as a light source (LS). Preprocessing of the spectrometer data included Gaussian smoothing ($\sigma=20$) to eliminate high-frequency noise. The reflection was extracted by normalizing the spectral brightness of tissues to the spectral illumination created by the source in the sample plane. To analyze the spectral features of healthy tissues and tissues with impaired spermatogenesis, the spectral reflectance curves were normalized to the maximum value and are presented in arbitrary units (a.u.). The spread of values was determined as the minimum and maximum value of the reflection coefficient among the obtained samples for each wavelength.

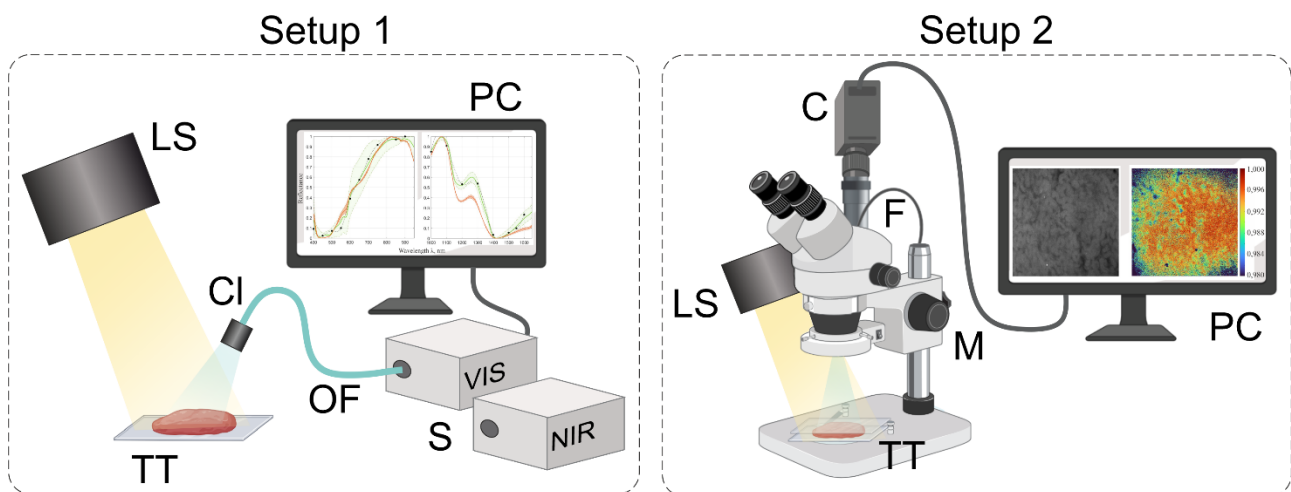


Figure 3: Assembled setups

To determine the spatio-spectral tissues characteristics, we assembled another setup consisting of a microscope (M) with a $5\times$ magnification, a digital camera (C) with a wide spectral sensitivity range TOUPCAM SWIR1300KMA (spectral sensitivity range 350 - 1700 nm, pixel size $5\times 5\ \mu\text{m}$, exposure time range 50 μs -3600 s), custom nozzle with a place for a filter (F) and a Dedolight DLH4 halogen lamp (150 W). The nozzle was fixed in front of the camera sensor to enable registration of spectral images. We used the Thorlabs FKB-VIS-10 and FKB-IR-10 filter sets, which allow filtering in the range of 350-850 nm with a step of 50 nm from the visible (VIS) to the near infrared (NIR) range and 900-1600 with a step of 100 nm from the NIR to short-wave infrared (SWIR) range. The width of the spectral channels of the filter sets is 10 nm. The microscope was focused on a tissue placed on a microscope stage and pressed against a glass slide to eliminate glare.

The experimental protocol included adjusting a filter in a special nozzle aperture, refocusing the microscope, and recording 50 spectral images of the sample at the same exposure for each filter. Then, with the same camera settings, spectral images of the reference plate were recorded with a uniform reflection close to 1. By dividing the multispectral cube of tissue images by the reference multicube, the spatial distribution of the spectral reflectance of the samples was obtained. The spectral reflectance was then averaged over the region and normalized to the maximum in the VIS-NIR and NIR-SWIR region for comparison with the spectral characteristics obtained by the fiber spectrometer. We also obtained correlation maps showing the degree of correspondence between the pixels of the sample reflectance multicube and the spectrum of healthy tissue.

3. Results and Discussion

The results of measuring the spectral characteristics of healthy tissues and tissues with impaired spermatogenesis are shown in the figure (Fig.4). Also, Fig. 4 shows the averaged reflectance spectra over the areas of healthy samples, obtained using multispectral imaging on a microscope. Discrete spectral data were interpolated by a spline. Multispectral imaging data does not go beyond the spread of reflectance values of a healthy tissue type, determined by the fiber spectrometer.

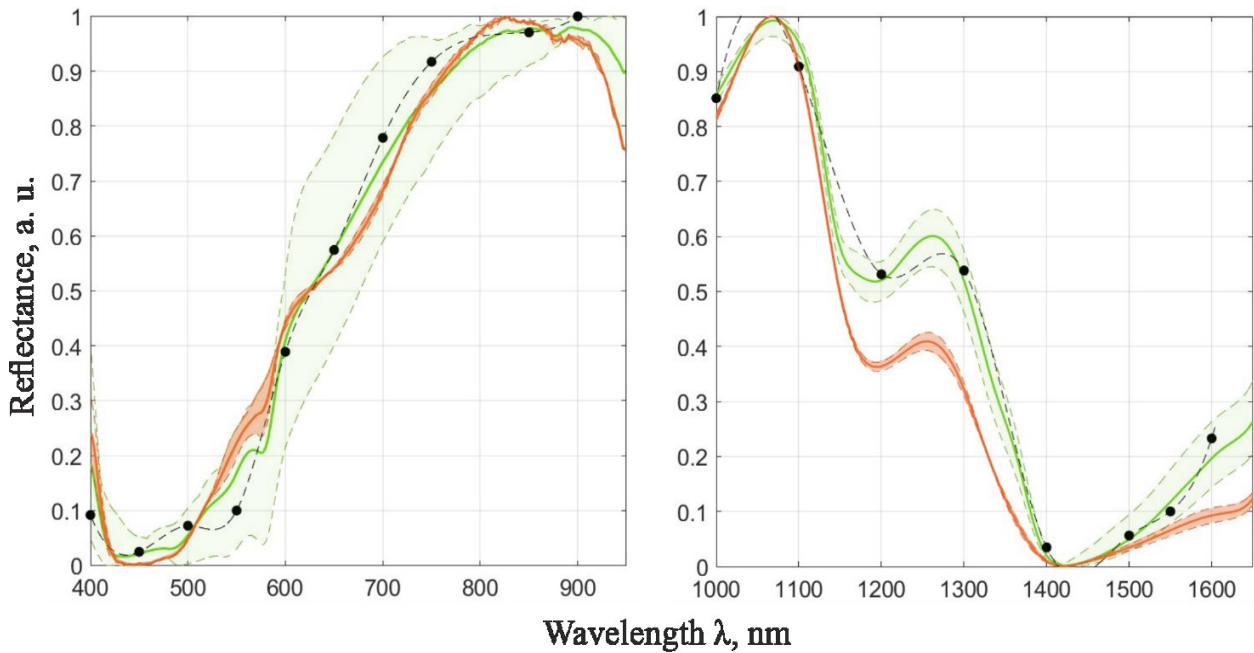


Figure 4: The average normalized spectral reflection coefficient of healthy tissues (green curve) and tissues with NOA (red curve) with a corresponding spread of values (colored areas) according to the samples. Black dots show averaged multispectral data from a healthy sample

As a result of the correlation analysis of the multispectral reflection cube of healthy tissue samples, maps were obtained (Fig. 5). Comparing the correlation map with the image at 550 nm, it can be concluded that the intertubular space and residual vignetting effects have a relatively low correlation with the spectrum of healthy tissue, which will avoid false negative classification. At the same time, most of the healthy tissue has a uniformly high spatial correlation (above 0,99) with the spectrum of healthy tissue.

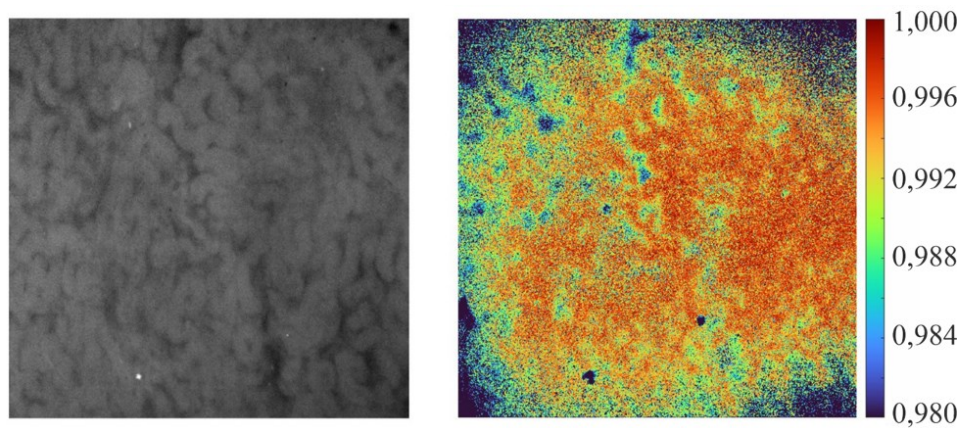


Figure 5: Tissue image (left) at 550 nm wavelength and correlation map with average spectrum of healthy tissue (right)

The high correlation of the reflection spectra of testicular tissues obtained from integral spectrometers and by averaging images with filters, as well as the uniformity of the correlation map constructed for healthy tissues, which corresponds to its morphological uniformity, confirm the reliability of the obtained data. These data suggest that in the infrared (IR) region of 1150-1400 nm there are noticeable differences in the spectral characteristics of seminal tubules with preserved and impaired spermatogenesis, which can be used for tissue differentiation in clinical practice. In the visible region of the spectrum, the differences in spectral characteristics are less pronounced and require further research and accumulation of statistical material.

The identification of spectral features characteristic of tissues with normal and impaired spermatogenesis is necessary for the subsequent design of hardware for spectral differentiation of tissues. Thus, the proposed surgical system can be built on the basis of multispectral imaging and contain active illumination at different, predetermined wavelengths or isolate the corresponding narrow spectral channels from broadband radiation reflected from the studied tissues using light filters. The images recorded by a monochrome video camera with high sensitivity and bitness in various channels will contain the spatial distribution of the spectral characteristics of the studied tissues and can be transmitted for processing and output to the surgeon's monitor during the operation with markers in the areas with the highest probability of the presence of spermatozoa. The safety of the proposed method is determined by the use of incoherent radiation of LEDs or xenon lamps used in modern surgical microscopes as light sources. We assume that the system should be autonomous, not requiring a surgical microscope.

4. Conclusion

In this study, the possibility of creating a spectral method of differentiation of testicular tissues with preserved and impaired spermatogenesis in patients with NOA during IVF-ICSI was considered. We proposed to use spectral characteristics as additional information about the histological structure of testicular tissue, in addition to the traditionally used diameter and consistency of the contents of the seminal tubules. To confirm the possibility of creating a spectral method for searching for spermatozoa in NOA, we implemented a number of *in vitro* experiments. Reflection spectra of testicular tissue samples of patients with varying degrees of spermatogenesis preservation were experimentally obtained by assembled setup. We presented the spatial distribution of the tissue spectral data, and showed differences between healthy tissue and tissue with impaired germ cell production in the near IR range. The obtained experimental outcomes can be useful for algorithms of visualization and automatic recognition of damaged tissues. The described approach to the analysis of testicular tissue can be non-contact, high-throughput, automated, safe for germ cells, intraoperative and thus appears to be a promising diagnostic tool for clinical practice.

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