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METHODS FOR AUTOFLUORESCENCE ANALYSIS OF UTERINE CAVITY WASHINGS

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The diagnostics of endometrial pathology can be done by obtaining information at the molecular level, e. g. using the autofluorescence-based technique. Thus, for the first time an experimental study was performed on waste material of uterine cavity washing specimens to evaluate suitability of the technique for diagnostics. The specimens were obtained from 32 patients who had a conventional uterine hydrososonography procedure. A portable Nd:YAG 355 nm microlaser was used to excite autofluorescence at the point of care. Various algorithms of multivariate curve resolution and artificial neural networks were utilized for spectra analysis. The spectra were classified according to histological and ultrasound diagnosis. Receiver operating characteristic (ROC) curve analysis was used to make statistical decisions. The results showed that it was possible to distinguish all compared groups: pathologic vs non-pathologic endometrium (sensitivity $97.3 \pm 5.2\%$, specificity $91.7 \pm 7\%$, AUC (area under the ROC curve) 0.96 ± 0.04), malignant endometrium vs endometrial polyps (sensitivity $100 \pm 0\%$, specificity $92.0 \pm 10.6\%$, AUC = 0.98 ± 0.07), and secretory menstrual cycle phase vs proliferative phase (sensitivity $87.5 \pm 13.2\%$, specificity $94.4 \pm 7.4\%$, AUC = 0.88 ± 0.10). To conclude, uterine cavity washing specimens could be used for endometrial pathology recognition using the autofluorescence-based technique in clinical setting. It will possibly speed up the treatment decision making for endometrial pathology.

Keywords: endometrium, tissue fluorescence, photodiagnostics, multivariate analysis

PACS: 02.50.Sk, 42.62.Be, 87.15.A-

1. Introduction

Fluorescence spectroscopy based on natural, intrinsic and endogenous fluorophores can be seen as an alternative or supplement for conventional diagnostic techniques [1, 2], but up to now the method has not found direct application in certain areas of medical practice due to problems associated with receiving reasonably high accuracy of diagnosis. This paper presents the way towards solving this issue.

Endometrial (i. e. inner mucous lining of uterus cavity) cancer is the most common form of cancer in women [3]. The earlier cancer is detected the easier it is to cure. However, confirmation of disease requires intervention: histological examination of a cut piece of tissue by a procedure called biopsy. Accuracy of other conventional diagnostic methods depends on technical shortcomings and qualification of an examiner [4–6].

Autofluorescence of endometrial tissue was investigated earlier in [2, 7–9], it was registered either from

a patient *in vivo* or from biopsied pieces of tissue *in vitro*. However, in our present case the autofluorescence is registered from specimens, which are taken in a non-invasive and less intervention requiring way [10, 11], and an example of these samples is a specimen of uterine cavity washings taken during hydrososonography [12]. It was proposed that the uterine cavity washings contain information about the metabolism of endometrium.

Methods based on the state-of-the-art optical technologies can be used to make diagnosis at a bedside, i. e. at a point of care. These methods are based on digital analysis, i. e. evaluation of autofluorescence spectra could be performed automatically in this way avoiding the factor of human error. The miniaturized equipment makes it easily moved from place to place. It allows performing a test in a physician's office during the first patient's visit which would save time and possibly lower expenses for a patient.

Thus the purpose of this experiment is to collect autofluorescence spectra in clinical conditions and to

create a method for analysis of autofluorescence in the medical specimens which are waste material from uterine cavity obtained during a conventional uterine hydrosonography procedure, and to compare spectra of several endometrial pathology groups as well as spectra in proliferative and secretory menstrual phases.

2. Methods

We present a microlaser-induced autofluorescence-based photodiagnostic method which is applied for dried washings (saline used for uterine hydrosonography) at the point of care for the first time. The research was performed in the Department of Obstetrics and Gynecology, Skåne University Hospital, Lund University, Malmö, Sweden, using a small mobile equipment (transported as a plain cabin baggage) from Vilnius University, Lithuania.

During ultrasound examination of woman's womb (this conventional medical procedure is called hydrosonography and is used to assess the uterine cavity examination of suspected uterine and endometrial pathology) saline is injected to uterine cavity as a contrast agent. For the purpose of this investigation the saline (mixed with biological matter) was drawn back from the cavity to a syringe. Several drops of this liquid were put on monocrystalline silicon substrates and dried (Fig. 1). The autofluorescence spectra of this material were excited using the 3rd harmonic (355 nm) of a pulsed (pulse duration ~0.5 ns, repetition rate 10 kHz) Nd:YAG micro-laser STA-01-TH (Standa Ltd., Vilnius, Lithuania), maximum power 11 mW, measured pulse power at the tip of optical light guide is approximately 2.2 μ J/pulse (i. e. roughly 2.2 mW on the average), the size of the laser beam spot varies from 1 to 3 mm in diameter. The autofluorescence was registered using an *Ocean Optics*

USB2000 spectrometer, spectral resolution 2048 pixels in the range from 340 to 1014 nm (on the average 3.04 pixel/nm) and spectra acquisition software OOIBase32 version 2.0, driver version 4.05 (*Ocean Optics, Inc.*). The laser beam is directed to the sample and the autofluorescence is collected through different channels of the same light guide. Light fibres of both channels are situated in parallel on the same light guide tip. Silicon substrate was used instead of conventional quartz plates to avoid a contribution of quartz and fluorescence of its surroundings that is rather complicated to remove from the experimental spectra [1]. To avoid a direct specular reflection of the laser beam to be collected, the angle between the plane of the substrate and the direction of the laser beam is chosen to be smaller than perpendicular, usually it is between 45–80°.

Three fluorescence spectra were registered from three separate points in the central part of the dried sample. Spectra of the same sample were slightly different compared to each other. The possible cause of this effect is a heterogeneous nature of biological matter. Each spectrum of the same sample was treated as a separate case with the same diagnosis. Spectra were neither summed nor averaged per patient.

Before the analysis the spectra were pre-processed: curves were smoothed using 11 adjacent points and normalized using the maximum peak between 485 and 515 nm. The data analysis consisted of component extraction (data dimensionality reduction), statistical Mann-Whitney test, receiver operating characteristic (ROC) curves analysis and application of artificial neural networks (ANN).

In the component extraction (spectra decomposition to components) phase the assumption that the experimental spectrum is a sum of several fluorescing constituents was made. Each experimental spectrum could be described by this equation [13]:

$$S = \sum_{i=1}^n C_i \cdot B_i + E, \quad (1)$$

where S is the experimental spectrum, C_i is the concentration (or in other words, an amplitude) of the i th component, B_i is the spectrum (spectral shape) of the i th component, E is the noise of the signal. The purpose of this type of decomposition is to find parameters B_i common for all experimental spectra and compare the spectra using parameters C_i .

Principal component analysis (PCA) was performed to extract initial spectral shapes of components. Based on the previous experiments [1, 2, 8], 8 components as fluorophores from the tissue plus one

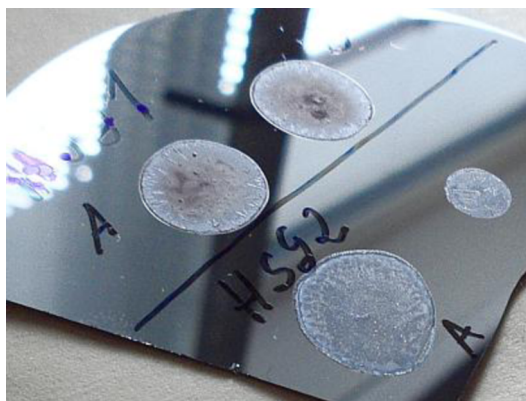


Fig. 1. Dried samples of uterine cavity washings on substrate.

extra component as a fluorescence spectrum of saline were chosen to be used for further analysis. Thus the first 9 PCA components were used for further analysis and rotated using the “varimax” method [13]. Other components were discarded as a noise (denoted as E in Eq. 1). The rotated components were used as initial conditions for the MCR-ALS (multivariate curve resolution using alternating least squares) algorithm in which the non-negativity constraints were set, i. e. the assumption that all intensity parts of every component should be non-negative was made. These components (optimized using the MCR-ALS algorithm) were considered to be “real” spectra of fluorophores of which the biological sample is composed.

The fluorescence spectra of medical samples were divided into groups according to the results of medical tests. The amplitudes of the extracted components were compared among the groups using the Mann-Whitney test. For the cases where statistically reliable differences were found, the ROC curve analysis was performed. Using the same extracted components, ANN was trained and the ANN output was used as one additional feature for the ROC curve analysis. Pairwise compared groups of different endometrial conditions were as follows: (1) pathologic vs non-pathologic, (2) malignant vs benign endometrial polyps, and (3) endometrium in proliferative menstrual phase vs that in secretory phase. The amount of samples was the following: non-pathologic (proliferative phase, 12 samples; secretory phase, 8 samples) and malignant endometrium (polyps, 8 samples; cancer, 4 samples). The groups were classified according to the histological diagnosis of pathological endometrium and accord-

ing to the assessment of sonoscopic ovulation signs to determine the menstrual cycle phase. Samples of the woman who used hormones were excluded from investigation.

3. Results

Averages of normalized fluorescence spectra divided into medical groups are presented in Fig. 2(a), and it is seen that the main relative difference of the groups was revealed in the spectral regions 400–470 and 550–600 nm. The spectra of all samples were divided into the components according to the way presented above and average-sized components are plotted in Fig. 2(b).

The endometrial autofluorescence consists of 9 components (Fig. 2), and they explain a major part of variation (the coefficient of determination $r^2 > 0.999$). Seven components which peak values are at 418, 441, 487, 496, 525, 596, 662 nm have closed, irregular Gaussian-like spectral shapes. The component at 496 nm was modelled as a spectrum of saline as its peak was most similar to the experimentally registered spectra of salt crystals from the dried saline. The fluorescence spectrum of this component was also experimentally registered and always used during the modelling process. Two components marked as 390* and 390** nm were not well defined. It was considered that laser scattering had some impact on these components and they were removed from further analysis. Thus seven components were statistically analysed.

Table displays the results of group discrimination in terms of AUC (area under the ROC curve). It was

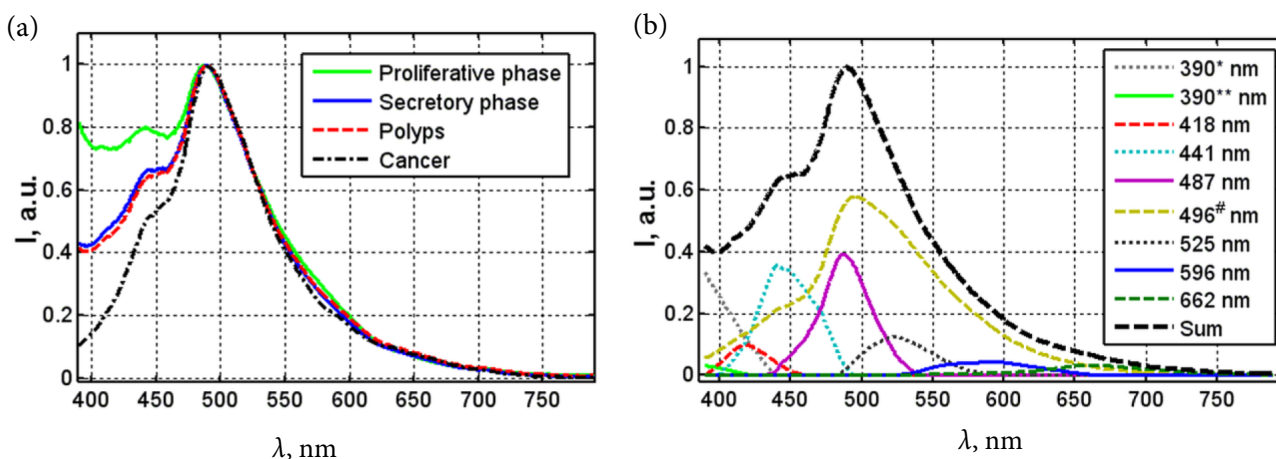


Fig. 2. Average experimental spectra in the medical groups (a) and average-sized components of the endometrial washing spectra model (b). The legend of components' figure indicates the peaks of components, “Sum” indicates the sum of components – an experimental spectrum. * and ** denote components that were not included in the analysis, # indicates the experimentally measured spectrum of dried saline. “Cancer” corresponds to “malignant endometrium”.

possible to distinguish all compared medical groups and the greatest AUC values achieved using ANN outputs as a feature to compare were these: pathologic endometrium vs non-pathologic endometrium $AUC = 0.96 \pm 0.04$, sensitivity (Se) = $97.3 \pm 5.2\%$, specificity (Sp) = $91.7 \pm 7\%$, overall classification accuracy (Acc) = 93.8% , secretory menstrual cycle phase vs proliferative phase $AUC = 0.88 \pm 0.10$, Se = $87.5 \pm 13.2\%$, Sp = $94.4 \pm 7.4\%$, Acc = 91.7% and malignant endometrium vs endometrial polyps $AUC = 0.98 \pm 0.07$, Se = $100 \pm 0\%$, Sp = $92.0 \pm 10.6\%$, Acc = 94.6% . AUC values calculated using individual components were smaller (see Table). However, reliable differences (comparing accuracy obtained using individual components versus ANN output values) within the margins of 95% confidence interval (CI) were found only comparing samples of pathologic vs non-pathologic endometria as the lower bound of ANN output AUC CI is 0.92 and the upper bound of single component AUC CI is 0.91 (Fig. 3). The width of confidence intervals is always narrower using ANN outputs as compared parameters.

In the case of using amplitudes of separate components the best differentiation between pathologic vs non-pathologic endometrium as well as between the malignant endometrium vs endometrial polyps is received using the component at 525 nm. Components with peaks at 496 and 487 nm most efficiently differentiate groups of endometrial samples in the proliferative menstrual cycle phase vs secretory phase. However, the AUC values calculated using amplitudes of these and other components coincide within 95% CI, i. e. these differences are not statistically reliable.

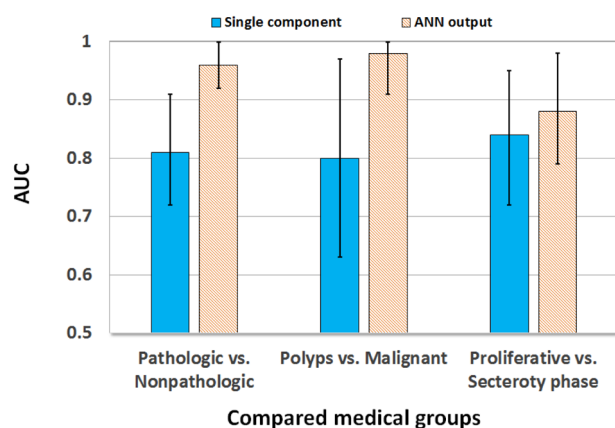


Fig. 3. Comparison of test performance using single component's amplitude (best AUC value) vs artificial neural network (ANN) output value. The labels below graphs indicate the groups of endometrial samples that are compared together.

Table. Components significant for medical group discrimination. Sorted in an ascending order according to AUC values. Cases where AUC values are above 0.75 are in bold. Sign '-' indicates cases where the Mann-Whitney test failed to find differences between the compared groups. CI is the confidence interval. AUC is the area under the receiver operating characteristic curve. "Malignant vs polyps" stands for "malignant endometrium vs endometrial polyps". "Proliferative vs secretory phase" stands for "proliferative menstrual cycle phase vs secretory phase".

Feature	AUC		
	Value	±	95% CI
Pathologic vs non-pathologic endometrium			
596 nm	0.67	±	0.11
418 nm	0.67	±	0.11
662 nm	0.69	±	0.11
441 nm	0.71	±	0.11
487 nm	0.77	±	0.10
496 nm	0.77	±	0.10
525 nm	0.81	±	0.09
ANN out.	0.96	±	0.04
Malignant vs polyps			
418 nm	–	–	–
596 nm	–	–	–
662 nm	–	–	–
441 nm	0.74	±	0.18
487 nm	0.77	±	0.17
496 nm	0.78	±	0.17
525 nm	0.80	±	0.17
ANN out.	0.98	±	0.07
Proliferative vs secretory phase			
596 nm	–	–	–
662 nm	–	–	–
418 nm	0.69	±	0.14
525 nm	0.74	±	0.13
441 nm	0.75	±	0.13
496 nm	0.83	±	0.11
487 nm	0.84	±	0.11
ANN out.	0.88	±	0.10

4. Discussion

The 355 nm radiation is suitable for fluorescence excitation of materials such as NADH, elastin, collagen, porphyrins, flavin, lipopigments [14], lipofuscin [15], natural oestrogens, cholesterol, dehydroergosterol (DHE) [16]. Considering possible causes of the variations in amplitudes of autofluorescence spectra (i. e. statistically compared parameter), it should be taken into account that in woman's body different hormones dominate during the secretory and proliferative phases of the uterine cycle. Researches show that changes concerned with maturation of uterine cervix which are caused by hormonal fluctuations

during pregnancy (e. g. before delivery) can be detected in the cervical mucus and foetal membrane rupture fluid fluorescence spectra at 420 nm while using 355 nm excitation [17]. It is reasonable to speculate that hormones-induced spectral differences arise not only during pregnancy but also in menstrual cycles. Amplitudes of the washing spectral component at 418 nm slightly differed among groups with non-pathologic and pathologic endometrium ($AUC = 0.67 \pm 0.11$) and endometrium in secretory vs proliferative phases of the menstrual cycle ($AUC = 0.69 \pm 0.14$). On the other hand, the spectral component at 418 nm can be related to fluorescence of other materials. Elastin has fluorescence maxima at 400–420 nm [8, 14, 15]. This material can be related to pathological processes in biological tissues [18], as well as changes in elasticity. It is an active component in cervical softening and opening in delivery [19].

In this investigation of uterine cavity washings the maximum differences between endometria in the proliferative vs secretory phases of the menstrual cycle were found comparing components at 487 and 496 nm. According to the *in vitro* research of the spectra of oestrogen derivatives [20], it can be speculated that one or both of the mentioned components can be related to the spectra of either sex hormones or their derivatives. Difference at 480 nm (355, 375 excitation) was the second significant wavelength in the foetal membrane rupture fluid and cervical mucus maturation detection in our earlier mentioned investigation, 480–520 nm was the phospholipide and lipid fluorescence zone [21]. Oestrogen fluorescence under 375 nm excitation is at 460–480 nm [22, 23], riboflavin fluorescence is at 495 nm [24].

The component at 496 nm was modelled as an experimentally registered sodium chloride (NaCl) spectrum. Detailed information about fluorescence of this compound could be found in literature [25]. Sodium cations and chorine anions can be found in blood, tissue fluids and in uterus secretions as well. Thus sodium and chorine could get into the sample not only from physiological liquid but also from uterine tissues. The experiments with laboratory rats [26] show that the concentration of sodium/calcium/chlorine ion carriers in uterine cell membranes fluctuates in accordance with concentration of sex hormones. If an analogical phenomenon occurs in humans, then during different phases of the menstrual cycle different quantity of Na^+ or Cl^- ions can be dissolved in physiological liquid with uterine cavity washings and foetal fluid. The concentration of these ions can also differ as the quantity of saline injected into womb varies depending on the patient.

The component peak at 441 nm might correspond to the NADH (nicotinamide adenine dinucleotide) fluorescence maxima, which in literature [15] are indicated at 440 and 460 nm. This material can be used as a biomarker of a cancerous condition *in vivo*. However, Schomacker and colleagues [27] showed by investigating the fluorescence spectra of intestinal polyps, cancer (adenocarcinoma) and normal tissue that the spectra *in vivo* and *in vitro* were different: after resection of the tissue the intensity of NADH fluorescence band decreases exponentially over time due to decreasing concentration of NADH. Therefore, one can assume that in our medical samples the concentration of NADH is too small for the material fluorescence spectra to be recorded.

Lipofuscin can also fluoresce in this spectral region (441 nm) as it absorbs radiation at 340–395 nm radiation and has fluorescence maxima at 430–460 and 540 nm [15]. This pigment is a byproduct of unsaturated fatty acid peroxidation. It can interfere with the renewal of cells and indicate a wear-off [28, 29].

The component at 525 nm could be related to the fluorescence of flavins, which has the peak at 535 nm [15], and/or to carotene [8]. The component at 596 nm is closest to the fluorescence of porphyrins. Gavryushin and colleagues [8] modelled the peak of porphyrins at 610 nm. In the region over 600 nm not only the fluorescence of porphyrins but also the fluorescence of collagen VI is possible [1, 30].

As the purpose of this research was to assess the possibility of fluorescence spectroscopy to be used for the diagnostics of uterine tissue, all ideas about possible fluorophores are only theoretical speculations as a biochemical analysis of specimen composition was not performed. These speculations are based only on the comparison of spectral peak positions and other aspects such as spectral shape and width of spectral bands/components were not taken into account. The issue of exact biochemical causes of autofluorescence and differences in fluorescence spectra was beyond the scope of this paper and it could lay the ground for further investigations.

The results of the current investigation can be compared to earlier works. In the analysis of our research team [2], a 353 nm laser was used to examine biopsied endometrial tissues of 3 women groups: with non-pathologic endometria, with endometrial polyps and with malignant endometria. The authors claimed that the identification accuracy (Acc) of the group with non-pathologic endometria is 65%, Acc of the group with endometrial polyps is 71%, and Acc of the group with malignant endometria is 80%.

The other investigation [7] in which 353 nm laser radiation was also utilized showed that it was possible to identify a cancerous group with sensitivity (Se) of 80% and specificity (Sp) of 98%, and to identify neoplastic (cancer + polyps) vs normal with Se = 78%, Sp = 68%. The investigation presented in this article shows a possibility to identify non-pathologic (corresponds to “normal” in the article [7]) vs malignant (corresponds to “neoplastic”) endometrium with Se = $97.3 \pm 5.2\%$, Sp = $91.7 \pm 7\%$, Acc = 93.8%; polyps vs cancer Se = $100 \pm 0\%$, Sp = $92.0 \pm 10.6\%$, Acc = 94.6%. It should be mentioned that in two previous investigations more invasive methods gave lower results than the less invasive method in this investigation.

Positive results were achieved by application of the analysis method. However, several drawbacks of this investigation should be mentioned as well in order to indicate directions for further improvement of the method.

First, the optical filter which cuts the laser radiation and prevents it from reaching the detector was not used. Thus it was not correct to compare amplitudes of mathematically extracted components which were in the short-wave side of the spectrum (390–400 nm). On the one hand, a compound called collagen-I could fluoresce around 395 nm. This material can be diagnostically valuable as it reflects structural changes [1]. On the other hand, while investigating washings but not biological tissues strong fluorescence of stromal layer collagen which could complicate the analysis is avoided [1].

Second, the model is based on the assumption that the same materials fluoresce in all the samples, the spectral shapes of their fluorescence are constant and the only measure that varies is the amplitudes of the components. In this way possible changes of spectral shapes were not modelled. The investigation of spectral shifts might be possible if the data base would be larger.

Third, the sample size of specimens is quite small. Thus, the results of this investigation should be treated as a tendency, but not as an undeniable conclusion, and the results indicate that it is worth continuing the investigation of uterine cavity washings. A larger number of specimens as well as more different endometrial pathologies should be investigated in order to get more reliable results.

5. Conclusions

The method for investigation of autofluorescence of uterine cavity washings was created. The analysis that included decomposition of autofluorescence spectra

provided investigators with qualitative (positions of component peaks allowed researchers to make assumptions about possible chemical composition of samples) and quantitative (relative amplitudes of components enabled researchers to statistically compare various groups of samples) information. Thus, natural changes of endometrium and secretion molecular (including fluorescing ones) composition and quantity fluctuations during menstrual cycle phases or in cases of pathology can be identified by fluorescence spectra. The medical group discrimination rate (in terms of AUC >0.75, sensitivity and specificity) calculated during this research was high enough to be acceptable for medical practice.

To sum up, positive results obtained in this research reveal the trend that the method based on autofluorescence spectroscopy has the potential to be used for medical diagnostics. However, broader investigations are needed in order to validate the created model and to get statistically more reliable results.

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GIMDOS ERTMĖS NUOPLOVŲ AUTOFLUORESCENCIJOS ANALIZĖS METODIKA

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Santrauka

Gimdos gleivinės patologiją parodo molekulinio lygio pakitimai, kuriuos galima nustatyti naudojant informaciją, gaunamą autofluorescencijos reiškinių pagrįstais metodais. Šiame darbe pirmą kartą atlikta naujo tipo bandinių – nuoplovų nuosėdų, gaunamų po standartinio kontrastinio ultragarsinio gimdos tyrimo (hidrosonografijos), – analizė. Darbo tikslas – išbandyti gimdos gleivinės ligų diagnostikos metodiką. Eksperimentai atlikti tiriant 32 pacienčių gimdos ertmės nuoplovas. Fluorescencija žadinta 355 nm nešiojamojo lazerio spinduliuote. Spektrų analizei naudoti pagrindinių komponentų bei kiti spektrų skaidymo į komponentus metodai, dirbtinių neuroninių tinklų algoritmas. Statistiniam sprendimui priimti naudota stebėtojo operacinės charakteristikos (ROC) kreivių analizė.

Tikrosios medicininės diagnozės nustatytos ištyrus histologijos ir ultragarso tyrimais. Taikyta metodika buvo galima identifikuoti visas tarpusavyje lygintas medicininės grupes: patologinius gimdos gleivinės pakitimus atskirti nuo sveikos gleivinės (jautrumas $97,3 \pm 5,2$ %, specifiškumas $91,7 \pm 7$ %, AUC (t. y. plotas po ROC kreive) – $0,96 \pm 0,04$), piktybinius pakitimus nuo polipų (jautrumas 100 ± 0 %, specifiškumas $92,0 \pm 10,6$ %, AUC – $0,98 \pm 0,07$), sekrecinę mėnesinių ciklo fazę nuo proliferacinės (jautrumas $87,5 \pm 13,2$ %, specifiškumas $94,4 \pm 7,4$ %, AUC = $0,88 \pm 0,10$). Rezultatai rodo, kad metodika potencialiai galėtų būti naudojama endometriumo ligoms greitai ir preliminariai diagnozuoti, t. y. būtų atliekama pacienčių apsilankymo pas gydytoją metu.