Abstract. Cervical smear material contains endo and exocervical cells, mucus and inflammative, immune cells in cases of pathology. Just not destroyed keratinocytes lay on the glass for microscopy. Liquid cytology supernatant apart other diagnostics could be used for photodiagnostic. The spectroscopic parameters suitable for Normal and HSIL cytology groups supernatant differentiation are demonstrated.

The dried liquid PAP supernatant fractions - sediment and liquid were investigated. Excitation and emission matrices (EEM), supernatant fluorescence decay measured under 280 nm diode short pulse excitation and fluorescence spectroscopy by excitation with 355 nm laser light were analyzed.

The differences between Normal and HSIL groups were statistically proven in the certain spectral regions. Fluorescence decay peculiarities show spectral regions consisting of few fluorophores. Obtained results on fluorescence differences in Normal and HSIL groups’ supernatant shows the potency of photodiagnosis application in cervical screening.

Keywords. Tissue fluorescence, fluorescence decay, EEM, photodiagnosis, liquid PAP, cervical screening.

INTRODUCTION

Cervical cancer is frequent type of cancer in women and it’s becoming more frequent cause of women mortality. With the introduction of the Papanicolaou (Pap) smear, however, early detection and treatment of preinvasive disease became possible. Further efforts at early detection and prevention, however, are likely to produce even more significant gains [1]. But confirmation of cervical cancer or pre-cancer diagnosis requires a biopsy of the cervix, which is indicated when cervical cytology shows suspicious cells. [2]. While the brush turns around endo/ectocervix superficial cells and mucus are collected. They are transferred to the fixative liquid. After centrifugation a part of full size keratinocytes, glandular and inflammation cells are separated for the microscopy evaluation. The bigger amount of epithelial and inflammative, immune cells, mucus remain in the supernatant. Supernatant is usually used for Human papilloma virus DNA detection and other genomics, proteomics investigations. Spectroscopy of supernatant gives information on cells and liquid fraction fluorophores.

This work is devoted to a study of possibility to apply luminescence method as alternative or supplement for cervical cancer diagnostics. The research based on comparison of cervical smear content fluorescence in Normal and HSIL cytology groups. These groups have different cell structures, mucus composition and because of this, not the same composition of materials that leads to luminescence spectra variations.

SAMPLES AND METHODS

Samples. The PAP smear material from cervix was taken by brush and put into the transportation liquid. Low concentration outwash of cervix liquid PAP samples was obtained after the filtering of cells for the cytology and then dried on quartz substrate. Supernatants are kept in test-tubes in a fridge (at
temperature +5°C). Every sample is taken with separate syringe. On the bottom of test tube there is dense fraction of supernatant and higher there is liquid one. The drops of liquid and a drop of dense mass of the smear were put on one side of quartz in different zones and a clean quartz area was left for its properties control. The samples were sorted into two groups after cytological analysis: I group normal cytology, no pathology; II group pre-cancer state HSIL (high-grade squamous intraepithelial lesion) both in cytology and pathology; Before using, quartz trays are disinfected with alcohol and cleaned.

The research based on comparison fluorescence of fixed in liquid cervical smear (cells and mucus) in normal and pre-cancer states. These groups have different cell structures and mucus composition and not the same composition of materials that leads to luminescence spectra variations.

2.1 Fluorescence measurement

It was performed measurements of fluorescence spectrum and fluorescence decay spectral distribution. The fluorescence excitation spectrum were recorded by a fluorescence spectrometer (f-4500, Hitachi) furnished with a photomultiplier as a detector and a 150 W Xe lamp as an excitation source.

The measurements of fluorescence excitation spectra confirmed the earlier data [3] that the excitation by 280 nm light excites fluorescence in all spectral region and the excitation by 355 nm fits to main excitation band of the samples (Fig.1). Both wavelength were used for detail analyze of fluorescence properties.

Fig.1. Fluorescence excitation matrix in supernatant samples. The left side (a,c) is the fluorescence of the dense part of smear sample and the right side (b, d) is from the dried liquid part of the smear. A space between the dark lines correspond a change of fluorescence intensity in 1% of the scale.
Therefore the 280 nm light emitting diode emission was used for the fluorescence decay measurement by time-correlated single-photon counting. Fig. 2 presents the examples of fluorescence decay in Normal sample (Fig.2a) and the spectral distribution of the fluorescence and of decay time constants (Fig.2b).

Fig. 2. Fluorescence decay at different wavelength (given in the inset) (a) and fluorescence spectra (luminescence) and decay time constants spectral dependencies (t1) and (t2)

The detail comparison of different groups of samples were performed by Nd:YAG microlaser STA-01-TH third harmonic 355 nm light throw 0.1 mm fiber. The laser beam is directed to optical waveguide. Through this path it reaches the surface of the sample (the end optical path and the sample are in 1-2 millimeters distance) and induces fluorescence of it. The luminescence emission comes back to the same optical path (it has 2 fibers) and through the second fiber goes to spectrometer. Spectrophotometers OceanOptics USB2000 and Avantes were used for spectra collection. Mathematical analysis of spectra was performed by ORIGIN multiple Gaussian peaks analysis. Because of ultraviolet radiation, high quality quartz is required for this experiment. While irradiating, not only sample but also quartz fluorescence was observed that was subtracted from the sample spectrum.

RESULTS

The example of spectrum of sample after the substraction of the quartz fluorescence signal is shown on Fig.4. Further analyze was performed by normalizing of all Gaussian components of spectrum according their amplitude to the peak at 410 nm value and the data obtained for NORMAL group were compared with the data of HSIL group by the statistical t-test analyze using the Gaussian component area data. The peak values were slightly different in different samples therefore the comparison was performed taking into account the similar peaks or the peaks from a certain spectral regions. The distribution of peaks area in different spectral regions and their statistical comparison are presented in Table 2.

Six spectral regions were recognized in Normal group, additional 450nm spectral region in HSIL group and they are given in the tables.

Table 1. Comparison of the Normal and HSIL groups by t-test and normalized to the peak area at 410 nm the peak area values in different spectral regions
<table>
<thead>
<tr>
<th>Spectral region, nm</th>
<th>Normal group</th>
<th>HSIL group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t-test P two tail</td>
<td>Mean area, normalized units (n.u.)</td>
</tr>
<tr>
<td>~410</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>~440</td>
<td>0.61</td>
<td>0.26</td>
</tr>
<tr>
<td>~450</td>
<td>0.0226</td>
<td>0</td>
</tr>
<tr>
<td>470</td>
<td>0.533</td>
<td>0.34</td>
</tr>
<tr>
<td>470-620</td>
<td>0.0025</td>
<td>0.31</td>
</tr>
<tr>
<td>650-695</td>
<td>0.099</td>
<td>0.11</td>
</tr>
<tr>
<td>&gt; 695</td>
<td>0.099</td>
<td>0.011</td>
</tr>
</tbody>
</table>

It is found the 450 nm and 470-620 nm regions are different in Normal and HSIL groups. Two sub regions found in 470-620 nm region in Normal and HSIL group: below and above 515 nm. No differences were found in area of sub regions in Normal and HSIL groups. HSIL has a shift to the left (max 490 nm) comparing with Normal (max 502 nm). The area of peaks in case of Normal cytology versus HSIL is bigger (<515 nm ± 8, 3, ± 37, 5) (>515 nm ±15, 3, ±80), respectively.

DISCUSSION

Spectroscopy of liquid cervical smear supernatant allows discriminating Normal from HSIL cytology groups. Microlaser 355 nm excitation of the dried sediment gives spectra with differences in Gaussian components. The main difference appears in region 470-620 nm (p<0, 01), also in region around 450 nm (p<0, 05).

Normal group do not have 450 nm peak. Region 470-520 nm is formed by two sub peaks in both groups, but HSIL has a shift of the first sub peak (max 490 nm) to the left in comparison to the Normal. Normal group peaks are sharp, HSIL- shallow. 440 nm and >650 nm regions are similar in both groups.

Data obtained from spectral analysis of Liquid PAP supernatant reveals differences between Normal and HSIL groups in the spectral region of the cell membrane components: cholesterol and dehydroergosterol (DHE) [4]. In literature Fluorescence excitation (λex = 220–380 nm) and emission spectra (λem = 350–550 nm) of DHE in a DHE/MCD complex were recorded at an emission wavelength λem = 376 nm and an excitation wavelength λex = 326 nm, respectively [5]. Chosen for presented investigation Nd:YAG micro laser STA-01-TH with 355 nm excitation is suitable for cholesterol, DHE, riboflavin and natural estrogens (Premarin) fluorescence emission [6]. Cholesterol is an important constituent of cellular membranes playing a fundamental role in many biological processes. This sterol affects membrane permeability, lateral lipid organization, signal transduction and membrane trafficking. DHE is an intrinsically fluorescent analog of cholesterol in living cells [5], DHE can completely redistribute from the basolateral to the canalicular membrane during prolonged incubation at reduced temperature [7]. DHE in certain temperature regimes expose crystallization phenomenon [8]. Dehydroergosterol fluorescence is temperature dependent and sensitive to lateral phase separations. There are data on possible link between oncogenes and inositol lipids [9].

Secretory cells produce cervical mucus. Cervical mucus contains cholesterol, concentration is highest before estrus [10], cholesterol forms crystal structures [11]. Cervical mucus contain mucins which are high molecular weight glycoproteins forming complex mesh-type structures. These present with different biochemical characteristics along the menstrual cycle. The change occurs in response to variations in the physiological levels of oestradiol and progesterone during a cycle, particularly over the ovulatory period [12].

CONCLUSION
Spectral analysis of Normal and HSIL groups supernatant sediment reveals the differences in spectral region which is representative for the fluorescence of cell membrane and mucus components that are related to sexual hormonal changes. It is necessary to point out the carotenoids, sexual hormones dysbalance have a link with cervical cancer development and Human papilloma virus clearance [13].

REFERENCES
