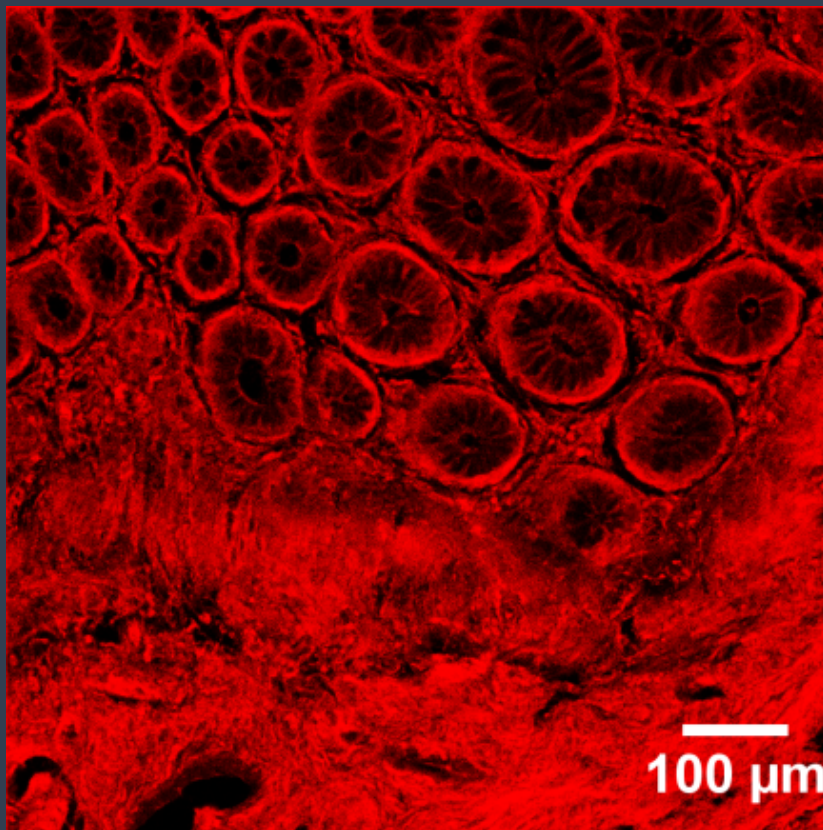


Special Issue

"Biophotonics Technologies"

Journal of Biomedical Photonics & Engineering



Special Issue Editors

Alexey N. Bashkatov, Elina A. Genina,
and Valery V. Tuchin

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Special Issue

“Biophotonics Technologies”

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Preface to the Special Issue on Biophotonics Technologies

We are pleased to present the special issue of J-BPE, which focuses on biophotonics technologies for different biomedical applications. The issue contains selected papers presented at Saratov Fall Meeting 2017 (SFM-17) – International Symposium on Optics and Biophotonics - V (September 25-29, 2017, Saratov, Russia) and includes eight representative papers that well characterize the major topics of SFM-17.

Invited paper by **Khaksari and Kirkpatrick** is devoted to the development of the speckle contrast imaging technique. Basing on the analysis of the first and second order statistics of a single speckle image (single frame) authors give recommendation how to carrying out the speckle measurements.

Invited paper by **Carneiro** and co-authors discusses the wavelength dependences of the refractive index of healthy and pathological human colorectal tissues. Authors underline that refractive index, which can be measured directly, is a significant optical parameter for the development of optical technologies applicable in clinical practice since it allows one to discriminate healthy and pathological tissues.

The paper by **Solovyyev** et al. deals with investigations of fluorescence of green fluorescent proteins. In this study the authors show for the first time that V127T SAASoti – FP without any point mutations in the chromophore environment can be reversibly photoswitched from the green ‘on’ to the dark ‘off’ state that can be useful for development of methods of sub-diffraction microscopy.

Genova et al. present results of investigation and comparison of the macro and micro fluorescence characteristics of *ex vivo* fresh and fixed tissue samples by fluorescence and synchronous fluorescence spectroscopies, and laser scanning confocal fluorescence microscopy, respectively, in application to differentiation of cancerous and healthy gastrointestinal tissues.

The paper by **Bashkatov** et al. presents results of *ex vivo* studies on control of absorption and scattering properties of the human gastric wall mucosa. These studies are able to optimize the laser radiation penetration depth and tissue absorbance, hence, to deliver optimal laser energy for more safe and less invasive therapeutics.

Maryakhina et al. investigate spectral characteristics of benign and malignant breast tissues at different stages of diseases in order to improve the optical techniques of breast cancer diagnostics. As a cheaper and quicker method, it can be used potentially for the breast pathologies screening instead of traditional biopsy and histological examination.

In overall, papers collected in this special issue demonstrate well the exciting potential of optical technologies for biomedical studies and applications aiming medical diagnostics and treatment.

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Water Content and Scatterers Dispersion Evaluation in Colorectal Tissues

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Abstract. Optical clearing treatments reduce light scattering in biological tissues. Due to the heterogeneous composition of tissues, such high scattering is created by the refractive index mismatch between tissue fluids and scatterers. With the objective of collecting experimental data to predict and characterize the magnitude of the refractive index matching in optical clearing treatments, a series of studies were performed with colorectal tissues. The free water content in normal mucosa, muscle and pathological colorectal tissues was estimated, showing similar values for normal tissues and about 5% more in pathological tissue. The refractive index of the tissues was measured at various wavelengths between UV and NIR. This data was used to estimate the refractive dispersion curves for the various tissues, which were then combined with the free water contents to calculate the dispersion of scatterers in these tissues. These dispersions remain unchanged during optical clearing and by obtaining such spectral data, it is possible to quantify and characterize the refractive index matching mechanism in any optical clearing treatment to be applied in these tissues. The differences obtained between the experimental data of healthy and pathological tissues can also be used as a marker for cancer progression in ex vivo analysis. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: colorectal tissue; cancer; refractive index; light scattering; tissue water content; free and bound water; optical clearing.

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1 Introduction and theoretical background

The internal composition of biological tissues is in general heterogeneous and tissue's optical properties depend on such composition and internal arrangement. Typically, soft biological tissues contain cells and their organelles, proteins and extracellular fibers that are surrounded by the cell cytoplasm and interstitial fluid (ISF), respectively [1-5]. These liquids contain mostly water and a small quantity of dissolved salts, proteins and organic compounds [5-7]. Light scattering is usually very significant in tissues. In part, such high scattering is originated by the refractive index (RI) mismatch that exists between tissue fluids and the other tissue components, which are commonly designated as scatterers [5]. As an example, for 589.6 nm, typical values for the RI of ISF are 1.35-1.37, while the RI of scatterers ranges from 1.39 to 1.47 [5]. Higher RI mismatch is obtained if we consider tissue dry matter – solid tissue components without any water. Typical values for the RI of skin melanin and dry proteins in skeletal muscle have been reported as: 1.6 [5] and 1.58 [6], respectively.

There are two types of water in biological tissues – free (bulk) and bound water. Bound water, which is strongly connected to the other tissue components, can only be converted into free water when a strong or long-term stimulation is applied [6-7]. Scatterers are a combination of dry components and bound water. Free water is able to move freely inside the tissue or to the outside if stimulated to do so [8-9]. Free water can be found in the ISF or inside the tissue cells, and small

magnitude or short-term stimulations can initiate its movement.

Since, in a macroscopic scale, no physical boundaries are perceived between tissue components, a biological tissue is generally considered as a continuous material, with spatial variation in the RI [5].

The RI mismatch between tissue components can be reduced by the application of optical clearing (OC) treatments. Such technique has been studied in recent years [5, 10-14], and it operates through two main mechanisms: tissue dehydration and RI matching [15]. During tissue dehydration, only the free water in the tissue will flow out to allow for the optical clearing agent (OCA) to flow from the treating solution into the interstitial space of the tissue. OCAs are innocuous chemicals that have higher RI than water, better matched to the RI of tissue scatterers. Such chemicals are in general sugars, alcohols and contrasting agents [14]. Several OC studies have been reported in literature that use OCAs such as glucose [12, 16], glycerol [7, 17], dimethyl sulfoxide, mannitol, propylene glycol and x-ray contrast agents, such as TrazographTM and OmnipaqueTM [14, 18]. A significant decrease in tissue scattering properties or an increase in tissue depth and in image contrast when using imaging techniques has been demonstrated in these studies [10].

Due to the exchange between the free water in tissue fluids and the OCA in the treating solution, an increase in the RI of the ISF will occur. The RI of tissue scatterers will remain unchanged if no strong or no long-term treatment is applied. If the RI of scatterers is

known, the calculation of the RI change in the ISF can be made to characterize the RI matching for a particular treatment [19]. After calculating the time dependence for the RI of the ISF, the time dependence for the RI of the tissue as a whole can be calculated using Gladstone and Dale equation [20]. The same equation can also be used to calculate the RI of the dry matter or scatterers in the tissue, provided that the bound and free water contents are known. It is highly important to know the total and free water content in biological tissues to quantify light scattering and absorption and to plan efficient OC treatments to reduce light attenuation. Normal and malignant tissues have been reported to have different free and bound water contents [11, 21], and though the knowledge of these values can be considered as a marker of cancer progression in general. The refractive dispersion curves of tissue scatterers and dry matter are also necessary for treatment planning and characterization or even for diagnostic purposes.

Human colorectal tissues are of great interest for research due to the great incidence of colorectal cancer in worldwide populations [22]. Several studies have been recently performed with these tissues to evaluate their dispersion curves [23-24], to estimate their optical properties [25] or to quantify the OCA diffusion properties in OC treatments [11]. On the other hand, these tissues are not fully studied, and the quantification of free and bound water contents or the estimation of the refractive dispersion curves for scatterers and dry matter were not obtained so far.

Considering these aspects, it was our objective to perform experimental studies that would lead to the determination of the free water content in colorectal tissues and to the estimation of the dispersion curves of their scatterers. The free water content in soft tissues is typically around 60% (water volume per tissue volume) [11, 15-16]. To determine the correct water content in *ex vivo* tissues, one can use OC treatments. If a tissue is treated with a solution that contains the same amount of water as the free water in the tissue, there will be a water balance between the treating solution and the tissue and only the OCA in the solution will flow into the tissue. As we have done for other previous research studies with other tissues [11, 15-16], to identify the free water content, we have used as probe OCAs molecules of glucose, glycerol or ethylene glycol in water solutions with various concentrations between 20% to 60%. Since OCAs are hyperosmotic agents [18, 26], instead of using the OCA concentration in aqueous solution, one can use the osmolarity concept to identify a solution by its clearing strength. The molar concentration of the OCA in a solution is calculated as the number agent moles (n) per solution volume (V) [27]:

$$C_M = \frac{n}{V}. \quad (1)$$

The molar concentration described by Eq. (1) is usually represented in mol/l. On the other hand, if we know the molar mass (M) of an OCA and the density of

OCA-water solutions (ρ), one can re-arrange equation to calculate molar concentration:

$$C_M = \frac{\rho}{M}. \quad (2)$$

For nonelectrolytes, the molar concentration (1 mol/l) is equal to osmolarity (1 osmol/l) [28]. In the present study, we have used only glycerol to prepare the OC solutions to treat the colorectal muscle samples. The glycerol concentrations in water solutions and the calculated osmolarities, are presented in Sub-section 2.3.

Several and independent studies that include direct tissue RI measurements for some wavelengths were performed. The methods used in these studies are described in Section 2.

2 Materials and methods

The research presented in this study was performed using different methods. In the studies to obtain the refractive dispersions of tissues, normal and pathological colorectal tissues were used. In the OC studies to determine the free water content, only colorectal muscle tissues were used. The normal colorectal mucosa and the pathological tissues have been previously studied and we used these already published data [11] to calculate the free water content.

Sub-section 2.1 presents a description of the different tissues samples used in this research and the methods used to prepare them. Sub-section 2.2 describes the method to obtain the refractive dispersion curves for all tissues studied and Sub-section 2.3 presents a description of the method used to estimate the free water content in tissues. The calculation procedure to obtain the refractive dispersions for tissue scatterers is described in Sub-section 2.4.

2.1 Tissue samples

Human healthy and pathological colorectal samples used in this study were collected from 7 adult patients undergoing colorectal surgery at Portuguese Oncology Institute of Porto, Portugal. The patients have signed a written consent previous to the surgical procedures, allowing for subsequent use of surgical specimens for diagnostic and research purposes. Such agreement has been approved by the Ethics Committee of the Portuguese Oncology Institute of Porto, Portugal.

Fig. 1 represents a human colorectal histology section and the gross features of an invasive colorectal cancer.

After collecting the samples from surgical procedures, the pathological and healthy areas were separated and preserved frozen at -80 °C for a period of 12 to 24 hrs. After this period, the samples were prepared for each type of study as described below and immediately used. Prior to each set of measurements, the samples were kept in saline for 10 min to mimic natural hydration.

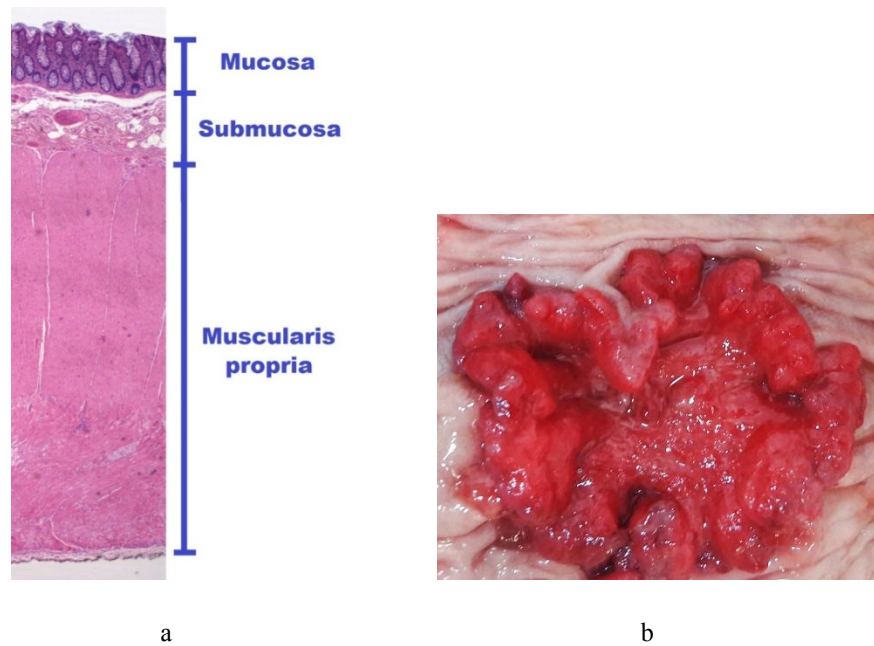


Fig. 1 Human colorectal histology section showing the distinct layers that compose its wall (a) and the gross features of an invasive colorectal adenocarcinoma viewed in a surgical specimen (b).

Two types of experimental studies were performed with normal colorectal mucosa, colorectal *muscularis propria* and pathological tissues.

The first of these studies was the RI measurement using the total internal reflection method and various lasers with different wavelengths between 400 and 850 nm [29-33]. The samples used in these measurements were prepared with an approximated rectangular shape ($\sim 4 \times 3 \text{ cm}^2$). These samples were flattened on one side to adhere perfectly to the prism surface. Three samples of each tissue type were used in these measurements to average results.

To estimate the free water content in the various tissues we have performed another study. For colorectal muscle samples under treatment with water-glycerol solutions with different osmolarities we measured the time dependence of collimated transmittance (T_c) spectra. Using this data and similar time dependencies for normal colorectal mucosa and pathological tissues that were already published [11], we have performed calculations to obtain the free water values in each tissue. The calculations with this data are described in Section 2.3. Similarly to the T_c study published in Ref. 11, the colorectal muscle samples to use in this study were prepared with circular form ($\phi \cong 1 \text{ cm}$) and 0.5 mm thickness. To prepare the samples with these dimensions and to flatten the ones for RI measurements, a cryostat from LeicaTM, model CM 1850 UV was used. Three samples of each type of tissue were used in each particular T_c study to average results, as indicated in Sub-section 2.3.

2.2 RI dispersion estimation

For each type of tissue considered in this research, the total internal reflection method [29-33] was used to measure the RI at different laser wavelengths within the UV-NIR range. In these measurements, we have used lasers with the following wavelengths: 401.4, 532.5, 668.1, 782.1, 820.8 and 850.7 nm [23].

After obtaining the mean RI values from each type of tissue at these wavelengths, we have used CFTOOL in MATLABTM to fit that data with the curves that are commonly used for biological tissues [34-35]. For all tissues we obtained the corresponding dispersion curves between 400 and 1000 nm and the best data fitting was found with the Cornu equation [34-35]:

$$n_{\text{tissue}}(\lambda) = A + \frac{B}{\lambda - C}. \quad (3)$$

In Eq. (3), λ represents the wavelength, to be used in nanometers and the Cornu coefficients A, B and C were estimated for each tissue during data fitting.

The calculated dispersion curves were later used to estimate the dispersion curves for tissue scatterers, as described in Sub-section 2.4.

2.3 Free water content estimation

To estimate the free water content in colorectal muscle, we have performed several OC treatments with glycerol solutions containing different osmolarities. The same procedure has been previously done for colorectal healthy and pathological mucosa under treatment with glucose solutions (see Ref. [11]). Colorectal muscle samples were treated with glycerol solutions with

concentrations and osmolarities as represented in Table 1. The osmolarities for the solutions were calculated using Eq. (2) and the molar mass of glycerol ($M = 92.0776 \times 10^{-3} \frac{\text{Kg}}{\text{mol}}$ [36]). In these calculations, the reported [37] densities for glycerol-water solutions at 20 °C were also used.

Table 1 Glycerol concentrations in water and solution osmolarities.

Glycerol concentration (%)	Osmolarity (osmol/l)
20	11.37
25	11.51
30	11.64
35	11.79
40	11.94
45	12.09
50	12.23
55	12.38
60	12.53

Considering these solutions, three studies were performed for each tissue/treatment combination to average final results. During these treatments, the T_c spectra of muscle tissues were acquired between 400 and 1000 nm. Since between 600 and 800 nm T_c increases linearly for the muscle, we calculated the T_c time dependence curves for individual wavelengths within this range [11].

Considering a particular tissue/treatment study, the averaged T_c time dependence for each particular wavelength was first displaced to have $T_c = 0$ at $t = 0$ (natural tissue) and then normalized to the highest T_c value observed at the beginning of the saturation regime. This displaced and normalized data was then fitted with Matlab's CFTOOL, with a curve as described by Eq. (2) [5, 11, 38]:

$$T_c(\lambda, t) \cong \left[1 - \exp\left(-\frac{t}{\tau}\right) \right]. \quad (4)$$

Eq. (4) indicates that the time-dependence of the T_c spectra ($T_c(\lambda, t)$) describes an exponential increase, which is created by the net effective flow involved in the treatment with a characteristic diffusion time, τ .

During the data fitting, a τ value was estimated for each wavelength. The mean and standard deviation for this parameter were calculated for each treatment.

The mean τ values obtained for each OCA concentration were represented as a function of OCA concentration in the treating solution. A smooth spline was created to interpolate between the experimental points and to show the dependence between τ and OCA concentration in solution. For any particular treatment, the estimated τ value represents the diffusion time for a combination of two fluxes: water flowing into (for low concentrated glycerol solutions) or out (for high concentrated glycerol solutions) and glycerol flowing only into the tissue. By calculating a spline curve that interpolates the data points, we will see that such curve

presents a maximum. Such maximum occurs for a particular glycerol concentration in solution. This particular solution is the one that will have the same water as the free water in the tissue and consequently no water flows in or out the tissue. Consequently, the T_c time dependence that would be measured for a treatment with such solution would be associated only to the glycerol flow into the tissue. Such time dependence has the slowest τ value that characterizes glycerol diffusion in the tissues and it corresponds to the maximum in the spline curve.

On the other hand, since the unique glycerol flow into the tissue occurs for a treatment with a solution that has the same water content as the free water in the tissue [11, 38], we can identify the free water content in the tissue from the graph. As example, if the peak in the graph occurs for a glycerol concentration of 40%, then the water in the treating solution is 60% (100% - 40%). This way the free water in the tissue will also be 60%. Using this knowledge, we estimated the free water content in each type of tissue.

2.4 Calculation of the dispersion for tissue scatterers

The estimated free water contents and dispersion curves from the various tissues were used to calculate the dispersions of tissue scatterers.

To perform these calculations and since our measurements were made at temperatures between 19 and 21 °C, we started by collecting the water dispersion data between 400 and 1000 nm at 20 °C from literature [39]. Using this data, we calculated the corresponding dispersion curves for tissue scatterers by subtracting the free water contribution from the experimental dispersion curves of the different tissues. In these calculations we used an adapted version of Gladstone and Dale equation [20]:

$$n_{\text{scatterer}}(\lambda) = \frac{n_{\text{tissue}}(\lambda) - n_{\text{water}}(\lambda)f_{\text{free-water}}}{1 - f_{\text{free-water}}}. \quad (5)$$

In Eq. (5), $n_{\text{tissue}}(\lambda)$ is the calculated dispersion for a particular tissue, $n_{\text{water}}(\lambda)$ is the water dispersion retrieved from literature [39] and $f_{\text{free-water}}$ represents the free water content in the tissue. All the results from the sequential calculation steps are presented in Section 3.

3 Results and discussion

As indicated in Section 2, our research is based on different measurements and involves some calculation procedures. Therefore, we have structured the presentation of results to show the sequential steps that were performed to obtain the results. The RI measurements and calculations to obtain tissue dispersion curves are presented in Sub-section 3.1. Sub-section 3.2 presents the T_c measurements and

calculations to estimate the free water content in the various tissues and Sub-section 3.3 presents the final calculations to obtain the dispersion curves for tissue scatterers.

3.1 RI dispersion of colorectal tissues

According to the description presented in Sub-section 2.2, the RI measurements made from the various colorectal tissues were made using the total internal reflection method and different lasers.

The samples to use in these measurements have been previously flattened with a cryostat, so they could be placed in perfect contact with the prism surface, without the creation of air bubbles in-between. Considering each laser used in these measurements, we have performed three sets of measurements from three individual samples of each tissue. These measurements were performed using 1° angular resolution and three reflectance curves at the prism/tissue interface were calculated. Fig. 2 presents these curves for the case of colorectal muscle and for the 532.5 nm laser:

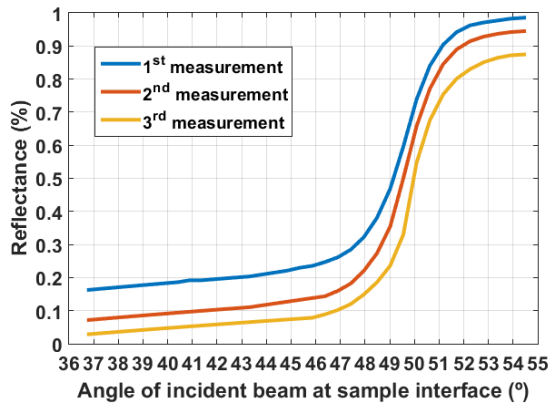


Fig. 2 Reflectance curves at the tissue/prism interface for the measurements with the 532.5 nm laser.

Calculating the first derivative for each curve in Fig. 2, we obtained the curves presented in Fig. 3, where a significant peak can be seen for each curve:

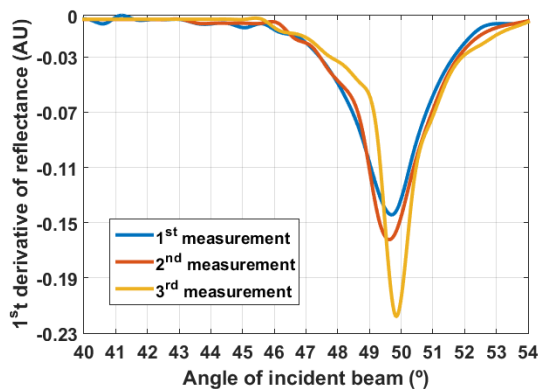


Fig. 3 1st derivative curves of the reflectance measurements presented in Fig. 2.

From Fig. 3, we have identified the angles where the peaks occur. These angles represent the critical reflection angles at the prism/tissue interface for each set of measurements. To calculate the RI values of the tissue $n_{\text{tissue}}(\lambda)$ for the wavelengths (λ) of the lasers used in these measurements, we have used Eq. (6) [23]:

$$n_{\text{tissue}}(\lambda) = n_1(\lambda) \times \sin(\theta_c). \quad (6)$$

In Eq. (6), $n_1(\lambda)$ represents the RI of the prism used in the measurements for the same wavelength of the laser and θ_c is the critical angle retrieved from the peaks in graphs like the one in Fig. 3.

A mean RI value and correspondent standard deviation (SD) value were calculated for the tissue at each laser wavelength. The mean RI and SD values for all tissues and wavelengths are presented in Table 2.

The data in Table 2 only corresponds to a wavelength range between approximately 400 and 850 nm. Since we wanted to obtain the dispersion curves for the tissues for a wider range, we have considered experimental data from another group [24] at 960 and 1551 nm to calculate the curves between 400 and 1000 nm. This procedure was made for the normal mucosa and pathological tissue. For the case of colorectal muscle, no data was available, so we have just extended the curve that fits our data.

The equations obtained for the dispersion curves of the three types of tissues for the wavelength range between 400 and 1000 nm are the following:

$$n_{\text{mucosa}}(\lambda) = 1.315 + \frac{16.73}{\lambda - 38.84}, \quad (7)$$

$$n_{\text{muscle}}(\lambda) = 1.3346 + \frac{19.48}{\lambda + 27.17}, \quad (8)$$

$$n_{\text{pathological}}(\lambda) = 1.315 + \frac{19.06}{\lambda - 49.42}. \quad (9)$$

All these equations are according to Cornu equation (Eq. (3)) [23], and were obtained with an R-square value of 0.999. The data points in Table 2 and the curves described by Eqs. (7), (8) and (9) are represented in Fig. 4.

The dispersion curves presented in Fig. 4 for normal mucosa and pathological tissue were calculated by neglecting the experimental points at 820.8 and 850.7 nm. Those experimental values are a little higher than the calculated curves, presenting the evidence of lipid content in these tissues [23, 25]. Since in this wavelength range we have performed measurements only with two lasers, it is not possible to make an accurate characterization of the wavelength dependence for the RI of these tissues due to the presence of lipids. As an alternative, we have represented the curves described by equations 7 and 9 with dashed lines between 784 and 870 nm [23]. In later calculations to

Table 2 Experimental RI data of healthy and pathological colorectal tissues at different wavelengths.

λ (nm)	Normal mucosa		Normal muscle		Pathological tissue	
	Mean RI	SD	Mean RI	SD	Mean RI	SD
401.4	1.3612	0.0012	1.3801	0.0012	1.3693	0.0017
532.5	1.3489	0.0013	1.3693	0.0024	1.3545	0.0011
668.1	1.3415	0.0010	1.3626	0.0014	1.3460	0.0012
782.1	1.3376	0.0012	1.3590	0.0018	1.3411	0.0008
820.8	1.3393	0.0012	1.3575	0.0014	1.3424	0.0009
850.7	1.3370	0.0012	1.3566	0.0014	1.3408	0.0011

estimate the dispersion curves for tissue scatterers, we will use the tissue dispersion curves as described by equations 7, 8 and 9 for the entire spectral range between 400 and 1000 nm.

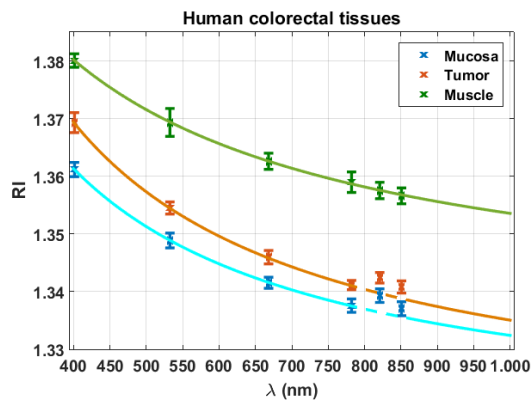


Fig. 4 Experimental RI data and calculated dispersion curves for colorectal tissues.

3.2 Determination of the free water content in colorectal tissues

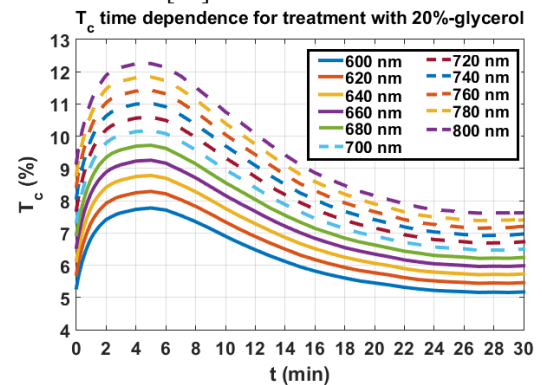
To calculate the dispersion curves for scatterers in colorectal tissues, the free water content is necessary. The evaluation of the free water content was made from measurements of tissue's T_c spectra during OC treatments.

As described in Sub-section 2.3, we have prepared several colorectal tissue samples with 0.5 mm thickness to use in these studies. The normal mucosa and pathological tissues were previously treated with water-glucose solutions with different osmolarities [11] and the muscle samples were treated in the present research with water-glycerol solutions, also with different osmolarities.

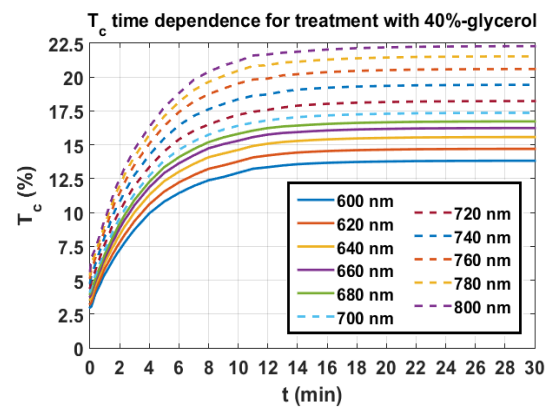
T_c time dependencies for individual wavelengths between 600 and 800 nm were obtained for all tissue/treatment studied. Fig. 5 presents those time dependencies for the treatments of muscle with 20%, 40% and 60%-glycerol:

For each particular treatment, the T_c time dependencies before the beginning of the saturation regime were vertically displaced to have $T_c = 0$ at $t = 0$ and then normalized to the highest value [11]. This rearranged data was fitted with a curve described by Eq. (4). When performing these fittings, the τ values were estimated for each wavelength within a particular treatment. Graphs representing the mean τ vs OCA

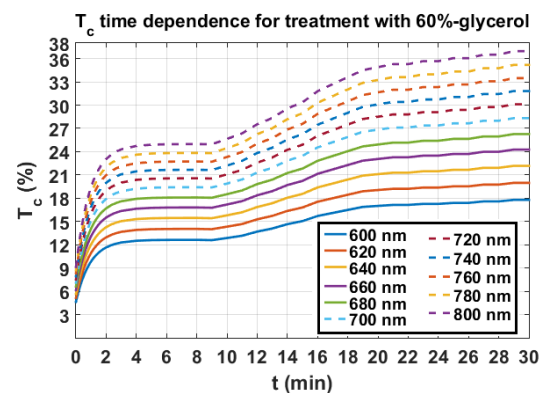
concentration were created for each tissue. The graphs for normal mucosa and pathological tissues are presented in Ref. [11].



a



b



c

Fig. 5 T_c Time dependencies for the treatments of colorectal muscle with: 20%-glycerol (a), 40%-glycerol (b) and 60%-glycerol (c).

Fig. 6 represents the case of colorectal muscle:

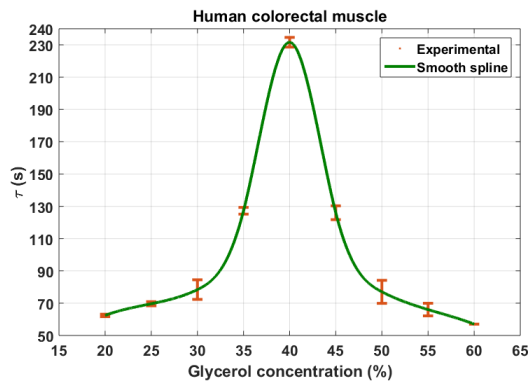


Fig. 6 Mean diffusion time as a function of glycerol concentration in the treating solution.

The peak in the graph of Fig. 6 corresponds to a unique diffusion of glycerol molecules into the muscle tissue. In this case the water in the solution is in perfect balance with the free water inside the tissue, meaning that by identifying the glycerol concentration in the graph of Fig. 6 as 40%, the free water content in the tissue can be calculated as: $100\% - 40\% = 60\%$. Similar estimations of the free water content in normal mucosa and pathological tissue were also made [11]. The estimated values were: 0.594 (normal mucosa), 0.644 (pathological tissue) and 0.600 (muscle).

These values can now be used with the dispersion curves presented in Sub-section 3.1 in calculations to obtain the dispersion curves of tissue scatterers.

3.3 Calculation of the dispersion curves for scatterers in colorectal tissues

Using the dispersion curves of the colorectal tissues that are described by Eqs. (7), (8) and (9) and the free water content values in Eq. (5), we have calculated the dispersion curves for the tissue scatterers. Considering each tissue, these calculations are described by Eqs. (10), (11) and (12):

$$n_{\text{scat-mucosa}}(\lambda) = \frac{n_{\text{mucosa}}(\lambda) - n_{\text{water}}(\lambda) \times 0.594}{1 - 0.594}, \quad (10)$$

$$n_{\text{scat-muscle}}(\lambda) = \frac{n_{\text{muscle}}(\lambda) - n_{\text{water}}(\lambda) \times 0.600}{1 - 0.600}, \quad (11)$$

$$n_{\text{scat-pathological}}(\lambda) = \frac{n_{\text{pathological}}(\lambda) - n_{\text{water}}(\lambda) \times 0.644}{1 - 0.644}. \quad (12)$$

The dispersion curves for tissue scatterers obtained in these calculations are presented in Fig. 7:

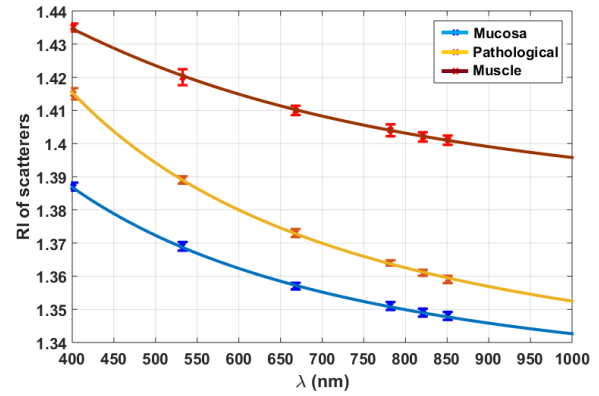


Fig. 7 Wavelength dependence of scatterers RI in colorectal tissues.

The dispersion curves presented in Fig. 7 show differences between the three tissues studied. First, comparing between normal mucosa and muscle, we see that the muscle dispersion shows higher values than mucosa. Such difference might be related to the different nature of the dry matter content in the two tissues, or also to the fact that mucosa might have higher bound water content than muscle. The dispersion for pathological colorectal tissue is also different from the other two, meaning that colorectal pathology is optically distinct from healthy tissues and *ex vivo* examination of the refractive dispersion may serve for diagnostic purposes.

These curves are also important for OC treatment planning in colorectal tissues. Since in the case of short-time OC treatments, only free water will be involved in the fluid exchange, the curves in Fig. 7 will remain unchanged during treatments. By performing some calculations with the data in curves of Fig. 7, it is possible to quantify and characterize the RI matching mechanism in OC treatments. Such calculations are described in literature [5], and in one case that we have studied recently for skeletal muscle under treatment with glucose and ethylene glycol [19].

4 Conclusion

The refractive dispersion curves for scatterers in colorectal tissues were obtained using a simple method based on direct RI and T_c time dependence measurements during OC treatments.

The T_c measurements performed during treatments allowed to calculate the free water contents in different colorectal tissues, and although values are in the same order of magnitude, the method is sensitive enough to discriminate between different tissues. We also observed that the pathological tissue presents considerably higher water content than normal tissues.

Combining these values with the experimental tissue dispersions, it was possible to calculate the dispersion curves of scatterers in colorectal tissues. These dispersion curves remain unchanged during OC treatments and may be used to evaluate the RI change in

tissue fluids or to characterize the RI matching mechanism and OC efficiency.

In the near future we plan to perform those calculations and perform similar research for different tissues.

Disclosures

The authors declare that there are no conflicts of interest related to this article.

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Laser Speckle Modeling and Simulation for Biophysical Dynamics: Influence of Sample Statistics

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Abstract. Laser speckle is a statistical phenomenon and should be treated as such when establishing experimental parameters for using laser speckle techniques to infer information about the dynamics of a coherently illuminated medium, such as biological tissue. Herein, we demonstrate that when sampling (imaging) speckle patterns, it is critical that the sampled speckle patterns are unbiased estimators of the underlying random speckle field. If this condition is not met, the quality of the results will be compromised. Specifically, this study examines the effects of first and second order spatial statistics of speckle intensity images on laser speckle contrast imaging results. Finally, it is recommended that when using speckle techniques such as laser speckle contrast imaging, investigators should examine the first and second order spatial statistics of a speckle image prior to collecting actual data. If this examination reveals that the imaged speckle intensity image is not an unbiased estimator of the underlying random speckle field, then adjustments should be made to ensure that the images taken are unbiased estimators of the true speckle field. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Laser speckle; speckle contrast; sample statistic; speckle imagings.

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1 Introduction

Laser speckle techniques are well established for investigating biophysical dynamics, including the motion of blood cells [1-3] and tissue mechanics [4-5]. Laser speckle techniques possess numerous advantages as tools for investigating dynamic biological systems, including but not limited to good spatial resolution, high sensitivity to motions, relative simplicity, and cost effectiveness. These same features, however, present a weakness of the techniques. That is, it is relatively simple to get data. The simple act of illuminating a scattering surface or volume with coherent radiation (*i.e.*, laser light) will result in the appearance of a speckle pattern in the observation plane. If the scattering medium is dynamic, then the observed speckle pattern will also appear to be temporally dynamic and estimates of this motion are easily made [6].

However, laser speckle is a statistical phenomenon and this fact must be accounted for in both the experimental parameters that are established to spatially and temporally sample the scattered speckle field and also in the approaches used to assess the speckle dynamics. In other words, the sample statistics of the observed intensity pattern must be unbiased estimators of the true scattered field. The result of not meeting this minimum sampling requirement can result in significant errors in the analysis of speckle dynamics. This paper will focus on the spatial sampling of the speckle intensity patterns and will not address temporal statistics.

The first and second order statistics of laser speckle patterns are well established for both ‘fully developed’ (polarized) and ‘partially developed’ (un-polarized) speckle patterns [7] and it is not necessary to present a full description of these statistics herein. Yet, a brief overview is necessary as the remainder of the present paper explores the effects of inadequate spatial sampling of laser speckle patterns such that the sample statistics are not unbiased estimators of the true underlying statistics.

We begin by noting that laser speckle may be viewed as the result of the coherent summation of randomly phased Huygen’s wavelets. Indeed, speckle as a general phenomenon arising from the coherent illumination of a scattering surface or volume can be viewed in this fashion regardless of the form of the radiation (e.g., coherent x-ray, ultrasound, etc.). Thus, for the remainder of this paper we will generalize and simply refer to the speckle phenomenon as speckle and drop the ‘laser’ modifier. It is left up to the reader to make the appropriate substitutions when necessary. These randomly phased wavelets can be viewed as arising from a real source in the case of imaged (or subjective) speckle or a virtual source in the case of non-imaged (or objective) speckle. Observing the speckle pattern intensity with a bare CCD or CMOS chip without an intervening system of imaging lenses would be an example of the latter case. Note that this configuration is equivalent to imaging at infinity. Regardless of the mode of observation, however, the physics of the speckle formation is identical and both imaged and non-imaged speckle display identical first and second order statistics. For this manuscript, first order statistics refer to the statistics at a single point in time and space, while second order statistics describe the relationships between neighboring points. First order statistics describe, for example, the intensity at a given pixel, while second order statistics describe the spatial structure of a speckle pattern [8].

1.1 First Order Statistics

Consider the situation displayed in Fig. 1. The desire is to describe the fields in some observation plane L distant from the illuminated surface. To do this, we consider the surface fields to be equivalent to a finite number of secondary Huygens’ wavelets. The ‘finite’ restriction will be subsequently relaxed. If we ignore the $1/R_n$ dependence of the individual wavelets and make the valid assumption that there is no systematic

variation in phases (i.e., the wavelets are truly randomly phased) then the fields at the surface can be expressed by

$$A = \frac{1}{\sqrt{N}} \sum_{n=1}^N \alpha_n e^{i\theta_n}, \quad (1)$$

where N is the number of summed wavelets.

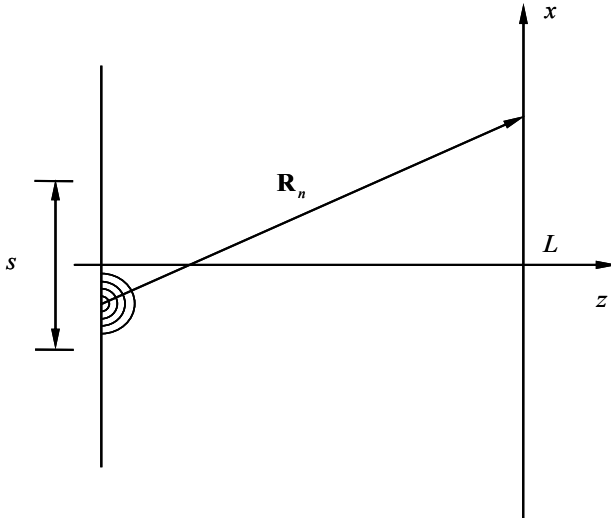


Fig. 1 Geometry of speckle observation. The observation plane is located at L and s is the extent of the illuminated portion of the surface.

Since we are treating the observation fields as the sum of randomly phased phasors, as $N \rightarrow \infty$ the sum becomes asymptotically circularly Gaussian. The joint probability density function (PDF) for the real and imaginary parts of a circular Gaussian complex variable is readily shown to be [7,8]

$$P_{RI}(r, i) = \frac{1}{2\pi\sigma^2} \exp\left\{-\frac{r^2 + i^2}{2\sigma^2}\right\}, \quad (2)$$

where $\sigma^2 = \sigma_r^2 = \sigma_i^2$ is the standard deviation of the underlying random process.

By integrating over the real and imaginary parts such that the integral of the joint PDF equals unity and switching to polar coordinates where

$$A = \sqrt{R^2 + I^2} \quad \text{and} \quad \theta = \tan^{-1} \frac{I}{R},$$

we see that

$$\int_0^{2\pi} d\theta \int_0^\infty da \, a \frac{1}{2\pi\sigma^2} e^{-\frac{a^2}{2\sigma^2}} = \int_0^\infty da \left[\frac{a}{\sigma^2} e^{-\frac{a^2}{2\sigma^2}} \right] = 1. \quad (3)$$

The term in the brackets of Eq. (3) is the PDF of the amplitude and defines the Rayleigh distribution when $a \geq 0$.

Since intensity, not amplitude, is what is observed in Fig. 1, by following the preceding approach for intensity, $I = a^2$, the integral becomes

$$\int_0^\infty \frac{di}{2\sqrt{i}} \left[\frac{\sqrt{i}}{\sigma^2} e^{-\frac{i}{2\sigma^2}} \right] = \int_0^\infty di \left[\frac{1}{2\sigma^2} e^{-\frac{i}{2\sigma^2}} \right] = 1. \quad (4)$$

The term in the brackets is the negative exponential PDF of intensity (Fig. 2). This PDF can be expressed by [9]

$$p_i(i) = \frac{1}{\mu} e^{-\frac{i}{\mu}}; \quad 0 \leq i. \quad (5)$$

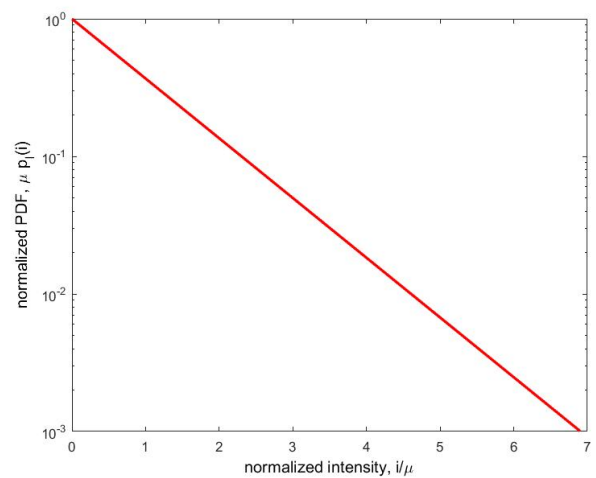


Fig. 2 Negative exponential PDF of intensity for a polarized laser speckle pattern. The expected value of the intensity in the speckle pattern is given by

$$E\{I\} \equiv \mu = \int_0^\infty i p_i(i) di = 2\sigma^2.$$

1.2 Laser Speckle Contrast

The contrast, c , of a laser speckle pattern is a first order property that is frequently investigated in order to infer properties related to the dynamics of a biophysical system. For example, laser speckle contrast imaging (LSCI) is a commonly employed modality used for the relative and qualitative imaging of blood flux [1, 2, 10]. LSCI and related modalities are relatively simple to implement, have wide fields of view, and relatively good spatial and temporal resolution. These features have allowed these techniques to be used as powerful tools in the measurement and monitoring of biophysical systems in near real-time.

In addition to the statistical considerations discussed below, LSCI results depend upon several other experimental considerations as discussed in the review

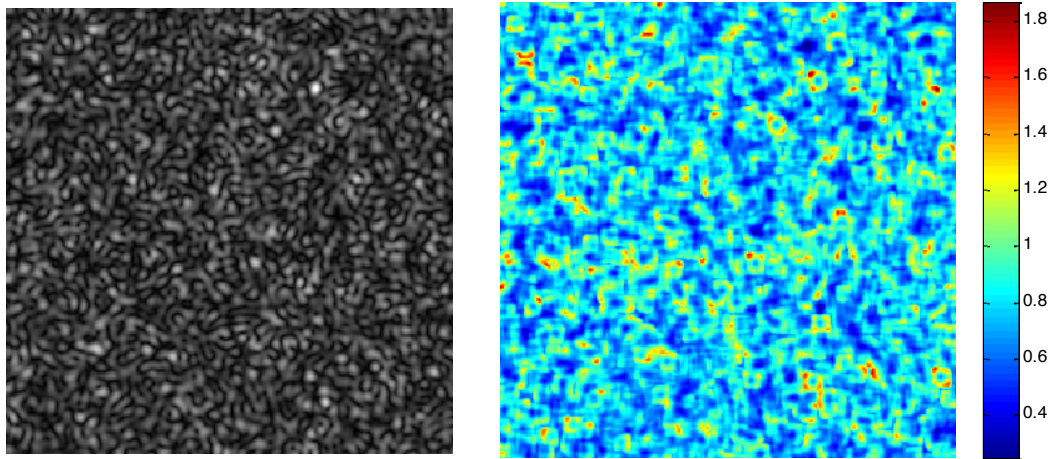


Fig. 3 Simulated speckle pattern (left) with a minimum speckle pattern – 4 pixels and corresponding contrast image (right). Contrast was calculated over a 7×7 sliding window. Calculated global contrast = 1.0.

by Briers et al. [2]. Recently, Khaksari and Kirkpatrick [10, 11] have demonstrated that ultimately, LSCI is sensitive to advective flux, scattering and absorption. Indeed, these three variables are challenging to disassociate from one another in terms of their influence on LSCI.

The contrast, c , of a speckle pattern is typically defined as the quotient of the standard deviation σ_I and the mean μ_I of the measured intensity:

$$c = \frac{\sigma_I}{\mu_I}. \quad (6)$$

For speckle patterns exhibiting an exponentially distributed intensity PDF (see Fig. 2), this value is exactly unity. The concept behind LSCI is to relate the motion of scatterers (blood cells) in the object space to the motion (and thus time integration) of speckles in the observation, or imaging space. Thus, the imaged speckle intensity pattern must be an unbiased estimator of the true dynamic speckle field arising from the moving scatterers. Otherwise, establishing this relationship between the moving scatterers and the observed speckle intensity is fundamentally impossible.

Thus, while contrast is defined in terms of expected values (see Eq. 6), in practice it is calculated over a local spatial (or temporal, or both) neighborhood in terms of the sampled statistics for the intensity:

$$\begin{aligned} C &= \frac{S}{M}; \\ M &= \frac{1}{N_s} \sum_{i=1}^{N_s} I_i; \\ S^2 &= \frac{1}{N_s - 1} \sum_{i=1}^{N_s} (I_i - M)^2, \end{aligned} \quad (7)$$

where M is the mean intensity of the sampled speckle and S^2 is the standard deviation of the sampled intensity, I . If it is assumed that the sample statistics (M and S^2) are unbiased estimators of the true field, then as the number of samples theoretically goes to infinity, then the estimate of the contrast approaches the theoretical value of unity. In practice, the number of samples does not need to approach infinity, it is sufficient at this point to note that the number of samples just needs to be ‘large’. Thus, we can write:

$$\frac{S}{M} \xrightarrow{N_s \rightarrow \infty} \frac{\sigma}{\mu}. \quad (8)$$

This is demonstrated in Fig. 3.

The details of how the speckle pattern in Fig. 3 was generated are given below in the Methods section, as is the calculation of minimum speckle size. As demonstrated by Duncan et al. [12], and is obvious from the contrast image in Fig. 3, the local values of contrast exhibit a distribution. In Fig. 3, the local contrast (calculated over a 7×7 sliding neighborhood window) values ranged from a minimum of approximately 0.2 to a maximum of approximately 1.7. An examination of the PDF of the contrast values reveals that the PDF of the local contrast is closely described by a log-normal distribution of the form

$$f_C(c) = \frac{1}{\sqrt{2\pi c \ln \sigma_g}} \exp \left\{ -\frac{\ln^2(c/c_m)}{2 \ln^2 \sigma_g} \right\}, \quad (9)$$

where c_m is the median value and σ_g is a dimensionless width parameter. The shape of this PDF is shown in Fig. 4 [12].

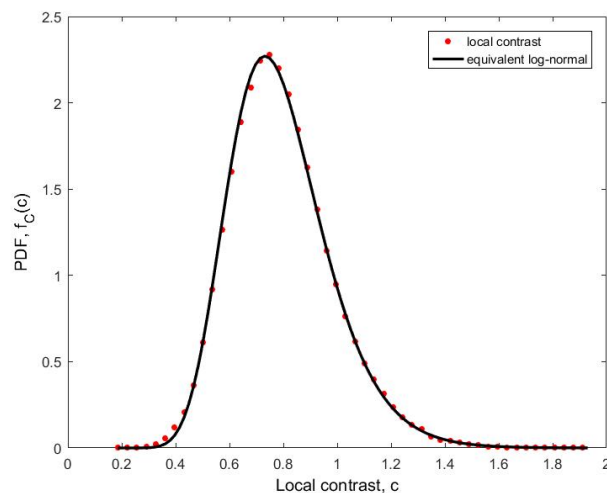


Fig. 4 Log-normal distribution of contrast values calculated over a 7×7 sliding window for a speckle pattern with a speckle size of 4 pixels.

It should be noted here, that typically when discussing sample statistics, the implicit assumption (although not required) is that the samples are statistically independent. Recall that in the example above, the speckles size equaled 4 pixels. This is 2x the spatial Nyquist criteria [13]. Thus in the example above, the speckle field was sampled above the spatial Nyquist limit and the smallest speckle extended over several pixels. Also note, that as is discussed below, the correlation length of the speckle pattern is on the order of the smallest speckle. Thus, the samples of intensity are not statistically independent.

This lack of statistical independence suggests that the median c_m and width σ_g parameters of Eq. 9 will depend upon the size of the neighborhood over which the contrast is computed and on the size of the speckles relative to size of the pixels in the observing camera. Absolute size of the speckles is not a consideration in and of itself, per se. It is the relative size of the speckles to the pixels that is of interest. These considerations are examined below.

1.3 Second Order Statistics

The spatial structure of a speckle pattern is described by the size of the observed speckles. Speckle size can be approximated as the square root of the coherence area of the speckle intensity pattern. The coherence area, in turn, is a function of the covariance of the intensity distribution [8]. The second order statistics of a speckle pattern describe, in essence, the coarseness [7] of the spatial structure. The distribution of speckle sizes in a speckle pattern has readily been found by either examining the full width at half-maximum (FWHM) of the autocorrelation function of the speckle intensity pattern or, alternatively, the spatial frequency distribution of the speckle pattern can be quantified by calculating the power spectral density (PSD) of the

intensity pattern [7, 14]. Either approach yields the same information as the autocorrelation function and the PWD constitute a Fourier transform pair.

An alternative approach of using spatial Poincaré plots with variable lag lengths was recently presented by Majumdar and Kirkpatrick [15]. This approach reduces some of the uncertainties associated with the approaches discussed above, particularly as the speckle size gets large and also provides a more physical connection with speckle size and the correlation length of the intensity pattern. The effects of spatially under sampling the speckle field is discussed in subsequent sections of this paper.

The remainder of this paper is devoted to a discussion of the effects of spatial sampling of laser speckle fields on laser speckle contrast images. In particular, the remainder of the manuscript focuses on the situations where the sample speckle image is not a statistically unbiased estimate of the true underlying scattered field. Both simulated speckle fields and actual, imaged speckle intensity patterns are used as examples.

2 Methods

2.1 Laser Speckle Numerical Simulation

Simulated band-limited speckle patterns were generated in the Matlab environment by filling a circular masks of radius R_r embedded in a larger zero-padded space with random complex numbers over the interval of $[0, 2\pi]$.

The spaces were Fourier transformed and multiplied by their complex conjugates, generating polarized speckle patterns exhibiting negative intensity PDFs (Fig. 2). The size of the speckles was altered by changing the relative size of the circular mask to the surrounding zero-padded space. When the speckle size equaled 2 pixels, the spatial Nyquist criteria was met [13]. This process for generating synthetic speckle patterns has been used extensively in the past [2, 6, 12, 13-15].

Using these speckle patterns, the effects of the neighborhood size over which speckle contrast was calculated was investigated as a function of speckle size and the results compared to those obtained when the sampled intensities were statistically independent from each other. That is, when the speckle size ≤ 1.0 pixels (see above). Neighborhood sizes ranging from 3×3 pixels up to 13×13 pixels were investigated. The largest speckle size investigated was $3 \times$ the Nyquist criteria size (i.e., 6 pixels) and the smallest (under-sampled) speckles were 1 pixel in size.

These simulations were used explicitly to examine what happens when the second order statistic (speckle size) of the sampled speckle intensity pattern is not an unbiased estimator of the underlying speckle field.

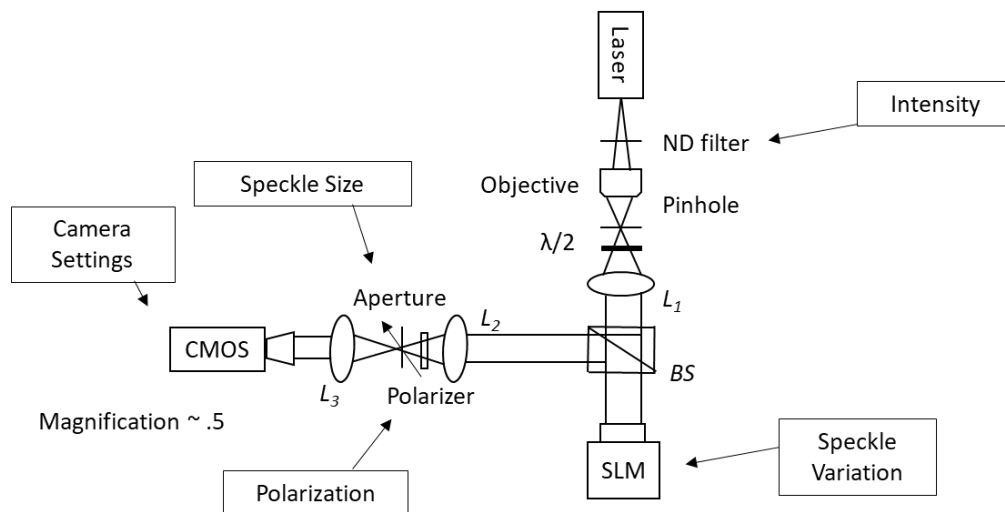


Fig. 5 Optical layout for using a nematic liquid crystal SLM for creating laser speckle patterns and simulated laser speckle contrast images. The intensity of the light incident on the SLM was varied by adjusting the ND filters in front of the stabilized 633 nm HeNe laser. (reproduced with permission from Ref. [16]).

2.2 Laser Speckle Contrast Imaging Simulation Using a Spatial Light Modulator

The effects of incorrect first order statistics on LSCI was investigated by simulating LSCI experiments with a nematic liquid crystal spatial light modulator (SLM) operated in the phase-only mode [16]. The focus of these experiments was to adjust the total intensity of the speckle patterns created via the SLM in the image plane of a CMOS camera and assess the resulting first order statistics of the speckle patterns. As the intensity PDFs of the speckle patterns changed as a result of altering incident intensity on the SLM, the effects on LSCI were investigated. Two separate analyses were performed to investigate the influence of first order statistics on LSCI. First, the global contrast of the speckle patterns generated with varying incident intensities was calculated in order to investigate whether the first order statistics had an influence on global contrast. Second, an analysis was performed on speckle patterns generated with the SLM that had a central region with a shorter decorrelation time, τ_{c_1} , than the surrounding speckle regions with a longer τ_{c_2} . The central region mimicked a vessel carrying blood flow while the surround speckle simulated more 'static' regions. While all other variables, such as camera integration time and speckle size were held constant, the intensity incident on the SLM was systematically varied. The difference in contrast between the 'fast' and 'slow' regions was compared and contrasted.

A detailed description of the procedures is given in Kirby et al. [16], so only a brief overview will be presented herein. Readers are referred to Kirby et al. [16] for details. The optical layout is presented in Fig. 5. The figure is reproduced with permission from reference [16].

In the SLM arrangement, the SLM (Boulder Nonlinear Systems XY Series, model P512) is illuminated by a 2.5 mW stabilized HeNe laser emitting at 633 nm. The resulting speckle patterns were imaged via a $4f$ lens system with an 8-bit CMOS camera (Thorlabs, DCC1545M High Resolution USB 2.0 CMOS Camera). The half-wave plate ($\lambda/2$ plate) in the incident arm combined with the analyzer in the imaging arm allowed the SLM to operate in the phase only mode and also to produce polarized speckle patterns. The magnification of the system was calculated and measured to be 0.5.

To generate speckle patterns from the SLM, a sequence of 50 phase screens was loaded to the SLM. The phase screens were generated in the Matlab environment by filling a 512×512 array with randomly generated complex numbers of unit amplitude with phases uniformly distributed over the interval $[0, 2\pi]$. A look-up table ensured that the phase modulation resulted in a linear relationship between the phase distribution, $[0, 2\pi]$, and the resulting gray values $[0, 255]$. The scattered light remained both temporally and spatially coherent, allowing for random interference to occur when the scattered light impinged upon the CMOS chip. Speckle patterns were thus produced.

Using the copula approach described by Duncan and Kirkpatrick [14], the phase screens, and thus the resulting speckle patterns, de-correlated in a user-defined fashion. In the present experiments the speckles decorrelated following a Gaussian decorrelation function. Regions that were made to simulate a blood vessel decorrelated approximately 16 times faster than the surrounding, more slowly moving speckles. This value was chosen through a purely empirical process so that the resulting laser speckle contrast images visually resembled actual speckle contrast images acquired in

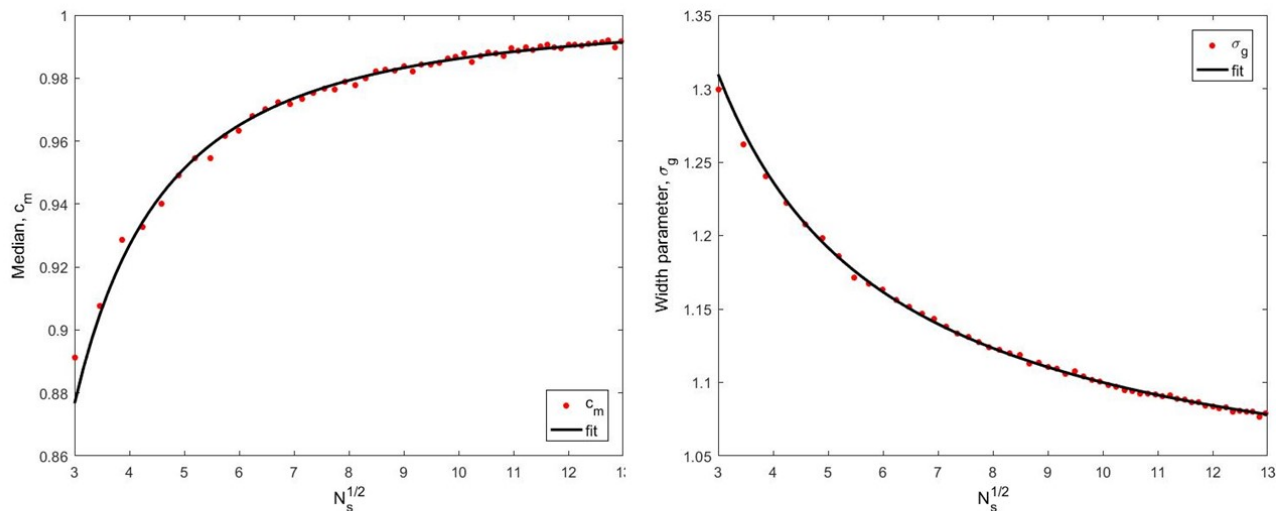


Fig. 6 Dependence on local neighborhood size for the first two moments of the local contrast. Median (left) and width parameter (right) for a sampled speckle pattern exhibiting an exponential PDF. Speckle size was 4 pixels/speckle.

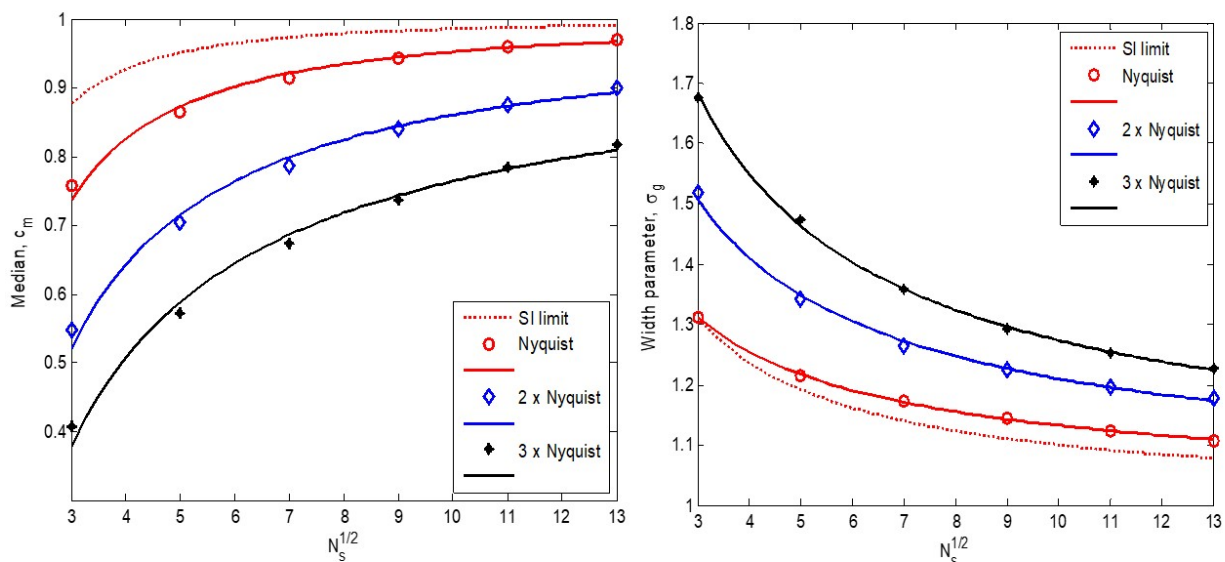


Fig. 7 Parameters of contrast distribution as a function of local neighborhood and speckle sizes. The results for the statistically independent situation is presented as well (dotted line).

the laboratory. The minimum speckle size in the imaged speckle patterns was approximately 3.25 pixels per speckle. Thus, the speckle size exceeded the minimum as determined by the (spatial) Nyquist criteria.

To create time integrated speckle patterns in order to create laser speckle contrast images, sequential frames were summed on an intensity basis to simulate the speckle blurring observed in an actual LSCI experiment when dynamic speckle imaged with a finite exposure time. The blurred speckle resulted in a reduced contrast as is seen in LSCI.

As the goal in these experiments was to alter the first order statistics of the sampled (imaged) speckle patterns, the incident intensity was varied over 4 orders of magnitude. This resulted in some speckle images with an excess of dark pixels (due to under-exposures) and at the other end of the spectrum, some speckle

images with an excess of saturated pixels. Both of these conditions alter the first order statistics of the speckle images.

3 Results

3.1 Effects of Neighborhood Size on LSCI – Second Order Statistics

To examine the effects of second order statistics on the PDF of a speckle contrast image, we first present the results of results of local neighborhood size over which speckle contrast is calculated [12]. As discussed above, the initial speckle size in this analysis was 4 pixels per speckle, or $2 \times$ the Nyquist lower limit. As can be seen in Fig. 6, as the neighborhood size increases, $c_m \rightarrow 1.0$

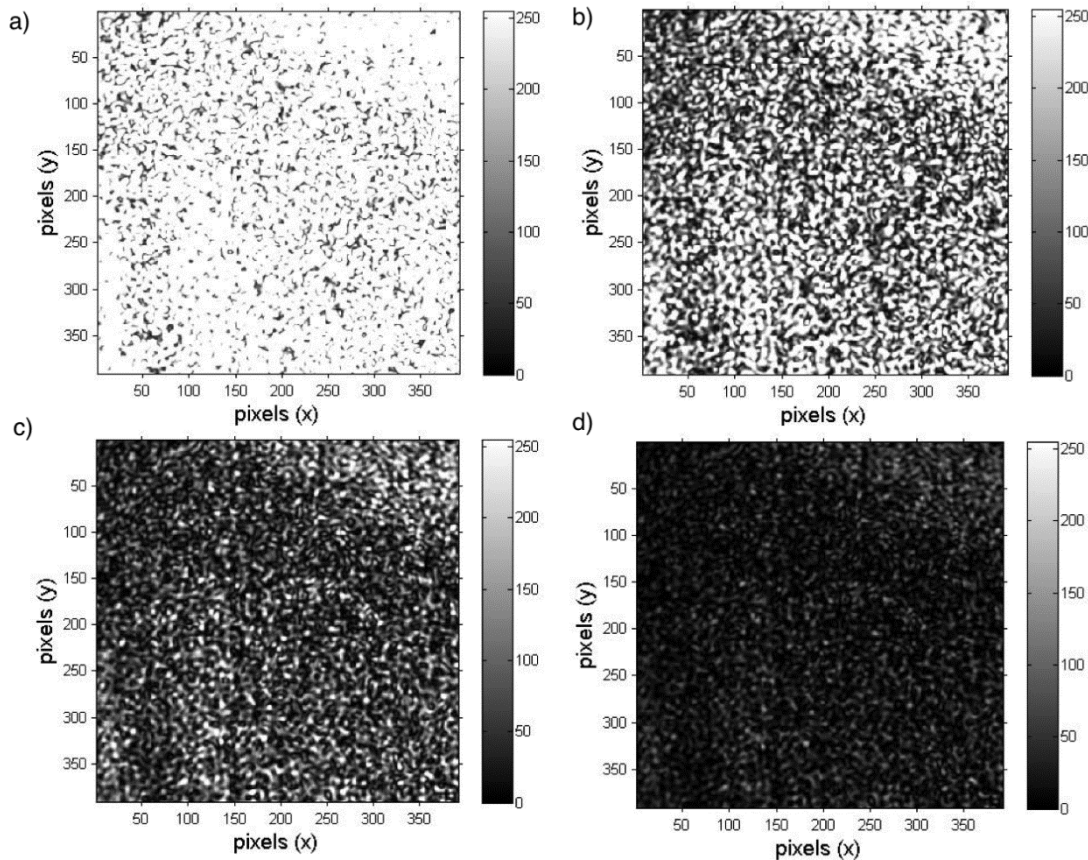


Fig. 8a-d Speckle patterns at various exposures. The top left image is over-exposed and the bottom image slightly underexposed.

and $\sigma_g \rightarrow 1.0$. Both of these results are consistent with the unbiased nature of the sample statistic for contrast.

To examine the effects of speckle size (which is a second order statistic) on the above results, the simulation was repeated for varying speckle sizes. The results are presented in Fig. 7 for speckle sizes at the Nyquist limit, at $2\times$ and $3\times$ the Nyquist limit. Additionally, the simulation was repeated when the sampled speckles were statistically independent of one another. In other words when the speckles were under-sampled and had sizes ≤ 1.0 pixel.

From a practical standpoint, these results indicate that in order to maintain statistical validity, the speckle pattern must be sampled spatially in a manner that meets the Nyquist criteria. That is, the minimum speckle size must be at least 2 pixels per speckle.

3.2 Effects of Intensity PDF on LSCI – First Order Statistics. Global Contrast

As discussed above, global contrast on a properly sampled speckle image should equal unity. In this section, the results of sampling the speckle field so that the intensity distribution is not an unbiased estimator of the true scattered field is examined. As discussed above, this was accomplished by either under or over exposing the speckle images.

Figures 8a-d and 8e-h display static speckle images at several exposures along with their respective intensity PDFs.

The top set of images in Figs. 8 were taken at 100% power from the laser and the bottom set of images were taken with a relative intensity of $0.1\times$ that maximum intensity (i.e., 4 orders of magnitude lower).

When the global contrast of speckle images with different exposures was calculated, it was found that those speckle images that had displayed a negative exponential intensity PDF had the highest contrast, while those that were either over exposed or under exposed, and thus did not display a negative exponential PDF, had a lower global contrast. These results are shown in Fig. 9.

Recalling that contrast is defined as the ration of the mean of the intensity to the standard deviation of the intensity (Eq. 6), then the results displayed in Fig. 9 can be explained by looking at each of these statistical descriptors individually. Figure 10 plots the means and standard deviations of intensity for the speckle images used to generate the data of Fig. 9. At the upper and lower ends of the plot, it is seen that the standard deviations decrease relative to the means as a result of pixel saturation (for over exposed images) or under exposure at the opposite end.

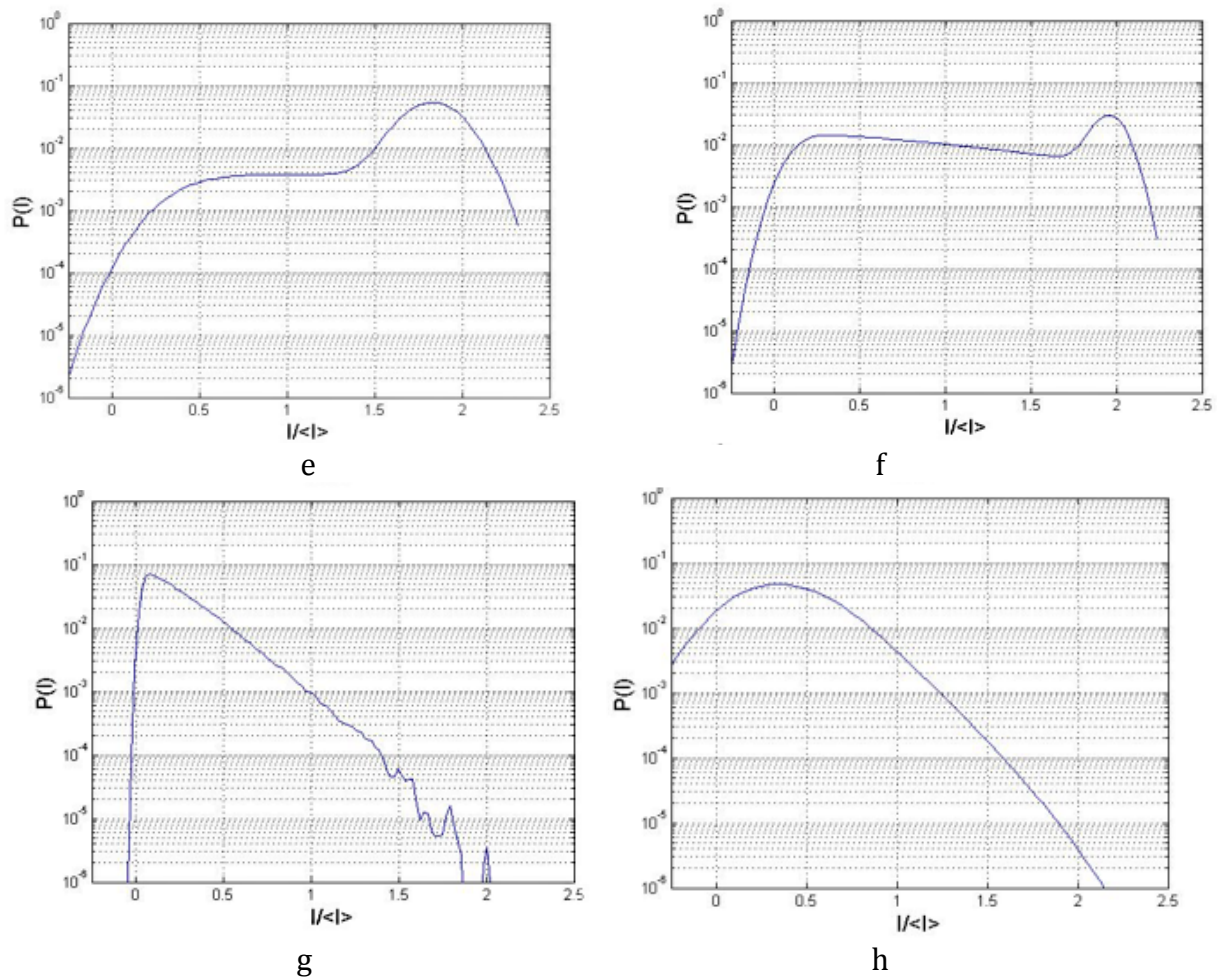


Fig. 8e-h Intensity PDFs of the speckle patterns shown in Figs 8a-d, respectively. The top left image is over-exposed and the bottom image slightly underexposed. Partially reproduced with permission from Ref. [16].

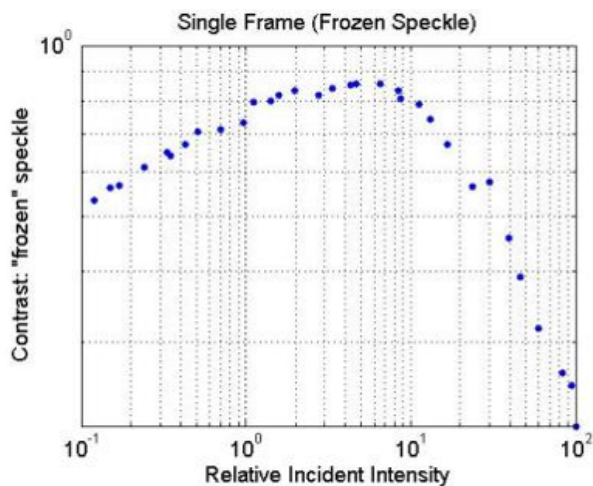


Fig. 9 Global contrast for static speckle images with different exposures that altered the intensity PDFs of the images. The highest contrast values were for speckle images that displayed a negative intensity PDF. Reproduced with permission from Ref. [16].

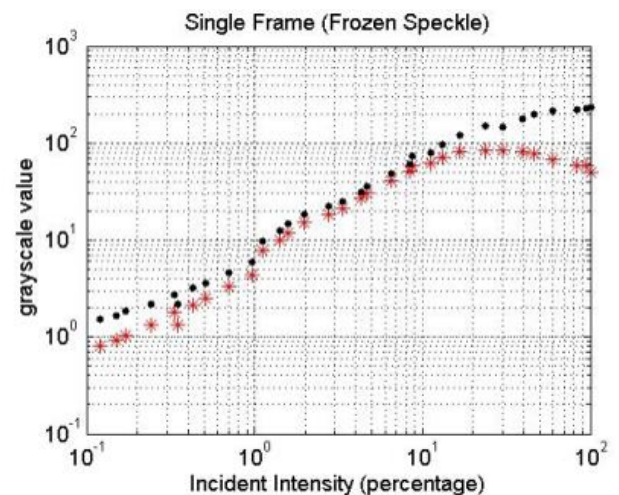


Fig. 10 Means and standard deviations of intensity for speckle images at different exposure levels. In the central portion of the plot, the means and standard deviations are nearly equal, giving a contrast of 1.0. Reproduced with permission from Ref. [16].

Likewise, for the simulated laser speckle contrast images, incident intensity that moved the intensity PDF away from a negative exponential PDF, resulted in a smaller difference between the ‘fast’ decorrelating area that mimicked a blood vessel and the slower decorrelating areas that mimicked the surrounding tissues. This is shown in Figs. 11a and 11b.

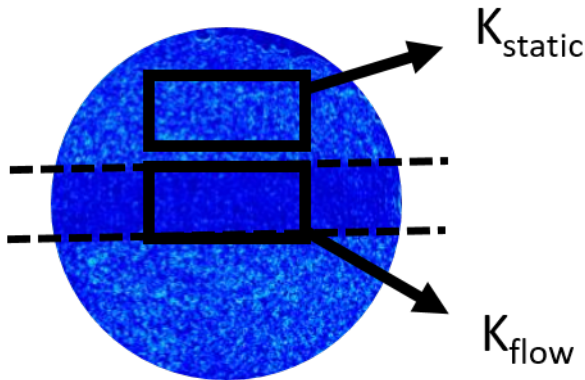


Fig. 11a Simulated LSCI image. The central ‘flow’ region can be seen and the had a decorrelation time approximately 16 times faster than the surrounding ‘static’ region.

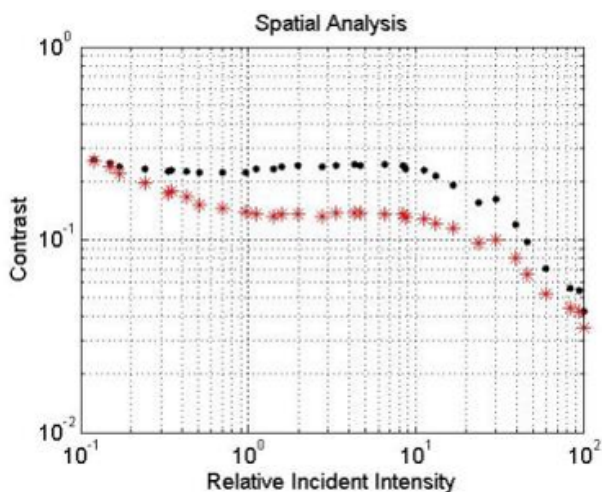


Fig. 11b The ability to discriminate between the flow and static regions in Fig. 11a decreased when the incident intensity was either too low or too high. Red stars display the contrast in the flow region, while the black dots display the contrast of the ‘static’ region. In the central region of the curves, the intensity PDFs of the speckle images followed a negative exponential behavior. At the ends the intensity PDFs were not negative exponentials. Reproduced with permission from Ref. [16].

When the ratio of the contrast in the static region to the contrast in the flow region was plotted as a function of incident intensity, the trend seen in the above simulations was repeated. That is, when the intensity PDF of the speckle images was a negative exponential, the ratio in contrast between the two regions was a

maximum. This is shown in Fig. 12. The ratio of contrast in the two regions was defined in decibels as

$$K_{ratio} = 20 \log_{10} \left(\frac{K_{static}}{K_{flow}} \right). \quad (10)$$

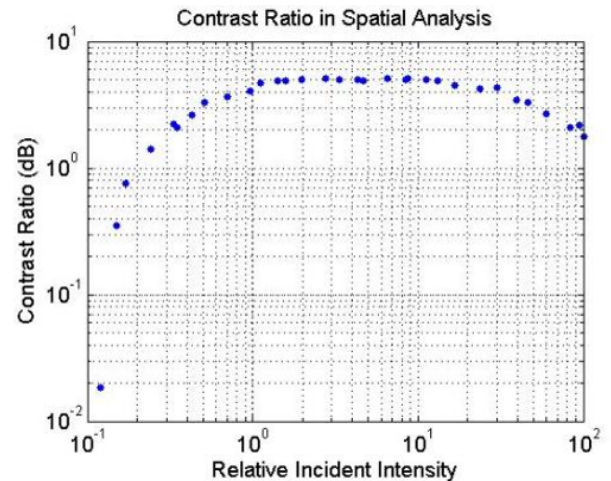


Fig. 12 Ratio between static and flow regions in the simulations as a function of relative incident intensity. As the ends of the curve, the ratio decreases. In the middle of the curve, the intensity PDFs of the speckle patterns were negative exponentials.

4 Discussion

The results of these simulations and experiments reinforce the fact that laser speckle is a statistical phenomenon and must be treated as such. The results further demonstrate that the sample speckle intensity must be an unbiased estimate of the true, underlying random process that speckle is. The results presented herein demonstrate that contrast is maximized the speckle field is sampled so that the sampled speckle intensity is described by a negative exponential PDF and so that the minimum speckle size is at least 2 pixels per speckle in order to satisfy the Nyquist criteria.

Frequently, the use of the so-called ‘coherence’ factor, β , has been invoked in LSCI for the purpose of correcting for insufficient spatial sampling of the field [1, 17, 18]. This factor originates from the Seigert relation that relates the intensity autocorrelation function to the field autocorrelation function in quasi-elastic light scattering (QLS). It should be noted that while LSCI and QLS are related techniques, their sampling requirements are very different. In particular, β is technically used to correct for under sampled speckles, that is, for speckle patterns where the minimum speckle size is below the Nyquist requirement. Roughly, β is equivalent to the inverse of the number of speckles per pixel. However, it is frequently invoked, perhaps inappropriately, as a universal ‘instrument’ factor to account for all sampling and instrument errors. The arguments above indicate

that it is statistically more appropriate to sample the underlying speckle field adequately in the first place, than to attempt to correct for this with a correction factor.

The results of this study lead to a recommendation that prior to taking speckle measurements, such as LSCI measurements, that a single speckle image (single frame) be acquired and the first and second order statistics analyzed to ensure that the sampled speckle pattern is an unbiased estimator of the true, underlying speckle field. If the acquired speckles are too small, or if the intensity distribution of the speckle image is anything but a negative exponential (for a polarized speckle pattern), then adjustments should be made to ensure that these conditions are met prior to acquiring data.

Disclosures

The authors declare that there are no conflicts of interest related to this article. Portions of this paper were presented as part of the 2017 Saratov Fall Meeting, Saratov Russia, September 26-29, 2017.

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Reversible photobleaching of photoconvertible SAASoti-FP

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Abstract. SAASoti is a green fluorescent protein suitable for use in super-resolution microscopy as it can be photoconverted to red fluorescence under 405 nm illumination. The green fluorescence of V127T SAASoti variant is reversibly photobleached under exposure to 470 nm light without photoconversion to the red form. The phenomenon can be explained by chromophore protonation that was confirmed by an increase in absorption at 400 nm (chromophore protonated form) and a decrease at 509 nm (anionic form). This light-induced photoswitching can be repeated with the same sample several times without loss of the initial fluorescence intensity. Subsequent sample exposures result in the same fluorescence recovery process. By changing the content of the H-form one can control photoswitching as only the protonated SAASoti may be converted to the red form. This property is extremely important for sub-diffraction microscopy. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: fluorescent proteins; photobleaching; reversible photoswitching.

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1 Introduction

Fluorescent proteins (FPs) have grown into indispensable tools for life science studies. Among them reversibly photoswitchable fluorescent proteins (RFPs) are of special interest as they can be applied to super-resolution fluorescence microscopy techniques [1].

Originally discovered in the *Stylocoeniella armata* coral, SAASoti FP was found to be irreversibly photoconvertible from green to red fluorescence under 405 nm illumination [2]. The process is accompanied by peptide bond break. Previously we have demonstrated the pH-dependence of this photoconversion and the presence of protonated and anionic chromophore forms [2]. Wild type SAASoti exists as a tetramer, while V127T point mutation led to its monomerization.

On the one hand, β -barrel structure of fluorescent proteins protects the chromophore from different types of fluorescence quenching, but on the other hand, neighboring amino acid residues facing inside the barrel interact with the chromophore and can therefore affect its photophysical properties. The effect of photoinduced bleaching is known for different GFP-like proteins [3], [4]. In some cases it is associated with *cis-trans* isomerization of the chromophore and a change of its protonation state, while in the case of Dreiklang FP the chromophore undergoes reversible hydration by a completely different mechanism [5]. For another green-to-red photoconvertible FP well-described today –

IrisFP – the photobleaching was shown to be reversible [4]. After X-ray structure analyses of the fluorescent ('on') and photobleached ('off') crystals the authors also determined amino acid residues that are crucial for stabilizing different states of the chromophore. Firstly, it was shown that the chromophore undergoes *cis-to-trans* isomerization during photobleaching. While the *cis*-configuration is stabilized by the E144-H194-E212 H-bonded triad, it is replaced by the E144-R66-E212 triad in the case of the *trans*-configuration [4]. Accordingly, either H194 or R66 stabilizes the chromophore by π -stacking or π -cation interactions, respectively [6]. As there exist FPs with *trans*- chromophore in their 'on' state [7] it should be emphasized that it is not the configuration (*cis*- or *trans*-) but its protonation state that is responsible for the fluorescence. Here we report the phenomenon of reversible photoswitching observed for V127T SAASoti-FP.

2 Materials and methods

2.1 Protein expression and isolation

The recombinant plasmid pET22b containing V127T-SAASoti gene (669 bp) was transformed into *E. coli* BL21 (DE3) cells, cultured at 37 °C and 200 rpm shaking in LB medium with 100 mg/ml ampicillin to an optical density of 0.5. Protein synthesis was induced

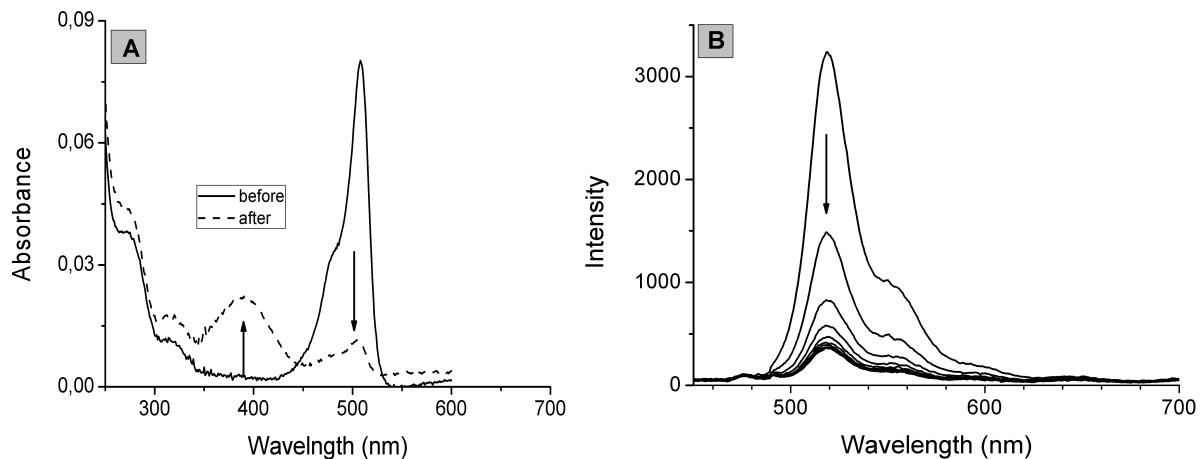


Fig. 1 (A) Absorbance spectrum of V127T SAASoti-FP variant before (solid line) and after (dashed line) 10 min 470 nm exposure (1 W/cm^2); (B) Fluorescence spectra under 470 nm (1 W/cm^2) exposure every minute during 10 min.

by addition of IPTG to the final concentration of 0.05 mM and subsequent incubation for 24 h at 20 °C and 200 rpm shaking. After *E. coli* disruption with a French press, the bacterial cell pellet was suspended in 20 mM Tris-HCL (pH 7.5) containing 150 mM NaCl, 1 mM PMSF, and 1 mkg/ml DNase. After ammonium sulfate was added to 50% saturation (v/v), the colored fraction was separated by centrifugation at 18 000 g and 4 °C. The colored precipitate was dissolved in 20 mM NaHCO_3 . After overnight dialysis against 20 mM NaHCO_3 solution, the V127T SAASoti solution was further purified with MonoQ-based ion-exchange chromatography. The protein was eluted with a linear 0 – 0.5 M NaCl gradient performed by mixing 20 mM NaHCO_3 anion-exchange buffer (A) and 20 mM NaHCO_3 containing 0.5 M NaCl anion-exchange buffer (B). AKTA purifier chromatographic system (GE Healthcare) with multiwavelength detection (260 nm, 280 nm and 509 nm) was used. Eluted fractions were analyzed by SDS-PAGE and concentrated.

2.2 Spectroscopic experiments

Prior to the spectroscopic experiments SAASoti was diluted in 200 mM sodium phosphate buffer pH 8.0 to the final concentration of 3 μM . The photobleaching and relaxation experiments were carried out in 3 mm Hellma quartz cuvettes. Illumination of the samples at 470/24 nm (1 W/cm^2) was performed by LED Lumencor Spectra X. Fluorescence kinetics data were obtained with a homebuilt linear CCD spectrometer based on Sony ILX511. Light power was verified with a Newport 2936-c power meter. Absorption spectra for thermal relaxation were recorded on a Cary 300 Bio spectrophotometer (Varian). Data analysis was performed with Origin 8.5 software package.

3 Results and discussion

After exposing the V127T SAASoti variant to 470 nm light (1 W/cm^2) for 10 min, we observed the

phenomenon of photobleaching. As it can be noticed from the absorption spectra in Fig. 1A, 470 nm exposure during 10 min leads to a 395 nm absorbance peak appearance while the absorbance intensity of the green form (509 nm) decreases. Previously it has been shown that the protonated chromophore absorbs at 395 nm while its anionic form shows a maximum absorbance at 509 nm. In other words, here we observe photoinduced switching between two different protonation states of the chromophore in the same buffer system (pH 8).

It is known for different FPs that during photobleaching *cis*-to-*trans* isomerization of the chromophore takes place along with its protonation [4], [8], [9]. The process can be reversed either by illumination at 405 nm or during thermal relaxation. We investigated thermal relaxation as V127T SAASoti can be irreversibly converted into the red fluorescent form by 405 nm illumination. Fig. 2A shows that photobleaching followed by thermal relaxation of the probe can be repeated with the same sample several times without any significant changes in the fluorescence intensity of the green form at 520 nm.

The photobleaching experiments with different fluorescent proteins exhibit bi- and mono-exponential fluorescence decay dependences [10], [11]. To characterize the photoswitching between ‘on’ and ‘off’ forms we recorded the photobleaching (Fig. 2A) and thermal relaxation (Fig. 3A) kinetics. A bi-exponential function (Eq. 1) fit the photobleaching experimental data well with $R^2 = 0.999$ and the random distribution of the residuals (Fig. 2A).

$$I = I_1 * \exp(-k_1 t) + I_2 * \exp(-k_2 t) + c, \quad (1)$$

where I_1 , I_2 – pre-exponential coefficients, c – background signal including residual SAASoti fluorescence. The calculated rate coefficients are $k_1 = 0.028 \text{ s}^{-1}$ and $k_2 = 0.011 \text{ s}^{-1}$ at 22 °C with the 1:1 ratio of the pre-exponential coefficients. The rate of

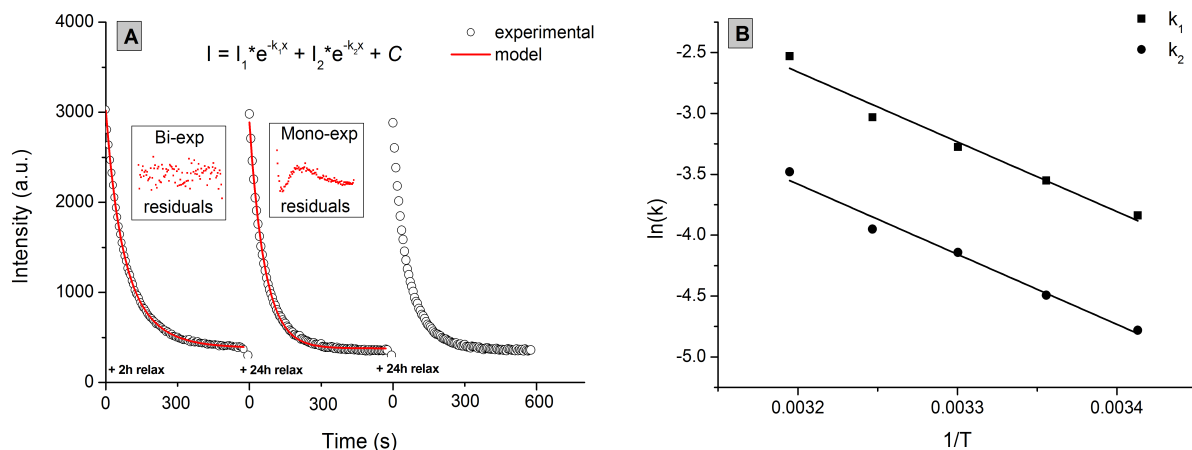


Fig. 2 (A) Photobleaching kinetics of V127T SAASoti-FP. Subsequent photobleaching cycles of the same sample including 470 nm exposure during 10 min (1 W/cm^2) and $\sim 24\text{h}$ thermal relaxation at 22°C . Fitting of the data to a bi-exponential model. (B) Arrhenius plots of rate coefficients for the photobleaching kinetics.

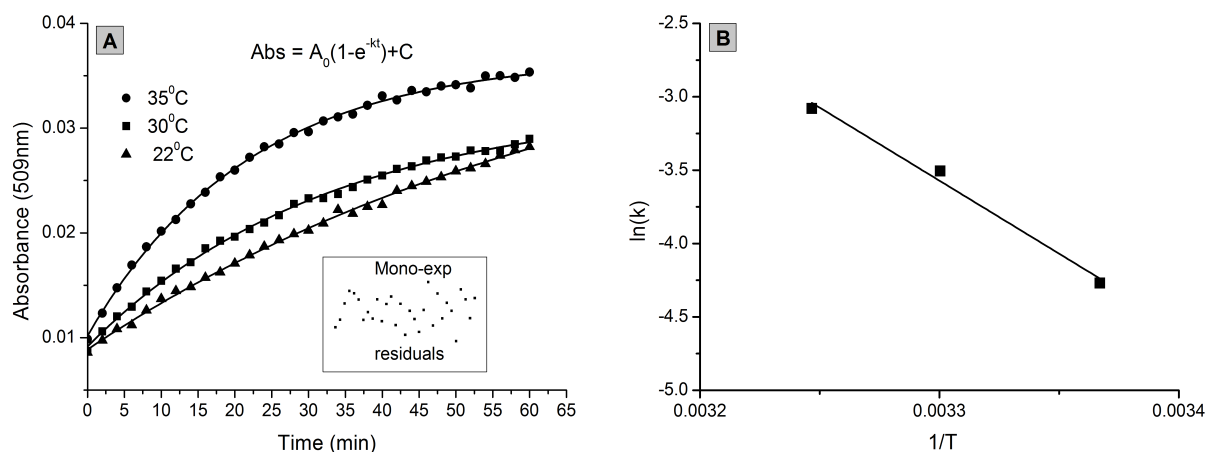


Fig. 3 (A) Thermal relaxation kinetics. The green form absorption recovery in time at different temperatures and fitting of the data to a mono-exponential dependency; (B) Arrhenius plot.

photobleaching certainly depends on the light exposure intensity. The phenomenon of bi-exponential fluorescence decay was explained by the existence of two protein populations in the excited state that can be reversibly converted to the 'off' state [11]. Previously, this 'multi protein populations' hypothesis was suggested by our group [12] when conducting experiments on KFP and its pH-dependency. Sometimes it is difficult to compare bleaching kinetics data for different FPs as the system parameters (illumination intensity, beam shape, geometry, and concentration of the sample) in different cases can vary dramatically. In order to calculate the photobleaching activation energy (E_a) we illuminated the sample with 470 nm light at different temperatures in the range of $20 - 50^\circ\text{C}$. E_a was calculated from the Arrhenius equation (Eq. 2) as 47 kJ/mol (Fig. 2B) for both protein populations.

$$k = A * \exp(-E_a/k_B T). \quad (2)$$

Fig. 3A shows the relaxation kinetics recorded as a time dependency of the absorption recovery at 509 nm for a photobleached sample. Experiments on thermal relaxation reveal the mono-exponential dependence ($R^2 = 0.998$ and the random distribution of the residuals) of the process (Eq. 3)

$$A = A_0 * (1 - \exp(-kt)) + c \quad (3)$$

with $k = 0.014 \text{ min}^{-1}$ at 22°C . The mono-exponential mechanism was also demonstrated in several works on FPs' thermal relaxation [13], [14]. Estimated E_a value for thermal relaxation (83 kJ/mol) (Fig. 3B) is obviously lower than that in the case of mTFP0.7 (106 kJ/mol) [15] and Dronpa (109 kJ/mol) [3] but slightly higher than 55 kJ/mol for a synthesized GFP-like chromophore [16]. KFP protein alternatively has a stable *trans* 'off' form and can thermally relax from the fluorescent *cis* 'on' form. Quantum mechanics/molecular mechanics

Sequence	Position	Sequence	Position
SAASoti	1	MALSKQYIPDDMELIFHMDGCVNGHYFTIVATGKAKPYEGKONLKATVTK	50
IrisFP	1	----MSAIKPDMMKINLRMEGNVNGHHEFVIDGDGTGKPFPEGKQSMDELVEKE	46
SAASoti	51	GAPLPFFSTDILSTVMHYGNRCIVHYPPGILDYFKQSFPEGYSWERTFAFE	100
IrisFP	47	GGPLPFAFDILTAFHYGNRVFAEYDPHIQDYFKQSFPGKYSWERSLTFE	96
SAASoti	101	DGGFCTASADIKLKDNCFIHTSMFHGTNFPADGPMQRKTIQWEKSIEKM	150
IrisFP	97	DGGICIRANDITMEGDTFYINKVRFHGVNFPANGPVMQKTKLKWEPSTEKM	146
SAASoti	151	TVSDGIVKGDITMFLLEGGGKYRCQFHTSYKAK-KVEMPOSHYVEHSI	199
IrisFP	147	YVRDGVLTGDIEMALLLEGNAHYRCDSTRTTYKAKEGKVLPGYHLVDHCI	196
SAASoti	200	E--RTNDDGTQFELNEHAVARLNE-----	221
IrisFP	197	EILSHDKDYNKVKLYEHAVAHSGLPDNARR	226

Fig. 4 Amino acid sequence alignment of V127T SAASoti with IrisFP. Chromophore forming residues are shown in green, E144* and E212 capturing the chromophore in the *cis*- and *trans*- configuration at the same time are grey shaded, whereas R66 (*trans*-) and H194 (*cis*-) are shaded blue-grey and red, respectively. (Program: needle, Matrix: EBLOSUM62). * - IrisFP amino acid numbering.

(QM/MM) simulations of KFP *cis-trans* isomerization predicts $E_a = 86$ kJ/mol (20.7 kcal/mol) [17] that is in good agreement with the previously published experimental data (71 kJ/mol) [9].

Thermal relaxation of V127T SAASoti occurs with a 50 min half-life ($\tau_{1/2}$) of the dark state at 22 °C. In the case of mTFP0.7 [15], the fluorescence intensity recovers in 30 min, thermal relaxation in the case of IrisFP [4] and Dronpa [18] occurs much slower with the $\tau_{1/2}$ values equal to 5.5 h and 14 h, respectively. Much faster relaxation kinetics in comparison with IrisFP and Dronpa along with the lower E_a values of SAASoti may indicate that V127T SAASoti is less stable in its ‘off’-state.

Amino acid sequence alignment of V127T SAASoti with well-studied IrisFP (Fig. 4) reveals over 64% sequence similarity and the same positions of the conserved amino acid residues – R66, E144, H194, and E212 (IrisFP a.a. numbering). Therefore, we suggest that the same residues capture the chromophore in the *cis*- and *trans*-configurations in the case of SAASoti-FP.

The F173S point mutation of green-to-red photoconvertible EosFP leads to the appearance of the reversible photoswitching phenomenon (IrisFP) [4]. By performing rational design of the chromophore environment (I157, M159, F173), the authors [19] later revealed that the same point mutation (F173S) in the case of Dendra2 also lead to the on/off photoswitching (NijiFP). Another promising mutation – M159A – in the case of EosFP resulted in the appearance of the on/off photoswitching along with a loss of the ability to be converted into the red form. The M159A variant of Dendra2 becomes photoswitchable and is still photoconvertible. Interestingly, in V127T SAASoti-FP, unlike EosFP and Dendra2, methionine occupies position 159 and phenylalanine – position 173, and the protein demonstrates on/off photoswitching.

4 Conclusions

Previously V127T SAASoti was shown to be irreversibly converted from green to red fluorescent form under 405 nm illumination. In this study we have shown for the first time that V127T SAASoti – FP without any point mutations in the chromophore environment – can be reversibly photoswitched from the green ‘on’ to the dark ‘off’ state. Studies of different photoswitching FPs have identified several amino acid residues crucial for formation of the on/off states of the chromophore. Some of them (R66, H194, E144, E212) are located at the same positions in the V127T variant of SAASoti. Curiously, V127T SAASoti is already photoswitchable without F173S and M159A substitutions, which are responsible for the appearance of the photoswitching phenomenon in the case of IrisFP and NijiFP. Although the mechanism of the photoswitching of V127T SAASoti has not been investigated in the study and remains unclear, it is most likely caused by *cis-trans* isomerization of the chromophore associated with the change of its protonation state.

We suggest that a further study of the different SAASoti-FP variants, on the one hand, can contribute a lot to the development of the super-resolution techniques, but on the other hand, will deepen our understanding of the photobleaching mechanisms.

Disclosures

The authors declare that there are no conflicts of interest related to this article.

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Study of the Changes of Gastric Wall Mucosa Optical Properties under the Impact of Aqueous Solutions of Haemoglobin and Glucose for Improving Conditions of the Laser Coagulation

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Abstract. The paper presents the results of *ex vivo* studies of the possibility to control the absorption and scattering properties of the human gastric wall mucosa. For the first time we obtained the increase of the tissue absorption coefficient in the range 350-1250 nm by 2-4.5 times under the injection of haemoglobin solution with the concentration of 70 g/L into the mucosa. The observed increase of the absorbed energy fraction by nearly 65-90% was accompanied by almost 50-60% decrease of the penetration depth at the wavelengths of the sources widely used for laser ablation and coagulation of gastric mucosa parapsasms. Under the injection of 40% glucose solution into the mucosa, we observed the reduction of the absorption coefficient in the spectral region of water absorption bands approximately by 20% and the reduction of the transport scattering coefficient by nearly 24-27% in the spectral range 350-2500 nm. Increasing of the depth of laser radiation penetration in this case amounted to 15-17% in the range 800-1100 nm. The performed studies show the possibility in principle to control the optical parameters of the gastric wall tissues from the point of view of both varying the laser radiation penetration depth and regulating the tissue absorbance and, hence, the power of laser radiation, which, in turn, will allow more safety and less invasion in the course of laser therapeutic procedures. © 2017 Journal of Biomedical Photonics & Engineering.

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1 Introduction

The contactless laser coagulation and ablation of pathologic formations in different organs is widely used in modern clinics [1-8]. The minor invasiveness of the procedure reduces the risk of postoperative complications. The sources used for this purpose include, particularly, the ytterbium (1075 nm) [3], CO₂ (9.4-10.6 μ m) [5], Nd:YAG (1064 nm) [1, 4], diode (980, 808, 810 nm) [2, 6, 7], and other lasers. The main advantage of lasers generating at the wavelengths that coincide with the absorption bands of water is the small depth of light penetration into tissues, which prevents the damage of the adjacent healthy tissue, underlying the affected area [5, 8]. However, in recent time the cheaper diode lasers become more and more widely used.

The radiation from infrared diode lasers in the range 800-1100 nm penetrates deep enough, since in this range the absorption of such tissue components as haemoglobin, melanin, proteins, and water is relatively small [9]. Thus, the danger of tissue damage or perforation arises. To prevent these complications, the authors of some papers propose to increase the thickness of the irradiated object. Thus, e.g., according to the data of Refs. [10-12], in order to protect the mucosa of stomach and intestinal wall in the course of laser endoscopic resection, the layers of mucosa and submucosa were separated by a certain spacing, filled with glycerol, sodium hyaluronate, or hydrogel.

An alternative possible approach is to optimise the laser impact by varying the optical parameters of the tissue. The control of optical parameters can be implemented both by enhancing the absorption properties of the object itself and by reducing the scattering in the tissues adjacent to the lesion focus. In the first case, on the one hand, the laser beam penetration depth is reduced and, on the other hand, the fraction of energy absorbed in the lesion focus is essentially increased, thus improving the laser coagulation efficiency. In the second case, the precision of laser radiation focusing is facilitated.

The absorption and scattering properties of the stomach wall mucosa are well-studied [13-16]. It has been shown that the use of biocompatible immersion agents, such as solutions of glycerol, propylene glycol, etc., results in efficient optical clearing (i.e., light scattering reduction) of the gastric tissues in the near IR spectral region [17-21]. Earlier we proposed to use haemoglobin in order to control the scattering properties of blood by creating a local haemolysis site in a blood

vessel [22]. In the present paper, we propose to use the blood haemoglobin as an absorbing agent.

The aim of the paper is to study the effect of aqueous haemoglobin solution and aqueous 40% glucose solution on the optical properties of gastric wall mucosa in order to improve the conditions for laser coagulation in the visible and near IR spectral ranges.

2 Materials and methods

The study has been performed with 15 samples of human gastric wall mucosa from different patients, obtained in the course of planned operations or anatomic investigations. The experimental studies were approved by the Ethics Committee of V.I. Razumovsky Saratov State Medical University. Immediately after the autopsy, the samples were placed in 0.9% solution of NaCl and were kept in it until the conduction of spectral measurements during 4-8 hours at the temperature $\sim 4^{\circ}\text{C}$.

Immediately before the measurements, the samples having the dimensions of nearly $15 \times 20 \text{ mm}^2$ were cut from the tissue. Five samples were used as a control group. They were not subjected to the impact of any agents. The next five samples were subjected to multiple injections of the standard aqueous solution of haemoglobin (OOO "AGAT-MED", Russia) with the concentration 70 g/L. The total volume of the solution injected into each sample amounted to $\sim 0.1 \text{ mL}$. Into the rest five samples the standard 40% aqueous solution of glucose (NPO "Microgen", Russia) was injected in a similar way. Multiple injection of preparation microdoses over the mucosa area before the laser exposure was intended to model the conditions of the endoscopic laser coagulation procedure. Then the samples were placed between two microscope slides without compression. The edges of the slides were fixed. The sample thickness was measured by means of a micrometer at five points. The accuracy of the measurement was $\pm 10 \mu\text{m}$. The obtained values were averaged. The resulting thickness of the samples varied from 1 mm to 2.5 mm.

The coefficients of total transmission and diffuse reflection were measured not later than in 10-15 minutes after the injections. The optical properties of the tissue were studied in the spectral range 350-2500 nm using the spectrophotometer LAMBDA 950 (PerkinElmer Ltd, UK) with integrating sphere, which is a double-channel monochromator with the built-in system of control and signal recording. The photometric accuracy of measurements amounted to $\pm 0.0006 \text{ Abs}$. As a source of radiation, we used a halogen incandescent lamp. The

spot dimensions of the light beam incident on the sample were $5 \times 5 \text{ mm}^2$. The scanning velocity was 2 nm/s .

To process the experimental results and to determine the optical parameters of mucosa we used a combined method. At the first stage of the method, the measurement data were processed using the inverse adding-doubling (IAD) method [23]. Then the accuracy of the obtained values of the absorption coefficient (μ_a) and transport scattering coefficient (μ_s') was improved using the inverse Monte Carlo method [24, 25] by minimising the target function

$$F(\mu_a, \mu_s') = (R_d^{\text{exp}} - R_d^{\text{calc}}(\mu_a, \mu_s'))^2 + (T_t^{\text{exp}} - T_t^{\text{calc}}(\mu_a, \mu_s'))^2.$$

Here R_d^{exp} , T_t^{exp} , R_d^{calc} , T_t^{calc} are the values of the diffuse reflection coefficient (R_d) and total transmission coefficient (T_t) experimentally measured and theoretically calculated using the Monte Carlo method [26], with the geometry of the studied medium and the experiment taken into account. As an iteration procedure, we used the Nelder-Mead simplex-method thoroughly described in Ref. [27]. The iteration procedure was continued until the agreement of the measured and the calculated data to a given accuracy ($<0.1\%$).

The light penetration depth is one of the most important characteristics for the correct determination of irradiation dose in photochemical and photodynamic therapy of different diseases, as well as the optical radiation dosimetry in the laser surgery of gastric ulcer. The depth of radiation penetration into the tissue (δ) was evaluated using the relation $\delta = 1/\sqrt{3\mu_a(\mu_a + \mu_s')}$ [28].

3 Results and discussion

Figures 1 and 2 present the spectra of the absorption coefficient (μ_a) and the transport scattering coefficient (μ_s') of the gastric wall mucosa before and after the injection of aqueous haemoglobin solution. The averaging was executed over five samples of the tissue.

From Fig. 1 it follows that in the spectral range from 350 to 1250 nm one can observe an essential increase of the absorption coefficient of the mucosa (by 2-4.5 times depending on the wavelength). In the spectral range 1250-2500 nm the increase is expressed essentially weaker. Such behaviour of the absorption coefficient is related to the characteristic absorption of haemoglobin in the visible wavelength range. From Fig. 2 it follows that the injection of the aqueous solution of haemoglobin has practically no effect on the scattering characteristics of the mucosa.

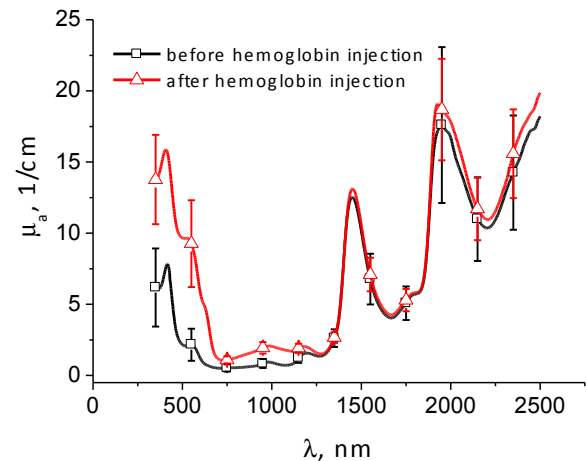


Fig. 1 Absorption spectrum of the human gastric wall mucosa measured before and after the injection of aqueous haemoglobin solution. Vertical bars show the root-mean-square deviation.

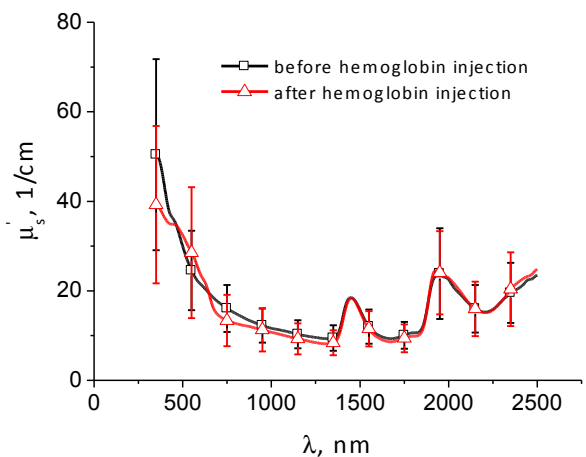


Fig. 2 Spectrum of transport scattering coefficient of human gastric wall mucosa measured before and after the injection of aqueous haemoglobin solution. Vertical bars show the root-mean-square deviation.

The result of estimating the depth (δ) of light penetration into the tissue is shown in Fig. 3. The used expression is applicable to the case when the tissue surface is uniformly illuminated by the radiation from a point source, located at some distance from the surface. This corresponds to the conditions of real laser surgery of gastric wall, since in this case the illuminating probe is introduced directly into the stomach cavity without a contact with the mucosa surface. The depth of light penetration into the mucosa was calculated using the values of the absorption coefficient (see Fig. 1) and the transport scattering coefficient (see Fig. 2).

Another important parameter is the fraction of the incident radiation energy absorbed in the mucosa. This parameter was calculated in the course of Monte Carlo modelling, and the result is presented in Fig. 3, too.

In this figure one can clearly see that the depth of laser radiation penetration essentially decreases in the

visible range of wavelengths (at the wavelength of Nd:YAG laser radiation (1064 nm) the penetration depth decreases by nearly 60%, whereas the fraction of absorbed energy at this wavelength increases by nearly 90%). The computer simulation allows the evaluation of the change of the absorbed energy fraction at the wavelengths of diode-based laser systems used for the photodestruction of tissue neoplasms. Thus, for the wavelength 810 nm the injection of haemoglobin aqueous solution leads to the increase of the absorbed energy fraction by nearly 80% with almost 50% reduction of the laser radiation penetration depth. For the wavelength 970 nm, the increase of the absorbed energy fraction amounts to nearly 65% with about 50% decrease of the laser radiation penetration depth.

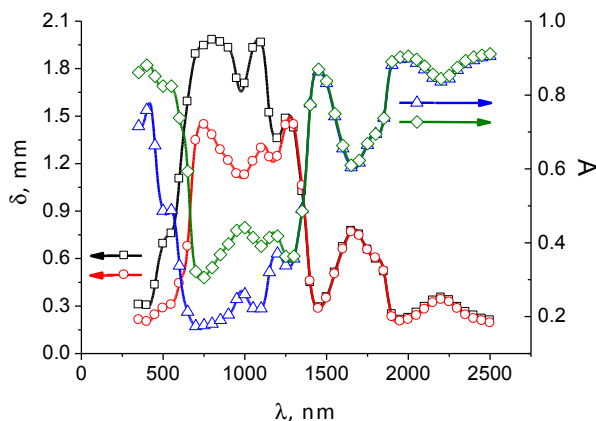


Fig. 3 Wavelength dependence of the depth of radiation penetration into the human gastric wall mucosa (δ) (black line, $\square$ – before the haemoglobin injection; red line, $\circ$ – after the haemoglobin injection), calculated from the experimental data of Figs. 1 and 2. Results of Monte Carlo simulation of the fraction of photons absorbed in the mucosa (A) versus the wavelength (blue line, Δ – before the haemoglobin injection; green line, $\diamond$ – after the haemoglobin injection).

Figures 4 and 5 present the spectra of absorption and transport scattering coefficients of the gastric wall mucosa before and after the injection of aqueous 40% glucose solution. The averaging was performed over five samples of tissue.

In Fig. 4, one can clearly see that the injection of glucose solution into the mucosa practically does not change its absorption properties, except the region of water absorption bands in the near IR spectral region with the peaks at 1450 and 1940 nm. This fact is due to the replacement of the mucosa interstitial fluid with the glucose solution and, hence, the decrease of water content in the tissue.

Figure 5 clearly demonstrates that the glucose injection leads to the essential (~20%) reduction of the transport scattering coefficient all over the range of wavelengths under study (24-27%), which leads to the increase of the laser radiation penetration depth (see Fig. 6).

The computer simulation shows that the growth of the penetration depth of laser radiation is about 15% for the wavelength 810 nm, about 17% for 970 nm, and about 16% for 1064 nm. Practically no increase of the fraction of photons absorbed in the mucosa is observed (see Fig. 6).

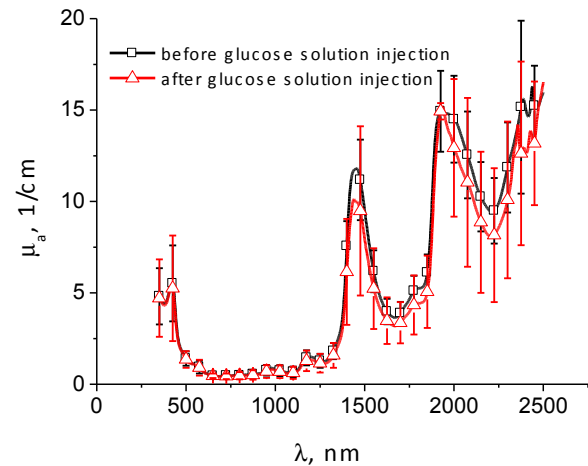


Fig. 4 Absorption spectrum of the human gastric wall mucosa, measured before and after the injection of aqueous 40% glucose solution. Vertical bars show the root-mean-square deviation.

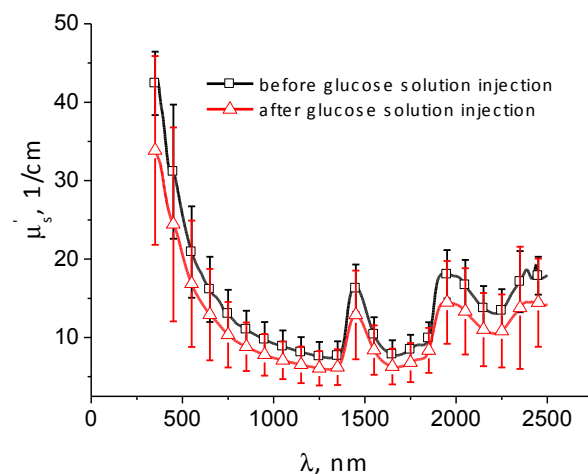


Fig. 5 Spectrum of transport scattering coefficient of the human gastric wall mucosa measured before and after the injection of aqueous 40% glucose solution. Vertical bars show the root-mean-square deviation.

The obtained results of the mucosa optical clearing agree well with the data of Refs. [17-21]. Thus, in Refs. [17, 18, 20] it is shown that the absorption coefficient of a fresh sample of porcine gastric mucosa at the wavelengths 1450 and 1936 nm decreases under the action of glycerol and dimethyl sulfoxide (DMSO), which is an evidence of water desorption caused by the applied agents. The authors observed the increase of the optical coherence tomography (OCT) probing depth at the wavelength 1300 nm using the glycerol and DMSO solutions as immersion clearing agents [17, 19]. The authors of Ref. [21] also obtained increased transparency of the murine gastric wall mucosa under

the application of 20% glucose solution in saline at the wavelength 830 nm in OCT studies.

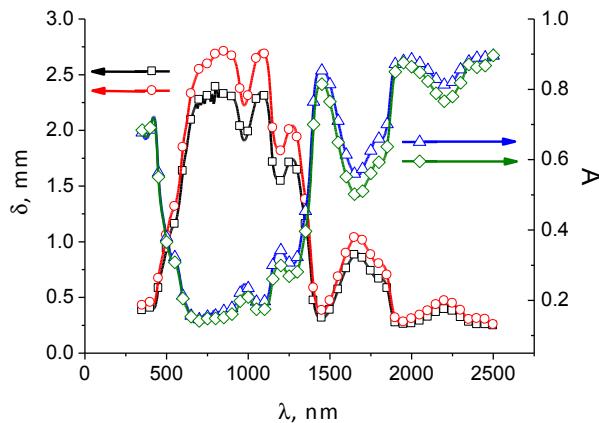


Fig. 6 Wavelength dependence of the depth of radiation penetration into the human gastric wall mucosa (δ) (black line, $\square$ – before the glucose injection; red line, $\circ$ – after the glucose injection), calculated from the experimental data of Figs. 4 and 5. The result of Monte Carlo simulation of the fraction of photons absorbed in the mucosa (A) versus the wavelength (blue line, $\triangle$ – before the glucose injection; green line, $\diamond$ – after the glucose injection).

4 Conclusion

As a result of the performed studies of the possibility to control the absorption and scattering properties of the gastric wall mucosa, we report the first experimental observation of the tissue absorption coefficient increase

in the range 350-1250 nm due to the injection of haemoglobin solution into the tissue.

After the injection of 40% glucose solution into the mucosa, we observed a decrease of the absorption coefficient in the region of water absorption bands and the decrease of transport scattering coefficient all over the range under study (350-2500 nm).

The performed studies show the possibility to control the optical properties of the gastric wall tissues from the point of view of both changing the depth of laser radiation penetration and regulating the tissue absorbance and, therefore, the incident radiation power. Increasing the absorption in the affected tissues will allow the reduction of the incident radiation power, which, in turn, will reduce the risk of damaging healthy tissues surrounding the lesion area, which can find application in planning the operations of gastric ulcer treatment using up-to-date laser technologies.

Disclosures

All authors declare that there is no conflict of interests in this paper.

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Macro and micro spectroscopy parameters of cancerous and healthy gastrointestinal tissues

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Abstract. New more sensitive diagnostic modalities, for either analysis of biopsy tissue samples or on site, in vivo microscopy tissue examination, emerge to aid the detection and accurate diagnosis of gastrointestinal cancer. One of the most intensively investigated techniques is the fluorescence spectroscopy approach.

We present our investigation of the detailed macro and micro fluorescence characteristics of ex-vivo fresh and fixed tissue samples. The fluorescence spectra of unprocessed cancerous and healthy gastrointestinal (GIT) tissue, obtained with two approaches fluorescence and synchronous fluorescence spectroscopy were compared with microspectroscopy performed with laser scanning confocal fluorescence microscopy (LSCFM) system in Lambda-scan regime over fixed tissue samples.

The evaluated optical macro- and micro- spectroscopy characteristics are presented and discussed in order to improve the current understanding of the origin of autofluorescence contrast between healthy and cancerous tissues and its application for improvement of the existing diagnostic modalities. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Autofluorescence; gastrointestinal tissues; colon carcinoma; confocal fluorescent microscopy (CFM).

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1 Introduction

The wide spread of oncologic diseases in gastrointestinal tract (GIT) and high mortality among the patients [1], entitle its early diagnosis and effective treatment as a primary public health concern.

The standard procedure for cancer detection in GIT includes endoscopic surveillance and biopsy. Their methodical application proves to be effective in reducing of mortality rates and improvement of patient's quality of life [2, 3]. However the rigorous biopsy protocols are costly and time consuming. The accuracy of the current diagnostic procedure relies entirely on the physicians' experience and it is limited by the high probability of miss rates [4]. Therefore new sensitive diagnostic modalities, for either analysis of biopsy tissue samples or on site, in vivo tissue diagnostic evaluation, are necessary. One of the most intensively investigated techniques, for overcoming the limitation of the standard diagnostic procedure, are the fluorescence spectroscopy and multispectral imaging approaches [4-12].

Recent advances in the understanding of the process of cancerogenesis and its footprints in tissue autofluorescence spectra allow the application of autofluorescence contrast in "red-flag" techniques for clinical diagnostics [5, 6, 7].

At general clinical application of autofluorescence contrast in GIT cancer diagnosis is based on the macroscopic manifestation of the difference in the autofluorescence of cancerous and healthy tissue, predominantly green fluorescence (450-550nm) of healthy mucosa and red fluorescence (600-700nm) of hyperplasic mucosa. Most often the diagnostic parameter is considered the ratio between the intensities of autofluorescence in the two regions, with different systems using different spectral ranges within the green

and red regions [6, 8, 9]. The origin of the difference in autofluorescence of cancerous and healthy GIT tissues is attributed to few main endogenous fluorophores that are affected by the process of oncogenesis: amino acids – tyrosine and tryptophan, co-enzymes – nicotinamide adenine dinucleotide (NAD(P)H) and flavine adenine dinucleotide (FAD), structural proteins collagen and elastin, as well as their cross-links, and porphyrins. In ex vivo studies of autofluorescence of normal and cancerous tissues with colorectal origin, reduced intensity of NAD(P)H's fluorescence maxima have been repeatedly observed [13-18]. NAD(P)H is fluorescent only in its reduced form, its fluorescence depends on the quantity, redox and free-bound state. Hypoxia in cancerous tissues could increase the fluorescence of NAD(P)H by ceasing its oxidation, however it is typical mostly for solid tumours. It was demonstrated that there are numerous metabolic pathways affecting the redox stated of NAD(P)H and also that an alteration in bound to free NAD(P)H ratio could cause decrease in NAD(P)H's fluorescence and also red shift in its maximum [19]. The intensity of the fluorescence of collagen is reduced due to disruption of intra and extracellular matrices, caused by the poor differentiation and excessive proliferation of cancerous cells [20]. Excessive amount of porphyrins is found in cancerous cells, as a result of bypassing effect of the inhibited activity of the enzyme ferrochelatases in cancerous cells [21]. It is considered that the combination between reduced fluorescence from collagen (400 nm, 405 nm) and increased fluorescence of porphyrins (630 nm, 690 nm) is the reason for the characteristic red fluorescence of cancerous tissues.

However, the current application of autofluorescence based diagnostic techniques is limited mostly by insufficient specificity and misjudging

between cancer and inflammation, due to higher porphyrins content characteristic for both pathologies [5, 22, 23].

The more efficient and robust application of autofluorescence based endoscopic techniques for detection of GIT cancer requires extensive and meticulous evaluation of autofluorescence spectral peculiarities characteristic for cancerous tissue fluorescence, their origin, significance and specificity.

In our previous works we have presented detailed investigations over the main fluorescence characteristics of gastrointestinal tissues, specific fluorophores and their significance for differentiation between cancerous and healthy GIT tissues [24-27]. We have also demonstrated implementation of synchronous fluorescence spectroscopy (SFS) [28] for tissue investigation and its superior to standard fluorescence spectroscopy, diagnostic potential [25-27].

Hereby we present our investigation and comparison of the macro and micro fluorescence characteristics of ex-vivo fresh and fixed tissue samples, through fluorescence and synchronous fluorescence spectroscopy, and laser scanning confocal fluorescence microscopy respectively. As a part of our work towards better understanding of the underlying autofluorescence contrast between cancerous and healthy gastrointestinal tissue and its application for cancer detection and diagnostics.

2 Materials and Methods

2.1 Unprocessed tissue samples

Investigated biological tissue samples originate from gastrointestinal tract, colon and rectum. The number of samples collected is as follows: eighteen colon adenocarcinomas, two tubulovillous adenomas (polyp) and four adenocarcinomas of rectum. The tissue samples are excised during standard surgical procedure for tumour removal and consist of tumour and surrounding healthy tissue – safety region, excised according protocols [29] and stored in safe-keeping modified Krebs solution (NaCl, KCl, glucose, taurine, hepes, pyruvic acid, calcium chloride dehydrate) and isothermal conditions prior the spectral measurements.

2.2 Processed tissue samples

Investigated fixed tissue samples are unstained, deparaffinized and rehydrated histology tissue slides. Two consecutive slides were cut for every sample. After cutting the tissue slides and positioning on the glass slides (Leica X-tra Adhesive) all slides were deparaffinized by xylene (soak for 5 min.).

According to the histopathology diagnosis, samples included in this study are five samples of colon adenocarcinoma, two samples of tubulovillous adenoma and two samples of adenocarcinoma of rectum.

All tissue samples were obtained with approval by Ethics committee of University Hospital “Tsaritsa Yoanna - ISUL” in the frames of collaboration between

the hospital and Institute of Electronics, Bulgarian Academy of Sciences.

2.3 Fluorescence spectroscopy

The fluorescence spectroscopy measurements were obtained using spectrofluorimeter FluoroLog 3 (HORIBA Jobin Yvon, France). For fluorescence spectroscopy the applied excitation wavelength is 405 nm, and emission was detected in the range of 415-800 nm. The SFS method is characterized with simultaneous scanning of both the excitation and emission wavelengths, while a constant wavelength interval (offset) is kept between them. For SFS excitation used was in the range of 280-440 nm, with a step of 1 nm and 60 nm and 90 nm offset. For each sample the normal and cancerous areas were measured spectroscopically.

2.4 Fluorescence Microspectroscopy

Imaging and microspectroscopy was performed with confocal laser scanning microscopy system Leica TCS SP (Leica Inc.), applying excitation at 405 nm excitation. Images were acquired using a 40x magnification objective with a numerical aperture of 0.75. The field of view for each image was 775×775 μm . In Lambda-scan® regime of the microscope the fluorescence spectra of histology tissue slides were evaluated over series of images throughout the slide thickness, which is 12 μm for healthy area and area with distinctive cancer alterations in the tissue pattern. The spectral and image detection eas the range of 420-680 nm.

3 Results and Discussion

Fig. 1(a) represents the obtained LSCFM image of colon adenocarcinoma, where the typical for oncogenesis distortion of the shape of crypts and their density is higher than normal with almost missing the natural lamina propria, fluorescence spectra of this area named “cancerous” and fluorescence spectra of healthy area on the sample (Fig. 1(b)).

Fig. 2(a) represents the obtained LSCFM image of tubulovillous adenoma (polyp), where the typical healthy crypts, “border” area and area with complete loss of differentiation is observed. Figure 3(a) represents the obtained LSCFM image of adenocarcinoma of rectum, where mostly complete loss of differentiation is observed.

For the fluorescence spectra of the fixed tissue sections, a slight red-shift for the autofluorescence of the cancerous areas is observed (Fig. 1(b); Fig. 2(b); Fig. 3(b)). Since this red-shift is very slight, box-whiskers plot with intensity values for fluorescence at 600nm for five cancerous and healthy parts of the included samples with the respective diagnosis are presented (Fig. 1(c); Fig. 2(c); Fig. 3(c)). For every plot the whiskers mark the standard deviation with 1.5 coefficient; upper and lower box ranges reflect the 25/75th percentiles, respectively; the mean value is

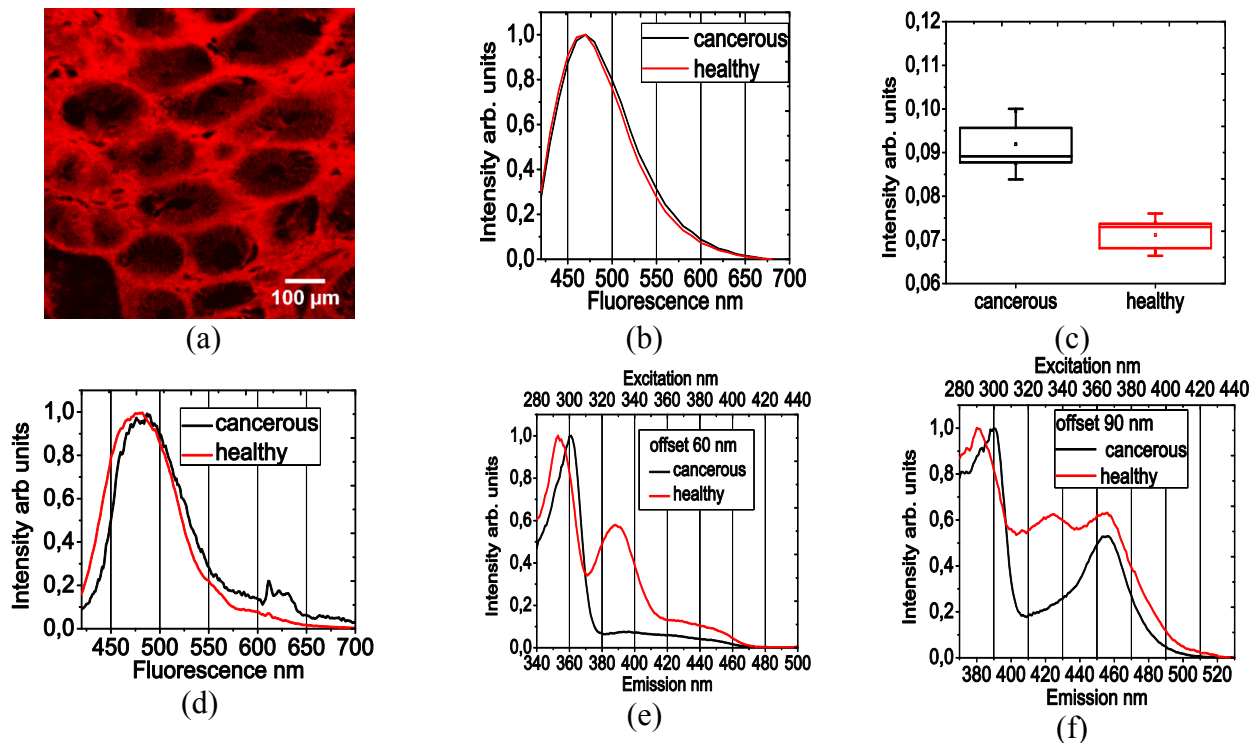


Fig. 1 Obtained macro and micro data for colon adenocarcinoma. LSCFM image of fixed tissue section for excitation of 405 nm (a); fluorescence spectra of fixed tissue section for excitation of 405 nm (b); intensity values at 600nm for 5 cancerous and healthy parts of the fixed tissue sample (c); fluorescence spectra of unprocessed tissue sample of colon adenocarcinoma for excitation of 405 nm (d); SFS spectra of unprocessed tissue sample of colon adenocarcinoma for offset of 60 nm (e) and 90 nm (f). All fluorescence spectra are from single sample and normalized according to their maximum.

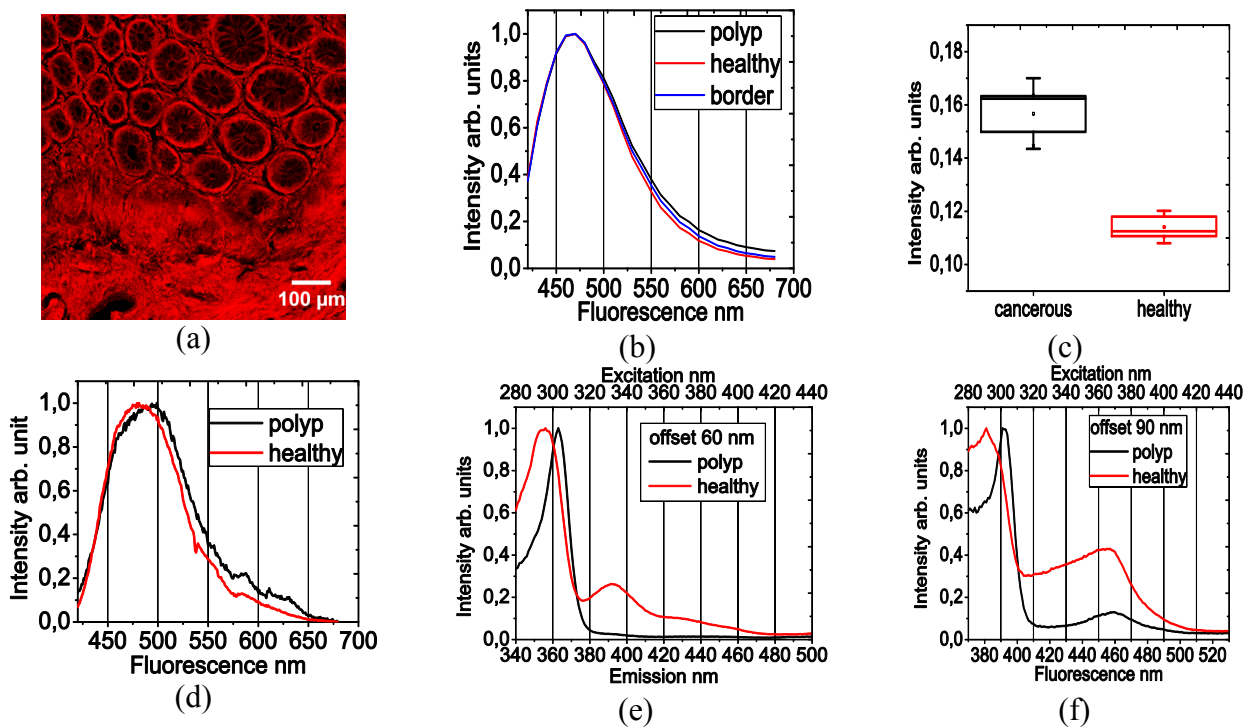


Fig. 2 Obtained macro and micro data for tubulovillous adenoma. LSCFM image of fixed tissue section for excitation of 405 nm (a); fluorescence spectra of fixed tissue section for excitation of 405 nm (b); intensity values at 600 nm for 5 cancerous and healthy parts of the fixed tissue sample (c); fluorescence spectra of unprocessed tissue sample of colon adenoma for excitation of 405 nm (d); SFS spectra of unprocessed tissue sample of colon adenoma for offset of 60 nm (e) and 90 nm (f). All fluorescence spectra are from single sample and normalized according to their maximum.

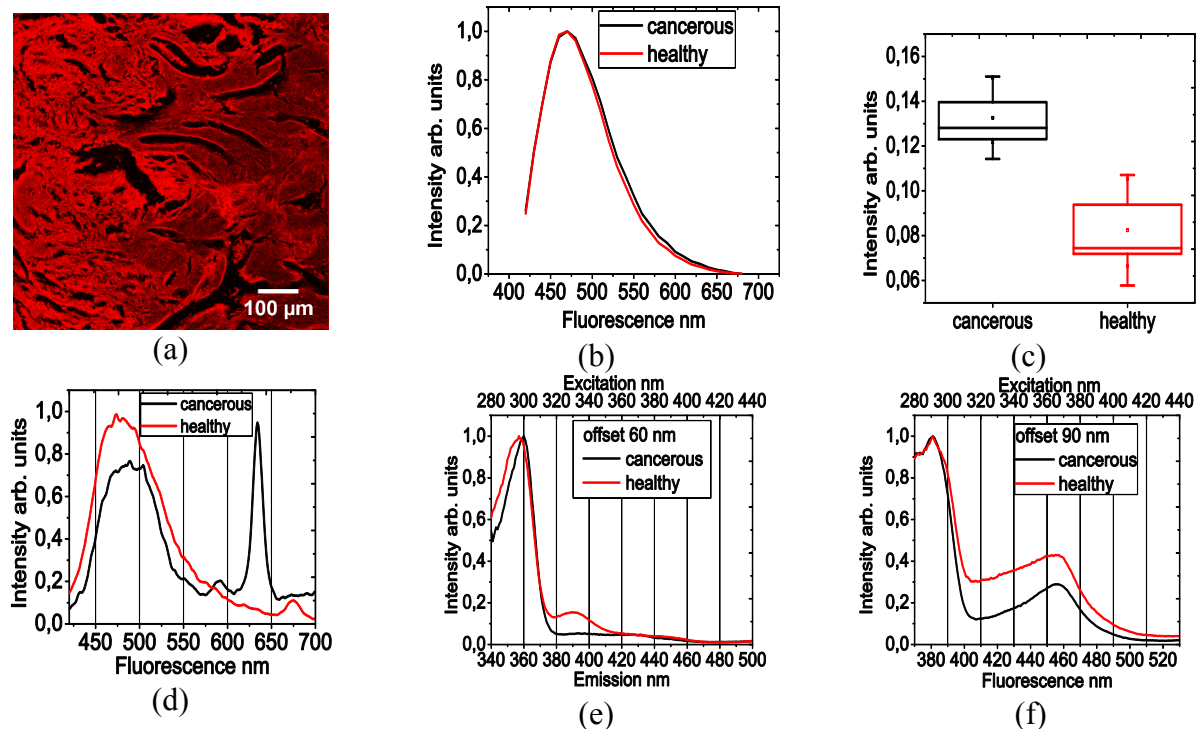


Fig. 3 Obtained macro and micro data for adenocarcinoma of rectum. LSCFM image of fixed tissue section for excitation of 405 nm (a); fluorescence spectra of fixed tissue section for excitation of 405 nm (b); intensity values at 600nm for 5 cancerous and healthy parts of the fixed tissue sample (c); fluorescence spectra of unprocessed tissue sample of colon adenocarcinoma for excitation of 405 nm (d); SFS spectra of unprocessed tissue sample of colon adenocarcinoma for offset of 60 nm (e) and 90 nm (f). All fluorescence spectra from single sample and are normalized according to their maximum.

represented with line across the box and the median value is represented with square. This representation of the data demonstrates that the observed red-shift of the fluorescence of cancerous tissues in fixed tissue samples is statistically significant. The detected fluorescence could be contributed to NAD(P)H, FAD, porphyrins and mostly the structural proteins collagen and elastin, since the applied excitation of 405 nm is adequate for their excitation, the other fluorophores are more affected by the tissue processing, also some of the fluorescence signal could be attributed to formalin fixation [30, 31]. The most discussed causes of the observed spectral shift towards longer wavelengths are an increase in the FAD/NAD(P)H ratio [32], alteration in the $\text{NAD(P)H}_{\text{bound/free}}$ ratio [33, 34] and the fluorescence of porphyrins [34, 35].

The fluorescence spectra of unprocessed tissue samples after excitation at 405 nm (Fig. 1(d); Fig. 2(d); Fig. 3(d)) exhibits one main fluorescence maxima due to the fluorescence of NAD(P)H and structural proteins and for the samples of colon adenocarcinoma and adenocarcinoma of the rectum (Fig. 1(d); Fig. 3(d)) characteristic fluorescence maxima of porphyrins around 630 nm that is typical for higher grade dysplasia.

Synchronous fluorescence spectra for excitation 280–440 nm and offset 60 nm (Fig. 1(e); Fig. 2(e); Fig. 3(e)) consists of two maxima for the healthy tissue fluorescence contributed to amino acids tyrosine and tryptophan and collagen and only the maxima of the

amino acids for cancerous tissue. The red-shift in the fluorescence maxima of the amino acids is consistent and prominent feature for the presented SFS spectra of cancerous tissues. The reason for this spectral shift could be the high sensitivity of tryptophan's fluorescence to the environment such as pH and local electric field [19, 34, 36] Red-shift was also observed for the fluorescence maxima of tryptophan in breast cancer tissue's fluorescence [37]. The lower fluorescence intensity of collagen for cancerous tissues is its typical spectral hallmark [24, 26, 27, 28] and in the presented results it is negligible low in comparison with collagen fluorescence maxima in healthy tissue spectra.

Synchronous fluorescence spectra for excitation 280 – 440 nm and offset 90 nm (Fig. 1(f); Fig. 2(f); Fig. 3(f)) have two prominent fluorescence maxima – first one is contributed to tryptophan and second one is NAD(P)H fluorescence maxima. Except for the colon adenocarcinoma (Fig. 1(f)) where and an additional maximum around 420 nm for healthy tissue fluorescence is distinguishable and could be attributed to collagen and its cross-links. For the maxima of NAD(P)H there is no prominent red spectral shift, the only difference between the spectra of cancerous and healthy fluorescence for this maxima is in the intensity, which is lower for cancerous tissues fluorescence and it was already observed [11, 28, 38].

4 Conclusions

Early detection of cancer plays an important role in global cancer control initiatives as it significantly increases the survival rate and improves quality of life of the patients. The fluorescence spectroscopy and/or imaging has already been proven to increase the image contrast and detect the subtle differences between healthy epithelial tissues and early precancerous changes of GI epithelium, that would remain invisible using conventional methods of diagnostics.

Fluorescence spectroscopy and microscopy approaches provide detailed information about fluorescence spectra peculiarities that differ cancerous and healthy tissues. Nevertheless for their application in clinical practice only the most distinctive and specific optical markers should be determined, so unnecessary data processing would be eliminated. Additionally the specificity of the observed red shift in cancerous tissues fluorescence and its advantages as diagnostic parameter should be determined.

In combination with other optical markers it could be applied through both techniques fluorescence

spectroscopy and microscopy, allowing a development of simplified systems, which could be used in clinical observations *in vivo*, or for retrospective and prospective analysis of histological slices and tissues *ex vivo*, as well for monitoring during surgical procedures for tumour removal.

Disclosures

The authors declare that there are no conflicts of interest related to this article.

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Fluorescent diagnostics of benign breast diseases and breast cancer

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Abstract. In the study the spectra of fluorescence excitation and autofluorescence of histological sections with different pathological processes in breast were measured. Additionally, element composition of the tissues was investigated by scanning electron microscopy. It has been shown that spectra of fluorescence excitation of benign breast diseases (fibroadenoma and fibrocystic breast disease) have low maximum at 232 nm and high maximum at 260 nm. Also, fluorescence spectra of histological samples change during tumor growth and have two maxima (335 and 420 nm). The registered spectra are result of emission of such fluorophores as fatty acids, tryptophan, NADH, vitamin B6, collagen and bilirubin. The obtained spectral data correlate well with data of element analysis. Selenium status of the tissues was investigated by scanning electronic microscope. High concentration of selenium was detected only in fibroadenoma. In breast cancer samples and fibrocystic breast disease low selenium content was detected. It may confirm higher risk for malignant transformation of fibrocystic breast disease than fibroadenoma. Our results may be helpful for cancer diagnostics and for prognosis prediction. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: breast cancer; benign breast disease; fluorescence; optical biomedical diagnostics.

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1 Introduction

Mortality and morbidity of breast cancer (BC) increase annually all over the World [1]. Survival of the patients is strongly associated with timely and high-quality diagnostics, the main goal of which is to detect tumor at early stage or to prevent malignant transformation of precancerous lesions [2]. Biopsy is considered to be mandatory procedure for verification of all pathological processes in breast, suspicious for cancer. Unfortunately, not all cases may be verified easily and unambiguously, especially if it is very small specimen of tissue, obtained through fine-needle or core biopsy

[3,4]. For histological sections of such difficult cases spectral techniques may become a new additional tool for investigation due to their high sensitivity.

Benign and malignant pathological processes in any organ differ not only by their histological structure, but also by different biochemical composition due to activation of catabolism and proliferative activity of the cells [5]. For example, due to tumor growth NADH concentration always increases [6], peptide and oxygen concentration decreases [7]. Also it is known that selenium level is low in malignant tumors [8] and this microelement has protective effect against cancer.

Biochemical proximity of benign pathological process in breast to biochemical composition of malignant tumor may explain its role in cancerogenesis or in some way may be helpful for patient prognosis prediction.

The aim of this study is to investigate spectral characteristics of benign breast disease (BBD) and breast cancer (BC) at different stages for improvement of the optical techniques of biomedical diagnostics.

2 Materials and methods of the research

2.1 Object of research

The objects of the investigation were tissue specimens from 86 women with different pathological processes in breast (average age 40 ± 5 years), treated in Smolensk Regional Oncological Dispensary during 2016. In the group of BBD cases of fibroadenoma (FA) and fibrocystic breast disease (FCBD) were included as diseases with minimal potential for malignant transformation [9]. The volume of operation was defined according to diagnosis, detected previously by core biopsy. Before operation all the patients signed an informed consent. The investigation was carried out with the agreement of ethical committee of Smolensk State Medical University. All the samples, obtained from surgically removed material, were treated with traditional histological paraffin-embedded technique. Tissue samples were put in 10% formalin for 24 hours at room temperature. Next, process for paraffin embedding schedule as following and performed in appropriate embedding cassettes: 70% Ethanol, two changes, 1 hour each; 80% Ethanol, one change, 1 hour; 95% Ethanol, one change, 1 hour; 100% Ethanol, three changes, 1.5 hour each; Xylene or xylene substitute (i.e. Clear Rite 3), three changes, 1.5 hour each; Paraffin wax (58-60 °C), two changes, 2 hours each; embedding tissues into paraffin blocks.

Table 1 Distribution of the cases in the groups according to diagnosis.

Diagnosis	Amount
Fibroadenoma	14
Fibrocystic breast disease	21
Cancer I stage	12
Cancer II stage	23
Cancer III stage	16

Two parallel sections 7 μ m in thickness were done, one of them was stained with hematoxylin and eosin and was investigated by pathologist for the diagnosis verification. Subdivision of the samples according to their diagnosis is shown in Table 1. The group of material from the patients with IV stage were absent in the investigation, because on this stage radical operation is not performed.

2.2 Fluorimetry

All histological sections without hematoxylin and eosin staining were placed on quartz glass and proceed by hexane for dewaxing. The unstained samples were used for experiment purity. Spectra of fluorescence excitation were measured on spectrofluorimeter SOLAR CM-2203 (Belarus) with additional equipment for measuring solid samples. Wavelength of registration was 410 nm after its excitation in UV region. Additionally, fluorescence spectra were measured. Wavelength of excitation was 300 nm. The diameter of sample excitation was 1 cm that is more then diameter of histological section. For the reason the obtained signal is total signal from all surface of sample.

2.3 Scanning electronic microscopy (SEM)

The microstructure of all dewaxed in hexane specimens was examined by scanning electronic microscope JEOL-6000 (Japan). The distribution of microelements (nitrogen, oxygen, carbon, selenium, sulfur) and their quantitative analysis were carried out under high vacuum with voltage 10 kV. We note, that we didn't measure absolute concentrations of chemical compounds, as all the samples have been possessed by paraffin-embedded technique and dewaxing, but compare samples from different pathologies of breast, that were prepared in the same way.

2.4 Statistical analysis

For statistical analysis (to compare elements concentration in the groups) non-parametrical criterion - Kruskal-Wallis test (H) - was used to compare multiple independent groups. The data were considered statistically significant when $p < 0.05$.

3 The results and their discussions

It is well known that tumors are characterized by different types of atypism, one of them is biochemical, that can be detected on spectra of fluorescence excitation from histological sections (Fig. 1). The spectra have several maxima at $233 \div 236$, $259 \div 268$, $290 \div 335$ nm. But all cases of BBD are characterized by three maxima when BC samples have four peaks. For their analysis the spectra were splitted by Gauss-Lorentz curves method on spectral components (Figs. 1b-1f). For every spectrum the square under the curve was calculated (Table 2). For better visualization the spectra were normalized on the maximum 330 nm. For this reason, squares of spectra components were analogically normalized on the square of curve with maximum 330 nm.

Cases of BBD have the smallest square under curve at 230 nm. The maximum is characterized by fatty acids absorption, that is the main constituent of adipose tissue in normal breast tissue and almost absent in fibrous stroma of FA and FCBD. This square is larger for BC on the 1st stage, as on this stage the normal

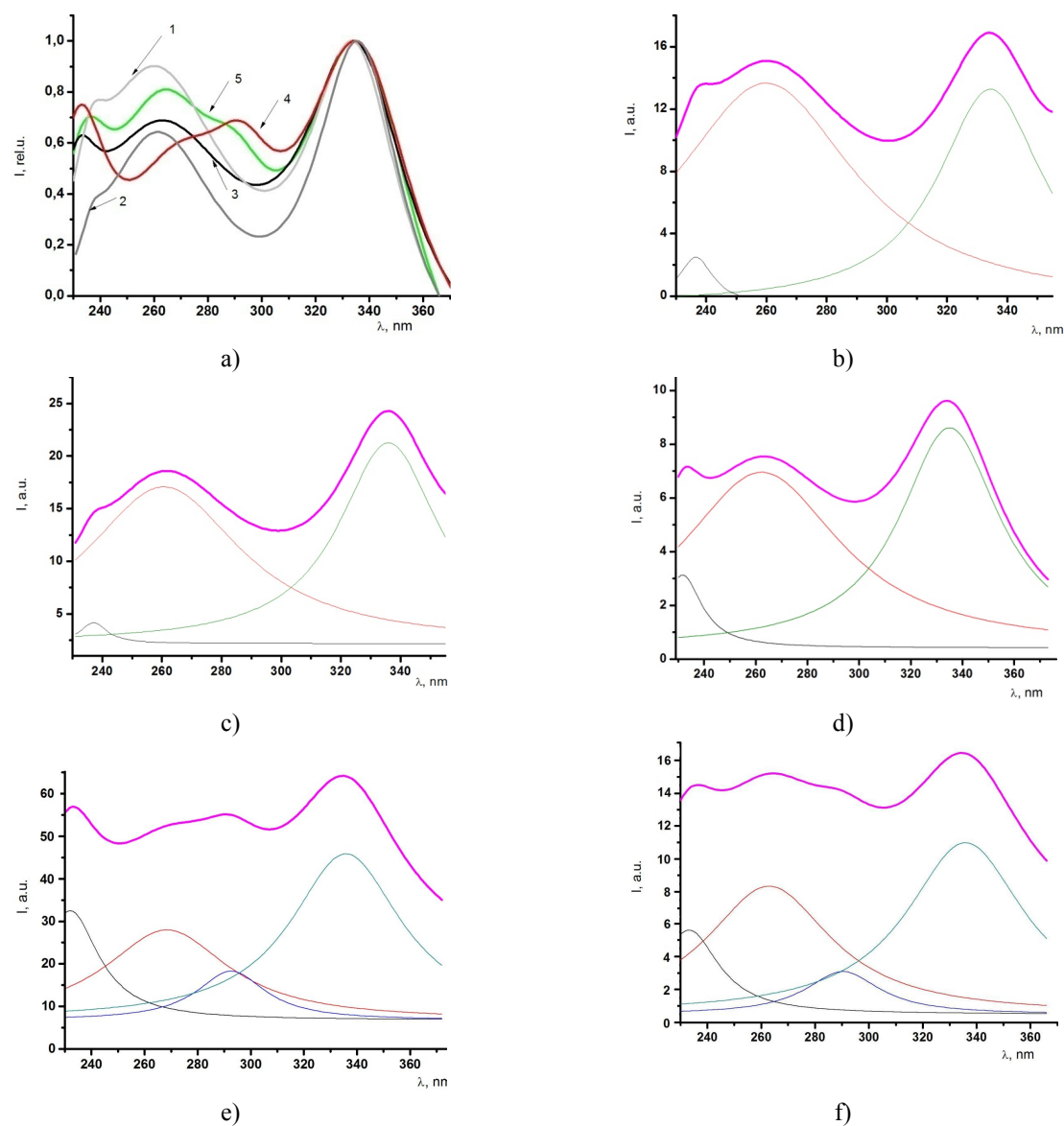


Fig. 1 The typical spectra of fluorescence excitation (a) of histological sections of different pathological processes in breast and their splitted curves. 1- FA (b), 2- FCBD (c), 3- stage I BC (d), 4- stage II BC (e), 5- stage III BC (f). Wavelength of fluorescence registration was 410 nm.

Table 2 The squares under the curves and wavelengths of fluorescence maxima of each group of fluorophores after excitation fluorescence spectra splitting into by Gauss-Lorentz curves method.

	Peak 1		Peak 2		Peak 3		Peak 4	
	λ, nm	S/S _{max4}	λ, nm	S/S _{max4}	λ, nm	S/S _{max4}	λ, nm	S/S _{max4}
FA	236	0.074	259	1.74	-	-	334	1
FCBD	238	0.027	261	1.22	-	-	336	1
Stage I BC	232	1.59	262	0.6	-	-	334	1
Stage II BC	232	0.33	268	0.67	292	0.18	335	1
Stage III BC	233	0.24	262	0.78	290	0.15	335	1

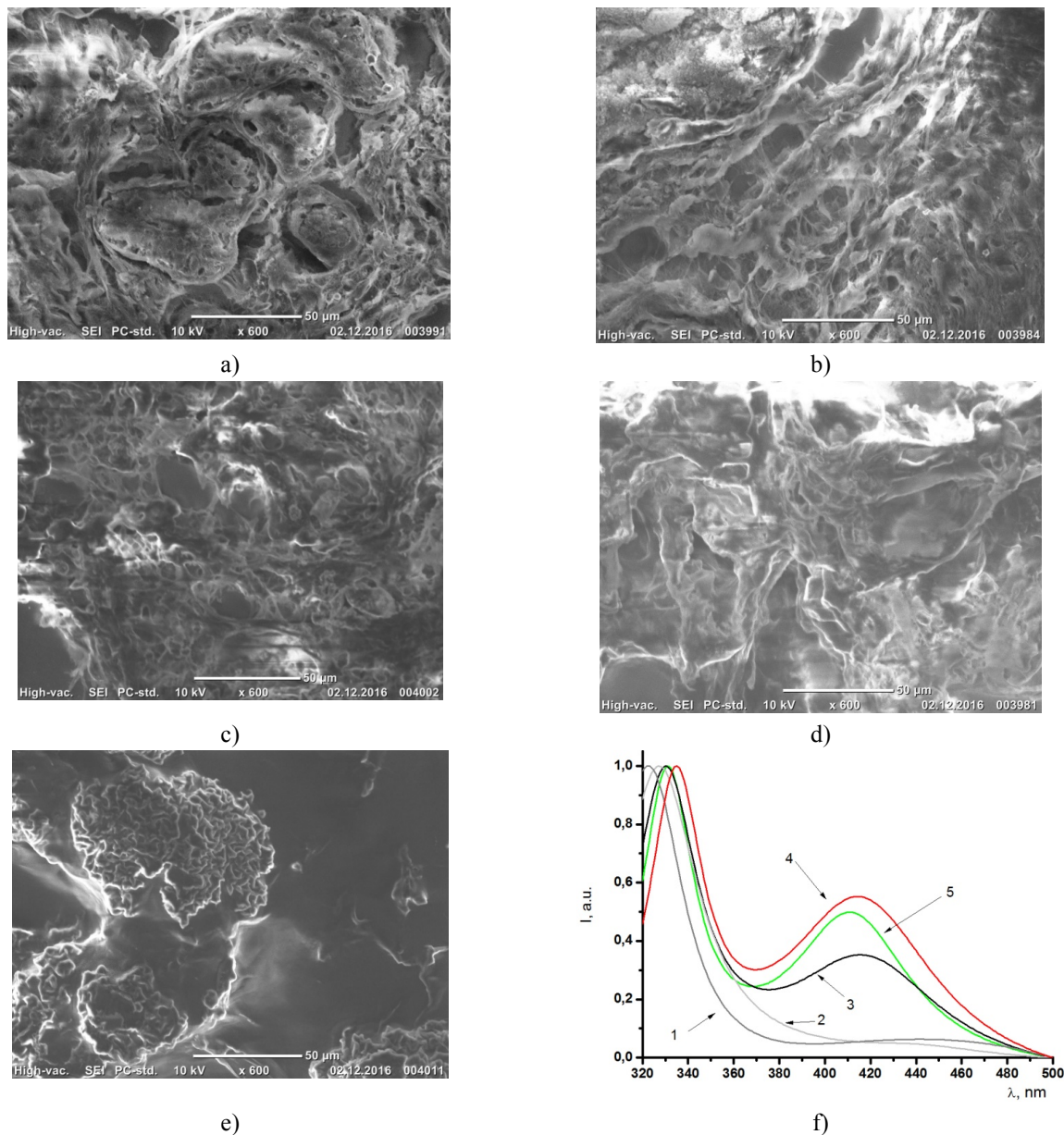


Fig. 2 The typical photo of SEM and fluorescence spectra (f). 1 - FA (a), 2 - FCBD (b), 3 - stage I BC (c), 4 - stage II BC(d), 5 - stage III BC (e). Wavelength of fluorescence excitation was 300 nm.

breast tissue is not damaged remarkably due to changed microenvironment [10]. Further progression of cancer (II and III stages) leads to decreasing absorption of fatty acids, because adipose tissue is replaced by fibrous tissue due to desmoplasia [11] and increased catabolism (the square of the maximum at III stage is 10 times higher than the same parameter for BBD). According to the element analysis (Table 3), the carbon concentration, that characterizes quantity of organic compounds, is higher in groups of BC in spite of fatty acids concentration decrease. It may be due to higher concentration of carbohydrates (but not proteins) because concentration of nitrogen and sulphur, related to amino acids concentration, in all groups of BC is remarkably lower than in BBD. It is known, that higher concentration of glucose increases invasiveness of

cancerous cells, that is the reason for cancer progression to more advanced stage [12].

The second maximum of these spectra is nearly at 260 nm, but it is higher for BBD, especially for FA. The peak is characterized by presence of tryptophan-containing peptides [13]. BC on the 1st stage is remarkably smaller square under the curve with maximum nearly at 260 nm. Due to progression from stage I to stage III the peak increases by 30%, but it is twice smaller, than in the group of BBD.

Cases with 2nd and 3rd stage of BC have additional peculiarity on their spectra - maximum at 290 nm. The peak is result of bilirubin presence [14] - the product of hemoglobin breakdown. Deposition of it in tissue of BC on later stages may be associated with hemolysis of erythrocytes due to changes in their membrane and

Table 3 Element composition of tissue with different pathological processes in breast and their comparison by Kruskal-Wallis test.

Diagnosis	Elements and their concentrations				
	W(C), % Rank sum	W(N), % Rank sum	W(O), % Rank sum	W(S), % Rank sum	W(Se), % Rank sum
FA	87.65 6	4.60 51	6.05 42	0.94 51	0.75 51
FCBD	86.68 51	4.03 42	8.96 51	0.33 41	0.01 42
Stage I BC	94.45 22	2.58 42	2.78 42	0.19 43	<0.01 26
Stage II BC	96.56 38	2.06 26	1.22 26	0.15 26	<0.01 26
Stage III BC	97.62 54	1.43 10	0.45 10	0.07 10	<0.01 26
Kruskal - Wallis test (H)	16.33; p=0.0026	16.33; p=0.0026	16.33; p=0.0026	16.12; p=0.0029	16.92; p=0.002

decreased life span [15]. Also it may be explained by death of endothelial cells through apoptosis for facilitation of metastasis with loss of vascular wall integrity and consequent hemolysis [16].

The fourth maximum of the spectra of fluorescence excitation (at 335 nm) reflects absorption of collagen in breast tissue [17].

Fluorescence spectra of histological samples are also different for benign and malignant breast diseases and changes during tumor growth. They have two maxima (335 and 420 nm) and depicted on Fig. 2f. Fluorescence at 420 nm could be defined by two fluorophores such as NADH and vitamin B6 [18]. Since fluorescence and absorption spectra of biological compounds are wide, we can partially see fluorescence of collagen excited to its absorption border. For FA and FCBD fluorescence of collagen may be remarkable due to its high concentration is stroma with lack of epithelial cells. In BC ratio between parenchyma and stroma changes due to increase of epithelial cells amount (Fig. 2 a-e). The picture shows that at 1st stage of BC around fragments of connective tissue there are pleomorphic epitheliocytes and adipocytes. Thus, at 1st stage BC the total fluorescence from collagen, vitamin B6 and NADH is visible. For BC on 2nd stage impact of fluorescence from vitamin B6 and NADH is remarkable due to increase of proliferative activity of the cells. On the 3rd stage of BC there is formation of rosette-like structures and tissue is getting compact due to increase of cells amount per square unit; adipose tissue diminishes (Fig. 2e). Concentration of collagen decreases under the influence of collagenases during tumor invasion and metastasing [19], and vitamin B6 gradually disappears from the tumorous tissue [20]. For this reason, the square of maximum at 420 nm gradually decreases for 3rd BC stage. The low level of vitamin B6 in blood was detected in many investigations for patients with advanced BC [20, 21]. Peak at 335 nm is

defined by tryptophan and tyrosine-containing peptides [18].

Element analysis detected lower oxygen concentration in BC tissue on the 1st stage, comparing with BBD (Table 3), with abrupt decrease of its concentration during cancer progression. This is the result of increasing hypoxia influence due to dysbalance between malignant cells proliferation and neoangiogenesis [7].

Previous investigations have shown protective effect of selenium against malignancies [22, 23]. The fact is also confirmed by our data (Table 3). The highest selenium concentration was detected in FA only, when selenium concentration in FCBD and BC was beyond the limit of the experimental device sensitivity. This fact may demonstrate higher potential for malignant transformation of FCBD, than FA.

4 Conclusion

In the article we demonstrated the theoretical substantiation for further improvement of optical techniques of breast cancer diagnostics based on biochemical atypism. These data may be useful in differential diagnostics of different breast pathologies or even become a screening instead of histological examination as cheaper and quicker method.

Disclosures

The authors declare that there are no conflicts of interest related to this article.

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