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PHYSICAL INSTRUMENTS FOR ECOLOGY, MEDICINE, AND BIOLOGY

An Improved System for in Vivo Fluorescent Analysis in Medicine

I. A. Raznitsyna[a,*], A. P. Tarasov[a,b], and D. A. Rogatkin[a]

[a] *Moscow Regional Research and Clinical Institute, Moscow, 129110 Russia*

[b] *Kotelnikov Institute of Radioengineering and Electronics of Russian Academy of Sciences, Moscow, 125009 Russia*

** e-mail: RaznitsynaIA@yandex.ru*

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Abstract—The design of individual elements of an improved system for laser fluorescence spectroscopy in vivo is described. The proposed technical solutions, in particular, the inclusion of a white light source in the system and the special design of the fiber optic probe and filter unit allow elimination of a number of drawbacks of the existing systems. In the described system, the diffuse reflection spectrum of the studied region is determined automatically after detecting the fluorescence spectra. The equality of the diagnostic volumes is achieved not only by automatically switching modes, but also by using a large number of lighting fibers located around the receiving fiber to uniformly illuminate the area of interest. The design of the filter unit, which allows one to adjust the transfer function of the device by adjusting the degree of overlap of the light beam by the filter, is shown. We believe that the inclusion of these elements in systems of laser fluorescence spectroscopy will make it possible to refine this method, unify the parameters of similar devices, and configure all devices in an identical way in the future.

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INTRODUCTION

Noninvasive fluorescence analysis methods are used in various fields of medicine [1]. The in vivo method of laser fluorescence spectroscopy (LFS) consists of detection of the spectra of secondary radiation that emerge from the surface of an object as a result of its exposure to narrowband radiation in the visible or near UV ranges. The recorded secondary fluorescence intensity spectrum of exogenous or endogenous fluorophores in a biological tissue carries information about its biochemical composition [2].

The best known application of LFS in medicine is photodynamic therapy (PDT) of tumors. This procedure involves the administration of drugs that increase the sensitivity of tissues to light (photosensitizers), which selectively accumulate in the field of pathology. To determine the boundaries of formation, as well as to control the dynamics of accumulation and excretion of photosensitizers, the fluorescence of the pathology and healthy tissue areas is evaluated [3].

The diagnostic systems for LFS in vivo work as follows. Radiation is delivered to the surface of an object to excite fluorescence from a narrowband source using the light fibers of a fiber-optic probe. Secondary radiation, in particular, diffusely reflected and fluorescent radiation, is delivered to a spectrometer via the receiving fiber of the probe. In this case, the recorded fluorescence signal is approximately 1000 times weaker than the diffusely reflected signal. Therefore, in order to detect and visualize the fluorescent part of the spectrum, the diffusely reflected part is attenuated by a spectral filter located in front of the spectrometer by $\beta \approx 1000$ times.

Devices that use LFS for the diagnosis and analysis of tumor fluorescence during PDT include the LESA-01-Biospec system described in [4]. Using this complex, fluorescence spectra can be observed in real time and special software allows one to evaluate their parameters (peak intensities, areas of individual spectral regions, etc.). Russian diagnostic systems for measuring the biomedical parameters of the skin and mucous membranes in vivo include those made by LAZMA LLC [5] with similar functions.

Further quantitative analysis of fluorescence spectra involves a number of difficulties when using these devices, as well as their foreign counterparts. In particular, attempts to correct the recorded fluorescence spectra taking the influence of instrumental characteristics and differences in the optical properties of biological tissues into account lead to the use of various normalization algorithms and unified indicators, for example, the tissue content index and the fluorescence contrast coefficient [2, 6, 7].

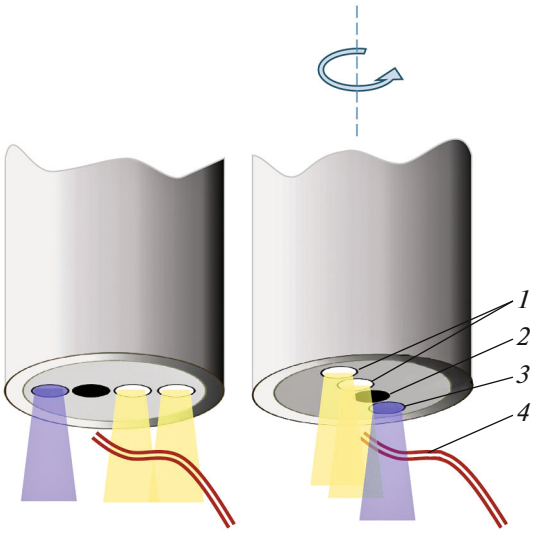

Fig. 1. The configuration of the fiber in the device described in [8], and an illustration of the possible sources of errors associated with the inequality of the diagnostic volumes and the rotation of the optical fiber around its axis. *1*, fibers delivering broadband radiation to the surface of the object; *2*, receiving fiber; *3*, fiber for delivering laser radiation; *4*, blood vessel.

When calculating these quantities, it is assumed that the absorbing properties of biological tissue at the wavelengths of excitation and fluorescence are the same, which is not always true. As well, to develop quantitative diagnostic criteria for the status of biological tissue, transition from the measured physical quantities to biomedical parameters expressed, for example, in accumulation levels or in relative concentrations of fluorophores, is important. Thus, the main drawback of the devices described above is the lack of a justified algorithm for the transition from measured physical quantities (spectra) to biomedical quantitative characteristics of the tissue, as well as the necessary hardware and software.

Systems exist that allow one to perform additional mathematical operations and adjust fluorescence spectra using certain approximations. A white light source is included in the design of the measuring device for this purpose; in addition to the fluorescence spectrum a diffuse reflection spectrum that characterizes the optical properties of the biological tissue depending on the wavelength is taken [8, 9]. In these instruments, the geometry of the fiber probe implies the presence of additional fibers for a broadband source.

Most often, these fibers are arranged in a row, which is due to the computational algorithms. In this case, the diagnostic volumes for studying fluorescence and diffuse reflection are not the same (Fig. 1). If a small vessel enters one of the studied areas it will lead to incorrect calculations, especially in the green part of the spectrum, where hemoglobin is the main absorbing substance. Accordingly, a difference in the diagnostic volumes for two measurements, as well as the rotation of the distal end of the fiber probe around its axis, can lead to errors in further calculations (see Fig. 1).

Metrological support of instruments to achieve uniform results and the required measurement accuracy is an important condition for the introduction of in vivo methods in clinics. We conducted a study in which the errors of measurements performed on different devices of the same manufacturer were evaluated [10]. It has been shown that the scatter of the recorded values, even on devices made by the same company, can exceed 25% for the LFS method.

When taking measurements on various instruments one can obtain completely different values due to the fact that there are no generally accepted standards and uniform measures for calibrating and adjusting instruments. As an example, the values of the fluorescence contrast coefficients, which are often used in studies [7, 8], are calculated via the formula

$$K_f = 1 + \frac{I(\lambda_f) - I(\lambda_e)}{I(\lambda_f) + I(\lambda_e)}, \quad (1)$$

where $I(\lambda_e)$ is the intensity of diffusely scattered radiation reduced by a filter by a factor of β; $I(\lambda_f)$ is the fluorescence intensity; and λ_e, λ_f are the wavelengths of excitation and fluorescence, respectively. The spectral optical filter of the system and $\beta(\lambda)$ have a significant effect on the value of this coefficient.

Based on this, it is necessary not only to carry out standard calibration procedures, but also to correct the transfer function of the device, thereby affecting the ratio of the maxima of diffuse reflection and fluorescence, which requires additional technical solutions.

In this work, a new version of a device for in vivo laser fluorescence spectroscopy in medicine is described, whose design features eliminate the above-described disadvantages of similar devices.

THEORETICAL SUBSTANTIATION

Due to the fact that different biological tissues may have different blood supplies, the fluorescence intensity $I(\lambda_f)$ may not be directly proportional to the concentration of fluorescent substances. In the general case, the dependence $I(\lambda_f)$ on the concentration of fluorophores C_f has a complex character [11]. The effect of the blood supply on the results of fluorescence analysis is most significant in the green wavelength range, since active absorption of light by hemoglobin occurs. Because of this, the comparison of $I(\lambda_f)$ in the green waveband in/for regions of even one location is not always correct.

We solved the problem of light propagation in a turbid medium with a uniformly distributed fluorophore [11] based on the modified Kubelk–Munk model and the solution of Kokhanovsky [12]. The expression for

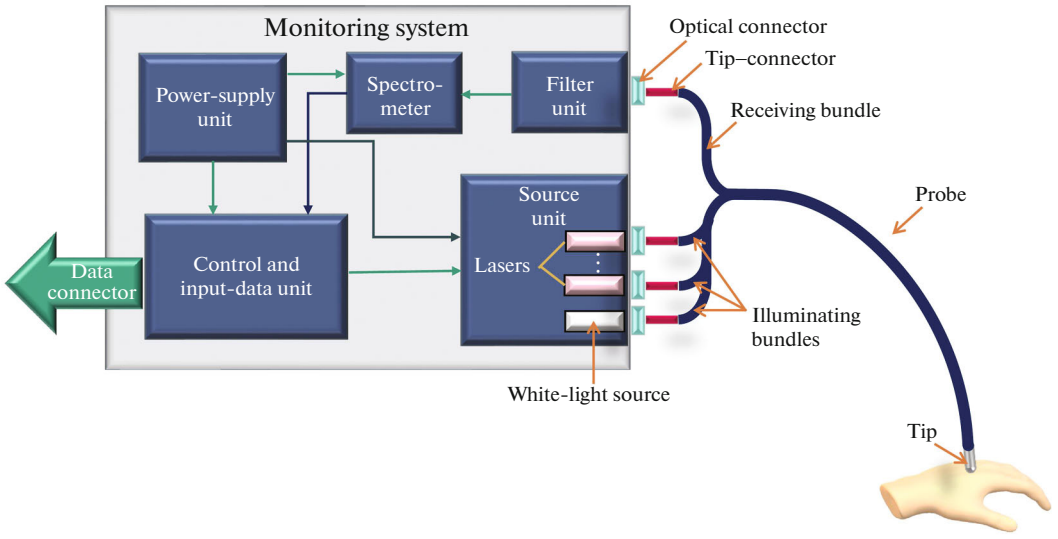

Fig. 2. A schematic diagram of the Multicom device for laser fluorescence spectroscopy.

the fluorescence flux J_f, W, on the surface of an object is as follows:

$$J_f = \Phi_0 A_f(\lambda_e)\varphi(\lambda_e,\lambda_f)\frac{(1+r_{\infty\lambda_e})(1+r_{\infty\lambda_f})}{2(\alpha(\lambda_e)+\alpha(\lambda_f))}. \quad (2)$$

Here, Φ_0, W is the exciting monochromatic flow incident on the boundary of the medium; $A_f(\lambda_e)$, mm^{-1} is the part of the exciting flow absorbed by the fluorophore on an elementary unit of length dx environment; $\varphi(\lambda_e, \lambda_f)$ is the fluorescence quantum yield; $r_{\infty\lambda}$ is the coefficient of diffuse reflection of the tissue at the wavelength λ, which determines the proportion of radiation emitted from the surface of a semi-infinite medium, relative to the exciting; and $\alpha(\lambda) = \sqrt{\beta_1^2(\lambda) - \beta_2^2(\lambda)}$, where $\beta_1(\lambda)$ and $\beta_2(\lambda)$, mm^{-1}, respectively, are the attenuation and backscattering coefficients for the test tissue.

The quantities $r_{\infty\lambda}$, $\beta_1(\lambda)$ and $\beta_2(\lambda)$ are the complex concentration dependence C_f of the fluorophore expressed in relative units ($0 \le C_f \le 1$) [11]. Therefore, to determine the relative concentrations of the fluorophore in the tissues, measuring the fluorescence spectra is not sufficient; it is necessary to know the diffuse reflection coefficients at the excitation wavelengths λ_e and fluorescence λ_f. In the general case, these coefficients cannot be taken as equal due to differences in the optical properties of the medium at different wavelengths. Therefore, the use of the backscattering peak value for any normalization (including in (1)) is not entirely correct. For measurement of $r_{\infty\lambda}$ for any of the visible wavelengths of interest, a broadband source must be included in the system.

DESCRIPTION OF THE DEVICE

To eliminate the aforementioned disadvantages, a new in-vivo LFS device called the Multicom was developed. Like similar devices, it contains an optical probe, at whose distal end there is a rigid tip that is in direct contact with the surface of the biological tissue (Fig. 2). At the proximal end there are for connecting the probe to the monitoring system.

The control system includes a block of primary radiation sources, a filter block, a spectrometer, a control and input-data unit that control all primary radiation sources, and a spectrometer, as well as a power supply unit. The monitoring system also has a number of optical connectors for connecting the probe through the tips–connectors. The control and input-data unit is connected to a standard personal computer with specialized software.

The operation of the device is performed as follows. The control and input data unit generates, receives, and processes two main control commands: *observation* and *measurement*. On the *observation* command the selected laser source is switched on in the continuous mode, after which the spectrometer continuously records the spectrum of secondary radiation. On the *measurement* command, the last recorded spectrum is stored in the memory of the device, the laser source is turned off, and the pulsed white light source is turned on for a short period of time. The spectrometer detects diffusely reflected radiation, after which all the measured spectra are transmitted to the control unit and input data for subsequent processing.

Unlike similar devices, the optical fiber probe of this system includes a group of lighting fibers (over 30) with a diameter of 100 μm and one receiving fiber with a diameter of 400 μm, through which secondary radi-

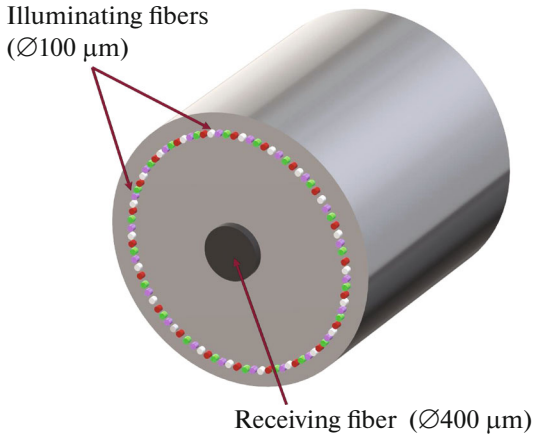

Fig. 3. The location of the lighting and receiving fibers in the fiber probe and the characteristic fiber sizes of the prototype device. The diameter of the probe tip is 3 mm.

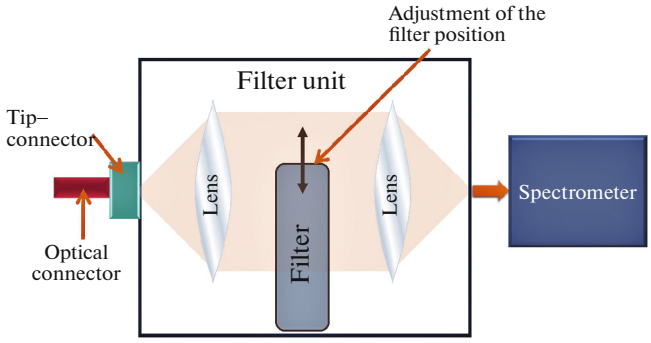

Fig. 4. A block diagram of the filter.

ation from biological tissue is delivered to the filter unit. The source unit contains several lasers for exciting fluorescence and a source of white light. The device uses sources at wavelengths of 365, 405, 515, and 632 nm and a standard source based on a xenon lamp.

The probe lighting fibers at the proximal end are assembled in separate bundles, each of which is connected to the source through an optical connector. Thus, several illuminating fibers are attached to each source. A separate bundle containing one receiving fiber is also attached to the filter unit. At the distal end, the illuminating fibers are arranged in a circle around the receiving fiber, uniformly illuminating the studied area (Fig. 3).

The use of a xenon flash lamp in this device, which is switched on by the user command, as well as the circular geometry of lighting, significantly reduce the systematic measurement error. This is due to the fact that for this device the data recording process is as automated as possible and it is not necessary to wait for the output to the white light source mode and to manually switch the sources. In addition, the use of a xenon flash lamp allows one to vary the amplitude of the received signal by changing the number of flashes.

To correct the transfer function, the filter block is made in the form of a collimating system of two collecting lenses, an attenuation optical filter placed between the lenses, and a mobile device that moves the optical filter perpendicular to the main optical axis of the lenses (Fig. 4).

The focusing lenses of the optical filter form a beam of large diameter (approximately 1–2 cm). An optical filter placed in a wide beam between the lenses attenuates the radiation at the fluorescence excitation wavelength a predetermined number of times ($\beta \approx 1000$), while the rest of the spectrum is attenuated slightly (by approximately 10%, depending on the filter). By varying the overlap area of the beam and the filter using a mobile element, the ratio of the backscattering and fluorescence peaks in the spectrum can be changed. The mobile element is a system of adjusting screws that allow one to move the filter in a wide parallel beam between the collimator lenses. This design will allow one to configure all devices in an identical way.

THE FEATURES OF DATA ANALYSIS

As a rule, the recorded diffuse reflection spectra are difficult to analyze without additional processing, since their appearance is mainly determined by the spectral characteristics of the radiation source itself. Therefore, for further analysis, normalization to diffuse reflectance spectra obtained from materials with known parameters is used. In optics, polytetrafluoroethylene (PTFE) is often used as such a material due to the fact that this material has a high degree of homogeneity, does not fluoresce, and actively scatters radiation in the entire visible range of the spectrum. Figure 5 shows the fluorescence and diffuse reflection spectra recorded using the new diagnostic system, as well as the calculated diffuse reflection coefficients.

Figure 5a shows the characteristic spectrum of the secondary radiation of a biological tissue obtained by exposure of its surface with laser radiation at a wavelength of $\lambda_e = 365$ nm. The spectrum has a pronounced diffuse reflection peak at the excitation wavelength reduced by the filter and a fluorescence peak with a maximum at a wavelength of 470 nm. Without additional analysis, it can be mistakenly concluded that the spectrum also contains two small peaks at wavelengths of approximately 560 and 590 nm. However, further analysis of the diffuse reflection coefficients (see Fig. 5c) shows that these maxima in the fluorescence spectrum are associated with a sharp change in absorption at given wavelengths. The dip observed in the graph (see Fig. 5c) in the green wavelength range corresponds to the absorption spectrum of oxyhemoglobin. Thus, additional analysis of the spectrum of diffuse reflection coefficients is necessary to eliminate possible inaccuracies and errors in the interpretation

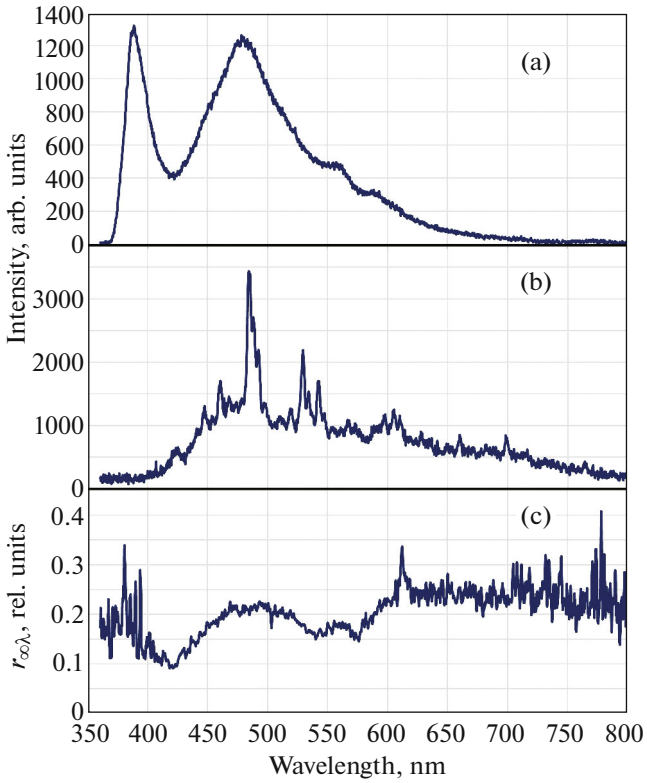

Fig. 5. An example of the spectra of a rat omentum: (a) recorded fluorescence spectrum, excitation wavelength $\lambda_e = 365$ nm; (b) recorded spectrum of diffuse reflection; (c) calculated diffuse reflection coefficients.

of the data. In addition, this spectrum can be used as an independent result, by which it is possible to assess the degree of blood supply to biological tissues.

THE MAIN CHARACTERISTICS OF THE DEVICE

It should be noted that the main characteristics of the device described above are determined by the characteristics of the selected components for certain tasks. As an example, in the Multicom system, which is designed to study the endogenous fluorescence of the skin and mucous membranes in the visible and near-infrared ranges, a spectrometer with an operating range of 350–800 nm is used. An AvaLight-XE pulsed xenon source (Avantes, Inc., United States) with a pulse energy of 39 μJ (the optical power in a single fiber with a diameter of 200 μm was 20 μW) has a maximum pulse repetition rate of 100 Hz. The number of flashes of a white light source for studying the properties of tissues is selected based on the maximum signal amplitude that can be detected by a spectrometer.

A fiber optic probe was manufactured (LLC "OPTOFIBER", RF RF) with 40 lighting and 1 receiving fiber for the model of the device using the manual assembly technology (see Fig. 3) [13]. It was

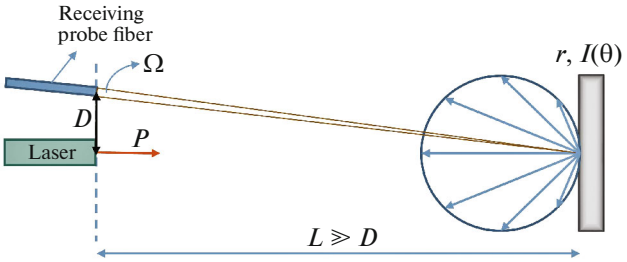

Fig. 6. The scheme for measuring low radiation power.

experimentally found that when using a probe with 19 illuminating fibers with a diameter of 100 μm and 1 receiving fiber with a diameter of 400 μm for a white light source, five flashes are sufficient for studying the skin and soft tissues and one flash is sufficient for measuring the diffuse reflection spectrum of a light-scattering standard (PTFE).

In a similar way, the values of the maxima of backscattering and fluorescence in the recorded spectrum were selected (see Fig. 5a), which varied directly by changing the laser radiation power and the filter used. The presented Multicom instrument was calibrated based on the task of reproducing the spectra obtained with the LAKK-M system (LLC LAZMA, RF). Fluorophore standards described in [14] were used for calibration. The radiation power at the output of the optical fiber was established: for a laser with a wavelength of 405 nm (seven illuminating fibers) it was 4.8 mW; at 515 nm (seven illuminating fibers) it was 5.8 mW; and at 635 nm (seven illuminating fibers) it was 3.3 mW.

An experiment was conducted whose scheme is shown in Fig. 6 to analyze the sensitivity of the device.

Laser radiation, with a power P, which was measured using a PD300 universal photosensor (OPHIR Ltd., Israel), impacted perpendicular to the surface of a light-scattering material with a given diffuse scattering coefficient r and the known dependence of the scattered radiation intensity on the scattering angle $I(\theta)$. Spectralon (Labsphere Ltd., New Hampshire, United States), which is close to an ideal Lambert surface with a diffuse reflection coefficient, was used as this material; $r = 0.75$ in the entire wavelength range that is of interest to us.

The receiving fiber was located at a distance $D < 5$ cm; the distance L from the laser source to the measured object should be much larger than D and more than 1 m. Thus, the secondary source in such a measurement geometry can be considered as a point source, the location of the receiving fiber is perpendicular to the surface of the object, and the solid angle Ω formed by the receiving fiber is small. The power of radiation that enters the receiving fiber can be estimated by the formula

$$P_{\text{det}} = P_{\text{las}} r \frac{\pi d^2}{4L^2}, \quad (3)$$

where d is the diameter of the receiving fiber.

The distance L and the filter (see Fig. 6) were selected in such a way that the signal arriving at the device was minimal, while being distinguishable from noise. It was determined that the threshold value of the radiation power that can be detected by the device is on the order of 10^{-12} W excluding fiber losses, which can be 5–10%.

CONCLUSIONS

This paper describes a device for laser fluorescence spectroscopy, with structural features of the elements that can significantly increase the accuracy of the method and provide additional information to a clinician. The special design of the filter unit allows one to select the transfer function of the device to reproduce the results of studies obtained using devices of other manufacturers. The design of the fiber optic probe eliminates errors associated with the mismatch of the diagnostic volumes when using multiple radiation sources, as well as with the rotation of the probe around its axis. We believe that after the development of standards for normalizing transfer functions based on this complex it will be possible to develop diagnostic methods and processing algorithms that are universal for devices of the entire class.

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