# Autofluorescence diagnostics of skin and mucosal tumors

I.A. NOVIKOV, Y.O. GRUSHA, N.P. KIRYUSHCHENKOVA

Research Institute of Eye Diseases of Russian Academy of Medical Sciences, Rossolimo st. 11AB, 119021, Moscow, Russian Federation

**Abstract**

The results are discussed of research on fluorescence diagnostics of skin and mucosal tumors carried out by a workgroup of the Research Institute of Eye Diseases, Moscow, between 2006 and 2012. The main achievement to be mentioned is development of an easy to perform, noninvasive, safe and cost-effective method of autofluorescent image acquisition and analysis that does not require induction of protoporphyrin IX or use of exogenous fluorophores. An original method of probabilistic tumor borders detection, valuable for surgical planning, has been also devised. A comprehensive approach has been suggested to analysis of autofluorescence pattern in the region of interest, which ensures high sensitivity and specificity of diagnostics. The results obtained are believed to be suitable for extrapolation to neoplasm of other, non-ocular localizations.

***Key words:*** *fluorescence diagnostics, autofluorescence, protoporphyrin IX, optical borders.*

**Vestnik oftal'mologii 2013; 129(5):147-153**

Skin cancer incidence today keeps growing. More than 130 thousand melanoma cases and 2—3 million malignant epithelial tumors are diagnosed each year. Basal cell carcinoma constitutes 75—97% of all malignant epithelial tumors [1—3]. It mostly affects head and neck zones (up to 70% of cases) [4, 5] including periorbital skin and eyelids. Despite scientific progress and high technology, there remains a need for simple and effective diagnostic techniques. Fluorescence diagnostics became an area of research in 1940s after the Wood's lamp introduction [6] and since then has achieved significant advances [7,8].

Human tissues contain a lot of natural fluorophores, mainly flavins, proteins, and porphyrins [9], each characterized by unique absorption and emission spectra **(Fig. 1)**. Emission spectra of different tissue constituents often overlap to a greater or lesser extent depending on the length of exciting radiation waves. The total fluorescence spectrum of the tissue is therefore confluent and demonstrates rather high versatility in its shape and range.

Ultraviolet fluorescence is characteristic of extracellular proteins, such as collagen and elastin. It can also be found in some aromatic amino acids – tryptophan, tyrosine, and phenylalanine, components of intracellular proteins. Blue and yellow-green fluorescence is much more common. It is seen in reduced pyridine nucleotides (NADH, NADFH) and oxidized flavoproteins (FMN, FAD), as well as in vitamins and products of metabolism including pyridoxals and folic acid derivatives, etc.

Red fluorescence, predominantly produced by endogenous porphyrins (Fig. 1b), is usually less intensive in comparison to ultraviolet and short-wave visible fluorescence [9-11]. Porphyrins are natural pigments consisting of tetrapyrrolic macrocycles and metal complexes. Protoporphyrin IX (PpIX), a precursor of heme, the non-protein component of hemoglobin, myoglobin, cytochromes, catalase, peroxidase, etc., is of the most interest for oncology **(Fig. 2)**. It is found that rapidly proliferating neoplastic cells are capable of selective accumulation of PpIX due to higher activity of enzymes responsible for initial stages of heme biosynthesis accompanied by iron and ferrochelatase deficiency [12—14]. This gave rise to development of fluorescent methods of tumor detection.

The main difficulty in early studies was the absence of analytic algorithms able to clearly identify weak fluorescence of endogenous PpIX in the total emission spectra of the inspected area.

Further development of fluorescence diagnostics was boosted by a discovery made by A.Policard concerning the capability of exogenously administered hematoporphyrin to induce red fluorescence of neoplastic tissue [12]. Due to high phototoxicity and long duration of the effect of exogenous fluorophores (hematoporphyrin derivatives, chlorins, etc.) they are now used mainly in photodynamic therapy [8], while diagnostics has turned back to evaluation of autofluorescence of endogenous protoporphyrins.

The next breakthrough was the development of autofluorescence induction through administration of PpIX precursor — 5-aminolevulinic acid (5-ALA) [15]. Today this method is the most popular [15—18]. However, 5-ALA administration can cause CNS side effects and severe phototoxic reactions even in young and otherwise healthy patients. It is also found that in some cases induction of PpIX fluorescence decreases diagnostic efficacy in general [19]. Thus, the need for an effective method of native (non-induced) autofluorescence examination has grown [20, 21].

In 2006 two methods of estimating skin and mucosal tumor proliferative activity were developed in the Research Institute of Eye Diseases of the Russian Academy of Medical Sciences, Moscow. One of them is based on local spectroscopy data, the other on fluorescent imag-

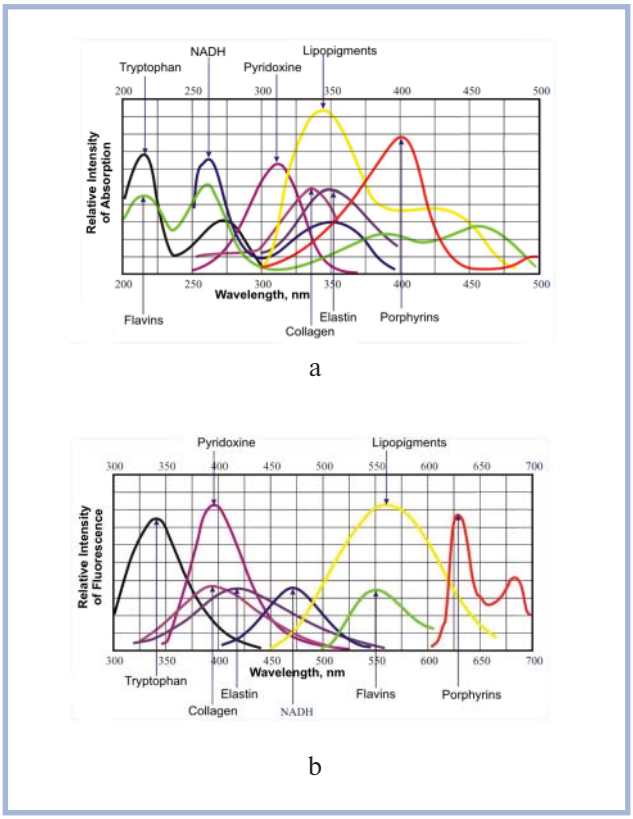


***Fig. 1.*** **Absorption (a) and emission (b) spectra of main fluorophores of biological tissues.**

Explanation in the text.

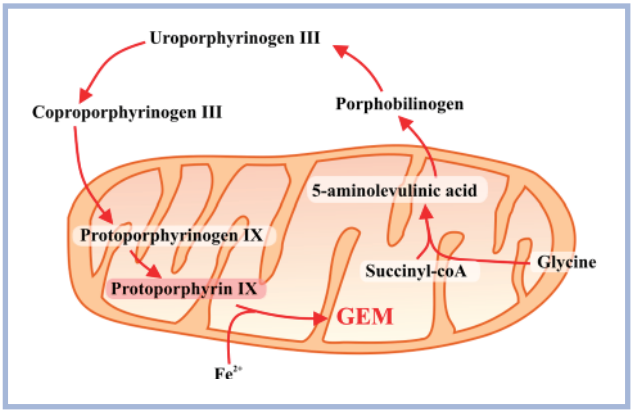


***Fig. 2.*** **Scheme of heme biosynthesis.**

Explanation in the text.

ing, both providing the opportunity to avoid any inducing agents or exogenous fluorophores (RU 2362489 and RU 64783) [19, 22].

The method of local spectroscopy data analysis works through modeling of a spectrum that is approximate to the total autofluorescence spectrum of the tissue. It was found that three substances (viz., collagen, keratin, and protoporphyrin) constitute 97% of the total autofluorescence spectrum of skin within the range of 650—800

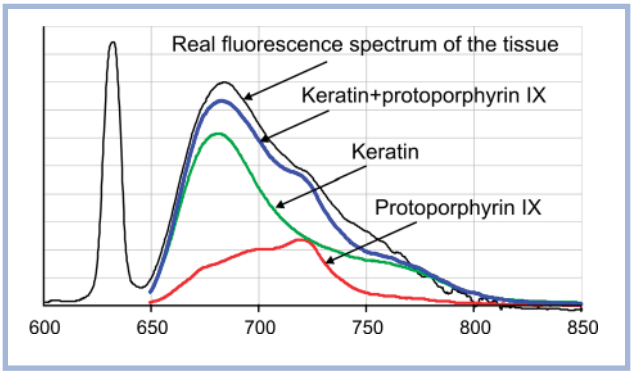


***Fig. 3.*** **Iterative approximation of the model autofluorescence spectrum (keratin+protoporphyrin IX) to the real spectrum acquired during spectroscopy.**

Explanation in the text.

nm upon light excitation at 632.9 nm [9]. Knowing reference fluorescence spectra of keratin and aqueous solution of protoporphyrin IX (collagen spectrum is not taken into account as it almost coincides with that of keratin within the indicated range), it is possible to evaluate contribution coefficients of each of the three substances within the total spectrum **(Fig. 3)**.

However, spatial mapping based on fluorescent imaging, shows more promise than local spectroscopy as it enables studying fluorophores distribution within a large area of skin, thus establishing borders of the pathologic process, while local spectroscopy reveals only the content of fluorophores in a given point. Borders detection is particularly important in cases of difficult tumor sites, for example, the inner corner of the eye, when one has to decide if proposed treatment method is appropriate or any adjustment of surgery extent is needed [23].

The new method of autofluorescent imaging and its analysis developed in the Research Institute of Eye Diseases, Moscow, is easy to perform and does not involve significant expenses. The full set required includes a special photoregistering device with color filters and their transmission bands corresponding to prevalent fluorophores of the tissue (i.e. skin and mucous). A more cost-effective option could be a domestic camera with acceptable characteristics of CCD-matrix (Canon EOS 300D in our case). Specialized analytic software was also developed for further processing of the acquired autofluorescent images (CancerPlot, RU 2007613931, 2007). It allows calculating percentages of the red channel contribution to color formation of each pixel, which corresponds to actual PhIX concentration in the area concerned, and is therefore called "proliferation factor". The results are displayed as proliferation factor isolines applied to the autofluorescent image **(Fig. 4)**. Delta of proliferation factor ($\Delta R$, %) serves as a diagnostic criterion. It is the difference between respective values for the growth and the surrounding tissues, which are taken for the background. Probability of tumor borders occurrence within the in-

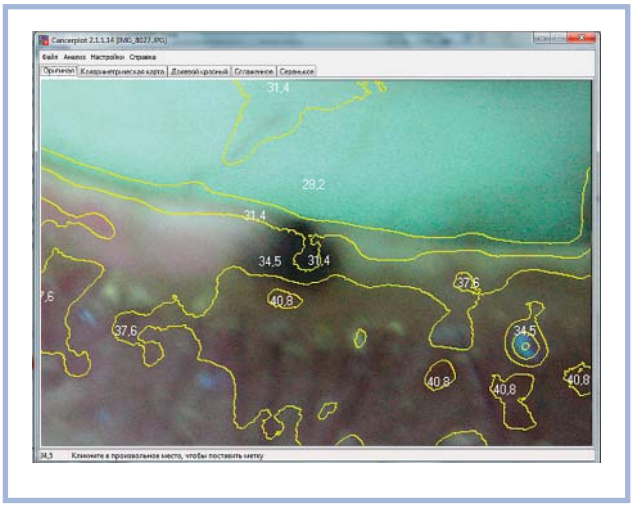


***Fig. 4.*** **CancerPlot interface.**
Proliferation factor isolines are shown in yellow (explanation in the text)

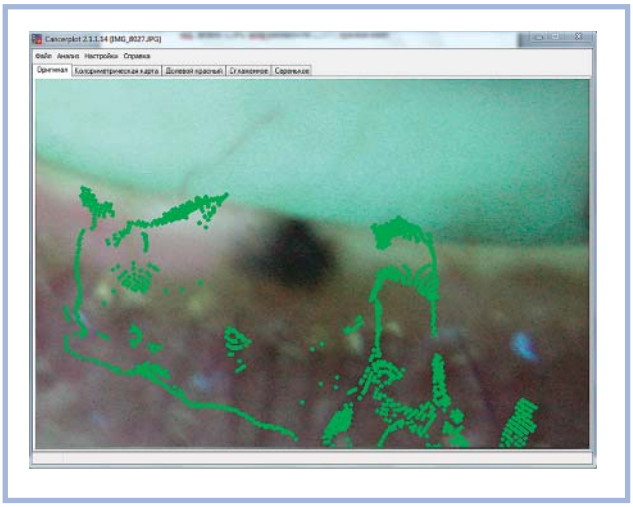

***Fig. 5.*** **CancerPlot interface.**
Green lines applied to the autofluorescent image represent probabilistic tumor borders (explanation in the text).

spected region is estimated separately (RU 2400265). The user manually chooses two zones of interest, one within the tumor, the other within the healthy tissues. The image is then scanned automatically and the program sorts out sections where autofluorescence patterns of both areas of interest are combined. This algorithm drastically differs from earlier approaches based only on analysis of tumor luminescence intensity. Visual borders are applied to the original autofluorescent image for the user's convenience **(Fig. 5)** and pass through the maximums of tumor border probability.

The described method of native autofluorescence examination of skin and mucosal tumors has proved practical possibility to avoid using fluorescence inducers and exogenous fluorophores without affecting the sensitivity of the diagnosis in general. Studies of the method specificity [24] conducted by the Research Institute of Eye Diseases, Moscow, revealed a so called «zone of uncertainty» where the distribution curves of proliferation factor delta for benign and malignant tumors overlapped **(Fig. 6)**. An additional criterion — the averaged reciprocal of tumor borders probability, named the coefficient of

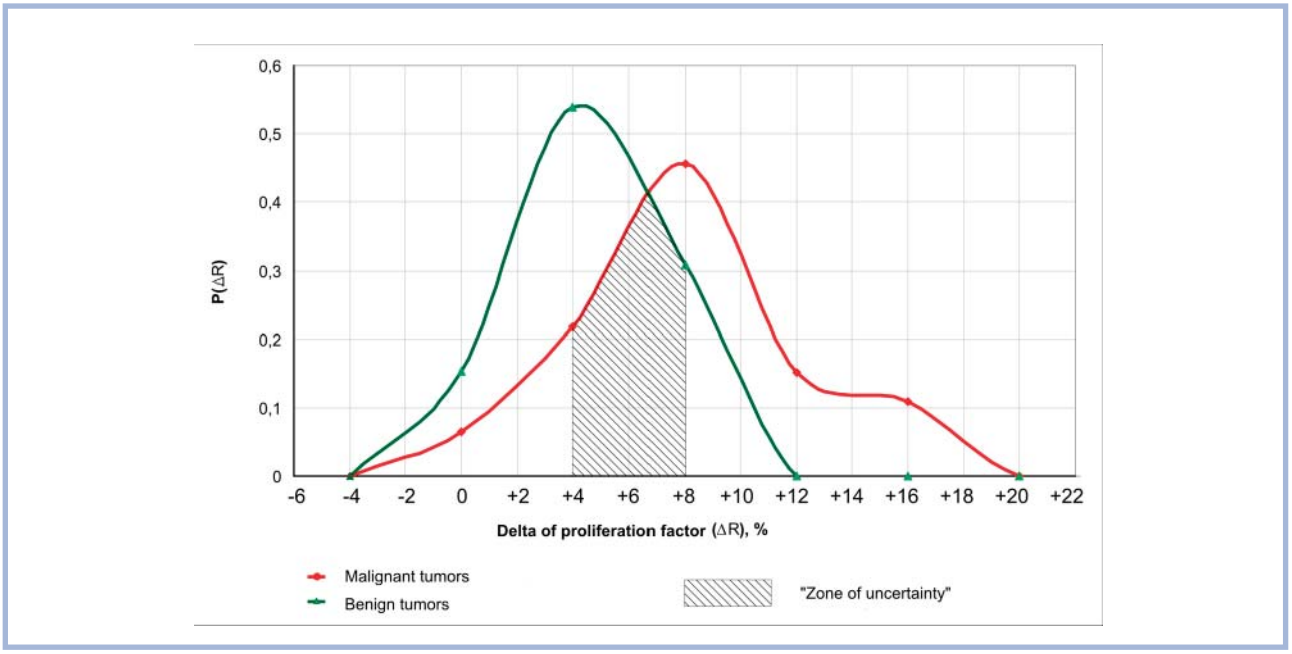


***Fig. 6.*** **Distribution of patients in the groups according to the delta of proliferation factor.**
Explanation in the text.

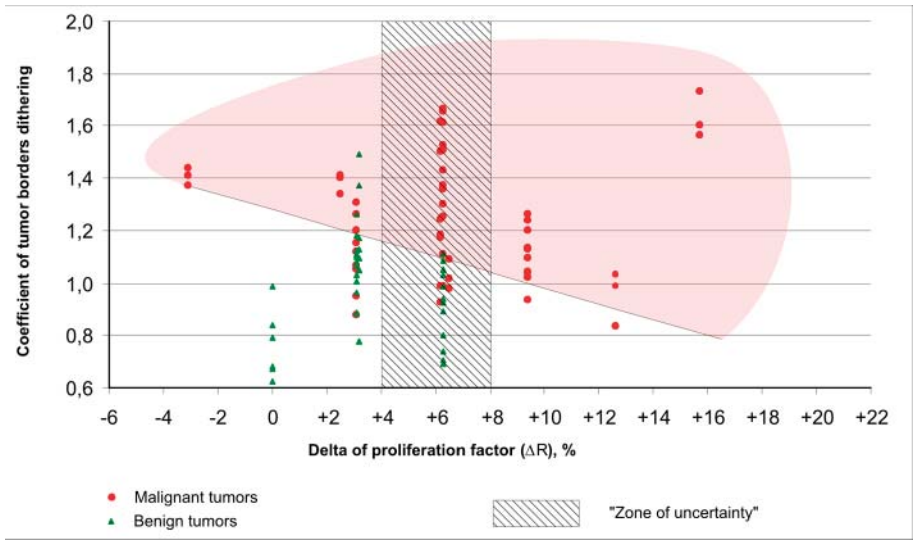


***Fig. 7.*** **Correlation between the coefficient of tumor borders dithering and the delta of proliferation factor. Red-colored region statistically corresponds to malignant process.**

Explanation in the text.

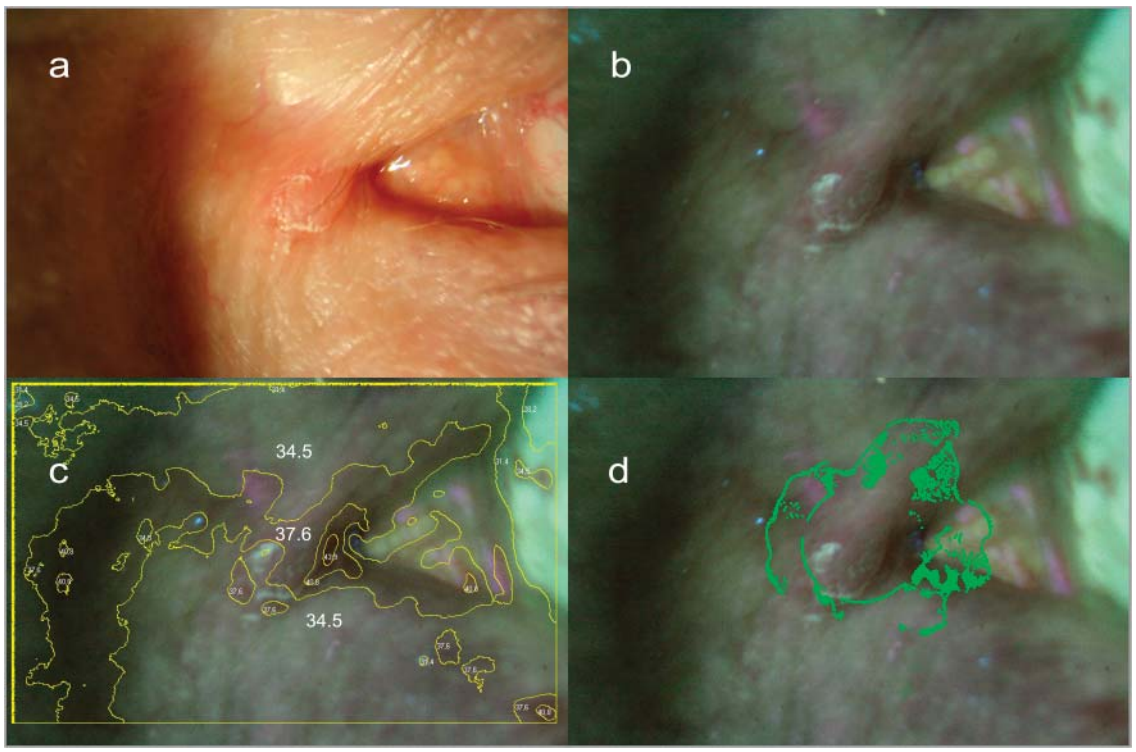


***Fig. 8.*** **Skin papilloma in the inner corner of the left palpebral fissure (histologically proven).**

a – macrograph, b — autofluorescent image, c – autofluorescent image with proliferation factor isolines (yellow), d – autofluorescent image with probabilistic tumor borders (green).

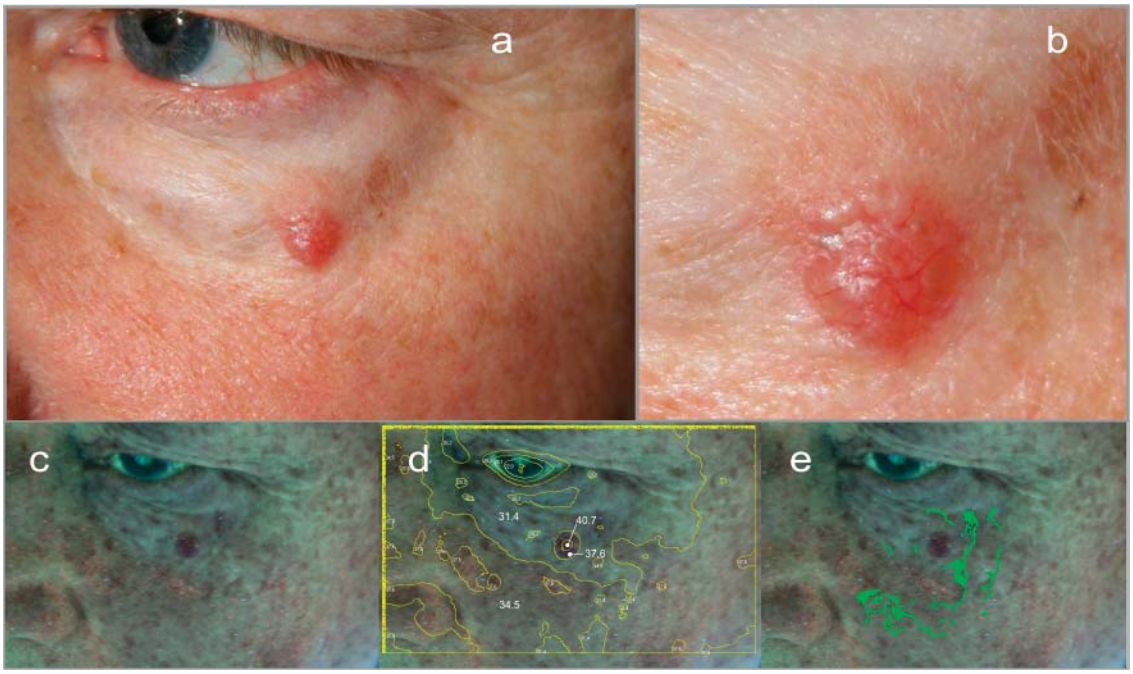


***Fig. 9.*** **Skin basal-cell carcinoma in the left periorbital region (histologically proven).**

a — macrograph, b — tumor macrograph, c — autofluorescent image, d — autofluorescent image with proliferation factor isolines (yellow), e — autofluorescent image with probabilistic tumor borders (green).

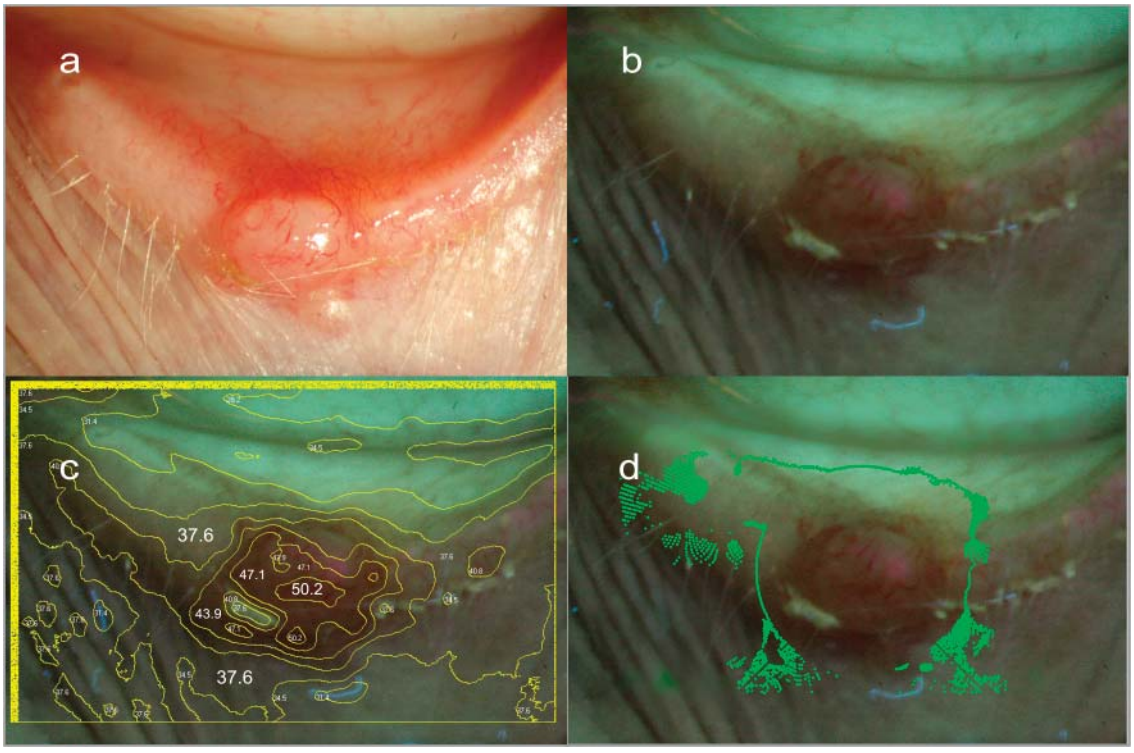


***Fig. 10.*** **Squamous cell carcinoma in the left lower eyelid margin involving palpebral mucous (histologically proven).**

a — macrograph, b — autofluorescent image, c — autofluorescent image with proliferation factor isolines (yellow), d — autofluorescent image with probabilistic tumor borders (green)

tumor borders dithering ($K_{dith}$), has been proposed as a solution. It represents the degree of functional atypism of neoplastic cells, possible invasion and occult growth. Owing to such comprehensive analysis of tumor fluorescence pattern, diagnostics specificity increased considerably (from 0.69 to 0.85). With the developments introduced, the method now enables attributing every case to either benign or malignant tumors with a high degree of statistical credibility **(Fig. 7)**.

Clinical cases are provided to illustrate the above.

Case 1. Patient K, female of 46, had skin growth of 2×1,5×3 mm in the inner corner of the left palpebral fissure (Fig. 8a). Autofluorescence diagnostics showed low proliferative activity at its base. The delta of proliferation factor was 3.2%, i.e. within the range of physiological values for this region [24]. Probabilistic optical borders of the growth could be detected almost throughout with a high degree of confidence. The coefficient of tumor borders dithering $K_{dith}$ was 0.99. According to the graph, Fig. 7, this growth matches the "green zone", i.e. can be considered as benign. Histological examination later revealed a skin papilloma.

Case 2. Patient V, male of 58, had skin tumor of 5x4x4 mm in the left periorbital region. An evident excess of proliferative activity of the tumor over that of the surrounding tissues was detected. The delta of proliferation factor was 6.2 and thus lied within the «zone of uncertainty» in Fig. 7. Unlike the previous case, probabilistic tumor borders could not be easily detected to the full extent, which conformed to the high coefficient of borders dithering — 1.42 Thus, the case could be attributed to malignant tumors («red zone» in Fig. 7). Histological examination diagnosed a basal-cell carcinoma.

Case 3. Patient N, female of 63, had tumor in left lower eyelid margin involving palpebral mucous, size of 5x3×2 mm. The delta of proliferation factor amounted to 12.6% indicating a considerable excess of proliferative activity of the tumor over that of the intact tissues. The coefficient of tumor borders dithering was 2.2.These combined data strongly suggested a malignancy (Fig. 7). Histological diagnosis was squamous cell carcinoma.

As it has been shown above, studies on fluorescence diagnostics carried out by the Research Institute of Eye Diseases of the Russian Academy of Medical Sciences in 2006—2012 have led to tangible results. A non-invasive, simple in application, safe and cost-effective method of autofluorescent image acquisition and analysis has been developed, that is not associated with the use of fluorescence inducing agents or exogenous fluorophores in primary examination of skin and mucosal tumors of periorbital region. An original technique of probabilistic tumor borders detection, highly valuable for surgical planning, was also worked out. A comprehensive approach of tumor autofluorescence pattern analysis, which demonstrated high sensitivity and specificity, has been put into clinical practice of the Institute, and a specialized diagnostic room for autofluorescence examinations has been arranged.

We believe that the results obtained can be extrapolated to neoplasm of other, nonocular localization. The future of the method is associated with improving its efficacy in differentiating malignancies and inflammation. Elaboration of a new CancerPlot interface to promote broadening of its clinical use is also being planned.

## REFERENCES


1. *Brovkina A.F.* Oftal'moonkologiya. Rukovodstvo dlya vrachej [Ophthalmic oncology. Guidelines for practitioners]. Moscow, Meditsina Publ., 2002, 424 p.
2. *Chissov V.I., Davydov M.I.* Onkologiya: natsional'noye rukovodstvo [Oncology: national guidelines]. Moscow, GEOTAR-Media Publ., 2008, 1060 p.
3. *Lomas A., Leonardi-Bee J., Bath-Hextall F.* A systematic review of worldwide incidence of nonmelanoma skin cancer: Epidemiology and Health Services Research. British Journal of Dermatology, 2012; 166(5).
4. *Erba P., Farhadi J., Wettstein R., Arnold A., Harr T., Pierer G.* Morphoeic basal cell carcinoma of the face. Scand J Plast Reconstr Surg Hand Surg., 2007; 41(4):184-8.
5. *Smith V., Walton S.* Treatment of facial Basal cell carcinoma: a review. Journal of Skin Cancer, 2011;2011:380371, doi: 10.1155/2011/380371.
6. *Costello M., Luttenberger L.* Fluorescence with the Wood's filter as an aid in dermatological diagnosis. New York State Journal of Medicine, 1944 Aug; 42, pp 1778-1784.
7. *Likhvantseva V.G., Osipova E.A.* Fluorescent studies in medical practice. Vestnik Oftalmologii — Annals of Ophthalmology, 2007(1), 48-52 (in Russian).
8. *Zellweger M.* Fluorescence spectroscopy of exogenous, exogenously-induced and endogenous fluorofores for the photodetection and photodynamic therapy of cancer. Lausanne: EPFL, 2000, doi: 10.5075/epfl-thesis-2126.
9. *Sinichkin Yu.P., Dolotov L.E., Zimnyakov D.A., Tuchin V.V., Utz S.R.* In vivo otrazhatel'naya i fluorestsentnaya spektroskopiya kozhi cheloveka [In vivo reflection and fluorescence spectroscopy of human skin]. Saratov, Saratov University Publ., 2003, 159 p.
10. *Gurinovich G.P., Sevchenko A.N., Solovyev K.N.* Spektroskopiya porfirinov [Spectroscopy of porphyrins]. Physics-Uspekhi. Moscow, UFN Publ., 1963 Feb; 79(2).
11. *Lakowicz J.R.* Principles of Fluorescence Spectroscopy. 3rd edition, Springer, 2007, 980 p. (Russ. ed.: Lakovich Dzh. Osnovy fluorestsentnoy spektroskopii. Moscow, Mir Publ., 1986, 496 p.)
12. *Auler H., Banzer G.* Untersuchung uber die Rolle der Porphyrine bei geschwulstkranken Menschen und Tieren. Z Krebsforsch 1942; 53, pp. 65-68.
13. *Meng J.W., Wang X.-J., Lin T., Ren X.G., Pang H.F., Zhou C.N.* The study of protoporphyrin IX metabolism in cell proliferation using photoluminescence method. J Luminesc, 01/1999; 83-84, pp. 271-273. doi:10.1016/S0022-2313(99)00110-6
14. *Rubino G.F., Rasetti L.* Porphyrin metabolism in human neoplastic tissues. Panminerva Medica, 1966 Jul-Aug; 8(7):290-2.
15. *Hinnen P., De Rooij F.W.M., Velthuysen M. L.F., Edixhoven A., Hillegersberg R., Tilanus H.W., Wilson J. H.P., Siersema P.D.* Biochemical basis of 5-aminolaevulinic acid-induced protoporphyrin IX accumulation: a study in patients with (pre)malignant lesions of the oesophagus. Br J Cancer, 1998 Sep; 78, pp 679-682.
16. *Hefti M., von Campe G., Moschopulosa M., Siegnerb A., Looserc H., Landolt H.* 5-aminolaevulinic acid-induced protoporphyrin IX fluorescence

in high-grade glioma surgery: a one-year experience at a single institutuion. Swiss Med Wkly, 2008; Mar 22; 138(11–12 ): 180-5, doi: 2008/11/smw-12077.

17. *Moan J, Bech O, Peng Q, Berg K.* Use of 5-aminolevulinic acid in photochemotherapy and fluorescence diagnostics. Tidsskr Nor Laegeforen, 1998 Mar; 118(8):1206-11.

18. *Smits T., Robles C.A., van Erp P.E., van de Kerkhof P.C., Gerritsen M.J.* Correlation between macroscopic fluorescence and protoporphyrin IX content in psoriasis and actinic keratosis following application of aminolevulinic acid. J Invest Dermatol, 2005 Oct; 125(4):833—9.

19. *Osipova E.A.* Fluorestsentnye metody issledovaniya opukholey vek i kon"yunktivy na osnove endogennykh i ekzogennykh fluoroforov. Diss. kand. med. nauk [Fluorescence techniques for eyelid and conjunctival tumors examination based on endogenous and exogenous fluorophores]. Moscow, Research Institute of Eye Diseases, RAMS, 2009, 121 p.

20. *Betz C.S., Mehlmann M, Ing D, Rick K, Stepp H, Grevers G, Baumgartner R, Leunig A.* Autofluorescence imaging and spectroscopy of normal and malignant mucosa in patients with head and neck cancer. Lasers Surg Med, 1999, 25:323-34.

21. *Onizawa K, Saginoya H, Furuya Y, Yoshida H, Fukuda H.* Usefulness of fluorescence photography for diagnosis of oral cancer. Int J OralMaxillofac Surg, 1999, 28:206-210.

22. *Likhvantseva V.G., Novikov I.A., Osipova E.A.* Investigation of autofluorescence spectrum of skin and mucous in norm and pathology. Sbornik materialov Vserossiyskoy nauchno-prakticheskoy konferentsii "Otechestvennye protivoopukholevye preparaty" [Proc. All-Russian scientific and technical conference "Domestic antineoplastic drugs"]. Moscow, Mar 2007.

23. *Novikov I.A., Grusha Ya.O., Kiryushchenkova N.P.* Options for improving efficacy of fluorescent diagnostics of eyelid and periorbital tumors in surgery extent evaluation. Sbornik materialov II Natsional'nogo kongressa "Plasticheskaya khirurgiya" [Proc. 2nd National congress "Plastic Surgery"]. Mocsow, Dec 2012, pp. 91-92.

24. *Novikov I.A., Grusha Ya.O., Kiryushchenkova N.P.* Options for improving efficacy of fluorescent diagnostics of eyelid and periorbital epithelial tumors. Vestnik Rossiyskoy akademii meditsinskikh nauk — Herald of Russian academy of Medical Sciences, 2012; 10, pp 62—70.