*Journal of Fluorescence, Vol. 14, No. 6, November 2004 (©2004)*

# Autofluorescence of Developing Plant Vegetative Microspores Studied by Confocal Microscopy and Microspectrofluorimetry

**Victoria V. Roshchina,[1,2] Valerii A. Yashin,[1] and Alexei V. Kononov[1]**

*Received September 15, 2004; revised July 6, 2004; accepted July 7, 2004*

Phenomenon of autofluorescence from vegetative microspores of spore-breding plant *Equisetum arvense* has been studied by methods of laser-scanning confocal microscopy (LSCM) and microspectrofluorimetry during the development of the cells. The microspores have demonstrated a difference between structures: blue-fluorescing cover and red-fluorescing chloroplasts. The fluorescence spectra of the studied cells was also measured by original microspectrofluorimeter. The character of the spectra and the color of fluorescence was changed during the microspores germination. The red fluorescence of the microspores was, mainly, due to the presence of chlorophyll and azulenes. The unicellular microspores may be recommended as natural probes of cellular viability and development.

**KEY WORDS:** Autofluorescence; chlorophyll; confocal microscopy; Equisetum arvense; fluorescence; microspectrofluorimetry; microspores; development.

## INTRODUCTION

The search of most suitable models-objects is actual for cellular biology. For this aim plant microspores, which serve for a plant breeding [1] could be recommended because their development and the sensitivity to various external factors are well-seen [2–5]. Main advantage of the objects is their autofluorescence excited by UV-light. There are vegetative (in spore-breeding plants such as horsetails, mosses and ferns) and generative (male gametophyte that named pollen, which is peculiar to Golosperms, mainly conifers, and blossomed species of Angiosperms) microspores. They are single cells covered by the multilayer cellulose wall that protects against unfavourable external factors of an environment. The microspores were found to fluoresce under luminescence microscope. Their emission may be first registered by microspectrofluorimeters during the development of the microspores [3]. Ealier, only histochemical staining [6–9] or electron microscopy [10] were used for the study of their development. Construction of a confocal microscope permits to observe cellular structures by the mode of a regulation of the depth of an object slice. Cells of algae and some non-secretory cells of higher plants were studied by the method [11,12]. Confocal imaging of secreting plant cells was studied for grass pollen analysis [13].

This paper is devoted to possibilities of the confocal microscopy and microfluorimetry in the study of the autofluorescence of the microspores during their development.

## EXPERIMENTAL

Objects of the study were vegetative microspores of *Equisetum arvense* collected in natural habitats in April-May 1996–2001 years. Germination of the microspores was studied by their cultivation on the object glasses put on the wet paper in Petri dishes [3]. The dry and wet samples

[1] Institute of Cell Biophysics RAN, Pushchino, Moscow region,142290, Russia.

[2] To whom correspondence should be addressed. E-mail: roshchina@icb.psn.ru

were studied. For the analysis under confocal microscope with the oil immersion, after the moistening the probes were dried by hot air at 50–70°C during 5–7 min. The measurements with water immersion by confocal and luminescence microscope of microspectrofluorimeters were done without a cover glass.

### Confocal Microscopy

The fluorescence was observed by both the oil and water immersion under laser scanning confocal microscope LSM 510 NLO "Carl Zeiss." The excitation of the emission was by three types of lasers: Argon/2 (λ 458, 488, 514 nm), HeNe1 (543 nm) and HeNe2 (λ 633 nm). Three photomultipliers can catch the autofluorescence, separately or simultaneously by use the pseudocolor effects. The image analysis was carried out with a help of the computer programs LSM 510 and Lucida Analyse 5. At the excitation by lasers with wavelengths 488, 543 and 633 nm, the registration of the autofluorescence was at 505–630 nm, 565–615 nm and 650–750 nm, relatively. Pseudocolors were according to the exciting wavelength blue for 488; green for 543 and red for 633. Summed image was seen when the images consisted of the pseudocolors were superposed and mixtured as shown on Fig. 1 for the microspores of *Equisetum arvense*.

### Microspectrofluorimetry

The sample's fluorescence (excited by UV-light 360–380 nm) was analysed using original-constructed microspectrofluorimeters 1) for the registration of fluorescence spectra [14] and 2) "Radical DMF-2" (Radical, Ltd) interfaced to PC/AT compatible computer for the measure the fluorescence intensities at two separate wavelength [15]. A special program "Microfluor" permits to obtain the histograms ,which are related to the fluorescence intensities and to perform statistical treatment of the data, using Student $t$-test. Autofluorescence of intact cellular surfaces induced by ultra-violet irradiation (360–380 nm) was observed under luminescent microscope *Fluoval* (*Carl Zeiss*), and the fluorescence spectra of the intact cells were measured with microspectrofluorimeter of original construction [14]. The diameters of optical probe: 100 $\mu$m, 20 $\mu$m, 2 $\mu$m, the objective: ×10, ocular: ×7. The registration time of XY recorder in a region 400–700 nm for the fluorescence spectra was 22 s. The error of the fluorescence measurement for the same sample was 1–2 relative units. The fluorescence intensity of microspores also measured by a duable-wavelength microspectrofluorimeter [15].

## RESULTS AND DISCUSSION

Figure 2 shows the views of the microspores studied under confocal microscope and their fluorescence spectra measured by microspectrofluorimeter. Under confocal microscope the rigid cover of the vegetative microspore is well-seen at the excitation 488 nm and registration at 505–635 nm. At the excitation 543 nm and 633 nm chloroplasts (red-orange colour) and other organelles (yellow colour).

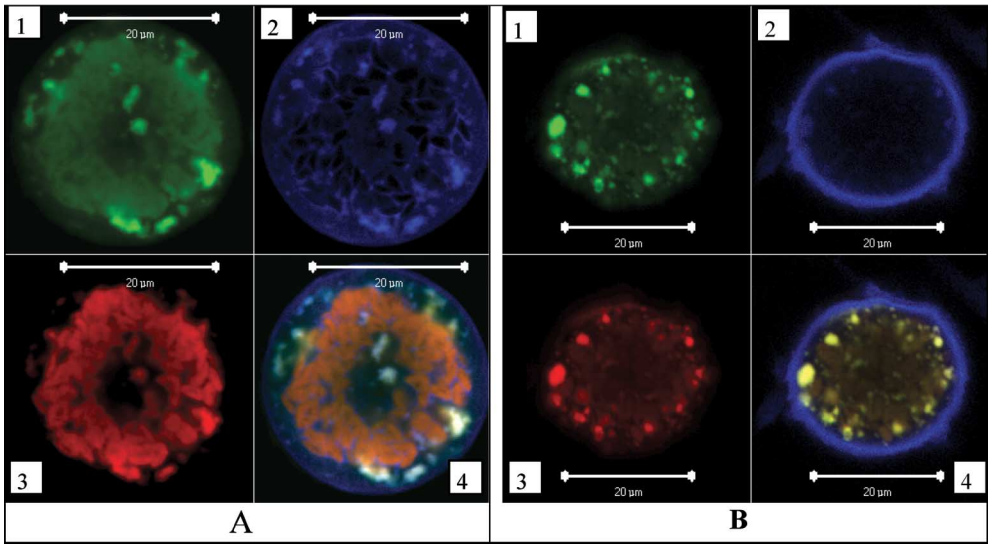


**Fig. 1.** LSCM view of dry (A) and moistened (B) microspores of *Equisetum arvense* under three laser excitation 1—channel 488 nm; 2—channel 533 nm; 3—channel 633 nm; 4—summed image with mixed (in a superposition) pseudocolors. 1 bar = 20 $\mu$m.

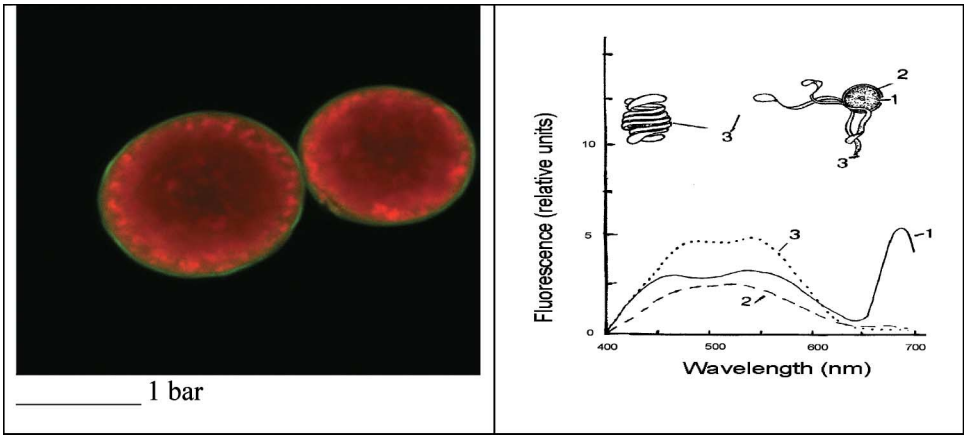


**Fig. 2.** The confocal LSCM images (left) and the fluorescence spectra (right) of the *Equisetum arvense* microspores. LSCM: channel 1—wavelength of excitation 488 nm (45%), pinhole: 56 $\mu$m. LSCM channel 2: wavelength of excitation 543 nm (49%), pinhole: 46 $\mu$m. 1 bar = 10 $\mu$m. In the fluorescence spectra measured by microspectrofluorimetry: 1—The middle part of the spore, 2—cover; 3—elathers served for the anchoring to a substrate (a soil). The excitation by UV-light (360–380 nm).

The fluorescence of vegetative microspores may be related to their cover (blue fluorescence) and chloroplasts (red fluorescence at 650–750 nm) that may be observed by confocal microscope (Figs. 2 and 3). A middle part of microspore has three maxima—460 nm, 550 nm and 680 nm. Maximum 680 nm is peculiar to chlorophyll fluorescence (Fig. 2). The cover of the microspore has no maximum 680 nm, as well as elathers (Fig. 2). The presence of chlorophyll is found in the dry microspores, and red fluorescence should be related to the pigment and, perhaps to azulenes [3].

The changes of the autofluorescence has been studied during the development of the *Equisetum arvense* microspore. The shifts dealt with first moistening are on Fig. 1. A diffusive distribution of the elements of cover and chloroplasts became to be seen more clear. The red fluorescence of chloroplasts had strengthened just at 15 min after moistening (Fig. 4). The cells are swelled and blue-fluorescing elathers are broken. Chloroplasts are moved from the centre to the cell wall. After 60 min of the development there is the back movement of red-fluorescing chloroplasts to the middle of the cell.

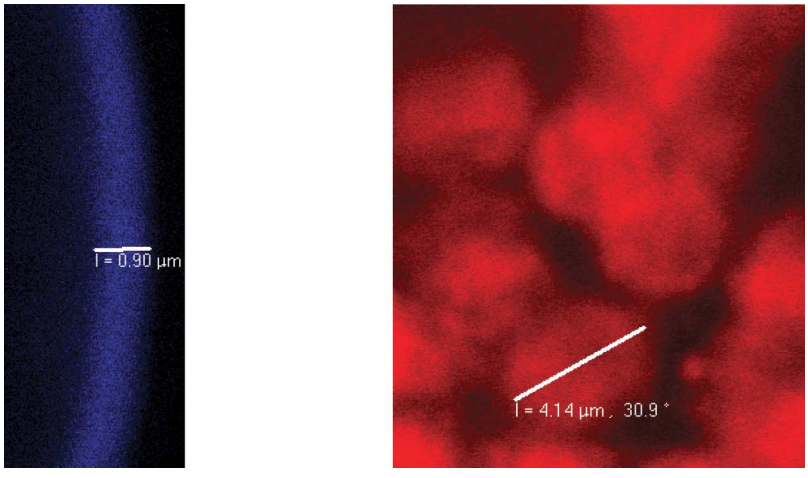


**Fig. 3.** The LSCM of the fluorescing compartments of the *Equisetum arvense* microspore. Left—Cover, 1 bar (thickness) = 0.9 $\mu$m; Right—chloroplast. 1 bar (diameter) = 4.14 $\mu$m. The channel excitation wavelength was for cover 488 nm, and for chloroplasts −633 nm.

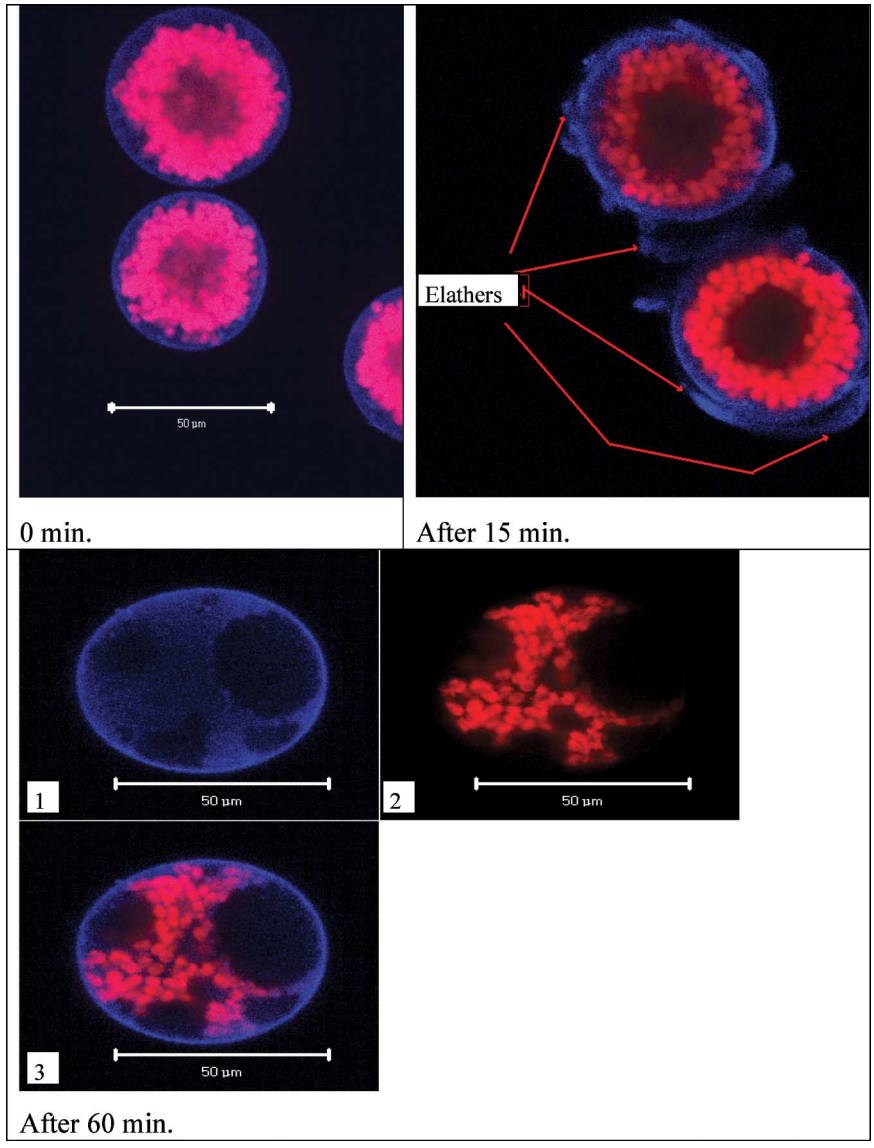


**Fig. 4.** The changes in the fluorescing microspores of *Equisetum arvense* during their development for first hour after moistening. 1 bar = 50 $\mu$m. In variants "0 min" and "after 15 min," the excitation by lasers with wavelengths 543 and 633 nm, the registration of the autofluorescence was at a superposition 565–615 and 650–750 nm, relatively. Elathers, structures for the anchoring of the spore to a substrate, are seen. In variant "after 60 min," the excitation by lasers with wavelengths 543 and 633 nm, the registration of the autofluorescence was at 565–615 nm (1), 650–750 nm (2) and at a superposition (3), relatively.

According to Nakazawa [9], it indicates the beginning of the cell division. To 120 min (sometimes to 24–72 hr) after moistening, the cell put off its blue-fluorescing rigid cover (Fig. 5). Red-fluorescing cell and its blue-fluorescing rigid cover are seen. The bright-lightening cover has maximum 460 and shoulder 500 nm in the fluorescence spectra whereas liberated cell-expressive maxima at 460 and 550 nm and high chlorophyll-related maximum at 680 nm. The cell without rigid cover divides, forming two cells, from one of which will form a multicellular tallus (Fig. 6). Other cell will be a rhizoid, which anchors the tallus to a substrate (a soil) and where chloroplasts will be decreased and, then completely, reduced. These data are correlated with the histochemical investigations with the staining by artificial dyes [6–9] and biochemical analysis of the content of chlorophyll and azulenes [3].

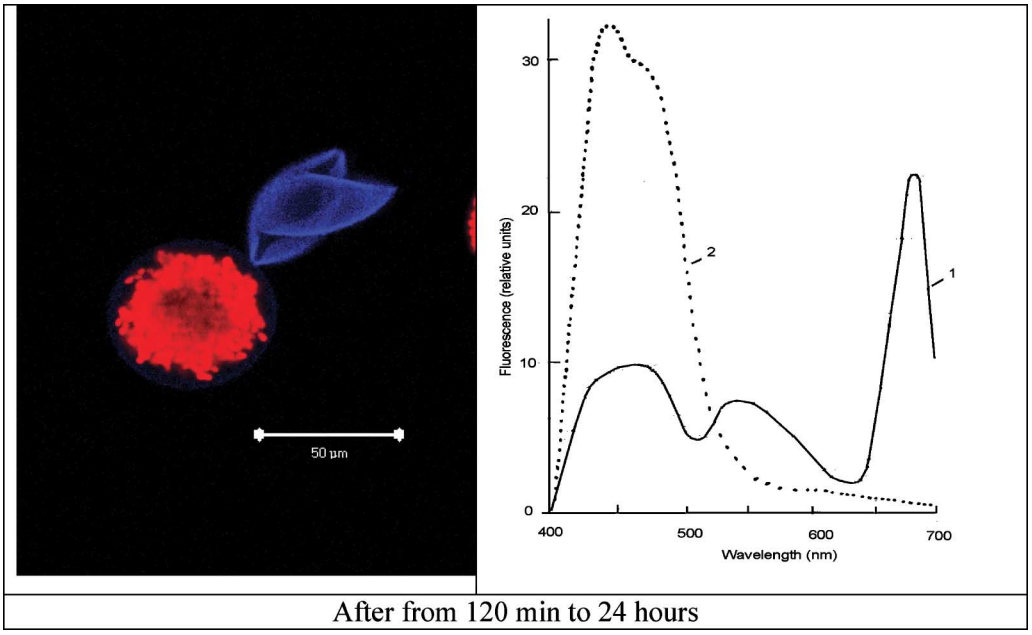


**Fig. 5.** The view under confocal microscope (left) and the fluorescence spectra (right) of the microspores of *Equisetum arvense* during their development from 120 min to 24 hr (in a dependence on the probe). 1 bar = 50 $\mu$m. Left—Red-fluorescing cell has been liberated from its blue-green fluorescing cover excited by lasers with wavelengths 458 nm and 633 nm, the registration of the autofluorescence was at 505–630 nm and 650–750 nm, relatively. Right: The fluorescence spectra of the developed cell (1) without cover and cover (2), which has been put off by the microspore. The excitation by UV-light (360–380 nm).

In more prolonged experiments, after 24 hr of the microspore development (Fig. 6), the intensive growth of tallus has been established that also confirmed by the strengthened red fluorescence of the probe studied. The tallus looks as fungi-like body, and its volume image is well seen.

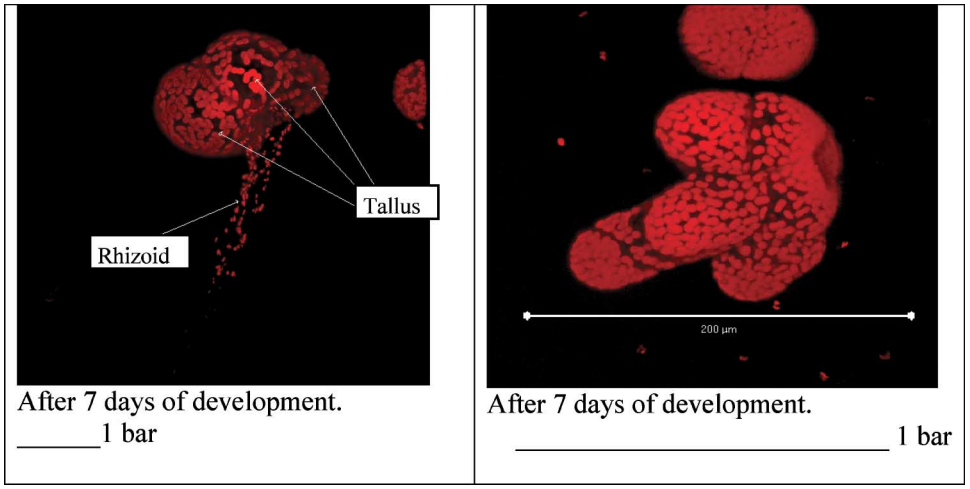


**Fig. 6.** The LSCM image of dividing cells derived from the microspores of *Equisetum arvense* after 24 hr and 7 days of development. The excitation by laser 633 nm, and the registration of the autofluorescence was at 650–750 nm. Left—The multicellular tallus starts to form after 24 hr. Rhizoid (the longest cell with smaller amount of chloroplasts) and chloroplasts'-enriched cells of tallus are seen. 1 bar = 50 $\mu$m. Right—The multicellular tallus has been formed after 7 days. 1 bar = 200 $\mu$m.

It should mark that all observations under both confocal and luminescent microscope of microspectrofluorimeter never damage the cells of microspores. They continue their development more, than 3 months. Periodically the researcher could analyse the probe directly on Petri dish.

## CONCLUSION

Autofluorescence of plant vegetative microspores (suitable cellular models) produced by horsetail *Equisetum arvense* could be recommended for the study of the cell development from a unicellular structure to a multicellular organism by methods of confocal microscopy and microspectrofluorimetry. All observations under both confocal and luminescent microscope of microspectrofluorimeter never damage the cells of microspores. They continue their development more, than 3 months.

## REFERENCES


1. K. M. Pryer, H. Schneider, A. R. Smith, R. Cranfill, P. G. Wolf, J. S. Hunt, and S. D Sipes (2001). Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. *Nature* **409**, 618–622.
2. V. V. Roshchina, E. V. Mel'nikova, L. I. Mit'kovskaya, and V. N. Karnaukhov (1998). Microspectrofluorimetry for the study of intact plant secretory cells. *J. Gen. Biol.* **59**(5), 531–554. (Russia).
3. V. V. Roshchina, E. V. Melnikova, V. A. Yashin, and V. N. Karnaukhov (2002). Autofluorescence of intact spores of horsetail *Equisetum arvense* L. during their development. *Biophysics* **47**(2), 318–324. (Russia).
4. V. V. Roshchina, A. V. Miller, V. G. Safronova, and V. N. Karnaukhov (2003). Reactive oxygen species and luminescence of intact cells of microspores. *Biophysics* **48**(2), 259–264. (Russia).
5. V. V. Roshchina and V. D. Roshchina (2003). *Ozone and Plant Cell*, Kluwer Acad. Publ., Dordrecht.
6. R. Beer (1905). The development of the spores of *Equisetum*. *New Phytol.* **8**, 261–266.
7. W. Nienburg (1924). Die Wirkung des Lichtes auf die Keimung der *Equisetum*-Spore. *Ber. Dtsch. Bot. Ges.* **42**, 95–99.
8. S. Nakazawa (1956). The latent polarity in *Equisetum* spores. *Bot. Mag* **69**, 506–509.
9. S. Nakazawa (1958). The rhizoid point as the basophilic center of *Equisetum* spores. *Phyton* **10**, 1–6.
10. B. M. Gulvag (1968). On the fine structure of the spores of *Equisetum fluviale* var. *media.-verticillatum* studied in the quiescent, germinated and non-viable state. *Grana Palynol.* **8**, 23–69.
11. P. C. Cheng and R. G. Summers (1990). Image contrast in confocal light microscopy. In J. B. Pawley (ed.), *Handbook of Biological Confocal Microscopy*, Plenum Press, New York, pp. 179–195.
12. J. G. White, W. B. Amos, and M. Fordham (1996). An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. In B. R. Masters (ed.), *Selected Papers on Confocal Microscopy*, SPIE Press, Bellingham, pp. 356–363.
13. J. A. Salih, D. Bass, and G. Cox (1997). Confocal imaging of exine for grass pollen analysis. *Grana*. **36**, 215–224.
14. V. N. Karnaukhov (1978). *Luminescent Spectral Analysis of Cell*, Nauka, Moscow, p. 208.
15. V. N. Karnaukhov, V. A. Yashin, V. I. Kulakov, V. M. Vershinin, and V. V. Dudarev (1982). Apparatus for investigation of fluorescence characteristics of microscopic objects. US Patent, N4, 354, 114, 1–14; V. N. Karnaukhov, V. A. Yashin, V. I. Kulakov, V. M. Vershinin, and V. V. Dudarev (1983). Patent of England 2.039.03 R5R.CHI.