World Applied Sciences Journal 26 (6): 712-718, 2013
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2013.26.06.13505

# Cell Staining by Novel Derivatives of Fluorescent Rhodamine Dyes

*[1]Sergei Yu Zaitsev, [1,2]Mikhail N. Shaposhnikov, [1]Daria O. Solovyeva and [2]Albert A. Rizvanov*

[1]Moscow State Academy of Veterinary Medicine and Biotechnology,
Akad Skryabin Str. 23, Moscow 109472, Russia
[2]Kazan Federal University, Kremlevskaya Str. 18, Kazan 420008, Russia

**Submitted:** Oct 13, 2013; **Accepted:** Nov 17, 2013; **Published:** Nov 23, 2013

**Abstract:** Two novel precursors of fluorescent dyes (PFD813 and PFD814) have been studied for their ability to photo-activation, transfer across the biomembrane and cells staining. The fluorescent dyes Rho813 and Rho814 formed by photo-activation of their precursors PFD813 and PFD814 inside cells were used for the optical detection of particular features *in vitro* (HaCat cells, human epithelial carcinoma A431, epidermoid carcinoma of the cervix HeLa and chinese hamster ovary CHO cells). One of the possibilities to visualize and track the pathways of macromolecules or organelles in a "living" cell is to monitor them after staining with these PFDs during the real time measurements. A bright fluorescent signal from the photoactivated dye molecules inside the small spot in the cell can be monitored during their movement into the cell dark region (where the dye was not activated and did not fluoresce). The obtained data are important for further application of these precursors of the fluorescent dyes ("caged" dyes) for microscopic probing of biological objects.

**Key words:** Fluorescent dyes % Photo-activation % Biomembranes % Monolayers % Cell staining

## INTRODUCTION

Caged fluorescent dyes (CFDs) are non-fluorescent precursors of fluorescent dyes (PFDs) [1-3]. Upon photo-activation such compounds form fluorescent dyes that have been used for cell staining and tracking of macromolecular movement in living cells [1]. Caged rhodamine dyes with particular functions have been used for super-resolution microscopy [2], photo-activated localization microscopy (PALM) [3], or imaging labile $Fe^{3+}$ ions in live neuronal cells [4]. Novel non-fluorescent precursors of rhodamine dyes have recently been introduced that are particularly useful for microscopic investigation of biological tissue by ultrahigh resolution optical microscopy (STED) [5-9]. Here, the precursor dye is non-fluorescent due to the incorporation of a group that may be photo-chemically eliminated thereby forming the fluorescent dye [10-13]. The precursor dye and conjugates have properties like charge distribution and solubility different from those of the fluorescent dyes [14, 15]. The incorporation of the caged dye in cells by transfer across the biomembrane may be more facile than that of the fluorescent dye, that has been shown by some model studies [16-18]. The design, membrane model study and applications of various styryl, pyridinium, anion-capped and butadienyl fluorescent dyes are the fascinating interdisciplinary directions between biophysics and biochemistry, cell biology and biomedicine [19-38]. The photochromic properties of such dyes are the reason they can transform during two photoinduced reversible reactions, namely, *trans-cis* C=C double-bond isomerization and [2 + 2] photocycloaddition to form substituted cyclobutanes [19-24]. For example, such dyes show the capability of forming stable monolayers at the air-water and water–alkali metal salt solution interfaces [25-30]. Langmuir monolayers and thin polymer films based on such dyes are not only unique models for the fundamental studies of molecular organization and recognition phenomena, but also prototype of chemosensoring materials on various small compounds (such as organic and inorganic molecules and ions) with optical signal detection [31-38].

The recent synthesis of novel "caged" rhodamine derivatives [6-8] (Fig. 1) and studies of their photo-isomerisation reactions with light-induced activation of fluorescence (open-state) and thermal

**Corresponding Author:** Sergei Yu. Zaitsev, Moscow State Academy of Veterinary Medicine and Biotechnology, Akad Skryabin Str. 23, Moscow 109472, Russia.

relaxation to the initial colorless substance (closed-state) [8] have triggered their application in modern confocal microscopy and nanoscopy [5-9].

The method of cell staining by the novel precursors of fluorescent dyes PFD813 and PFD814 has been tested with various examples of the human cell lines (human keratinocytes HaCaT, human epithelial carcinomas A431 and epidermoid carcinoma of the cervix HeLa) as well as with chinese hamster ovary CHO cells. Testing of novel compounds using transformed cell lines *in vitro* is widely accepted in many areas of scientific research [39].

The aim of this work was to prepare and study PFDs for cell staining with different types of cells before and after photo-activation of the PFDs.

## MATERIALS AND METHODS

**Materials:** The synthesis and properties of the "caged" dyes PFD813 and PFD814 have been described earlier [6-8]. The rhodamine dye GM140 have been used for permanent florescence (without photoactivation) as reference to Rho813 and Rho814 and described in our previous publications [9, 10, 16].

The cell samples were cultivated and handled in the Institute of Bioorganic Chemistry, Russian Academy of Sciences: human cells HaCaT, human epithelial carcinoma A431, epidermoid carcinoma of the cervix HeLa and chinese hamster ovary CHO [13]. Nuclear stain Hoechst 33342 was purchased from Sigma-Aldrich.

**Measurements:** The following PFD concentrations were used: PFD813 and PFD814 solutions from 0.02 till 10 µg/ml in the final media from the preformed solutions in DMF at 10 mg/ml and in DMSO at 2 mg/ml. The cells were incubated in $CO_2$-incubator. After 15 min incubation with fluorescent dyes the nuclei staining dye Hoechst 33342 was added and cells were incubated for additional 15 min. Then cells were washed with phosphate buffer (pH 7.0). These samples were fixed on glass by Mowiol 4.88 (Calbiochem, Darmstadt, FRG) and stored at 4°C overnight for the complete polymerization. All samples were analyzed on confocal microscope Eclipse TE2000 (Nikon) using a special program (EZ-C1 3.90 Free Viewer).

## RESULTS AND DISCUSSION

The method of cell staining by the novel precursors of fluorescent dyes PFD813 and PFD814 has been tested with various examples of the human cell lines (human keratinocytes HaCaT, human epithelial carcinomas A431 and epidermoid carcinoma of the cervix HeLa) as well as with chinese hamster ovary CHO cells. The corresponding samples for application in fluorescence microscopy have been prepared by standard procedures described above. Investigation of such cell preparations by fluorescence microscopy allows one to find the optimal PFD concentrations in various solvents (details have been published earlier [14]). Optimal concentration of PFD813 and PFD814 solutions for staining was 5 µg/ml, in all cases. High resolution images of HaCaT cells were obtained after light-induced activation of PFD813 (Fig. 1). In all microphotographs (Fig. 1-3) the red fluorescence in all cells after staining and subsequent photo-activation are due to the presence of the Rho813 or Rho814, respectively, whereas the blue fluorescence is due to the presence of the standard fluorescent dye Hoechst 33342 (to stain cell nuclei). The difference in the images before and after the light-induced activation of the PFD814 in the HeLa cell is clearly shown in the Fig. 2. The left image Fig 2a (before light-induced activation) presents only blue circular areas of cell nuclei stained by the standard dye Hoechst 33342, whereas PFD814 in the HeLa cell is non fluorescent (dark regions). The right image Fig. 2b presents the additional red regions which are correspond to the cell cytoplasm and some organelles stained by PFD814 and transformed to fluorescent Rho814 (after the light-induced activation).

It is important to underline that all cell preparations stained by PFD813 show easy photo-activation and bright fluorescence of the Rho813 in the selected area just after the first quick cell scan with actinic light. In contrast, cell preparations with PFD814 show extremely low fluorescence of Rho814 by activation in this way. Therefore, in order to obtain a bright image in the case of Rho814 (Fig. 2b) it is necessary to scan the cell sample (Fig. 2a) by a laser beam very slowly and several times. Consequently, dye precursor PFD814 appeared not suitable for further cell staining studies.

In other series of experiments, cells were stained with PFD813 and the rhodamine dye GM140 (the last one was described in our previous publications [9, 16]). The presence of GM140 is required to select areas of local photo-activation. A micrograph (Fig. 3a, left) without photo-activation shows the low intensity fluorescence of dye GM140 only due to its association with single cell layers. In contrast, the molecules of PFD813 present in the cells are not visible (being in the ring-closed form). After local photo-activation of two selected areas (small parts inside the cell were marked as a white squares

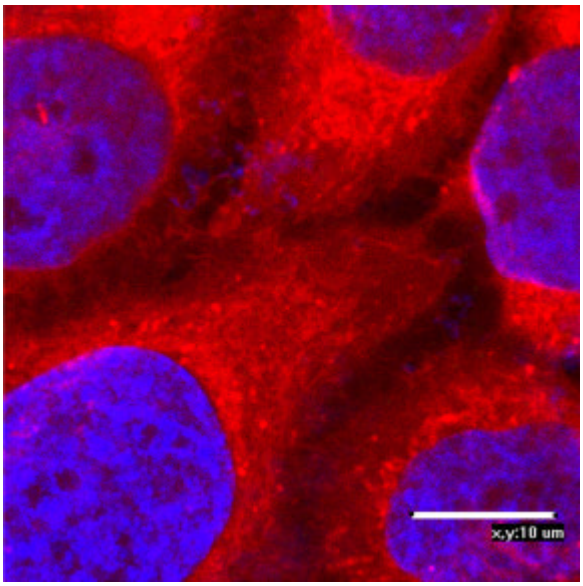


Fig. 1: Microphotograph of the HaCaT cells after staining by PFD813. HaCaT cells (image dimensions, 42 by 42 µm) after staining by PFD813 in concentration of 5 µg/ml in DMF solution (made after light-induced activation, red fluorescence) in comparison with the standard dye Hoechst 33342 (blue fluorescence)

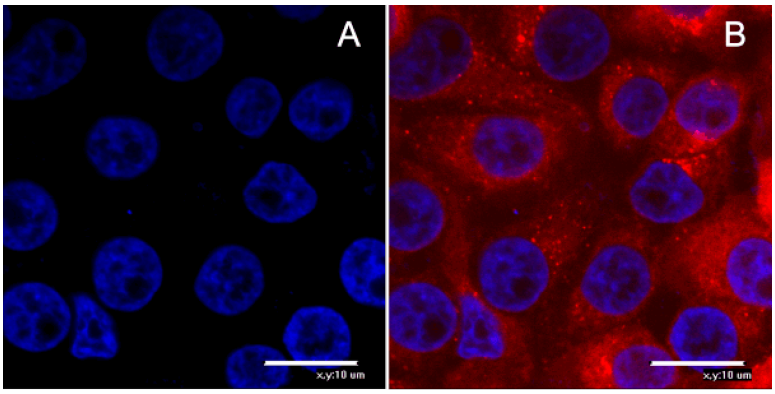


Fig. 2: Microphotographs of the HeLa cells. HeLa cells after staining by PFD814 (red fluorescence) at a concentration of 5 µg/ml in DMSO (image dimensions, 42 by 42 µm) in comparison with the standard dye Hoechst 33342 for staining of nuclei (blue fluorescence); left image: before light-induced activation (non fluorescent PFD814), right image: after light-induced activation (fluorescent Rho814)

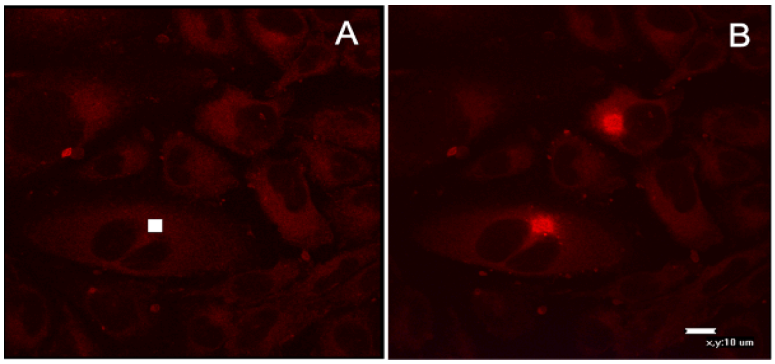


Fig. 3: Microphotographs of CHO cells. CHO cells after staining by PFD813 and GM140 at a concentration of 5 µg/ml and 3 µg/ml, respectively (image dimensions, 127 by 127 µm); a pre-selected photo-activation region (A) in the CHO cell and two bright fluorescent regions after two local photo-activations (B)

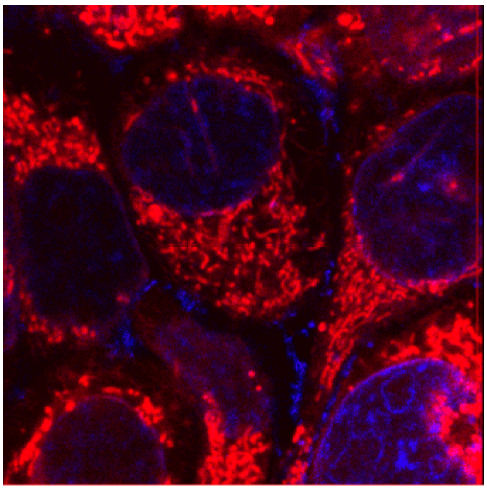

Fig. 4: Microphotograph of the A431 cells. “Living” À431 cells (image dimensions, 36 by 36 μm) stained by PFD813 after photo-activation

with area 3.2 μm$^2$) by laser light of wavelength $\lambda$ = 405 nm, the bright fluorescence of the dye Rho813 is seen in the micrograph in these two selected areas (Fig. 3b, right). Irradiation of the selected area was carried out for 1 second and this procedure can be repeated for other cells of monolayer. A series of 27 micrographs (in steps of vertical distance of 0.5 μm) was obtained, using a special program (EZ-C1 3.90 Free Viewer) in order to made 3D-reconstruction of cell monolayer with thickness of 13 μm. Such “volumetric layer” can be rotated using this program in order to observe intracellular staining at different angles.

Visualization of the intracellular content of so-called “living” cells is very important and interesting for biological applications. One of the possibilities to visualize and track the pathways of macromolecules or organelles in a “living” cell is to monitor them after staining with a PFD during the real time measurements. A bright fluorescent signal from the photo-activated dye in the small cell organelle can be monitored during its movement into the cell dark region (where the dye was not activated and does not fluoresce). High-speed video micro-photography makes it possible to calculate the diffusion velocity of the molecules. These methods allow one to track the movement of small organelles and biopolymers (protein and nucleic acid complexes) in cell cultures of different tissues. The best micrographs were obtained after the total and local photo-activation (Fig. 4) of “living” cells single layers stained with PFD813. This may be explained by the fact that in the “living” cells after photo-activation the positively charged dye Rho813 can accumulates into intermembrane space and held by the negatively charged membrane. The membrane potential disappears after fixation and the dye freely distributed in the cytoplasm. The advantages of such methodology (making preparations of living cells) are the following: the absence of the fixing agent (formaldehyde solution); replacement of the washed buffer by fresh culture medium; and the exclusion of a polymerizable material (e.g. “Mowiol”). The only disadvantage of such methodology is connected with the necessity of fast investigation of such samples because of the rapid evaporation of the culture medium between the glass plates.

As can be concluded from the obtained micrographs, PFD813 is concentrated mainly in the intracellular organelles in the “living” cells (Fig. 4) as compared to the fixed cells (Fig. 1). In our opinion, the main subcellular structures stained by PFD813 may be mitochondria and lysosomes.

## CONCLUSIONS

Properties of the novel PFDs before and after photo-activation to fluorescent rhodamine derivatives have been characterized and testing procedures have been developed. Novel dye PFD813 demonstrated better cell staining properties as compared to PFD814. Their potential for nanoscale optical resolution renders them particularly suited for special tasks in biology and bio-nanotechnology, human and animal biomedical applications.

## ACKNOWLEDGMENTS

Some parts of this work were supported by grants from Russian Foundation for Basic Research (13-04-97099), Russian Ministry of Education and Sciences (agreement 8461). AAR was supported in part by Russian Federation President Grant for Young Scientists M)-433.2013.4. The authors are thankful to Dr. Svirshchevskaya E.V. and Ph.D.-student Generalov A. A. for cell cultivation and suggestions on cell staining experiments; Dr. Belov V. N. for preparation of the precursors of fluorescent dyes.

## REFERENCES

1. Politz, J.C., 1999. Use of Caged Fluorochromes to Track Macromolecular Movement in Living Cells. Cell Biology, 9: 284-287.

2. Banala, S., D. Maurel, S. Manley and K. Johnsson, 2012. A Caged, Localizable Rhodamin Derivative for Superresolution Microscopy. ACS Chem. Biol., 7: 289-293.
3. Wysocki, L.M., J.B. Grimm, A.N. Tkachuk, T.A. Brown and E. Betzig, 2011. Facile and General Synthesis of photoactivable Xanthene Dyes, Angew. Chem. Int. Ed., 50: 11206-11209.
4. Wei, Y., Z. Aydin, Z. Liu and M. Guo, 2012. A Turn-On Fluorescent Sensor for Imaging Labile $Fe3^{+}$ in Live Neuronal Cells at Subcellular Resolution. Chem. Bio. Chem., 13: 1569-1573.
5. Hell, S.W., 2007. Far-field Optical Nanoscopy. Science, 316: 1153-1158.
6. Foelling, J., V. Belov, R. Kunetsky, R. Medda, A. Schoenle, A. Egner, C. Eggeling, M. Bossi and S.W. Hell, 2007. Photochromic Rhodamines Provide Nanoscopy with Optical Sectioning. Angew. Chem. Int. Ed., 46: 6266-6270.
7. Boyarskiy, V.P., V. Belov, R. Medda, B. Hein, M. Bossi and S.W. Hell, 2008. Photostable, Amino Reactive and Water-Soluble Fluorescent Labels Based on Sulfonated Rhodamine with a Rigidized Xanthene Fragment. Chem. Eur. J., 14: 1784-1792.
8. Belov, V., C.A. Wurm, V.P. Boyarskiy, S. Jakobs and S.W. Hell, 2010. Rhodamines NN: A Novel Class of Caged Fluorescent Dyes. Angew. Chem. Int. Ed., 49: 3520-3523.
9. Zaitsev, S.Y., 2010. Supramolecular Nanodimensional Systems at the Interfaces: Concepts and Perspectives for Bionanotechnology. Moscow, LENAND (in Russian).
10. Belov, V.N. and S.Y. Zaitsev, 2010. Photoactivated Fluorescent Dyes and the Prospects for their Use in Biomedical Research. Veterinary Medicine, 1: 10-13 (in Russian).
11. Shaposhnikov, M.N., S.Y. Zaitsev, D.B. Chudakov and A.A. Generalov, 2013. Determination of the Localization of a New Photoactivatable Fluorescent Dye in the A431 Cells Culture Using Selective Fluorescent Probe. Scientific Proceedings of the Bauman Kazan State Academy of Veterinary Medicine, 214: 483-488 (in Russian).
12. Shaposhnikov, M.N, M.S. Bartov and S.Y. Zaitsev, 2010. Innovative Method of Staining the Cells by New Photoactivated Fluorescent Dyes. Scientific Proceedings of the Bauman Kazan State Academy of Veterinary Medicine, 201: 360-365 (in Russian).
13. Zaitsev, S.Y., M.N. Shaposhnikov and E.V. Svirshchevskaya, 2010. Staining of Cells by New Photoactivated Fluorescent Dyes. Veterinary Medicine, No 3-4: 32-34 (in Russian).
14. Shaposhnikov, M.N., D.B. Chudakov, A.A. Generalov and S.Y. Zaitsev, 2012. Preparation of Chitosan Conjugate with Photoactivatable Fluorescent Dye and its Application in Cell Microscopy. Veterinary Medicine, 3-4: 32-35 (in Russian).
15. Shaposhnikov, M.N., D.B. Chudakov, A.A. Generalov, A.A. Savina and S.Y. Zaitsev, 2012. The Fluorescence Dependence of a New Photoactivatable Dye on the Environment Parameters. Fundamental Research, 9(2): 322-327. (in Russian).
16. Zaitsev, S.Y., V. Belov, G.Y. Mitronova and D. Möbius, 2010. Mixed Monolayers of a Novel Rhodamine Derivative. Mendeleev Commun., 20: 203-204 (in Russian).
17. Bartov, M.S., S.Y. Zaitsev and P.E. Volyn, 2010. Molecular Modeling of the Binding of Photoactivatable Fluorescent Dyes with the Lipid Bilayer of Lecithin Analog. Veterinary Medicine, No 3-4: 26-28 (in Russian).
18. Zaitsev, S.Y., M.N. Shaposhnikov, D.O. Solovyeva, I.S. Zaitsev and D. Mobius, 2013. Novel Precursors of Fluorescent Dyes. Interaction of the Dyes with Model Phospholipid in Monolayers. Cell Biochemistry and Biophysics, 66: 1-7.
19. Zaitsev, S.Yu., V.P. Vereschetin, S.P. Gromov, O.A. Fedorova, M.V. Alfimov, H. Huesmann and D. Mobius, 1997. Photosensitive Supramolecular Systems Based on Amphipilic Crown Ethers. Supramolecular Science, 4 (3-4): 519-524.
20. Zaitsev, S.Yu., V.P. Vereschetin, E.A. Baryshnikova, S.P. Gromov, O.A. Fedorova, M.V. Alfimov, H. Huesmann and D. Mobius, 1998. Photosensitive and Ionoselective Properties of the Amphiphilic Crown Ethers Dye in Monolayers. Thin Solid Films, 327-329: 821-823.
21. Zaitsev, S.Y., E.A. Baryshnikova, T.I. Sergeeva, S.P. Gromov, O.A. Fedorova, O.V. Yescheulova, M.V. Alfimov, S. Hacke, W. Zeiss and D. Möbius, 2000. Langmuir Films of the Novel Anion-Capped Amphiphilic Benzodithia-15-Crown-5 Dye. Thin Solid Films, 372(1): 230-236.

22. Zaitsev, S.Yu., T.A. Sergeeva, E.A. Baryshnikova, W. Zeiss, D. Möbius, O.V. Yescheulova, S.P. Gromov, O.A. Fedorova and M.V. Alfimov, 1999. Synthesis and Monolayer Study of a New Amphipilic Photochromic Crown-Ether. Materials Science and Engineering, 8-9: 469-473.
23. Zaitsev, S.Yu., E.A. Baryshnikova, T.A. Sergeeva, S.P. Gromov, O.A. Fedorova, O.V. Yescheulova, M.V. Alfimov, S. Hacke, W. Zeiss and D. Möbius, 2000. Monolayers of the Photosensitive Benzodithia-15-Crown-5 Derivative. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 171(1-3): 283-290.
24. Zaitsev, S.Yu., T.A. Sergeeva, E.A. Baryshnikova, S.P. Gromov, O.A. Fedorova, M.V. Alfimov, S. Hacke and D. Möbius, 2002. Anion-Capped Benzodithia-18-Crown-6 Styryl Dye Monolayers. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 198-200: 473-482.
25. Sergeeva, T.I., S.Yu. Zaitsev, M.S. Tsarkova, S.P. Gromov, A.I. Vedernikov, M.S. Kapichnikova, M.V. Alfimov, T.S. Druzhinina and D. Möbius, 2003. Monolayers of a Novel Ionselective Butadienyl Dye. Journal of Colloid and Interface Sci., 265(1): 77-82.
26. Zaitsev, S.Yu., T.I. Sergeeva, D. Möbius, A.I. Vedernikov, M.S. Kapichnikova, S.P. Gromov and M.V. Alfimov, 2004. Synthesis and Ion-Selective Properties of an Amphiphilic Butadienyl Dye. Mendeleev Communications, 14(5): 199-200.
27. Sergeeva, T.I., S.Yu. Zaitsev, S.P. Gromov, A.I. Vedernikov, M.S. Kapichnikova, M.V. Alfimov and D. Möbius, 2005. Organisation in Monolayers at the Air/Water Interface of Butadienyl Dyes Containing Benzodithiacrown-Ether or Dimetthoxybezene. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 264(1-3): 207-214.
28. Sergeeva, T.I., S.Yu. Zaitsev, S.P. Gromov, A.I. Vedernikov, M.S. Kapichnikova, M.V. Alfimov, V.T. Lieu, D. Möbius, M.S.Tsarkova, 2005. Influence of the Counter Anion on the Interaction of Cations with the Benzodithia-18-Crown-6 Butadienyl Dye in Monolayers. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 255(1-3): 201-209.
29. Turshatov, A.A., M.L. Bossi, D. Möbius, S.W. Hell, A.I. Vedernikov, S.P. Gromov, N.A. Lobova, M.V. Alfimov and S.Y. Zaitsev, 2005. Spectroscopic Properties of an Amphiphilic Styryl Pyridinium Dye in Langmuir-Blogett Films. Thin Solid Films, 476(2): 336-339.
30. Turshatov, A.A., D. Möbius, M.L. Bossi, S.W. Hell, A.I. Vedernikov, N.A. Lobova, S.P. Gromov, M.V. Alfimov and S.Y. Zaitsev, 2006. Molecular Organization of an Amphiphilic Styryl-Pyridinium Dye in Monolayers at the Air/Water Interface in the Presence of Various Anions. Langmuir, 22: 1571-1579.
31. Sergeeva, T.I., S.Yu. Zaitsev, S.P. Gromov, A.I. Vedernikov, M.S. Kapichnikova, M.V. Alfimov, D. Möbius. 2005. Mixed Langmuir Monolayers of an Amphiphilic Chromo-Ionophore and the Phospholipid Dmpc. Applied Surface Science, 246(4): 377-380.
32. Turshatov, A.A., D. Möbius, M.L. Bossi, S.W. Hell, A.I. Vedernikov, N.A. Lobova, S.P. Gromov, M.V. Alfimov and S.Y. Zaitsev, 2006. Molecular Organization of an Amphiphilic Styryl-Pyridinium Dye in Monolayers at the Air/Water Interface in the Presence of Various Anions. Langmuir, 22: 1571-1579.
33. Bossi, M.L., A.A. Turshatov, S.Y. Zaitsev, M.V. Alfimov, D. Moebius and S.W. Hell, 2007. Influence of Monolayer State on Spectroscopy and Photoizomerization of an Amphiphilic Styryl-Pirdinium Dye on a Solid Substrate. Langmuir, 23: 3699-3705.
34. Turshatov, A.A., D. Möbius, S.Yu. Zaitsev, S.K. Sazonov, A.I. Vedernikov, S.P. Gromov and M.V. Alfimov, 2008. Anion Effects on Monolayers of a New Amphiphilic Styryl-Pyridinium Dye at the Air-Water Interface Colloids and Surfaces A: Physicochemical and Engineering Aspects., 329(1-2): 18-23.
35. Zaitsev, S.Yu., E.N. Zarudnaya, V.V. Bondarenko, V.I. Maksimov, I.S. Zaitsev, D. Möbius, E.N. Ushakov, N.A. Lobova, A.I. Vedernikov, S.P. Gromov and M.V. Alfimov, 2008. Ultrathin Chemosensoring Films with a Photosensitive Bis (Crown Ether) Derivative. Mendeleev Communications, 18(5): 270-272.
36. Zaitsev, S.Y., A.A. Turshatov, D. Möbius, S.P. Gromov and M.V. Alfimov, 2010. Organization and Properties of a Novel Amphiphilic Crown-Ether Dye in Monolayers at the Air/Water Interface, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 354: 51-55.

37. Zaitsev, S.Yu., E.N. Zarudnaya, I.S. Zaitsev, M.S. Tsarkova, N.A. Lobova, A.I. Vedernikov and S.P. Gromov, 2011. Thin Films with immobilized bis-crown-ether dye. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 383: 120-124.
38. Zaitsev, S.Y., 2009. Membrane Nanostructureson the Basis of Biologically Acitive Compounds for Bionanotechnological Purposes. Nanotechnologies in Russia, 4: 379-396.
39. Blatt, N.L., R.N. Mingaleeva, S.F. Khaiboullina, V.C. Lombardi and A.A Rizvanov, 2013. Application of Cell and Tissue Culture Systems for Anticancer Drug Screening. World Applied Sciences Journal, 23(3): 315-325.