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Original Article

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Fluorescent dyes for the study of siliceous sponges

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ABSTRACT. New fluorescent dyes containing coumarine and rhodamine groups were applied as vital dyes capable of staining growing siliceous spicules of Baikal sponge *Lubomirskia baicalensis* (Pallas, 1773). Cultivation of the sponge primmorphs in the presence of fluorescent dyes allows the use of confocal microscopy to study the morphology of the spicules and, in the case of two-color staining, to draw some conclusions about the growth rate of the spicules. 5-6.5 months were estimated to be the time required for the full formation of the spicules.

Keywords: siliceous sponge, fluorescent dye, vital staining, confocal microscopy, spicules

1. Introduction

Sponges are the oldest invertebrates, and they play an important role in aquatic ecosystems. Sponge skeleton consists of composite needle-like spicules based on silica or calcium carbonate. Spicules are linked by organic material (Fig. 1), forming three-dimensional structures filled with sponge cells and numerous symbionts: zoochlorellas, bacteria, viruses, dinoflagellates, diatoms, and fungi (Taylor et al., 2007). These symbionts are an inexhaustible source of biologically active compounds (Blunt et al., 2018; Carroll et al., 2019; El-Demerdash et al., 2019; Khalifa et al., 2019). Spicules of siliceous sponges are considered as prospective biogenic constructions for optic applications and a source of ideas of bioinspired materials (Zhang et al., 2016; Mcheik et al., 2018). Sponges are motionless organisms that filter out a huge amount of water through their bodies and are thus highly susceptible to the influence of harmful environmental factors. A disease of Baikal sponges (Denikina et al., 2016; Khanaev et al., 2018; Zvereva et al., 2019) is the striking manifestation of crisis phenomena in the Baikal coastal zone.

Experiments with sponges or with sponge cultures (primmorphs, (Wilson, 1907; Custodio et al., 1998)) are important for environmental and biotechnological investigations. The proper formation of the sponge spicules is important evidence of the organism's viability. Counting the new spicules during an experiment is a difficult and unobvious task, as it is impossible to distinguish between old and new spicules. Another intriguing question in the sponge physiology is what the growth rate of the spicules is. The spicule growth continues for days, and there are no harmless methods for observing individual spicule. Recently, we proposed to apply vital fluorescent dye NBD-N2 (green fluorescence, Fig. 1) for staining the growing siliceous spicules (Annenkov et al., 2014). This dye is accumulated in bio-silica such as sponge spicules and frustules of diatom algae which are obtained from cultivation in the presence of the dye.

This work is aimed at the application of other vital dyes (Fig. 2): Q-N2 (blue fluorescence) and Rhod-N3 (red fluorescence) for staining siliceous spicules of Baikal endemic sponge *Lubomirskia baicalensis* (Pallas, 1773). Q-N2 was previously studied in staining diatom frustules, and we found a change of blue fluorescence in solution to cyan emission in siliceous materials. The availability of several fluorescent dyes allowed us to interchange dyes during sponge cultivation and estimate the growth rate of the spicules.

2. Materials and methods

2.1. Chemical reagents

Bottled Baikal water was used for primmorph cultivation. Fluorescent dyes NBD-N2 and Q-N2 were obtained according to Annenkov et al., 2010; 2019. Rhod-N3H (Annenkov et al., 2016a) was synthesized (Fig. 3) following the approach reported in (Kwon et al., 2005). Briefly, rhodamine B was converted to the chloroanhydride and further reacted with N,N-bis[3-(methyamino)propyl]methylaamine (N3) to give the target Rhod-N3. The product was purified on a flash chromatography column packed with C18-reversed phase silica gel ($H_2O/CH_3CN/HCOOH = 63.2/36.7/0$, yield 18%). Other chemicals were purchased from Merck, Fisher or Acros Chemicals and used without

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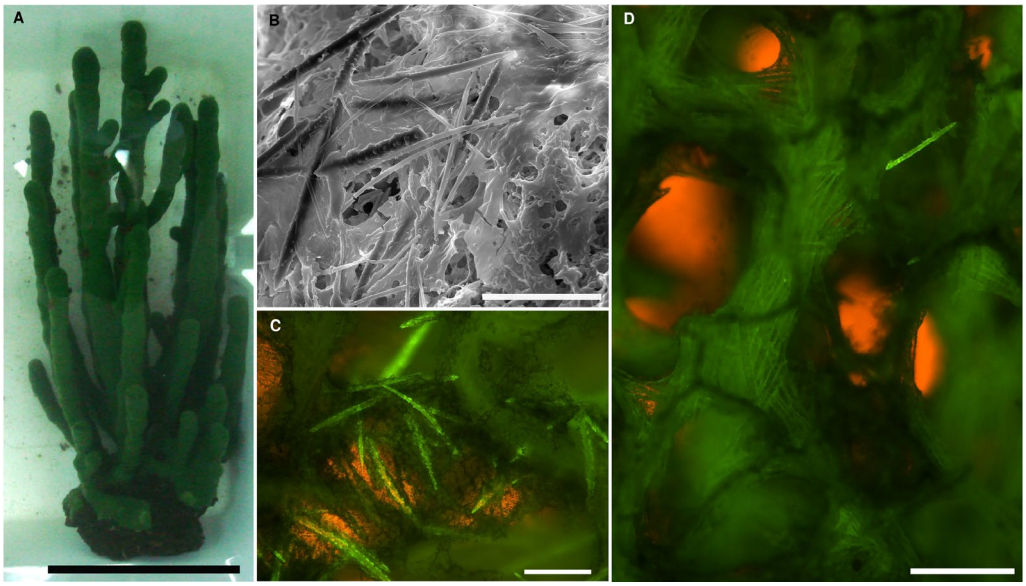

Fig. 1. Baikal sponge *L. baicalensis* collected for primmorph preparation (A); scanning electron microscopy (B) and combined light and fluorescent microscopy (C and D) images of the sponge spicules associated with organic material. Bright green spicules (C and D) are stained with NBD-N2 by the sponge cultivation in the presence of the dye during one month. Excitation was at 470 nm, and emission was observed above 515 nm. Scale bar represents 5 cm (A), 100 μm (B and C) and 200 μm (D).

further treatment.

2.2. Sponge samples and cultivation of primmorphs

Experiments with sponge samples and primmorphs were performed according to Annenkov et al., 2014; Annenkov and Danilovtseva, 2016b. Samples of *L. baicalensis* were collected near the village Bolshie Koty, in the southwestern part of Lake Baikal, from 10 m depth. Primmorphs were obtained similar to Custodio et al., 1998. Briefly, sponge samples were cut under Baikal water (3 °C) into ≈1-2 mm particles. The particles and water were transferred into 50 mL conical plastic tubes (sponge to water ratio ≈ 1: 20) and gently shaken for 15 min with a rotatory shaker. Then, the suspension was filtered through a 100 μm nylon net. The cells obtained were harvested by sedimentation (1 h, 3 °C) and washed again with Baikal water. The cell suspension was placed into 400 ml plastic containers with 200 ml of Baikal water containing 0.002 % of ampicillin. The containers were kept in the same conditions as sponge samples under cultivation. Every day during two weeks, 75% of the water was replaced by fresh water containing the antibiotic. After two weeks, the primmorphs obtained (1 mm and more in diameter) were moved to the new containers with water and antibiotic and 75% change of the water was performed weekly. At the same time, fluorescent dyes were added to the cultivation medium in 0.5 μM concentration. Experiments on the weekly change of the dyes were performed with triple washing of the primmorphs with the culture medium.

2.3. Study of Rhod-N3 toxicity

Experiments aimed at determination of the toxicity of Rhod-N3 towards diatoms were performed similar to (Annenkov et al., 2010). The synchronized culture of *Ulnaria ferefusiformis* (M.Kulikovskiy & H.Lange-Bertalot, 2016) was diluted with DM medium to a final concentration of 1000-3000 cell/mL and 90 μL of the suspension was added into every well of 96-well microcultivation flat bottom plate. Then, 10 μL of an appropriate solution of Rhod-N3 in the DM medium was added to every well. Experiments with each concentration of Rhod-N3 were repeated 5-6 times. The cultivation was performed at 18°C. A luminescent lamp was switched on and off at 12 h intervals and the light intensity at "daytime" was 16 μmoles/m^{-2}·sec^{-1}. The growth of the cells was observed during 7 days.

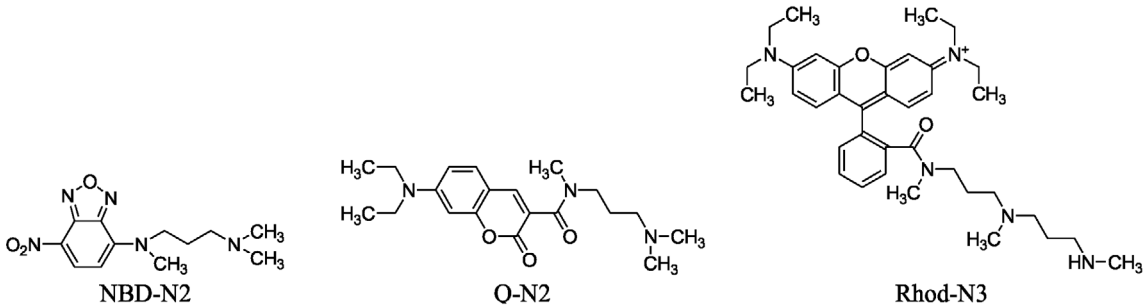

Fig. 2. Structures of the fluorescent dyes.

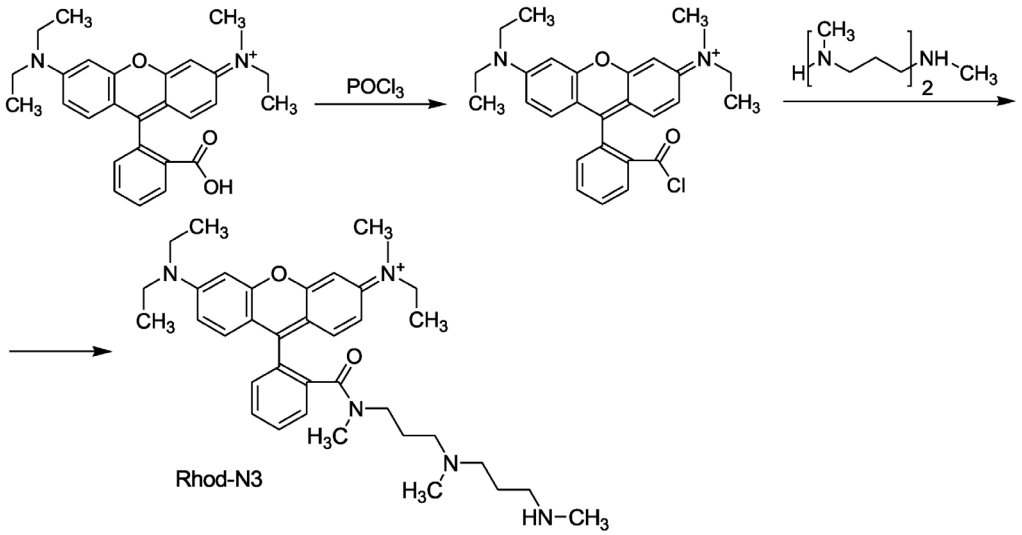

Fig. 3. Scheme of Rhod-N3 synthesis.

2.4. Instrumentation

UV–Vis absorption measurements were carried out using a Perkin Elmer Lambda 950 spectrophotometer. Photoluminescence spectra were recorded at 25 °C using a Perkin-Elmer LS-55 instrument. For absorption and fluorescence measurements the path length of the quartz cuvette was 1 cm.

Fluorescent microscopy was performed using an inverted microscope Motic AE-31T with an HBO 103 W/2 OSRAM mercury lamp. Excitation was at 470 nm, and emission above 515 nm was observed. Confocal images were obtained using a Zeiss LSM710 microscope with the following parameters: excitation 405, 488 or 561 nm; detector slit 410-585, 501-572 or 566-620 nm for Q-N2, NBD-N2 and Rhod-N3 dyes respectively with a ×63 oil immersion objective. For double color Q-N2 – NBD-N2 images: excitation 405 and 488 nm, detector slit 409-491 and 519-620 nm, respectively. For double color QN_2 – Rhod-N3 images: excitation 405 and 561 nm, detector slit 410-523 and 566-620 nm respectively.

3. Results and discussion

Absorption and fluorescence of new dye Rhod-N3 do not depend on pH in the 5.5-9.18 interval (Fig. 4). Fig. 5. Summarizes the fluorescent properties of NBD-N2, Q-N2, and Rhod-N3 dyes are summarized. NBD-N2 and Q-N2 do not have a significant effect on the growth of diatom algae in the 1 μm concentration (Annenkov et al., 2010; 2019), and 0.5 μm Rhod-N3 slightly depresses the diatom culture (Fig. 6).

Primmorphs of the Baikal sponge *Lubomirskia baicalensis* were cultivated in the presence of 0.5 μM Q-N2 and Rhod-N3 giving rise to fluorescent tagged spicules (Fig. 7). The formation of fluorescent vesicles in silicifying sponge cells (sclerocytes) was observed on the first day after adding the dye to the culture medium (Fig. 8). 350-450 nm stained layer was found in spicules after three days of cultivation, and a week growth in the presence of fluorescent dye resulted in a 550-650 nm stained layer.

Cultivation of the primmorphs with the addition of two dyes that were changed every week enabled to obtain layer-by-layer stained spicules which were studied with confocal microscopy. The parameters for confocal microscopy of the bicolored spicules were chosen from the fluorescent spectra of siliceous diatom frustules stained with the corresponding dyes (Fig. 9, Fig. 10). The confocal images were recorded using alternately two excitation lasers and two detector slits which corresponded to almost separate emission from

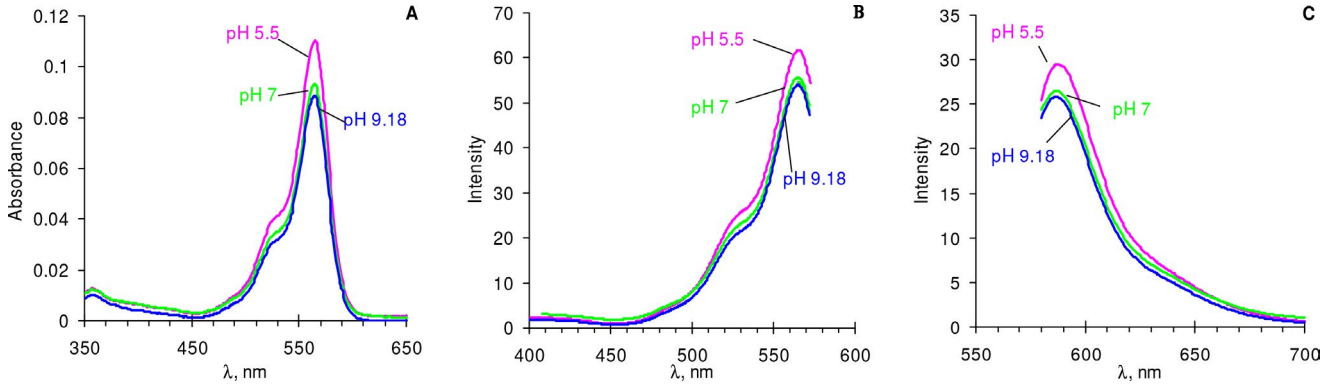

Fig. 4. The absorption (A), excitation (B) and emission (C) spectra of Rhod-N3 dye in aqueous solutions at pH 5.5, 7 and 9.18. The concentration of Rhod-N3 is 2.08 μM. Excitation spectra were recorded for emission at 587 nm, and 565 nm excitation was applied for emission spectra.

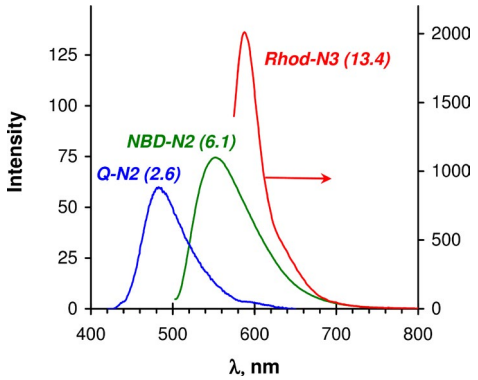

Fig. 5. Emission spectra and quantum yields (Q, %, in brackets) of NBD-N2, Q-N2 and Rhod-N3 dyes at pH 7. Solution concentration and excitation wavelength are 1, 1.5 and 1 μM, 490, 411 and 565 nm, respectively.

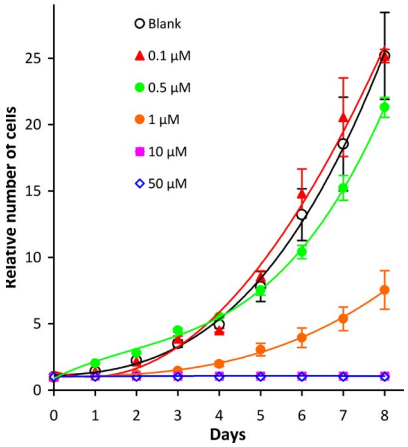

Fig. 6. Growth curves of *U. ferefusiformis* in the presence of Rhod-N3 dye. The concentration of the dye is presented on the chart.

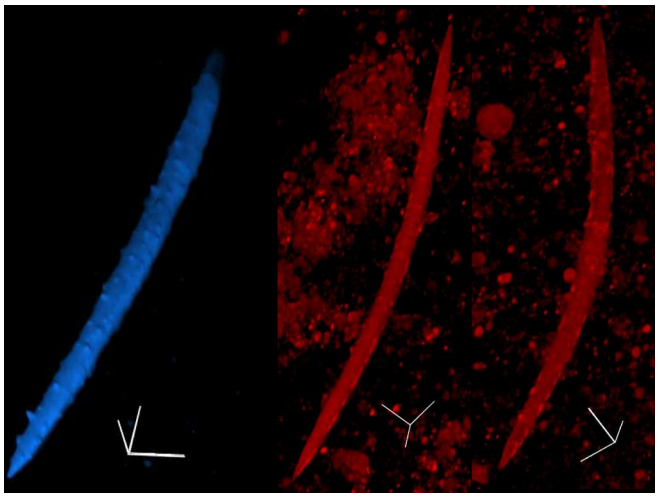

Fig. 7. 3D confocal images of *L. baicalensis* spicules obtained from primmorphs cultivated in the presence of Q-N2 (blue) and Rhod-N3 (red) dyes. Scale bar represents 10 μm.

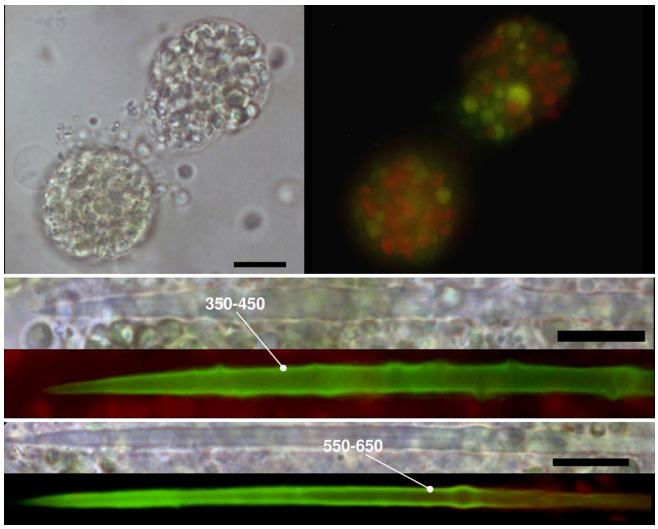

Fig. 8. Fluorescent images of cells in *L. baicalensis* primmorphs after one-day growth in the presence of 0.5 μM NBD-N2 (top) and spicules obtained on third (middle) and seventh (bottom) days after adding the dye to culture medium. Excitation was at 470 nm, and emission was observed above 515 nm. Scale bar corresponds to 10 μm.

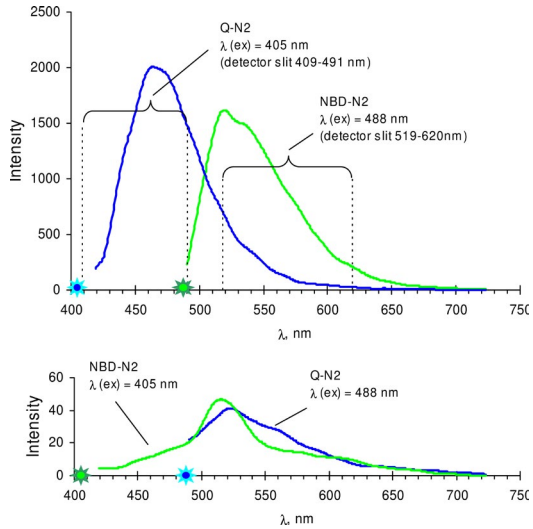

Fig. 9. Determination of excitation wavelength and detector slit for simultaneous observation of objects stained with Q-N2 and NBD-N2 dyes. *U. ferefusiformis* frustules stained by cultivation in the presence of these dyes were applied.

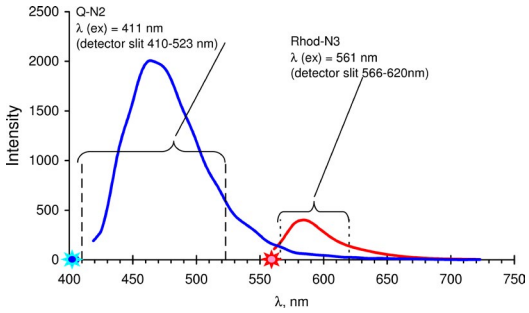

Fig. 10. Determination of excitation wavelength and detector slit for simultaneous observation of objects stained with Q-N2 and Rhod-N3 dyes. *U. ferefusiformis* frustules stained by cultivation in the presence of these dyes were applied. Q-N2 and Rhod-N3 do not show noticeable fluorescence at 561 and 411 nm excitation, respectively.

the dyes.

Images from the confocal microscopy (Fig. 11) allow estimating the thickness of the alternate silica layers that formed during one week in the presence of the corresponding dye. We conclude that inner weekly layers are 450-800 nm, and, taking into account 13 μm thickness of mature spicules (Veynberg, 2009), this corresponds to 2-3.5 months needed for complete maturation of the *L. baicalensis* spicules. This is a very rough estimation for several reasons: the growth rate possibly depends on the spicule age; fluorescent dye is possibly accumulated in the primmorph bodies and in sclerocytes, which lengthens the weekly interval of the dye change; the rate of spicule growth in natural sponges may differ from that in primmorphs. At the same time, long-time experiments with living *L. baicalensis* are impossible due to poor viability of the sponges in aquariums, and it is difficult to distinguish between such stages as a healthy sponge, an oppressed sponge or the onset of sponge cell atrophy. 3.5-6.5 monthly estimates of the growth of the spicules in primmorphs seem to be realistic since in experiments with different dyes we observed only a few 8-10 μm stained spicules after four months of primmorph cultivation.

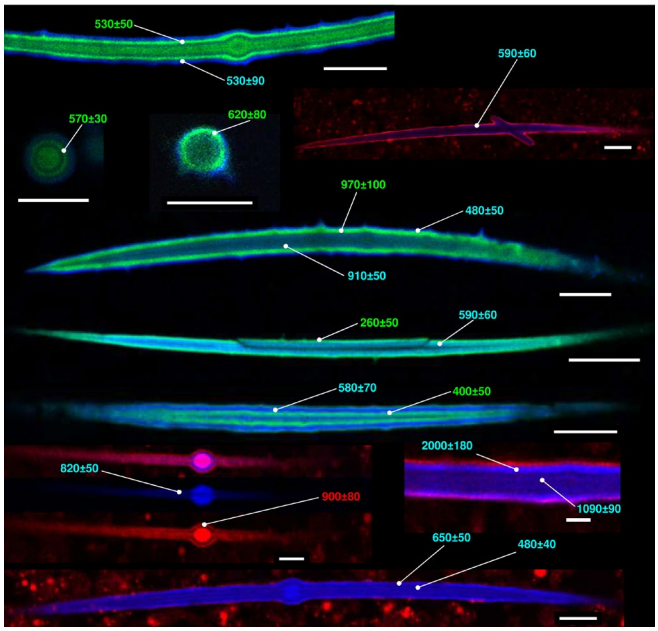

Fig. 11. Representative confocal microscopy images of *L. baicalensis* spicules stained with two dyes that were changed weekly during 4 months of primmorph cultivation. Green fluorescence - NBD-N2, blue - Q-N2 and red - Rhod-N3. Numbers correspond to the thickness of the stained layer, nm. Scale bars correspond to 10 μm.

4. Conclusions

Thus, new fluorescent dyes Q-N2 and Rhod-N3 are effective in vital staining of the growing siliceous spicules in sponge primmorphs. Cultivation of the sponge in the presence of these dyes allows for the use of confocal microscopy to study the morphology of the spicules and, in the case of two-color staining, to draw some conclusions about the growth rate of the spicules.

Acknowledgments

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