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ORIGINAL PAPER

# Fluorescent Amines as a New Tool for Study of Siliceous Sponges

**Vadim V. Annenkov · Ol'ga Yu. Glyzina · Ol'ga N. Verkhozina · Elena N. Danilovtseva**

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**Abstract** Siliceous sponges (Hexactinellida and Demospongiae classes) are aquatic invertebrates which are important both for marine and freshwater ecology and also as the source of biologically active compounds. The sponge skeleton consists of spicules - needle-like or branched composite structures based on silicon dioxide. Mechanisms of silicon assimilation and synthesis of high-ordered glass-like structures at ambient temperatures by sponges are intriguing for biologists, chemists and nanotechnologists. Fluorescent amines are *in-vivo* dyes that stain growing siliceous frustules of diatom algae so the use of these agents for the sponge study was attempted. We found that cultivation of the *Lubomirskia baicalensis* (Pallas, 1773) sponge in the presence of fluorescent tracers of biosilica - $N^1$,$N^3$,$N^3$-trimethyl-$N^1$-(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine and $N^1$,$N^3$-dimethyl-$N^1$-[3-(dimethylamino)propyl]-$N^3$-(7-nitro-2,1,3-benzoxadiazo-4yl)propane-1,3-diamine results in the staining of growing siliceous spicules. This finding shows that amine dyes accompany silicon from the environment to sponges spicules which opens a new way to study of silicon assimilation by sponges. Fluorescent staining of the growing spicules following with the confocal microscopy can be a powerful tool for morphological studies, revealing information about the dynamics of spiculogenesis and for bio-fabrication of new fluorescent materials.

V. V. Annenkov (✉) · O. Yu. Glyzina · O. N. Verkhozina · E. N. Danilovtseva
Limnological Institute of the Siberian Branch of the Russian Academy of Sciences, 3, Ulan-Batorskaya St., P.O. Box 278, Irkutsk, 664033, Russia
e-mail: annenkov@lin.irk.ru; annenkov@yahoo.com

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## 1 Introduction

Sponges (Spongia) are aquatic invertebrates of the phylum Porifera which forms a blind branch near the bottom of phylogenetic tree of multicellular organisms. They are the oldest multicellular animals known from the Precambrian era and their active development was observed in the Mesozoic era [[1, 2] and refs. cited in these articles]. Sponges are harvested from water reservoirs and they are produced in aquaculture not only as bath sponges but also to extract numerous biologically active compounds [3]. The sponge skeleton consists of spicules - needle-like or branched composite structures based on silica (Hexactinellida and Demospongiae classes) or calcium carbonate (Calcarea). Spicule size varies from micrometers to centimeters [4, 5]. Demospongiae spicules contain an axial protein filament surrounded with silicon dioxide. Individual spicules can fuse or interlock with each other giving rise to more complex constructions. Spicules are formed with the aid of special cells – sclerocytes, and the secretion of spicules is intra- or extracellular process, depending on the spicule size and function (reviewed in [4]). When the mature spicule is formed, it lies in mesogloea where it adheres to other spicules, giving rise to the sponge skeleton. Such spicules are called megascleres as contrasted to microscleres, which do not play a visible role in skeleton organization [4, 5]. The formation of well-ordered siliceous spicules as well as diatom siliceous frustules is the object of keen interest as a route to new siliceous and glass materials obtained at moderate temperatures [6–13].

In spite of large efforts, the molecular mechanism of silicon assimilation by sponges is poorly understood. There is no comprehensive theory for the main stages of this process: primary capture of silicic acid from the environment, silicon storage and transport in the cell cytoplasm, and building of the spicules. Our understanding of silicification processes in sponges is hampered by difficulties in observation on siliceous structures in the living cultures. Silicon dioxide and less condensed oligosilicates are optically transparent substances and so hypotheses are often built on the basis of electron microscopy of dissected samples and indirect data from *in vitro* experiments.

Specific fluorescent dyes capable of *in vivo* staining are the powerful tools for study of the intracellular processes, especially in the case of silica structures which are colorless and transparent to visible light. Basic dyes starting from rhodamines are used for *in vivo* staining of diatom siliceous frustules [14–21]. These dyes stain acidic vesicles in eukaryotic cells and also SDVs – Silica Deposition Vesicles in which parts of new frustules are synthesized. Fluorescent staining of the growing siliceous structures was also described for Parmales, Silicoflagellates, Choanoflagellates and Radiolarians [22, 23]. *In vivo* staining of the diatom frustules is considered as a biotechnological approach to fluorescent nanomaterials [19]. Siliceous sponge spicules are considered as a material for design of optical elements [24, 25] and so biotechnological introduction of fluorescent dyes in to spicules is of practical interest. Recently we have obtained two new dyes based on 7-nitro-2,1,3-benzoxadiazol fluorescent fragment – $N^1$,$N^3$,$N^3$-trimethyl-$N^1$-(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine (NBD-N2) and $N^1$,$N^3$-dimethyl-$N^1$-[3-(dimethylamino)propyl]-$N^3$-(7-nitro-2,1,3-benzoxadiazo-4yl)propane-1,3-diamine (NBD-N3) [26]. NBD-N2 is able to stain silicon-containing particles only and it was used to monitor the initial stages of silicon assimilation by diatoms [27].

The use of fluorescent amines for staining of diatom biosilica is based on the discovery of long-chain polyamines [28] associated with siliceous frustules. Similar substances were found in the siliceous spicules [29]. There has been only one attempt to use a fluorescent dye in the study of silicon assimilation by demosponge cells [30]. Sponge primmorphs (aggregates of proliferating cells) were cultivated in the presence of PDMPO dye and fluorescent inclusions were found in the cytoplasm when an elevated silicon amount was applied. This experiment shows that non-specialized sponge cells can accumulate PDMPO dye but the important question remains unanswered: can the known silica tracers pass the whole silicon route in sponges, from the environment to spicules? A positive answer to this question would allow us to use the corresponding dyes for study of the molecular mechanisms of spicule formation.

The objective of this work is to study the ability of NBD-N2 and NBD-N3 dyes to stain siliceous spicules during cultivation of mature sponge. We used the Lake Baikal freshwater endemic sponge *Lubomirskia baicalensis* (Pallas, 1773), suborder Spongillina, order Haplosclerida. These sponges form long branched stem-like constructions (Fig. 1). The growth of *L. baicalensis* is relatively slow, 1-3 mm *per* year [31].

## 2 Experimental

*Sponge samples and cultivation:* Samples of *L. baicalensis* were collected near the village Bol'shie Koty, in the southwestern part of the Lake Baikal, at 10 m depth. Cultivation temperature was 10±1°C. Luminescent lamps (color temperature 6500 K) were used for illumination of the aquariums with the light intensity 200 μmol photons $m^{-2}$ $s^{-1}$ in 12h/12h light/dark cycle. Sponge specimens (4-5 cm length) were placed into an aquarium with flowing Baikalian water after 3 h after sampling and were kept there for 14 days in order to achieve acclimatization. After that the sponges were cultivated in a 6 L aquarium with daily water exchange during two weeks. The fluorescent dyes were added to the cultivation water to attain an 0.5 μM concentration. Other cultivation details are described in [32, 33].

*Chemical reagents:* Bottled Baikalian water was used for the sponge cultivation. The chemical composition of this water is described in [34]. NBD-N2 and NBD-N3 were obtained according to [26]. Household bleach (7 % sodium hypochlorite solution in water) was used. Other chemicals were purchased from Sigma-Aldrich, Fisher or Acros Chemicals and used without further treatment.

*Preparation and study of the siliceous spicules:* The sponge samples were air-dried and washed with organic solvents (2-propanol: dichloromethane = 1: 1) 3–5 times (until the

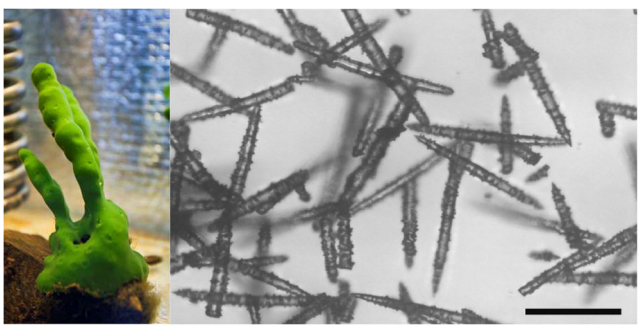

**Fig. 1** Baikalian endemic siliceous sponge *L. baicalensis* in the aquarium and the sponge spicules.The scale bar represents 100 $\mu$m

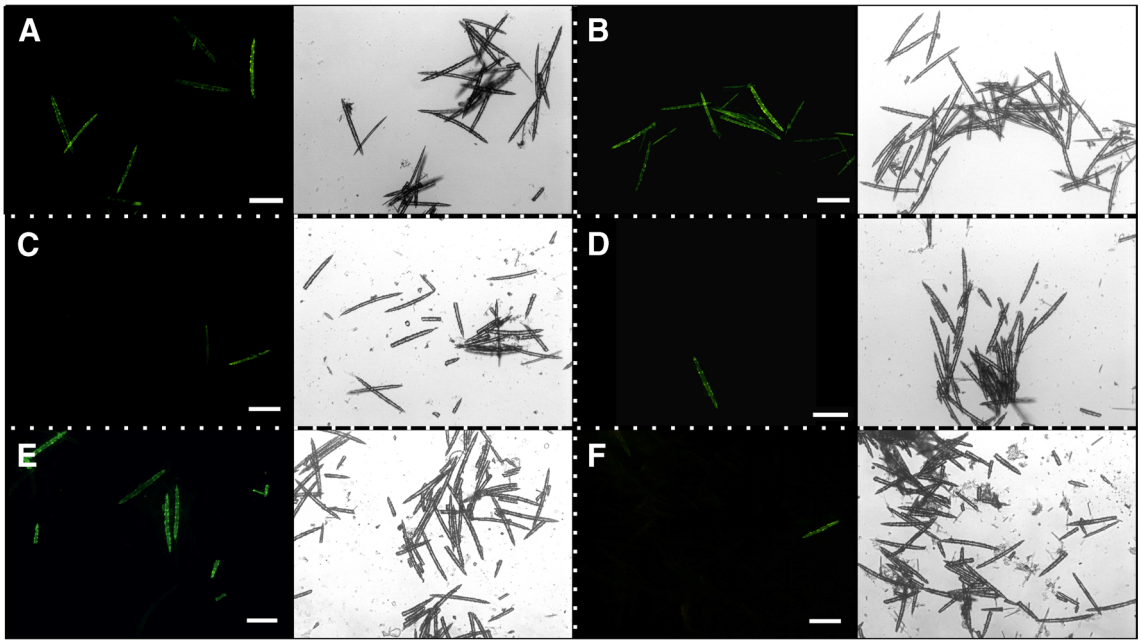


**Fig. 2** Fluorescent and optical microphotographs of *L. baicalensis* spicules after growth in the presence of fluorescent amines. Spicules were obtained from the apex (A, B, E) and the middle (C, D, F) of the sponge body. The sponges were cultivated in the presence of NBD-N2 (A-D) and NBD-N3 (E, F). The scale bar represents 50 $\mu$m

washes were colorless) by vortexing during 10 min and centrifugation (3000 g, 5 min). After that the samples were air-dried and oxidized with the bleach (sodium hypochlorite) during 2 h followed by water washing. This treatment allows the removal of all organic substances from the spicule surface.

We used an Axiovert 200 microscope (Zeiss) with the mercury lamp HBO 50W/AC ASRAM. Excitation was performed at 450–490 nm and emission was observed at 515 nm with beam splitting at 510 nm. A Zeiss LSM710 microscope was used to obtain confocal images with the following parameters: excitation 488 nm, detector slit – 501–572 nm.

## 3 Results and discussion

The sponge samples were cultivated for 2 weeks in the presence of two fluorescent dyes NBD-N2 and NBD-N3 [26]. 1 mm slices were cut after cultivation from the various parts of the sponge body: apex, middle, bottom and core. The obtained slices were treated with organic solvents and bleach with the aim to remove any organic substances and to obtain clean siliceous spicules according to the known procedures of the sponge spicules [35] and diatom frustules [36, 37] treatment.

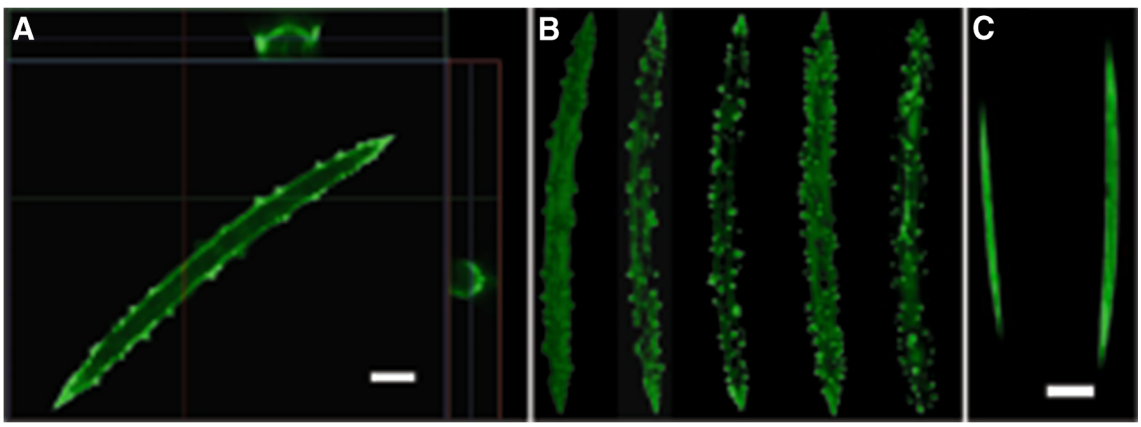


**Fig. 3** Confocal microscopy images of *L. baicalensis* spicules stained by cultivation in the presence of NBD-N2. A – a central cross-section with orthogonal projections; B – 3D reconstructed images of megascleres and C - cross-section images of or immature spicules. Scale bar represents 20 $\mu$m

We found that the sponge growth in the presence of fluorescent dyes results in formation of stained spicules (Fig. 2). The comparison of optical and fluorescent images shows that only a few spicules are stained. The fraction of stained spicules is maximal in the slices from the apex and no fluorescent objects were found in the bottom or core of the sponges. We did not see any differences between the staining action of NBD-N2 and NBD-N3. The prevalence of non- fluorescent spicules in the samples is the evidence of specific staining of the growing spicules because the sponge growth proceeds on the surface only, especially from the apex end.

Higher resolution images were obtained by confocal microscopy. This method allows us to obtain sharp images at high magnification from relatively large objects by acquisition of thin cross-section images which can be combined into 3D images. The confocal method is applicable to fluorescent samples only and before this work it had not been used for study of siliceous spicules. The cross-section images and orthogonal projections (Fig. 3) show staining of the surface layer of the spicules, 1-2 μm in depth. We also observed small spicules without spinules which possibly are immature (young) spicules because microscleres are not known for the Baikalian sponges [31, 38]. All of the small smooth spicules found were stained, which points to their formation during cultivation with the fluorescent dye and thus confirms their nature as immature spicules, not microscleres.

The ability of fluorescent amines NBD-N2 and NBD-N3 to stain growing siliceous spicules that we found allows us to apply these reagents for monitoring the silicification process in sponges. The simultaneous staining of incipient and almost mature spicules points to the similarity of these processes in some key stages. Recently, the use of fluorescent dyes resulted in finding submicrometer silicon-containing particles in the diatom cytoplasm at the initial stage of silicon assimilation [27]. Similar dyes were used for visualization of the growing silica structures (valves, girdle bands) in complex experiments including various specific staining agents [21]. We hope our data will stimulate corresponding investigations with siliceous sponges.

Another promising application of these fluorescent amines is in staining the sponge spicules for fluorescent microscopy, especially for confocal techniques. Spicules are usually used in sponge taxonomy because size, shape, and spicule arrangement are species-specific [4], but all these parameters are strongly dependent on the environmental conditions (silicic acid concentration, temperature, insolation, etc.) which can lead to wrong conclusions. Sponge cultivation in the presence of fluorescent amines will allow standardization of the environmental conditions and thus more reliable phylogeny data. 3D images built from the confocal data can provide new information for taxonomy studies.

In addition *in vivo* staining opens new possibilities for ecological experiments, i.e., for study of the influence of environmental conditions on the sponge's growth. Fluorescent staining allows us to distinguish new spicules that appeared during an experiment from the old ones, which is impossible by other methods. We can also obtain a collection of spicules from various staining (growth) stages and thereby obtain information about the dynamics of spiculogenesis. Moreover, the relatively low growth rate of the spicules allows us to expect formation of sophisticatedly stained siliceous structures by alternating cultivation in the presence of assorted fluorescent dyes.

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