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Authors: Natalia V Povarova, Snizhana O Zaitseva, Nadezhda S Baleeva, Alexander Yu Smirnov, Ivan N Myasnyanko, Marina B Zagudaylova, Nina G Bozhanova, Dmitriy A Gorbachev, Kseniya K Malyshevskaya, Alexey S Gavrikov, Alexander S Mishin, and Mikhail S. Baranov

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Red-shifted substrates for FAST fluorogen-activating protein based on the GFP-like chromophores

Natalia V. Povarova,[a] Snizhana O. Zaitseva,[a] Nadezhda S. Baleeva,[a] Alexander Yu. Smirnov,[a] Ivan N Myasnyanko,[a] Marina B. Zagudaylova,[a] Nina G. Bozhanova,[a] Dmitriy A. Gorbachev,[a] Kseniya K. Malyshevskaya,[a] Alexey S. Gavrikov,[a] Alexander S. Mishin[a] and Mikhail S. Baranov[a,b]*

Abstract: In this paper, we present a novel genetically encoded fluorescent tag for live cell microscopy. This tag is composed of previously published fluorogen-activating protein FAST and a novel fluorogenic derivative of GFP-like chromophore with red fluorescence. The reversible binding of the novel fluorogen and FAST is accompanied by three orders of magnitude increase in red fluorescence (580-650 nm). The proposed dye instantly stains target cellular proteins fused with FAST, washes out in a minute timescale, and exhibits higher photostability of the fluorescence signal in confocal and widefield microscopy, in contrast with previously published fluorogen:FAST complexes.

In modern fluorescence microscopy, various GFP-like fluorescent proteins (FPs) are widely used as genetically encoded tags for visualization in living systems.[1] The recent decades have been devoted to the development of numerous FPs which differ in color, fluorescence lifetime, phototoxicity, photoswitching ability, and other properties.[2] However, all these FPs have a serious drawback: they require time and oxygen for maturation.[3] Moreover, all of them are highly prone to the irreversible destruction of the chromophore core due to photobleaching.[4]

The recently discovered fluorogen-activating proteins (FAPs) are very promising for overcoming abovementioned problems.[5] FAPs form fluorescent complexes with fluorogens - substances that do not have pronounced fluorescence by themselves, but acquire it when they bind to the pocket of FAP (Figure 1A).[6] Thereby FAPs labeling do not require the presence of oxygen or any additional time for maturation except for the protein folding. Fluorogenic dye (and hence the fluorescence signal) can be introduced or washed out on demand. Due to the noncovalent nature of the interaction, a photobleached fluorogen molecule can be replaced with another molecule from the solution.[7]

Derivatives of FPs chromophores are promising source for fluorogenic compounds.[8] High structural and color diversity of these compounds, as well as their small size, high hydrophilicity, and simple synthetic protocols make them attractive fluorogenic partners.[9] Most of the chromophores are non-fluorescent in solution due to the mobility of their benzylidene fragment which can dissipate their excited state energy.[10] However, they become highly emissive when the flexible substituent is fixed by an external influence[11] or by an internal lock.[12] Previously they were successfully used as fluorogens for various proteins,[13] nucleic acids,[14] and whole cell organelles.[15]

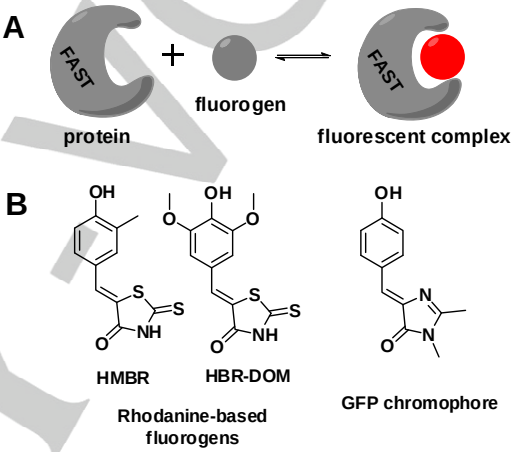

Figure 1. (A) Principal scheme of fluorogen-activating protein action. (B) Previously presented rhodanine derivatives and their structural analogy with GFP chromophore.

Various rhodanine derivatives[16] are close structural analogs of the FPs chromophores (Figure 1B). Several hydroxybenzylidene-rhodanines were previously used for fluorescent labeling in complex with the fluorogen-activating protein FAST ("Fluorescence-Activating and absorption-Shifting Tag"),[17] an engineered variant of the photoreceptor from *Halorhodospira halophila* - Photoactive Yellow Protein (PYP).[18] The high similarity between rhodanines and FPs chromophores prompted us to test the latter as substrates for the FAST. Despite the close resemblance between arylidene rhodanine derivatives and FPs chromophores, a larger size of sulfur atoms provides a noticeable geometric difference. For this reason, at the first stage of this work, we decided to test the most diverse library of the FPs chromophores derivatives containing both close analogs of the FPs chromophores and those which include additional heteroatoms, as well as larger cycles (1st Library, Figure 2, and Table S2.1). Screening of the first library using protein immobilized on beads of TALON metal affinity resin (SI, Part 4) identified a number of compounds exhibiting an increase in the fluorescence intensity upon an interaction with FAST (Figure S4.1).

[a] Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117997 Moscow, Russia
E-mail: baranovmikes@gmail.com
[b] Pirogov Russian National Research Medical University, Ostrovitianov 1, Moscow 117997, Russia

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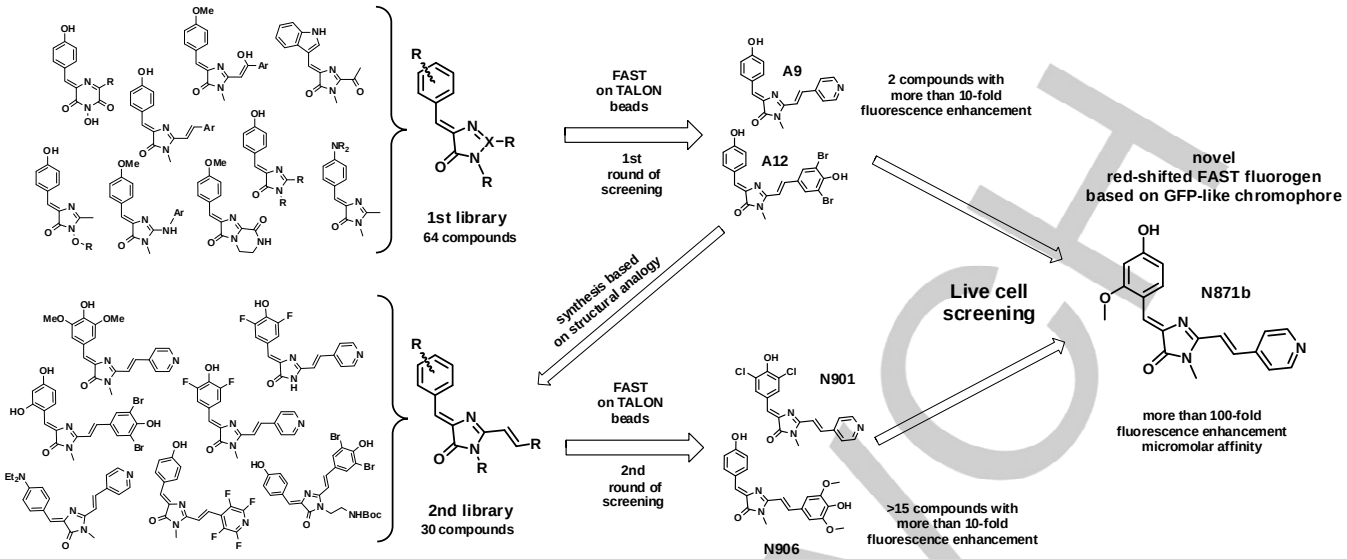

Figure 2. Chromophores screening pipeline and a novel effective fluorogen for FAST protein, compound **N871b**.

Among these substances, compounds **A9** and **A12** (Figure 2) provided the most noticeable fluorescence enhancement with a small background signal. Testing of these compounds on the purified protein showed that both of the substances demonstrate approximately 10-fold enhancement of the fluorescence signal (see SI, part 3). Compound **A12** showed a smaller fluorescence increase with tighter binding, while **A9** showed a weaker fluorescence signal in a free form (Tables 1, S3.1, S6.1, and S8.1). Such low fluorescence increase will hinder the use of these substances as effective fluorogens. However, their structures could guide the synthesis of a new library.

The change in the fluorescence spectra occurring upon binding of the **A9** and **A12** chromophores to FAST suggested possible deprotonation of the phenol group and consequently its participation in binding (see Figure S12.1C). Therefore, we prioritized this residue in the second library. We also kept the styrene fragment in the second position of the imidazolone cycle which was presented in both structures. In this regard, only substituents in aryl groups, as well as at the nitrogen atom, were subjected to variation (Figure 2, Table S3.1). We additionally introduced groups that could red-shift the absorption and emission maxima: electron-donating groups in the benzylidene fragment and electron-withdrawing groups in the styrene one.[19]

The second library screening using protein immobilized on TALON beads (SI, Part 4) revealed more than a dozen of new substances with the same or higher fluorescence enhancement compared to **A9** and **A12** (Figure S4.2, Table S3.1).

However, high fluorescence increase and effective binding *in vitro* do not guarantee suitability of the fluorogen for microscopy of living systems due to the possibility of untargeted staining, poor diffusion through the membrane, etc. In this regard, on the next stage, we proceeded directly to the live-cell experiments.

We found that most of the identified substances failed to label the nucleus of cells transfected with the fusion of histone H2B and FAST proteins. A detectable but weak signal was visible for the chromophores **A9**, **N901**, and **N906**, while a prominent signal was observed only when the **N871b** chromophore was added. We showed that **N871b** fluorogen was effective for the fluorescent labeling of the cells transiently transfected with various FAST fusions (Figure 3). A bright red signal was observed for different subcellular localization without nonspecific staining. Compound **A9** can also be used for staining, but showed only weak signal visible only under very carefully selected conditions (SI, part 11).

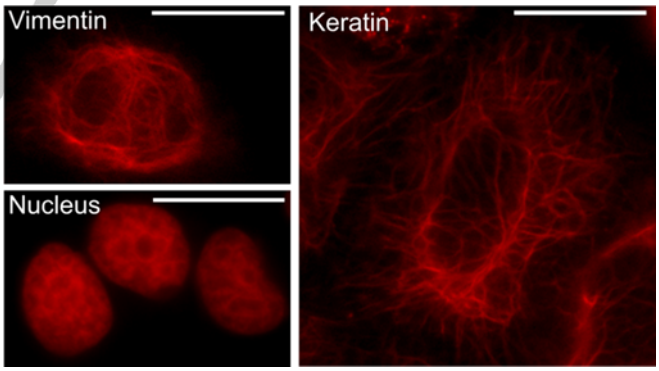

Figure 3. Widefield fluorescence microscopy of HeLa Kyoto cells transiently transfected with various FAST fusions labelled with **N871b**. Cells expressing FAST fused with H2B (nucleus) were labelled with 5 μM of fluorogen. Vimentin and keratin filaments were labelled with 10 μM of **N871b**. Scale bars 20 μm.

The optical spectra of the **N871b** chromophore in complex with the FAST protein resemble the ones of the previously described complex with **HBR-DOM**[17] chromophore (Table 1, Figure 4). However, despite the similar emission maximum, the wide shape of the emission spectrum makes it possible to detect the emission in the region of 650 nm (Figure 4).

Table 1. Optical properties of chromophores and their complexes with FAST protein.

Compound	Absorbance maxima position, nm[a]			Emission maxima position, nm[a]			ε, $M^{-1}\cdot cm^{-1}$	Fluorescence quantum yield (FQY), %		Fluorescence enhancement (=FQY_{bound}/FQY_{an})	Brightness (=ε·FQY)	pK_a	K_d, μM
	neutral	anionic	bound	neutral	anionic	bound	bound	anionic	bound				
HMBR	400	461	483	493	560	540	45000	0.2[17]	33[17]	165	14850	8.7[17]	0.20[b] 0.13[17]c
HBR-DOM	406	486	520	530	600	600	39000	0.14[b]	31[17]	220	12000	8.3[17]	1.14[b] 0.97[17]c
A9	430	506	537	545	600	604	–[d]	0.03	–[d]	~20	–[d]	7.6	~50
N871b	455	528	562	570	630	606	23000	0.022	25	1140	5750	7.4	0.25

a – characteristics of neutral forms were investigated in 0.1M phosphate buffer pH 6, anionic forms – in 0.1M carbonate buffer pH 10
b – characteristic was measured in current work;
c – the difference is probably occurs from the use of different phosphate buffers with different total electrolyte concentrations (i.e. different ionic strength);
d – characteristic was not obtained due to the low affinity.

We observe that the interaction with FAST resulted in the complete deprotonation of the phenolic residue characterized by a noticeable red-shift for all the chromophores tested in this work as well as all previously published rhodanines (Table 1, Figure 4). However, the spectra of the complexes do not always coincide with the ones of anionic form of unbound chromophores in water, but rather resembled the spectra of anionic form in less polar solvent (Table S6.1). Hydrophobic environment of the binding pocket may cause these considerable spectral shifts due to the pronounced solvatochromism of the chromophores (SI, Part 6). Indeed, according to the X-ray data for the original PYP protein (PDB ID: 1NWZ), a cavity which is believed to accommodate a chromophore, contains several lipophilic residues (62Phe, 31Ile, 96Ile, and 66Val, Figure S12.1B).

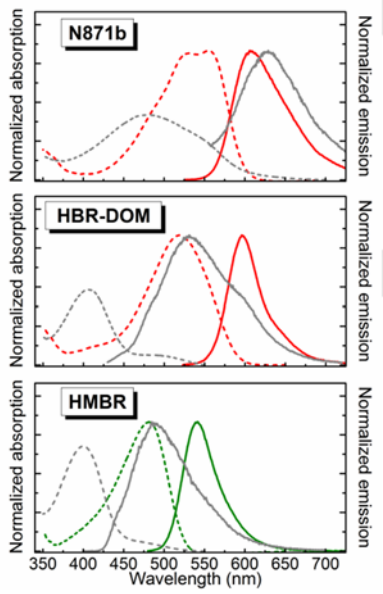

Figure 4. The absorption (dashed lines) and emission (solid line) spectra of free chromophores (grey) and chromophore:FAST complexes (colored). The ratio of the absorption intensity of the complex and free chromophore corresponds to the real change in the extinction coefficient and shape of the spectra in PBS. The emission spectra of the complex and free chromophore are scaled to the equal height.

The level of a background fluorescence is influenced by the efficiency of an unbound chromophore excitation. At physiological pH, the fraction of the free chromophore in anionic form for **N871b** is higher, in comparison with the **HBR-DOM** and **HMBR** (Table 1, SI Part 7). However, the unbound **N871b** is dimmer and exhibits three orders of magnitude higher increase in fluorescence upon binding with FAST than the **HBR-DOM** or **HMBR**, ensuring excellent imaging contrast.

The dissociation constant of the **N871b**:FAST complex is 0.25 μM (SI part 8), which is intermediate between the constants of the published complexes of fluorogens **HBR-DOM** and **HMBR**.[17] So, as in the case of the rhodanines, **N871b** can be easily added and washed out (Figure S10.1). The rate of **N871b** washing out was comparable with the rate of washing out of the **HMBR** fluorogen (Figure 5 A, B), which indicates the similar values of K_{off} and K_{on}. The addition of **HMBR** and **N871b** together to the cells already stained with **N871b** led to an equilibrium displacement (Figure 5 C, D), which confirmed the interaction with the same binding pocket within the protein (see also SI part 12, Figure S12.1A).

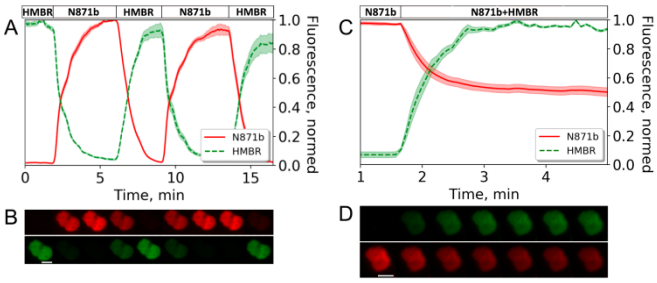

Figure 5. Fluorescence microscopy of HEK293NT cells expressing H2B-FAST in a flow of chromophore solutions. (A, B) Alternating action of **N871b** and **HMBR** chromophores (5 μM both). Cells were imaged with TxRed and GFP filter sets for **N871b** and **HMBR** respectively (SI Part 10). (C, D) Displacement of the **N871b** chromophore by adding an equal concentration of **HMBR** (5 μM both). Scale bars 10 μm.

We compared target staining and the background fluorescence signals of **N871b**:FAST and **HBR-DOM**:FAST complexes by staining the same cell sequentially with both dyes. The intermediate filaments visualized in the cells transiently expressing keratin-FAST show very close amplitude of the signal and similarly low out-of-cell fluorescence (Figure S 10.2, see SI part 10).
However, a closer study of the TALON beads (Figures SI 9.1 and 9.2) and HeLa Kyoto cells expressing H2B-FAST fusion protein (Figure 5, SI Part 9) revealed that **N871b**:FAST complex exhibited strikingly better photostability than complexes of previously proposed fluorogens **HMBR** and **HBR-DOM** under intensive green and blue light irradiation (Figure 6).

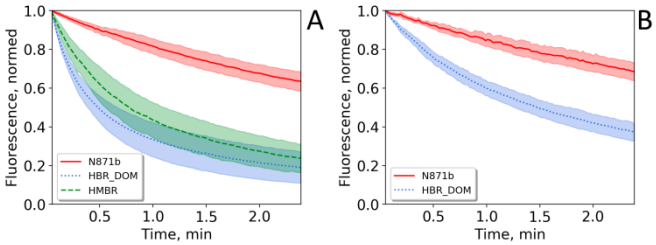

Figure 6. Photobleaching behavior of fluorogen:FAST complexes with compounds **HMBR**, **HBR-DOM**, and **N871b**. (**A**) Photobleaching curves of HeLa Kyoto cells expressing H2B fused with FAST stained with 10 μM chromophores in a confocal setup. 6-10 nucleus was scanned with 9,5 μW 488 nm laser for 2,5 minutes. (**B**) Photobleaching curves of HeLa Kyoto cells expressing H2B fused with FAST stained with 10 μM chromophores in a confocal setup. 6-10 nucleus was scanned with 7,5 μW 543 nm laser for 2,5 minutes.

The dye appears to be non-toxic at the 10 μM concentration: the cells continue to divide in the presence of **N871b** in the cultural media at the same rate (see SI part 13) which makes it suitable for live-cell imaging applications.

To conclude, we report on the development of a new efficient substrate for the fluorogen-activating FAST protein. The novel fluorogen **N871b** forms complex with FAST which is three orders of magnitude brighter than the free fluorogen and is characterized by red-shifted emission (580-650 nm). The **N871b** instantly stains the target cellular proteins fused with FAST and washes out in a timescale of minutes. The complex of compound **N871b** with FAST exhibits superior photostability in comparison with the previously published rhodanine fluorogens, making **N871b** the fluorogen of choice for prolonged time-lapse imaging and light-intense microscopy modalities. The similar affinity and exchange rates of the dyes suggest that the chemical nature of the fluorogen may be an important factor limiting the overall photostability of FAP labeling system.

Conflicts of interest

There are no conflicts to declare.

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Keywords: fluorescence • chromophore • fluorogen-activating protein • fluorescent labelling • GFP

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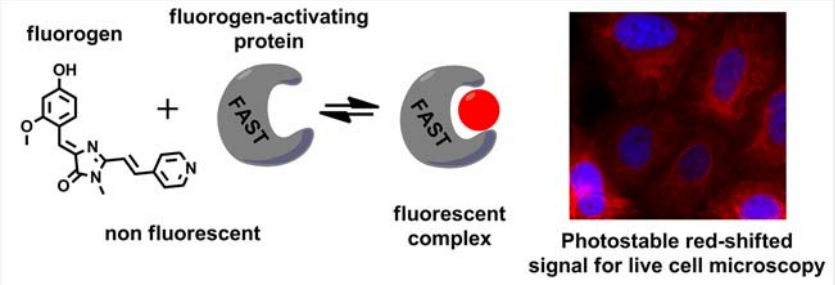

*Natalia V. Povarova,[a] Snizhana O. Zaitseva,[a] Nadezhda S. Baleeva,[a] Alexander Yu. Smirnov,[a] Ivan N Myasnyanko,[a] Marina B. Zagudaylova,[a] Nina G. Bozhanova,[a] Dmitriy A. Gorbachev,[a] Kseniya K. Malyshevskaya,[a] Alexey S. Gavrikov,[a] Alexander S. Mishin[a] and Mikhail S. Baranov[a,b]**

Red-shifted substrates for FAST fluorogen-activating protein based on the GFP-like chromophores

Novel genetically encoded fluorescent tag with red signal capable for live cell microscopy is presented. The tag is based on previously published fluorogen-activating protein FAST and a novel fluorogenic derivative of GFP-like chromophore. The proposed dye instantly stains proteins fused with FAST, washes out in a minute timescale, and exhibits high photostability of the fluorescence signal.