A Surprising Photoactivity of Blue Fluorescent Protein TagBFP Allows for Super-Resolution Microscopy

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The aim of this research was to study photobehavior of a popular blue fluorescent protein TagBFP and apply this marker for super-resolution microscopy.

Materials and Methods. Photoactivation of TagBFP was examined both in protein solution *in vitro* and in living cells. Subdiffraction imaging was performed using total internal reflection fluorescence microscopy followed by super-resolution radial fluctuations or single-molecule localization analysis.

Results. We show that TagBFP exhibits blinking behavior upon 405 nm light illumination. Moreover, photoactivation to red-emitting state is occurring in the conditions typically used for TagBFP imaging. The red (photoactivated) form of TagBFP possesses spectral properties similar to TagRFP — a close homologue of TagBFP. We show that both blinking and photoactivation of TagBFP can be utilized for super-resolution imaging. We conclude that photoactivation of TagBFP to red-emitting form should be taken into account in the design of multi-channel imaging experiments involving high-power or prolonged UV illumination.

Key words: TagBFP; photoactivation; blue-to-red photoconversion; blinking; super-resolution microscopy.

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Introduction

Fully genetically encoded labels — fluorescent proteins of the green fluorescent protein (GFP) family enable real-time visualization of various structures and events in live cells [1]. They provide a rich information in multicolor imaging regime that makes it possible to establish a relationship between different cellular targets in space and time. Unlike other parts of the visible spectrum, the palette of widely used blue fluorescent proteins is limited to only a few bright and photostable monomeric proteins, namely Azurite, EBFP2, and TagBFP [2–4]. Therefore, these proteins are often included as a blue tag in multicolor imaging experiments.

Regular fluorescence microscopy provides the maximum resolution of 200–350 nm that makes many important fine features of a target structure hidden. This basic problem has been recently solved by super-resolution fluorescence microscopy techniques, which ensure subdiffraction resolution down to 10–20 nm [5]. Subdiffraction imaging methods based on single-molecule localization microscopy provide the highest resolution but require special fluorescent labels [6]. Only some types of these probes are suitable for fast imaging of live cells [7].

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We have previously reported that TagRFP and several closely related red fluorescent proteins exhibit fast reversible photoconversion between dark and bright states, which could be utilized for super-resolution imaging [8]. We, therefore, sought to assess the photoconversion in a closely related fluorescent protein — TagBFP [4], which is a blue-emitting variant of TagRFP.

The aim of this research was to study photobehavior of TagBFP and apply this genetically encoded fluorescent marker for super-resolution microscopy.

Materials and Methods

Plasmids and protein purification. We used actinin-TagBFP and Lifeact-TagBFP plasmids from Evrogen (Russia). TagBFP in pQE-30 vector with 6His-tag at the N-terminus was used for protein expression. After sonication of the bacterial suspension, the protein was purified from soluble fraction using TALON metal-affinity resin (Clontech, USA) according to the manufacturer's protocol. The protein solution (in phosphate-buffered saline) was illuminated with 405 nm light (~1 W/cm^2 from X-Cite XLED1 light source) (Excelitas Technologies, USA) for 30 min. Absorbance and fluorescence spectra of TagBFP were recorded using a Cary 100 spectrophotometer (Varian, USA) and a Cary Eclipse fluorescence spectrophotometer (Varian, USA).

Cell culture. HeLa (human cervical carcinoma) cells and NIH 3T3 (mouse embryo fibroblasts) cells were cultured in DMEM (PanEco, Russia) supplemented with 10% fetal bovine serum (HyClone, USA), 2 mM L-glutamine (PanEco, Russia), and antibiotics (50 U/ml penicillin plus 50 μg/ml streptomycin) (PanEco, Russia) at 37°C in humidified atmosphere with 5% CO_2. Cells were passaged every 3 days.

Total internal reflection fluorescence (TIRF) microscopy and data analysis. TIRF imaging was carried out on an Eclipse Ti N-STORM microscope (Nikon, Japan) with NIS-Elements 4.13.01 Software and 100× oil-immersion TIRF objective (Apo TIRF/1.49; Nikon, Japan). Time series were acquired with Andor iXon3 DU-897 camera (Andor, UK), EM gain of 200 or 296, and pre-amplifier gain of 5.1× or 2.4×, with a pixel size of 160 nm. The samples were illuminated using 405 or 561 nm laser lines. Data processing and image reconstruction for super-resolution radial fluctuations (SRRF) were performed as described [9] with 100–500 raw frames. Single-molecule localization analysis was performed with ThunderSTORM [10] software.

Results and Discussion

Fast reversible photoconversion of TagBFP. We found that TagBFP, similarly to TagRFP, exhibits robust frame-to-frame pixel intensity fluctuations with exposures between 10 to 100 ms and illumination intensities as low as 0.1 W/cm^2 of 405 nm light illumination. We revealed that further increase in illumination intensity does not increase the variance-to-mean ratio of fluctuation. We then processed the raw data with recently developed SRRF algorithm [9], which took the advantage of temporally correlated fluctuations resulting from multiple blinking events of overlapping fluorophores. A representative image demonstrating an improvement in spatial resolution compared to TIRF microscopy is shown in Figure 1.

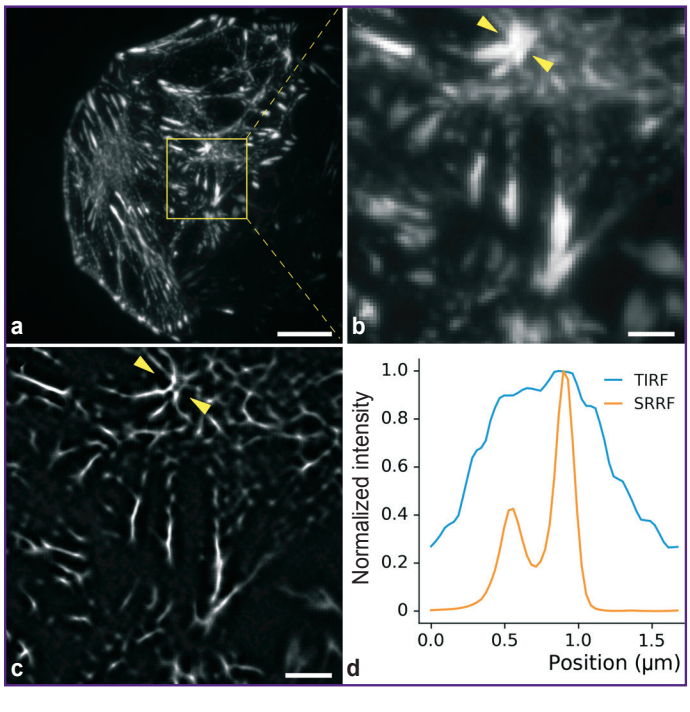

Figure 1. Super-resolution imaging with TagBFP
Live HeLa cells labeled with actinin-TagBFP. Image reconstruction by SRRF (super-resolution radial fluctuations) algorithm from 300 consequent frames at 50 ms camera exposure time, 405 nm light excitation at 0.1 W/cm^2. (a) TIRF (total internal reflection fluorescence) image, scale bar 10 μm; (b) enlarged fragment from panel (a), scale bar 2 μm; (c) SRRF image of the same region, scale bar 2 μm; (d) intensity profiles between yellow arrows on panels b — *blue lines* and c — *orange lines*

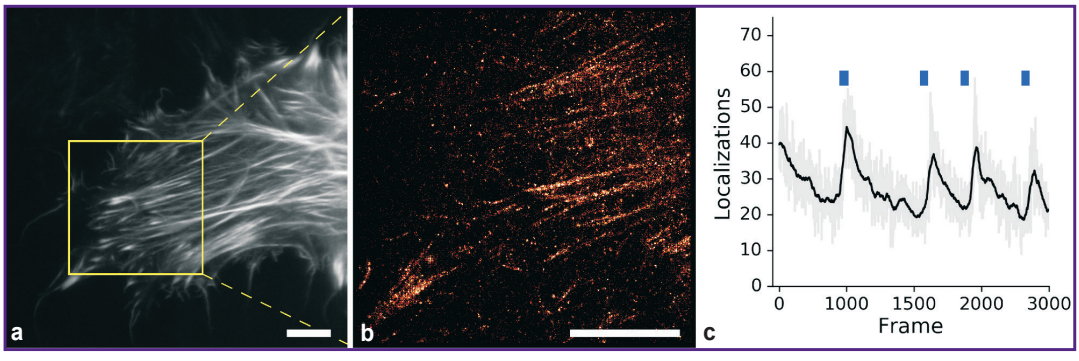

Figure 2. Photoactivation of TagBFP to red-emitting form
Live NIH 3T3 cells labeled with Lifeact-TagBFP. (a) TIRF (total internal reflection fluorescence) image, 405 nm excitation, scale bar 10 μm; (b) super-resolution image, 561 nm excitation (60 W/cm^2), the total of ~110,000 localizations are shown, scale bar 10 μm; (c) dependence of the number of single-molecule localizations on the 405-nm photoactivation (*blue squares*, ~1-s pulse of 0.3 W/cm^2). Smoothed (*black*) line is added to aid the visualization

In cellulo photoactivation of TagBFP. The TagBFP-like chromophore is believed to be a precursor of DsRed-like chromophores of red fluorescent proteins, and also represents the dark states of some irreversibly photoactivated fluorescent proteins [11, 12]. To test this idea, we illuminated TagBFP-labeled live cells with 405 laser of varying intensity, followed by imaging with 561 nm excitation. The increase in red fluorescence was clearly visible right after ~1-s pulses of 405 laser (0.3–1.0 W/cm^2). The photoconversion efficiency of TagBFP was at least an order of magnitude lower than the one of Dendra2 (not shown) under similar conditions. Nonetheless, we localized more than 100,000 single-molecule bursts of red fluorescence in a 500 μm^2 region of the cell in less than 2.5 min with a total of 5 s of 0.3 W/cm^2 405-nm laser illumination (Figure 2).

In vitro photoactivation of TagBFP. We detected that purified TagBFP in a solution shows a sharp increase in ~555 nm absorbance after 30-min illumination with high-power 405-nm light-emitting diode (Figure 3). The absorbance of the photoproduct (555 nm) coincides almost perfectly with published TagRFP absorbance spectra. Surprisingly, we did not observe a significant increase in the fluorescence from the same sample (data not shown). Presumably, the emerging red form appears initially in the dark state. Therefore, the photoconversion of TagBFP demonstrated for the first time in current work may have been overlooked by previous studies.

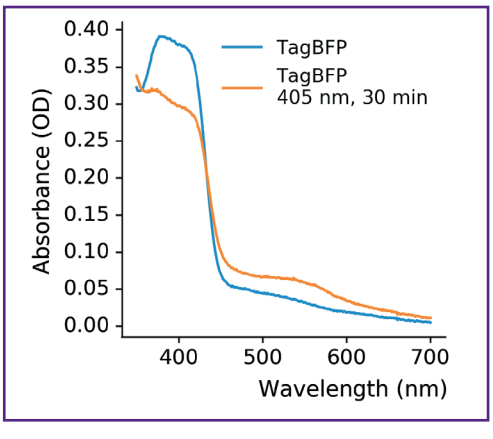

Figure 3. *In vitro* photoactivation of TagBFP
Absorbance spectra of a TagBFP solution (phosphate-buffered saline, pH 7.4) before (*blue lines*) and after (*orange lines*) photoactivation with high-power 405-nm light-emitting diode

Conclusion

The photoconversion of TagBFP into red-emitting form is a particularly disturbing phenomenon for the design of super-resolution experiments. Specifically, the red-emitting form of TagBFP could be easily confused with red-emitting forms of other photoactivated proteins, such as Dendra2. In many high-resolution experimental setups, the optical components are specifically designed to maximize photon count of detected localization, with little or no spectral filtering (for instance, filter-cubes with multiband dichroic mirrors, without any suppression filters). While we found the efficiency of blue-to-red photoactivation of TagBFP insufficient for dedicated single-molecule localization imaging, the possible artifacts should be considered with any combination of TagBFP with UV-induced photoactivatable fluorescent proteins, such as Dendra2, mEos3.2, PA-GFP, PA-TagRFP, PA-mCherry, etc. In contrast, the blinking behavior of TagBFP allowing for super-resolution microscopy gives an additional fluorescent tool for researchers.

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Conflict of Interests. The authors declare no conflict of interests.

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