Fluorescence microscopy for visualization of mutual localization of RecA and RecN proteins inside *E. coli* cells

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To visualize the SOS response proteins inside bacteria, the *E. coli* were edited to create strains with fluorescently labeled *recA* and *recN* in their genome. The *recA* gene was fused with the mNeonGreen fluorescent protein, and the *recN* gene was fused with the mScarlet fluorescent protein. Fluorescence visualization of the resulting strain showed the synthesis of filaments and foci by the RecA protein during the bacterial SOS response, which in some cases colocalize with the RecN protein foci.

Keywords: SOS response, RecA protein, RecN protein, CRISPR-Cas9, fluorescence microscopy.

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Fluorescence microscopy is a powerful method used in molecular biology, medicine, and diagnostics to obtain important data on living objects and visualize molecular processes in real time. This is made possible by fluorescent labeling of individual proteins or cellular structures with dyes or fluorescent proteins and subsequent excitation and detection of their fluorescence. The rapid development of fluorescence microscopy driven by joint efforts of physicists, chemists, and biologists has led to the emergence of new methods. Conventional diffraction-limited fluorescence microscopy is applied alongside with various approaches that provide an opportunity to enhance the image resolution significantly.

In the present study, wide-field fluorescence microscopy [1] is used to visualize the mechanism of interaction between RecA and RecN proteins in the SOS response of bacteria caused by damage of their DNA.

The RecA protein is a recombinase that is crucial for the SOS response and repairs double-stranded DNA breaks through homologous recombination. Another important contributor to the SOS response and homologous recombination is the RecN protein, which is one of the key partners of RecA. However, it remain unclear how exactly does RecN facilitate the search for homologous DNA to restore a break. It has already been shown that RecN is recruited to DNA repair centers together with the RecA protein, which is the main enzyme of homologous repair and activator of the SOS response of bacteria [2]. In our earlier studies, we have demonstrated ATP-dependent *in vitro* binding of RecN to predominantly single-stranded DNA. It has also been revealed via fluorescence microscopy that, outside of the SOS response, RecN is localized in the center of actively dividing cells and at their poles, but does not colocalize with DNA [3]. However, these data were obtained using proteins artificially expressed in cells even without DNA damage; thus, we could not clarify completely the molecular mechanisms of functioning of the RecN protein. In the present study, genome editing methods with CRISPR-Cas [4] and homologous recombination techniques were used to create a strain of *E. coli* that carries in its chromosome the *recA* and *recN* genes fused with the genes of fluorescent proteins mNeonGreen and mScarlet, respectively. The resulting strain with a modified genome allows to visualize the localization of RecA and RecN proteins in the process of bacterial SOS response.

The aim of the study was to obtain a strain of *E. coli* with fluorescently labeled proteins RecA::mNeonGreen and RecN::mScarlet. For this purpose the BL21AI strain of *E. coli* was used.

The CRISPR-Cas9 system, which is one of the simplest and best known CRISPR-Cas systems, was used for genome editing. Its effector complex is formed by the Cas9 protein and guide RNA, which directs Cas9 to the desired DNA sequence. The Lambda-Red recombination system, which allows for *in vivo* genome modification through homologous recombination, was also used. The CRISPR-Cas9 system introduced a double-strand break at the chosen insertion site of the target sequences. Homologous recombination was then initiated, and a sequence from the recombination template was inserted.

DNA constructs encoding guide RNA were assembled using the circular polymerase extension cloning (CPEC) method. Fragments for recombination templates were obtained by PCR and assembled using the Gibson method.

Thus, the following constructs were obtained for the *recA* gene: the pKDsgRNA-ack(*recA*) plasmid encoding guide RNA; the (*recA*+mNeonGreen) recombination template, which is a linear fragment containing the mNeonGreen fluorescent protein sequence flanked by a small part of *E. coli* genome sequences. The corresponding pKDsgRNack(*recN*) and (*recN*+mScarlet) constructs performing the same functions were created for *recN*.

To test how the CRISPR-Cas system targets the genes of interest, we first grew cell cultures carrying plasmids

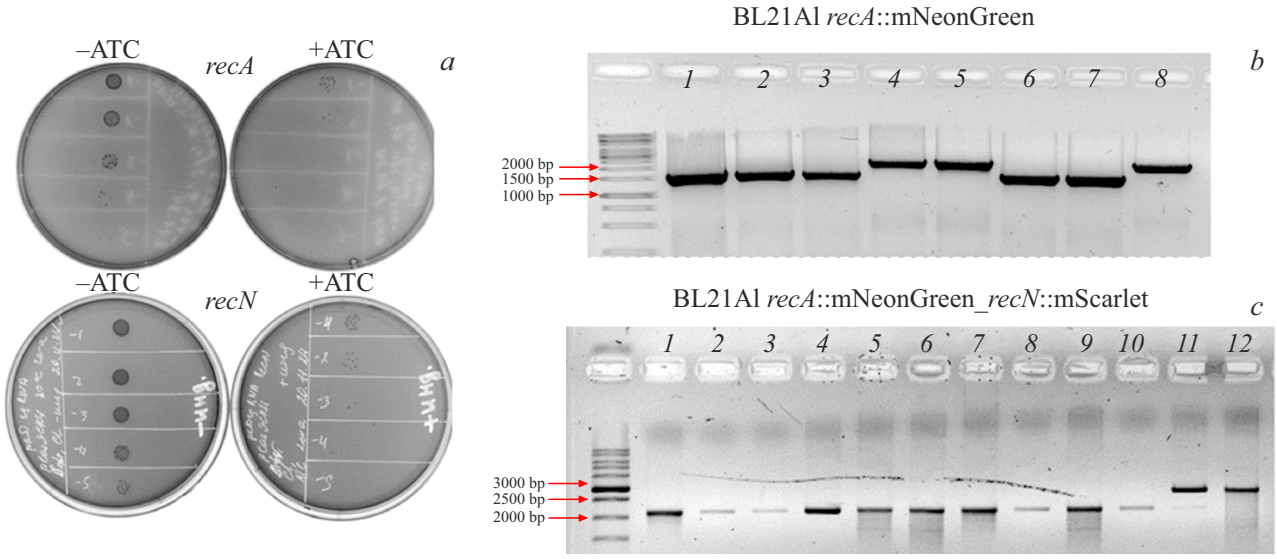

Figure 1. Results of genome editing experiments. *a* — Targeting of the CRISPR-Cas system to the genes of interest; *b* — PCR screening of *E. coli* colonies with edited *recA*::mNeonGreen gene (*1–8* — colony numbers); and *c* — PCR screening of *E. coli* colonies with *recN*::mScarlet gene (*1–12* — colony numbers).

coding the CRISPR-Cas system and the needed guide RNA. The cell culture was then diluted by 1–6 orders of magnitude, and the dilutions were applied in 5 μl droplets to Petri dishes with 1.5 % LB agar medium (per 500 ml of water: 5 g of tryptone, 2.5 g of yeast extract, 5 g of sodium chloride, and 7.5 g of microbiological agar) with and without the anhydrotetracycline (ATC) inducer and incubate in a thermostat (BD 53, Binder) at a temperature of 30 °C for 24 hours.

At the first stage of genome editing, genetic constructs required for fluorescent labeling of *recA* were introduced sequentially into cells by electroporation. Arabinose (50 mM) was then added to the culture to induce the Lambda Red recombination system and ATC to express the Cas9 nuclease. Next, cell culture dilutions were applied to Petri dishes with the addition of ATC. The edited colonies were then analyzed by PCR screening.

Prior to editing the *recN* gene, we needed to remove the pKDsgRNA-ack(*recA*) plasmid containing guide RNA to the *recA* spacer, in order to pKDsgRNA-ack(*recN*) plasmid could be introduced into the cells. Since pKDsgRNA-ack plasmids are temperature-sensitive and are removed from cells at 37 °C. The edited cells were incubated at this temperature without the addition of the selective antibiotic (spectinomycin) because of the plasmid will be removed.

The second stage of genome editing was similar to the first: genetic constructs required for fluorescent labeling of the *recN* gene were introduced into the cells. As before, the edited colonies were analyzed by PCR screening.

Genome editing was followed by fluorescence microscopy studies, which revealed the localization of RecA::mNeonGreen in cells under normal conditions and in the SOS response state. Colocalization of RecA::mNeonGreen and RecN::mScarlet proteins in *E. coli* under normal conditions and in the SOS response state was also visualized. To activate the SOS response, a 1 ml sample of the bacterial culture was irradiated with ultraviolet light for 1 min. The cultures were then incubated for 20 min in a TS-100 (BioSan) thermal shaker at 37 °C.

Slides for imaging were prepared in the following manner: 500 μl o 1.5 % agarose dissolved in 1X TAE buffer (40 mM of tris, 20 mM of acetic acid, and 1 mM of EDTA per 1l of water, pH = 8) were applied to a glass slide and pressed down with a second glass slide to form a smooth substrate. The top glass slide was removed after hardering of the agarose. The cell culture (1 μl) was then applied to the formed substrate and dried, and a cover slide was placed on top. An inverted Nikon Eclipse Ti-E microscope with an 100× objective with oil immersion was used for imaging in three channels: in the transmitted light mode, in the green fluorescence channel for recording the fluorescence of RecA::mNeonGreen (excitation at a wavelength of 506 nm, detection at 517 nm), and in the red fluorescence channel for RecN::mScarlet (excitation at 569 nm, detection at 594 nm). All images were obtained using a Zyla 4.2 (Andor) sCMOS camera.

As the result the CRISPR-Cas system was targeted successfully at *recA* and *recN* genes. The images in Fig. 1, *a* make it clear that the addition of the ATC inducer significantly reduced the number of colonies in each dilution, indicating that the CRISPR-Cas system was targeted as intended.

E. coli strains containing only the *recA*::mNeonGreen gene and both *recA*::mNeonGreen and *recN*::mScarlet were obtained.

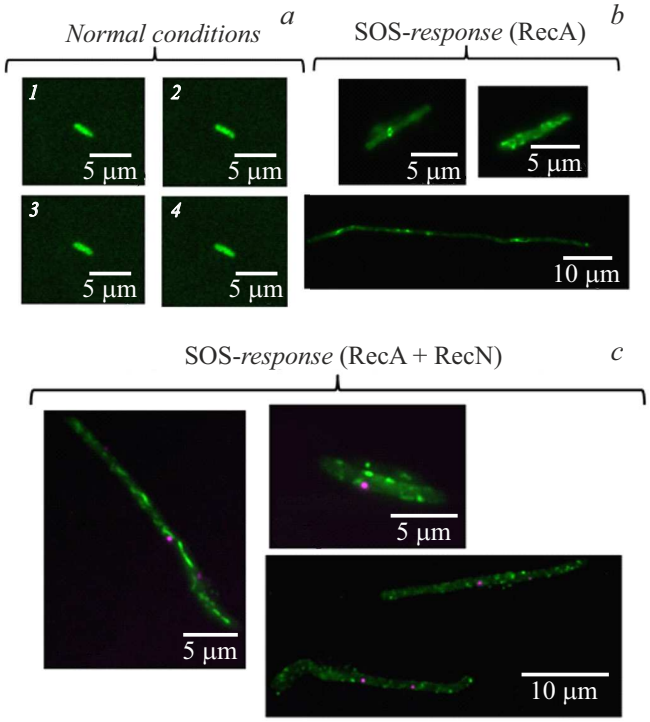

Figure 2. Visualization of RecA::mNeonGreen (green) in normal *E. coli* cells (*a*) and in *E. coli* cells in the SOS response state (*b*). *c* — Localization of RecA::mNeonGreen (green) and RecN::mScarlet (purple) in *E. coli* cells in the SOS response state. A color version of the figure is provided in the online version of the paper.

The results of the first stage of genome editing are presented in Fig. 1, *b*. Cell colonies with the edited *recA* gene were analyzed by PCR screening. The edited colonies should have a PCR fragment length of 2000 bp. It can be seen that the editing was successful in colonies *4*, *5*, and *8*.

The results of the next part of genome editing are presented in Fig. 1, *c*. Cell colonies with the edited *recN* gene were also analyzed by PCR screening. The edited colonies should have a PCR fragment length of 2700 bp. It can be seen that the editing was successful in colonies *11* and *12*.

Fluorescence microscopy images of BL21AI RecA::mNeonGreen_RecN::mScarlet *E. coli* cells were obtained under normal conditions and in the SOS response state. It is evident from Fig. 2, *a* that the RecA protein is also expressed in cells outside the SOS response state and moves actively around them (sequential frames *1–4* obtained for the same cell with 1 s intervals are shown). When the SOS response is activated (Fig. 2, *b*), it could be seen how the RecA protein forms filaments inside the cells. Images of cells with two fluorescently labeled proteins RecA and RecN were also obtained in the SOS response state (Fig. 2, *c*). The RecN protein is not observed without activation of the SOS response and during the SOS response, the amount of RecN in the cell is much smaller than the amount of RecA. Also it is shown that the proteins are colocalized in certain cases.

Thus, a strain of *E. coli* with fluorescently labeled RecA and RecN proteins was obtained. The localization of proteins of interest were obtained under normal conditions and in the SOS response state was visualized via fluorescence microscopy. Under normal conditions, the RecN protein is not found in cells, while the RecA protein moves actively around them. In the SOS response state, the RecA protein forms filaments inside cells; at the late stages of the SOS response, RecA foci are visualized. The RecN protein also forms foci in the process of SOS response. In certain cases, RecN foci are colocalized with RecA foci. The amount of RecN protein present in cells is much smaller than the amount of RecA.

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Conflict of interest

The authors declare that they have no conflict of interest.

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