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CHARACTERIZATION OF FLUORESCENT PROTEINS FOR STUDYING THE MORPHOLOGICAL REARRANGEMENTS INSIDE SINGLE BACTERIAL CELLS DURING INFECTION WITH phiKZ BACTERIOPHAGE

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Abstract. This work evaluates the key properties of some fluorescent proteins (FPs) in order to use them for observation of redistributing the target proteins between the cytoplasm and pseudonucleus forming in bacterial cell infection by bacteriophage phiKZ. Four FPs, namely, mNeonGreen, mCherry, ECFP and EYFP, have been investigated using fluorescence microscopy. It was established that EYFP, unlike other objects, localized in the pseudonucleus during the infection. This phenomenon gave grounds to exclude EYFP from further experiments. As a result of analyzing the values of FP key parameters, a set of FPs suitable for studying the process of bacterial cells infection by fluorescence microscopy was found.

Keywords: fluorescent protein, optical microscopy, bacterial cell, phiKZ bacteriophage

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ХАРАКТЕРИЗАЦИЯ ФЛУОРЕСЦЕНТНЫХ БЕЛКОВ ДЛЯ ИЗУЧЕНИЯ МОРФОЛОГИЧЕСКИХ ПЕРЕСТРОЕК ВНУТРИ ОДИНОЧНЫХ БАКТЕРИАЛЬНЫХ КЛЕТОК ПРИ ИНФЕКЦИИ БАКТЕРИОФАГОМ phiKZ С ПОМОЩЬЮ ОПТИЧЕСКОЙ МИКРОСКОПИИ

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Аннотация. В работе проведена оценка ключевых свойств флуоресцентных белков (ФБ) с целью их использования при наблюдении процессов перераспределения целевых белков между цитоплазмой и псевдоядром, которое образуется в ходе инфицирования бактериальной клетки бактериофагом phiKZ. С помощью флуоресцентной микроскопии были изучены четыре ФБ: mNeonGreen, mCherry, ECFP и EYFP. Установлено, что белок EYFP, в отличие от остальных, локализуется в псевдоядре во время инфекции, что послужило основанием для его исключения из дальнейших опытов. В результате анализа полученных значений ключевых параметров ФБ был выявлен набор ФБ, перспективных для исследования процесса инфицирования бактериальных клеток.

Ключевые слова: флуоресцентный белок, оптическая микроскопия, бактериальная клетка, бактериофаг phiKZ

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Introduction

While the genomes of giant bacteriophages have a great coding potential, most genes do not have detected homologous sequences in other organisms, so it is difficult to determine their functions. The reason for the keen interest towards phiKZ-like bacteriophages is that they form a spherical structure called the pseudonucleus in the center of the cell throughout their life cycle [1].

The pseudonucleus is coated with a protein shell protecting the bacteriophage's DNA from the defense systems of the infected cell, such as restriction-modification systems and CRISPR/Cas aimed at destroying foreign DNA [2, 3]. The pseudonucleus is localized through the phage-encoded cytoskeletal structure produced by the tubulin-like protein TubZ [1, 4] . It was established in [1] that during infection, the pseudonucleus separates not only phage DNA but also the transcription factory from the cell cytoplasm, while the translation processes are localized to the cytoplasm. Furthermore, enzyme transport into the lumen of the pseudonucleus was found to be selective: experiments with phage non-virion RNA polymerase confirmed that proteins can translocate through the proteinaceous shell post-transcriptionally, suggesting a mechanism for selective transport [5, 6].

Nuclei of eukaryotic cells have the same properties. In view of this, it can be hypothesized that the predecessors of phiKZ-like viruses, capable of assembling pseudonuclei, might have played the key role in evolutionary nucleogenesis of modern eucaryotic cells [7].

Further studies into the mechanisms behind the phage pseudonucleus assembly, the protein product translocation into the lumen, selective transport and other processes of intracellular growth in bacteriophages can contribute towards drawing comparisons with the life cycle of eucaryotic cells, subsequently gaining deeper insights into the evolutionary pathways of multicellular life on Earth.

We focused on the phiKZ phage, which is the first gigantic bacteriophage ever described [8] (its genome size is 280 kilobase pairs (kbp)); this bacteriophage infects *Pseudomonas aeruginosa*-like bacteria [8]. The pseudonucleus of this phage is still poorly characterized, in contrast to its relative 201phi2-1 [5].

Fluorescent microscopy is an effective method for studying bacterial cells. Fluorescence imaging allows to monitor the changes occurring to the cell infected by a bacteriophage. A report on gigantic phages [1] fusing the red fluorescent protein (FP) mCherry to the target protein, detected that the proteinaceous shell of the pseudo-nucleus essentially consists of the Gp54 protein. This largely determined our approach to studying the proteins of the phiKZ bacteriophage throughout the cellular infection. However, it was also discovered that the mutated GFP, GFPmut1, are capable of translocating into the pseudonucleus of the phiKZ bacteriophage [3]. This means that different FPs should be selected and tested for further experiments.

We examined the expression in several FP genes taken for the experiments in bacteriophage-infected cells to select the optimal variants on the progression of the phiKZ bacteriophage in *P. aeruginosa* cells. We managed to detect one more FP (EYFP) capable of translocation through the proteinaceous pseudonucleus shell.

Fluorescence methods

Fluorescence techniques are widely used to research cellular compartments, proteins, and DNA. Different methods can be applied for fluorescent tagging depending on the samples considered. Organic dyes with low molecular weight are actively used for staining cell components. For example, lipophilic dyes such as Nile Red are used to stain cell membranes, MitoRed and MitoTracker Red [9] are used to stain mitochondrial membranes, while DAPI, Propidium Iodide, YOYO-1 and others are often used to stain DNA in the cell [1]. Some dyes can stain only dead cells, allowing to estimate the number of viable bacteria in the population. The range of dyes for particular tasks is limited, so it is impossible to selectively stain any biomolecules in most cases.

Selective fluorescent tagging is often performed with antibodies that have highly specific binding to the target. Antibodies can be modified by a fluorophore molecule, allowing to track the position of the antibody (and, respectively, its target) to in the cell via fluorescence microscopy [10]. However, this method also has limitations: in particular, the target can only be observed in fixed dead cells, antibodies specific to the molecule under consideration should be produced (the terms 'molecule of interest' (MOI) or 'protein of interest' (POI) are used for brevity from now on), and the modified antibodies are expensive.

FPs are extremely popular for imaging protein structures. There is a wide variety of FPs with different excitation and emission spectra, different brightness, maturation time, sensitivity to pH, etc. This way, researchers are able to select the proteins most appropriate for each particular experiment. FP genes are fused in a single reading frame with POI genes to synthesize fusion proteins. Expression of such a gene produces a protein macromolecule containing both the POI and the FP. This allows to detect the location of fluorescent protein, and therefore the POI by fluorescence microscopy of living cells over long time periods. However, in the case of fusion proteins, the FP should reach the correct conformation in the cell so that it does not prevent the POI from performing its function. An important criterion for further experiments is the option for tracking the POI within living cells over long time periods, so we chose fluorescent tagging of the POI.

Selection of fluorescent proteins

To monitor synthesis and localization of proteins in real time over a long period, we had to select the FPs with the optimal characteristics that could be convenient for simultaneously tracking several proteins in a single cell.

We considered the following FPs: ECFP, EGFP, mNeonGreen, EYFP, TagRFP, mCherry, and FusionRed (Table 1); data were extracted from the FPbase fluorescent protein database [11].

The most important characteristics of the FP in our study were the *in vivo* maturation time, fluorescence brightness, and stability.

In terms of brightness, calculated as the multiplication of the molar extinction coefficient and quantum fluorescence yield, mNeonGreen, EYFP, and TagRFP FB turned out to be the most intense. However, the red fluorescent protein TagRFP proved largely inferior to others in terms of maturation time, also serving as an essential characteristic, since cultivating bacterial colonies for excessively long periods can distort the results obtained. Another red protein, FusionRed, did not fit this requirement either, despite its high pH stability. Thus, mCherry appeared to be the best red protein, sufficiently stable and fast-maturing, even though it is still inferior to TagRFP in terms of brightness.

The proteins mNeonGreen and EYFP are better than the protein EGFP in terms of fluorescence brightness and also have comparably shorter maturation, so we excluded the green protein EGFP from analysis. Even though the yellow protein EYFP has weak pH stability (high pKa), we still decided to test it since its other parameters are at acceptable levels. The protein ECFP is a stable monomer, however, it has a rather low brightness, so it was not considered for further experiments.

Table 1

Key characteristics for several fluorescent proteins

Parameter	Parameter value for FP						
	ECFP	EGFP	mNG	EYFP	TagRFP	mCherry	FR
Excitation peak, nm	434	488	506	513	555	587	580
Emission peak, nm	477	507	517	527	584	610	608
EC, $M^{-1} \cdot cm^{-1}$	32.5	55.9	116	67.0	100	72	94.5
QY	0.40	0.60	0.80	0.67	0.48	0.22	0.19
Calculated intensity	13.00	33.54	92.80	44.89	48.00	15.84	17.95
pKa	4.7	6.0	5.7	6.9	3.8	4.5	4.6
Maturation time, min	–	60	10	9	100	15	130
In vivo structure	M	WD	M	WD	WD	M	M

Notations: EC is the molar extinction coefficient at maximum absorption, QY is the quantum fluorescence yield, pKa is the pH value at which the fluorescence intensity drops to 50% of the maximum, M refers to the monomer, WD to weak dimerization. Note. The brightness is calculated as the product of EC × QY.

Based on this reasoning, we selected only three FPs from the list for testing: mNeonGreen, EYFP, and mCherry. We prepared gene constructs containing the genes of these FPs for subsequent expression in cells.

Experimental procedure

Fluorescent microscopy allows to visualize the locations of FPs in cells. However, a small amount of FPs may complicate subsequent image processing and analysis of protein localization in the cell. To improve image quality, cells with the above-mentioned gene constructs were grown in the presence of an inducer in a medium activating protein synthesis in the cell. Thus, the cells already contain some amount of FP by the start of the experiment. Cells were infected with a bacteriophage, immediately placed on a slide with an agarose pad containing inducer and culture medium, and sealed with a cover glass. Uninfected cells were used as controls. The sample was placed under a microscope for subsequent imaging. An inverted Nikon Eclipse Ti-E microscope (Japan) equipped with an incubator was used for fluorescence microscopy under transmitted light as well as red and yellow/green fluorescence. A set of xRed-4040C bandpass filters was used to detect red fluorescence, YFP-2427B to detect yellow or green fluorescence (the filters were produced by Semrock (USA) in both cases). An ND8 filter (Fuijimi, Japan) was used during the imaging to avoid FP destaining in cells. The exposure was the same for all proteins, selected in such a way that the signal from the FP was not too strong to prevent overshooting. Images were acquired at 1 frame per 10 min over 3 h at 37 °C using the MicroManager script.

Results and processing

The images acquired were processed in ImageJ software. First, the background was subtracted from the images, then the relative brightness intensity was measured in the cells outside the pseudonucleus (I_0) and inside it (I_1) for each FP tested. The localization of the pseudonucleus in the cells was confirmed by DNA staining with the DAPI fluorescent dye (Beckman Counter Life Sciences, USA). Since the absolute amount of FPs is individual for each cell, we calculated its relative amount as the ratio I_1/I_0 in the same infected cell. Next, we found the mean value and the standard deviation among the infected cells considered for this ratio. The computational results are summarized in Table 2.

Table 2

Quantitative computations for the contents of selected fluorescent proteins in infected cells

Fluorescent protein	Number of cells considered	Mean value I_1/I_0	Standard deviation
EYFP	45	2.10	0.50
mNeonGreen	37	0.80	0.11
mCherry	50	0.66	0.14

Notation: I_1/I_0 is the ratio of relative fluorescence brightnesses in the cell inside the pseudonucleus (I_1) and outside it (I_0).

Notably, FPs are distributed uniformly in uninfected cells, while the behavior of the protein in the cell varied throughout the infection, depending on the type of protein (Fig. 1). The fluorescent protein EYFP was redistributed to the pseudonucleus throughout the infection, so that the mean fluorescence intensity inside the pseudonucleus was twice as high than that outside it. Admittedly, this behavior of EYFP limits the potential for using it in further studies. On the other hand, the proteins mNeonGreen and mCherry did not penetrate the pseudonucleus, as the fluorescence intensity inside it decreased by an average of 20–35% (depending on the protein type) under similar conditions.

Brightness of proteins in the cells is another crucial parameter for analyzing the data obtained by fluorescence microscopy. The higher the protein's fluorescence intensity, the easier it is to process and analyze the images. The mean fluorescence intensity was calculated for each FP from 80 uninfected cells, along with the corresponding standard deviation in the first frame. The results of these calculations are as follows (in relative units):

Fluorescent protein	Mean fluorescence intensity, rel. units
mNeonGreen	550 ± 390,
mCherry	210 ± 110,
EYFP	250 ± 100.

The results present clear evidence that the protein mNeonGreen best meets the requirements for further experiments, since it does not redistribute into the pseudonucleus and also exhibits the highest brightness among the proteins considered. Even though the mean fluorescence intensity of the protein EYFP was slightly higher than that of mCherry, the former, unlike the latter, was redistributed to the pseudonucleus, so it was rejected. Protein destaining in this experiment does not impose considerable limitations, since the imaging rate was fairly low (1 frame per 10 min), so it was possible to synthesize the new FP over the time between frames.

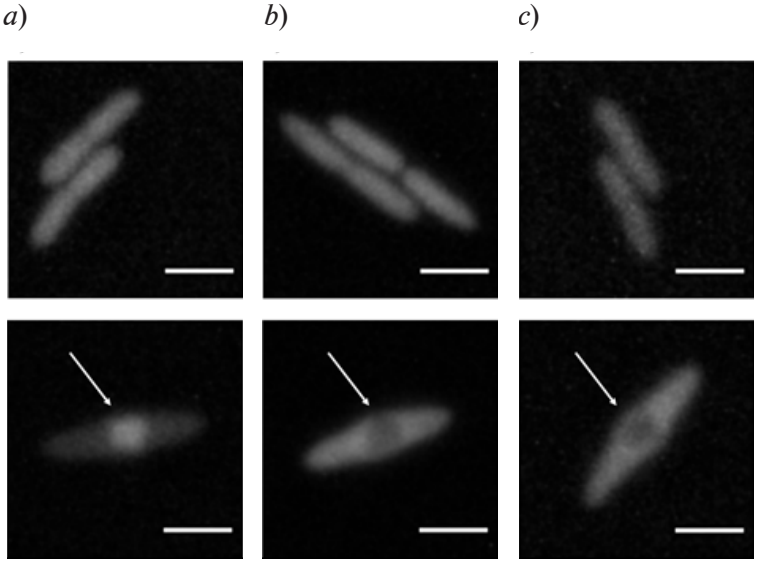

Fig. 1. Micrographs of uninfected (top row) and infected (bottom row) cells containing fluorescent proteins EYFP (*a*), mNeonGreen (*b*) and mCherry (*c*) 60 min after the onset of infection. The white arrows point to the pseudonuclei. The scale bars are 2 μm long

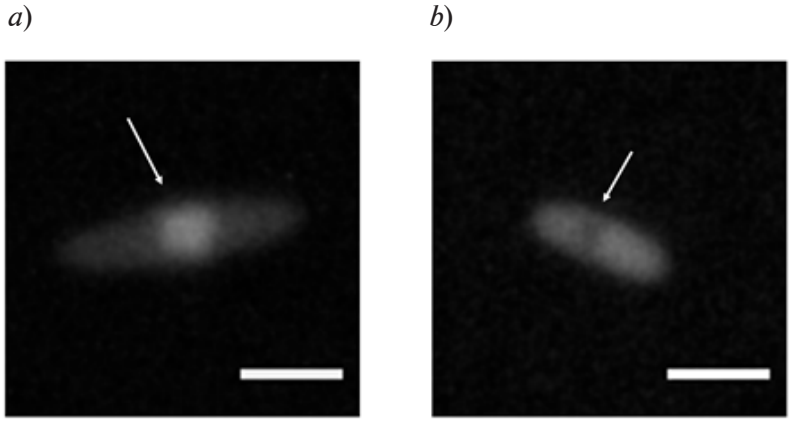

Fig. 2. Micrographs of infected cell preparations containing fluorescent proteins EYFP (*a*) and ECFP (*b*). The white arrows point to the pseudonuclei The scale bars are 2 μm long

Estimating the relationship between FP amino acid sequences and their localizations within bacteriophage-infected cells

In contrast to other types of the initial GFP (specifically, sfGFP and pMutinGFP), the protein GFPmut1 that redistributes to the pseudonucleus contains amino acid substitutions at positions 99, 153, and 163. Serine was replaced by phenylalanine (99F) at position 99, tyrosine by methionine (153M) at position 153, alanine by valine (163V) at position 163, while 99-phenylalanine and 153-methionine made the greatest contributions to redistribution to the pseudonucleus according to [3]. EYFP, GFPmut1, and ECFP PBs are all derived from the same protein. As reported in [3], unlike the other two proteins, ECFP is not redistributed to the pseudonucleus. To verify this result, we prepared an expression plasmid containing the ECFP gene and analyzed its localization under the given experimental conditions. According to the data obtained, the protein ECFP indeed does not penetrate the pseudonucleus (Fig. 2). We also analyzed its amino acid sequence and aligned it with the proteins EYFP and GFPmut1 (Fig. 3).

As seen from Fig. 3, the protein EYFP contains both amino acids in its sequence; according to [3], these amino acids are responsible for redistributing the FP inside the pseudonucleus, which is in apparent agreement with our results. However, the protein ECFP, which also contains phenylalanine at position 99, does not redistribute to the pseudonucleus, remaining in the cytoplasm. The protein ECFP carries the same amino acid at position 153 as the proteins EYFP and EGFP.

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EYFP        MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT   60
ECFP        MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT   60
GFPmut1     -MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT   59
             :************************************************************

EYFP        LVTTFGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL   120
ECFP        LVTTLTWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL   120
GFPmut1     LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL   119
            ****: :*:***:*********************************************
                                  99
EYFP        VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA   180
ECFP        VNRIELKGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLA   180
GFPmut1     VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA   179
            ************************ ******.*********.****************
                                     153       163
EYFP        DHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK   239
ECFP        DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK   239
GFPmut1     DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK   238
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Fig. 3. Alignment of amino acid sequences for related fluorescent proteins EYFP, ECFP, and GFPmut1. The amino acids 99, 153 and 163 of the protein GFPmut1 are underlined and tagged. All three types have phenylalanine (F) at position 99

Thus, the data we have obtained suggest that the mechanism behind the FP distribution between the pseudonucleus and the cytoplasm does not depend on the presence of particular amino acids at certain positions, but is most likely associated with the general changes in the structure that they cause.

Conclusion

The study aimed to find fluorescent proteins (FPs) suitable for monitoring their redistribution between the cytoplasm and the pseudonucleus resulting from infection of a bacterial cell by the phiKZ bacteriophage.

In view of this, we selected FPs for the experiments on imaging living cells. The FPs were then tested for brightness levels, with distribution of FPs in infected cells subsequently constructed. The experiments indicate that the ECFP, mNeonGreen and mCherry proteins do not enter the pseudonucleus, while the protein EYFP is redistributed to the pseudonucleus during infection and is therefore unsuitable for further experiments. Moreover, further experiments allowed to disprove the traditional theory about the relationship of specific mutations in fluorescent proteins with their position relative to the pseudonucleus.

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