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# Generation and characterization of some UnaG mutants

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**ABSTRACT**

*UnaG protein from Japan eel (Anguilla japonica) is a novel fluorescent protein with binding domain that acquires fluorescence when bound to unconjugated bilirubin (UC-BR). In this study, several point mutations (F17M, N57D, N57E, N57R, L41F, Y99F_Y134W, Y99M_Y134M, and W9F_W103F) were made on the UnaG nucleotide sequence via using a method for sequence and ligation independent cloning (SLIC). The aim of the mutations on UnaG is to figure out the change in fluorescence properties. The new mutagenic vector was transformed into the commercial competent cells (E. coli Mach1) by using heat shock at 42 °C for 2 minutes. Transformed cells were grown on and selected from the LB agar plate with ampicillin. (1:1000). The DNA sequencing results show that all these mutations have done correctly. The expression of the mutant proteins was made in the pTOLT expression system by inducing with IPTG. Cells were collected with high speed centrifugation.*

**Key words**: UnaG, mutagenic vector, pTOLT expression system

## INTRODUCTION

UnaG protein from Japan eel (Anguilla japonica) is a novel fluorescent protein with binding domain that acquires fluorescence when bound to unconjugated bilirubin (UC-BR). In this study, several point mutations (F17M, N57D, N57E, N57R, L41F, Y99F_Y134W, Y99M_Y134M, and W9F_W103F) were made on the UnaG nucleotide sequence via using a method for sequence and ligation independent cloning (SLIC). The aim of the mutations on UnaG is to figure out the change in fluorescence properties. The new mutagenic vector was transformed into the commercial competent cells (E. coli Mach1) by using heat shock at 42 °C for 2 minutes. Transformed cells were grown on and selected from the LB agar plate with ampicillin. (1:1000). The DNA sequencing results show that all these mutations have done correctly.

The expression of the mutant proteins was made in the pTOLT expression system by inducing with IPTG. Cells were collected with high speed centrifugation. Before disrupting the cells, lysozyme enzyme was added to make break up the cells easier, some protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine) were added for the protection from proteases of the protein and DNase and RNase were added on the cell pellet to avoid the DNA and RNA contaminations. Ultracentrifugation was applied on the cell lysate. Ni- NTA affinity chromatography system was used to get the pure mutant proteins from supernatant. SDS-PAGE and semi-dry Western blot were applied on the protein for the qualitative analyse. The pure protein bands were observed on the SDS-PAGE gel image.

Additionally, the spectroscopic features of purified mutant proteins were measured after adding fresh UC-BR on fluorescence spectrophotometer. Excitation and emission spectra of the mutant proteins are similar; even so they have different fluorescence intensity at the same concentration. This study suggests that mutant UnaG proteins can be used to detect UC-BR level of cells/tissue. We introduced enhanced UnaG (eUnaG), a ligand-activatable fluorescent protein, for conventional and super-resolution imaging of subcellular structures in the mammalian cells. eUnaG is a V2L mutant of UnaG with twice brighter bulk fluorescence. We previously discovered the reversible fluorescence switching behavior of UnaG and demonstrated the high photon outputs and high localization numbers in single-molecule localization microscopy (SMLM). In this study, we showed that the fluorescence of eUnaG can be switched off under blue-light illumination, while a high concentration of fluorogenic ligands in the buffer can efficiently restore the fluorescence, as in UnaG. We demonstrated the capacity of eUnaG as an efficient protein label in mammalian cells, as well as for SMLM by utilizing its photoswitchable nature.

While cytosolic UnaG proteins showed aggregated patches and fluorescence reduction at high expression levels, eUnaG-labeled protein targets successfully formed their proper structures in mammalian cells without notable distortion from the endogenous structure in the majority of transiently expressing cells. In particular, eUnaG preserved the vimentin filament structures much better than the UnaG. eUnaG provided similarly high single-molecule photon count distribution to UnaG, thus also similarly high resolution in the super-resolution images of various subcellular structures. The sampling coverage analysis of vimentin filaments in SMLM images showed the improvement of labeling efficiency of eUnaG. eUnaG is a high-performance fluorescent protein for fluorescence and single-molecule localization imaging in green emission with minimal labeling artifact. Fluorescent proteins (FPs) are workhorses in live-cell fluorescence microscopy due to the facile and specific labeling of the target proteins. Likewise, FPs have been extensively used in single-molecule localization microscopy (SMLM). The first demonstrations of SMLM used photoactivatable fluorescence proteins along with organic dyes. The on–off transition of fluorescence emission is required in the SMLM for the temporal separation of individual molecules within the diffraction-limited area, allowing high precision localization of the individual molecules. The quality of the resultant super-resolution image is determined by two photophysical characteristics of the fluorophores. The photon number emitted from the fluorescent state determines the localization precision of determining the centroid position of a single fluorophore. The number of switching cycles and the fraction of time spent in the fluorescent state, termed as the on–off duty cycle, are related to the labeling density and the Nyquist resolution. Most of the FPs offer lower photon numbers than the organic fluorophores, hence resulting in lower spatial resolutions. Furthermore, FPs often show irreversible fluorescence transition that restricts the spot density or transition between two different emission states that complicates multicolor applications. Recently, we introduced UnaG protein as an efficient SMLM probe for multicolor live-cell imaging. UnaG is a ligand-activatable FP with a fluorogenic ligand, bilirubin. The ligand and apoUnaG protein are non-fluorescent in solution and become fluorescent upon binding to form holoUnaG. Photoswitching of UnaG is mediated by repetitive binding of bilirubin after photooxidation of the ligand followed by detachment of the damaged bilirubin from the protein. UnaG offers the highest photon numbers among blue-absorbing FPs and easily controllable switching kinetics.

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