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Development of a Novel Blue Fluorescent Gene-encoded Calcium Indicator Modified from GCaMP3

Chorok Yum¹ · Taekyoung Ahn¹ · Won-Sik Shim^{1,2,3}

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Abstract

Intracellular calcium can be monitored by various calcium-specific fluorescent dyes including gene-encoded calcium indicators (GECI). GCaMP is a widely-used GECI that emits green fluorescence proportional to the level of intracellular calcium. However, since many tagging proteins also emit green fluorescence, GCaMP cannot be used with another green fluorescent protein. Therefore, it would be ideal to develop a GECI that has a distinct color profile other than green. In this regard, we developed a novel blue fluorescent calcium indicator modified from GCaMP called Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. Specifically, a simple threonine to histidine substitution to a green fluorescent Cys³³⁰-GCaMP3 successfully changed its fluorescence to blue (Cys³³⁰-BCaMP3, B for blue). Furthermore, a couple of additional amino acid substitutions resulted in more enhanced blue fluorescence intensity. Among other Cys³³⁰-BCaMP3 variants, it was found that Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 exhibited the strongest blue fluorescence intensity. When Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 was co-expressed with TRPA1 – a non-selective cation channel – in HEK293T cells, it showed moderate blue fluorescence. One of the drawbacks of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 was that the fluorescence intensity was not enough when cells were cultured under 37°C. However, this limitation was circumvented by lowering cell culture temperature to 28°C, allowing much more enhanced blue fluorescence. Although Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 mandates further optimization, the present study has found a promising blue fluorescent GECI that is derived from GCaMP3.

Keywords Calcium dye · Genetically encoded calcium indicators · GCaMP3 · Green fluorescent protein · Blue fluorescent protein

Introduction

Calcium, as a second messenger, plays a significant role in various signaling pathways at cellular level [1]. As cellular responses are often initiated by abrupt changes in intracellular calcium levels, measuring the calcium changes are vital to understand intricate signaling cascades in cells. Fortunately, measurement of calcium levels can be easily achieved by calcium-specific fluorescent indicators known as “calcium dyes”. These calcium indicators share common characteristics

that (a) fluorescence is only emitted in the presence of intracellular calcium, and (b) the intensity of fluorescence is proportional to the calcium levels. Most of the popular calcium dyes are small molecule compounds such as Fura-2 and Fluo-3 [2, 3]. However, there also is a different class of indicator based on proteins that can emit fluorescence, known as genetically encoded calcium indicators (GECI). GCaMP, one of the popular GECI, is widely used for various research fields both *in vitro* and *in vivo* experimental conditions [4].

GCaMP is a calcium-specific fluorescent protein composed of multiple domains derived from various sources. In detail, these regions include enhanced green fluorescent protein (EGFP), calmodulin (CaM) and the Ca²⁺/CaM-binding “M13” peptide (M13pep) [5]. Original green fluorescent protein (GFP), which was cloned from the jellyfish *Aequorea victoria*, and has its unique dual excitation peaks (395 nm and 475 nm) and single emission peak (504 nm) profile that results in its unique green fluorescent color. This dual excitation peak of GFP is corrected by the single mutation (Ser65Thr) to GFP, which is named as EGFP, has a single excitation maximum peak at 488 nm and emission at 504 nm [6, 7].

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Since after the initial development of GCaMP, various attempts were made to enhance overall features of GCaMP. As a result, numerous modifications were made to the GCaMP, and various enhanced derivatives such as GCaMP3 were discovered. However, it was found that GCaMP3 can be further optimized by additional alterations to certain amino acids, resulting in much more enhanced fluorescence [5]. Indeed, our group had reported earlier that the single Thr330Cys substitution in GCaMP3 can exhibit improved characteristics as a calcium indicator compared to the original GCaMP3 [8]. From our previous study, we realized that GCaMP3 has a fundamental structure that can be easily optimized and/or modified to enhance its calcium-specific fluorescence.

It is not surprising that green fluorescence is the most widely used color in various microscopic images due to the popularity of EGFP. However, since many fluorescent indicators have the same green color profile, there may be “conflict” in the color if the researchers want to detect more than two target-of-interests simultaneously. For instance, if the protein-of-interest is tagged with EGFP, another green fluorescent dye cannot be used at the same time. To circumvent this problem, researchers have found that simple mutations of EGFP can induce different fluorescence colors. For instance, blue fluorescence protein (BFP) is simply made by replacing tyrosine with histidine at position 66 of EGFP [7, 9]. Therefore, it is believed that simple amino acid substitution can completely change the color profile of fluorescent proteins [10, 11].

If we scale up this “color-conflict” issue to intracellular calcium measurement cases, it is evident that the activity of an EGFP-tagged calcium-permeable ion channel cannot be measured with GECI such as GCaMP3 simultaneously. In this scenario, it would be ideal if there is a different type of GECI with non-green fluorescent profiles. Because the green color can be changed into blue by simple amino acid substitution of EGFP, we sought to shift the green fluorescence of Cys³³⁰-GCaMP3 into blue, followed by subsequent optimization of the fluorescence intensity based on site-directed mutagenesis approach in the present study.

Materials and Methods

Genes

The full-length cDNA of GCaMP3 subcloned into pEGFP-N1 backbone was purchased from Addgene (Plasmid #22692, Cambridge, MA, US). Human TRPA1 cDNA was subcloned in the pcDNA3.1 mammalian expression vector. All cDNAs were verified by sequencing and showed 100% identity to NCBI Genbank sequences.

Site-directed Mutagenesis

Four different site-directed mutations were performed using site-directed mutagenesis kit, following the manufacturer's protocol (Muta-Direct™ Site Directed Mutagenesis Kit, iNtRON, Seongnam, Korea). Primer sequences used in the mutagenesis are summarized in Table 1.

Cell Culture and Gene Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and Zell Shield (Minerva biolabs, Berlin, Germany). Cells were grown at either 37 or 28°C in a humidified atmosphere of 5% CO₂. Cells are subcultured every 3 or 4 days. Genes were transfected with FuGENE®HD (Promega, Madison, WI, USA) according to manufacturer's protocol. Imaging experiments were performed 24 or 48 h after transfection.

Calcium Imaging With Fluorescent Microscope

Intracellular calcium was measured using a calcium imaging technique with an inverted fluorescent microscope (DMi8; Leica, Wetzlar, Germany) by a previously described method. Briefly, HEK293T cells were cultured on a lysine-coated eight-well chamber (Lab-Tek; Naperville, IL, USA). The cells were transfected with genes of interest. A day after transfection, the culture medium was replaced with normal bath solution NBS [140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)]. Fluorescent microscopic images were captured by LAS X (Leica). Fluorescence intensity was measured by ImageJ.

Statistical Analysis

Data were presented as mean ± SEM. For comparison between the two groups, the student's t-test was applied by GraphPad Prism.

Results

Tyrosine to Histidine Substitution in GCaMP3 Changed Green Into Blue Fluorescence

As mentioned above, it has been reported that a single amino acid substitution of EGFP at position 66 allows shift of its fluorescent color [7, 12]. In detail, replacement of tyrosine at position 66 with histidine (Tyr66His) changes the green fluorescence into blue. To verify if Tyr66His substitution indeed alters the fluorescence color, site-directed mutagenesis was performed on EGFP. As shown in Fig. 1a, Tyr66His

Table 1 Primer sequences used for introduction of BCaMP3 variants

Name	Ser ²²² -Ala ²²⁹ -Cys ³³⁰ -BCaMP3	Ala ²²⁹ -Cys ³³⁰ -BCaMP3	Ser ²²² -Cys ³³⁰ -BCaMP3	Cys ³³⁰ -BCaMP3
Forward	GACCACCCTGAGCCACGG CGTGCAGTGC TTCGCCCGCTACCCC	GACCACCCTGACCCACGG CGTGCAGTGTCTCGCCCGCTACCCC	CTCGTGACCACCCT GAGCCACGG CGTGCAGTGCTTC	CTCGTGACCACCCT GACCCACGG CGTGCAGTGCTTC
Reverse	GGGGTAGCGGGCGAAG CACTGCACGC CGTGGCTCAGGGTGGTC	GGGGTAGCGGGCGAAG CACTGCACGCCGTGGGTCAG GGTGGTC	GAAGCACTGCACGC CGTGGCTCAG GGTGGTCACGAG	GAAGCACTGCACGC CGTGGGTC AGGGTGGTCACGAG

substitution indeed changed the green fluorescence of EGFP into blue. Thus, it was confirmed that Tyr66His is sufficient to evoke a shift into blue from its original green fluorescence.

As shown in Fig. 1b, GCaMP3 – a gene-encoded calcium indicator with green fluorescence – also has the same amino acid sequences that are found in EGFP, although the tyrosine 66 in EGFP corresponds to position 223 in GCaMP3. As we previously found that Cys³³⁰-GCaMP3 exhibits much stronger fluorescence, this Cys³³⁰-GCaMP3 was used as a backbone for Tyr223His substitution and observed if the fluorescence color indeed changed or not. As shown in Fig. 1c, the Tyr223His substitution of Cys³³⁰-GCaMP3 indeed changed fluorescence emission from green to blue (referred to as Cys³³⁰-BCaMP3, as “B” stands for blue), indicating that Tyr223His substitution is effective for the fluorescence shift. However, the intensity of blue fluorescence from Cys³³⁰-BCaMP3 was relatively weak and faint, and the blue fluorescence did not last enough probably due to photobleaching.

Additional substitutions to Cys³³⁰-BCaMP3 Resulted in Significant Increase of the Blue Fluorescence Intensity

We then investigated if additional amino acid substitutions in Cys³³⁰-BCaMP3 can further improve overall intensity and stability of its blue fluorescence. As mentioned earlier, Ser65Thr of GFP is the critical mutation that allowed development of EGFP from the original GFP. This is because the mutation resulted in prevention of chromophore protonation of EGFP, which changed the excitation profile from dual to single excitation peak at 488 nm. However, threonine substitution at this position is linked to low blue fluorescence quantum yield, suggesting that this position could be responsible for the reduced blue intensity of Cys³³⁰-BCaMP3 [7]. Thus, it is expected that the position corresponding to the Ser65Thr of EGFP might be a critical residue that determines the blue fluorescence intensity in Cys³³⁰-BCaMP3 as well. Moreover, numerous attempts were made to improve the fluorescence intensity of GFP-derived proteins. Among various mutations, it was reported that Ser72Ala substitution on both

EGFP and EBFP did improve the protein folding and fluorescence brightness [7]. Therefore, it was investigated if mutations at position 222 and 229 in Cys³³⁰-GCaMP3 (the position 222 and 229 correspond to the position 65 and 72 in EGFP, respectively).

As shown in Fig. 2, introduction of T222S mutation to Cys³³⁰-BCaMP3 (Ser²²²-Cys³³⁰-BCaMP3) improved blue fluorescence intensity compared to Cys³³⁰-BCaMP3. Moreover, the double mutations at T222S/S229A in Cys³³⁰-BCaMP3 (Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3) also resulted in intense blue fluorescence intensity compared to that of Cys³³⁰-BCaMP3. In fact, the double T222S/S229A mutations in Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 exhibited much enhanced fluorescence intensity than that of Ser²²²-Cys³³⁰-BCaMP3. However, S229A mutation alone in Cys³³⁰-BCaMP3 (Ala²²⁹-Cys³³⁰-BCaMP3) exhibited did not show appropriate blue fluorescence and suffered from photobleaching. Taken together, these results demonstrate that additional mutations (T222S/S229A) in Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 exhibited the strongest blue fluorescence intensity compared to other Cys³³⁰-BCaMP3 variants.

Co-transfection of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 with TRPA1 Exhibited Calcium-dependent Fluorescence Changes

Although the green-to-blue shift was successfully achieved by Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3, it was still questionable whether it can still emit fluorescence based on intracellular calcium changes. Therefore, TRPA1, a non-selective cation ion channel, was transiently transfected in HEK293T cells along with Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3, and verified if transient intracellular calcium changes can be measured after AITC, a specific agonist of TRPA1 [13]. When Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 and TRPA1 was used 1:1 ratio (0.5 µg DNA, respectively) for the co-transfection, however, it was found that overall blue fluorescence signal was slightly weak (data not shown). Nevertheless, these results demonstrated that Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 is able to detect intracellular calcium changes, although the sensitivity and intensity of the blue fluorescence may not be ideal yet.

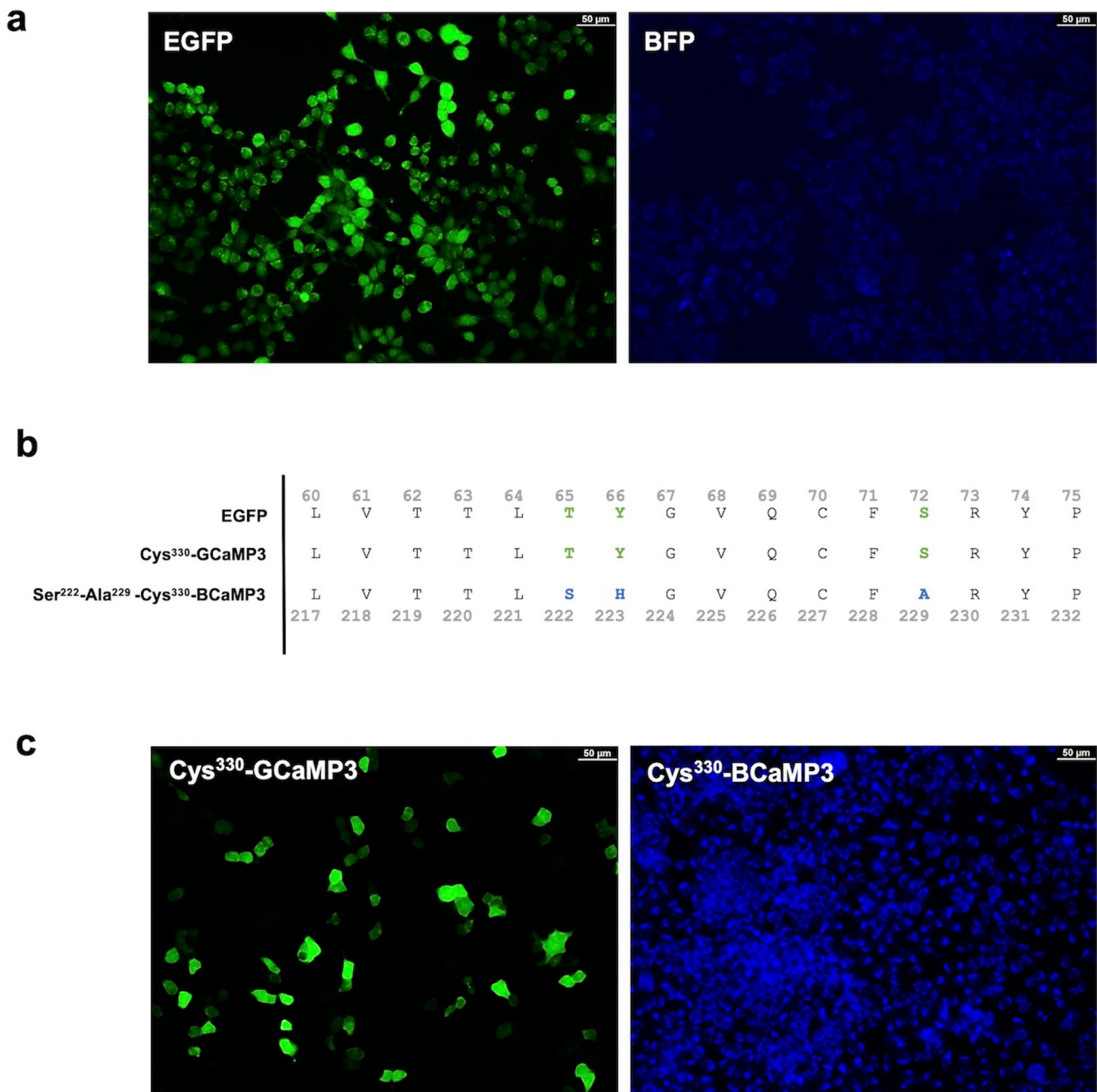


Fig. 1 Blue fluorescence of Cys³³⁰-BCaMP3 is obtained by Y223H substitution of Cys³³⁰-GCaMP3. **a** Tyr66His substitution of EGFP changed its green fluorescence (left) into blue fluorescence (right), verifying that the specific position determines the fluorescence color. **b** Sequence alignment of EGFP, Cys³³⁰-GCaMP3 and Ser²²²-Ala²²⁹-

Cys³³⁰-BCaMP3. T65, Y66 and S72 residue on EGFP corresponds to T222, Y223 and S229 of Cys³³⁰-GCaMP3, respectively. **c** Unmodified Cys³³⁰-GCaMP3 emitted green fluorescence (left), whereas Cys³³⁰-BCaMP3 (Y223H mutation of Cys³³⁰-GCaMP3) showed blue fluorescence (right)

Low Temperature Condition for Cell Culture Significantly Improved Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 Expression and Activity

To further improve the fluorescence property of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 as a calcium indicator, we finally investigated if the cell culture temperature can impact overall features of the blue fluorescence [14, 15]. For this experiment, we again

transiently co-transfected the two genes, both TRPA1 and Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. In detail, HEK293T cells were cultured under two different temperature conditions, either 37°C vs. 28°C while BCaMP3:TRPA1 ratio was fixed to 1:1. As shown in Fig. 3, it was found that cells cultured under 28°C showed increased overall blue fluorescence intensity as well as a high number of cells emitting blue fluorescence. Among other BCaMP3 variants, Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 showed the

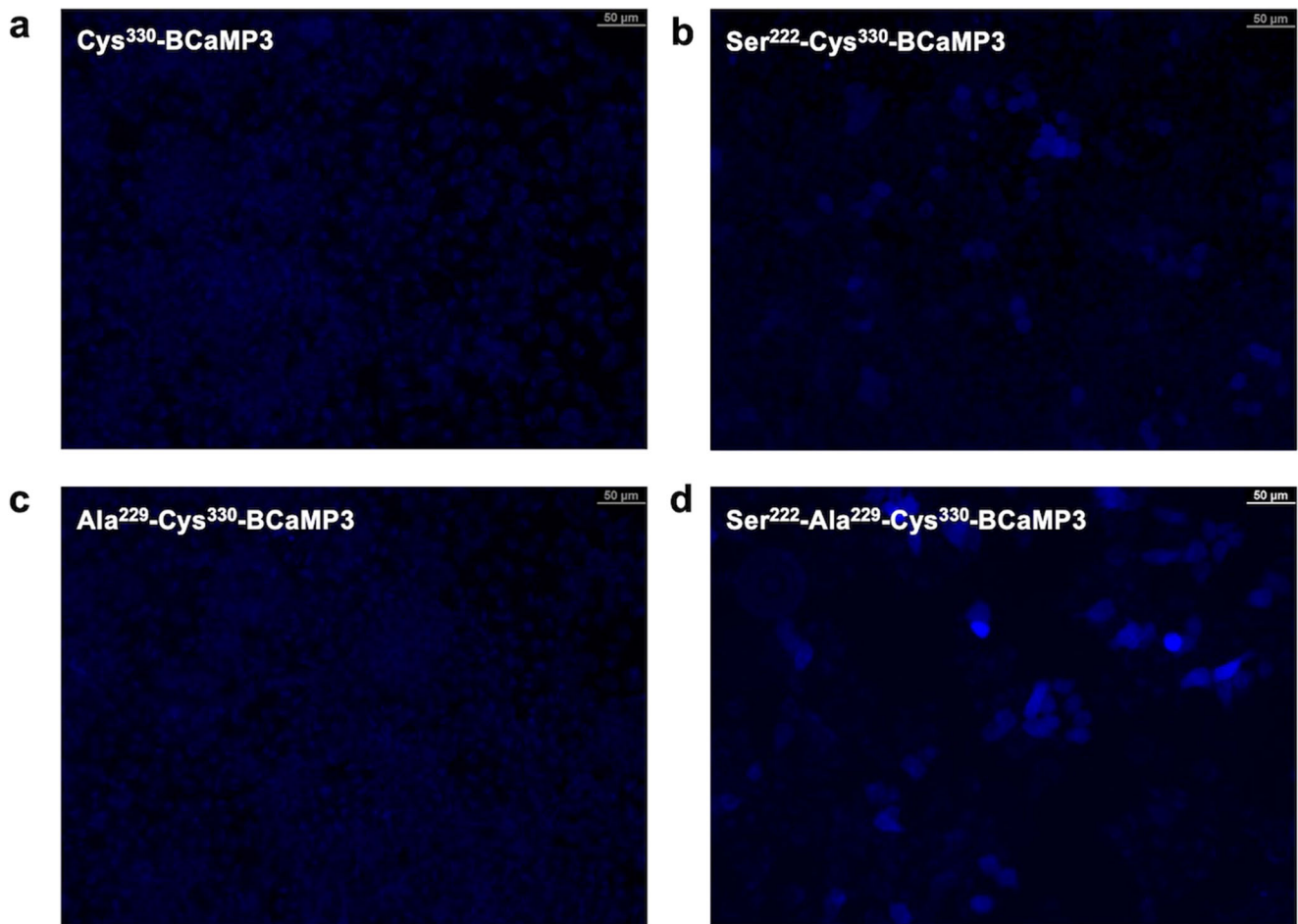


Fig. 2 Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 showed the strongest fluorescent intensity among other variants. Blue fluorescence intensities of Cys³³⁰-BCaMP3 variants were inspected. **a** Cys³³⁰-BCaMP3; **b** Ser²²²-Cys³³⁰-

BCaMP3; **c** Ala²²⁹-Cys³³⁰-BCaMP3; **d** Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. Cells were cultured under 37°C after transfection

strongest blue fluorescence, proving the concept that multiple amino acid substitutions to BCaMP3 were required to improve the overall property as a calcium indicator.

Discussion

The present study discovered that a single amino acid replacement (Tyr223His) of GCaMP3 was essential to induce the green-to-blue fluorescence change. Although this color shift has already been originally reported on EGFP, the present study for the first time verified that the similar mutation was able to change green to blue fluorescence shift in GCaMP3 as well.

However, it is widely known that BFP emits relatively weak fluorescence and suffers from fast photobleaching [7, 16]. To overcome these shortfalls, several attempts have been made to improve BFP. Some of the mutations are known to improve chromophore formation (F64L), protein folding (S72A, V163A, and S175G), solubility (M153T and V163A), or to abolish a tendency to dimerize (A206K) [6, 7, 17–19]. Based on these previous reports, we designed several primer pairs for

introductions of various point mutations on Cys³³⁰-GCaMP3. As a result, we prepared four different BCaMP3 referred to Cys³³⁰-BCaMP3, Ser²²²-Cys³³⁰-BCaMP3, Ala²²⁹-Cys³³⁰-BCaMP3, and Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3.

As shown in Fig. 2, the blue fluorescence intensity was most profound in Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. On the other hand, only dim blue fluorescence was found in Ala²²⁹-Cys³³⁰-BCaMP3. Therefore, it is assumed that both T222S and S229A substitutions were essential to enhance blue fluorescence intensity especially in transient expression condition. It is speculated that this double substitution might have improved its folding and/or enhanced chromophore formation.

The present study also found that a 1:1 ratio for co-transfection of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 and TRPA1 was not suitable for proper expression in HEK293T cells. One theory is that TRPA1 has been dominantly expressed in HEK293T cells so that the expression levels of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 had to be decreased compared to TRPA1. Another plausible explanation would be due to the lack of Y302F in all BCaMP3 variants used in the present study. In fact, the Y302F mutation is known to correct a codon usage

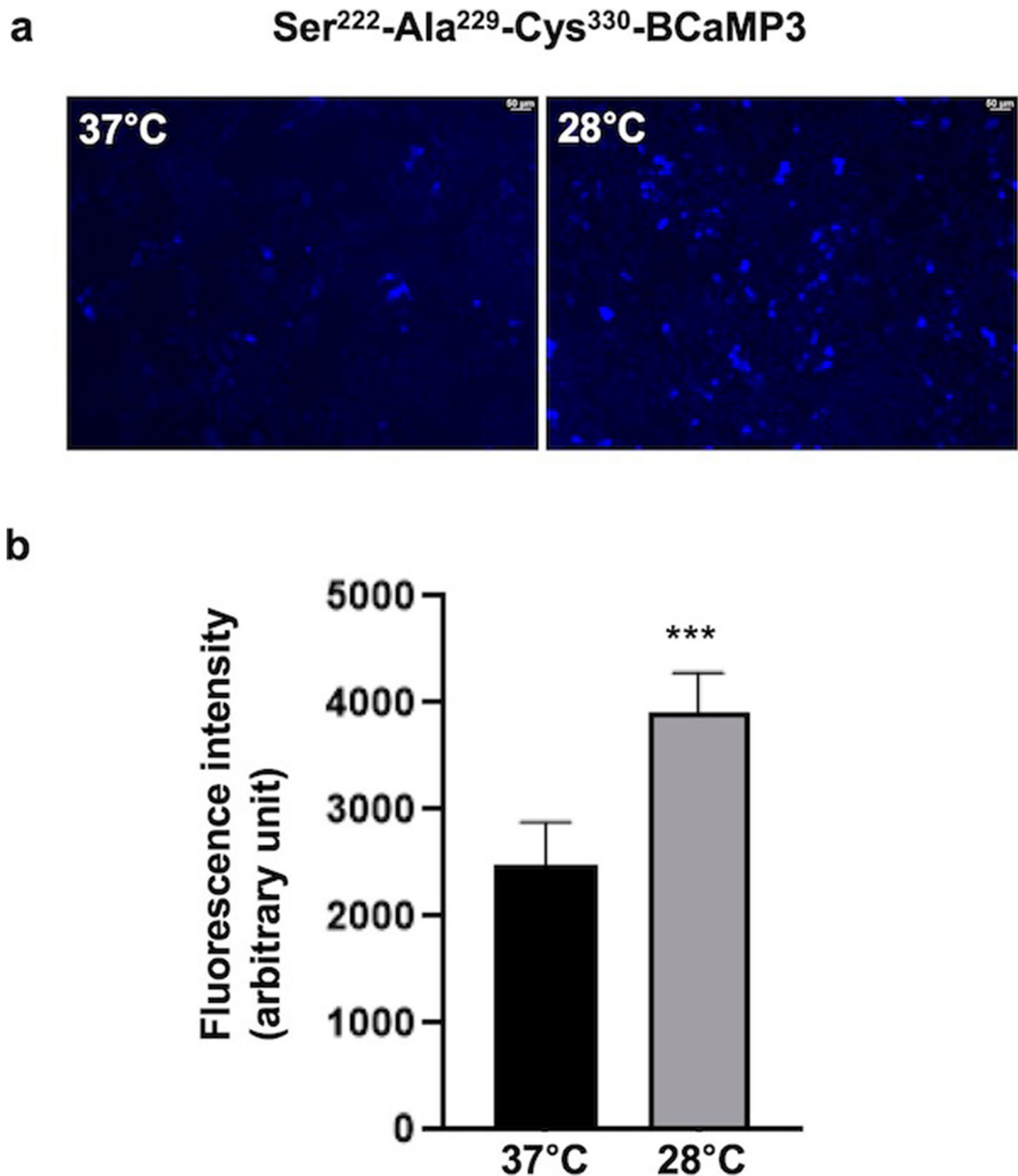


Fig. 3 Effect of cell culture temperature for fluorescence intensity of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. **a** Fluorescence of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 after incubation at 37°C for 24 h or at 28°C for 48 h after transfection. **b** Fluorescence intensities of HEK293T cells co-

transfected with Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3-BCaMP3 and TRPA1 were compared between the different incubating conditions. *** $p < 0.001$

optimized for proper expression in mammalian cells [7]. It will be intriguing to see if Y302F can also improve

protein synthesis and increases the rate of chromophore formation of all the BCaMP3 variants used in the present

study as well. We are planning to do additional mutation studies in the near future.

It was interesting that lowering the cell culture temperature greatly enhanced the overall expression and sensitivity of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3. In general, it is believed that lower temperature helps increase the correct folding of fluorescent protein, especially chromophore formation. Indeed, it was reported that a mild hypothermia can increase cellular productivity of recombinant proteins in HEK293T cells [15]. However, culturing cells under low temperature is not ideal, since many cell incubators are set to 37°C as a default temperature. To overcome this temperature limitation, further mutations to Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 might be effective. In fact, there were already similar attempts to circumvent the temperature-dependency by simple amino acid mutations. For example, one study has reported that a single amino acid substitution to the GFP variant allowed stronger fluorescence emission under 37 [20]. Another study has claimed that two additional amino acid substitution to BFP results in enhanced fluorescence in both bacteria and yeast at 37 [21]. Thus, a possibility cannot be ruled out that the activity of Ser²²²-Ala²²⁹-Cys³³⁰-BCaMP3 may be further improved without the necessity of low-temperature conditions by additional mutations.

In conclusion, the present study observed that amino acid substitutions at position 222, 223 and 229 on Cys³³⁰-GCaMP3 accompanied by low culture temperature are essential for changing overall property as a blue calcium indicator expressed in mammalian cells. Considering the advantage of blue fluorescent calcium indicator, it warrants further study to further improve its protein expression and fluorescence intensity.

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