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A Novel Bicistronic Expression Strategy for Neuronal Calcium Imaging in *C. elegans**

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Abstract Calcium imaging has been widely used to monitor the activity of various neurons in *C. elegans*. However, it is a challenge to determine the calcium transient in a freely moving worm for two reasons. One reason is the challenge in ensuring the co-expression of the genetically encoded calcium indicator and reference fluorescent protein in the same target neurons. Another reason is the common problem with spectrum cross-talk between the fluorescent pair used most often: G-CaMPs (calcium indicator) and DsReds (the reference fluorescent protein). Spectrum cross-talk occasionally introduces artifacts in the calcium transient measurements. Herein, we developed a novel bicistronic expression strategy to ensure co-expression efficiency and simplify the labeling for the target neurons. Moreover, we presented a new fluorescent protein pair for calcium imaging, mKate2 and G-CaMPs, which have less spectrum cross-talk. We successfully tested our new labeling strategy in the *C. elegans* ASH sensory neuron. We anticipate that this improved technique will facilitate neural circuit studies in *C. elegans*.

Key words bicistronic expression, calcium imaging, *C. elegans*
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Animal behaviors are coordinated by neural circuits[1]. How the brain controls behaviors has been a research hotspot for decades. Unlike complex invertebrates or other vertebrates, the nematode *Caenorhabditis elegans* is a perfect model organism for studying this problem because an adult *C. elegans* hemaphrodite only has 302 neurons and engages in various behaviors, such as feeding, locomotion, and chemosensation. Calcium is as a second messenger that is directly related to neuronal activity. Because its body is transparent, calcium imaging has been used to monitor activity and signaling in a variety of *C. elegans* cell types[2].

In the past few years, many types of genetically encoded calcium indicators (GECI for short) have been developed to study neural functions in worms[3-4]. GECI are fluorescent proteins that are sensitive to calcium concentration [2]. GECI can be grouped into two major classes: FRET-based proteins, such as cameleons, and non-FRET proteins (also intensity-based indicators), including G-CaMP, camgaroo, and pericam.

In brief, both indicator types are suitable for measuring the change in calcium intensity for a living worm. However, the intensity-based proteins, G-CaMP for instance, are advantageous because the dynamic range is typically greater than the FRET-based proteins. In addition, enhanced intensity-based proteins have been generated, such as the GEM-GECO1 [4]. Thus, the non-FRET calcium indicators are increasingly used by scientists. The primary advantage of FRET-based proteins is a ratio-metric readout with

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a better signal-to-noise ratio, which has also been produced in non-FRET indicators through the co-expression of a photo-stable fluorescent protein as a reference in the same cell. Thus, the calcium measurements are more precise in neurons. Worm researchers are increasingly using this method[5-6].

However, questions remain. First, the co-expression of two different fluorescent proteins in the same cell is not always successful. Further, the expression efficiency of two proteins is difficult to control; therefore, the fluorescence intensity of the calcium indicator is often dimmer than the reference protein. Second, it is important to use a reference fluorescent protein that has less spectrum cross talk with the calcium indicator. The most commonly used pairs are DsRed2/G-CaMP and mCherry/G-CaMP. In this study, a bicistronic expression strategy was developed to co-express two different fluorescent proteins under the control of a single promoter, which ensures co-expression. Moreover, a new far-red fluorescent protein, mKate2, was presented as a better choice for a reference protein in calcium imaging because it has less spectrum cross talk with G-CaMP and better photo-stability. Finally, the ASH neuron activity was successfully measured using the fluorescent protein pair G-CaMP2.0/mKate2 and monitored the avoidance response in a wild-type worm upon exposure to Cu^{2+}.

1 Materials and methods

1.1 Molecular biology

A 3.8 kb promoter for *sra-6* was inserted into the pPD95.75 *Sbf* Ⅰ and *Xma* Ⅰ sites to generate the plasmid P*sra-6*::GFP. The GFP coding sequence in the plasmid P*sra-6*::GFP was then replaced with a PCR fragment of G-CaMP2.0 or mKate2 to generate the plasmid P*sra-6*::G-CaMP2.0 or P*sra-6*::mKate2. The SL2 trans-splicing signal used in this study was a minimal size gpd-2 and gpd-3 intergenic sequence that was approximately 160 bp long[7-8]. This sequence was amplified through PCR using genomic DNA from *C. elegans* Bristol N2 strain worms and fused to the gene of interest through overlap extension PCR to produce a PCR fragment of G-CaMP2.0-SL2-mKate2. The PCR fragment was then inserted into the *Age* Ⅰ and *Eco*R Ⅰ sites in the plasmid P*sra-6*::GFP to generate the plasmid P*sra-6*::G-CaMP2.0-SL2-mKate2.

1.2 Preparing the *C. elegans* and germline transformation

Wild type (N2) worms were kindly provided by CGC (Caneorhabditis Genetics Center). The worms were cultured following standard methods [1]. To compare the traditional co-injection method and novel bicistronic expression strategy, P*sra-6*::G-CaMP2.0 (50 mg/L) and P*sra-6*::mKate2(50 mg/L) were co-injected, and P*sra-6*::G-CaMP2.0-SL2-mKate2 (50 mg/L) was injected separately.

1.3 Confocal imaging

The imaging was performed using a Fluoview FV500, which is a classic laser scanning confocal microscopy system (Olympus Corporation, Tokyo, Japan). The worms were picked from the NGM plate and immobilized on a drop of agarose with 10 mmol/L sodium azide before imaging [1]. The fluorescence images were produced using the Olympus software with a Zeiss 60×1.39 NA oil-immersion objective lens (Carl Zeiss MiroImaging Inc., Göttingen, Germany).

1.4 Neuronal calcium imaging for crawling *C. elegans*

Calcium activity in the sensory neuron ASH was monitored using a self-designed automated neuron tracking system [5, 9]. This system is performed on a IX81 inverted microscope (Olympus Corporation, Tokyo, Japan), and the images were acquired using a Andor Luca EMCCD (Andor Technology plc, Belfast, Northern Ireland) at a 10 Hz frame-rate. Before imaging, the young adult worm was picked from the NGM plate and added to the fresh new agar plate; the agar plate was then transferred to a 0.17 mm cover glass. During the imaging process, the worm was automatically tracked to maintain the neuron in the middle of the camera field [5, 9]. When two color fluorescence signals were recorded, a beam splitter was used before the EMCCD. The left half of the view is the green channel that measures dynamics changes in G-CaMP2.0, and the right half of the view is the red channel that measures the reference protein mKate2. The final data for ASH activity is the ratio of the two channels. A 40×1.3 NA oil-immersion objective lens (Olympus Corporation, Tokyo, Japan) was used for the calcium imaging experiment.

2 Results

2.1 Bicistronic expression of G-CaMP2.0 and mKate2 in *C. elegans*

To validate the bicistronic strategy, the *gpd-2-gpd-3* intercistronic region was used as the SL2 trans-splicing signal herein. A vector containing G-CaMP2.0-SL2-mKate2 was constructed as shown in Figure 1a. Using this new bicistronic expression strategy, neuron labeling is faster and more efficient. The ASH-neuron-expressing G-CaMP2.0 and mKate2 bicistronic expression strategy or co-injection of the plasmids P*sra-6*::G-CaMP2.0 and P*sra-6*::mKate2 is shown in Figure 1b.

As mentioned above, the commonly used reference protein for intensity-based G-CaMP is DsRed2. Figure 1c shows that the DsRed2 emission spectrum generates cross-talk with G-CaMP2.0. This cross-talk sometimes produces an artifact. To decrease such artifacts, the far-red fluorescent protein mKate2 was used as a new reference protein for the G-CaMP. In Figure 1c, the cross-talk area of the emission spectrum for G-CaMP2.0 and mKate2 is smaller than for DsRed2.

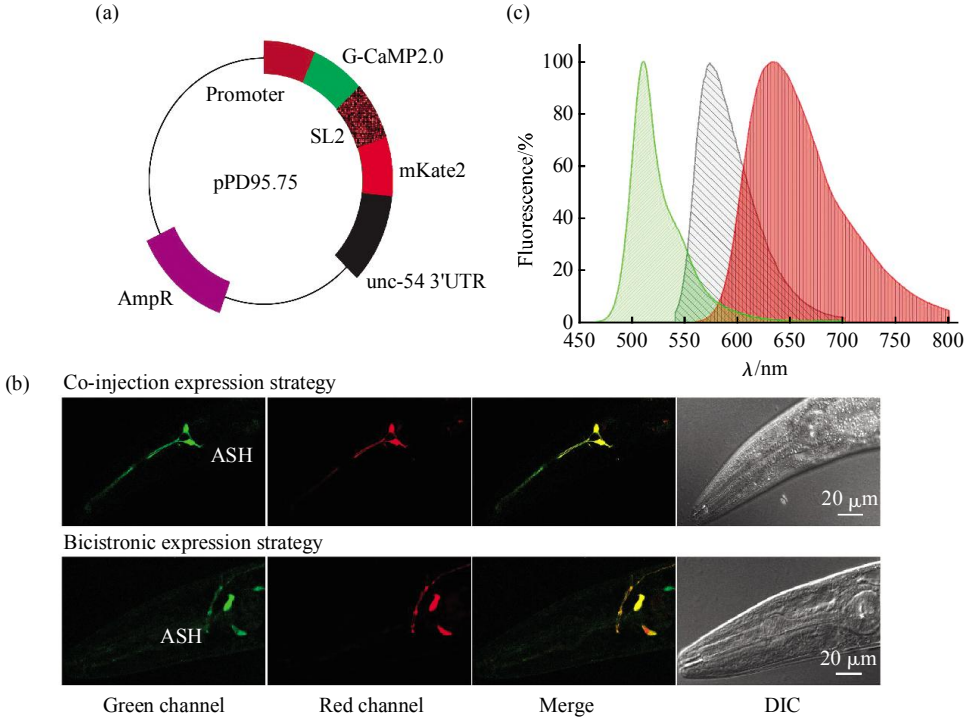

Fig. 1 Bicistronic expression of mKate2 and G-CaMP2.0 in a worm ASH sensory neuron

(a) Schematic diagram of the bicistronic expression construct. The *sra-6* promoter is used for specific expression in ASH neurons. (b) Emission spectra for G-CaMP2.0 (Green), mKate2(Red) and DsRed2(Light gray). (c) Confocal images of an ASH neuron co-expressing the plasmids P*sra-6*::G-CaMP2.0 and P*sra-6*::mKate2 (upper) and confocal images of an ASH neuron expressing G-CaMP2.0-SL2-mKate2 through the bicistronic strategy (lower). ——: DsRed2, Em; ——: mKate2, Em; ——: G CaMP2.0, Em.

2.2 Recording the activity of ASH neurons in response to Cu^{2+} stimulation

As shown in Figure 2a, in the calcium measuring system, both the G-CaMP2.0 and mKate2 signals were simultaneously recorded. First, the mKate2 photo-stability under laser illumination was measured. mKate2 produced low levels of bleaching after an ~140 second light illumination, which was suitable for a reference protein in calcium imaging (Figure 2b). This process was repeated in 3 different worms to verify mKate2 stability (Figure 2b).

To test the neurons, the widely studied sensory neuron ASH was recorded to measure signal transients upon stimulation. ASH neurons sense external clues, such as volatile chemicals. As previously reported[10–12], our data showed significant changes in calcium levels upon Cu^{2+} incubation. In Figure 2c, a 10 mmol/L $CuSO_4$ application induced a 3～5 folds increase in the ASH neuron G-CaMP/ mKate2 ratio signal (blue line). In Figure 2c, the red line and the green line showed the individual change of G-CaMP2.0 and mKate2 respectively. As a result of the movement of the worm, the single channel of G-CaMP2.0 and mKate2 both showed some noise. However, it shows a significant improvement of signal-to-noise after calculate the ratio of the G-CaMP2.0 and mKate2. The result suggests that it would be necessary to use a reference as previously reported [10–12]. The co-injection transgenic worms produced similar results compared with SL2 transgenic worms (data not shown).

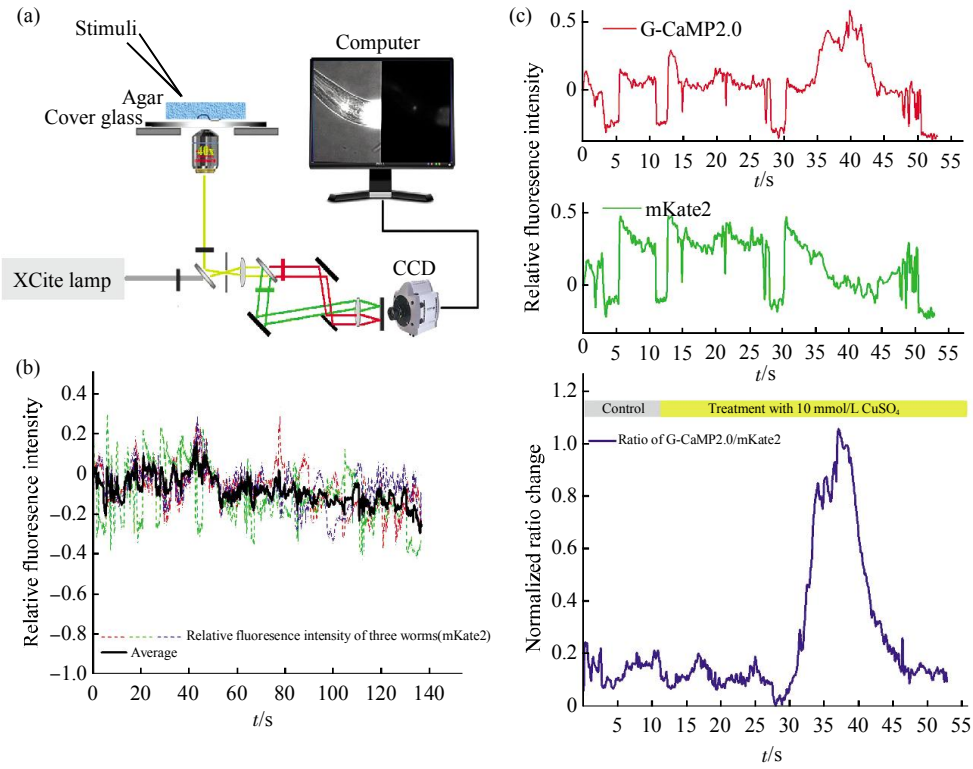

Fig. 2 Recording the avoid response of ASH neuron to Cu^{2+} (Treatment with 10 mmol/L $CuSO_4$)

(a) It is the schematic diagram for the calcium imaging system. (b) Stability test for mKate2 as a reference protein for G-CaMPs. The red, green and blue lines are three different worms' fluorescence intensity data of mKate2. The black line is the average intensity for the three worms. (c) The graph shows the ASH neuron response to Cu^{2+} stimulation. The red line is the relative fluorescence intensity of G-CaMP2.0. The green line is the relative fluorescence intensity of mKate2. The blue line is normalized ratio change of G-CaMP2.0 and mKate2.

3 Discussion

In this study, a novel bicistronic expression strategy for calcium imaging in *C. elegans* neurons was used. The new fluorescent protein pair investigated in this study, mKate2 and G-CaMP2.0, is ideal for neuronal calcium imaging. The calcium signal recorded herein is stronger than using previous methods. The novel bicistronic expression strategy both simplifies plasmid construction and produces better results. This bicistronic expression strategy is a promising tool for further studies on *C. elegans* neural circuits. Although it is a valuable tool, the bicistronic expression strategy has limitations. One of the primary limitations is that rarely there will be expression in intestine by the bicistronic expression strategy (data not shown). Fortunately, it does not interfere with recording the signal in the neurons. In general, the new

pair of calcium indicators and the bicistronic expression strategy is an alternative and better way to study a worm brain's control over complex behaviors.

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References

[1] Liu Y, LeBeouf B, Guo X, *et al*. A Cholinergic-regulated circuit coordinates the maintenance and bi-stable states of a sensory-motor behavior during *Caenorhabditis elegans* male copulation. PLoS Genet, 2011, **7**(3): e1001326

[2] Kerr R A. Imaging the Activity of Neurons and Muscles. WormBook, 2006: 1-13

[3] Tian L, Hires S A, Mao T, *et al*. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Meth, 2009, **6**(12): 875-881

[4] Zhao Y, Araki S, Wu J, *et al*. An expanded palette of genetically encoded Ca^{2+} indicators. Science, 2011, **333**(6051): 1888-1891

[5] Ben Arous J, Tanizawa Y, Rabinowitch I, *et al*. Automated imaging of neuronal activity in freely behaving *Caenorhabditis elegans*. Journal of Neuroscience Methods, 2010, **187**(2): 229-234

[6] Zheng M, Cao P, Yang J, *et al*. Calcium imaging of multiple neurons in freely behaving *C. elegans*. Journal of Neuroscience Methods, 2012, **206**(1): 78-82

[7] Chen M R, Yang S, Niu W P, *et al*. A novel fluorescent timer based on bicistronic expression strategy in *Caenorhabditis elegans*. Biochemical and Biophysical Research Communications, 2010, **395**(1): 82-86

[8] Huang T, Kuersten S, Deshpande A M, *et al*. Intercistronic region required for polycistronic pre-mRNA processing in *Caenorhabditis elegans*. Molecular and Cellular Biology, 2001, **21**(4): 1111-1120

[9] 张海宁, 黄文明, 付家骏, 等. 计算机视觉在线虫进食行为自动分析中的应用. 生物化学与生物物理进展, 2013, **40**(2): 188-194
Zhang H N, Huang W M, Fu J J, *et al*. Prog Biochem Biophys, 2013, **40**(2): 188-194

[10] Faumont S, Lockery S R. The awake behaving worm: simultaneous imaging of neuronal activity and behavior in intact animals at millimeter scale. Journal of Neurophysiology, 2006, **95**(3): 1976-1981

[11] Chokshi T V, Bazopoulou D, Chronis N. Probing the physiology of ASH neuron in *Caenorhabditis elegans* using electric current stimulation. Applied Physics Letters, 2011, **99**(5): 53702

[12] Thiele T R, Faumont S,Lockery S R. The neural network for chemotaxis to tastants in *Caenorhabditis elegans* is specialized for temporal differentiation. J Neurosci, 2009, **29**(38): 11904-11911

双顺反子表达技术在线虫神经元钙成像中的应用 *

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摘要 在线虫中，钙成像技术已被广泛用于检测不同神经元的活性．然而，对于准确记录爬行中的活体线虫神经元钙信号仍然存在许多挑战．其中一个困难即来自于标记目标神经元．在同一个目标神经元中共同表达基因编码的钙指示蛋白和常量参考值荧光蛋白常常具有无法共表达的不确定性．另外，光谱的串扰影响存在于目前最常用的绿色钙指示蛋白系列 G-CaMP 与其参考值荧光蛋白 DsRed 系列之间．光谱的串扰有时会给信号记录带来假阳性结果．综上所述，本文首次提出应用双顺反子表达技术用于同一神经元的双蛋白标记，这不仅提高了共表达效率，更简化了线虫神经元标记的工作量．同时，本文还首次采用 mKate2，一种与 G-CaMP 没有串扰的红色荧光蛋白作为参考量．以上改进已在感觉神经元 ASH 中得到验证．希望本文提出的方法能给线虫神经回路的研究提供一个更为方便、有效的途径．

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