

RESEARCH ARTICLE

# An improved inverse-type $\text{Ca}^{2+}$ indicator can detect putative neuronal inhibition in *Caenorhabditis elegans* by increasing signal intensity upon $\text{Ca}^{2+}$ decrease

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**Data Availability Statement:** All relevant data are within the paper, its Supporting Information files, and figshare. Plasmids for IP2.0 can be obtained from Addgene. The raw data of Fig 2D is available in figshare (<https://doi.org/10.6084/m9.figshare.5976067.v1>). The raw data of Fig 3B and 3D is available in figshare (<https://doi.org/10.6084/m9.figshare.5976610.v1>). The raw data of Fig 4 is available in figshare (<https://doi.org/10.6084/m9.figshare.5976619.v1>). The raw data of S4 Fig is available in figshare (<https://doi.org/10.6084/m9>).

## Abstract

Sensory processing is regulated by the coordinated excitation and inhibition of neurons in neuronal circuits. The analysis of neuronal activities has greatly benefited from the recent development of genetically encoded  $\text{Ca}^{2+}$  indicators (GECIs). These molecules change their fluorescence intensities or colours in response to changing levels of  $\text{Ca}^{2+}$  and can, therefore, be used to sensitively monitor intracellular  $\text{Ca}^{2+}$  concentration, which enables the detection of neuronal excitation, including action potentials. These GECIs were developed to monitor increases in  $\text{Ca}^{2+}$  concentration; therefore, neuronal inhibition cannot be sensitively detected by these GECIs. To overcome this difficulty, we hypothesised that an inverse-type of GECI, whose fluorescence intensity increases as  $\text{Ca}^{2+}$  levels decrease, could sensitively monitor reducing intracellular  $\text{Ca}^{2+}$  concentrations. We, therefore, developed a  $\text{Ca}^{2+}$  indicator named inverse-pericam 2.0 (IP2.0) whose fluorescent intensity decreases 25-fold upon  $\text{Ca}^{2+}$  binding *in vitro*. Using IP2.0, we successfully detected putative neuronal inhibition by monitoring the decrease in intracellular  $\text{Ca}^{2+}$  concentration in  $\text{AWC}^{\text{ON}}$  and ASEL neurons in *Caenorhabditis elegans*. Therefore, IP2.0 is a useful tool for studying neuronal inhibition and for the detailed analysis of neuronal activities *in vivo*.

## Introduction

In the central nervous system, sensory information is co-ordinately processed by excitatory and inhibitory neuronal activities. These neuronal activities have been studied by electrophysiology [1, 2] and live imaging using fluorescent chemicals [3–6] and genetically encoded  $\text{Ca}^{2+}$  indicators (GECIs) [7–12]. GECIs, including FRET (Förster Resonance Energy Transfer)-based indicators such as Cameleons [13–15] and single-fluorophore indicators, such as the

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GCaMP family, make it possible to analyse neuronal activities of specific neurons *in vivo* by using gene promoters that express the proteins in specific types of neuron [16–18]. In addition, recent improvements to GCaMPs enables single action potentials in living animals to be detected, and red fluorescent Ca<sup>2+</sup> indicators, such as R-GECO and RCaMPs, have been also developed [19–23]. These GECIs are sufficiently sensitive to increases in Ca<sup>2+</sup> concentration that neuronal excitation can easily be detected. On the other hand, most GECIs have difficulty in detecting decreases in Ca<sup>2+</sup> concentration from the resting phase, because they have been optimized to monitor increases in Ca<sup>2+</sup> concentration. For example, the fluorescence of GCaMPs under the resting phase is very dim and thereby the signal to noise ratio is low. Therefore, Ca<sup>2+</sup> concentration lower than that at the resting phase cannot be reliably detected. This is mainly because the  $K_d$  values of most GECIs do not correspond to lower Ca<sup>2+</sup> concentrations. Moreover, a decrease in fluorescence intensity can incidentally occur with a change of intracellular conditions, such as pH [24]; therefore, a decrease in fluorescence is not always caused by a decrease in Ca<sup>2+</sup> concentration. Therefore, in addition to the ordinary GECIs, a new type of GECI that can monitor decreases in Ca<sup>2+</sup> concentration from the resting phase is needed to investigate neuronal function because it would reflect neuronal inhibition. The development of sensitive GECIs that detect decreases in Ca<sup>2+</sup> concentration may lead to a new chapter of Ca<sup>2+</sup> imaging studies for the investigation of neuronal processing *in vivo*.

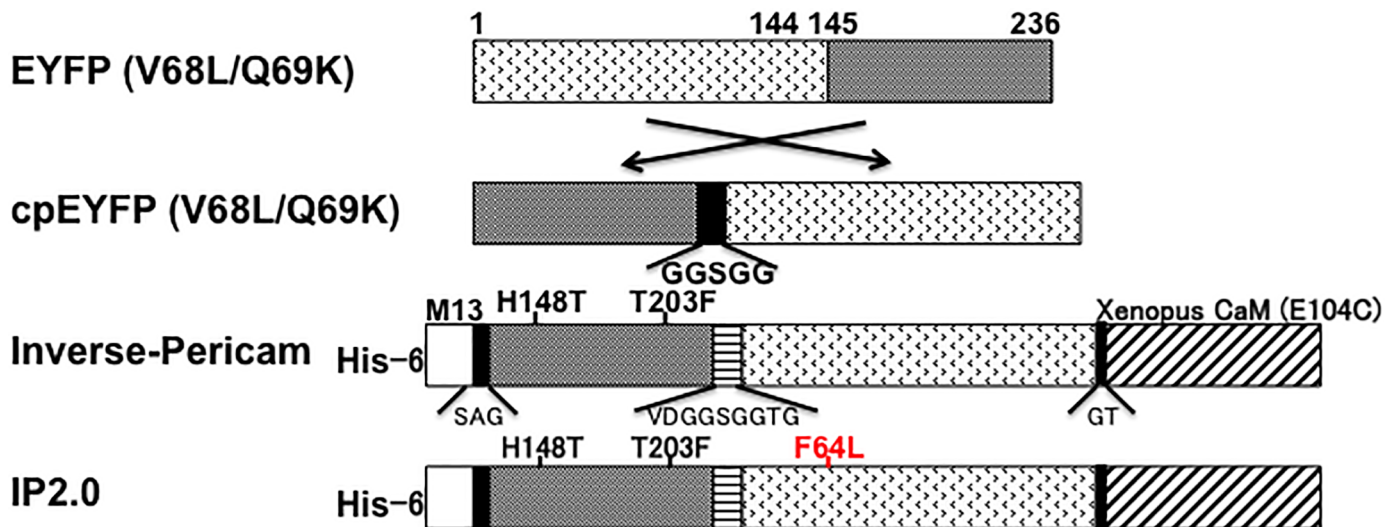
We previously reported various “pericam” types of indicators that are based on circularly permuted yellow fluorescent protein (cpYFP) fused to calmodulin and calmodulin-target peptide, M13 [8]. Among these, “inverse-pericam” has a unique property of being a quenching type Ca<sup>2+</sup> indicator; its fluorescence intensity becomes 7-fold dimmer upon Ca<sup>2+</sup> binding [8]. This inverse-pericam and another inverse-type Ca<sup>2+</sup> indicator, Y-GECO1 have been already used for Ca<sup>2+</sup> imaging to detect Ca<sup>2+</sup> change in brain slices [25, 26]. But these indicators have not been used to monitor the decrease of Ca<sup>2+</sup> concentration in neurons of living animals.

Here we report an improved inverse pericam 2.0 (IP2.0), generated by mutagenesis of the original inverse-pericam. IP2.0 can sensitively monitor Ca<sup>2+</sup> oscillation in HeLa cells. Furthermore, by expressing IP2.0 in *Caenorhabditis elegans*, we succeeded in monitoring both decreases and increases in Ca<sup>2+</sup> concentration in the chemosensory neurons, AWC<sup>ON</sup> and ASEL. These responses may represent inhibition and excitation of these sensory neurons, respectively. Therefore, IP2.0 is useful for monitoring changes in Ca<sup>2+</sup> concentration *in vivo* and may enable the sensitive and simultaneous detection of neuronal excitation and inhibition in neuronal circuits *in vivo* by combined monitoring with conventional red GECIs, such as RCaMPs.

## Results

### Development of improved inverse-pericam

Neuronal excitation in living animals is often analysed using GCaMPs, whose fluorescence intensities increase as intracellular Ca<sup>2+</sup> concentration increases. We considered that Ca<sup>2+</sup> indicators that monitor decreases in Ca<sup>2+</sup> concentration through increasing fluorescence intensities could complement GCaMP-type GECIs to enable analysis of both neuronal excitation and inhibition. Therefore, we sought to improve inverse-pericam, one of the various pericams, whose fluorescence increases as Ca<sup>2+</sup> concentration decreases [8]. Inverse-pericam is a GECI with a M13 peptide, cpYFP and calmodulin domain-like GCaMP. Its green fluorescence, at 500 nm excitation, decreases by 15% with Ca<sup>2+</sup> [8]; however, it has not been used for the sensitive detection of intracellular Ca<sup>2+</sup> concentration *in vivo*. To obtain improved inverse-type Ca<sup>2+</sup> probes from inverse-pericam, we screened colonies expressing inverse-pericam mutants generated by error prone PCR (see [Methods](#)). After screening about 20,000 colonies,



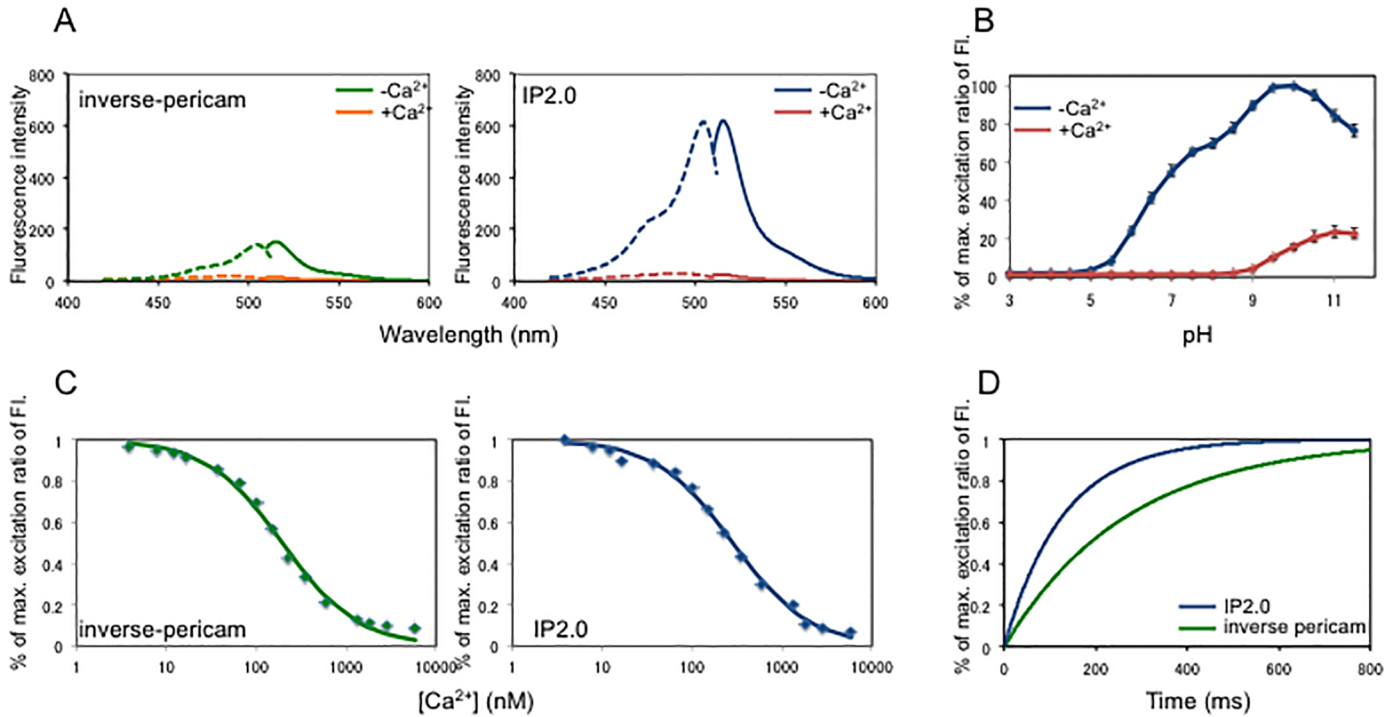
**Fig 1. Schematic structures and sequences of inverse-pericam and IP2.0.** Sequences of linkers and amino and substitutions are shown below and above the bars, respectively. His-6: the polyhistidine tag.

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we obtained an improved inverse-type Ca<sup>2+</sup> indicator, named IP2.0, which has only one substitution, F64L, compared with inverse-pericam (Fig 1). IP2.0 exhibited similar excitation and emission spectra to inverse-pericam [8]. IP2.0, excited at 488 nm, showed an emission peak at 514.5 nm without Ca<sup>2+</sup>, which is close to that of inverse pericam at 515 nm. The intensity at the fluorescence spectrum peak of IP2.0 was approximately three times higher than that of inverse-pericam in the absence of Ca<sup>2+</sup> (Fig 2A and Table 1) and the dynamic range at 515 nm between the presence and absence of Ca<sup>2+</sup> was approximately two times larger for IP2.0 than for inverse-pericam (Fig 2A, Table 1 and S1 File). Indeed, IP2.0 excited for the optimal emission peak showed 25-fold greater fluorescence at 520 nm without Ca<sup>2+</sup> compared to that with Ca<sup>2+</sup> (Fig 2A). Both Ca<sup>2+</sup>-bound and Ca<sup>2+</sup>-free IP2.0 were pH-titrated in a similar way and the Ca<sup>2+</sup>-free protein was about 7-fold brighter than the Ca<sup>2+</sup>-bound form in the ionized state (pH>8.0) (Fig 2B and S2 File) [8]. In the neutral pH region, the fluorescence of IP2.0 appeared bright enough for *in vivo* studies. From the Ca<sup>2+</sup> titration curve, the K<sub>d</sub> value of IP2.0 could be calculated as 284 nM (Fig 2C and S3 File) and it was lower than that of inverse-pericam (Table 1) and slightly higher than that of GCaMP3 [17, 18, 27], or GCaMP6f [18]. Ca<sup>2+</sup>-binding to inverse-pericam and IP2.0 was accompanied with Hill coefficients close to 1.0, similar to values shown for GCaMP families [18, 21]. To measure the response kinetics of inverse-pericam and IP2.0, we used stopped-flow fluorometry. In a stepped reduction of free Ca<sup>2+</sup> concentration from 10 μM to zero (<10 nM), both inverse-pericam and IP2.0 responded with a double exponential time course (Fig 2D). The dissociation constant k<sub>off</sub> was calculated as 280 ms<sup>-1</sup> for inverse-pericam and as 128 ms<sup>-1</sup> for IP2.0 (Table 1) and these responses were similar to those of GCaMP variants [17, 18]. These results indicate that IP2.0 is a good candidate for the measurement of intracellular Ca<sup>2+</sup> *in vivo*, especially for decreases in Ca<sup>2+</sup> concentration.

### Imaging in HeLa cells expressing inverse-pericam and IP2.0

We examined whether IP2.0 could be used to analyse intracellular Ca<sup>2+</sup> concentration using HeLa cells. We transfected cDNA for inverse-pericam or IP2.0 into HeLa cells and monitored the change of intracellular free Ca<sup>2+</sup> concentration after histamine stimulation. Stimulation of HeLa cells by histamine induces Ca<sup>2+</sup> oscillations; therefore, the various GECIs



**Fig 2. In vitro properties of IP2.0.** A, Normalized fluorescence excitation (dashed lines) and emission (solid lines) spectra in Ca<sup>2+</sup>-free (green and blue lines) and Ca<sup>2+</sup>-saturated (orange and red lines) states. B, pH-dependency of normalized amplitudes at the 515 nm excitation peak in Ca<sup>2+</sup>-free (blue line) and Ca<sup>2+</sup>-saturated (red line) states. C, Ca<sup>2+</sup> titration curve of inverse-pericam (left) and IP2.0 (right). D, Fluorescence rise response of inverse-pericam (green line) and IP2.0 (blue line) to a stepped decrease in [Ca<sup>2+</sup>]<sub>free</sub> from 10 μM to < 10 nM. The raw data of Fig 2D is available in figshare (<https://doi.org/10.6084/m9.figshare.5976067.v1>).

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have been tested in HeLa cells [8, 19]. Similarly to inverse-pericam, we observed oscillations in the fluorescence intensity of IP2.0 following histamine stimulation (Fig 3). The response of IP2.0 was about three times larger than that of inverse-pericam and the changes in fluorescence seemed to be opposite to those of flash-pericam [8] or GECO variants [19]. We performed additional experiments using 44 HeLa cells expressing IP2.0. S1 Fig showed the ratios of the fluorescent intensity of the first spike after histamine stimulation to the basal intensity (S4 File). This analysis revealed that the intensity of IP2.0 consistently changed to the stimulation without being affected by the difference of the expression level among cells. From these results, we suggest that IP2.0 can be used as a practically useful inverse type of Ca<sup>2+</sup> indicator in living animals.

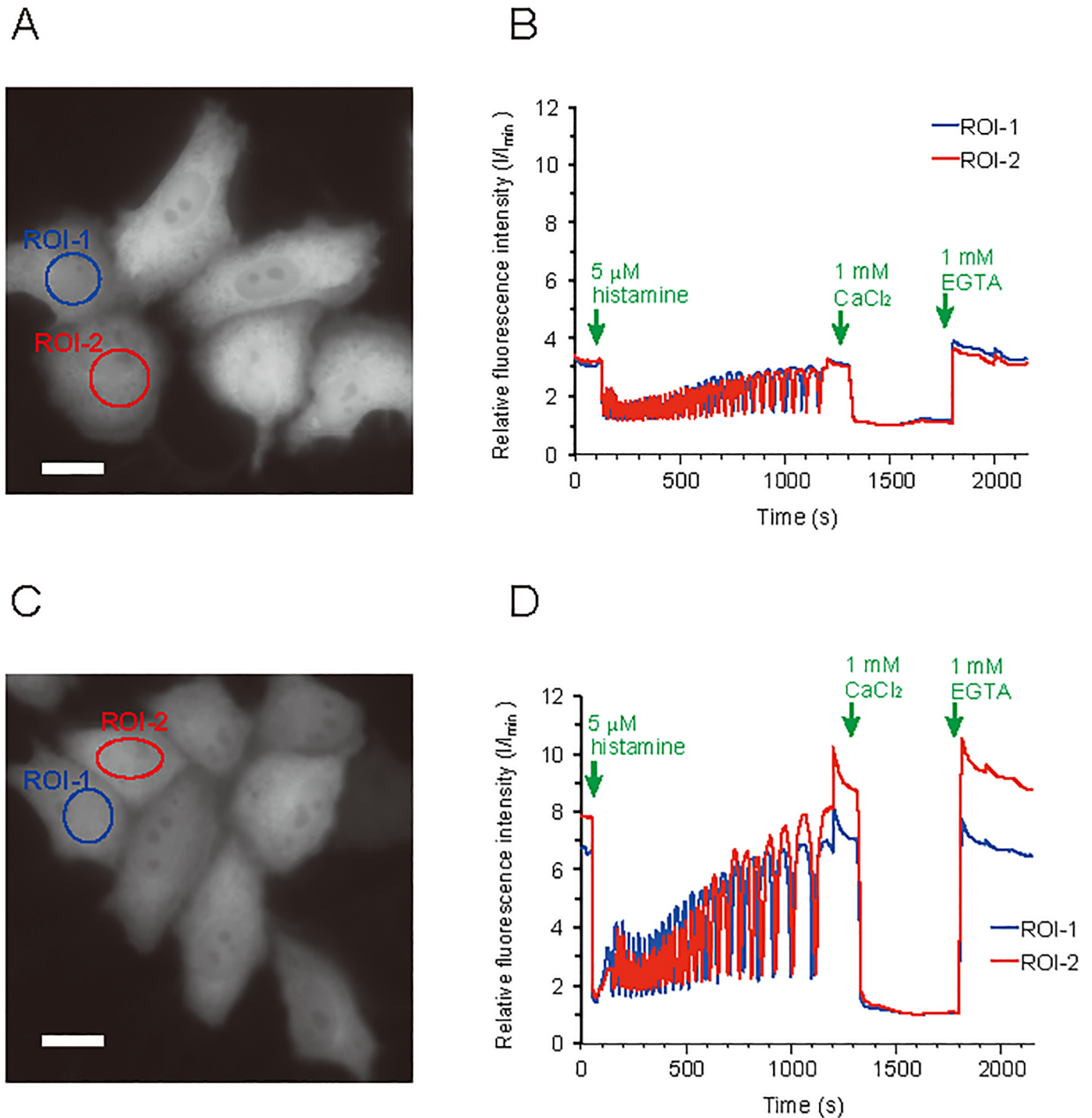
**Table 1. Spectral characteristics of inverse-pericam and IP2.0.**

Variant	Mutation <sup>a</sup>	K <sub>d</sub> for Ca <sup>2+</sup> (n) <sup>b</sup>	Relative F <sub>max</sub>	Dynamic Range (F <sub>max</sub> -F <sub>min</sub> /F <sub>min</sub> )	Rise t <sub>1/2</sub>
Inverse-pericam	H148T T203F	196 nM (1.03)	1.00	6.7	280 msec
IP2.0	H148T T203F F64L	284 nM (1.02)	2.95	25.0	128 msec

<sup>a</sup>Substitutions from primary sequence of EYFP (V68L/Q69L) are given as the single-letter code for the amino acid being replaced, its numerical position in the sequence, and the single-letter code for replacement.

<sup>b</sup>K<sub>d</sub> values for Ca<sup>2+</sup> were measured from fitted curves in Fig 2C. n in parentheses is the Hill coefficient.

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**Fig 3. Representative  $\text{Ca}^{2+}$  imaging in HeLa cells.** Fluorescence images of HeLa cells (A, C) and fluorescence intensity vs. time traces (B, D) in the ROIs of fluorescence images. Images were taken of HeLa cells transfected with inverse-pericam (A, B) and IP2.0 (C, D). Scale bar: 20  $\mu\text{m}$ . The raw data of Fig 3B and D is available in figshare (<https://doi.org/10.6084/m9.figshare.5976610.v1>).

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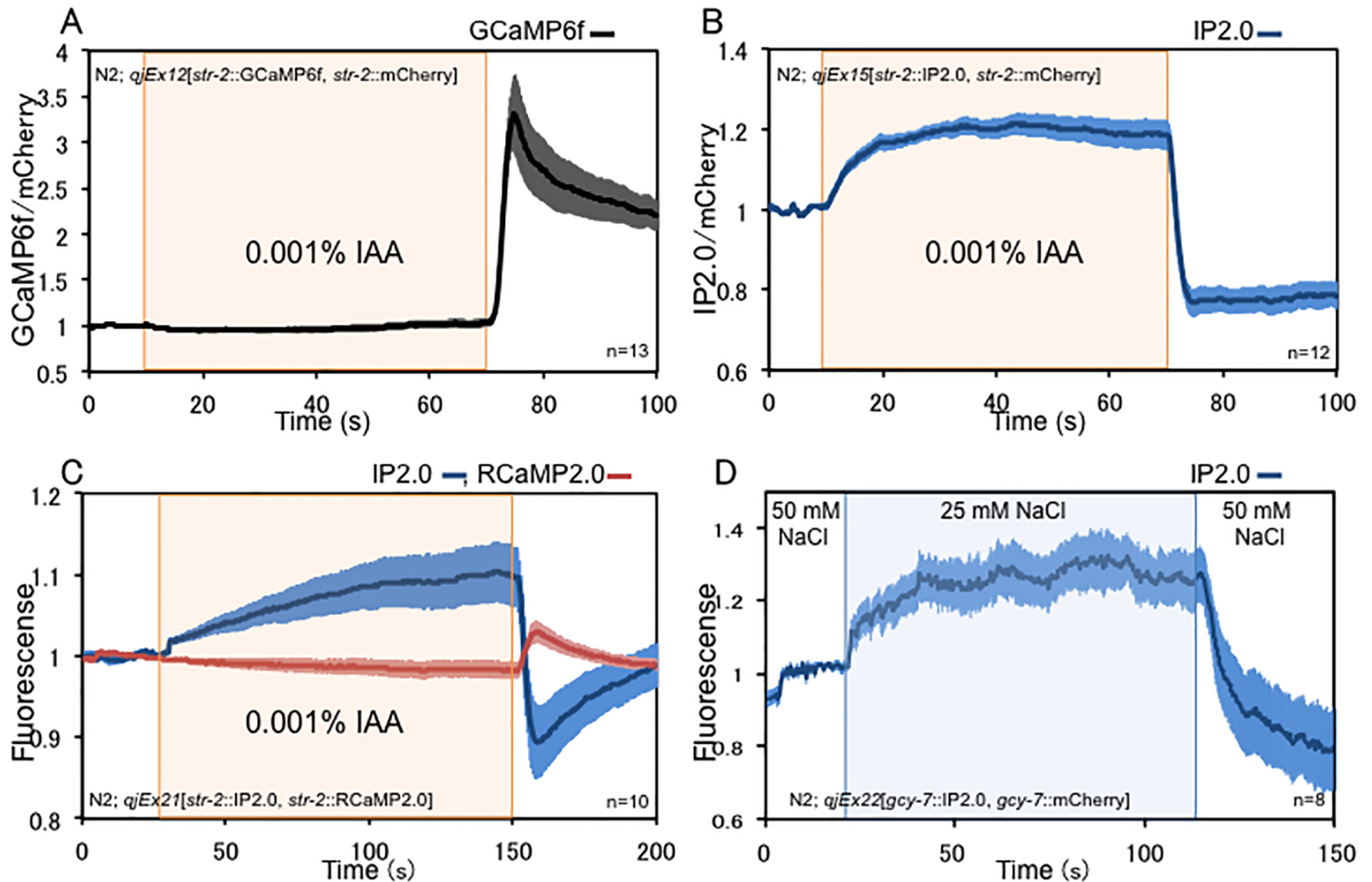
## Imaging of $\text{Ca}^{2+}$ concentration in *C. elegans* neurons

Next, we analysed neuronal activity using IP2.0 in living *C. elegans* to test its *in vivo* performance. We used unanaesthetised *C. elegans* to monitor the activity of the  $\text{AWC}^{\text{ON}}$  chemosensory neuron, in which the fluorescence of GCaMPs decreases in response to odour stimulation and increases with odour removal (S2 Fig) [28, 29]. IP2.0 or GCaMP6f [18] was expressed in  $\text{AWC}^{\text{ON}}$  neurons together with mCherry, which was used as an internal fluorescent standard to prevent motion artefacts by calculating the fluorescence ratios of IP2.0 or GCaMP6f to mCherry. Before  $\text{Ca}^{2+}$  imaging, we checked whether transgenic worms are affected by the expression of GECIs and found that worms expressing GCaMP6f or IP2.0 in  $\text{AWC}^{\text{ON}}$  exhibited normal AWC-dependent chemotaxis (S3 Fig and S4 File), suggesting that the expression of GECIs did not affect the behaviour in these strains. Since  $\text{AWC}^{\text{ON}}$  neurons expressing GCaMP respond to 0.01–0.0001% isoamylalcohol (IAA) [28, 30], individual worms were imaged in a microfluid chamber [31] during addition and after removal of 0.001% IAA, which the  $\text{AWC}^{\text{ON}}$  neuron senses. We confirmed that IP2.0 also detected neuronal activities of  $\text{AWC}^{\text{ON}}$  neurons responding to 0.01–0.0001% IAA (S4 Fig). The increase of GCaMP6f fluorescence can be observed in  $\text{AWC}^{\text{ON}}$  neurons upon IAA removal as reported using GCaMP3 [29], whereas a decrease in fluorescence upon IAA stimulation was not evident in two independent lines (Fig 4A and S5A Fig). This result is very similar to that using other GCaMPs [28–30, 32, 33] suggesting that GCaMP6f, is suitable for monitoring the increase of  $\text{Ca}^{2+}$  concentration and for analysing neuronal excitation. On the other hand, the decrease of  $\text{Ca}^{2+}$  concentration upon IAA stimulation can be clearly monitored by increased IP2.0 fluorescence (Fig 4B and S5B Fig), suggesting that IP2.0 can sensitively detect neuronal inhibition. When IP2.0 was simultaneously expressed with RCaMP2.0 [21] in the  $\text{AWC}^{\text{ON}}$  neuron, the fluorescence intensity of RCaMP2.0 was increased by removal of IAA, similar to GCaMP6f, while the fluorescence intensity of IP2.0 was decreased by removal of IAA and increased by IAA stimulation. When two different types of  $\text{Ca}^{2+}$  indicator were expressed in an  $\text{AWC}^{\text{ON}}$  neuron simultaneously, the changes of intracellular  $\text{Ca}^{2+}$  concentration seemed more reliable and easier to be understood (Fig 4C).

Next, we analysed the neuronal activities of ASEL neurons, which respond to NaCl concentration, by expressing IP2.0. The ASEL neuron is known to respond to an increase in NaCl concentration [34]; however, general calcium probe did not detect a change in  $\text{Ca}^{2+}$  concentration in response to a decrease in NaCl concentration. The fluorescent intensity of IP2.0 increased in response to a decrease in NaCl concentration, indicating that  $\text{Ca}^{2+}$  concentration in the ASEL neuron decreased in response to the decrease in NaCl concentration (Fig 4D). Next, we carried out the simultaneous measurement of the fluorescent changes of IP2.0 and RCaMP2.0 in ASEL neurons of another independent transgenic line. The fluorescent changes of RCaMP2.0 can be observed only when ASEL responded to the decrease in NaCl concentration, whereas those of IP2.0 were observed when ASEL responded to both of increase and decrease in NaCl concentration (S5C Fig). These results suggest that IP2.0, which can be used with various red fluorescent  $\text{Ca}^{2+}$  probes, is suitable for studying sensitive neuronal activities that cannot be detected by GCaMPs or RCaMPs.

## Discussion

For the last decade, GECIs, especially indicators with one fluorophore, such as GCaMPs, have been actively developed. They have found different applications in *in vivo* studies depending on their characteristics, such as colour, affinities to  $\text{Ca}^{2+}$ , optimal pH or  $\text{Ca}^{2+}$  binding speeds [35–37]. Accordingly,  $\text{Ca}^{2+}$  dynamics have been analysed in cultured cells and in many species *in vivo*, including, mouse, rat, *Drosophila*, zebrafish and *C. elegans* [11, 17–23, 29, 32, 38–44].



**Fig 4. In vivo imaging of  $\text{Ca}^{2+}$  responses in *C. elegans*.** (A) GCaMP6f  $\text{Ca}^{2+}$  response to isoamylalcohol in  $\text{AWC}^{\text{ON}}$  ( $n = 13$ ). (B) IP2.0  $\text{Ca}^{2+}$  response to isoamylalcohol in  $\text{AWC}^{\text{ON}}$  ( $n = 12$ ). (C) Dual-colour of RCaMP2.0 and IP2.0  $\text{Ca}^{2+}$  responses to isoamylalcohol in  $\text{AWC}^{\text{ON}}$  ( $n = 10$ ). (D) IP2.0  $\text{Ca}^{2+}$  response to change of NaCl concentration in ASEL ( $n = 8$ ). The values are shown as relative to  $F_0$  and error bars represent SEM. The raw data of Fig 4 is available in figshare (<https://doi.org/10.6084/m9.figshare.5976619.v1>).

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However, these fluorescent  $\text{Ca}^{2+}$  indicators are used mainly for detecting neuronal excitation, because their fluorescence increases when the expressing neuron is excited. To understand the informational processing in neuronal circuits, which are finely controlled by the combination of excitation and inhibition, simultaneous analysis of not only neuronal excitation but also neuronal inhibition may be helpful.

We developed an inverse-type  $\text{Ca}^{2+}$  indicator, IP2.0, from inverse-pericam. We introduced random mutations into inverse-pericam by error-prone PCR. By comparing the fluorescent intensities of colonies expressing mutagenized inverse-pericam between conditions with and without  $\text{Ca}^{2+}$ , we screened about 20,000 clones and isolated IP2.0. IP2.0 becomes dimmer with increasing of  $\text{Ca}^{2+}$  concentration and brighter with decreasing of  $\text{Ca}^{2+}$  concentration, which makes it possible to monitor neuronal inhibition at least in *C. elegans*. IP2.0 was changed from inverse pericam by only one substitution, F64L. F64L is important for chromophore formation and for brightness, and this substitution was also introduced into EGFP, when EGFP was developed from GFP [45]. The structural study on EGFP made it clear that replacement of Phe64 with Leu in EGFP causes subtlety of the hydrophobic core packing to the chromophore and reduces surface exposure of two hydrophobic residues [46].

The  $K_d$  value of IP2.0 is 285 nM, which is close to that of other indicators, including GCaMPs and YC3.60 [15]; therefore, IP2.0 can be a good  $\text{Ca}^{2+}$  indicator for monitoring intracellular  $\text{Ca}^{2+}$  concentration.

The most unique characteristic of IP2.0 is that it is bright at low  $\text{Ca}^{2+}$  concentrations; neurons expressing IP2.0 can be easily observed even at resting state, and thereby subtle changes in fluorescence intensity can be sensitively monitored even at low  $\text{Ca}^{2+}$  concentrations. This is in contrast to most  $\text{Ca}^{2+}$  indicators, which are so dark at the resting state that it is difficult to identify the cells expressing the  $\text{Ca}^{2+}$  indicator.

IP2.0 differs from inverse-pericam in various *in vitro* characteristics (Table 1) and the change in fluorescence intensity of IP2.0 by histamine stimulation of HeLa cells was about three times larger compared with that of inverse-pericam. Our *in vivo* studies using *C. elegans* showed that IP2.0 is a unique GECI for the sensitive detection of neuronal inhibition. We detected not only neuronal inhibition during IAA stimulation in AWC<sup>ON</sup> neurons (Fig 4A–4C) but also a change of intracellular  $\text{Ca}^{2+}$  concentration in ASEL neurons depending on a decrease in NaCl concentration (Fig 4D). Though ASEL neurons are well known to respond to an increase in NaCl concentration [34], it has not been previously reported that it also responds to a decrease in NaCl concentration. According to these results, IP2.0 may be possible to monitor the decrease of intracellular  $\text{Ca}^{2+}$  concentrations coinciding with neuronal inhibition in many other neuronal subtypes where other GECIs have been unable to detect decreasing intracellular  $\text{Ca}^{2+}$  concentrations. Moreover, IP2.0 will be useful for the large-scale recording of neuronal activities in unanaesthesia *C. elegans*, which will aid investigations of how populations of neurons generate animal behaviour [47–49].

IP2.0 emits green-yellow fluorescence; therefore,  $\text{Ca}^{2+}$  probes emitting other colours need to be chosen for simultaneous imaging. However, compared with GFP-based  $\text{Ca}^{2+}$  probes, such as the GCaMP series, only a few red or cyan  $\text{Ca}^{2+}$  probes have been developed that can be used in neurons *in vivo* [20–22]. Inverse-pericam fused with DsRed2 has been used for monitoring the intracellular  $\text{Ca}^{2+}$  concentration in pharyngeal muscles of *C. elegans* [50]. Furthermore, Hasen et al. succeeded in production of transgenic mouse with inverse-pericam and showed the change of intracellular calcium concentration [26]. This report suggests that IP2.0, which is a more sensitive indicator, may be functional in mammalian systems. We also propose that development of  $\text{Ca}^{2+}$  probes may be helpful for dual-colour imaging together with inverse-type  $\text{Ca}^{2+}$  probes in the same region to measure simultaneously neuronal excitatory and inhibitory activities.

We succeeded in the detection of neuronal inhibition in *C. elegans* with high sensitivity and we suggest that IP2.0 may enable the detection of neuronal inhibition in other species. Although conventional GECIs, such as GCaMPs, are useful for the detection of spike firing, they are barely capable of detecting neuronal inhibition [51, 52]. Therefore, the coexpression of IP2.0 with conventional one fluorophore GECIs, such as RCaMPs, may lead to sensitive and simultaneous detection of neuronal inhibition and excitation. Another quenching type GECI, Y-GECO1, may be able to detect neuronal inhibition *in vivo* and differences in its fluorescence characteristics, including the emission colour, may be useful for the analysis of neuronal activities. Y-GECO1 has a main excitation peak at 525 nm and an additional excitation peak around 413 nm in the presence of  $\text{Ca}^{2+}$ ; therefore, it might be difficult to use Y-GECO1 with other fluorescent proteins, including CFP, because of cross excitation, especially for monitoring fast changes of higher  $\text{Ca}^{2+}$  concentrations.

We predict that this kind of application will be helpful for understanding fast spiking neurons in the mammalian cortex, where action potentials are produced in the resting state and that inverse-type GECIs, such as IP2.0, will help unravel neuronal circuit activities in the brain.



## Materials and methods

### Construction of an inverse-pericam mutants library

The TorA protein export plasmid (pTorPE) (Invitrogen) was constructed by inserting a DNA fragment encoding TorA-6xHis-inverse-pericam into EcoRI and HindIII sites. Primers FW-CCTCGCCACAGAATTCATGGTCGACTCATCAATGAA and RW-CAAAACAGCCAAGCTTGTTACCATTTCGCACGCTTAC were used for error-prone PCR to introduce random mutations into inverse-pericam. The resulting PCR products were digested with EcoRI and HindIII and ligated with similarly digested pTorPE.

### Screening of inverse-pericam mutant library

Competent One Shot Top10 *E. coli* (Invitrogen) were transformed with plasmids encoding the mutant library and cultured on nitrocellulose filters (ADVANTEC) overlaying LB-agar supplemented with 0.1 mM  $\text{CaCl}_2$ , 0.0016% (wt/vol) L-arabinose and 50  $\mu\text{g}/\text{ml}$  carbenicillin overnight at 37°C. Filters were transferred onto 1.5% agarose supplemented with 10 mM EGTA, 0.0016% L-arabinose and 50  $\mu\text{g}/\text{ml}$  carbenicillin (EGTA-agarose plate) and incubated for 4 h at 4°C. Before and after EGTA-agarose plate incubation, images were captured and colonies exhibiting a greater than ten times change in fluorescence intensity between the two images were picked. To compare images, we developed an image processing procedure using the MATLAB program and screened approximately 20,000 colonies (20 nitrocellulose membrane filters).

### Protein purification and characterisation of purified proteins

Recombinant fluorescent proteins with a polyhistidine tag at the N-terminus expressed in One Shot Top10 *E. coli* were subjected to Ni-NTA Sepharose purification and eluted as described [19]. The purified proteins in 30 mM MOPS (pH7.2) and 100 mM KCl were concentrated using Amicon Ultra-15 Centrifugal Filter Devices (Millipore) to make final concentration of 8  $\mu\text{g}/\mu\text{l}$  and used for *in vitro* characterization. Purified proteins of IP2.0 and inverse pericam were characterized in 30 mM MOPS (pH7.2) and 100 mM KCl containing either 10 mM EGTA ( $\text{Ca}^{2+}$ -free buffer) or 10 mM CaEGTA ( $\text{Ca}^{2+}$  buffer) (Molecular Probes by Life Technologies). Fluorescence emission (488 nm excitation) and excitation (515 nm emission) spectra were measured with 5 nm slits (JASCO FP-8200. Fluorescence Spectrometer).

For pH titrations, a solution containing 30 mM trisodium citrate and 30 mM borax was adjusted to pH 11.5 and HCl was then added dropwise to make solutions with pH values ranging from 11.5 to 4. One  $\mu\text{l}$  of concentrated protein in  $\text{Ca}^{2+}$ -free buffer (30 mM MOPS (pH7.2), 100 mM KCl, 10 mM EGTA) or  $\text{Ca}^{2+}$ -containing buffer (30 mM MOPS (pH7.2), 100 mM KCl, 10 mM  $\text{CaCl}_2$ ) was added into 100  $\mu\text{l}$  of each of the buffers described above. The fluorescent intensities were normalized to the maximum value in  $\text{Ca}^{2+}$ -free buffer (S2 File).  $\text{Ca}^{2+}$  titrations were performed by reciprocal dilution of a 1  $\mu\text{l}$  of concentrated protein solution into a series of 100  $\mu\text{l}$  of buffers mixed with  $\text{Ca}^{2+}$ -free and  $\text{Ca}^{2+}$ -saturated (39  $\mu\text{M}$ ) buffers (Molecular Probes by Life Technologies). The fluorescent intensities were normalized to the maximum value (S3 File). The  $\text{Ca}^{2+}$ -titration fluorescence was fit to the Hill's equation to extract the Hill coefficient and  $K_d$  for inverse-pericam and IP2.0.

$k_{\text{off}}$  was determined from a single exponential fit to the fluorescence increase following rapid mixing of the protein samples in 30 mM MOPS (pH 7.2), 100 mM KCl, 10 mM EGTA- $\text{Ca}^{2+}$ , 10 mM KOH and buffer with 10 mM MOPS (pH 7.2), 100 mM KCl and 10 mM EGTA using a stopped-flow device coupled to a fluorometer (JASCO J1500 with SFC). The fluorescent intensities were normalized to the maximum value and were fit to the equation of

1-exp (-t/tau). These raw data can be accessed in figshare (<https://doi.org/10.6084/m9.figshare.5976067.v1>).

## HeLa cell culture and imaging

The culture and imaging of HeLa cells were performed as described before [8, 18]. Briefly, HeLa cells (40–60% confluent) grown on collagen-coated 35-mm glass bottom dishes (Mastromami) were transfected with 1  $\mu\text{g}$  of plasmid DNA and 4  $\mu\text{L}$  SuperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After incubation for 3 h the media was exchanged to DMEM containing 10% foetal bovine serum and the cells were incubated for an additional 24 h at 37°C in a  $\text{CO}_2$  incubator. Immediately prior to imaging, cells were washed twice with Hank's balanced salt solution (HBSS) and then 1 mL of 20 mM HEPES buffered HBSS (HHBSS) was added.

Cell imaging was performed with an inverted Eclipse Ti-E microscope (Nikon) equipped with an electron multiplying (EM) iXon3 CCD camera (Andor). MetaMorph imaging software (Molecular Devices) was used for automated microscope and camera control. For determination of dynamic ranges in live cells, cells were imaged with a Plan Apo 60 $\times$  1.40 NA oil-immersion objective lens (Nikon). For excitation the samples were illuminated with light from a 100 W mercury arc lamp that was passed through 25% and 12.5% neutral density filters and a 497/16 nm bandpass filter. The emission filter was 535/22 nm. All imaging was performed at room temperature.

For imaging of histamine-induced  $\text{Ca}^{2+}$  dynamics, cells were imaged with a 100 ms exposure (2 $\times$ 2 binning) acquired every 5 s for a duration of 20 min. Approximately 30 s after the start of the experiment, histamine was added to a final concentration of 5  $\mu\text{M}$ . Once the measurement had ended, cells were washed twice with HHBSS, and then incubated for 10 min in 1 mL HHBSS to allow histamine-induced oscillations to subside. Cells were then imaged as described above, with exposures every 10 s for a duration of 10 min. Approximately 1 min after imaging was started, 1 mL of 2 mM  $\text{CaCl}_2$ , 10  $\mu\text{M}$  ionomycin in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HHBSS [HHBSS(-)] was added to the dish via a peristaltic pump. After measurements were completed, cells were washed 3 times with HHBSS(-) and 1 mL of HHBSS(-) was added. Approximately 2 min after imaging was started, 1 mL of 2 mM EGTA and 10  $\mu\text{M}$  ionomycin in HHBSS(-) was added and cells were imaged with exposures every 10 s for a total of 8 min. These raw data can be accessed in figshare (<https://doi.org/10.6084/m9.figshare.5976610.v1>).

## Preparation and imaging of transgenic *C. elegans*

cDNA sequences encoding IP2.0 and GCaMP6f were optimized and three introns inserted for effective expression in *C. elegans* [53]. The modified cDNAs encoding IP2.0, GCaMP6f and mCherry (donated by Dr. Jorgensen, University of Utah) were used to construct a destination vector using the Gateway system (Invitrogen). Each destination vector was used to make an expression plasmid fragment by an LR reaction with an entry vector containing the *str-2* promoter or *gcy-7* promoter. We used the following transgenic worms that were constructed by microinjection of the DNA mixture [54]. N2; *qjEx11*[20 ng/ $\mu\text{L}$  *pstr-2::GCaMP6f*, 5 ng/ $\mu\text{L}$  *pstr-2::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*], N2; *qjEx12*[20 ng/ $\mu\text{L}$  *pstr-2::GCaMP6f*, 5 ng/ $\mu\text{L}$  *pstr-2::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*], N2; *qjEx15*[20 ng/ $\mu\text{L}$  *pstr-2::IP2.0*, 5 ng/ $\mu\text{L}$  *pstr-2::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*], N2; *qjEx17*[20 ng/ $\mu\text{L}$  *pstr-2::IP2.0*, 5 ng/ $\mu\text{L}$  *pstr-2::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::rfp*], N2; *qjEx21*[20 ng/ $\mu\text{L}$  *pstr-2::IP2.0*, 75 ng/ $\mu\text{L}$  *pstr-2::RCaMP2.0*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*], N2; *qjEx22*[20 ng/ $\mu\text{L}$  *pgcy-7::IP2.0*, 10 ng/ $\mu\text{L}$  *pgcy-7::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*], N2; *qjEx23*[20 ng/ $\mu\text{L}$  *pgcy-7::IP2.0*, 75 ng/ $\mu\text{L}$  *pgcy-7::mCherry*, 5 ng/ $\mu\text{L}$  *plin-44::gfp*]. A young adult hermaphrodite expressing fluorescent proteins in an AWC<sup>ON</sup> neuron or an ASEL neuron was put into a polydimethylsiloxane

(PDMS) microfluidic chip for  $\text{Ca}^{2+}$  imaging. For imaging  $\text{Ca}^{2+}$  responses of an  $\text{AWC}^{\text{ON}}$  neuron, 0.001% IAA was added in imaging buffer (50 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.001% gelatine and 25 mM HEPES, pH 6.0) and perfused onto the top of the worm's head. A buffer (25 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.001% gelatine and 25 mM HEPES, pH 6.0) was prepared and used for the analysis of ASEL neurons. Optical recordings were performed on a Zeiss Axioplan upright compound microscope fitted with an image splitting optics W-VIEW GEMINI (Hamamatsu Photonics) with bandpass filters, FF01-512/25 and FF01-650/60-25 (Semrock) and a dichroic mirror, FF580-FDi01-25x36 (Semrock). Fluorescence images were acquired using HSIImage/HSR software (Hamamatsu Photonics) at 5 frames/second. The fluorescence intensity ratios of IP2.0 against mCherry were calculated using our MATLAB program. For imaging  $\text{Ca}^{2+}$  responses from two kinds of  $\text{Ca}^{2+}$  probe (IP2.0 and RCaMP2.0), an Olympus BX53-F microscope equipped with a 60 $\times$  objective and an ORCA-D2 (Hamamatsu) was used with bandpass filters, FF01-650/60-25 (Semrock) and a dichroic mirror, 570 (Hamamatsu Photonics). The fluorescence intensity in a period before stimulation (time = 1–10 s for Fig 4A and 4B, time = 1–25 s for Fig 4C and time = 1–30 s for Fig 4D) was averaged and defined as  $F_0$ . The change in fluorescence value of ROI relative to  $F_0$  was plotted for all stacks. All time series data relative to  $F_0$  can be accessed in figshare (<https://doi.org/10.6084/m9.figshare.5976619.v1>).

## Supporting information

**S1 Fig. Representative  $\text{Ca}^{2+}$  imaging in HeLa cells expressing IP2.0.** Each dot was the ratio of fluorescent intensity of the first spike responding to histamine to the initial fluorescent intensity.

(TIFF)

**S2 Fig. *C. elegans* expressing GCaMP6f in an  $\text{AWC}^{\text{ON}}$  neuron.** The region of interest (ROI) was defined by a square.

(TIFF)

**S3 Fig. Effects of transgenic genes of GCaMP6f or IP2.0 in  $\text{AWC}^{\text{ON}}$  on chemotaxis toward IAA.** Chemotaxis toward IAA was analyzed on 9 cm chemotaxis assay plates as described previously (Bargmann CI et al, 1993), except that the assay plates contained 50 mM NaCl. The chemotaxis index was calculated as  $(A-B) / N$ , where A was the number of animals within 1.5 cm of the IAA spot, B was the number of animals within 1.5 cm of the control spot, and N was the number of all animals. A, 0.033%-0.33% IAA was spotted on assay plates, and 2  $\mu\text{l}$  of 1 M sodium azide were placed on both the IAA spot and the control spot to anesthetize animals when they reached either spot. In the behavioral assays, the chemotaxis indexes of both the transgenic animals (*qjEx15* and *qjEx17*) and wild type animals were measured on the same assay plates. To distinguish these two, when we counted the number of animals on assay plates, *Plin44::gfp* or *plin44::rfp* was used as injection markers for carrying transgenes. Prior to the behavioral assays, adult worms were washed twice with S-basal buffer (100 mM NaCl, 50 mM  $\text{K}_2\text{HPO}_4$  [pH 6]) containing 0.02% gelatin, and once with water containing 0.02% gelatin. B, Chemotaxis indexes of worms expressing GCaMP6f (*qjEx12*) or IP2.0 (*qjEx15*) toward 0.33% IAA was analyzed. Error bars represent SEM (n = 4).

(TIFF)

**S4 Fig. Concentration dependency of IAA on IP2.0  $\text{Ca}^{2+}$  responses in  $\text{AWC}^{\text{ON}}$ .** IP2.0  $\text{Ca}^{2+}$  imaging was performed as described in Fig 4A (see [Materials and methods](#)). Bar graphs show fluorescence changes during the 60 seconds after stimulation of various concentration of IAA (10–70 sec. in Fig 4). The values are shown as relative to  $F_0$  (see [Materials and methods](#)) and

error bars represent SEM ( $n = 5$ ). These raw data can be accessed in figshare (<https://doi.org/10.6084/m9.figshare.5976634.v1>).

(TIFF)

**S5 Fig. *In vivo* imaging of  $\text{Ca}^{2+}$  responses in *C.elegans*.** (A) GCaMP6f  $\text{Ca}^{2+}$  response to isoamylalcohol in  $\text{AWC}^{\text{ON}}$  (*qjEx11*) ( $n = 6$ ). (B) IP2.0  $\text{Ca}^{2+}$  response to isoamylalcohol in  $\text{AWC}^{\text{ON}}$  (*qjEx17*) ( $n = 7$ ). (C) Dual-colour of RCaMP2.0 and IP2.0  $\text{Ca}^{2+}$  responses to change of NaCl concentration (*qjEx23*) ( $n = 10$ ). The values are shown as relative to  $F_0$  and error bars represent SEM. These raw data can be accessed in figshare (<https://doi.org/10.6084/m9.figshare.5976643.v1>).

(TIFF)

**S1 Table. Transgenic worms used in this work.**

(DOCX)

**S1 File. Data of excitation and emission spectra in  $\text{Ca}^{2+}$ -free and  $\text{Ca}^{2+}$ -saturated states of inverse-pericam and IP2.0.**

(PDF)

**S2 File. Data of fluorescent spectra under the various pH-condition at the 515 nm excitation peak in  $\text{Ca}^{2+}$ -free and  $\text{Ca}^{2+}$ -saturated states.**

(PDF)

**S3 File. Data of fluorescent spectra under the various  $\text{Ca}^{2+}$ -concentration of inverse-pericam and IP2.0.**

(PDF)

**S4 File. Data of fluorescent intensity-change of IP2.0 after histamine stimulation in HeLa cells.**

(PDF)

**S5 File. Data of chemotaxis assay toward IAA using transgenic worms shown in S2 Fig.**

(PDF)

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