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## Cellomics approach for high-throughput functional annotation of *Caenorhabditis elegans* neural network

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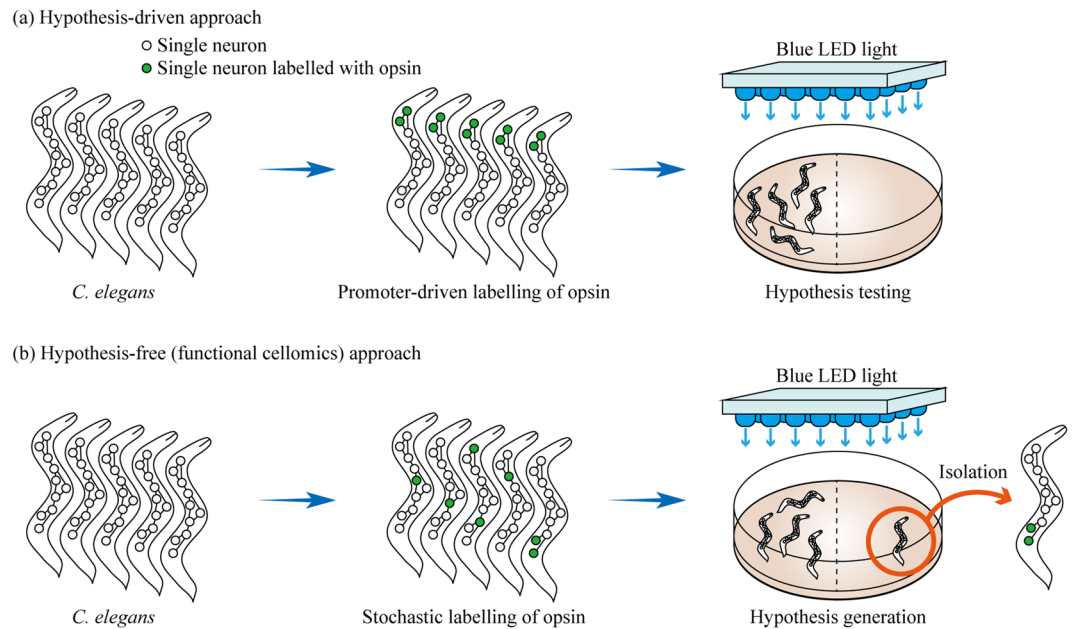
In *Caenorhabditis elegans*, which has only 302 neurons, relationships between behaviors and neural networks are not easily elucidated. In this study, we proposed a novel cellomics approach enabling high-throughput and comprehensive exploration of the functions of a single neuron or a subset of neurons in a complex neural network on a particular behavior. To realize this, we combined optogenetics and Brainbow technologies. Using these technologies, we established a *C. elegans* library where opsin is labeled in a randomized pattern. Behavioral analysis on this library under light illumination enabled high-throughput annotation of neurons affecting target behaviors. We applied this approach to the egg-laying behavior of *C. elegans* and succeeded in high-throughput confirmation that hermaphrodite-specific neurons play an important role in the egg-laying behavior. This cellomics approach will lead to the accumulation of neurophysiological and behavioral data of the *C. elegans* neural network, which is necessary for constructing neuroanatomically grounded models of behavior.

One of the primary objectives of neuroscience is to understand how computations are implemented across neural networks. Hermaphrodites of *Caenorhabditis elegans*, for instance, have a simple neural network consisting of 302 neurons, and their connectome, a comprehensive map of neural connections, has already been clarified<sup>1,2</sup>. However, even in *C. elegans*, the relationships between behaviors and neural networks have not been comprehensively revealed.

Data-driven science, in which an enormous amount of data is accumulated in a hypothesis-free manner, has deepened our understanding of complex biological processes. For example, the applications of state-of-the-art mass spectrometry<sup>3</sup> and next-generation sequencers<sup>4</sup> have been streamlining the process of understanding diverse biological processes. However, even with such omics analyses, it is still difficult to gain deeper insights into individual-level biological processes engendered by complex neural networks. To understand such biological processes, it is important to develop a new methodology for collecting omics data at the cellular level (cellomics) and implement data-driven science at the individual-level. In recent studies, “descriptive” cellomics, has been gradually changing the landscape of neuroscience. Serial electron microscopy<sup>5</sup> has been promoting comparative connectomics<sup>6,7</sup>. In addition, new approaches, such as Brainbow<sup>8</sup> or Optobow<sup>9</sup> were developed in an attempt to reveal brain structures using optical microscopy.

Structural information is important to understand the workings of the brain, but that alone is still insufficient. It is considered essential to accumulate comprehensively neurophysiological and behavioral data on what functions a particular part of the brain has, in an effort to construct neuroanatomically grounded models of behavior. In *C. elegans* research, various methodologies for collecting neurophysiological data are available, such as calcium imaging<sup>10</sup>, optical and electrophysiological recording<sup>11</sup>, and laser ablation<sup>12</sup>. However, no single methodology

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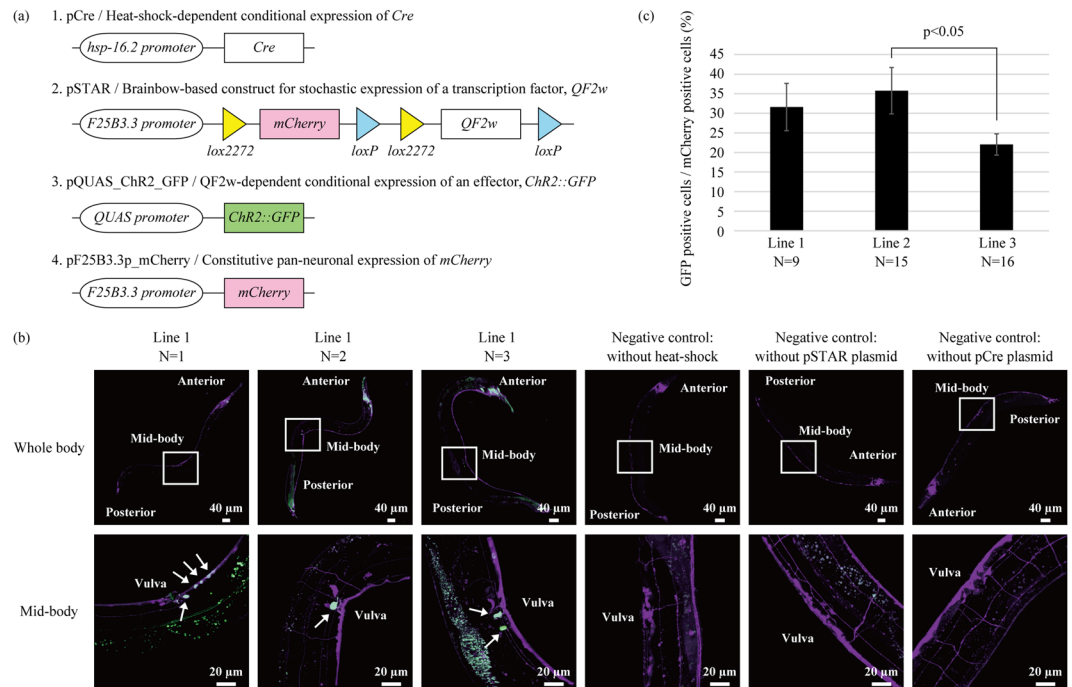
**Figure 1.** Comparison between hypothesis-driven and hypothesis-free (functional cellomics) approaches for annotation of neural networks. **(a)** Experimental scheme for hypothesis-driven neural network analysis. First, a hypothesis is formulated as to which neuron or a subset of neurons is responsible for a certain behavior. Next, only specific neurons are allowed to express an effector (opsin in this figure) by using appropriate promoters. Finally, behavioral experiments are conducted with this transgenic *C. elegans* to test whether the initial hypothesis is correct. **(b)** Experimental scheme for hypothesis-free (functional cellomics) approach for neural network analysis. In the method that we proposed, it is determined in a stochastic manner whether or not each single neuron is labeled by an effector. By performing behavioral experiments on a *C. elegans* library in which neurons are labeled in various patterns, one can expect to find individuals that differ behaviorally from wild-type *C. elegans*. By isolating these individuals and examining which neuron was producing opsin, one may be able to discover a new relationship between neural networks and behavior.

realizes comprehensive accumulation of neurophysiological and behavioral data on what functions a particular part of a neural network has in a high-throughput and systematic manner.

In this context, we considered establishing a novel methodology called “functional” cellomics, which allows comprehensive exploration of what functions a single neuron or a subset of neurons in a complex neural network has on a certain behavior. A methodology to realize functional cellomics is required to allow researchers to manipulate a neuron or a subset of neurons in a high-throughput, hypothesis-free, single-cell-resolution, and simple manner and to quantify the effect of such manipulation on a behavior. To achieve this approach, we fused optogenetics, Brainbow technologies, and behavioral analysis. Optogenetics is a technique that enables on-demand photoregulation of neural activity through the expression of opsin genes<sup>13</sup>. However, with conventional optogenetics, it is essential to determine in advance which promoters should be used to produce an opsin in specific neurons<sup>14</sup>. Therefore, this approach is hypothesis-driven, meaning that it is effective in precisely testing existing hypotheses, but is not conducive to establishing entirely new ones (Fig. 1a).

To address this issue, we designed a new optogenetic experimental scheme to accomplish hypothesis-free neural network annotation (Fig. 1b). Specifically, we attempted to develop a system that stochastically determines whether the effector is produced in each single neuron and to acquire a *C. elegans* library in which the effector is labeled in diverse patterns. To achieve this stochastic labeling, we adopted Brainbow technologies based on the Cre-lox system<sup>8</sup>. If a *C. elegans* library with a random labeling pattern of the effector can be obtained through the use of Brainbow technologies, it would be possible to uncover hitherto unknown relationships between neural networks and behaviors in a high-throughput manner by performing behavioral experiments with the library under light illumination. This approach is hypothesis-free because individuals showing abnormal behavior are first detected and responsible neurons are then identified. By analogy, functional cellomics can be regarded as a cell level application of the forward genetics concept, in which random mutations are introduced across the whole genome and genes altered in mutants showing some phenotypic changes are then identified.

In the present study, we successfully demonstrated the possibility that neurons affecting a behavior of interest can be identified by the combined use of optogenetics and Brainbow technologies. The results substantiated the basic concept of functional cellomics that enables functional annotation of neural networks in a high-throughput, hypothesis-free, single-cell-resolution, and simple manner.



**Figure 2.** Stochastic labeling of neurons based on Brainbow technologies. **(a)** One of the constructs (pCre) is designed to produce Cre recombinase in a heat-shock-dependent manner. Another construct (pSTAR) is based on Brainbow technologies, with the *lox* sequences, *mCherry*, and the transcription factor *QF2<sup>w</sup>* encoded downstream of the pan-neuronal promoter (*F25B3.3p*). In the initial state, pSTAR and pF25B3.3p\_mCherry produces mCherry in all neurons. When an excision occurs between *lox2272* sequences, *QF2<sup>w</sup>* is produced, and the production of mCherry continues from pF25B3.3p\_mCherry even after Cre recombination. pQUAS\_ChR2\_GFP expresses the effector *ChR2::GFP* in a *QF2<sup>w</sup>*-dependent manner. **(b)** Stochastic labeling of neurons with ChR2–GFP. A brief heat shock was applied to the transgenic *C. elegans* that carries the four constructs as extrachromosomal (Ex) arrays, and each sample was observed using confocal laser scanning microscopy 12 h later. For negative control experiments, the transgenic *C. elegans* without heat shock, the transgenic lines without pSTAR plasmid or pCre plasmid with heat shock were used. The whole body was observed at a magnification of 10 $\times$ , and the mid-body section (framed in white in the whole body picture), where neurons can be counted with relative ease, was observed at a magnification of 40 $\times$ . The 2D images shown are the maximum-intensity projection reconstructed from the z-stacks of the images acquired with the confocal microscope. The fluorescence of ChR2–GFP is presented in green, and that of mCherry in magenta. The cells producing both fluorescent proteins are presented in white and indicated by an arrow. The white-dyed cells differed from one individual to another, indicating the success of stochastic labeling. **(c)** The ratio of GFP positive cells/mCherry positive cells. We established three transgenic lines harboring all plasmids shown in Fig. 2a, and quantified the ratio of GFP positive cells using at least nine individuals from each line. In each individual, we counted 12–26 fluorescent cells. In this experiments, we counted fluorescent cells of the mid-body and tail sections, because cytoplasmic production of mCherry made it difficult to precisely count the number of fluorescent neurons around the head ganglia. The data show mean  $\pm$  standard deviation, and Tukey’s test was used to test the significance of differences between all of pairs of the result.

## Results

**Design of strategy for optogenetic functional cellomics.** Stochastic labeling of individual neurons with an effector gene that induces cell autonomous activity provides a high-throughput and hypothesis-free means of functional cellomics at a single-cell resolution. Brainbow technologies<sup>8</sup> refer to systems that can stochastically determine whether a certain gene is expressed in a certain cell through the application of the Cre-lox recombination system. In Brainbow technologies, multiple *lox* variants (e.g., *loxP* and *lox2272* sequences) are inserted alternately downstream of one promoter and two other genes are interposed between these *loxP* sequences (Fig. 2). If Cre recombinase is allowed to act on this sequence, excision occurs exclusively either between *loxP* sequences or between *lox2272* sequences. Consequently, it becomes possible to determine which of these two genes is expressed in a Cre-dependent manner.

To implement functional cellomics, we designed four plasmids (Fig. 2a). The plasmid pCre expresses Cre recombinase in response to a heat shock. In the plasmid pSTAR, *lox* sequences, *mCherry*, and a transcription factor (*QF2<sup>w15</sup>*) were inserted downstream of a pan-neuronal promoter (*F25B3.3p*). The plasmid pQUAS\_ChR2\_GFP expresses an arbitrary effector gene in a *QF2<sup>w</sup>*-dependent manner. In the present study, we adopted channelrhodopsin-2 fused with GFP (*ChR2::GFP*). Since the constructs producing a transcription factor or an effector are modularized, it is easy to use not only opsin but also various other effectors. We also constructed pF25B3.3p\_mCherry, which continuously produces mCherry even after Cre recombination.

When all of these plasmids are introduced into *C. elegans*, all neurons produce only mCherry at the initial state. After a heat shock is applied to induce Cre recombinase, QF2<sup>m</sup> is produced if an excision occurs between *lox2272* sequences, and the production of mCherry continues from pF25B3.3p\_mCherry even after Cre recombination. In neurons producing QF2<sup>m</sup>, Chr2–GFP is produced as an effector, enabling the on-demand activation of these neurons by light illumination. Since GFP is fused to Chr2, it is easy to identify which neurons are producing opsin following a behavioral experiment.

**Stochastic labeling of neurons at single-cell level.** We introduced the above-mentioned four plasmids into *C. elegans* and established three lines (Lines 1–3) of the AYK338 strain (*aykEx338 [hsp-16.2p::Cre, F25B3.3p::lox2272::mCherry::loxP::lox2272::QF2<sup>m</sup>::loxP, QUAS::Chr2::GFP, F25B3.3p::mCherry]*). After this *C. elegans* strain had propagated, a brief heat shock was applied to determine whether Chr2–GFP was labeled stochastically in each *C. elegans* individual. After isolating at least nine individuals, we observed their mid-body sections where the neuron density was low at a magnification of 40x. The results revealed that all of heat-shock-treated animals showed production of Chr2–GFP, and the labeling pattern of Chr2–GFP differed from one individual to another (Fig. 2b). In Fig. 2b, we showed micrographs of three representative animals from Line 1. We quantified the average ratio of cells labeled with Chr2–GFP. Though we observed that production of Chr2–GFP was randomized using any lines, the labelling ratio slightly differed probably because of differences in structures of extrachromosomal (Ex) arrays (Fig. 2c). We compared the labelling probability of Chr2–GFP at the mid-body region and the tail region. Using the same dataset in Fig. 2, we found that transgenic *C. elegans* showed the GFP-labelling probability  $32 \pm 12\%$  (mean  $\pm$  standard deviation) at the tail region and  $31 \pm 16\%$  (mean  $\pm$  standard deviation) at the mid-body region. Though we found no significant difference using *t*-test, the mid-body region showed higher variation. In addition, we carried out three types of negative control experiments using the transgenic *C. elegans* without heat shock, the transgenic lines without pSTAR plasmid (substrate for Cre) or pCre plasmid with heat shock. In each negative control experiment, we established three lines, and observed ten animals from each line. As a result, we found no green fluorescence in neurons (Fig. 2b). These results indicated production of Chr2–GFP was dependent on induction of Cre by heat shock.

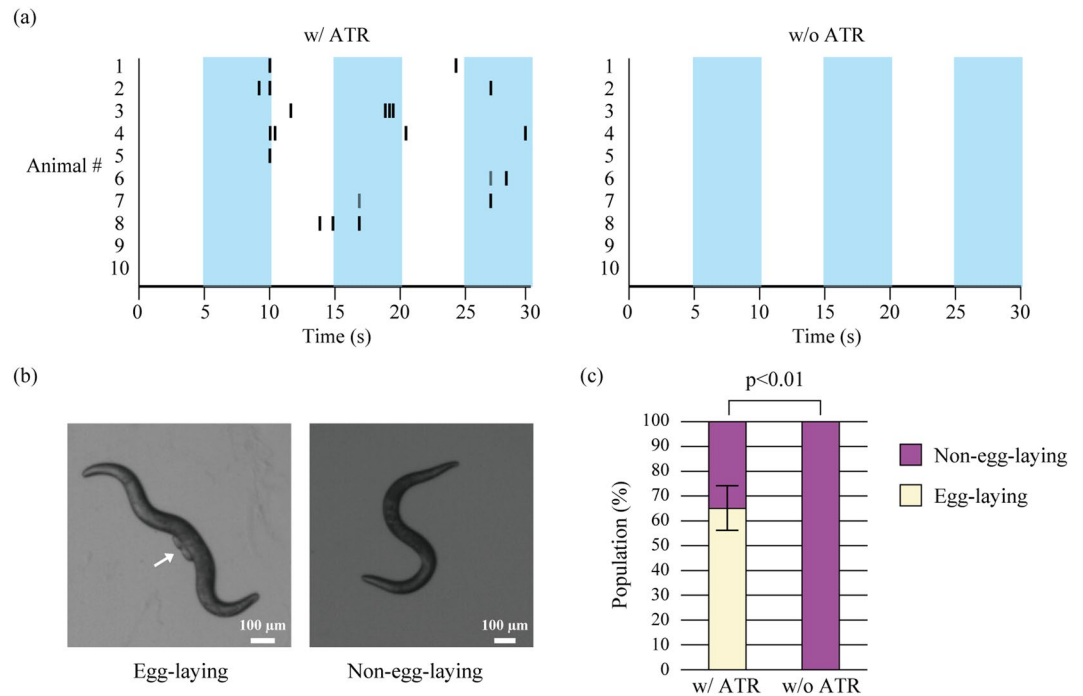
**Identification of neurons responsible for egg-laying behavior.** In functional cellomics, stochastic labeling of an effector gene makes it possible to explore the relationships between neural networks and behaviors in a hypothesis-free and comprehensive manner. To demonstrate the feasibility of functional cellomics, we selected the egg-laying behavior of *C. elegans* as a model. It is known that a relatively simple neural network is responsible for controlling the egg-laying behavior of this nematode. In this behavior, two hermaphrodite-specific neurons (HSNs: HSNR and HSNL) play a central role through direct excitation of vulval muscles and ventral C neurons (VCs)<sup>16–18</sup>. Moreover, it is known that the activation of the HSNs by Chr2 induces egg-laying behavior<sup>19</sup>. If the HSNs can be identified by functional cellomics in a high-throughput manner, it shows that this strategy actually works.

We constructed a *C. elegans* library in which Chr2–GFP is stochastically labeled. Individuals of this transgenic *C. elegans* were filmed for 30 sec while illuminated with blue light (Fig. 3a). Among the filmed individuals, 65% laid eggs in a light-dependent manner, whereas 35% did not (Fig. 3b,c and Supplementary Information 1). When a similar experiment was conducted without all-*trans* retinal (ATR), a cofactor of Chr2, no egg-laying behavior was observed. These results indicate that the egg-laying behavior observed in this experiment is Chr2-dependent, and that individual nematodes exhibiting the target phenotype can be readily obtained through the stochastic labeling of an effector.

Next, we used confocal laser scanning microscopy to determine whether Chr2–GFP was produced in the HSNs. After isolating egg-laying and non-egg-laying individuals and observing the vicinity of the vulva, we confirmed that Chr2–GFP was produced in the HSNs in the all of egg-laying individuals (Fig. 4a), but it was not in the non-egg-laying individuals (Fig. 4b). In the representative individual shown in Fig. 4a, the production of Chr2–GFP was seen in HSNR but not in HSNL. Around 80% of non-egg-laying individuals showed fluorescence of mCherry in HSN. The GFP-labelling probability is  $31 \pm 11\%$  (mean  $\pm$  standard deviation,  $N = 10$ ) and  $29 \pm 14\%$  (mean  $\pm$  standard deviation,  $N = 10$ ) in egg-laying and non-egg-laying animals similar to the data in Fig. 2, and we found no significant difference using *t*-test. Quantitative profiling of fluorescence intensity showed that production of Chr2–GFP was detected in HSNR but not in other neurons adjacent to HSNs in the egg-laying individual (Fig. 4c). A previous study demonstrated that killing one HSN by laser ablation did not markedly affect the egg-laying behavior of the nematode, whereas killing both HSNs resulted in strong inhibition of the egg-laying behavior<sup>17</sup>. Our result that the egg-laying behavior was induced sufficiently by activating only one HSN is consistent with that of this previous study. However, our results don't exclude the possibility that egg-laying behavior can be activated by Chr2-dependent activation of other neurons. In this paper, cytoplasmic production of mCherry in all neurons and the high probability of Chr2–GFP labelling made it difficult to precisely annotate neurons other than HSNs which might have a certain role on egg-laying behavior. In future work, we will improve our system by using nuclear-localization signal and/or decreasing the probability of Chr2–GFP labelling.

## Discussion

Although various methodologies have been established to explore the properties of neural networks, no single methodology satisfies all of the criteria necessary for realizing the conceptual framework of functional cellomics. One typical example of existing methods, which is very easy yet effective, is to induce the production of effectors using cell-type-specific promoters. However, this approach is basically hypothesis-driven because one needs to select specific promoters a priori, meaning that it is not suitable for establishing entirely new hypotheses. Moreover, it is also difficult to analyze neural networks at single-cell resolution because *C. elegans* has few single-cell-specific promoters. Another example is laser ablation, which enables hypothesis-free and single-cell-resolution analysis<sup>12</sup>. Although this is a powerful technique applicable to any species, its low

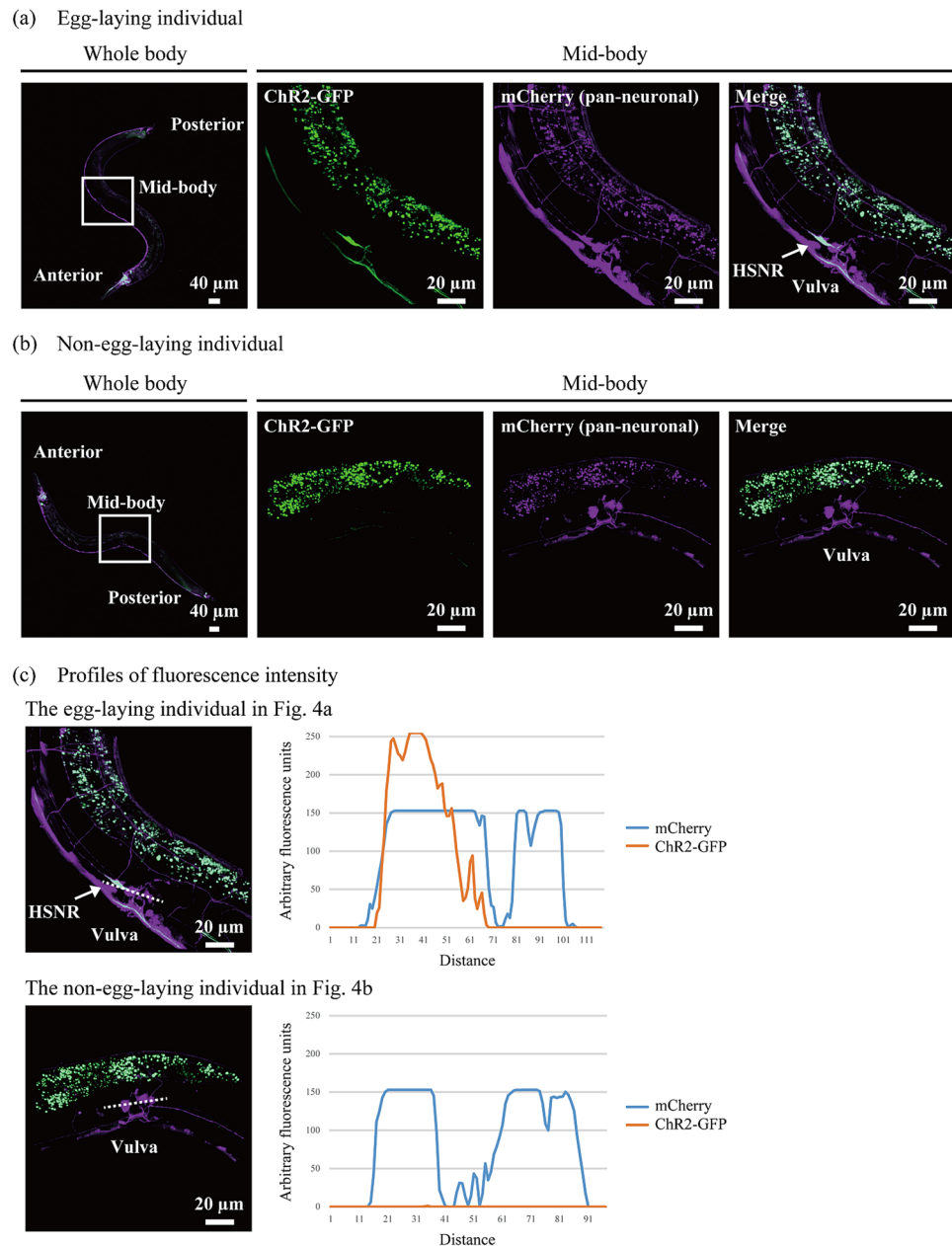


**Figure 3.** Acquisition of individuals exhibiting egg-laying behavior in a light-dependent manner. **(a)** Egg-laying data in raster graph format. The behavior of each nematode was filmed for 30 sec. During the filming, blue light was turned on and off at 5-second intervals. Each black line shows an egg-laying event. **(b)** Images captured from the representative footage during the behavioral experiment, showing an individual that exhibited egg-laying behavior in a light-dependent manner (left) and an individual that did not (right). The arrow indicates where the eggs are. **(c)** Proportion of individuals that exhibited light-dependent egg-laying behavior. In the presence of all-*trans* retinal (ATR), a cofactor of opsin, 65% of adult individuals exhibited egg-laying behavior under blue light (N = 20). A negative control experiment was performed identically without ATR, in which all adult individuals turned out to be non-egg-laying (N = 20). In these experiments, at least six individuals from each line were used. Fisher's exact test was used to test the significance of differences. The error bar shows 95% confidence interval.

throughput makes it difficult to conduct experiments involving various patterns of intervention. Additionally, laser ablation lacks expandability in that it cannot activate or suppress neurons. A recent study has suggested the possibility that contradictory results are generated depending on the mode of intervention<sup>20</sup>, indicating the need to compare results obtained from various modes of intervention (activation, suppression, killing, etc.). Another method enabling analysis at single-cell resolution involves application of a heat shock to only specific cells to induce effectors<sup>21,22</sup>. This method is capable of manipulating neurons in many ways; however, similar to laser ablation, it is also restricted by its low throughput capacity. The patterned illumination technique using digital micromirror devices, whose development has been advancing in recent years, allows for a high degree of freedom in experimental design and has a relatively high throughput<sup>19</sup>. However, it is still difficult to perform accurate analysis at single-cell resolution with this method because multiple adjacent neurons may be illuminated simultaneously, unless sufficiently sparse expression patterns of effectors are provided<sup>23</sup>.

Functional cellomics described in this study is the first approach to combine all properties necessary for achieving individual-level cellomics, that is, high-throughput, hypothesis-free, single-cell resolution, and simplicity. In fact, by applying functional cellomics to the egg-laying behavior of *C. elegans*, we managed to establish the proof of concept of this approach.

Compared with the existing methodologies, this system has advantages in terms of throughput, resolution, and expandability. First, it can be easily implemented in any laboratory without requiring any specialized equipment. Second, having no limitations in feasible labeling patterns, it is completely hypothesis-free, facilitating easy labeling at single-cell resolution, even for bilaterally symmetrical neuron pairs with almost identical gene expression patterns. Third, with one transgenic *C. elegans* individual capable of propagating many other individuals with different labeling patterns, it is both simple and high-throughput. Fourth, it can intervene in neural networks in various fashions. Besides opsin, which was employed in the present study, any effectors can be used as long as they cause either loss of function or gain of function in neurons. This enables a variety of interventions, such as cell killing<sup>24,25</sup>, suppression<sup>26</sup>, activation<sup>27</sup>, and gene expression control<sup>28,29</sup>. Our approach will be more useful if it could determine which neurons are required for a target behavior. Fifth, multitudes of experimental designs are available using *C. elegans* promoterome<sup>30</sup> and bipartite gene expression systems such as QF2<sup>w</sup> and Gal4<sup>31</sup>. For example, instead of a pan-neuronal promoter, one can use a promoter specific to a subset of neurons for more “focused” functional cellomics. In addition, simultaneously employing QF2<sup>w</sup> and Gal4, one can label multiple effectors in a stochastic fashion.



**Figure 4.** Identification of HSNs. The whole body was observed with a 10 $\times$  objective, and the mid-body section with a 40 $\times$  objective. The 2D images shown are the maximum-intensity projection reconstructed from the z-stacks of the images acquired with a confocal microscope. (a) Fluorescence images of an egg-laying individual. The fluorescence of ChR2-GFP is presented in green, and that of mCherry in magenta. The cells that are producing both fluorescent proteins are presented in white. In this particular individual that laid eggs in a light-dependent manner, the expression of ChR2-GFP in HSNR was observed. (b) Fluorescence image of a non-egg-laying individual. Light did not induce egg-laying behavior in this individual, and no production of ChR2-GFP was observed in neurons around the vulva. (c) Profiles of fluorescence intensity. Fluorescence profiles of dotted lines were quantified by ImageJ.

Although promising, there are three points of concern regarding functional cellomics. One is robustness of the heat-shock promoter used in this study. Though previous studies showed the promoter causes robust induction of proteins in the nervous system<sup>32–34</sup>, it has not been proved that it works equally in all types of neuronal cells. To address this issue, it will be necessary to use various cell-type-specific pSTAR plasmids and to quantify the probability of *ChR2::GFP* labelling in each cell types. The second point is the high probability of effector labeling, which makes it difficult to calculate the labeling rate accurately. To perform a well-designed experiment, it is necessary to achieve strict control of the probability of effector labeling, as is the case with forward genetics in which the mutation rate is predetermined. Figure 3b shows that a large population of *C. elegans* (65%) displayed egg-laying behavior, and this was probably because of high copy number of the pSTAR plasmid. The high copy number

increases the chance of excising the *lox2272* sequence by Cre, resulting in a large proportion of neurons labeled by opsin. When too many neurons are labeled by effectors, the effectors cause high level of neuronal activation and it becomes difficult to determine which neurons are responsible for the target behavior. Concomitant use of single-copy integration and *lox* variants may be able to control the probability of labeling only a desired number of all 302 neurons of a *C. elegans* hermaphrodite. In our laboratory, we are proceeding with the construction of a more sophisticated system that will help improve this issue. The third point is how to ensure that the obtained results are reproducible. If functional cellomics implies that a certain labeling pattern may affect the target behavior, it is still necessary to verify this by other methods. To reproduce the labeling pattern, methodologies that can evoke gene expression in arbitrary cells, such as use of a pulsed infrared laser<sup>21</sup> and multi-step optogenetics<sup>22</sup>, may be applied to verify the results relatively easily. Besides, the intersectional Cre-*lox* strategy<sup>35</sup> and multiple-feature Boolean logic<sup>36</sup> may also be applicable to reproducing the labeling pattern.

In conclusion, we have demonstrated for the first time the possibility of identifying neurons responsible for a target behavior by randomizing the labeling patterns of effector genes based on Brainbow technologies. Though several improvements and additional data are necessary to prove the utility of our approach on various behaviors, the results substantiated the basic concept of functional cellomics, which enables functional annotation of neural networks of *C. elegans* in a high-throughput, hypothesis-free, single-cell-resolution, and simple manner. Since its connectome information is already mapped and available, *C. elegans* is highly compatible with functional cellomics. By overlaying the connectome information with the results of cyclopedic intervention experiments by functional cellomics, we should become able to construct neuroanatomically grounded models of behavior that can explain how complex neural networks implement computation.

## Methods

**Construction of plasmids.** To construct pCre, *SV40NLS-Cre* was amplified from pPGK-Cre-bpA deposited by Klaus Rajewsky (Addgene plasmid #11543). The amplified fragment was inserted into pPD49\_78 deposited by Andrew Fire (Addgene plasmid #1447).

To construct pSTAR, a backbone plasmid with *lox* and *QF2<sup>w</sup>*<sup>15</sup> sequences was synthesized (Thermo Fisher Scientific, MA, USA). In addition, *mCherry* was subcloned from pGH8<sup>37</sup> deposited by Erik Jorgensen (Addgene plasmid #19359) and a pan-neuronal promoter, *F25B3.3p*, was cloned from the *C. elegans* genome. These two fragments were inserted into the backbone plasmid.

To construct pQUAS\_ChR2\_GFP, *ChR2 (H134R)* and *GFP (S65C)* were amplified from pAAV-Efla-vCreDIO hChR2(H134R)-EYFP deposited by Karl Deisseroth (Addgene plasmid #55643) and L2680 deposited by Andrew Fire (Addgene plasmid #1516), respectively. In addition, a *QF2<sup>w</sup>*-dependent promoter sequence, *QUAS::Δpes-10*<sup>38</sup>, was synthesized (Thermo Fisher Scientific). These three fragments were inserted into pPD49\_78.

To construct pF25B3.3p\_mCherry, *F25B3.3p* and *mCherry* were subcloned from pSTAR. These fragments were inserted into pPD49\_78.

Full sequences of these plasmids are provided in Supplementary Information 2.

**Culture conditions.** Worms were cultivated on nematode growth medium (NGM) plates with *Escherichia coli* OP50. Specifically, the OP50 plates were prepared with 250 μL of OP50 seeded into 6-cm NGM plates. The worms were maintained at 20 °C, with care taken to ensure that the temperature shifted as little as possible. To perform optogenetic experiments, 300 μL of 500 μM ATR (Sigma-Aldrich, MO, USA) was added to solid NGM plates with *E. coli*, and the samples were allowed to dry while shielded from light by aluminum foil.

**Transgenic strains.** Injections into the nematodes were performed with the aid of a stereomicroscope (SZX10; Olympus, Tokyo, Japan) equipped with a Femtojet 4i (5252 000.021; Eppendorf, Hamburg, Germany) and Femtotips II (1501040; Eppendorf). The strain AYK338 (*aykEx338 [hsp-16.2p::Cre, F25B3.3p::lox2272::mCherry::loxP::lox2272::QF2w::loxP, QUAS::ChR2::GFP, F25B3.3p::mCherry]*) was generated by co-injecting the four plasmids constructed in this study (50 ng μL<sup>-1</sup> each in water) into the *C. elegans* N2 background. The injection was performed on 20 nematodes with an N2 background, and three *mCherry*-expressing lines were obtained. Previous researches showed that blue-light illumination on the N2 background don't affect egg-laying behavior<sup>39</sup>.

To carry out negative control experiments, we generated the strain AYK339 (*aykEx339 [F25B3.3p::lox2272::mCherry::loxP::lox2272::QF2w::loxP, QUAS::ChR2::GFP, F25B3.3p::mCherry]*, 50 ng μL<sup>-1</sup> each in water) and AYK340 (*aykEx340 [hsp-16.2p::Cre, QUAS::ChR2::GFP, F25B3.3p::mCherry]*, 50 ng μL<sup>-1</sup> each in water) by microinjection into the N2 background.

**Induction of Cre recombinase for stochastic labeling of ChR2.** Transgenic worms were placed on NGM plates with or without ATR. The worms were incubated at 37 °C for 30 min for the induction of Cre recombinase by heat shock, after which they were placed in an incubator at 20 °C. At 12 h after the heat shock, the worms were examined by egg-laying assay and/or fluorescence microscopy.

**Fluorescence microscopy.** A 5% agarose pad (01149-05; Nacalai Tesque, Kyoto, Japan) was prepared, onto which 5 μL of 50 mM sodium azide (830011; Nacalai Tesque) was placed. *C. elegans* worms were picked up and placed onto the agarose pad with sodium azide, over which a cover glass was placed gently. Fluorescence was observed by confocal laser scanning microscopy (LSM700; Carl Zeiss, Oberkochen, Germany). Fluorescence of GFP and *mCherry* was observed using 488 nm and 561 nm lasers, respectively. Acquired images were processed using Zen Lite, Imaris, or ImageJ<sup>40</sup> software.

**Egg-laying assay.** The worms were observed under a stereomicroscope (SZX10; Olympus) equipped with a camera (HAS-L1; DITECT, Tokyo, Japan). The SZX10's halogen lamp (410849; PHILIPS, Amsterdam,

the Netherlands) was fitted with an optical filter (Asahi Spectra, Tokyo, Japan) that blocks wavelengths below 600 nm to prevent ChR2-GFP from being activated within the worms during observation. To activate ChR2-GFP, the worms were illuminated using blue light (LDL2-98X30BL2; CCS, Kyoto, Japan) powered by PD3-5024-4-PI (CCS). To prevent the LED's blue light from being detected by the camera, the object lens was fitted with an optical filter (Asahi Spectra) to block wavelengths below 570 nm.

For an egg-laying assay to examine light-dependent behavior modulation, each worm was transferred to a 6-cm agar NGM plate without *E. coli* and filmed for 30 sec. During this filming, the blue light was turned on and off at 5-second intervals, and the individuals that exhibited egg-laying behavior during 30 sec were defined as egg-laying individuals. Irrespective of the absence or presence of *E. coli*, the activation of the HSNs induces egg-laying behavior<sup>19</sup>.

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## Author Contributions

W.A. conceived the project. W.A., H.M., Y.Y., and H.Y. performed experiments and data analysis. K.H., R.S. and M.U. provided advice on method development. The manuscript was prepared by W.A. and edited by all co-authors.

## Additional Information

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