Video Article

A Molecular Readout of Long-term Olfactory Adaptation in *C. elegans*

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Abstract

During sustained stimulation most sensory neurons will adapt their response by decreasing their sensitivity to the signal. The adaptation response helps shape attention and also protects cells from over-stimulation. Adaptation within the olfactory circuit of *C. elegans* was first described by Colbert and Bargmann[1,2]. Here, the authors defined parameters of the olfactory adaptation paradigm, which they used to design a genetic screen to isolate mutants defective in their ability to adapt to volatile odors sensed by the Amphid Wing cells type C (AWC) sensory neurons. When wildtype *C. elegans* animals are exposed to an attractive AWC-sensed odor[3] for 30 min they will adapt their responsiveness to the odor and will then ignore the adapting odor in a chemotaxis behavioral assay for ~1 hr. When wildtype *C. elegans* animals are exposed to an attractive AWC-sensed odor for ~1 hr they will then ignore the adapting odor in a chemotaxis behavioral assay for ~3 hr. These two phases of olfactory adaptation in *C. elegans* were described as short-term olfactory adaptation (induced after 30 min odor exposure), and long-term olfactory adaptation (induced after 60 min odor exposure). Later work from L'Etoile *et al.*,[4] uncovered a Protein Kinase G (PKG) called EGL-4 that is required for both the short-term and long-term olfactory adaptation in AWC neurons. The EGL-4 protein contains a nuclear localization sequence that is necessary for long-term olfactory adaptation responses but dispensable for short-term olfactory adaptation responses in the AWC[4]. By tagging EGL-4 with a green fluorescent protein, it was possible to visualize the localization of EGL-4 in the AWC during prolonged odor exposure. Using this fully functional GFP-tagged EGL-4 (GFP::EGL-4) molecule we have been able to develop a molecular readout of long-term olfactory adaptation in the AWC[5]. Using this molecular readout of olfactory adaptation we have been able to perform both forward and reverse genetic screens to identify mutant animals that exhibit defective subcellular localization patterns of GFP::EGL-4 in the AWC[6,7]. Here we describe: 1) the construction of GFP::EGL-4 expressing animals; 2) the protocol for cultivation of animals for long-term odor-induced nuclear translocation assays; and 3) the scoring of the long-term odor-induced nuclear translocation event and recovery (re-sensitization) from the nuclear GFP::EGL-4 state.

Video Link

The video component of this article can be found at http://www.jove.com/video/4443/

Protocol

1. Construction of GFP Tagged EGL-4 Expressing Animals

1. Clone the translational fusion GFP::EGL-4 under the promoter for the *odr-3* gene (use 2678 bp directly upstream of the start codon): *(p)odr-3*::GFP::EGL-4. The *odr-3* promoter drives expression in the amphid neuron pairs: AWA; AWB; AWC; and weakly in ASH.
2. Inject the plasmid *(p)odr-3*::GFP::EGL-4 into wildtype (N2) animals at 50 ng/µl with the co-injection markers using standard germ-line transformation techniques[8]: *(p)ofm-1*::GFP[9] (25 ng/µl), and *(p)odr-1*::dsRed[10] (25 ng/µl). The *odr-1* promoter drives expression in the AWC and AWB amphid neuron pairs, and the *ofm-1* promoter drives expression in the coelomocytes.
3. Subject the transgenic animals expressing the extrachromosomal arrays detailed in Step 1.2 to the Ultra Violet (UV)/trimethylpsoralen (TMP) integration protocol[11].
4. Bleach synchronize the animals[12] and cultivate them on Nematode Growth Media (NGM) plates containing OP50 *E. coli* to the L4 stage. Wash L4 animals off NGM plates with M9 buffer to remove OP50 *E. coli* bacteria.
5. Remove M9 buffer and add 50-100 µl of 30 µg/ml TMP working solution onto the worm pellet in the 1.5 ml microcentrifuge tube. Wrap the 1.5 ml microcentrifuge tube in foil to block ambient light, and agitate gently on a rotator for 15 min in the foil-wrapped 1.5 ml microcentrifuge tube. Transfer the worms in the TMP/worm solution to a large unseeded NGM plate.
6. Expose the worms on the NGM plate to UV 350 µJ(X100) using a Stratalinker 2400. Then add worms to an OP50 containing NGM plate and incubate in the dark for 5 hr.
7. Pick 50 transgenic worms to approximately 10 seeded plates (2-5 worms each plate). Transfer P0's to new plates every 24 hr for 3 days.

8. Pick 250 clonal F1 animals (1 animal per plate). Clone out 2-4 F2 animals from each F1 plate. Check F3 animals to look for a 100% transmission line by looking at *(p)ofm-1*::GFP expression pattern under a dissecting fluorescent microscope.
9. Outcross the final integrated line(s) with wildtype N2 animals 5 times. We will refer to the final integrated line as *pyIs500*, and the GFP-tagged EGL-4 molecule as GFP::EGL-4.

2. Cultivation and Maintenance of Animals for Nuclear Translocation Assays

1. Cultivate the *pyIs500* animals at 25 °C on standard 10 cm NGM plates (see below for recipe) seeded with OP50 *E. coli* bacteria[13] (**Figure 1**).
2. On Day 1, pick four to five L4 *pyIs500* animals from plates cultivated at 25 °C onto five separate 10 cm OP50 *E. coli* seeded plates and incubate at 25 °C (that is, 4-5 L4 animals per plate).
3. On Day 4, wash adult populations of *pyIs500* animals off the large NGM plates using S-Basal buffer (see below for recipe). Use disposable glass pipettes to transfer animals, as they will stick to the side of plastic pipette tips.

3. Long-term Odor Induced Nuclear Translocation Assays

1. Wash the *pyIs500* animals 3 times in S-Basal to remove bacteria. Do not centrifuge animals between washes; rather allow the animals to settle by gravity in a stationary 1.5 ml microcentrifuge tube. It is critical to ensure all bacteria is removed, and that NGM pates are free from contamination, as residual bacteria will negatively affect the outcome of the translocation assay. Also, we have found that autoclaving the 1.5 ml microcentrifuge tubes produces a "sticky" property on the interior walls of the tube that causes animals to stick to the sides, and so use non-autoclaved 1.5 ml microcentrifuge tubes.
2. While animals are settling between washes, make up the adaptation solution. Add 100 ml of S-Basal buffer to a 100 ml graduating cylinder. Add adapting odor to the S-Basal and seal the cylinder using a strip of Parafilm. If performing benzaldehyde adaptation add 7.5 µl of benzaldehyde to 100 ml of S-Basal, for butanone adaptation add 11 µl of 2-butaone to 100 ml of S-Basal. Gently invert (do not shake) the graduating cylinder containing S-Basal and odor 30 times to create a uniform emulsion.
3. After the final wash in S-Basal, allow the animals to settle and remove all liquid. Label one tube "+ve" or "adapt" and the other tube "-ve" or "unadapt". Then add 1 ml of the adaptation mix to the adaptation microcentrifuge tube (+ve), and add 1 ml of S-Basal to the unadapted control microcentrifuge tube (-ve). Try to keep numbers of animals between 100 and 200 in each tube. With some practice, it is possible to estimate the approximate number of animals in a pellet by eye.
4. Place a 1.5 ml microcentrifuge tube cap protector to each tube (prevents lids from popping open) and place the tubes on a rotator for 80 min. We have found that odor exposure between 60 min and 80 min yields similar results (both result in long-term adaptation responses). However, 80 min exposure provides more consistent results in our hands. Thus all our long-term adaptation assays are standardized by using an 80 min odor exposure (**Figure 2**).
5. After 80 min, wash animals 3 times in S-Basal. Allow animals to settle by gravity between each wash.

4. Short-term Odor-induced Nuclear Translocation Assays

1. In the case of short-term odor adaptation, use the same protocol as per long-term odor-induced translocation assays (Steps 3.1-3.4), however instead of incubating the *pyIs500* animals in adapting odor for 80 min, use a 30 min exposure. The quantification of odor-induced nuclear translocation assays outlined below (Step 5) is the same for long-term and short-term exposures.

5. Scoring the Odor-induced Nuclear Translocation Event

1. Make 2% agarose pads containing 5 mM sodium azide (NaN_3) by dissolving gel electrophoresis agarose in dH_20. Place a single drop of molten agarose containing NaN_3 on a glass microscope slide. Immediately place another glass microscope slide on the first slide to flatten the drop of molten agarose. Leave for 30 sec, and then gently tease apart the microscope slides leaving one microscope slide with a flat agarose pad of ~1 mm.
2. After the final wash in S-Basal, allow the worms to settle in the 1.5 ml microcentrifuge tube and remove all liquid. Then add the animals drop-wise onto the agarose pads of several microscope slides. It's important to keep the numbers of animals per slide to between 50 and 100. Too many animals per slide will complicate scoring and often create a scattering glow after blue-light excitation. Excess liquid may be wicked from the agarose pad by using a small Kim-wipe. Place a 0.5 mm glass cover-slip onto the pad and let stand for 2 min to allow for the NaN_3 to immobilize the animals. Note, animals can be recovered from the slides after scoring. The animals will recover from the NaN_3 anesthetic after ~1 hr if transferred to a seeded NGM plate into a drop of M9 Buffer.
3. At this point it is imperative that each experimenter devise a key to track which slide is the experimental (+ve) slide and which slide is the control (-ve) slide, and pass the slides to another person that will blindly score the GFP:EGL-4 localization pattern. This will control for any experimenter bias.
4. Score between 50 and 100 animals per slide using an upright fluorescent microscope with 10X, 40X and 63X magnification objectives and GFP/RFP filters. Firstly, locate animals under 10X magnification using bright-field illumination. Secondly, locate the AWC neuron by turning off the bright-field illumination and using the RFP filter. The *(p)odr-1*::dsRed drives expression in AWB and AWC neurons. The AWC neurons have distinctive oval shaped cell bodies as compared with AWB cell bodies which are smaller and circular in shape (**Figure 2**).
5. Once the AWC cell body has been located, switch to the GFP filter and record the localization of GFP:EGL-4 as nuclear or cytoplasmic. Using a counter allows the experimenter to keep looking into the eyepiece while scoring the slide. Note: Steps 5.1-5.5 are repeated at least 3 times on separate days to produce statistically significant data (**Figures 1-2**).

6. Monitoring GFP-tagged EGL-4 during the Recovery from Long-term Odor Adaptation

1. After Step 3.5, divide the populations of *pyIs500* animals into multiple aliquots and score for nuclear versus cytoplasmic GFP::EGL-4 in the AWC at time point zero (that is, after 80 min exposure) and multiple subsequent time points (**Figure 3**).

2. Transfer both the control (-ve) and experimental (+ve) animals using disposable glass pipettes onto NGM plates.
3. Allow animals to recover for various lengths of time and re-examine the localization of GFP::EGL-4in AWC at each time point as in Steps 5.1 to 5.5.

7. Materials

NGM plates: For 1 L add to 1 L ddH_20: 21 g Bacto-Agar; 3 g Sodium Chloride (NaCl); 2.5 g Bacto-Peptone. Autoclave Media. After autoclaving, cool the agar until the temperature reads 54 °C. Add the following solutions: 25 ml 1M Potassium Phosphate Buffer; 1 ml 1 M Magnesium Sulfate ($MgSO_4$); 1 ml 1 M Calcium Chloride ($CaCl_2$); 1 ml Cholesterol in Ethanol (5 mg/ml stock).

1 M Dibasic K_2HPO_4 174.2 g/mol: For 500 ml: In 400 ml ddH_2O, dissolve 87.1 g Dibasic K_2HPO_4. Adjust volume to 500 L with ddH_2O. Filter sterilize using a 500 ml Sterile Filter Unit.

1 M Monobasic KH_2PO_4 136.1 g/mol: For 1 L: In 800 ml ddH_2O, dissolve 136.1 g Monobasic KH_2PO_4. Adjust volume to 1 L with ddH_2O. Filter sterilize using a 500 ml Sterile Filter Unit.

1 M Potassium Phosphate (K_3PO_4) Buffer pH 6.0: For 1 L: In a 1 L Sterile Filter Unit, add 868 ml of 1 M Monobasic KH_2PO_4. Allow unit to filter through, then add 132 ml of 1 M Dibasic K_2HPO_4. Allow unit to filter all the liquid. Remove filter unit and adjust pH to 6.0. Store at room temperature.

S-Basal: For 1 L: add 50 ml 1M K_3PO_4 Buffer, pH ~6.0; and 20 ml 5M $NaCl_{aq}$. Fill to 1 L with ddH_2O. Autoclave Solution.

M9: For 1 L: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O. Fill to 1 L with ddH_20. Sterilize by autoclaving.

Representative Results

An example of the localization pattern of GFP::EGL-4in the AWC before and after prolonged odor exposure is shown in **Figure 2**. Prior to prolonged odor exposure, GFP::EGL-4 is localized to the cytosol of the AWC (**Figure 2B**), and after 80 min odor exposure GFP::EGL-4 is localized to the nucleus of the AWC (**Figure 2D**). At the behavioral level, animals with cytosolic GFP::EGL-4 in AWC are attracted to a point source of odor (**Figure 2C** graphs representative results from chemotaxis assays of unadapted animals - note the chemotaxis index[3] is close to 1). Animals exhibiting nuclear GFP::EGL-4 in the AWC are not attracted to a point source of the adapting odor (**Figure 2E** graphs representative results from chemotaxis assays of adapted animals - note the chemotaxis index is close to zero). To generate statistically significant data from odor-induced nuclear translocation assays, the experiments should be run at least 3 times and on separate days. For a detailed description of the chemotaxis assay it may be useful to refer to *JoVE* article 2490[14].

Once EGL-4 enters the nucleus of the AWC neurons it causes stable and long-lasting changes in the AWC physiology that persist even when EGL-4 is no longer in the nucleus (**Figure 3**). After 80 min odor exposure animals will ignore a point source of the adapting odor (**Figure 3** upper panel, light bar on left), and this adaptation will persist even after 120 min of recovery (**Figure 3** upper panel, light bar on right). After 80 min of odor exposure GFP:EGL-4 is observed in the nucleus of the AWC (**Figure 3** lower panel, light bar on left). This nuclear entry is both necessary and sufficient to induce long-term adaptation in the AWC[5]. After 120 min recovery, these animals no longer exhibit nuclear GFP::EGL-4 (**Figure 3** lower panel, light bar on right) yet will still ignore a point source of the adapting odor benzaldehyde at the behavioral level.

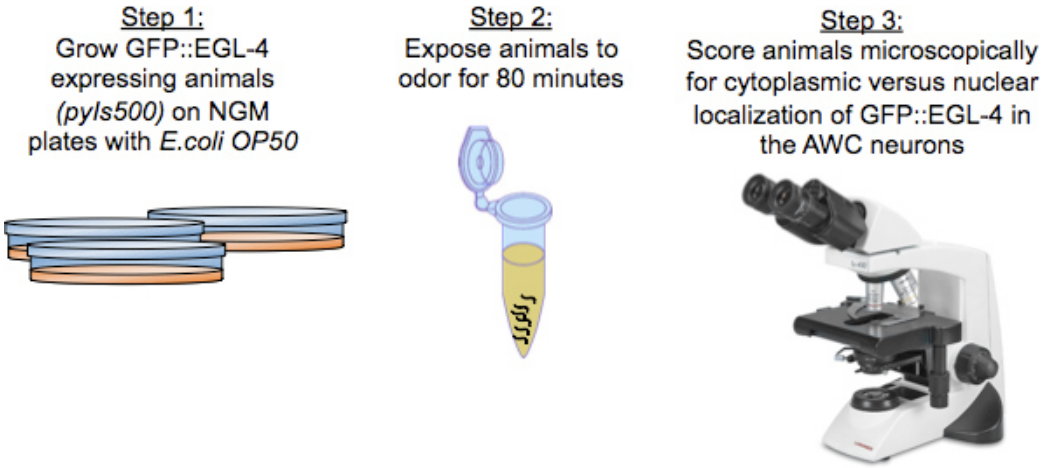

Figure 1. Overview of protocol for long-term odor-induced nuclear translocation assays. Firstly, animals expressing GFP::EGL-4 (*pyIs500* animals) are cultivated at 25 °C on NGM plates seeded with OP50 *E. coli*. Secondly, animals are exposed to an odor adaptation mix (experimental group) or S-Basal buffer (control group). Thirdly, animals are scored blindly by counting the number of animals exhibiting nuclear GFP::EGL-4 in the AWC and the number of animals exhibiting cytoplasmic GFP::EGL-4 in the AWC.

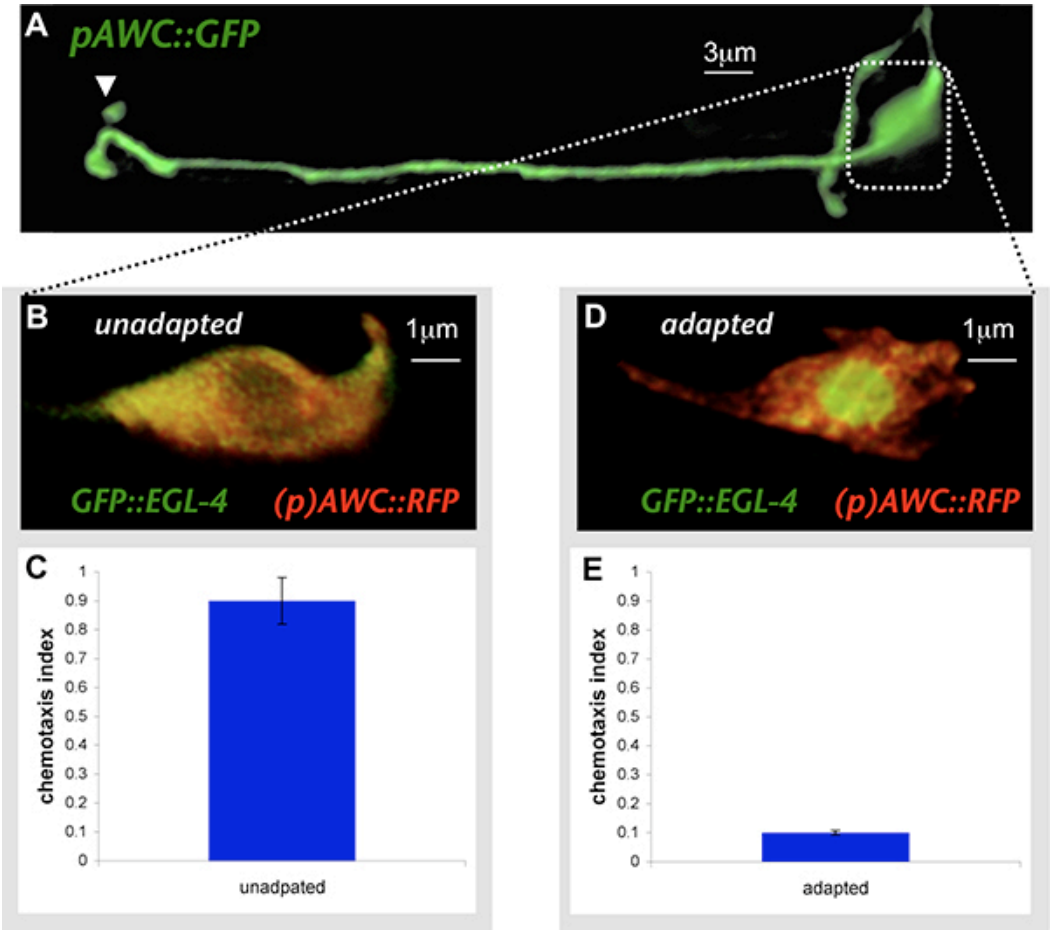

Figure 2. An example of the localization pattern of GFP::EGL-4 in the AWC before and after prolonged odor exposure is shown. (A) Confocal image of the AWC neuron expressing soluble GFP. (B) Confocal image of a naïve (unexposed) animal exhibiting GFP::EGL-4 throughout the cytosol of the AWC neuron. (C) Unadapted animals exhibit a high chemotaxis index close to 1 when behaviorally assayed to a point source of odor. (D) Confocal image of an animal exposed to odor for 80 min exhibiting GFP::EGL-4 in the nucleus of the AWC. (E) Animals that exhibit nuclear GFP::EGL-4 exhibit a chemotaxis index close to zero (normally ~0.2) when behaviorally assayed to the adapting AWC odor. This figure is modified with permission from O'Halloran *et al.*[6] **Figure 2B.** Prior to prolonged odor exposure, GFP::EGL-4 is localized to the cytosol of the AWC. **Figure 2C.** At the behavioral level, animals with cytosolic GFP::EGL-4 in AWC are attracted to a point source of odor. This graph was generated from representative results from chemotaxis assays of unadapted animals. Note the chemotaxis index is close to 1. **Figure 2D.** After 80 min odor exposure GFP::EGL-4 is localized to the nucleus of the AWC. **Figure 2E.** Animals exhibiting nuclear GFP::EGL-4 in the AWC are not attracted to a point source of the adapting odor. This graph was generated from representative results from chemotaxis assays of adapted animals. Note the chemotaxis index is close to zero.

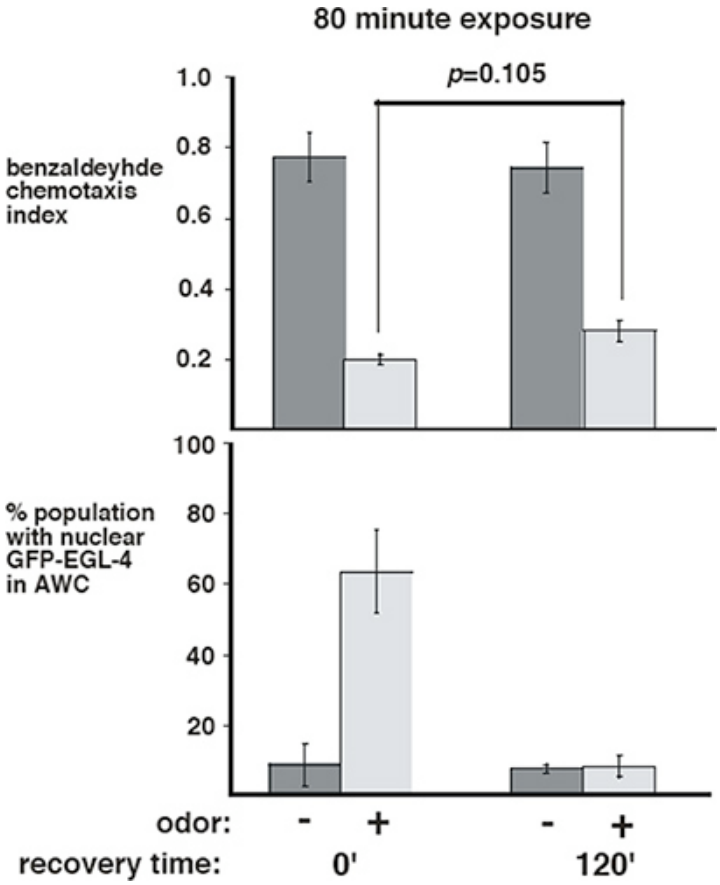

Figure 3. Once EGL-4 enters the nucleus of the AWC neurons it causes stable and long-lasting changes in the AWC physiology that persist even when EGL-4 is no longer in the nucleus. Animals exposed to odor (80 min) are allowed to recover from the long-term odor exposure and scored for cytoplasmic versus nuclear localization of GFP::EGL-4. (Upper Panel) After 120 min recovery the animals that were exposed to odor for 80 min are still adapted at the behavioral level. (Lower Panel) After 120 min recovery animals that were exposed to odor for 80 min no longer exhibit nuclear GFP::EGL-4. Thus, nuclear EGL-4 invokes stable long-lasting changes in the physiology of the AWC that persist after EGL-4 is no longer in the nucleus. Figure is modified with permission from Lee *et al.*[5]. **Figure 3 upper panel**. After 80 min odor exposure animals will ignore a point source of the adapting odor, and this adaptation will persist even after 120 min of recovery. **Figure 3 lower panel**. After 80 min of odor exposure GFP::EGL-4 is observed in the nucleus of the AWC. This nuclear entry is both necessary and sufficient to induce long-term adaptation in the AWC. After 120 min recovery, these animals no longer exhibit nuclear GFP::EGL-4 yet will still ignore a point source of the adapting odor benzaldehyde at the behavioral level.

Discussion

The odor-induced nuclear entry of a GFP-tagged EGL-4 molecule described here provides a robust molecular readout of olfactory adaptation in *C. elegans*. The odor-induced nuclear translocation assays are straightforward and require only a few days of preparation time. The *pyIs500* animal that we have constructed for these assays, expresses a marker that illuminates the AWC neuron as well as expressing the GFP-tagged EGL-4 protein. Thus, we feel that an experimenter with little to no experience working with this class of neuron can very rapidly (perhaps after examining a few dozen animals) begin to feel comfortable identifying the correct cell and scoring for nuclear versus cytoplasmic EGL-4. It may be useful for the reader to refer to *JoVE* article 835 that describes GFP analysis in *C. elegans*[15].

To ensure that the data generated is of the highest quality we suggest the following guidelines: 1) all scoring should be done blindly; 2) cultivation plates containing ANY trace of contamination (fungal or bacterial) cannot be used; 3) worms that experienced starvation during cultivation should never be used; and 4) adaptation mixes must be made fresh on the day of experimentation.

All sensory systems exhibit examples of neuronal plasticity. The transmission of signals to the nucleus is a highly conserved feature of stable forms of neuronal plasticity[16]. By developing a molecular readout of olfactory plasticity that represents a nuclear translocation of a PKG in stimulated neurons, we provide a platform for rapid and detailed dissection of neuronal plasticity within an *in vivo* model. Using our molecular readout of olfactory adaptation we have begun to describe some of the events that shape olfactory adaptation in *C. elegans*[5,6,7]. However, this is just a beginning, and by studying this process as a function of age, sex, infection, or diet we may uncover important themes of neuronal plasticity at the circuit and systems level.

Disclosures

No conflicts of interest declared.

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