

FIONA on *Caenorhabditis elegans*[†]

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ABSTRACT: Despite much work, subcellular neurons of *Caenorhabditis elegans* have not been studied at nanometer resolution with millisecond time resolution. Nor has there been an effective way to immobilize *C. elegans*. Here we show that, without using anesthetic or paralyzing agents, fluorescence imaging with one-nanometer accuracy (FIONA) can be successfully applied to fluorescently labeled molecules within *C. elegans* nerves. GFP- and DENDRA2-labeled ELKS puncta can be localized with sub-10 nm accuracy in ~5 ms. Our results show that the protein ELKS is occasionally transferred by microtubule-based motors. This is the first example of FIONA applied to a living organism.

Because of its transparent body and overall simplicity, *Caenorhabditis elegans* has been used as a model organism in medicine, physiology, and neuroscience (1). For example, the nervous system is just 302 neurons and approximately 7000 synapses, but the ability to localize various proteins with nanometer accuracy and millisecond time resolution, as was done previously, in vitro (2) and in vivo, i.e., in cultured mammalian cells (3), has not been done in situ, i.e., in a living organism.

We have chosen to isolate the protein ELKS, which is found in the six mechanosensory touch neurons. ELKS (which is named for its amino acid composition) plays a role in regulating synaptic development and colocalizes with other synaptic active proteins at synapses (4). This can be seen when other synaptogenic pathways are compromised (5), although many of the features of ELKS are not well established. For our use, ELKS was fused with either green fluorescent protein (GFP) or DENDRA2 (a photoconvertible fluorescent protein) (6) (see the Supporting Information for details about constructs).

The three criteria for being able to perform in situ fluorescence imaging with one-nanometer accuracy (FIONA) are met with the GFP–ELKS construct. First, previous studies have shown that ELKS fused with GFP and its derivatives can be observed as very bright puncta in *C. elegans* neurons both in processes and at synapses (5). The ELKS process puncta may represent an invertebrate counterpart to active zone precursor vesicles with which numerous vertebrate active zone proteins have been shown

to associate during axonal transport to the synapses (7). Second, all of the *C. elegans* touch receptor neurons are very close to the cuticle (~100 nm) (8); the neuronal processes are aligned directly adjacent to the coverslip. This makes it possible to excite them with regular TIR microscopy and thus achieve a high signal-to-noise ratio. Third, we have come up with a way to immobilize *C. elegans*.

The existing procedure for immobilization is to glue the worms to glass. Unfortunately, this takes a considerable amount of time (~2 min per worm) since the worms have to be glued one at a time with cyanoacrylate glue (9). Another technique is to anesthetize the worms with sodium azide (10). However, this drastically alters the metabolism of worms, especially in the presence of ATP (11, 12). Other drugs such as levamisole and muscimol are also used for immobilizing *C. elegans* (13). They stabilize the worms to a great extent, but there are still some spasmodic movements that make it impossible to perform nanoscale recordings.

We have used a straightforward technique to stabilize multiple worms simultaneously in the absence of any paralyzing drugs or microfluidic devices (14, 15). In our experiments, the worms are pipetted away from the NGM (nematode growth medium) plates into Eppendorf tubes with M9 buffer [3 g of KH₂PO₄, 6 g of Na₂HPO₄, 5 g of NaCl, 1 mL of MgSO₄ (1 M), and 1 L of deionized water]. Then 5–10 μ L of buffer (containing the worms) is placed on a 2 in. glass bottom dish (WillCo Wells BV, GWST 5040, Amsterdam, The Netherlands). A coverslip (Fisher Scientific, 12-548-5A, 30 mm \times 22 mm) is placed atop the drop to sandwich the worms between the two surfaces as the buffer disperses due to the capillary effect. Approximately 10 min later, as the water evaporates from the edges, the distance between the two surfaces becomes narrower, immobilizing ~90% of the worms (thicker worms; adults and L4s can be stabilized instantly). After this point, evaporation has to be slowed by closing the lid of the dish since excessive pressure turns out to be lethal for adult and L4 stage worms. When the pressure is released by careful addition of buffer to the dish, the worms start moving again, verifying their vitality.

To test how effectively this procedure can immobilize the worms, we have used a strain which is cultured in NGM plates that were seeded with 1 μ m diameter fluorescent beads (Fluo-Spheres F8819, Molecular Probes/1000-fold diluted, 1 mL for a 9 cm diameter plate). In Figure 1, the beads swallowed by the worms decorate the intestinal lumen and can be used as stable markers for determining the positions of the worms with high accuracy. After sandwiching the worms, we imaged the

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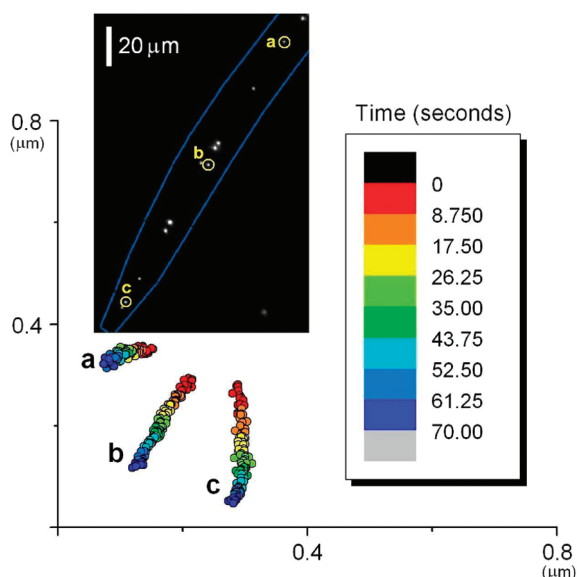


FIGURE 1: Movement of fluorescent beads in immobilized *C. elegans* (at the L2 stage). (a) Beads at the anterior side are shown in the inset. The beads move ~ 300 nm at most in 65 s. (b) Beads at the posterior side are shown in the inset. The beads move ~ 100 nm at most in 65 s. Beads move in different directions and different amounts, showing that the movement is not due to a stage drift but the movement of the worm itself. See the movie in the Supporting Information showing how worms start moving when the pressure is released.

fluorescent beads that were trapped in the lumen with epifluorescence. We observed that the beads move extremely slowly (~ 300 nm in 1 min), suggesting that the immobilization is very strong. The fact that the intestinal lumen is situated micrometers above the coverslip shows that the whole animal is immobilized. After the recording, we released the pressure to verify the vitality (see the movie in the Supporting Information). Also, this technique can immobilize the worms that were previously treated with levamisole or muscimol, enabling high-resolution recordings in the absence of spasmodic movements. Previously, a similar approach was used to take pictures of formaldehyde-fixed (dead) *C. elegans* at different orientations where a few Sephadex G-100 particles between the two surfaces were used to glide the coverglass gently to roll the animals to the desired position (16).

We find that fluorescently labeled ELKS punctae are found in the form of bright clumps that are distributed with a distance of ~ 5 μm between them within the neuronal processes of the touch neurons (Figure 2A). We also find that fluorescent punctae found in touch neurons of live *C. elegans* can be tracked by FIONA with sub-10 nm spatial resolution within ~ 5 ms (Figure 2B). This is similar, though not quite as good, as FIONA detected on GFP-labeled organelles in cultured cells, where we found 1.5 nm and 1 ms resolution.

We have observed that GFP-ELKS punctae are motionless most of the time. However, we have recorded some rare, long-range directed movements (Figure 3A). This suggests that, at times, GFP-ELKS punctae are intact and can move as a whole within the axon. The high-resolution tracking of moving GFP-ELKS spots shows that the average traveled distance increases linearly over time, with an average velocity of 0.35 $\mu\text{m/s}$ (Figure 3B). This shows that the spots are not diffusing but are being transported by molecular motors.

We find that GFP-ELKS spots are distributed in a surprisingly even way along the microtubule cells. Except for a few cases, we observed that these bright spots do not change their positions in

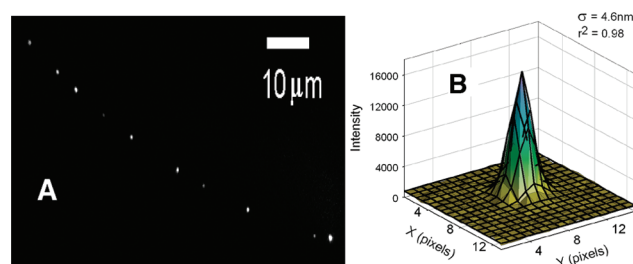


FIGURE 2: (A) Stable DENDRA2-ELKS spots that are distributed in a punctual way along the touch neuron process. (B) Two-dimensional Gaussian fit to the emission pattern of a single DENDRA2-ELKS puncta. The peak can be localized within 4.6 nm in 4 ms.

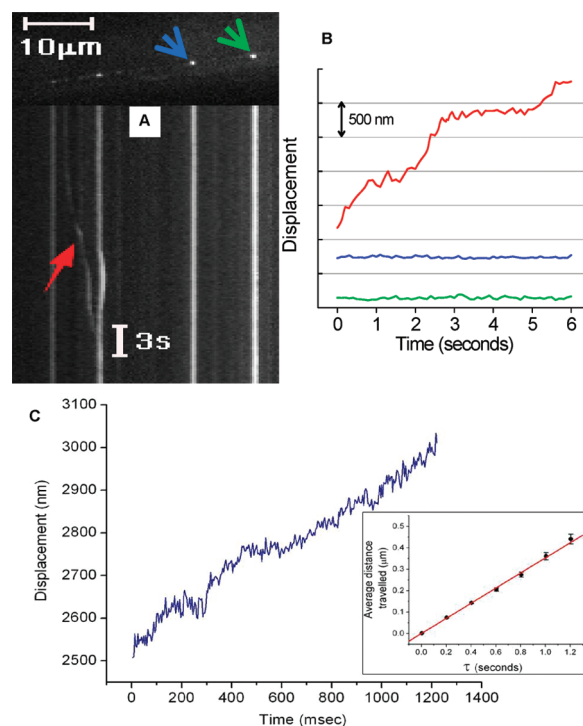


FIGURE 3: (A) Kymograph showing the movement of a GFP-ELKS puncta (shown with a red arrow). Nonmoving punctae (shown with blue and green arrows) found in the same process confirm that the touch neuron is motionless during the movie. (B) Traces of puncta shown in panel A. Red, blue, and green traces correspond to the kymograph shown with the red arrow and the spots shown with blue and green arrows, respectively. (C) Trace of a GFP-ELKS spot imaged with a temporal resolution of 4 ms. The average distance traveled is shown in the inset.

the neuronal processes. We used several different mutant strains of *C. elegans* in an attempt to understand why the distribution of ELKS spots does not change. The first is a *mec-7* mutant, in which the 15-protofilament microtubules are replaced by 11 protofilaments. Mechanosensory neurons have microtubule bundles of 15 protofilaments rather than 11 protofilaments which are found in all other microtubules of the worm. When the 15-protofilament microtubules are disrupted or are replaced by 11 protofilament microtubules by the mutation of the *mec-7* gene (MECHANOSENSORY abnormality), the touch sensitivity is lost (17). The second strain consists of *mec-4* mutants in which degenerin channel formation is disrupted. Mechanosensory transduction in microtubule cells is assumed to be mediated by degenerin/epithelial Na^+ channels (18), which are believed to be encoded by mechanosensory abnormality genes, *mec-4*, *mec-10*, and *mec-6* (19). Previous work has revealed that MEC-4 proteins are distributed

in a punctuate pattern (20), indicating that the degenerin channels are assembled heterogeneously along the touch neuron processes. This punctate distribution is dependent on the MEC-1 and MEC-5 extracellular matrix proteins which are thought to link the degenerin channel complexes to the epidermis (cuticle). We have used the *mec-1*, *mec-4*, *mec-5* mutant strain to observe if ELKS punctae are associated with degenerin channel clusters.

We have applied in situ FIONA tracking to DENDRA2–ELKS spots in both *mec-7* and *mec-4* mutants. In these two strains, we have observed no significant difference either in the mobility or in the distribution of ELKS punctae. Furthermore, epifluorescence microscopy revealed that GFP–ELKS puncta were not differentially distributed in *mec-1* or *mec-5* mutants in contrast to that which is observed for the degenerin channels. These findings imply that ELKS dynamics does not depend on 15-protofilament microtubule structure or the localization of degenerin channels.

In conclusion, our findings are an important step in extending the current techniques for high-resolution real-time imaging of cultured cells to a widely used multicellular model organism. We have shown that fluorescently labeled ELKS proteins accumulated in touch neurons of live *C. elegans* can be tracked at sub-10 nm resolution in a few milliseconds using the technique of FIONA. Furthermore, *C. elegans* can be immobilized without affecting the biochemistry of the nematode for high-resolution imaging experiments. Generally, ELKS punctae positions are stable. On the other hand, seldom long-range directed movements mediated by motor proteins imply that the ELKS proteins either are aggregated on membranous vesicles that are carried by motor proteins or are a part of some complexes which interact with molecular motors. Our results suggest that replacing the 15 protofilament microtubules with 11 protofilaments does not affect the motor activity on the ELKS punctae in touch neurons. Moreover, ELKS punctae distribution is not affected by the loss of degenerin channels or extracellular matrix components required for degenerin channel clustering, suggesting that ELKS does not interact with mechanosensory channels.

SUPPORTING INFORMATION AVAILABLE

ELKS constructs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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