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An optogenetic application of proton pump ArchT to *C. elegans* cells

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Abstract

Application of novel light-driven ion channel/pumps would benefit optogenetic studies of *C. elegans*.A recent study showed that ArchT, a novel light-driven outward proton pump, is >3 times more light-sensitive than the Arch proton pump. Here we report the silencing effect of ArchT in *C. elegans* cells. ArchT expressed by using a body-wall muscle-or pan-neuronal-promoters caused a quick and reliable locomotion paralysis when worms were illuminated by green light. Unlike the report on mouse neurons, however, light sensitivity of ArchT is similar to that of Arch in *C. elegans*. ArchT-mediated acute silencing of serotonergic neurons quickly triggered backward locomotion. This response was abolished in the presence of exogenously added serotonin, suggesting that, in a normal situation, serotonin is secreted in a constitutive fashion to repress backward movement.

**Introduction**

The nematode *Caenorhabditis elegans* (*C. elegans*), in which the position of neurons and the connectivity of neural circuits are fully elucidated, is used widely as a model organism for studying the nervous system ( [White et al., 1986] and [Jorgensen et al., 2011]). *C. elegans* is particularly suitable for optogenetic analysis using transgenes expressing exogenous light-driven ion pumps/channels, since a broad array of genetic tools, such as various neuron-subtype specific promoters, are available for this animal and highly specialized light-irradiation devices are not required for illumination on target cells in its small transparent body ( [Nagel et al., 2005] and [Zhang et al., 2007]).

Several optogenetic tools have been successfully applied to *C. elegans*. The light-driven Cl-pump Halorhodopsin (NpHR) (Schobert, et al., 1982), which is widely used for silencing cells (Han et al., 2007), has been employed to alter a variety of worm’s behaviors ( [Zhang, 2007], [Guo et al., 2009] and [Kuhara et al., 2011]). However, NpHR is not always effective in silencing cells because it can mediate only modest inhibition and enters into a long inactivation state rapidly after the onset of light illumination.

Recently reported archaerhodopsin-3 (Arch), a light-driven proton pump derived from halobacteria *Halorubrum sodomense*, has an advantage over NpHR with its stronger quieting effect and higher resistance to inactivation. When Arch is activated by green light illumination, H+ pours out of the cell, whereby the excitation is silenced (Chow et al., 2010). We have succeeded in applying Arch to *C. elegans*. Under green light illumination, worms expressing Arch in body-wall muscles or neurons of the entire body stopped locomotion for a period of 30 sec, and Arch expressed in different motor neuron subsets mediated distinct defects in locomotion (Okazaki et al, 2012).

More recently, another light-driven proton pump, ArchT from *Halorubrum* sp. TP009, which can silence mammalian neurons with 3.3 times higher light sensitivity than Arch, was reported (Han et al., 2011). Here we apply ArchT to *C. elegans* cells and compared the silencing activity of ArchT with Arch by examining locomotion of worms. We also utilize ArchT for analyzing *C. elegans* serotonergic neurons, whose modulatory roles in locomotion will be discussed.

**Materials and Methods**

*C. elegans* culture conditions

Worm strains expressing Arch and ArchT were grown on Nematode Growth Medium (NGM) plates (Brenner, 1974) seeded with solution of Escherichia coli OP50 and 500 µM all-trans-retinal (ATR) (Sigma-Aldrich, USA). Animals were maintained at 20°C in the dark unless indicated otherwise. *C. elegans* wild-type strain N2 was used.

Heat-shock induction of Arch and ArchT

All worms tested were F1 roller progeny of P0 roller adults picked onto NGM plates with ATR three days before experiments. Late L4 larvae were transferred to NGM plates with ATR. The culture plates containing worms were placed in a bacteria culture incubator at 37°C for 30 minutes, and then were returned to the worm incubator at 20°C. The room was maintained at 25°C.

Locomotion assay

Locomotion assay under illumination and measurement of light intensity were carried out as described previously (Okazaki et al., 2012). Animals were observed under a fluorescence stereo microscope (SZX12 or MVX10, Olympus, Tokyo, Japan), and were illuminated with a 100W HBO mercury lamp through an excitation filter for either Red Fluorescent Protein DsRed (545±10nm) or Y-excitation (562.5±17.5nm).

For locomotion assay of animals expressing ArchT::GFP with the *tph-1* promoter, an animal was transferred to a new 30mm NGM supplemented with or without ATR. Worms expressing GFP or mCherry in the pharynx were chosen as transgenic animals. Each worm was observed under a fluorescence stereo microscope MVX10 and was scored for frequency of backward locomotion in 1 minute.

Serotonin treatment of animals

Serotonin treatment was performed as previously described (Sawin et al., 2000). Solution of serotonin creatinine sulfate complex (50mM, Sigma) were prepared fresh in M9 buffer (Brenner 1974) and 400μl of this solution was added to each 60mm plate containing -10ml of agar and bacterial lawn (final concentration of serotonin is 2mM). Then lids of the plates were opened and kept at room temperature for 1 hr to dry up the liquid. Animals of each strain were transferred to each plate, incubated on the plate at room temperature for 2 hrs, and assayed.

Plasmid construction

The plasmids were made using the TOPO cloning kit and the Gateway system (Invitrogen, USA). *ArchT::gfp* cDNA was amplified from AAV-FLEX-ArchT-GFP (Han et al., 2011) (Addgene, Cambridge, MA, USA) by PCR using 5’CACCGGTAGAAAAAATGGACCCCATCGCTCTGCAGGCTGG3’ and 5’TTGGTACCTTACTTGTACAGCTCGTCCA3’ as primers, and was cloned into pENTR/D by TOPO reaction to generate pOKA004 following the manufactures’ instruction. pDEST*- tph-1p,* the destination vector containing the *tph-1* promoter was constructed by inserting the PCR-amplified genomic fragment into the *Sph*I site of pDEST-PL (gift from Hidehito Kuroyanagi) using the following primers: 5’TTGCATGCAAGCTTCTGATGCAAGTGTTCTCTTTATTGAC3’ and 5’TTGCATGCATGATTGAAGAGAGCAATGCTACCTAAAAAC3’. pOKA004 was recombined by LR reaction into destination vectors pDEST-*aex-3p*, pDEST-*F25.B3.3p*, pDEST-*myo-3p*pDEST*-hsp16-2p* (Kuroyanagi et al., 2010) and pDEST*- tph-1p*.

Transgenic strains

Transgenic animals were generated by microinjection of DNA into the distal arms of gonads of N2 hermaphrodites as previously described (Mello and Fire., 1995).

pOKA059 (*hsp16-2p::ArchT::gfp, 120ng/*μ*l*) and pRF4 (*rol-6d,* 190ng/μl), which is a transgenic marker conferring the Roller phenotype, were injected together to create the line *ncEX3043[pOKA059 (hsp16-2p::ArchT::gfp), pRF4(rol-6d)]*; pOKA060 (*aex-3p::ArchT::gfp, 170ng/*μ*l*) and pRF4 (*rol-6d,* 285ng/μl) to create the line *ncEX3048[pOKA060 (aex-3p::ArchT::gfp), pRF4(rol-6d)]*; pOKA061 (*myo-3p::ArchT::gfp, 155ng/*μ*l*) and pRF4 (*rol-6d,* 380ng/μl) to create the line *ncEX3050[pOKA061(myo-3p::ArchT::gfp), pRF4(rol-6d)]*; pOKA062 (*F25B3.3p::ArchT::gfp, 170ng/*μ*l*) and pRF4 (*rol-6d,* 380ng/μl) to create the line *ncEX3054[pOKA062(F25B3.3p::ArchT::gfp), pRF4(rol-6d)]*. pOKA086(*tph-1p::ArchT::gfp,* 192ng/μl),pCFJ90(50ng/μl)and pBlueScript (368ng/μl) were used to create the line *ncEX3090[pOKA086( tph-1p::ArchT::gfp), pCFJ90 (myo-2p::mCherry)]*, in which pharyngeal expression of mCherry was used as a transgenic marker. Transgenic lines expressing Arch: *ncEx3026[aex-3p::Arch::gfp]*, *nc3031Ex[myo-3p::Arch::gfp]*, *ncEx3034[F25B3.3p::Arch::gfp]* and *ncEx3003[hsp-16.2*p*::Arch::gfp],*were used (Okazaki et al., 2012)*.*

Microscopic observation of GFP-expressing worms

For observation of GFP fluorescence, a microscope Axioplan2 (Zeiss, Oberkochen, Germany) with filter set #10 with a 100W HBO mercury lamp was used. Images were acquired with a digital CCD camera Cool SNAP HQ2 (Photometrics, Tucson, AZ, USA) using PM Capture Pro software (Nippon Roper, Japan), and processed using ImageJ public domain software. For more detailed observation, the confocal laser scanning microscope system FV300 consisting of an Ar laser (488nm, 10mW) and a microscope BX50WI (Olympus, Tokyo, Japan) were used.

**Results and Discussion**

Light intensity-dependence of silencing activity of Arch and ArchT

In order to investigate the utility of ArchT as an optogenetic tool in *C. elegans*, ArchT::gfp was expressed by using organ specific promoters. We examined the light intensity needed for Arch and ArchT to stop locomotion of the transgenic worms under green light illumination (544±10nm).

We have used 2 promoters, *F25B3.3p* and *aex-3p,* for driving pan-neuronal expression of ArchT. Animals carrying either the transgene *F25B3.3p::ArchT::gfp* or *aex-3p::ArchT::gfp* stopped locomotion immediately when illuminated with green light. We found that *F25B3.3p::ArchT::gfp* animals and *F25B3.3p::Arch::gfp* animals reacted to green light in a similar intensity-dependent manner. Arch appears to confer a slightly higher sensitivity to light on animals than ArchT, but there was no statistical difference except at 0.21 mW/ mm2 (P=0.04; two-tailed Student’s *t*-test) (Fig 1A). With ArchT and Arch driven by the *aex-3* promoter, there was no statistical difference in light intensity-dependence of locomotion arrest between ArchT and Arch except at 0.37mW/mm2 (P=0.04; two-tailed Student’s *t*-test) (Fig 1B).

We have used the *myo-3* promoter for expressing ArchT in body-wall muscles. Animals carrying the transgene *myo-3p::ArchT::gfp* stopped locomotion when illuminated with green light. Higher light intensities were required for locomotion arrest by silencing body-wall muscle cells compared with silencing neurons. Animals carrying *myo-3p::ArchT::gfp* and those carrying *myo-3p::Arch::gfp* reacted to green light in a similar manner. There was no statistical difference in light intensity-dependence of locomotion arrest between ArchT and Arch except at 1.3 mW/mm2 (P=0.04; two-tailed Student’s *t*-test) (Fig 1C).

Finally we investigated the light intensity-dependence of locomotion arrest with ArchT and Arch expressed via the heat-shock promoter (Fig 1D). The locomotion of the transgenic worms was examined 24 hours after heat shock. There is no statistical difference in paralyzing activity except at 0.97 mW/mm2 between Arch and ArchT (P=0.04; two-tailed Student’s *t*-test).

To summarize, ArchT can be expressed without noxious effects in *C. elegans* and silences excitable cells efficiently to stop worm’s locomotion under green light illumination. However, contrary to the report on mammalian neurons showing that ArchT is capable of mediating a photocurrent 3.3-fold stronger than Arch under low light intensity (<20mW/ mm2) (Han et al., 2011), we have failed to detect a clear difference in silencing activity between ArchT and Arch throughout the entire intensity range tested (<35mW/ mm2).

Wavelength dependence of silencing activity of Arch and ArchT

It was reported that the ArchT action spectrum has the peak at a longer wavelength (580nm) compared with Arch (550nm), and that hyperpolarizing currents induced by ArchT under low light intensities at 575nm are greater than that of Arch ( [Chow et al., 2010] and [Han et al., 2011]). Wondering whether green light may not be optimal for activation of ArchT, we next used yellow-green light, which may be more suited for activation of ArchT, to examine the light intensity required for locomotory paralysis.

Animals expressing ArchT::gfp and Arch pan-neuronally via *F25B3.3p* responded to yellow light almost similarly. It seems that ArchT exhibited a slightly lower sensitivity to light than Arch, though there is no statistical difference (P>0.1; two-tailed Student’s *t*-test) (Fig 2A). Animals expressing ArchT::gfp and Arch via *myo-3p* in muscle cells also responded similarly to yellow light. Arch appears to confer a higher sensitivity to yellow-green light on muscle cells than ArchT, but there was little statistical difference except at 0.77 mW/mm2 and 4.0 mW/mm2 (P=0.02; two-tailed Student’s *t*-test) (Fig 2B). Finally expression of ArchT and Arch driven by the heat-shock promoter (Fig 2C) also resulted in similar light intensity-dependence of locomotory paralysis (P>0.2; two-tailed Student’s *t*-test).

Thus, unlike the report on mammalian neurons by Han et al. (2011), our experiments failed to show that worms expressing ArchT have higher responsiveness to light than those expressing Arch under illumination of yellow-green light as well. It is possible that the peak wavelength of the spectrum of the yellow-green light may not be long enough to generate a detectable change in responsiveness between ArchT and Arch. Alternatively, in *C. elegans* cells, ArchT and Arch might respond similarly to green and yellow-green light.

ArchT::GFP expression in *C. elegans* cells.

The silencing activity of ArchT and Arch is expected to depend on their expression levels on the cell membrane. Therefore, the poorer than expected performance of ArchT in this study might suggest a lower expression level of ArchT::GFP on the cell membrane compared with Arch::GFP. To address this, we examined the localization of ArchT and Arch in details by using a confocal microscopy.

With the promoter of the *myo-3*gene encoding the myosin heavy chain expressed in body-wall muscles and vulval muscles (Fig 3A), ArchT::gfp was expressed strongly at the muscle cell boundaries (Fig 3I), and a spotted fluorescence pattern of GFP was observed along the myofibril-like structures in body wall muscles. The expression level of ArchT was similar to that of Arch in muscles, and Arch::gfp and ArchT::gfp showed a similar localization pattern (Fig3A, B).

With the promoter of the *aex-3* gene (Fig 3C) and the *F25B3.3* gene (Fig 3E), both of which drive pan-neuronal expression, ArchT::gfp was expressed on the cell membrane and in the cytoplasm (Fig 3J). In the cell soma excluding the nucleus, strong dotted GFP expression was observed among weak fluorescence of fine granules (Fig 3K). There was strong and uniform GFP expression in axons (Fig 3L). As with the promoter of the *myo-3*gene, the expression level and the localization pattern of ArchT driven by the pan-neuronal promoters were similar to that of the Arch (Fig 3C, D, E, F).

We also used the promoter of the *hsp16-2*gene encoding a heat-shock protein. Twenty four hours after heat-shock, ArchT::gfp was expressed in the whole body of *C. elegans* similarly to Arch::GFP expressed with the same promoter (Fig 3G, H)*.*

To determine the difference of the expression level between ArchT::GFP and Arch::GFP, we compared the average of the highest brightness values of each fluorescence image which was acquired with the same excitation light-intensity and exposure time. With the *F25B3.3* promoter, ArchT::GFP expression was stronger than that of Arch::GFP (5297±340.9 and 7303±241.2, respectively. P=0.0013, n=10). However, there was no statistically significant difference in brightness between ArchT::GFP and Arch::GFP expressed with either the *aex-3* promoter, the *myo-3*promoter, or the *hsp16-2* promoter (24 hours after heat-shock) (P>0.1, n=10). Thus, our analysis failed to show that ArchT is expressed at a lower level than Arch. Nevertheless, we cannot completely rule out the possibility that ArchT and Arch are expressed differently. As transgenes used for expression of ArchT and Arch in this study were all carried as extrachromosomal arrays, there is considerable variability in the expression level and the localization pattern among strains as well as among individuals within a strain, which makes it difficult to compare the expression levels of ArchT and Arch exactly.

In conclusion, ArchT can be used as an optogenetic tool for *C. elegans*. Our data, however, showed no clear difference between ArchT and Arch in their expression as well as their responsiveness to light. It is not clear why our results using *C. elegans* cells appear to differ in some respects from those of the study using cultured mammalian neurons (Han et al., 2011). Several unique electrophysiological properties, whichare not seen inmammals, have been reported for *C. elegans* ( [Goodman et al., 1998] and [Beg and Jorgensen, 2003]). Therefore, it might be that there are unknown differences between the intracellular environment of *C. elegans* and that of mammals, which would have distinct influences on the performance of ArchT and Arch.

Optical silencing of serotonergic neurons with ArchT

Finally, we investigated the effect of silencing a small number of selected neurons by using ArchT. In *C. elegans,* serotonin modulates a variety of behaviors, such as egg laying, pharyngeal pumping and locomotion, in response to environmental changes ( [Chase and Koelle, 2007], [Horvitz et al., 1982] and [Wakabayashi et al., 2005]). While the modulatory roles of serotonin has been established through analysis of serotonin deficient mutants, or analysis of wild-type animals exposed to exogenously added serotonin, acute effects of silencing serotonergic neurons have not been investigated previously. We tried to express ArchT::GFP in worms by using the promoter of the *tph-1* gene, which encodes tryptophan hydroxylase, an essential enzyme for serotonin synthesis, expressed in serotonergic neurons; NSM, ADF and HSN (Sze et al., 2000). In animals carrying the transgene *tph-1p::ArchT::gfp*, an intense GFP signal was observed in NSM neurons (Fig. 1L) and weak GFP signal was observed in ADF neurons occasionally. Some animals expressed GFP in both of the bilateral pair of NSMs, but most animals expressed GFP only unilaterally.

Freely moving *C. elegans* worms crawl forward on agar in a sinusoidal motion, which was occasionally interrupted by brief backward movements. We found that, immediately after illuminated with the green light, animals carrying *tph-1p::ArchT::gfp* stopped forward locomotion, apparently being relaxed instantaneously, and then started backward locomotion (Fig 4A). This backward locomotion continued only for a short while, and animals resumed forward locomotion again, usually in an altered direction. Under continued green light illumination, the frequency of backward locomotion increased (Fig 4B). Though the wild type animals as well as those carrying *tph-1p::ArchT::gfp* grown without ATR sometimes stopped movement when irradiated with green light, they did not start backward locomotion, proving that backward locomotion of animals carrying *tph-1p::ArchT::gfp* under the green light illumination is evoked specifically by silencing of serotonergic neurons expressing ArchT. Although the *tph-1* promoter drives gene expression specifically in serotonergic neurons, some of them are known to release neurotransmitters other than serotonin. For example, NSM neurons release glutamic acid and neuropeptide-like proteins [ (Lee et al., 1999) and (Nathoo et al, 2001)]. To clarify whether the light-triggered changes in the locomotory behavior was caused by inhibition of serotonin secretion, we examined the locomotion of animals in the presence of exogenously added serotonin. With exposure to exogenous serotonin, whereby animals slowed down their locomotion as reported previously (Horvitz et al., 1982), green light illumination did not trigger backward locomotion (n=10, 3 trials for each animal). Thus, it is likely that, in a normal situation, serotonin is secreted in a constitutive fashion to repress backward movement.

On a plate seeded with a thick layer of food *E. coli*, wild type animals tended to dwell on a spot; they alternated frequently between forward and backward locomotion, and sometimes stopped moving briefly. While worms carrying *tph-1p::ArchT::gfp* also tended to dwell on a spot on a thick layer of food, the speed of locomotion appeared to increase under green light illumination (data not shown). Thus, the worms tended to move away from their starting point under illumination, despite the frequent alternation between forward and backward locomotion.

Our finding of the increased frequency of backward movement under green light illumination seems to be consistent with the previous study showing that serotonin deficient *tph-1* mutants are defective in roaming behavior on a food, which consists of straight displacement of sustained sinusoidal forward movement (Arous et al., 2009). The finding that silencing serotonergic neurons by switching on illumination quickly triggers backward movement is novel, and suggests that light-triggered rapid depletion of serotonin lead to this behavior. Although serotonin, like other biogenic amines, could function as a humoral factor, worms’ quick response to light suggests that serotonin acts via synapses for repressing backward movement.

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Figure legends

Fig. 1. Dependence of locomotion paralysis on intensity of green light

ArchT::gfp and Arch::gfp were expressed by (A) the *F25B3.3* promoter, (B) the *aex-3* promoter (C) the *myo-3* promoter, and (D) the *hsp16-2* promoter. With the transgene under the control of the *hsp16-2*, animals were tested 24 hours after heat-shock. Animals were illuminated with green light (545±10nm) of various intensities. (mean±SEM, n=3)

Fig. 2. Dependence of locomotion paralysis on intensity of yellow-green light ArchT::gfp and Arch::gfp were expressed by (A) the *F25B3.3* promoter, (B) the *myo-3* promoter (C) the *hsp16-2*promoter. With the transgene under the control of the *hsp16-2* promoter, animals were tested 24 hours after heat-shock. Animals were illuminated with yellow-green light (562.5±17.5nm) of various intensities. (mean±SEM, n=3)

Fig.3. Expression of ArchT::GFP and Arch::GFP in *C. elegans*by various promoters

(A) A fluorescent micrograph showing expression of ArchT::GFP in an *nc3061Ex[myo-3p::ArchT::gfp]* animal.

(B) Expression of Arch::GFP in an *nc3031Ex[myo-3p::Arch::gfp]* animal.

(C) Expression of ArchT::GFP in an *nc3060Ex[aex-3p::ArchT::gfp]* animal*.*

(D) Expression of Arch::GFP in an *nc3026Ex[aex-3p::Arch::gfp]* animal*.*

(E) Expression of ArchT::GFP in an *nc3062Ex[F25B3.3p::ArchT::gfp]* animal.

(F) Expression of Arch::GFP in an *nc3034Ex[F25B3.3p::Arch::gfp]* animal.

(G) Expression of ArchT::GFP in an *nc3043Ex[hsp-16.2p::ArchT::gfp]*animal. ArchT::GFP is expressed in the entire body.

(H) Expression of Arch::GFP in an *nc3003Ex[hsp-16.2p::Arch::gfp]*animal. Arch::GFP is expressed in the entire body. Anterior is toward the right.

(I) An enlarged view of body wall muscles in an *nc3061Ex[myo-3p::ArchT::gfp]* animal. ArchT::GFP was detected on the cell membrane (arrow). The fluorescence signal was also detected in the cytoplasm as small granules or lines of puncta. Vesicular structures are also observed (arrow head).

(J) An enlarged view of the cell body of a neuron in the head of an *nc3060Ex[aex-3p::ArchT::gfp]* animal. ArchT::GFP is expressed in vesicular and granular structures and on the cell membrane of the soma (arrow) but not in the nucleus (arrowhead).

(K) An enlarged view of the cell body of a neuron in an *nc3062Ex[F25B3.3p::ArchT::gfp]* animal. ArchT::GFP is accumulated in the cytoplasm (arrow).

(L) An enlarged view of an axon of a motor neuron in an *nc3060Ex[aex-3p::ArchT::gfp]* animal (arrow). ArchT::GFP was also detected in the ventral nerve cord.

(M) An enlarged view of head in an *nc3090Ex[tph-1p::ArchT::gfp]* animal. ArchT::GFP is expressed in the NSM neurons.

(Scale bar A to H =100μm, I to L = 5μm, and M=50μm)

Fig.4 Changes in locomotion caused by silencing serotonergic neurons

(A) The fraction of animals in which backward locomotion was triggered immediately after the green light was turned on (545±10nm, 21 mW/mm2). ArchT::gfp was expressed with the *tph-1* promoter. Animals were raised with or without ATR. (mean±SEM, n=3, 10 animals were examined for each trial.)

(B) The frequency of backward locomotion under continuous green light illumination (545±10nm, 21 mW/mm2). ArchT::gfp was expressed with the *tph-1* promoter. Animals were raised with or without ATR. The average number of occurrence of backward locomotion during 3 one-minute observation periods for ten animals in each trial is shown. (mean±SEM, n=3).

Figure 1

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Figure 2

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Figure 3.

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Figure 4.

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