

Chapter 2

A Method to Map Spatiotemporal pH Changes in a Multicellular Living Organism Using a DNA Nanosensor

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Abstract

Environmental pH has a determining role in the structure of biomolecules, thus playing an important role in regulating cellular activities. Eukaryotic cells must, therefore, strive to stringently regulate pH in various intracellular organelles so as to confer normal functioning at the level of whole organism. Several pH-sensitive probes have been reported, each of which can be used to map the pH dependence of an in vivo process. However, these probes suffer from some inherent drawbacks. Here we demonstrate the utility of an externally introduced, pH-triggered DNA nanomachine inside the multicellular eukaryote *Caenorhabditis elegans*. The nanomachine uses FRET to effectively map spatiotemporal pH changes associated with endocytosis in coelomocytes of wild type as well as mutant worms, in a variety of genetic backgrounds. It shows highest dynamic range in the pH regime 5.3–6.6 and has a half-life of ~8 h, thus positioning it well to interrogate a variety of pH-correlated biological phenomena in vivo.

Key words I-switch, pH sensor, *Caenorhabditis elegans*, Coelomocytes

1 Introduction

Protons have a determining role in the charge and, in turn, structure of biomolecules. Hence, proton concentration plays an important role in regulating cellular and, in turn, organismal activities. Eukaryotic cells, thus, have stringently controlled pH in their various intracellular organelles (1). Perturbation of cytoplasmic and organellar pH has been shown to lead to defects in receptor-mediated endocytosis, intracellular targeting of newly synthesized lysosomal proteins, calcium homeostasis, protein processing and sorting, and degradation of neurotransmitters (2). In vivo, these cellular defects are manifested in terms of tumor metastasis (1), growth defects (2), neurodegeneration (3), lysosomal storage disorders (4), defects in embryogenesis, spermatogenesis, and excretion (5), to name a few. Hence, pH is an important correlate of biological processes in vivo.

Due to the molecular and cellular complexity encountered in the environment of a whole organism, there are only few reports of pH-responsive probes *in vivo*. Most pH measurements in organisms have relied on genetically encodable, pH-sensitive GFP variants termed pHluorins, which can be fused to a specific protein and, thus, used to mark a pathway of interest (6). pHluorins enable ratiometric imaging, which helps to cull differences in protein expression. These properties have led to its use in a variety of systems, right from bacteria to mice. In bacterial systems like *Escherichia coli* and *Listeria*, this probe has been used to measure stress response to optical tweezers (7). On the other hand, Dittman et al. have elegantly used pHluorins to identify genes regulating abundance of vesicular SNAREs (soluble NSF attachment protein receptors) at *Caenorhabditis elegans* cholinergic motor neuron synapses (8). In another report, Poskanzer et al. have used synaptopHluorins to study the dynamics of resting pools of synaptic vesicles in the *Drosophila* neuromuscular junction (9). Despite such promising applications, pHluorins suffer from some issues such as (1) fixed wavelength, which limits its use in the background of GFP-expressing transgenics; (2) simultaneous tracking of two pathways is not possible; (3) quenching of its fluorescence at acidic pH values that leads to difficulties in visualization in highly acidic compartments; and (4) its fixed pH sensitive regime given that many physiological processes often operate at pH regimes beyond the pH sensitivity of pHluorins.

Less widely used pH-sensitive probes *in vivo* are fluorescein and its derivatives fluorescein isothiocyanate (FITC), 2',7' bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and carboxyfluorescein (10). Rare examples are the use of BCECF to measure the pH of interstitial space in neoplastic tumors of the rabbit ear (11) and carboxyfluorescein to study intracellular pH in the bacterial species *Lactobacillus delbrueckii* and *Listeria innocua* (12). However, their inability to be targeted specifically to a pathway of interest, fixed wavelength, and suboptimal photophysical properties has precluded their wider applicability in whole organisms.

Thus, there is a need for a pH probe that combines robust fluorescence properties, targeting, and the ability to manipulate wavelengths as well as pH regimes. Here, we describe the functioning of a rationally designed pH-sensitive DNA nanomachine, called the I-switch (13), *in vivo*. This FRET-based pH sensor utilizes bright and stable fluorophores and can be used at different wavelengths, positioning it well in the context of fluorescent-protein-expressing transgenics. It functions autonomously and reversibly in the organismal milieu, making it amenable to a variety of mutant backgrounds. The structure and charge of the DNA backbone also makes it facile for targeting. It has highest dynamic range in the pH regime 5.3–6.6, enabling it to map spatiotemporal pH changes occurring during endosomal maturation (14).

Importantly, due to its rational design, it is possible to change its dynamic range as well as pH-sensitive regime, positioning it well to track pH changes in other physiological processes *in vivo*.

2 Materials

2.1 Oligonucleotides and Sample Preparation

1. All oligonucleotides (obtained from MWG Biotech, Germany, or IBA GmbH, Germany) are high-performance liquid chromatography (HPLC) purified and lyophilized (Table 1). Oligonucleotides are dissolved in Milli-Q (MQ) water to prepare 200 μM stocks, aliquoted, and all aliquots stored at -20°C , until further use. Fluorescently labeled oligonucleotides are subjected to ethanol precipitation prior to use to remove any traces of free dye.
2. Ethanol, absolute.
3. 3.0 M potassium acetate solution: 2.94 g CH_3COOK dissolved in 10 mL MQ water and pH adjusted to 5.2.
4. Phosphate buffer – 100 mM KH_2PO_4 : 1.36 g KH_2PO_4 dissolved in 10 mL MQ water. 100 mM K_2HPO_4 : 1.74 g K_2HPO_4 dissolved in 10 mL MQ water. Each potassium phosphate solution is diluted to 10 mM. Add 10 mM KH_2PO_4 and 10 mM K_2HPO_4 to obtain a buffer of pH 5.5.
5. 3.0 M potassium chloride solution: 2.23 g KCl dissolved in 10 mL MQ water.
6. Heat block and water bath.

2.2 Fluorescence Spectroscopy

1. FluoroMax-4 instrument (Horiba Jobin Yvon, Japan), equipped with a mercury–Xe lamp as the light source.
2. Clamping buffer – 20 $\times$ salt solution: 3.57 g KCl, 0.116 g NaCl, 0.044 g CaCl_2 , and 0.081 g MgCl_2 dissolved in 20 mL MQ water. 20 $\times$ HEPES solution: 1.9 g HEPES dissolved in 20 mL MQ water.

Table 1
Oligonucleotide sequences used for the I-switch

Sequence
5'-CCCCAACCCCAATACATTTTACGCCTGGTGCC-3'
5'-CCGACCGCAGGATCCTATAAAACCCCAACCCC-3'
5'-TTATAGGATCCTGCGGTTCGGAGGCACCAGGCGTAAAATGTA-3'
5'-Alexa 488-CCCCAACCCCAATACATTTTACGCCTGGTGCC-3'
5'-CCGACCGCAGGATCCTATAAAACCCCAACCCC-Alexa 647-3'

3. 10 mL of 1 N HCl and 1 N NaOH solutions.
4. Prior to use, clamping buffers of desired pH values (5.0, 5.5, 6.0, 6.5, and 7.0) are made by mixing the salt and HEPES solutions and diluting to make 1× solution. The pH is then adjusted by using 1 N HCl or 1 N NaOH.
5. Samples are diluted to 100 nM using clamping buffers of various pH.
6. 1 cm quartz cuvette.

2.3 *C. elegans* Maintenance and Strains

1. *C. elegans* is grown at 22°C on nematode growth medium (NGM) containing a lawn of OP50 bacteria.
2. All strains have been obtained from Caenorhabditis Genetics Center (University of Minnesota, USA).
3. Wild-type strain: *C. elegans* isolate from Bristol (strain N2).
4. Mutant strains: *rme-1(b1045)*, *rme-4(b1001)*, *rme-5(b1013)*, and *rme-6(b1014)*.
5. Transgenic strains: *arIs37* [*pmyo-3::ssGFP*], *cdIs131* [*pcc1::GFP::RAB-5 + unc-119(+)* + *myo-2::GFP*], *cdIs66* [*pcc1::GFP::RAB-7 + unc-119(+)* + *myo-2::GFP*], and *pwlIs50* [*lmp-1::GFP + Cb-unc-119(+)*].
6. BOD incubator at 22°C for maintenance of nematode stocks.

2.4 *Coelomocyte* Labeling and pH Clamping

1. 5 μM I-switch sample containing acceptor label only (I_{A647}) and both donor and acceptor labels ($I_{A488/A647}$) is diluted to the desired concentration (see Subheading 3.1) in 1× medium 1.
2. 10× medium 1: 4.37 g NaCl, 0.18 g KCl, 0.055 g CaCl₂, 0.1 g MgCl₂, and 0.95 g HEPES dissolved in 50 mL MQ water and pH adjusted to 7.3. This is filter-sterilized using 0.22 μm membrane filter.
3. TE2000-S inverted microscope, equipped with a 40×, 0.75 NA objective (Nikon, Japan), and microinjection setup (Narishige, Japan).
4. Borosilicate glass capillaries.
5. 2.0% agarose pads, made on 22 × 50 mm glass coverslips.
6. 2.0% agarose: 0.2 g agarose dissolved in 10 mL MQ water.
7. Halocarbon oil.
8. 10 mM nigericin: 1.0 mg nigericin (Sigma-Aldrich, USA) is dissolved in 133 μL of absolute ethanol. 10 μL aliquots are made and stored at −20°C.
9. 10 mM monensin: 1.0 mg monensin (Sigma-Aldrich, USA) is dissolved in 120 μL of absolute ethanol. 10 μL aliquots are made and stored at −20°C.

10. Clamping buffers: See Subheading 2.2 above. To each of these buffers, 10 mM monensin and 10 mM nigericin are added to obtain final concentration of 100 μ M each.

2.5 Competition Experiments

1. Maleic anhydride.
2. Bovine serum albumin (BSA).
3. BSA solution: 6 mg/mL solution in 0.1 M sodium carbonate bicarbonate buffer (pH 9).
4. BSA solution: 6 mg/mL solution in 1 $\times$ phosphate-buffered saline (PBS) of pH 7.4.
5. 1 $\times$ PBS: For 1 L, dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄; adjust pH to 7.4; and bring to 1 L with MQ water.
6. Dextran, 10 kDa MW.
7. Dextran solution: 3.2 mg/mL solution in 1 $\times$ PBS of pH 7.4.
8. Dextran sulfate, 9–20 kDa MW.
9. Dextran sulfate solution: 4.4 mg/mL solution in 1 $\times$ PBS of pH 7.4.
10. Heparan sulfate, 30 kDa MW.
11. Heparan sulfate solution: 2.0 mg/mL solution in 1 $\times$ PBS of pH 7.4.
12. Unlabeled I-switch, diluted to 100 nM using 1 $\times$ medium 1.

2.6 Fluorescence Microscopy and Image Analysis

1. Axiovert Apotome microscope (Zeiss, Bulgaria), equipped with 40 $\times$, 1.3 NA objective, metal halide lamp (Zeiss, Bulgaria), and filters suitable for each fluorophore.
2. TE2000-U inverted microscope (Nikon, Japan), equipped with 60 $\times$, 1.4 NA objective, mercury arc illuminator (Nikon, Japan), filters suitable for each fluorophore (Chroma, USA), and Cascade II CCD camera (Photometrics, USA).
3. Image acquisition software: MetaMorph (Universal Imaging, USA).
4. Fluoview 1000 confocal microscope (Olympus, Japan), equipped with argon ion laser (Spectra-Physics, USA) for 488 nm excitation and He–Ne laser (Spectra-Physics, USA) for 633 nm excitation and a set of excitation, emission, and dichroic filters suitable for each fluorophore (Olympus, Japan).
5. Image analysis software: ImageJ ver. 1.42 (NIH, freely available from website: <http://rsbweb.nih.gov/ij/>).
6. 40 mM sodium azide: 13 mg NaN₃ dissolved in 5 mL M9 buffer.

7. 1× M9 buffer: 0.6 g Na_2HPO_4 , 0.3 g KH_2PO_4 , 0.5 g NaCl, and 0.1 g NH_4Cl dissolved in 100 mL MQ water and pH is adjusted to 7.3. The buffer is filter-sterilized through a 0.22 μm membrane filter.

3 Methods

We describe the use of a rationally designed synthetic DNA assembly called the I-switch that functions as a pH sensor to map spatiotemporal pH changes in a multicellular living organism. The I-switch consists of two DNA duplexes connected to each other by a flexible hinge and bearing cytosine-rich single-stranded overhangs at the duplex termini. Upon protonation, these C-rich overhangs base pair with each other to form an I-motif, causing a structural transition at acidic pH (13, 14). At neutral pH, the I-motif dissociates and entropic forces as well as electrostatic repulsion between the duplex arms cause the reversal of the structural transition (Fig. 1a). This forms the molecular basis of a FRET-based pH sensor using the I-switch.

Endocytosis is known to be accompanied by changes in pH as vesicles mature from the early endosomes to the lysosomes, via the late endosomes (15). These pH changes, measured in cultured cells, are known to be in the regime of 6.0–6.2 in early endosomes to ~5.5 in late endosomes and ~5.0 in lysosomes (16). Given that this shows a good match with the pH-sensitive regime of the I-switch, we describe its use in mapping spatiotemporal pH changes associated with endosomal maturation in vivo. As an example, we use it to map the same along the anionic ligand-binding receptor (ALBR) pathway in the coelomocytes of wild-type *C. elegans*. We show that this pH sensor can map variations in pH in different genetic backgrounds such as mutants and RNAi knockdowns that perturb pH homeostasis. In a model organism like *C. elegans*, where pathways are elucidated primarily by genetics, this is of special importance, since it demonstrates the utility and non-perturbative nature of the I-switch in this model organism.

3.1 Sample Preparation

1. The I-switch is generally prepared at 5 μM concentration in a volume of 50 μL . 1.25 μL of O1, O2, and O3 (each from a 200 μM stock) with 1.65 μL of 3 M KCl are mixed. The volume is made up to 50 μL by adding 10 mM potassium phosphate buffer of pH 5.5 (see Note 1). The solution is briefly vortexed.
2. The solution is heated at 90°C for 5 min in a heat block and then cooled to room temperature at a rate of 1°C per 3 min. Samples are then equilibrated at 4°C overnight. Fluorescently labeled I-switch is prepared in a similar manner with fluorophore-labeled oligonucleotides. Samples are used within 7 days of annealing.

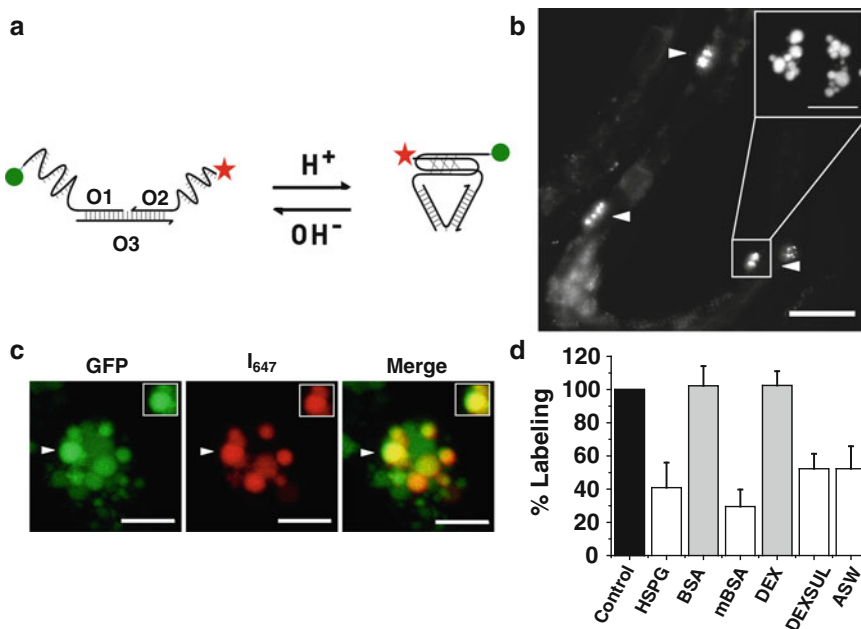


Fig. 1 I-switch marks endosomes of the anionic ligand-binding receptor (ALBR) pathway in coelomocytes of *Caenorhabditis elegans*. **(a)** Schematic showing the principle of the I-switch. **(b)** Epifluorescence image of wild-type *C. elegans* hermaphrodite microinjected with I_{A647} . Arrowheads indicate labeled coelomocytes. Scale bar: 50 μ m. Inset: confocal image of I_{A647} -labeled coelomocyte. Scale bar: 5 μ m. **(c)** I-switch specifically marks coelomocytes upon injection in the pseudocoelom of *arls37* hermaphrodites. Scale bar: 10 μ m. Inset shows a typical image of one such endosome, showing co-localization between GFP and I_{A647} . **(d)** Competition assay where I_{A647} is co-injected with 300 equivalents of various endocytic markers to establish mode of uptake of I-switch in coelomocytes (HSPG: heparan sulfate proteoglycan, BSA: bovine serum albumin, mBSA: maleylated bovine serum albumin, DEX: dextran, DEXSUL: dextran sulfate, ASW: Competing DNA). Error bars indicate s.e.m. ($n=20$ worms)

3. Prior to use, samples are diluted in 1 $\times$ medium 1 and vortexed to enable mixing. They are centrifuged at 9300 rcf for 20 min.

3.2 Microinjections and Coelomocyte Labeling

1. For coelomocyte labeling, I-switch made from Alexa 647-functionalized O2 (I_{A647}) is used. 5 μ M stock solution of I-switch sample is diluted to 100 nM using 1 $\times$ medium 1.
2. One-day old hermaphrodites grown on NGM plates (+OP50) are mounted on a 2% agarose pad containing a droplet of halo-carbon oil.
3. Injections are performed, using borosilicate capillaries, at 50–55 psi in the dorsal side of the pseudocoelom, just opposite the vulva.
4. Injected worms are released using 1 $\times$ M9 buffer and transferred to NGM plates (+OP50). Plates are incubated at 22°C for 1 h.

5. After 1 h, injected worms are mounted on a glass slide containing a 2.0% agarose pad, anesthetized using 40 mM NaN_3 in M9 buffer, and imaged.
6. Wild-type hermaphrodites, when injected with I_{A647} and imaged on an Axiovert Apotome microscope, show bright puncta in coelomocytes (Fig. 1b). Uptake is quantified by percentage of coelomocytes labeled postinjection.
7. Confirmation that the I-switch marks coelomocytes in *C. elegans* is obtained by injecting I_{A647} in the strain *arIs37*. Co-localization between GFP and I_{A647} is performed by merging images taken on an Olympus Fluoview confocal microscope (Fig. 1c).
8. For confirming the mode of endocytosis, competition experiments are performed with an excess of anionic ligands, which are known to bind ALBRs with high affinity. I_{A647} is mixed with competitor ligands mBSA, dextran sulfate, heparan sulfate, and unlabeled DNA in a 1:300 ratio, such that the final concentration of I_{A647} is 100 nM. As a control, injections with the neutral molecules BSA and dextran at the same molar ratios are also performed. Imaging is performed on an Axiovert Apotome microscope, equipped with a 40 $\times$, 1.3 NA objective.
9. Uptake is quantified by percentage of coelomocytes labeled postinjection; all values are normalized to uptake in hermaphrodites injected with I_{A647} alone (Fig. 1d).

3.3 pH Clamping

1. For pH measurements, doubly labeled I-switch ($\text{I}_{\text{A488/A647}}$) is used.
2. First, an in vitro pH calibration curve is generated by diluting 5 μM fluorescently labeled I-switch to 100 nM in clamping buffer of the desired pH, ranging from pH 5.0 to 7.0. All samples are vortexed and equilibrated for 30 min at room temperature. The samples are excited at 488 nm, and emission is collected between 500 and 700 nm with a bandwidth of 1 nm (for excitation) and 10 nm (for emission) and spectral scan speed of 1 nm/s.
3. Fluorescent intensities at 520 nm (D) and 665 nm (A) are obtained, and then D is divided by A for every pH value to generate an in vitro pH response curve.
4. For in vivo pH measurements, 5 μM stock solution of $\text{I}_{\text{A488/A647}}$ is diluted to 500 nM using 1 $\times$ medium 1.
5. The functionality of a sensor in vivo is determined by assessing the fold change of its donor/acceptor (D/A) ratio in the dynamic regime and comparing this to the in vitro fold change. Good correspondences of the fold change values indicate the sensor integrity in the given environment.

6. One-day old wild-type hermaphrodites are injected with $I_{A488/A647}$, incubated at 22°C for 1 h, and then kept in a dish containing 1 mL clamping buffer of the desired pH for 75 min. This buffer contains the ionophores nigericin and monensin at a final concentration of 100 μ M each, which equilibrate the intra-coelomocyte pH to that of the external buffer. Using this method, the pH of coelomocytes is clamped at pH 5.0 in ten worms and at pH 7.0 on ten different worms. Prior to soaking in clamping buffer, the cuticle of the worms is perforated in three regions (anterior, middle, and posterior) with a microinjection needle. After 75 min in the clamping buffer, the worms are mounted on a glass slide using the same clamping buffer (with nigericin and monensin) and the coelomocytes imaged (see Note 2). This is done three times independently, on ten worms each (see Note 3).
7. For each endocytic vesicle, fluorescence intensity at 520 nm (D) is divided by the intensity at 665 nm (A). This gives the D/A ratio for that vesicle. Cells clamped at pH 5.0 show a low D/A ratio, while those clamped at pH 7.0 show elevated values (Fig. 2a, b).
8. Fold change is calculated by dividing the D/A ratio at the higher end of the dynamic regime to that obtained at the lower end (pH 7.0 and pH 5.0, respectively, in this case). This fold change is then compared to the in vitro fold change in order to assess the performance of the sensor (Fig. 2c).
9. pH is then clamped at intermediate values (5.0, 5.5, 6.0, 6.5, and 7.0) to obtain the standard calibration curve (Fig. 2d). This will now be used to calculate pH values from D/A ratios in the system under study (see Note 4).

3.4 Spatiotemporal pH Mapping

1. In order to assess the pH changes occurring during endocytic maturation as a function of time, temporal regimes of the residence times of the I-switch in different populations of vesicles are determined. This is done by performing co-localization studies, as a function of time, of I_{A647} in the GFP-expressing transgenics *cdIs131*, *cdIs66*, and *pwIs50*. These strains express GFP fusions of Rab-5 (early endosomal marker), Rab-7 (late endosomal/lysosomal marker), and Lmp-1 (lysosomal marker).
2. I_{A647} is diluted to 500 nM using 1 $\times$ medium 1.
3. I_{A647} is injected in wild-type hermaphrodites and, after the requisite time (from 5 to 60 min, at increments of 5 min for the first 30 min), transferred to chilled NGM plates (+OP50), and kept on ice. This method efficiently stops endocytosis and trafficking of vesicles (see Note 5).

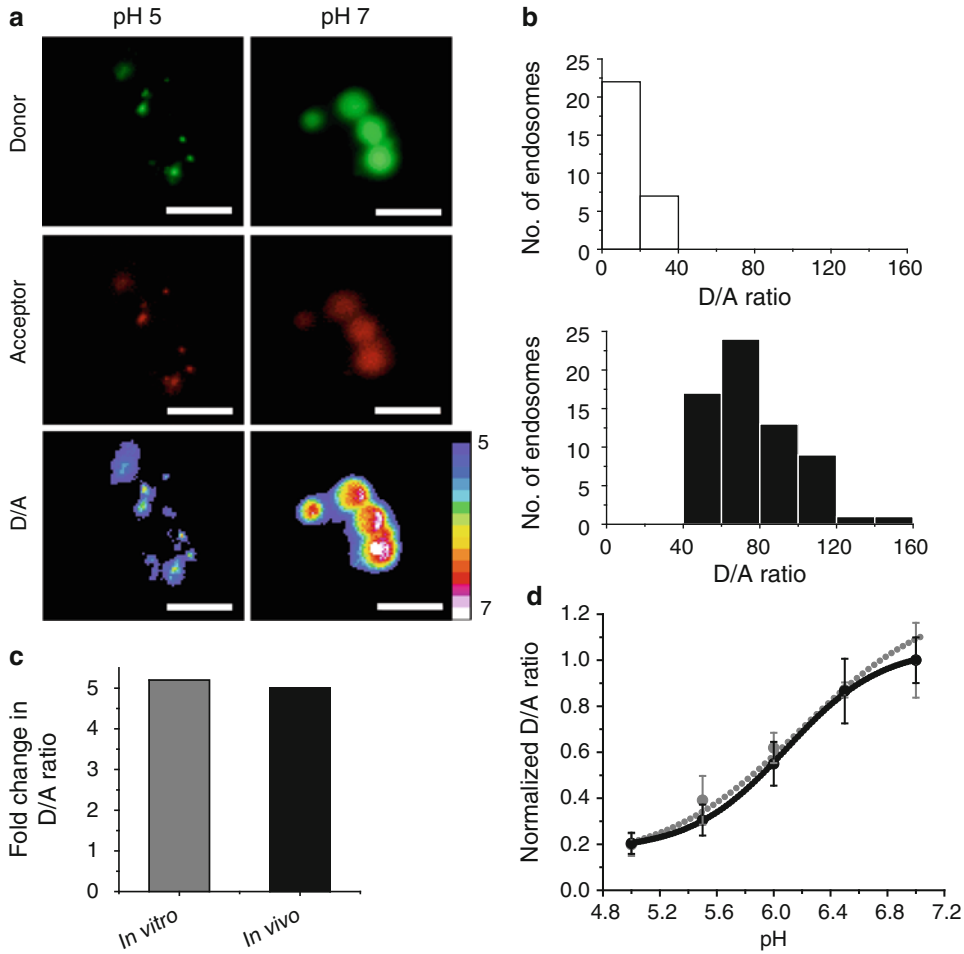


Fig. 2 In vivo characterization of the I-switch. **(a)** Donor channel (D), acceptor channel (A), and respective pseudocolor D/A images of coelomocytes labeled with $I_{A488/A647}$ and clamped at pH 5.0 and 7.0. Scale bar: 10 μ m. **(b)** Histograms showing typical spread of D/A ratios of endosomes clamped at pH 5.0 (*white bars*) and pH 7.0 (*black bars*) ($n = 25$ endosomes). **(c)** In vitro and in vivo fold change in D/A ratios of $I_{A488/A647}$ between pH 7.0 and pH 5.0. **(d)** pH calibration curve of $I_{A488/A647}$ in vivo (*black trace*) and in vitro (*gray trace*) showing normalized D/A ratios versus pH. Error bars indicate s.e.m. ($n = 50$ endosomes)

- Coelomocytes are imaged on the Fluoview 1000 confocal microscope. Co-localization of GFP and I_{A647} is determined by counting the numbers of I_{A647} -positive puncta that co-localize with GFP-positive puncta and expressing them as a percentage of the total number of I_{A647} -positive puncta. The time point where the I-switch shows maximal co-localization with an endocytic marker is chosen for pH measurements in that particular endocytic vesicle (Fig. 3a–d).

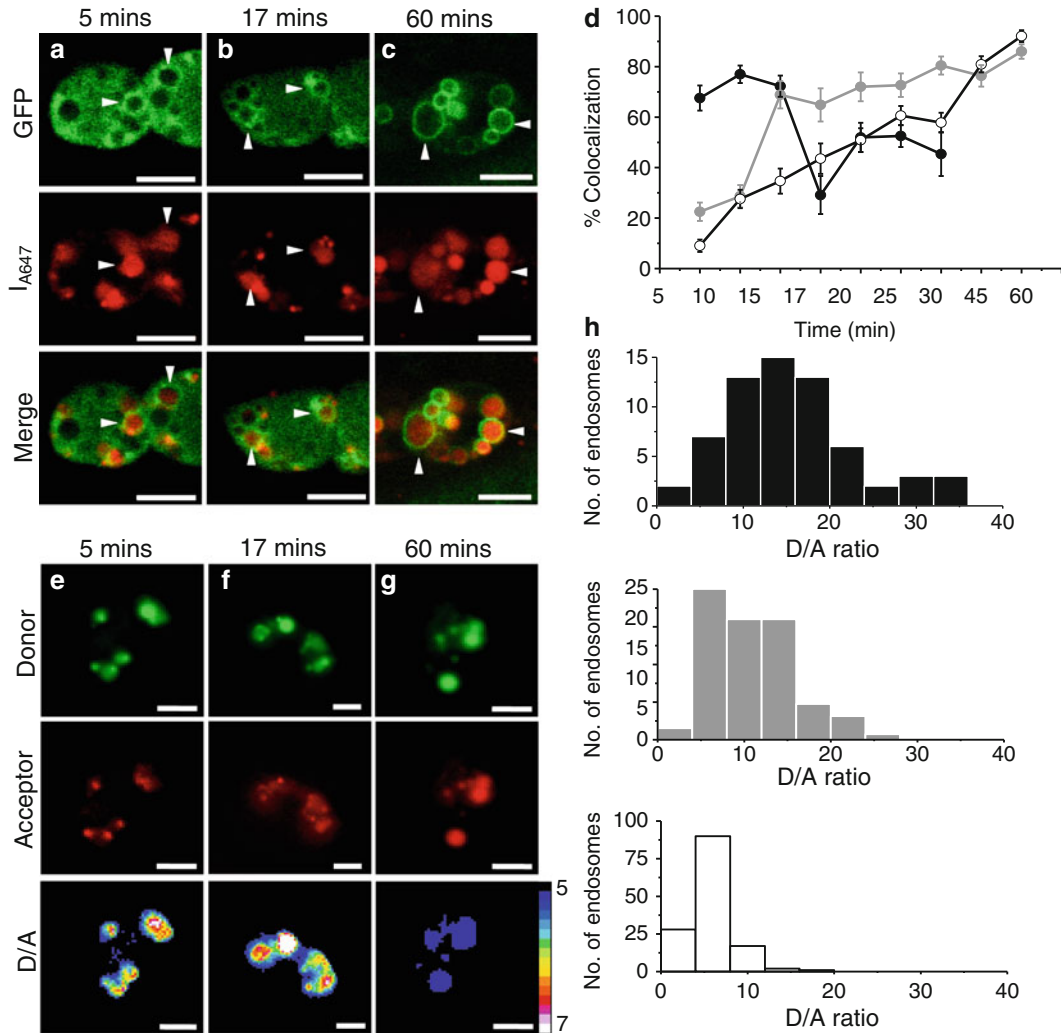


Fig. 3 Spatiotemporal mapping of pH in coelomocytes. Confocal images showing co-localization of I_{A647} with (a) GFP::RAB-5 at 5 min. (b) GFP::RAB-17 at 17 min. (c) LMP-1::GFP at 60 min. Scale bar: 5 μ m. (d) Trafficking of endocytosed I_{A647} . Percentage co-localization of I_{A647} with GFP-tagged endosomal markers (RAB-5, black circles; RAB-7, gray circles; LMP-1, black open circles) at indicated times ($n \sim 75$ endosomes). (e–g) Representative pseudocolor D/A images of $I_{A488/A647}$ -labeled coelomocytes in wild-type hermaphrodites at indicated times. Scale bar: 5 μ m. (h) Histograms of D/A ratios of maturing endosomes; early endosomes at 5 min (black bars), late endosomes at 17 min (gray bars), and lysosomes at 60 min (white bars) ($n \sim 100$ endosomes)

5. Now, to map the pH of a particular population of vesicles, 500 nM $I_{A488/A647}$ is injected in wild-type hermaphrodites. After the requisite time (chosen according to the co-localization studies), the worms are transferred to chilled NGM (+OP50) plates. The worms are anesthetized using 40 mM NaN_3 in M9 buffer, and the coelomocytes are imaged.

3.5 Ratiometric Microscopy and Image Analysis

1. All ratiometric images are collected using a Nikon TE2000-U epifluorescence microscope. Coelomocytes are located and imaged so as to focus maximal number of puncta. Fluorescence images of the cells are obtained by exciting Alexa 488 and collecting emission using the 530 ± 15 nm emission filter. This yields a donor image (D). The cells are then re-excited at 488 nm, and emission of the acceptor is acquired using a 710 ± 40 nm filter. This is the FRET image (A). A third image is obtained by directly exciting the acceptor and collecting emission at acceptor emission wavelength. This is the acceptor image (I).
2. Autofluorescence of each image (D, A, and I) is calculated by measuring mean pixel intensity over an adjacent cell-free area in that image. This autofluorescence is subtracted from the corresponding image, prior to all image processing.
3. Each endosome in the donor is selected by the ROI plug-in within ImageJ program, and total and mean intensities in each endosome are measured and recorded.
4. Each saved ROI is recalled, and total and mean intensities of the corresponding vesicles in the FRET image are measured.
5. Dividing the mean intensity of each endosome in the donor image by the corresponding intensity in the FRET image provides a donor/acceptor (D/A) ratio for that endosome. This is repeated for all the cells imaged (each reading is obtained from coelomocytes of ten worms) to obtain a spread of D/A ratios for that time point (Fig. 3e–h). These values are then used to calculate a mean D/A ratio for the corresponding time point.
6. The standard in vivo calibration curve is then used to convert this D/A ratio to its corresponding pH value (Table 1).

3.6 pH Mapping in Mutants

1. The I-switch is now used to measure pH in genetic backgrounds that perturb the endocytic pathway (Table 2). Two methods are chosen to induce this perturbation: one, a genetic knockout in the *rme-1* gene (this gene functions to recycle internalized receptors to the plasma membrane) and, the other, a knockdown of the VHA-8 protein (this protein is a compo-

Table 2
Mean endosomal pH ($\pm$ s.e.m.) at various time points postinjection

Strain	5 min	17 min	60 min
Wild type	6.4 ± 0.12	6.0 ± 0.09	5.4 ± 0.03
<i>rme-1</i>	6.1 ± 0.06	6.0 ± 0.1	5.7 ± 0.05
<i>vha-8</i> (RNAi)	5.7 ± 0.1	5.2 ± 0.07	6.0 ± 0.04

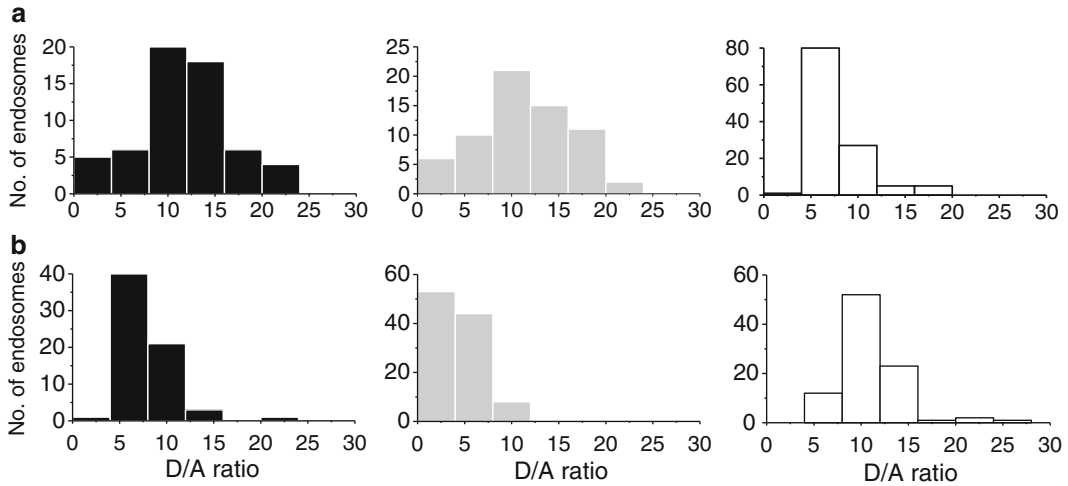


Fig. 4 pH measurements in various genetic backgrounds using the I-switch. (**a, b**) Histograms of D/A ratios of endosomes undergoing progressive maturation, from early endosomes at 5 min (*black bars*) to late endosomes at 17 min (*gray bars*) to lysosomes at 60 min (*white bars*) in *rme-1(b1045)* and *vha-8* (RNAi) hermaphrodites, respectively ($n=10$ cells, ^{3} 60 endosomes)

ment of the V-ATPase complex that maintains pH of organelles).

2. Ten hermaphrodites of each strain are injected with 500 nM $I_{A488/A647}$. After the requisite time (according to the co-localization studies in wild-type hermaphrodites), the worms are transferred to chilled NGM plates. The worms are anesthetized using 40 mM NaN_3 in M9 buffer and the coelomocytes are imaged (Fig. 4a, b).
3. Imaging and analysis is performed as outlined in Subheading 3.5, and pH values are calculated from the standard calibration curve (Table 1).

3.7 Targeting the I-Switch to Other Pathways

1. The native I-switch enters coelomocytes via the ALBR pathway. It can be induced to enter other endocytic pathways by saturating the ALBRs with mBSA and tagging the I-switch with the appropriate endocytic ligand. This can be done by injecting the I-switch with mBSA at molar ratios greater than 1:500.

4 Notes

1. It is imperative that the I-switch sample is always annealed in phosphate buffer of pH 5.5 to minimize variability.
2. Coelomocytes are present in the pseudocoelom, and accessibility of the cells to the clamping buffer is sometimes an issue.

Hence, there may be a few cells whose pH may not be efficiently clamped. These cells are discarded from the final analysis.

3. Coelomocytes clamped at pH 5.0 are very distinctive and can immediately be distinguished from those clamped at pH 7.0 due to their small sizes.
4. The in vitro and in vivo calibration curve is normalized by dividing the D/A values at every pH by the D/A value at pH 7.0.
5. Worms should be grown and maintained at 22°C. Maintenance at lower temperatures slows down all physiological processes, including endocytosis, which may alter temporal regimes of trafficking.

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