Video Article

Detecting Protein Subcellular Localization by Green Fluorescence Protein Tagging and 4',6-Diamidino-2-phenylindole Staining in *Caenorhabditis elegans*

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Abstract

In this protocol, a green fluorescence protein (GFP) fusion protein and 4',6-diamidino-2-phenylindole (DAPI) staining are used to track protein subcellular localization changes; in particular, a nuclear translocation under a heat stress condition. Proteins react correspondingly to external and internal signals. A common mechanism is to change its subcellular localization. This article describes a protocol to track protein localization that does not require an antibody, radioactive labeling, or a confocal microscope. In this article, GFP is used to tag the target protein EXL-1 in *C. elegans*, a member of the chloride intracellular channel proteins (CLICs) family, including mammalian CLIC4. An integrated translational *exl-1::gfp* transgenic line (with a promoter and a full gene sequence) was created by transformation and γ-radiation, and stably expresses the gene and *gfp*. Recent research showed that upon heat stress, not oxidative stress, EXL-1::GFP accumulates in the nucleus. Overlapping the GFP signal with both the nuclei structure and the DAPI signals confirms the EXL-1 subcellular localization changes under stress. This protocol presents two different fixation methods for DAPI staining: ethanol fixation and acetone fixation. The DAPI staining protocol presented in this article is fast and efficient and preserves both the GFP signal and the protein subcellular localization changes. This method only requires a fluorescence microscope with Nomarski, a FITC filter, and a DAPI filter. It is suitable for a small laboratory setting, undergraduate student research, high school student research, and biotechnology classrooms.

Video Link

The video component of this article can be found at https://www.jove.com/video/57914/

Introduction

A change of protein subcellular localization is a common mechanism in response to internal or external signals such as heat stress, starvation, oxidative stress, apoptosis, protein phosphorylation, and others. For example, heat stress induces a FOXO member DAF-16 nuclear translocation[1,2], and the pro-apoptotic BCL-2 protein BID translocates to the mitochondria upon receiving death signaling[3,4]. Various techniques are available to detect these changes. A combination of western blotting and biochemically isolating subcellular structures (*e.g.*, mitochondria or the nuclei) could well achieve the goal[3]. However, it requires a specific antibody against the protein of interest. Thus, a well-established antibody becomes the key to the success. An alternative approach is to label different subcellular structures or organelles with various markers such as green fluorescence protein (GFP), red fluorescence protein (RFP), yellow fluorescence protein (YFP), and mCherry, and meanwhile label the protein of interest with other markers. Then, observe them under a confocal microscope to localize the targets[5,6]. Radioactive isotopes are an alternative choice for labeling target proteins and then detecting their subcellular localization[7]. However, this method requires proper training and handling of radioactive wastes. Under circumstances such as the lack of a specific antibody, the absence of a proper marker, or the scarceness of equipment such as a confocal microscope, an alternative approach needs to be considered. To identify a protein nuclear translocation, it is attractive to only label the target proteins with a marker and to stain nuclei with the chemical reagent 4',6-diamidino-2-phenylindole (DAPI) since this only requires a regular fluorescence microscope.

Immunolabeling *C. elegans* with antibodies is challenging due to the low permeability of either the eggshell or the collagenous cuticle surrounding the animal. Meanwhile, since *C. elegans* proteins are significantly divergent from their vertebrate orthologs, a few commercial companies provide *C. elegans* with specific products. It is difficult for a small laboratory to generate *C. elegans* antibodies for themselves. Researchers in the community often use tagged proteins as markers to demonstrate a protein localization or gene expression. This article uses EXL-1::GFP as an example to track a protein nuclear translocation under heat stress[8]. An integrated translational *exl-1::gfp* into the animal genome is used to stably express the gene with *gfp* fusion. Research showed that *exl-1* is expressed in intestine, body wall muscle, and other subcellular structures[8]. This protocol demonstrates how to synchronize worms to the fourth larval (L4) stage, perform a heat stress experiment, and conduct DAPI staining, ethanol fixation, acetone fixation, and imaging under a regular fluorescence microscope.

Protocol

1. Solutions

1. **NGM plates**
    1. In a 2 L Erlenmeyer flask, add 3 g of NaCl, 17 g of agar, 2.5 g of Peptone, and 975 mL of dH_2O. Cover the mouth of the flask with aluminum foil. Autoclave the flask for 50 min. Cool it for 20–30 min.
    2. Then add sterilized solutions: 1 mL of 1 M $CaCl_2$, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M KPO_4 (pH 6.0) buffer. Swirl the solutions to mix them well.
    3. Using a liquid dispenser, dispense the NGM solution to 60 mm Petri plates. Fill the plates 2/3 full of agar. Store them in an air-tight container at 4 °C for future use.

2. **1 M KPO_4 buffer (pH 6.0)**
    1. Dissolve 10.83 g of KH_2PO_4 and 3.56 g of K_2HPO_4 in dH_2O, then adjust the total volume to 100 mL. Autoclave the solution for 15 min.

3. **M9**
    1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in dH_2O, add 1 mL of 1 M $MgSO_4$, and adjust the total volume to 1 L.

4. **10 µg/mL DAPI**
    1. Add 1 µL of 10 mg/mL DAPI into 999 µL of dH_2O.

5. **95% alcohol (v/v)**
    1. Measure 95 mL of 100% alcohol and add distilled water to adjust the volume to 100 mL.

6. **30% acetone (v/v)**
    1. Add 30 mL of acetone to dH_2O and adjust the total volume to 100 mL.

2. Heat Stress

1. Generate an extrachromosomal array line with a translational construct of target genes[9,10]. Alternatively, obtain such lines from the Caenorhabditis Genetics Center (CGC), which is a public resource for *C. elegans* research, or from a previously published research laboratory.
2. Integrate the extrachromosomal lines into the genome by exposing worms to 3,800 Rad γ-radiation[9]. Then, select the stably expressed lines so that each animal expresses a marker protein such as a GFP or roller.
3. To remove mutations generated during the radiation, outcross the lines at least 2x. Use this transgenic line to detect protein expression pattern changes under stress.
4. Prepare NGM worm plates as described in step 1.1. Streak an OP50 clone and culture the bacteria in liquid LB broth overnight. Seed the NGM plates with liquid OP50 culture and incubate the plates in a 37 °C incubator overnight to grow a bacteria lawn as a food source for the worms. Cool down the plates at room temperature for at least 30 min before use.
5. Grow the transgenic line at 20 °C without starvation for at least 2 generations prior to the heat stress assay.
6. Pick 8–10 young gravid adults (uterus filled with eggs) to a new worm plate, then let them lay eggs for about 3–5 h and remove all the adults from the plates.
7. To synchronize the worms, let the eggs hatch and grow the larvae for approximately 48 h to the fourth larval (L4) stage. Examine their vulva structure (a half-moon structure) to identify the L4 stage (**Figure 1**, arrow)[11].
8. Place the worm plates with the L4 larvae in a 35 °C incubator for 2–5 h to achieve heat stress. Place a thermometer in the incubator to accurately measure the temperature.

3. DAPI Staining

1. **Ethanol fixation**
    1. Add 10 µL of the M9 buffer in the center of a glass slide.
    2. Pick the heat-shocked worms from step 2.8 and put them into the M9 buffer on the glass slide.
        1. Alternatively, wash off the plate with M9, centrifuge it at 1,000 x *g* for 1 min at room temperature, and discard the supernatant. Add the worms to the glass slide.
    3. Use a soft tissue to drain any excess liquid. Watch this step under a dissecting microscope.
    4. Add 10 µL of 95% alcohol onto the worms and let them air dry. Watch the process under a dissecting microscope, and do not let the animals get too dry. Immediately after the ethanol has evaporated from the animals, proceed to the next step.
    Note: Ethanol evaporates very quickly.
    5. Repeat step 3.1.4 twice so that the worms are fixed.
    Note: It is normal for the animals to look darker and distorted.
    6. Add 10 µL of DAPI (10 µg/mL) to the worms. Immediately cover them with a coverslip and seal the slide with transparent nail polish gel.
    7. View the animals after around 10 min on a fluorescence microscope.

2. **Acetone fixation (an alternative approach)**

1. Wash off the heat-shocked plate from step 2.8 with M9, centrifuge it at 1,000 x *g* for 1 min at room temperature, and discard the supernatant. Wash the pellet with 1 mL of dH_2O, collect the pellet, and discard the supernatant.
2. Add 400 µL of 30% acetone for 15 min. Centrifuge at 1,000 x *g* for 1 min at room temperature and discard the supernatant. Use 500 µL of dH_2O to wash the animals 2x. Discard the supernatant.
3. Add 200 µL of DAPI (10 µg/mL), or 4x the amount of the total worms' volume, in the tube for 15 min.
4. Centrifuge at 1,000 x *g* for 1 min and discard the supernatant. Wash the pellet 2x with 500 µL of dH_2O. Place the worms on glass slides, cover them with coverslips, seal the slide with transparent nail polish gel, and observe the animals under a fluorescence microscope.

4. Microscopic Imaging

1. Turn on the UV light source and a fluorescence microscope. Turn on the computer connected to the microscope.
2. Load the slide on the fluorescence microscope. Use a low power objective lens (10X) to locate the worms, increase the magnification power to 400X, and focus on the specimen.
3. Open the software provided with the microscope. Click on "Acquisition" in the menu; click on "Camera" and select the camera connected to the microscope; this allows the selected camera to communicate with the software.
   Note: Different microscopes may vary in operation.
4. Under the same "Acquisition", click on "Multidimensional Acquisition". This opens a new "Multidimensional Acquisition" navigation window. Click the "Multichannel" button, and all available channels will appear.
5. Choose blue, green, and DIC (differential interference contrast) to observe the specimen. In the "Multidimensional acquisition" navigation window, leave the "Blue-DAPI", "Green-GFP", and "Grey-DIC", "Nomarski" channels on; right-click on other channels, such as "Red-rhodamine" and "White-bright field", to close them.
6. **First, measure the exposure time for each channel:**
   1. Left-click the "Blue-DAPI" channel to select it and then click on "Measure" in order to take a live image under the DAPI channel with the automated exposure time. A new window with a live image will appear.
   2. On the left side of the live image window, manually adjust the exposure time if needed to make the image as desired.
   3. Finally, click on **"**OK" in the live image window. This sets the exposure time under the DAPI channel.
7. Repeat step 4.6 for the other channels, "Green-GFP" and "Grey-DIC", "Nomarski", to set up the exposure times for those channels.
8. In the "Multidimensional acquisition" navigation window, click on "Start" to automatically collect 3 images under the 3 different channels in order (see above) with specific exposure times as set.
9. To save the file, go to the main menu, click on "File" then on "Save as". Input file name and the folder name. Save the file as the default file format to preserve the detailed imaging information for future reference.
10. **To export the file in other formats:**
    1. Go to "File" and click on "Export". This will open up an export setting window.
    2. Set file name and folder. Check "Create a project folder" to better organize the data. The software also allows the user to specify 4 other options: "Generate merged images", "Use color for channel images", "Use channel names", and "Merged image only".
    3. Choose a desired file type from the drop-down menu, such as "TIF", "BMP", "PSD", or "JPG" and other types. Then click on "Start" to export.

Representative Results

Chloride intracellular channel proteins (CLIC) are multifunctional proteins that are highly conserved across species[12]. Much research shows that CLICs regulate cellular stress, autophagy, apoptosis, carcinogenesis, angiogenesis, and the macrophage innate immune response in the mammalian system[13,14,15,16,17]. There are two CLIC homologs in *C. elegans*: *exl-1* and *exc-4*. Our recent study showed that *exl-1* regulates animal stress management[8]. We integrated a translational *exl-1::gfp* construct into an animal genome and created transgenic lines, which stably express gene *exl-1* with a GFP as a marker (**Figure 2A** and **2B**). Under heat stress, EXL-1::GFP accumulates in the nucleus in the intestinal region since the strong GFP signal overlaps with the nucleus structure (**Figure 2C-E**). To confirm the subcellular localization, DAPI staining was used as an approach to stain the nuclei. Using ethanol fixation for the DAPI staining shows clear nuclei in the animal body (**Figure 3B**). Acetone fixation also achieves similar results (**Figure 3E**). The overlapping GFP and DAPI signals confirm that EXL-1::GFP indeed accumulated in the nuclei in the intestinal region (**Figure 3C**, **F**).

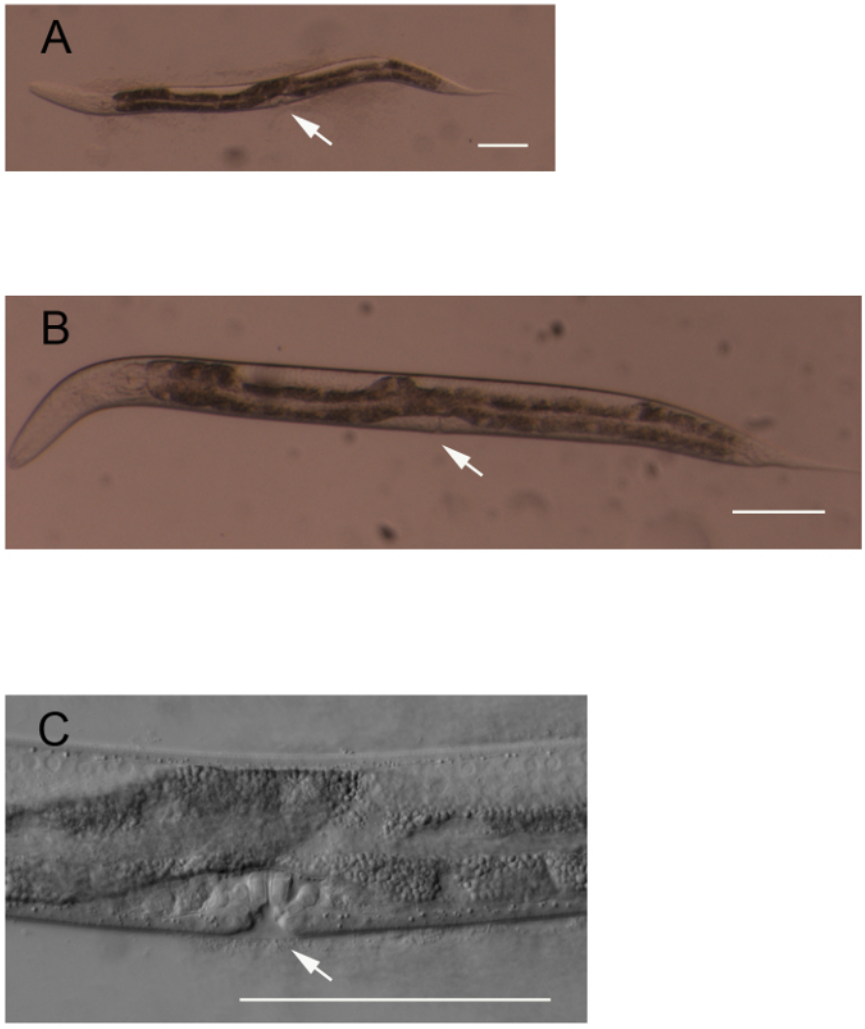

Figure 1: Images of a fourth larval (L4) stage *C. elegans* under different magnification powers. (A) 63X, **(B)** 115X, and **(C)** 400X magnification power. Arrows point to the vulva of a L4 worm; note half-moon structure. Scale bar = 100 µm in all images. Please click here to view a larger version of this figure.

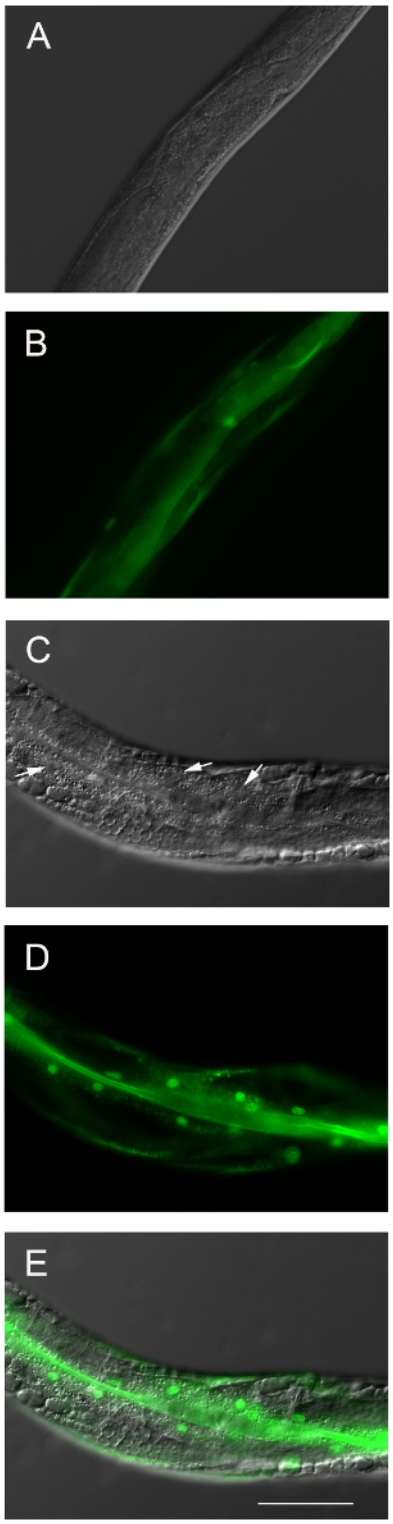

Figure 2: Under heat stress, EXL-1::GFP accumulates in the nuclei of intestine. (A) Normaski image of an intestinal region before heat shock. **(B)** A strong GFP signal was observed in the instestinal region before heat shock. **(C)** Normaski image of intestinal region after heat shock (Arrows point to the nuclei of the intestinal region). **(D)** A strong GFP signal accumulates after heat shock. **(E)** Overlapping image of GFP and Normaski. All pictures are taken with 400X magnification power. Scale bar = 50 µm in all images. Please click here to view a larger version of this figure.

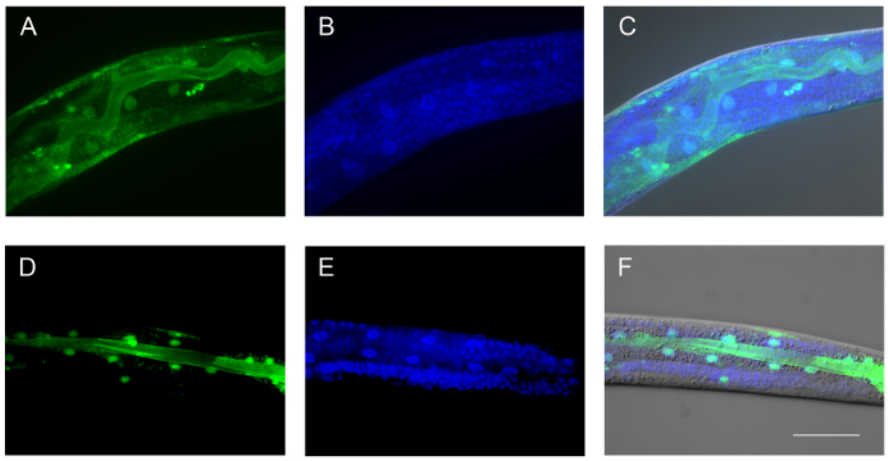

Figure 3: EXL-1::GFP nuclear translocation is confirmed by DAPI staining. (A-C) Using ethanol fixation for DAPI staining. **(D-F)** Using acetone fixation for DAPI staining. In **A** and **D**, green color shows GFP signal in intestinal region. In **B** and **E**, blue color shows nuclei by DAPI staining. **C** and **F** show overlapping images of GFP, DAPI, and Normaski. Scale bar = 50 µm in all images. Please click here to view a larger version of this figure.

Supplementary Figure: Acetone fixation preserves the specimen and 60 days after acetone fixation, GFP and DAPI signals are still observable. (A) Green is for GFP. **(B)** Blue is for DAPI signal. **(C)** Overlapping image of GFP, DAPI, and DIC. All images are taken under 400X magnification power. Scale bar = 50 µm. Please click here to download this file.

Discussion

This article presented a fast and efficient method to verify protein subcellular changes from the cytoplasm to the nucleus. The protein expression was shown by a GFP fusion, while the nucleus structure was verified by DAPI staining (**Figure 3**). Since immunostaining *C. elegans* proteins is challenging, most *C. elegans* protein subcellular localizations are characterized by tagging them with marker proteins such as GFP, Laz, mCherry, and others[18,19,20,21]. In this article, *gfp* was used to tag the target gene *exl-1* in order to demonstrate its protein cellular localization. To confirm the nuclear localization, DAPI staining was used. Two alternative methods were presented for the DAPI staining. Both efficiently showed strong nuclei in worms within a decent working time frame (less than 1 h). The ethanol fixation is suitable for a small sample collection such as a quality observation (collecting less than 50 worms). Preventing the worms from drying completely is the key. Once the 95% alcohol evaporates from the worms, proceed to the next step immediately. The acetone fixation is suited for a large-scale sample collection such as a quantitative analysis (collecting over 50 animals). However, due to the multiple steps of washing the sample, some specimen will be lost. Thus, it is highly recommended to carefully remove the supernatant and retain as many worms as possible. Once they are sealed, slides resulting from acetone fixation can be stored for a week at 4 °C.

This protocol can be adjusted to other proteins of interest to track subcellular localization changes. In addition to the heat stress presented here, other stress assays could be assessed, such as oxidative stress, heavy metal stress, dietary restrictions, and others. Protein subcellular localization change could also be examined by changing the genetic background of the protein of interest. For instance, the transgenic line can be crossed into a different mutant's background, so that the novel genetic interaction may be identified. RNA interference (RNAi) could also be applied to knock down specific genes and investigate the resulting protein subcellular localization change. These applications are of high interest to identify novel regulatory components. For an unknown protein, various experimental conditions should be tested, such as the testing reagent concentrations, the duration of the stress time, and the specific developmental stages of the worms. In our study, we tried different durations, from 30 min to 5 h, and different heat stress temperatures. A heat shock at 35 °C for 3 h clearly demonstrates a protein nuclear translocation.

Additional applications of DAPI staining include the examination of the meiosis and mitosis process in worms, especially in germline cells[22,23,24,25], and a count of the number of nuclei in a genetic mutant's background[20,26,27].

Disclosures

The authors have nothing to declare.

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