Journal of Visualized Experiments

Observation and quantification of telomere and repetitive sequences using fluorescence in situ hybridization (FISH) with PNA probes in C. elegans

--Manuscript Draft--

Manuscript Number:	JoVE54224R3
Full Title:	Observation and quantification of telomere and repetitive sequences using fluorescence in situ hybridization (FISH) with PNA probes in C. elegans
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Telomere; alternative lengthening of telomeres (ALT); fluorescence in situ hybridization; Caenorhabditis elegans; colocalization; cell division; immunofluorescence; PNA probes; imaging; quantification; Molecular Biology
Manuscript Classifications:	5.5.200.500.607.512.240: Fluorescent Antibody Technique; 5.5.200.500.620.670.325.350: In Situ Hybridization, Fluorescence (FISH); 5.5.200.500.620.760.720: Tissue Fixation
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Abstract:	Telomere is a ribonucleoprotein structure that protects chromosomal ends from aberrant fusion and degradation. Telomere length is maintained by telomerase or an alternative pathway, known as alternative lengthening of telomeres (ALT)1. Recently, C. elegans has emerged as a multicellular model organism for the study of telomere and ALT2. Visualization of repetitive sequences in the genome is critical in understanding the biology of telomeres. While telomere length can be measured by telomere restriction fragment assay or quantitative PCR, these methods only provide the averaged telomere length. On the contrary, fluorescence in situ hybridization (FISH) can provide the information of the individual telomeres in cells. Here we provide protocols and representative results of the method to determine telomere length of C. elegans by fluorescent in situ hybridization. This method provides a simple, but powerful, in situ procedure that does not cause noticeable damage to morphology. By using fluorescently labeled peptide nucleic acid (PNA) and digoxigenin-dUTP-labeled probe, we were able to visualize two different repetitive sequences: telomere repeats and template of ALT (TALT) in C. elegans embryos and gonads.
Author Comments:	
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Dear Editor,

We would like to submit our final revision of the manuscript entitled "Observation and quantification of telomere and repetitive sequences using fluorescence *in situ* hybridization (FISH) with PNA probes in *Caenorhabditis elegans*" for publication in ***Journal of visualized experiments***.

We tried our best to improve the manuscript by satisfying all the requests. We now sincerely hope that you find the manuscript ready for publication in Journal of Visualized Experiments.

Thank you for your attention.

Sincerely yours,

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TITLE:
Observation and quantification of telomere and repetitive sequences using fluorescence *in situ* hybridization (FISH) with PNA probes in *Caenorhabditis elegans*

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KEYWORDS:
Telomere; alternative lengthening of telomeres (ALT); fluorescence *in situ* hybridization; *Caenorhabditis elegans*; colocalization; cell division; immunofluorescence; PNA probes; imaging; quantification; molecular biology

SHORT ABSTRACT:
We report a concise procedure of fluorescence *in situ* hybridization (FISH) in the gonad and embryos of *Caenorhabditis elegans* for observing and quantifying repetitive sequences. We successfully observed and quantified two different repetitive sequences, telomere repeats and template of alternative lengthening of telomeres (TALT).

LONG ABSTRACT:
Telomere is a ribonucleoprotein structure that protects chromosomal ends from aberrant fusion and degradation. Telomere length is maintained by telomerase or an alternative pathway, known as alternative lengthening of telomeres (ALT)[1]. Recently, *C. elegans* has emerged as a multicellular model organism for the study of telomere and ALT[2]. Visualization of repetitive sequences in the genome is critical in understanding the biology of telomeres. While telomere length can be measured by telomere restriction fragment assay or quantitative PCR, these methods only provide the averaged telomere length. On the contrary, fluorescence *in situ* hybridization (FISH) can provide the information of the individual telomeres in cells. Here, we provide protocols and representative results of the method to determine telomere length of *C. elegans* by fluorescent

in situ hybridization. This method provides a simple, but powerful, *in situ* procedure that does not cause noticeable damage to morphology. By using fluorescently labeled peptide nucleic acid (PNA) and digoxigenin-dUTP-labeled probe, we were able to visualize two different repetitive sequences: telomere repeats and template of ALT (TALT) in *C. elegans* embryos and gonads.

INTRODUCTION:

Telomere protects chromosomal ends from aberrant fusion and degradation. Mammalian telomere is composed of G-rich hexameric repeats, TTAGGG, and shelterin complexes. The telomere repeat sequence of the nematode is similar to those of mammals (TTAGGC). Most eukaryotes utilize telomerase to add telomere repeats to their chromosomal ends. However, 10-15% of cancer cells utilize telomerase independent mechanism, known as Alternative Lengthening of Telomeres (ALT)[3]. Previously, we reported that telomere repeats and its associated sequences, named as TALT, were amplified in the telomeres of telomerase mutant lines that survived critical sterility[2].

Telomere length was measured by quantitative PCR or by Southern blot, which provides average length of total telomeres[4,5,6,7]. Read count of telomere repeat in whole genome sequencing data is also an indicator of total telomere contents[8]. Although Single TElomere Length Analysis (STELA) could provide the length of a single telomere, it cannot provide spatial information of telomeres[9]. While POT-1::mCherry reporter protein provides the spatial information of telomeres *in vivo,* it cannot represent lengths of double-stranded telomeres, as POT-1 is a single-strand telomere binding protein[10].

While aforementioned methods provide the averaged information of repetitive sequences, fluorescence *in situ* hybridization (FISH) allows to observe the amount and spatial pattern of individual sequences of interest on a chromosomal scale. Instead of purification of DNA, tissues or cells are fixed to preserve the native spatial information in FISH. Thus, FISH is a both quantitative and qualitative tool for observation of individual repeat sequences, such as telomere repeats.

This protocol provides an efficient method for simultaneous detection of both telomere and other repeats based on improvements from previously described methods [11,12]. *C. elegans* larvae or adults are multicellular organism with highly differentiated cells. The heterogeneity of cells impedes on the quantitative analysis of a large number of telomere spots. To maximize the number of cells analyzed, embryos are isolated and spread on the polylysine-coated slides for FISH. In addition, this protocol can also be combined with immunofluorescence.

As a proof that the protocol works, we show that it is possible to observe and quantify two different repetitive sequences. DNA probe against TALT1 was generated with simple PCR incorporating digoxigenin-dUTP. Then this TALT1 probe and fluorescence-labeled telomere PNA probe were hybridized simultaneously. Subsequently, digoxigenin was detected by canonical immunofluorescence methods. We present here the representative images where TALT1 colocalized with the telomere in *trt-1* survivors.

PROTOCOL:

1. Labeling probes with digoxigenin-dUTP by PCR

1.1) Perform PCR labeling with 10x dNTP mix containing digoxigenin-dUTP as previously described[13].

1.2) Purify PCR product with spin-column purification according to manufacturer's instruction.

1.2.1) If the probe is shorter than 200 bp, remove free digoxigenin-dUTP with spin-column chromatography from the reaction mixture rather than spin-column purification.

2. Preparing polylysine coated slides

Note: The entire procedure takes about 2 hours. Most of the steps are done at room temperature except for the drying step.

2.1) Cleaning the slides

2.1.1) Place the slides in a plastic container and rinse the slides briefly with distilled water (DW). Remove the water and fill the container with DW containing 1% glass cleaner.

2.1.2) Agitate the slides for 15 min at 50 rpm at room temperature (RT). Wash the slides with DW 3 times for 5 min each at RT.

2.1.3) Wash the slides with 70% ethanol for 15 min with agitation. Discard 70% ethanol and place the slides on a 65 °C dry block and air-dry for 15 min.

2.2) Polylysine coating of multi-well glass slides

Note: Polylysine coating of slide glass is an important step, since it provides the sample adhesion throughout the staining procedure. Poorly coated slide will result in the loss of sample.

2.2.2) Dilute the polylysine stock solution to 0.01% (w/v) in distilled water. Add 20 µL of the diluted 0.01 % (w/v) polylysine to the wells of a clean glass slide.

2.2.3) Incubate the glass slide for 5 min at RT.

2.2.4) Place the slides on a 65 °C dry block and air-dry for 1 hour.

2.2.5) Store the polylysine slides in the dust free box.

3. Fixation of worms on the slide glass (Figure 1)

3.1) Preparing embryos for FISH

Note: Harvest worms before all the bacterial food is consumed by watching the growth media under microscope. Starvation reduces egg production of adult worms and increases egg hatching. Detailed methods are described in [14,15].

3.1.1) Grow the worms in 50 mm petri-dish according to standard methods[14].

3.1.2) After all the bacterial food is consumed, cut the agar media in quarter with spatula. Sterilize

the spatula before cutting to prevent contamination.

3.1.3) Put all the piece of agar on the 100 mm nematode growth media (NGM) plate. Turn the agar piece upside down for the worms to reach the fresh bacterial food.

3.1.4) After 48 to 72 hr, collect the worms with M9 buffer. Add 3-5 mL of M9 buffer on the NGM plate. Pipette M9 buffer on the surface of NGM to wash the worms.

3.1.5) Collect the liquid with worms and add to a 15 mL tube.

Note: If there is agar debris after harvest, centrifuge the worms in a 30% sucrose solution. While debris are pelleted, worms float on the surface.

3.1.6) Add M9 buffer to make up the volume to 15 mL. Pellet the worms by centrifugation at 300 x g for 3 min and remove most of the M9 buffer. Repeat this step 2 more times.

Note: If the worms are still floating after centrifugation, set the brake level of the centrifuge to value =1.

3.1.7) Aspirate M9 buffer. Add bleaching solution to the worms. Per 0.5 mL of worms, add 7.5 mL of DW, 2 mL of hypochlorite, 1 mL of 5M KOH.

3.1.8) Incubate the worms with rocking at RT for 3 min at 50 rpm. Vortex worms for 15 seconds to mechanically shear the worms and expose the eggs.

3.1.9) Observe the tube under a dissection microscope during bleaching. Make sure that worms are cut in half and eggs are released. When the most of the adult body is dissolved, add M9 buffer to make up the volume to 15 mL.

3.1.10) Centrifuge at 300 x g for 3 min. Aspirate most of the M9 and add fresh M9 buffer to make up the volume to 15 mL. Repeat wash step 3 times.

Note: Avoid using excessive amount of worms, as they hinder the bleaching process. Keep the overall reaction time less than 8 minutes until the wash. Over-bleached eggs produce strong autofluorescence.

3.1.11) Add phosphate buffered saline with polysorbate-20 (PBST) up to 200 µL and 200 µL of 4% paraformaldehyde (PFA) to make 2% PFA.

Caution: Since PFA is carcinogenic, wear protective clothing, gloves and eye shield before using PFA.

3.1.12) Add 40 µL of the eggs in 2% PFA onto the well of polylysine coated slide.

3.1.13) Place the slides in a humid chamber and incubate for 15 min at RT. Close the humid chamber right after the slides are placed inside.

Note: The eggs settle to the bottom of slide while being fixed.

3.2) Preparing dissected gonads for FISH

3.2.1) Harvest adult worms grown on 50 mm NGM plate by pipetting 1 mL of M9 buffer. Harvest worms before bacterial food is depleted.

3.2.2) Wash the worms from any bacteria with M9 buffer, 2 times. Note: Residual bacteria may interfere with the dissected gonads from sticking to polylysine coated slides.

3.2.3) Pellet the worms by centrifugation at 300 x g. Remove M9 and transfer the worms to the empty NGM plate by micropipette.

3.2.4) Add 30 µL of M9 buffer containing 2 mM levamisole on a well of polylysine treated slide.

3.2.5) Add 500 µL of M9 buffer to a 1 mL tube. Use this buffer to transfer the worms by mouth pipette.

3.2.6) Fill the tip of the mouth pipette with M9 buffer by placing a capillary in the 1 mL tube containing the M9 buffer.

3.2.7) Under dissecting microscope, put the tip of mouth pipette just in front of the head of adult worm and drag mouth pipette so that the head of worms enters the mouth pipette. Once the head of worms enters the tip, the entire body of worm will be drawn into the tip.

3.2.8) Transfer the worms to the polylysine coated slide using mouth pipette.

3.2.9) Using a razor, cut off the head or the tip of the tail of worms on the slide. When the worm is cut, the gonads will pop out. Gonads will stick to the slides.

3.2.9.1) Prepare at least 30 worms in one well. More wells can be used for another 30 worms.

3.2.10) Put the slide in the humid chamber and aspirate off the M9 buffer with mouth pipette.

3.2.11) Fix the sample by adding 20 µL of 2% PFA at RT for 15 min in the humid chamber.

4. Fixation and Permeabilization

4.1) Place an aluminum block on dry ice and store it in a deep freezer (-80 °C). Store methanol and acetone in -20 °C.

4.2) After PFA fixation step 3.1.15 or 3.2.11, remove fixative using micropipette leaving ~5 µL of the fixative.

4.3) Put another polylysine coated slide on the sample slide. Remove the fixative with paper towel if the solution is excessive. Do not move the slides once they are stuck together.

4.4) Freeze the slides on the aluminum block for at least 15 min.

Note: The samples can be stored for at least 2-3 days.

4.5) While the slides are being frozen, put the jars containing cold methanol and acetone on ice.

4.6) Take the slides out and twist them to freeze-crack the sample. Discard the upper slide. Immediately soak the slide into the ice-cold methanol for 5 min.

4.7) Transfer the slides to ice-cold acetone for 5 min.

4.8) Wash the slides 3 times with PBST for 5 min to remove residual fixative. Proceed to the next step or store the slides in 100% ethanol at 4 °C.

Note: The samples can be stored for at least 2-3 days.

5. Hybridization of fixed cells

5.1) Add 20 µL of RNase solution (PBST containing 10 µg/mL RNase A). Incubate the slide in the humid chamber at 37 °C for 1 hour.

5.2) Wash the slide twice in 2X saline and sodium citrate with polysorbate-20 (2X SSCT) for 15 minutes each.

5.3) Add 20 µL of hybridization solution and put the humid chamber in the 37 °C incubator. After 1 hour, remove the hybridization solution by pipetting.

5.4) Before removing hybridization solution, prepare the probe. If the probe is double stranded DNA, denature the probes by heating at 95 °C for 5 min on a dry block. After heating, cool the probe on ice briefly.

5.5) Add 10 µL of hybridization solution containing probes to the sample. For PNA probe, use concentration at a ratio of 1: 2000 and for dig-labeled probe, use concentration at a ratio of 1:200. Cover the sample with cover glass.

5.6) Put a paper towel soaked with water on the heat block (80 °C). Put a plastic box cover on the heat block to preserve the humidity and temperature.

5.7) After the temperature of the heat block has stabilized (to 80 °C), place the sample slide on the heated paper towel and cover the samples with the plastic box cover. Denature the sample for 3 min.

5.8) Incubate the slides in a humid chamber overnight at 37 °C.

6. Washes and immunofluorescence

6.1) Warm up the hybridization wash solution (2X SSC, 50% formamide) to 37 °C.

6.2) Wash the sample in the PBST twice at RT for 5 min. Remove the cover glass.

6.3) Wash the sample in hybridization wash solution at 37 °C for 30 min.

6.4) Wash the sample slide in PBST 3 times at RT. Note: Perform all the subsequent steps in humid chamber at RT.

6.5) Add 20 µL of blocking solution and incubate for 1 hour at RT in the humid chamber.

6.6) Remove blocking solution and add FITC conjugated anti-digoxigenin antibody solution (1:200) for 3 hour at RT or overnight at 4 °C.

7. Mounting and observation

7.1) Wash the sample slide with PBST 2 times for 15 minutes each.

7.2) Add 10 µL of mounting solution with DAPI. Put the cover glass and press gently. Remove any excess solution with a paper towel.

7.3) To prevent evaporation of mounting solution, seal the edges of the cover glass with nail polish.

7.4) Observe under confocal microscope. Exclude embryos with high background. Focus on a field with 4 - 20 nuclei.

7.5) Take images according to manufacturer's instruction with 100X objective lens. Note: Excite sample with 405 nm laser for DAPI, with 555 nm laser for cy3, with 488 nm laser for FITC.

8. Quantification of telomere signal

Note: Quantification was done as described previously[16]. All the images that are to be compared should be taken with same setting including exposure time and light source.

8.1) Export the image in .tif format.

8.2) Download and install the image analysis software.

8.3) Execute the image analysis software and click agree button.

8.4) Click open button. Open the images with telomere FISH by double-clicking the image file.

8.5) Click [edit] – [select processing region], select region of interest by left-click and dragging. Exclude all the non-specific staining.

8.6) Click [measure] – [spot optical densities], select the channel with telomere signal and enter the file name to save the results in .txt file.

Note: The column of results are in the following order: Fluorescence of spot, background intensity of spot and area of spot.

8.7) Copy the values and subtract background intensity of spot from fluorescence of spot. The values can now be statistically analyzed.

REPRESENTATIVE RESULTS:

It was previously reported that ALT survivor can emerge from telomerase-deficient mutant, *trt-1(ok410)*, in low frequency by replicating internally localized 'Template of ALT' (TALT) sequences for telomere maintenance[2]. Using PNA probe, we were able to visualize telomeres in the dissected gonads (Figure 2A). The faint telomere signal was detected both in *trt-1(ok410)* and ALT survivor. The fuzzy signal was overlapped only with DAPI, suggesting that they may not be autofluorescence. Interstitial telomere-like repeat (ITR) is consistently observed in TRF assay in the study of *C. elegans* telomere[4,10]. Considering high specificity of PNA probe, they are likely to be the ITR dispersed throughout the genome.

The number of telomere spots was approximately 9 per pachytene nucleus in *trt-1 (ok410)*. In the previous study, 12 foci was observed by POT-1::mCherry protein, which binds to single stranded telomere DNA[10]. Maximum of 24 foci per nucleus was observed in the wild type embryos[17]. The result suggest that mCherry reporter method is better for the experiment where the number of telomeres should be counted. However PNA FISH is able to detect double-stranded telomere DNA as well as single-stranded telomere DNA in proportion with the telomere length. In contrast, the number of telomere spots was approximately 7 in ALT survivor, which have fused chromosomes (N=3) [2]. This result is consistent with the prediction that telomere spots would be 6 in ALT survivor. We concluded that the signal intensity of ALT survivor was sufficient to be observed.

Telomere signal was colocalized with TALT1 in the ALT survivor, suggesting that TALT1 is used as copy template for telomere in the absence of telomerase (Figure 3). Telomere signal of ALT survivors increased compared to that of parental *trt-1(ok410)* mutant, indicating that telomere is robustly maintained in ALT survivors without telomerase (Figure 4). The signal of PNA probe was greater than that of digoxigenin-labeled probe (Figure 4). Designing probes with PNA oligomer might result in stronger signal than digoxigenin-labeling.

Figure Legends:

Figure 1: Overview of FISH experiment. Eggs are harvested by bleaching adult worms and fixed in 2% PFA on a polylysine-coated slide. Samples are freeze-cracked and permeabilized with methanol and acetone for probe penetration. Probes are added to the sample and hybridized overnight at 37 °C. Digoxigenin-labeled probe is detected by immunofluorescence. The samples are imaged and then quantified by the image analysis software.

Figure 2: Telomere FISH in the dissected gonads. (A). Telomere (red) was detected by cy3-PNA-$(TTAGGC)_3$ in the distal tip of gonads (arrowhead). The intensity of ALT survivor is greater than that of *trt-1(ok410)*. Z-stack image was rendered with maximum projection. Nuclei indicated by white arrow is blown-up on the upper right corner. Scale bar, 10 μm. (B) Number of telomere spots per nucleus in pachytene stage was measured by visual inspection. N = 50. Error bars, SEM.

Figure 3: Telomere and TALT1 FISH in the embryos. A representative image of telomere (red) and TALT1 (green) FISH. Telomere and TALT1 probe were hybridized to embryos simultaneously. DNA was counterstained with DAPI (blue). Scale bar, 10 μm.

Figure 4: Quantification of FISH data. Telomere and TALT1 intensity from Figure 3 were quantified in the image analysis software (a gift from Dr. Peter Lansdorp). Each spot was quantified with threshold level over 15 to exclude non-specific background. T-test was used for

evaluating statistical significance. (*$p < 0.001$). Error bars, SEM.

DISCUSSION:

The main advantage of our protocol is the simplicity of the procedure without noticeable damage to the morphology of cellular structure. Several steps were optimized for *C. elegans* FISH in this protocol. The critical steps for successful FISH include labeling of probes, fixation of embryos and penetration. Digoxigenin-dUTP labeling method provides an easy-to-use labeling method by PCR or nick-translation. To label long target sequence, nick-translation is preferred. In this case, the probes should be digested with appropriate restriction enzyme to facilitate the penetration of probes. Biotin-dUTP tag is not recommended because biotin-labeled probes produced excess amount of background signal from the cytoplasm. Although endogenous biotin blocking reagent is commercially available, it was not attempted.

This protocol uses isolated embryos to increase the density of cells for efficient quantification. Intestinal nuclei of *C. elegans* are large in size and are polyploid, which contribute excessive number and intensity of telomere spots compared to the rest of somatic nuclei. For this reason, whole worm is not suitable for quantification of telomeres in *C. elegans*. In contrast, embryos are appropriate for evaluation of telomere length as they provide homogeneous cells without effect of polyploidy.

This protocol uses 2% PFA fixative that worked fine for telomere FISH. Although glutaraldehyde is reported to result in a lower background signal and harder fixation in RNA *in situ* hybridization, glutaraldehyde increased autofluorescence significantly[18]. This excess background was not abolished after treatment of sodium borohydride, which reduces unreacted aldehyde group. For this reason, glutaraldehyde was not used. If the signal-to-noise ratio is low, time of prehybridization can be extended up to several hours to block non-specific binding sites. In addition, time of stringent washing can be increased to decrease background level.

Staining technique in *C. elegans* can be a challenge for proper permeabilization treatment. *C. elegans* contains thick cuticular exoskeleton which inhibits penetration of antibodies and probes. Traditional antibody staining method involves the treatment of collagenase, which requires much time and optimization process. The enzymatic penetration method also damages the morphology of the worms in exchange of penetration efficiency. Freeze-crack and methanol-acetone treatment was used to facilitate probe penetration. Freeze-crack is simple and rapid compared to chitinase or yatalase treatment[19]. Dehydration or rehydration of methanol series did not seem to affect the quality of FISH. These steps were simplified in this protocol. Although it was reported that proteinase K digestion was required for RNA *in situ* hybridization[19], obvious difference was not observed between telomere FISH results with and without proteinase K treatment. In addition, freeze-cracking of embryos provided an easy-to-use method for visualizing many cells simultaneously on a single focal plane for large quantitative analysis.

By using PNA probe, telomere signal was significantly increased compared with that obtained with a DNA probe. This might be due to higher binding affinity for its complementary target and its smaller size (3 repeat compared to 4 repeat). Fluorescently labeled PNA probe also directly binds to its target, minimizing subsequent steps. Strong affinity maintained in the following immunofluorescence steps makes the post-fixation unnecessary, which can avoid background

noise.

However, some limitations exist in this technique. One is that the permeabilization step slightly damages the morphology at the cost of efficient probe penetration. Treatment of gonads and embryos with methanol and acetone distorted circularity of nuclei compared to untreated control. For experiments that require perfectly preserved morphology, different permeabilization method should be attempted. Another is that the telomere signal is quantified in arbitrary units. This is mainly due to variations among independent experiments. Ribosomal DNA may be considered as an internal control to normalize each sample. More useful methods can be found in reference [20].

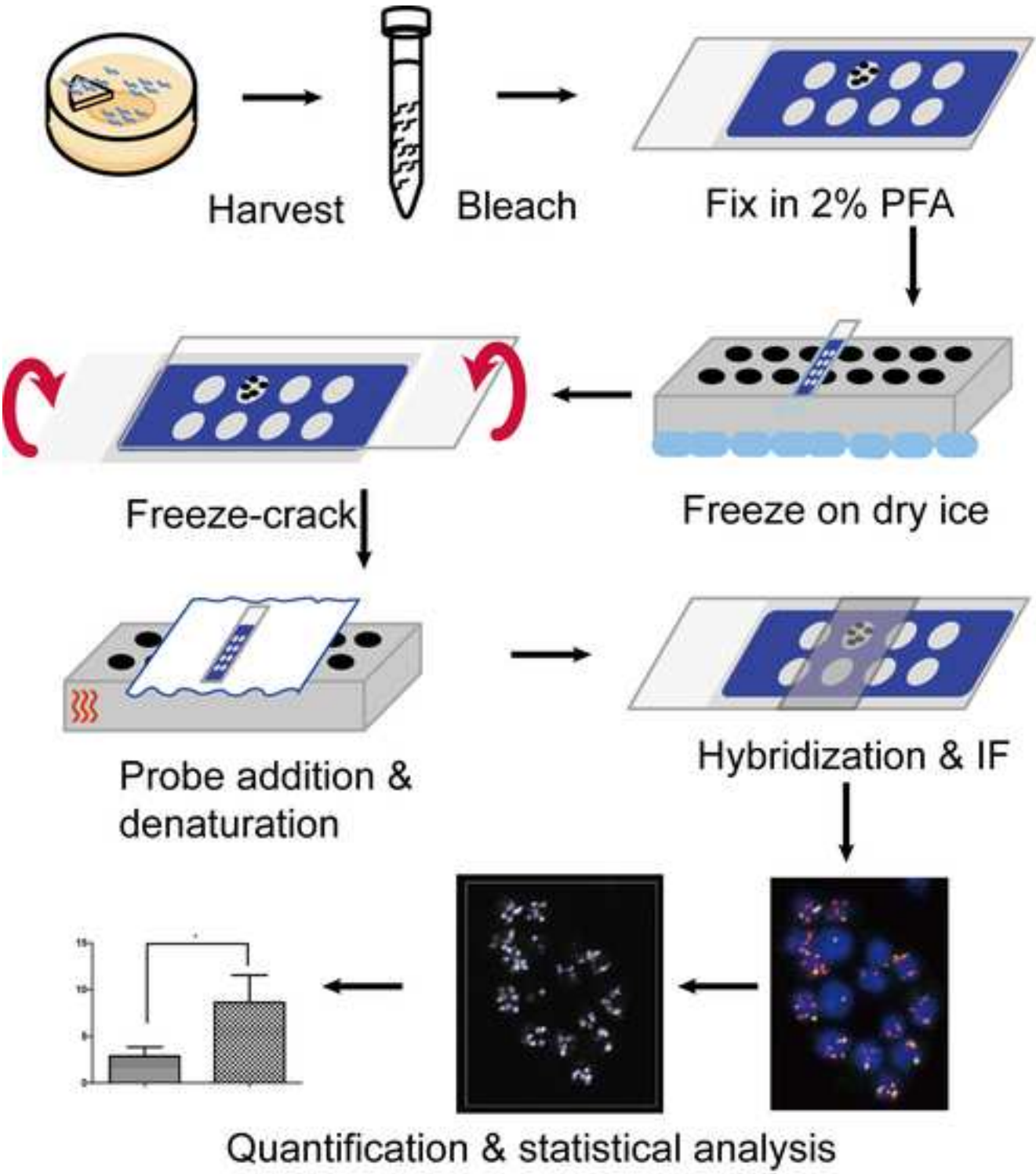
A simple telomere FISH protocol is described here, which requires minimum steps. Many steps are reduced for analysis of large amount of embryos. However, further modification can be made for stronger signal, such as chitinase treatment for penetration, and other innovative trials. In combination with cell culture method, super-resolution imaging may be possible. This protocol may help to discover the novel telomere maintenance mechanism in *C. elegans*.

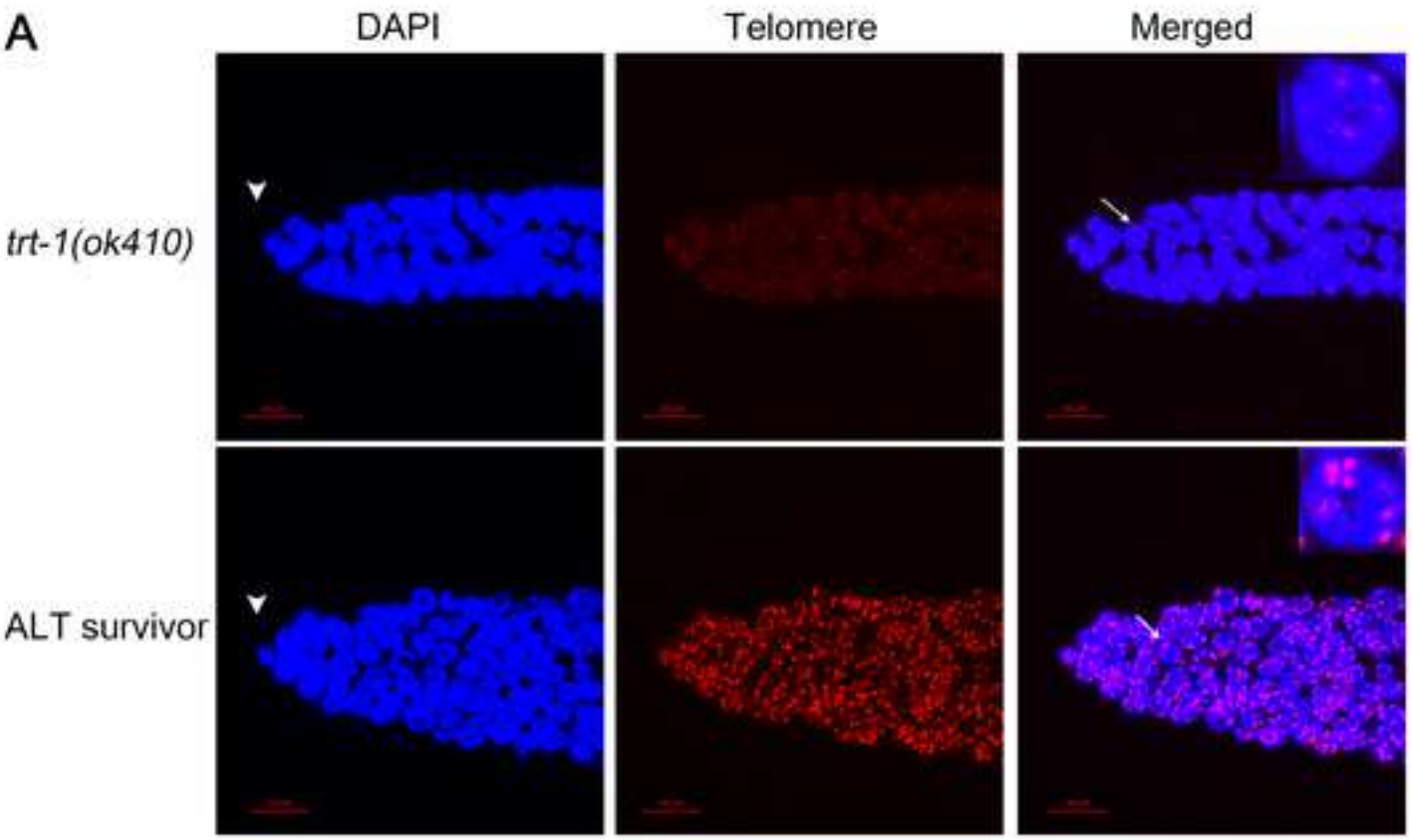
ACKNOWLEDGMENTS:
Mutant worm strains were kindly provided by the Caenorhabditis Genetics Center. This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C1277).

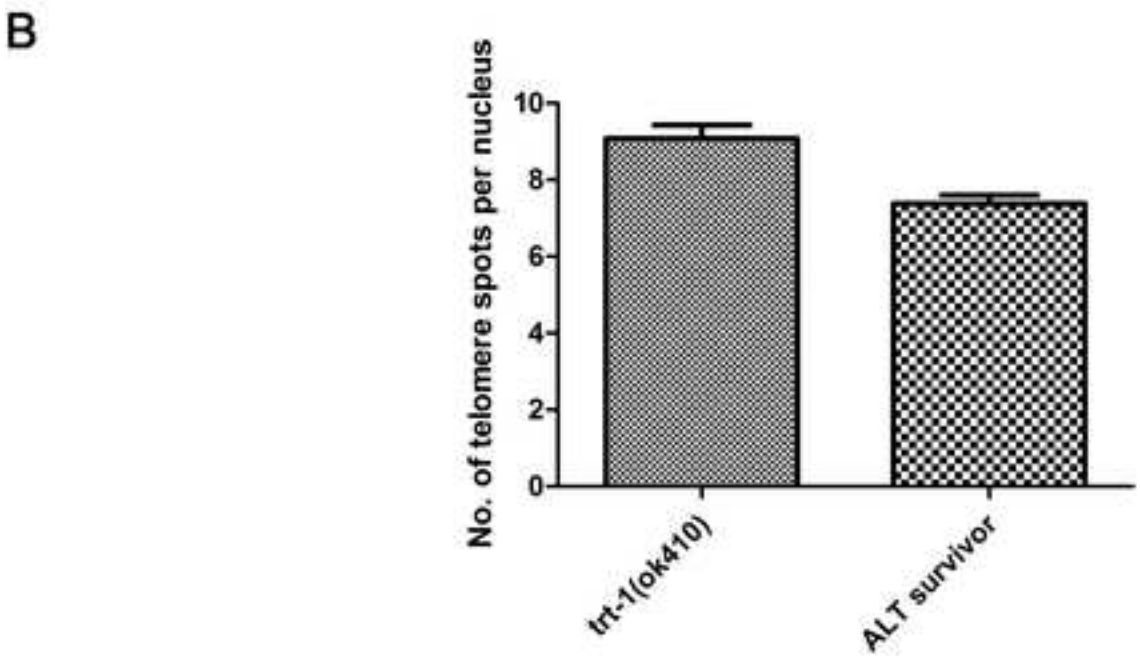
DISCLOSURES:
The authors have nothing to disclose.

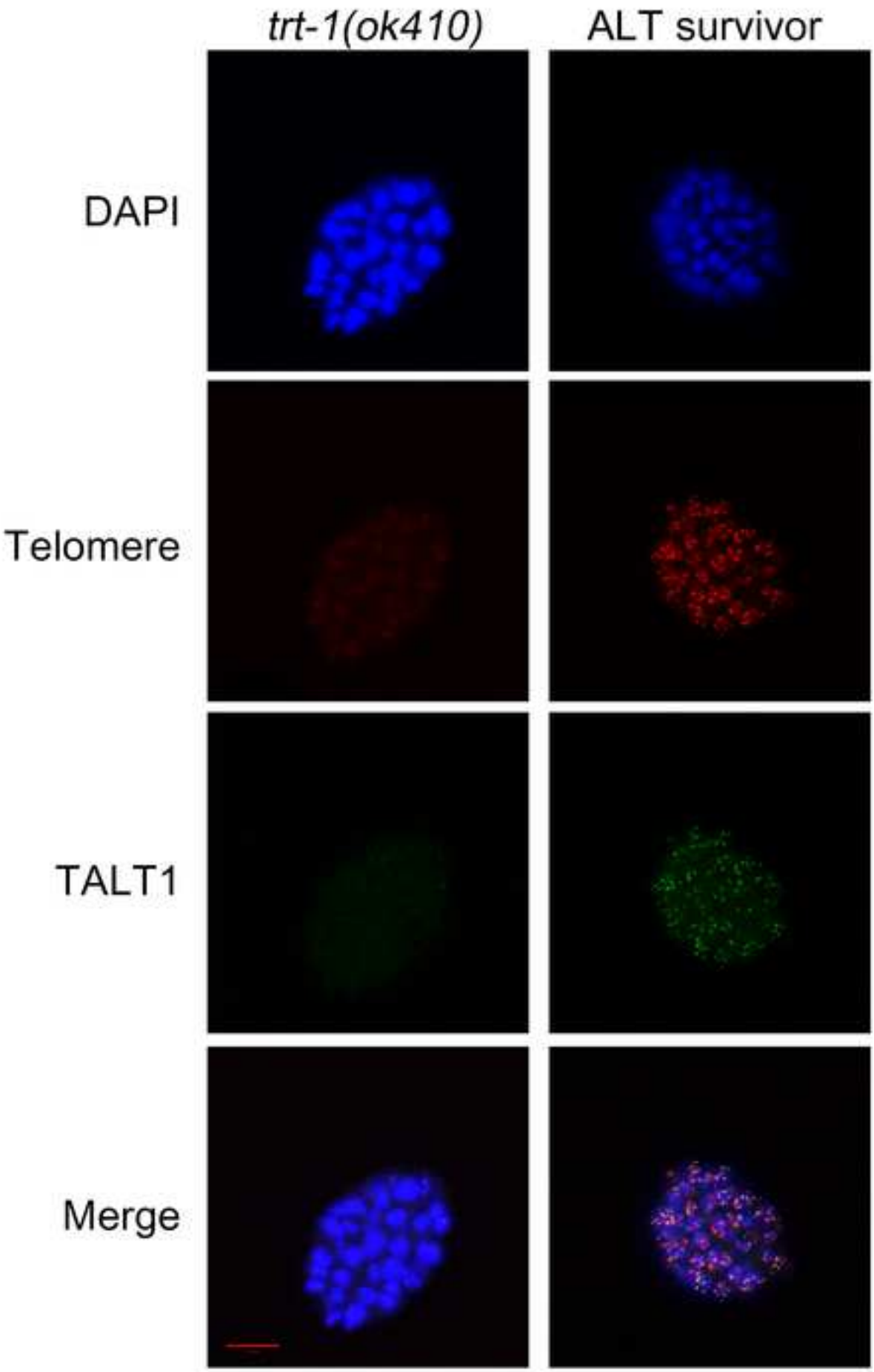
REFERENCES:
1 Reddel, R. R., Bryan, T. M. & Murnane, J. P. Immortalized cells with no detectable telomerase activity. A review. *Biochemistry-Moscow+* **62**, 1254-1262 (1997).
2 Seo, B. *et al.* Telomere maintenance through recruitment of internal genomic regions. *Nat Commun* **6**, 8189, doi:10.1038/ncomms9189 (2015).
3 Cesare, A. J. & Reddel, R. R. Alternative lengthening of telomeres: models, mechanisms and implications. *Nat Rev Genet* **11**, 319-330, doi:10.1038/nrg2763 (2010).
4 Meier, B. *et al.* trt-1 is the Caenorhabditis elegans catalytic subunit of telomerase. *Plos Genetics* **2**, 187-197, doi:ARTN e1810.1371/journal.pgen.0020018 (2006).
5 Cawthon, R. M. Telomere measurement by quantitative PCR. *Nucleic Acids Res* **30**, e47 (2002).
6 Raices, M., Maruyama, H., Dillin, A. & Karlseder, J. Uncoupling of longevity and telomere length in C. elegans. *PLoS Genet* **1**, e30, doi:10.1371/journal.pgen.0010030 (2005).
7 Southern, E. M. Detection of Specific Sequences among DNA Fragments Separated by Gel-Electrophoresis. *Journal of Molecular Biology* **98**, 503-&, doi:Doi 10.1016/S0022-2836(75)80083-0 (1975).
8 Lee, M. *et al.* Telomere extension by telomerase and ALT generates variant repeats by mechanistically distinct processes. *Nucleic Acids Res* **42**, 1733-1746, doi:10.1093/nar/gkt1117 (2014).
9 Cheung, I. *et al.* Strain-specific telomere length revealed by single telomere length analysis in Caenorhabditis elegans. *Nucleic Acids Res* **32**, 3383-3391, doi:10.1093/nar/gkh661 (2004).

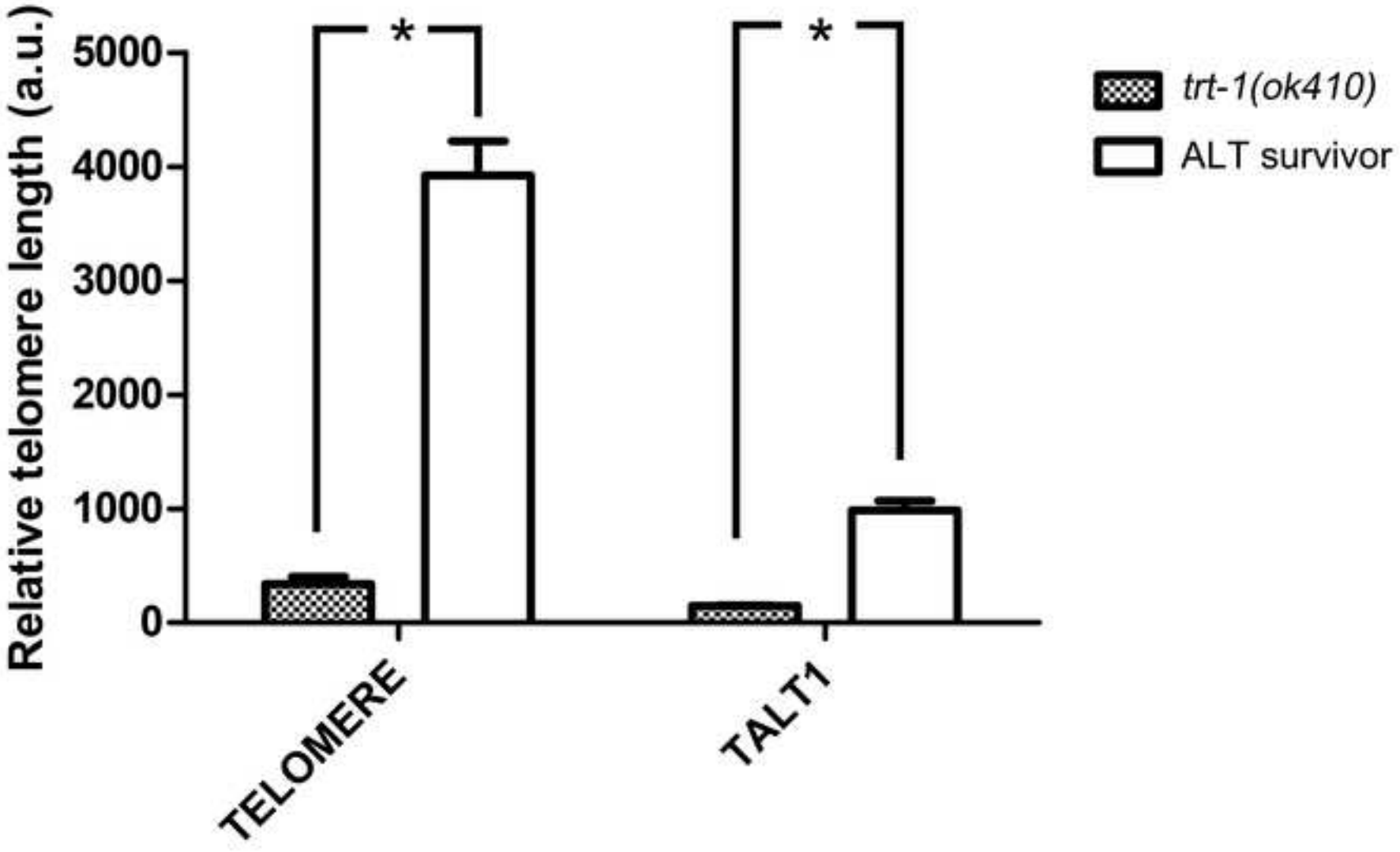
10 Shtessel, L. *et al.* Caenorhabditis elegans POT-1 and POT-2 repress telomere maintenance pathways. *G3 (Bethesda)* **3**, 305-313, doi:10.1534/g3.112.004440 (2013).
11 Duerr, J. Immunohistochemistry. WormBook (The C. elegans Research Community). *WormBook*, doi:doi/10.1895/wormbook.1.105.1 (2006).
12 Phillips, C. M., McDonald, K. L. & Dernburg, A. F. in *Meiosis* 171-195 (Springer, 2009).
13 Emanuel, J. R. Simple and efficient system for synthesis of non-radioactive nucleic acid hybridization probes using PCR. *Nucleic acids research* **19**, 2790 (1991).
14 Stiernagle, T. Maintenance of C. elegans. *WormBook*, 1-11, doi:10.1895/wormbook.1.101.1 (2006).
15 Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A. & Ceron, J. Basic Caenorhabditis elegans methods: synchronization and observation. *J Vis Exp* **81**, e4019, doi:10.3791/4019 (2012).
16 Poon, S. S. S., Martens, U. M., Ward, R. K. & Lansdorp, P. M. Telomere length measurements using digital fluorescence microscopy. *Cytometry* **36**, 267-278, doi:Doi 10.1002/(Sici)1097-0320(19990801)36:4<267::Aid-Cyto1>3.0.Co;2-O (1999).
17 Ferreira, H. C., Towbin, B. D., Jegou, T. & Gasser, S. M. The shelterin protein POT-1 anchors Caenorhabditis elegans telomeres through SUN-1 at the nuclear periphery. *J Cell Biol* **203**, 727-735, doi:10.1083/jcb.201307181 (2013).
18 Lee, M. H. & Schedl, T. RNA in situ hybridization of dissected gonads. *WormBook*, 1-7, doi:10.1895/wormbook.1.107.1 (2006).
19 Tabara, H., Motohashi, T. & Kohara, Y. A multi-well version of in situ hybridization on whole mount embryos of Caenorhabditis elegans. *Nucleic Acids Res* **24**, 2119-2124, doi:doi: 10.1093/nar/24.11.2119 (1996).
20 Poon, S. S. & Lansdorp, P. M. Quantitative fluorescence in situ hybridization (Q-FISH). *Curr Protoc Cell Biol* **Chapter 18**, Unit 18 14, doi:10.1002/0471143030.cb1804s12 (2001).











Name of Material/ Equipment	Company	Catalog Number	Comments/Description
PNA probe	PANAGENE	custom order	
Anti-Digoxigenin-Fluorescein, Fab fragments	Roche	11207741910	use 1:200 diluted in PBST
Digoxigenin-dUTP	Roche	11573152910	
Bovine serum albumin	SIGMA-ALDRICH	A-7906	
Paraformaldehyde	SIGMA-ALDRICH	P-6148	prepare 4% paraformaldehyde by heating in DW with few drops of NaOH. add 0.1 volume of 10x PBS.
Vectashield	Vector Laboratories	H-1200	
Hybridizaiton solution			3X SSC, 50% formamide, 10% (w/v) dextran sulfate, 50 ug/ml heparin, 100 ug/ml yeast tRNA , 100ug/ml sonicated salmon sperm DNA
Hybridizaiton wash solution			2X SSC, 50% formamide
Formamide	BIONEER	C-9012	toxic
Methanol	Carlo Erba		
Acetone	Carlo Erba		
Heparin	SIGMA-ALDRICH	H3393	make 10 mg/ml for stock solution
Dextran sulfate	SIGMA-ALDRICH	67578	
10X PBS			For 1 Liter DW : 80 g NaCl, 2.0 g KCl, 27 g Na_2HPO_4:$7H_2O$, 2.4 g KH_2PO
PBST			1X PBS, 0.1% tween-20
Polysorbate 20	SIGMA-ALDRICH	P-2287	Commercial name is Tween-20
Poly-L-Lysine solution (0.1 % w/v)	SIGMA-ALDRICH	P-8920	prepare fresh 0.01 % w/v solution before use
M9			3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 L

Bleaching solution			20% sodium hypochlorite, 0.5 M KOH
Antibody buffer			1X PBST, 1mM EDTA, 0.1% BSA, 0.05% Sodium azide (toxic)
Blocking solution			Antibody buffer with 5% bovine serum albumin (BSA)
illustra Microspin G-50	GE healthcare	27-53310-01	
20X SSC			To make 1L, 175.3 g of NaCl, 88.2 g of sodium citrate, H2O to 1 L, adjust pH to 7.0
2X SSCT			2X SSC, 0.1 % tween-20
10x digoxigenin-dUTP mix			1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.65mM dTTP, 0.35mM DIG-11-dUTP
PCR purification columns	Cosmo genetech	CMR0112	
Glass cleaner / ULTRA CLEAN	Dukssan pure chemicals	8AV721	
Multi-well glass slide	MP biomedicals	96041205	
Nematode growth media			to make 1 L, 3 g of NaCl, 17 g of agar, 2.5 g of peptone, H2O to 974 mL. Autoclave and cool the flask. Add 1 mL of 1M CaCl2, 1 ml of 4 mg/mL cholesterol in ethanol, 1 ml of 1 M MgSO4, 25 mL of 1 M KPO4.
Levamisole	SIGMA-ALDRICH	196142	
Razor	Feather	blade No. 11	
Rnase A	Enzynomics		
BSA	SIGMA-ALDRICH	A7906	
Equipments			
Confocal microsope	Zeiss	LSM 510	EC Plan-Neofluar 100x was used as objective lens.
Dry block / aluminum block	Labtech	LBH-T03	Set temperature to 80℃
Humid chamber			Plastic box filled with paper towel soaked in DW

Image Analysis Software	Dr. Peter Landsdorp	TFL-telo	http://www.flintbox.com/public/project/502

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Author(s): Beomseok Seo, Junho Lee

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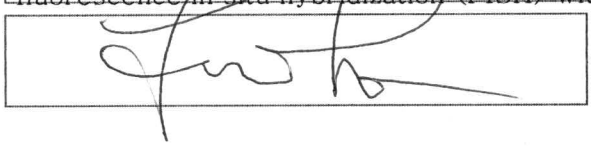
Signature:	 Date: Oct. 13th 2015

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Responses to comments

• The Note starting section 2 should be a step with more details such as concentration/time/temp. Editor adjusted the numbering of protocol section 2 to comply with the JoVE format. Please ensure that all references to particular protocol steps are updated to reflect this change.

We followed the instruction and revised the manuscript accordingly (line 99-115).

• Step 2.1: Please provide the concentration of polylysine used to coat the glass slides. Please provide additional details on how to coat the slides. For e.g., Add xx mL/uL of yy ug/mL polylysine in PBS to the glass slides and incubate for 90 min on a dry block set at 65 C. etc...

We added details in the revision.

• Figure 2A- Please provide scale bars for the figures in the bottom row.

Scale bar was added.

• Discussion- Lines 343-349 (Limitations): Please copy-edit this paragraph for grammar.

We copy-edited the paragraph.

• The highlighted portion of your protocol is slightly over our 2.75 page highlighting limit. Please adjust the highlighting to identify a total of 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We reduced the highlighted portion of section 2 (line 98-115).