bio-protocol

Published: Mar 20, 2022

TUNEL Labeling to Detect Double-stranded DNA Breaks in *Caenorhabditis elegans* Gonads

Peter A. Kropp*, Kyle Rhodehouse[$] and Andy Golden

Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA
[$]Present address: Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, USA
*For correspondence: peter.kropp@nih.gov

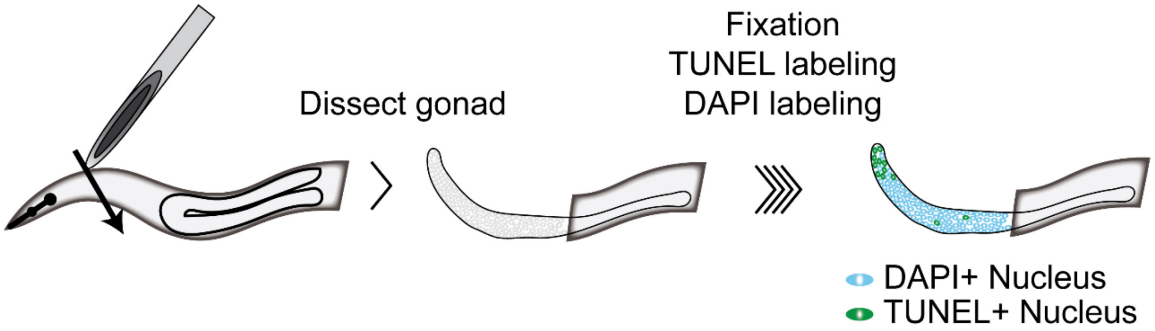
Abstract

Analysis of DNA double strand breaks (DSBs) is important for understanding dyshomeostasis within the nucleus, impaired DNA repair mechanisms, and cell death. In the *C. elegans* germline, DSBs are important indicators of all three above-mentioned conditions. Although multiple methods exist to assess apoptosis in the germline of *C. elegans*, direct assessment of DSBs without the need for a reporter allele or protein-specific antibody is useful. As such, unbiased immunofluorescent approaches can be favorable. This protocol details a method for using **t**erminal deoxynucleotidyl transferase d**U**TP **n**ick **e**nd **l**abeling (TUNEL) to assess DNA DSBs in dissected *C. elegans* germlines. Germlines are co-labeled with DAPI to allow for easy assessment of DNA DSBs. This approach allows for qualitative or quantitative measures of DNA DSBs.

Keywords: *C. elegans*, Germline, TUNEL, Double strand breaks

This protocol was validated in: PLoS Genet (2021), DOI: 10.1371/journal.pgen.1009771

Graphic abstract:



Schematic for TUNEL labeling of *C. elegans* germlines.

Background

Many stresses, nuclear or otherwise, can result in germline phenotypes in *C. elegans,* including reduced brood size or complete sterility. When trying to understand the mechanism resulting in germline defects, it is necessary to investigate the possibility of increased DNA double strand breaks that are likely to lead to apoptosis. Although techniques are well established to assess germline apoptosis [*e.g.*, analysis of cell corpses; CED-3::GFP transgenes (Chen *et al*., 2016)] and DNA damage [*e.g.*, LacZ reporters transgenes (Pontier and Tijsterman, 2009); immunolabeling for DSB repair markers, such as RAD-51 and RPA-1 (Bae *et al*., 2020; Hinman *et al*., 2021)] in the *C. elegans* germline, inclusion of a genetically-encoded reporter can be complicated or impossible in the case of genetic linkage. Therefore, biochemical approaches may offer more flexibility in assessment of DNA DSBs. As presented here, TUNEL labeling can identify DNA DSBs in nearly all *C. elegans* genetic backgrounds (excluding those without a gonad). This approach not only eliminates the need to generate new mutant strains containing the reporter, but allows for parallel preparation and analysis of multiple samples. This protocol is an adaptation of a protocol used in Parusel (2006) and exactly as described in Kropp *et al*. (2021).

Materials and Reagents

1. 25 × 75 mm Superfrost Plus Slides (Diagger, catalog number: EF15978Z), store at room temperature
2. 25 × 25-1 Microscope Cover Glass (Fisherbrand, catalog number: 12-542-C), store at room temperature
3. 23 G 1¼ Precision Glide Needle (BD Bioscience, catalog number: 305120), store at room temperature
4. KimWipes (Kimtech Science, Kimberly-Clark Professional, catalog number: 05511), store at room temperature
5. M9 Buffer (IPM Scientific, catalog number: 11006-517), store at room temperature
6. 10× PBS (KD Medical, catalog number: RGF-3210), store at room temperature
7. Poly-L-lysine solution (Sigma Life Science, catalog number: P8920-100mL), store at room temperature
8. Paraformaldehyde Powder (Sigma-Aldrich, catalog number: 158127), store at 4°C
9. Triton X-100 (Sigma Life Science, catalog number: X100-100mL), store at room temperature
10. Sodium Citrate tribasic dihydrate powder (Sigma-Aldrich, catalog number: C8532), store at room temperature
11. TUNEL Assay Kit – FITC (Abcam, catalog number: ab66108), store at 4°C and -20°C
12. DAPI powder (Sigma-Aldrich, catalog number: D9542), store at 4°C
13. Vectashield (Vector Laboratories, Inc, catalog number: H-1000), store at 4°C
14. Immersion oil for microscopy (Cargille, catalog number: 16482), store at room temperature

Stock solutions to be made

1. 4% paraformaldehyde (PFA) solution (see Recipes).
2. PTX solution (see Recipes)
3. 100 mM sodium citrate buffer (see Recipes)
4. DAPI solution (see Recipes)
5. TUNEL reaction mix from TUNEL Assay kit (see Recipes)
6. 1 M Sodium citrate (see Recipes)

Equipment

1. SMZ645 stereomicroscope (Nikon, model: SMZ645-L)
2. Digital Heat block (VWR Scientific Products, catalog number: 13259-050)
3. Spinning disk confocal microscope
   a. Nikon Eclipse Ti2-E inverted microscope
   b. Yokagawa CSUZ-1 spinning disk
   c. Photometrics Prime95B camera
   d. Nikon Plan Flour 40×/1.3 oil immersion objective

Software

1. Elements Advanced Research (Nikon, https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research)

Procedure

A. Working reagent preparation

1. Prepare Poly-L-lysine coated slides
   a. Add 3–5 µL of Poly-L-lysine to the center of a Superfrost Plus Slide.
   b. Spread the drop of Poly-L-lysine solution into an even layer ~1 cm in diameter with the side of a pipet tip.
   c. Dry at room temperature (RT) overnight. Slides can be stored at 4°C for up to one month once dry.
   d. Allow slides to come to RT prior to the procedure.
2. Prepare 4% paraformaldehyde (PFA) solution (see Recipes).
3. Prepare PTX solution (see Recipes).
4. Prepare 100 mM sodium citrate buffer (see Recipes).
5. Prepare DAPI solution (see Recipes). Excess solution can be stored at 4°C for up to one month, protected from light.

B. Sample preparation

1. From a synchronous population of *C. elegans* (L4 or adult), manually pick individual animals into a watch glass containing M9 solution, to remove excess bacteria (Figure 1A and 1B).
2. Pipet 10 µL of M9 solution to the Poly-L-lysine-coated region of a slide.
3. Individually pick *C. elegans* from the watch glass into the droplet of M9 on the slide (Figure 1C and 1D). Add ~10 animals to the droplet in total.

Note: When picking worms from the watch glass, gently scoop them up individually with your pick. The surface tension of the M9 will allow you to pick up individual worms. Remove them from the pick by gently sweeping the pick through the droplet of M9 on the slide.

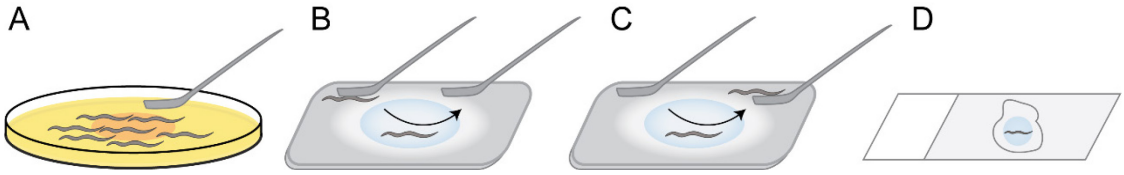


Figure 1. Picking animals from plate to slide.
(A) Using a platinum wire pick, pick the animal(s) from the culture plate to a watch glass containing M9 buffer. (B) Using a sweeping motion, gently remove the animal(s) from the pick into the M9 buffer in the watch glass. (C) Using a sweeping motion, gently lift individual animals from the watch glass and transfer them to a slide containing a drop of M9 buffer. (D) Representative schematic of a slide with an individual animal contained within a droplet of M9 buffer on a dried bed of poly-L-lysine. Representations are not to scale.

4. On the slide, dissect the animals' gonads
   a. Using the edge of a needle, cut the head and/or tail from *C. elegans* on the slide. Gonads will automatically extrude and stick to the Poly-L-lysine coating (Figure 2).

Notes:
1) Some people prefer to use two needles and a scissoring motion, although we found that to be unnecessary.
2) Addition of an anesthetic such as 1mM levamisole can be helpful.
3) A video protocol can be found at https://www.jove.com/v/20131/c-elegans-gonad-extrusion-a-rapid-dissection-technique (Gervaise and Arur, 2016).

   b. Work quickly, as all gonads should be dissected within two minutes.

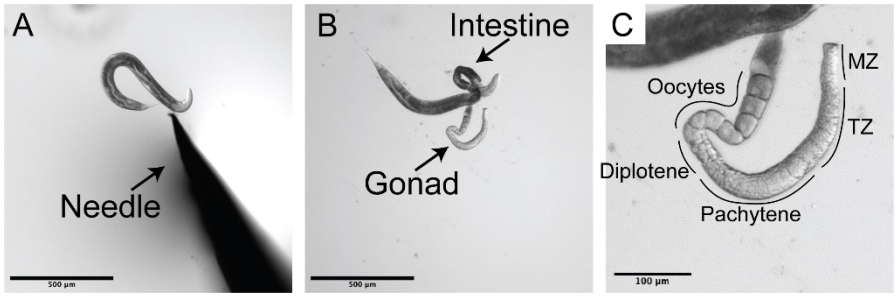


Figure 2. Gonad dissection from adult *C. elegans*.
(A) Representative image through a dissecting microscope of a day-one adult *C. elegans* with a 23 G 1¼ needle for scale. The tip of the needle is adjacent to the junction of the pharynx and intestine (also indicated by change from light to dark color). This is where the incision should be made. (B) Representative image of an extruded gonad as viewed through a dissecting microscope. The intestine will also extrude. Note that the head was not completely removed in this instance. (C) Enhanced image (from B) of extruded day-one adult gonad with regions labeled. MZ: Mitotic zone; TZ: Transition zone. Scale bars (A and B): 500 µm; (C) 100 µm.

5. Fix sample with PFA
   a. Wick away excess M9 solution with the edge of a KimWipe (Figure 3).
   b. Pipet 25 µL of 4% PFA onto the sample. Gently add cover glass to prevent evaporation.
   c. Incubate at RT for 20 min.
   d. Carefully remove cover glass. Carefully remove PFA with a pipet and dispose in an appropriate receptacle.

> *Notes:*
> *1) It may be necessary to use a razor blade to create a separation between the cover glass and the slide. If used, be sure not to damage the sample.*
> *2) PFA is a hazardous waste material. Dispose of it in accordance with your institution's guidelines. All PTX washes immediately after PFA fixation should be assumed to contain PFA and disposed of with the PFA.*

   e. Wash the sample with 25–50 µL of PTX solution three times for three min each. Use the same pipetting technique as used for PFA, to avoid damaging the samples. Dispose of PTX washes in the same receptacle as PFA.

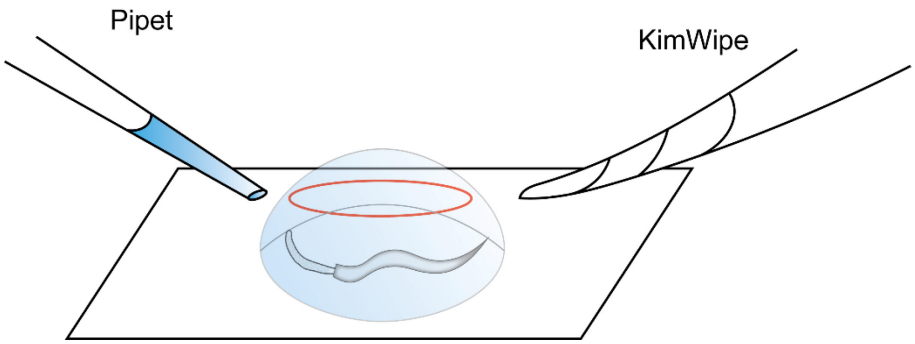


Figure 3. Schematic for fixing, washing, and staining extruded gonads.
The animals will be contained within a droplet of buffer. Pipet or wick away (with a KimWipe) buffers at the end of each step. Add buffers with a pipet at the beginning of each step. To avoid disrupting the samples, pipet or wick from the edge of the buffer droplet as indicated by the area in the red oval. If necessary, tilt the slide to make wicking easier. Representations not to scale.

6. Incubate sample in prepared sodium citrate buffer to improve antigen retrieval
   a. Pipet 25 µL of 10 mM sodium citrate solution on the sample and cover with cover glass.
   b. Place the slide on a heat block prewarmed to 65°C. Incubate for 20 min.
   c. Carefully remove cover glass. Wick away sodium citrate solution with the edge of KimWipe.
   d. Wash three times with PTX, as in step 5e. The PTX wash solution does not need special disposal at this point.
7. Incubate sample in TUNEL reaction mix
   a. Prepare TUNEL reaction mix from TUNEL Assay kit (see Recipes).
   b. Pipet 51 µL of reaction mix onto the sample. Add cover glass to prevent evaporation.
   c. Incubate in a humid chamber at 37°C for 60 min. Protect the slide from light from this point forward.

> *Note: Any plastic container with a damp paper towel is sufficient to make a humid chamber. If there is no natural way to keep the slide from sitting directly on the paper towel, inclusion of something to prop up the slide (such as two parallel pieces of a serological pipet) works well.*

d. Wash three times with PTX as in step 5e. The PTX wash solution does not need special disposal at this point.

8. DAPI co-labeling
   a. Wick away PTX solution with the edge of a KimWipe.
   b. Add 25 µL of DAPI solution to sample and incubate at RT protected from light for 5 min
   c. Wash three times with PTX as in step 5e. The PTX wash solution does not need special disposal at this point.
9. Mounting and cover slipping
   a. Wick away any remaining PTX solution with the edge of a KimWipe.
   b. Add one drop of Vectashield directly to the sample.
   c. Gently lower a clean cover glass onto the sample. To avoid bubbles, lower one edge first, then slowly lower the rest of the cover glass.

Note: If bubbles are present, gently press on the bubble with a toothpick to move it to the edge of the cover glass.

   d. Wick away any excess Vectashield with a KimWipe. Seal cover glass with nail polish.
   e. Sample can be imaged immediately or the following day. Imaging the following day tends to yield better results. If waiting to image, store the slide at 4°C.

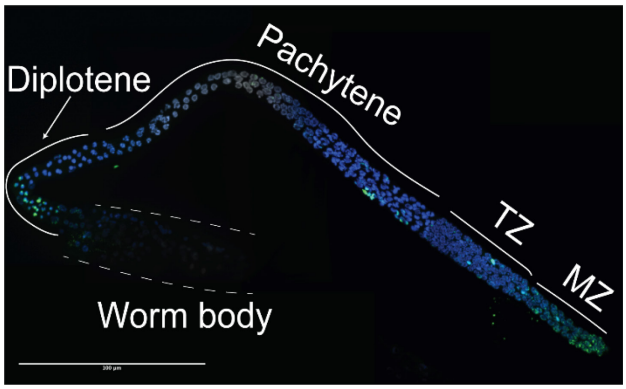


Figure 4. Representative maximum intensity projection of a wild-type L4 *C. elegans* gonad co-labeled with DAPI and TUNEL.
DAPI-stained nuclei are labeled in blue and TUNEL-positive nuclei are labeled in green. As this is an L4 gonad, no oocytes are present. The different regions of the gonad are discernable based on nuclear (DAPI) structure. Due to crossover formation during meiosis (diplotene), nuclei will label with TUNEL. Artifactual labeling of mitotic nuclei with TUNEL has been reported in other systems, including human tissue sections (Labat-Moleur *et al*., 1997) and *Drosophila* ovaries (Qi and Calvi, 2016). DNA nicks during replication are likely the cause (Mets and Meyer, 2009). Apparent pale labeling in the middle pachytene region is an artifact from stitching two z-stacks into a single image. Scale bar: 100 µm.

C. Confocal Imaging

1. Preparation
   a. Ensure that all appropriate lasers (405 nm and 488 nm) and light sources are turned on and warm. Set the 405 nm laser to 35% power and 100 ms exposure. Set the 488 nm laser to 25.5% power and 100 ms exposure.
   b. Mount the slide onto the microscope stage.

c. If using an oil immersion objective, add a small drop of oil onto the cover glass.
d. Find the desired sample.

2. Image acquisition
   a. Set z-slice thickness to 0.3 µm.
   b. Define z-stack to encompass the entire germline diameter.
   c. Capture the z-stack.
   d. If necessary, capture second z-stack to image entire germline.

Data analysis

Data analysis should be performed at the discretion of the experimenter depending on ultimate end use. We qualitatively assessed TUNEL labeling, but quantitative measurements could be generated by determining the percentage of TUNEL-positive nuclei as a function of total nuclei ($\frac{\#\ \text{TUNEL+}}{\#\ \text{DAPI+}}$) or the percentage of TUNEL-positive nuclei within a specific region of the gonad (*e.g.*, pachytene). Comparison of the percentage of TUNEL-positive nuclei between wild-type and mutant samples would be appropriate.

Notes

A positive control to increase incidence of DSBs is exposure of *C. elegans* to UV light. We achieved this by placing plates with *C. elegans* on a standard UV light box for 20 min. Plates were seeded with OP50 bacteria, although we do not believe this is explicitly necessary.

Recipes

All buffers were purchased. Preparations of incubation and wash buffers are indicated in below.

1. **4% paraformaldehyde (PFA) solution**
   In H_2O, from 16% stock. Use fresh. May require heating. Store at 4°C.

2. **PTX solution**
   10× PBS stock and Triton X-100. 1× PBS + 0.4% Triton X-100. Use fresh.

3. **100 mM sodium citrate buffer**
   1 M stock + 0.1% Triton X-100. Use fresh.

4. **DAPI solution**
   10 µg/mL in H_2O.

5. **TUNEL reaction mix from TUNEL Assay kit**
   10 µL of Reaction Buffer, 0.75 µL of TdT Enzyme, 8 µL of FITC-dUTP, and 32.25 µL of ddH_2O.

6. **1 M Sodium citrate**
   In H_2O. May require heating. Store at room temperature.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases (A.G.). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. This protocol is an adaptation of a protocol used in Parusel (2006) and exactly as described in Kropp *et al*. (2021).

Competing interests

The authors declare no competing interests.

References

Bae, W., Park, J. H., Lee, M. H., Park, H. W. and Koo, H. S. (2020). Hypersensitivity to DNA double-strand breaks associated with PARG deficiency is suppressed by exo-1 and polq-1 mutations in *Caenorhabditis elegans*. *FEBS J* 287(6): 1101-1115.

Chen, X., Wang, Y., Chen, Y. Z., Harry, B. L., Nakagawa, A., Lee, E. S., Guo, H. and Xue, D. (2016). Regulation of CED-3 caspase localization and activation by *C. elegans* nuclear-membrane protein NPP-14. *Nat Struct Mol Biol* 23(11): 958-964.

Gervaise, A. L. and Arur, S. (2016). Spatial and Temporal Analysis of Active ERK in the *C. elegans* Germline. *J Vis Exp*(117): 54901.

Hinman, A. W., Yeh, H. Y., Roelens, B., Yamaya, K., Woglar, A., Bourbon, H. G., Chi, P. and Villeneuve, A. M. (2021). *Caenorhabditis elegans* DSB-3 reveals conservation and divergence among protein complexes promoting meiotic double-strand breaks. *Proc Natl Acad Sci U S A* 118(33): e2109306118.

Kropp, P. A., Wu, J., Reidy, M., Shrestha, S., Rhodehouse, K., Rogers, P., Sack, M. N. and Golden, A. (2021). Allele-specific mitochondrial stress induced by Multiple Mitochondrial Dysfunctions Syndrome 1 pathogenic mutations modeled in *Caenorhabditis elegans*. *PLoS Genet* 17(8): e1009771.

Labat-Moleur, F., Guillermet, C., Lorimier, P., Robert, C., Lantuejoul, S., Brambilla, E. and Negoescu, A. (1998). TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement. *J Histochem Cytochem* 46(3): 327-334.

Mets, D. G. and Meyer, B. J. (2009). Condensins regulate meiotic DNA break distribution, thus crossover frequency, by controlling chromosome structure. *Cell* 139(1): 73-86.

Parusel, C. T. (2006). URI-1 is required for DNA stability in *C. elegans*. *Development* 133(4): 621-629.

Pontier, D. B. and Tijsterman, M. (2009). A robust network of double-strand break repair pathways governs genome integrity during *C. elegans* development. *Curr Biol* 19(16): 1384-1388.

Qi, S. and Calvi, B. R. (2016). Different cell cycle modifications repress apoptosis at different steps independent of developmental signaling in *Drosophila*. *Mol Biol Cell* 27(12): 1885-1897.