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Nematode Autofluorescence and Its Use as an Indicator of Viability

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Abstract: Representatives of 15 nematode genera were viewed with 450–490-nm epi-illumination and found to autofluoresce. The autofluorescence was limited to 1–5-μm-d globules in the intestinal cells of live nematodes. When adult *Pratylenchus penetrans* or *Caenorhabditis elegans* were killed with formaldehyde, freezing, or heat, autofluorescence dispersed throughout the body. Mixed stages of *P. penetrans* were killed by freezing at several different temperatures. Estimates of survival based on autofluorescence dispersal matched estimates based on mobility more closely than did estimates based on the vital stain, eosin-y.

Key words: autofluorescence, lipofuscin, nematode viability, secondary lysosome.

Many areas of nematological research require the ability to distinguish live from dead nematodes. Vermiform-stage mobility, spontaneous or induced, is an obvious indicator of viability. The lack of mobility, however, does not indicate that a nematode is dead. This distinction is especially important in research on population dynamics and pesticide efficacy. In the first case, seasonal changes in nematode physiology such as extreme quiescence or diapause may drastically affect the ability to move. In the case of pesticides, especially those that are acetylcholinesterase inhibitors, sublethal doses may temporarily affect mobility (11).

Stains have been used with variable success to distinguish live from dead nematodes (1,2,6,8,9,12–14). Stains work only if the event inducing death results in cuticle permeability (2,8,12). Consequently, the reliability of any particular stain is affected by the event inducing mortality, the harshness of that event, and the length of time between death and staining. Since it is dependent on cuticle permeability, the reliability of a stain can be expected to vary among species and among life stages within a species.

Nematodes accumulate the fluorescent compound lipofuscin (5,10) and are therefore autofluorescent. Clokey and Jacobson (3) identified autofluorescent globules in the intestinal cells of *Caenorhabditis elegans* as secondary lysosomes. We observed that the pattern of autofluorescence in dead nematodes appeared different from the pattern in live nematodes (7). Bird (1) stained live and dead nematodes with fluorescein diacetate and found fluorescence limited to globules in live nematodes and dispersed in dead nematodes. The fluorescence was attributed to hydrolysis of fluorescein diacetate by esterases, which dispersed upon nematode death. Davis et al. (4) treated *Meloidogyne* spp. juveniles with fluorochrome-lectin conjugates and found fluorescence in the lip region of live specimens and throughout the body of dead specimens.

The purpose of this research was to determine if the dispersal of autofluorescence is a reliable indicator of death in nematodes.

Materials and Methods

Representatives of 15 genera (*Aphelenchus, Caenorhabditis, Criconemella, Ditylenchus, Helicotylenchus, Heterodera, Hoplolaimus, Meloidogyne, Pratylenchus, Rotylenchus, Longidorus, Trichodorus, Tylenchus, Mononchus,* and *Rhabditis*) were viewed with a compound microscope (Zeiss Standard GFL) equipped for epi-illumination. Only second-stage juveniles of *Heterodera* and *Meloidogyne* were observed. The light source and filter system consisted of a 50-watt mercury vapor bulb, band pass filter for 450–490-nm excitation, FT510 chromatic beam splitter, and barrier filter for 520 nm

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and above. In addition to the 450–490-nm excitation, several specimens of *C. elegans* were viewed with a filter system that resulted in 365-nm peak excitation. Nematodes were photographed with black and white film (Kodak, ASA 400) and an exposure time of 5 seconds.

To determine if autofluorescence dispersed when nematodes were killed, adult female *Pratylenchus penetrans* and adult hermaphrodites of *C. elegans* were hand picked into 0.5-ml microcentrifuge tubes containing 0.2 ml tap water. Ten replicate tubes containing 30 *P. penetrans* and six tubes containing 100 *C. elegans* were administered one of three lethal treatments: 1) 60 C for 75 seconds, 2) −20 C for 12 hours, or 3) 0.2 ml of 10% formalin, resulting in a 5% final concentration. The formalin was removed after 30 minutes by centrifuging, removing the supernatant, and resuspending in tap water. Controls were left at room temperature (ca. 24 C) for 12 hours. Twenty-four hours after the completion of each treatment, nematodes were viewed at 100× using epi-illumination. The percentage of nematodes with dispersed autofluorescence was recorded.

Estimates of survival based on dispersed autofluorescence of mixed stages of *P. penetrans* frozen at three different temperatures were compared with estimates based on mobility and staining with eosin-y. In order to start the experiment with 100% live specimens, the nematodes were twice required to move through tissue on a Baermann funnel; survivors were then suspended to a density of approximately 250 individuals/ml tap water. One milliliter of the suspension was pipetted into each of 36 test tubes and the tubes were divided into four groups of nine. Three groups were frozen at −2, −4, or −8 C by placing each in a refrigerated ethylene glycol bath at one of the temperatures and adding a small ice crystal to each tube to initiate freezing. The fourth group served as unfrozen controls.

After 24 hours at −2, −4, or −8 C, the samples were placed at room temperature and 1.0 ml of 1.0% eosin-Y was added to three samples from each group. After an additional 24 hours, the stain was removed by twice adding 5.0 ml tap water, allowing the nematodes to settle, and then removing excess solution. Each sample was then viewed with a stereo microscope at 70×, and the percentage of each stage taking up stain was determined. On the same day, three of the unstained samples from each group were viewed with a stereo microscope at 70×, and the percentage of each stage not exhibiting any mobility was determined. This included not only spontaneous mobility, but any movement detected after the nematodes were tapped several times near the nerve ring with a fiberglass pick. The remaining three samples from each group were viewed with epi-illumination at 100×, and the percentage of each stage exhibiting dispersed autofluorescence was recorded.

The data for each stage were analyzed separately as a split-plot design, with freezing temperature as the main factor and assay type as the subfactor. Least significant differences were used for comparison of means.

Results

All nematodes viewed with 450–490-nm epi-illumination exhibited autofluorescence in the form of globules in the intestinal region (Fig. 1). Spicules and the lip region also fluoresced. The color, intensity, and quantity of autofluorescent globules differed slightly between genera as well as between individuals of a given species. The globules ranged in color from pale yellow to green. In *C. elegans,* the globules ranged in diameter from ca. 1 μm to 5 μm and fluoresced blue when viewed at 365-nm peak epi-illumination. Autofluorescent globules were faint in second-stage juveniles of *Pratylenchus, Meloidogyne,* and *Heterodera.*

Regardless of how adult *P. penetrans* females or *C. elegans* hermaphrodites were killed, autofluorescence was dispersed in at least 97% of the individuals (Table 1). The

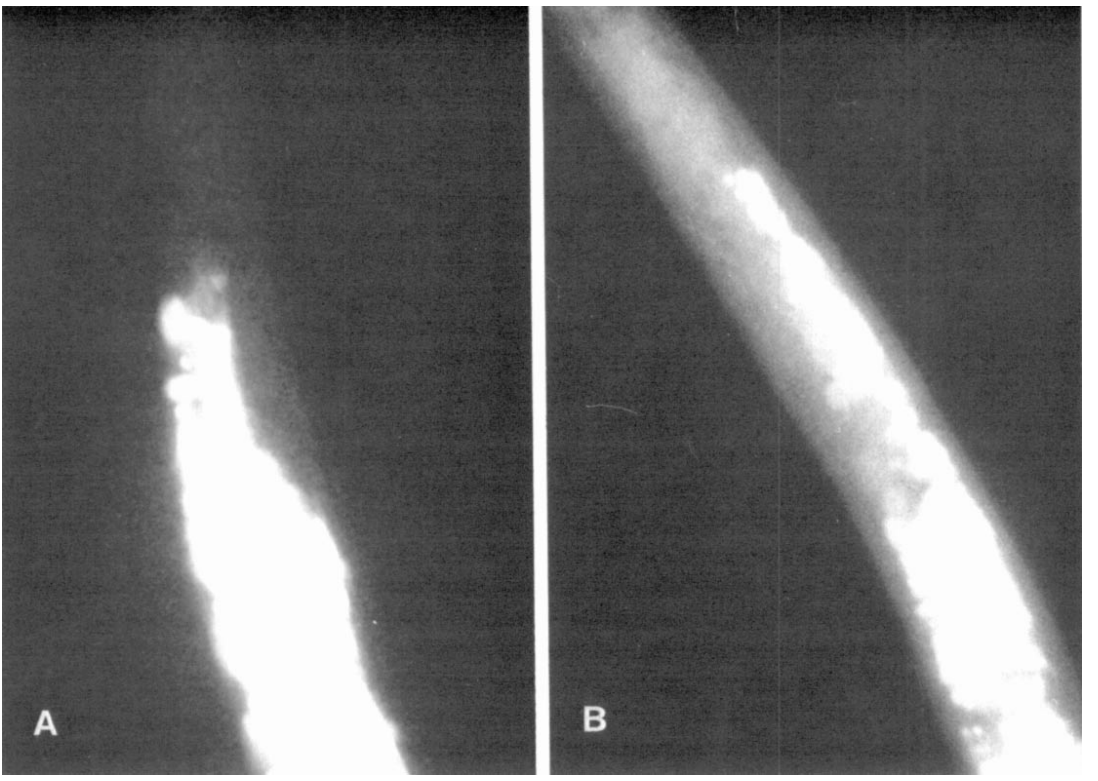

FIG. 1. Autofluorescence at junction of esophagus and intestine of an adult female *Pratylenchus penetrans* viewed with 450–490-nm epi-illumination. A) Alive. B) Immediately after freezing at −20 C for 30 minutes and thawing.

autofluorescence was dispersed in less than 2% of untreated controls. On the basis of lack of mobility, individuals with dispersed autofluorescence were classified as dead.

Estimates of survival based on autofluorescence were significantly lower ($P = 0.05$) than estimates based on staining for every combination of stage and freezing temperature except third-stage juveniles frozen at −2 C (Fig. 2). Estimates of survival based on autofluorescence were significantly higher than estimates based on mobility for second-stage juveniles frozen at all three temperatures, fourth-stage juveniles frozen at −4 C, and females frozen at −4 C. For all but the second juvenile stage, there was a significant ($P < 0.01$) interaction between the type of viability assay and temperature of freezing. For all three methods of assessing survival, the estimates for unfrozen controls were always 100%.

DISCUSSION

At 365-nm peak epi-illumination, the size, distribution, and color of autofluorescent globules in *C. elegans* match those described by Clokey and Jacobson as secondary lysosomes (3). The globules observed at 365-nm peak epi-illumination are the same size as those observed using 450–490-nm epi-illumination.

That all nematodes viewed so far exhibit

TABLE 1. Percentage of female *Pratylenchus penetrans* (Pp) and hermaphrodites of *Caenorhabditis elegans* (Ce) with dispersed autofluorescence after lethal treatments.

Treatment	Pp	Ce
Freezing	99.6	97.7
Formaldehyde	98.9	99.2
Heat	97.4	100.0
Controls	0.4	1.2

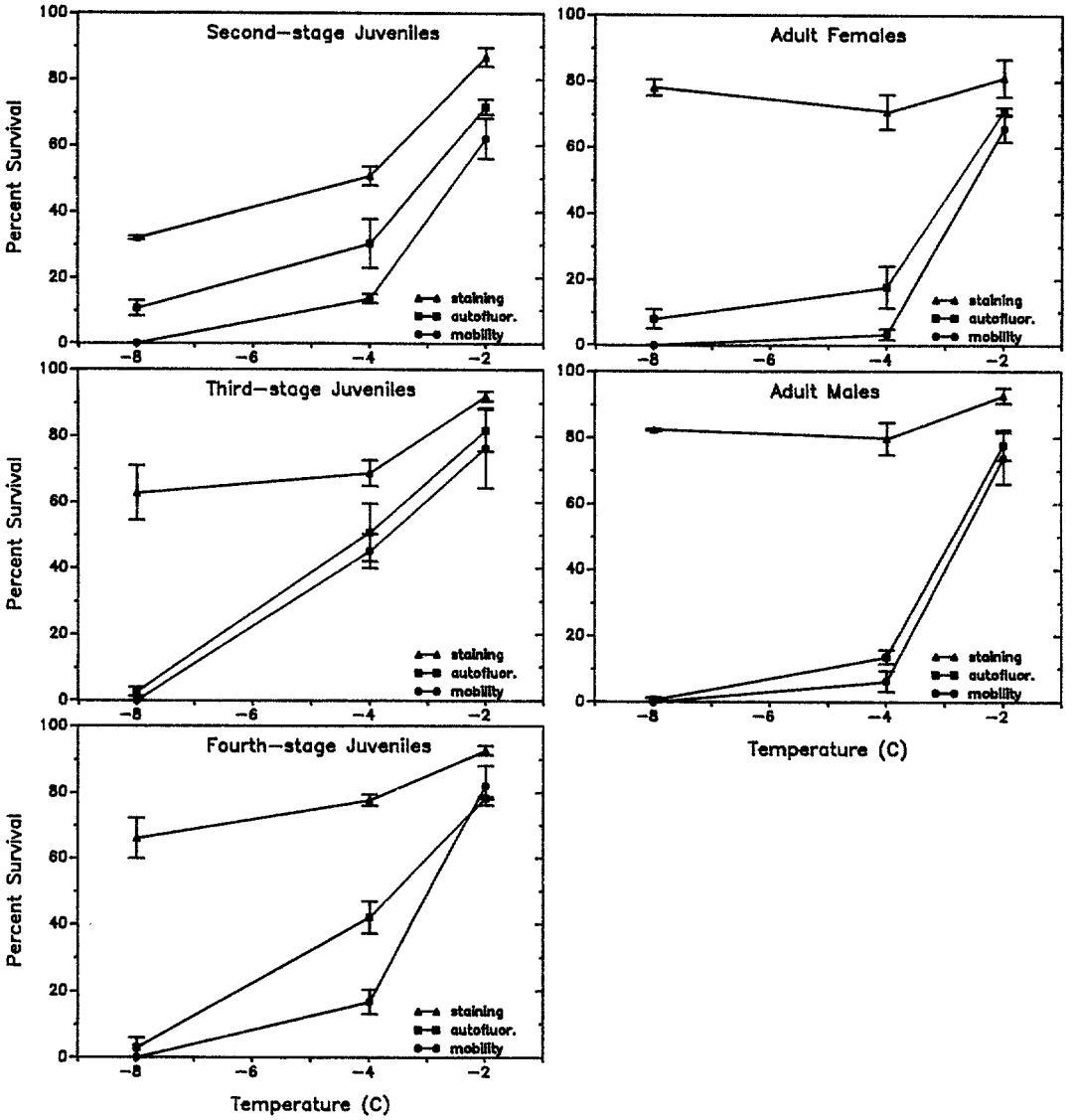

FIG. 2. Relationship between temperature of freezing and estimates of survival (± SE) based on staining, autofluorescence, and mobility for each stage of *Pratylenchus penetrans*.

autofluorescence is not surprising, since the accumulation of lipofuscin is known to occur in many cell types throughout the metazoa (3,5). Lipofuscin accumulation is related to age (3,5,10), which may explain why the juveniles do not fluoresce as intensely as older stages of the same species. The association between death and dispersal of autofluorescences suggests that death may result in the breakdown of lysosomal membranes, allowing the lipofuscin to disperse. Death may also result in the dispersal or activation of enzymes or other molecules that cause biochemical reactions leading to autofluorescence in areas besides the secondary lysosomes.

In addition to showing that a method of estimating survival works when the nematodes have been killed by at least several different events, it is also informative to determine how the method compares with other methods across several levels of survival. Estimates of survival based on autofluorescence dispersal paralleled estimates based on mobility more closely than those based on staining.

No mobile nematodes have been observed with dispersed autofluorescence. Since mobility is an absolute indicator of viability, autofluorescence does not err by categorizing live nematodes as dead. If and when mobility errs as a basis for estimating survival, it is because live nematodes are categorized as dead. It is likely that autofluorescence can distinguish between living nonmobile and dead nematodes. Nonmobile nematodes have been observed that do not exhibit dispersed autofluorescence. This may explain why estimates of survival based on autofluorescence were slightly higher than those based on mobility.

Nonetheless, under the conditions of this experiment, mobility should provide highly accurate estimates of survival. Although low temperatures result in reduced metabolic rates and mobility (i.e., quiescence), we have found that mobility of *P. penetrans* resumes within an hour of returning to room temperature (Forge, unpubl.). In other words, there is no evidence for a deep quiescence induced by short-term exposure to subzero temperatures. All other factors affecting the accuracy of estimates based on mobility should be reflected in the unfrozen controls, which always had values of 100% survival.

Eosin-y was chosen for this comparison because it was previously reported to be effective in staining free living nematodes killed by freezing (2). If the accuracy of autofluorescence or mobility is accepted, then it is clear that eosin-y is not effective in staining *P. penetrans* killed by freezing. The discrepancy between survival estimates based on staining and estimates based on autofluorescence or mobility was least for second-stage juveniles and greatest for the adult stages. This phenomenon may be due to cuticle differences between stages, because staining efficacy is dependent on cuticle permeability.

We have shown that a wide variety of nematodes are autofluorescent and that the autofluorescence is consistently dispersed in *P. penetrans* or *C. elegans* killed by formaldehyde, freezing, or heat. In addition, we have shown that estimates of survival based on autofluorescence closely parallel estimates based on mobility over a range of survival levels. Although mobility is an indisputable indicator of viability, it must often be induced by prodding nematodes and can be time consuming. Dispersed autofluorescence is a reliable indicator of nematode mortality which can be quickly and easily determined.

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