Fluorescent live-cell staining methods for tardigrades

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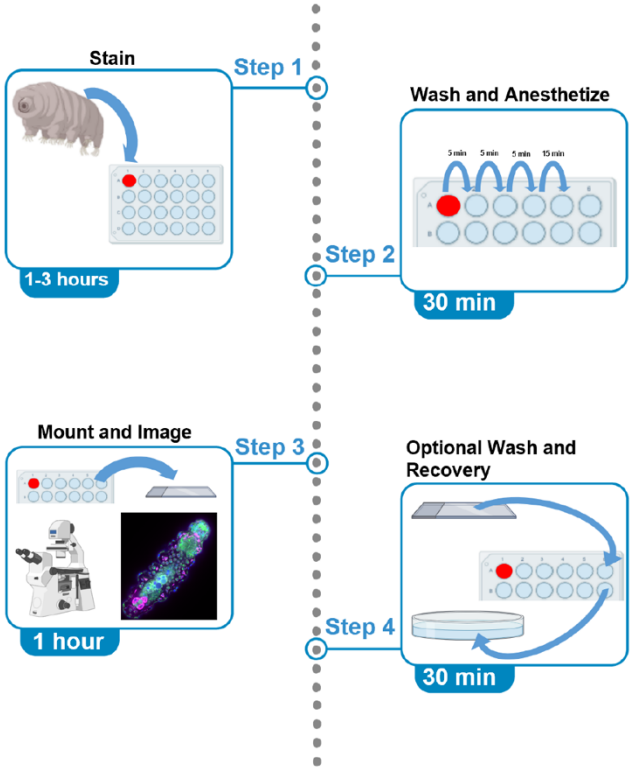
Summary

Tardigrades are microscopic organisms with exceptional resilience to environmental extremes, making them of interest in multiple fields, including biotechnology, astrobiology, and zoology. Most protocols to visualize internal anatomy of tardigrades rely on fixation, hampering our understanding of dynamic changes to organelles and other subcellular components. Here, we provide protocols for staining live tardigrade adults and other postembryonic stages, facilitating real-time visualization of structures including lipid droplets, mitochondria, lysosomes, and DNA. These procedures enable imaging and detailed analysis of live animals.

Graphical abstract



Before you begin

Tardigrades, also known as water bears, stand out as a valuable emerging model organisms in scientific research due to their unusual resilience[1]. Some tardigrades can survive extreme conditions that are lethal to most other animals, such as desiccation, high levels of radiation, pressure, and extreme temperatures[2–6]. This exceptional ability to withstand harsh environments makes them an ideal model for studying stress tolerance; indeed, this ability makes tardigrades suitable for some research that is not possible in traditional model organisms. Furthermore, tardigrades' relatively simple anatomy, coupled with their evolutionary position in the Ecdysozoa along with the well-studied model organisms *Caenorhabditis elegans* and *Drosophila melanogaster,* renders them an ideal model for comparative biology questions in general, for example for investigating evolutionary developmental questions[1]. There is value in studying many species of tardigrades, but presently, many tools and methods have been developed in the species *Hypsibius exemplaris*[7–10]*.* Although protocols have been outlined for differential interference contrast (DIC) microscopy of tardigrades, DIC imaging is not amenable to distinguishing many subcellular structures[11]. Other methods such as immunostaining and transmission electron microscopy allow for the visualization of the internal anatomy but cannot be carried out on live animals[12,13]. Studying organelle structures can provide crucial insights into the functions of cells as distinct arrangements and compositions of structures can correlate with specialized roles, such as protein synthesis, energy production, or organelle destruction, which can be used to infer underlying mechanisms driving cell physiology or function[14,15]. Fluorescence microscopy can be used to investigate internal cellular and anatomical structures without compromising the viability of the animal. Expression of transgenes in tardigrades provides a powerful method for visualizing fluorescent proteins in living tardigrades but relies on microinjection coupled with electroporation[16]. In contrast, many fluorescent stains are commercially available and offer the possibility of rapid visualization of subcellular compartments. Fluorescent staining methods for the embryos of tardigrades have been previously described, including methods for marking lysosomes, mitochondria, and nuclei, but to our knowledge no such protocol exists for postembryonic stages including adults[17]. We consider it important to share newly developed methods without delay, in order to advance the pace of ongoing research on an emerging model organism where new methods can be invaluable, and also to share methods that can be used in diverse research and teaching environments.

Here, we present protocols we developed for staining different subcellular components in live tardigrades, offering another set of tools for the study of this emerging model system. We tested each of the stains here at multiple concentrations and for different lengths of time, and we also monitored animal viability after staining; optimal methods that we identified are presented here. Many of these staining methods can be conducted simultaneously to visualize multiple subcellular components (Figure 1). As such, these protocols may be employed in isolation or in combination.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BODIPY™ 493/503	Invitrogen	D3922
Deer Park spring water	Staples	705032
Dimethyl sulfide (DMSO)	Macron	4948
Immersion oil (Type A)	Nikon	MXA20233

Lanolin	Fisher Scientific	AAA1690230
LysoTracker™ Green	Invitrogen	L7526
Levamisole	MilliporeSigma	L9756
MitoTracker™ Green	Invitrogen	M7514
MitoTracker™ Red CMXros	Invitrogen	M7512
NucBlue	Invitrogen	R37605
Paraffin	Fisher Scientific	22900700
PKmito Red	SpiroChrome	SC052
TMRE (Tetramethylrhodamine, Ethyl Ester, Perchlorate)	Invitrogen	T669
Vaseline	MilliporeSigma	16415
Experimental models: Organisms/strains		
Chorococcum hypnosporum, living	Carolina Biological Supply Co.	152091
Hypsibius exemplaris (strain Z151)	Carolina Biological Supply Co.	133960
Software		
Metamorph	Molecular Devices	7.10.5.476
Other		
1 ml syringe	BD (Becton Dickinson)	309659 BR78054
1.5 ml microcentrifuge tube	MilliporeSigma	BR78054
24 Well Cell Culture Plate	Corning	CLS2524
25.60 µM glass beads	Whitehouse Scientific	MS0026
35 mm vented Petri dishes	Fisher Scientific	FB0875711YZ
Aluminum foil	Fisher Scientific	15-078-291
Aspirator tube assembly	MilliporeSigma	A5177
Borosilicate glass capillaries	World Precision Instruments	1B100F-4
Confocal or epifluorescence microscope	various	
Cover Glass thickness 1 1//2 18 x18	Corning	2850-18
Crayola watercolor brush set size 8	Crayola	05-1127-008
Disposable Micro Pipets	Fisher Scientific	21-164-2E

Dry bath incubator	Fisher Scientific	11-718
Open air rocker	Fisher Scientific	88-861-025
PrecisionGlide™ Needle 25G x 5/8	BD (Becton Dickinson)	305122
Stereo microscope	Leica	MZ9.5
Vistavision™ microscope slides	VWR	16005-106
X-ACTO® precision knives	MilliporeSigma	Z407704

Note: Product information provided cites identifiers available in the United States. These may vary internationally.

Materials and Equipment

VALAP

To prepare VALAP to be used for sealing coverslips, combine equal parts vaseline, lanolin, and paraffin. Heat the mixture to melt components while stirring.

Reagent	Final concentration	Amount
Vaseline	33.3%	50 g
Lanolin	33.3%	50 g
Paraffin	33.3%	50 g
Total		150 g

[Once prepared, VALAP can be stored at room temperature (~20-22 °C). in a sealed container. Smaller aliquots (~1mL) can be scraped from this stock with a metal spatula and added to an Eppendorf tube to be melted on a heat block for staining experiments.]

Step by Step Methods Details

Specimen collection

In this section, we describe methods for culturing and isolating tardigrades.

Timing <1 hr

1. Grow *Hypsibius exemplaris* (Z151) cultures in 35 mm vented plastic petri dishes in commercial spring water. Tardigrades should be fed *Chlorococcum* algae, and animals should be moved to fresh dishes with new algae and water every two weeks[18].
2. To isolate individual tardigrades, an aspirator pipette is required (Figure 2). This aspirator pipette can be assembled according to the protocol outlined by McGreevy *et al.*[17].
    a. Pull a glass microcapillary tube using a needle puller or flame. Heat the center of the capillary tube while gently pulling apart with consistent force so that as the glass melts, the capillary will separate into two tapered needles.

b. Use a precision knife to apply pressure and break the tapered end of the needle to a width slightly larger than the length of the tardigrades you will transfer (approximately 200-250 µm diameter).
c. Place the wider end of the needle into the aspirator tube assembly.
d. Fold the tubing furthest away from the needle, and clamp using a binder clip.
Note: Aspiration can be controlled by squeezing the tubing between a thumb and a finger.

3. Use the aspirator needle to transfer animals to a 35 mm plastic petri dish filled approximately half full with spring water.
Note: Animals will be drawn into the aspirator needle by capillary action and can be expelled by gently applying pressure to the tube (Figure 2).
4. Use the aspirator needle to remove any excess algae from the petri dish as algal autofluorescence can interfere with imaging.

Specimen staining

In this section, we describe steps for staining tardigrades with fluorescent dyes.

Timing 1-3 hrs

5. Prepare 1 ml of staining solution in spring water. The table below presents the minimum recommended concentrations and time of staining:

Target	Stain	Excitation wavelength	Emission wavelength	Concentration	Recommended staining time
Lipids					
	Bodipy 493/503	493 nm	503 nm	0.5 µM	1 hour
Nucleus					
	NucBlue	360 nm	460 nm	4 drops to 1mL	3 hours
Mitochondria					
	MitoTracker Green	490 nm	516 nm	3 µM	3 hours
	TMRE	549 nm	574 nm	1 µM	1 hour
	PKmito Red	549 nm	569 nm	3 µM	3 hours
	MitoCMXros	579 nm	599 nm	1 µM	3 hours
Lysosomes					
	LysoTracker Green	504 nm	511 nm	3 µM	3 hours

Table 1. Recommended staining times and concentrations. We suggest preparing fresh working solutions from concentrated stocks immediately before each staining experiment.

6. Use an aspirator needle to transfer animals into 1 ml of the staining solution.
Note: We recommend using a 24-well plate for convenient staining and washing. Alternatively, a 35 mm petri dish or similar may also be used for this portion of the protocol. To minimize photobleaching, the 24-

well plate or petri dish containing tardigrades should be covered with aluminum foil throughout staining and washing steps.

7. Place the plate or dish containing tardigrades in stain solution on a rocker for the recommended staining time (Table 1).
   Note: Rocking the animals at a 7-degree tilt at a speed of 21 tilts per minute is sufficient for mixing the stains and water. Staining and washing steps should be done at room temperature (~20-22 °C).

Specimen washing and anesthetization.

In this section, we describe steps for washing excess stain from the tardigrades and immobilizing them for imaging.

Timing 30 min

Washing and anesthetization are performed simultaneously, with 3 washes for 5 minutes each followed by a 15-minute wash to ensure animals are anesthetized.

8. To anesthetize tardigrades for imaging, prepare 5 mL of anesthetic solution (20 mM levamisole in spring water) for each staining condition. Aliquot 1 mL of solution to 4 consecutive wells in a row of the 24 well plate.
9. Wash the animals in the first well for 5 minutes. Tardigrades should be transferred with an aspirator needle into the next well of the 24 well plate containing 1 mL of anesthetic solution. Repeat this step two times.
10. Transfer the tardigrades to 1 ml of anesthetic solution for 15 minutes to ensure anesthetization.

Mounting and imaging

In this section, we describe steps for preparing slides and imaging stained tardigrades.

Timing 1 hr

VALAP sealant should be prepared in advance by mixing and heating equal parts Vaseline, lanolin, and paraffin according to the protocol outlined in Cold Spring Harbor Protocols[19].

11. Melt VALAP sealant in advance by filling a test tube with solid VALAP and placing it in a heating block at 68˚C. We recommend using a metal spatula to transfer solid VALAP to the test tube.
12. Pipette a small droplet (~10 µl) of anesthetic solution onto the center of an uncoated glass slide.
13. Use an aspirator needle to transfer the stained tardigrades into the droplet of anesthetic solution.
14. To prevent the coverslip from crushing the tardigrades, glass beads should be applied to the droplet. To do so, dip a pipette tip into a tube of 25.60 µm glass beads. While keeping the tip in the tube, lightly tap the blunt end to shake excess beads off. Dip the pipet tip into the edges of the droplet on the slide. Use a syringe needle to center the animals in the droplet of anesthetic solution.
15. Gently place a glass coverslip over the droplet containing the tardigrades and beads.
16. Dip a small paintbrush into melted VALAP and dab each corner of the coverslip to adhere it to the slide. Then, seal each side of the coverslip with a single stroke of the VALAP-coated paintbrush.
    Note: It is important to apply melted VALAP efficiently, as it will solidify quickly at room temperature (~20-22 °C).. Identify any holes in the VALAP seal under a dissection microscope and fill them with melted VALAP to limit evaporative loss of the solution on the slide.
17. Image the animals mounted on slides using a suitable fluorescence microscope.

Note: This protocol was optimized using a variety of microscopy settings and microscopes – both epifluorescence and confocal. However, all images in this manuscript were captured with a Hamamatsu Orca-

Quest2 (C15550-22CU) qCMOS camera mounted on a Nikon Eclipse inverted spinning disk confocal microscope with a Yokogawa CSU-X1 spinning disk scan head. All images were collected at 20-22 °C using a Plan Apo 60x 1.4 NA oil immersion lens and illuminating with 404 nm, 488 nm, and 561 nm coherent lasers.

Note: When imaging, we recommend first locating the animals at lower magnification (such as 10x) before progressing to higher magnification objectives.
Note: To minimize photobleaching, we suggest beginning imaging with low illumination power and exposure time and increasing as needed to optimize conditions for visualizing fluorescence.

Rescuing tardigrades (optional)

In this section, we describe steps to recover tardigrades from glass slides following imaging.

Timing 30 min

18. Under a dissection microscope, use a precision knife to cut through the VALAP seal around the cover slip. Use the precision knife to carefully lift and remove the coverslip, leaving a VALAP square on the slide.
19. Pipette 200 µl of spring water into the VALAP square.
20. Locate the specimens and use an aspirator needle to transfer animals to 1 ml of spring water. The tardigrades can be returned to culture dishes as they regain mobility (typically in ~15-30 min).
21. For these experiments, tardigrades should be exposed to levamisole solution for no longer than 2 hours before being rescued.
    Critical: Viability of the animal should be assessed based on the animal's motility and ability to reproduce after being returned to spring water.

Expected outcomes

Implementation of this protocol should result in high quality images of cellular components including mitochondria, lipid droplets, nuclei, and lysosomes. We note that each of these stains exhibits some variability in efficacy across tissues. Observations and expected outcomes for each of these stains are offered below:

BODIPY 493/503

This stain is used as an indicator for neutral lipids. Fluorescence could be seen primarily in the epithelial cells, storage cells and oocytes (Figure 3). In some samples, diffuse fluorescence was observed in the ganglia, brain, claw glands, and Malpighian tubules but not in a pattern typical of lipid droplets. We interpret this as non-specific staining.

NucBlue

NucBlue is a cell permeable DNA stain that marked the nuclei of epithelial cells, brain, salivary glands, ganglia, claw glands, Malpighian tubules, and storage cells (Figure 4).

Mitochondrial stains

All the mitochondrial stains we tested produced strong signal in the epithelial cells and Malpighian tubules. Signal was also observed in muscles as well as storage cells (Figure 5 and Figure 6).

Lysotracker green

Lysotracker staining reproducibly labeled lysosomes in epithelial cells, salivary glands, Malpighian tubules, the ovary, and storage cells (Figure 7). Diffuse fluorescence was often observed in several tissues including muscles,

ganglia, and claw glands but not in a punctate pattern typical of stained lysosomes. We interpret this as non-specific staining (Figure 4).

Other tardigrade species

We developed and optimized this protocol for staining *H. exemplaris*; however, this approach can likely be applied to other species. For example, we observed similar outcomes when visualizing cellular structures in *Ramazzottius varieornatus* (strain YOKOZUNA-1) (Figure 8). While the staining times and concentrations we suggest may work for other species, we suggest some optimization testing when applying these methods to other tardigrades.

Limitations

While these protocols offer reliable methods for visualizing subcellular components in live tardigrades, there are several limitations to consider when interpreting staining results. First, it is important to note that there is diminishing image quality as distance from the microscope's objective lens increases. This could possibly be due to signals from deeper planes dispersing as light passes through the organism, or from an inability of dyes to permeate deeper into the tardigrade. Given this limitation, before applying the coverslip it is important to position the tardigrade, using a precision needle, in an orientation that will position the structures of interest closest to the lens. Another point of caution is that some structures in the tardigrades exhibit autofluorescence under multiple wavelengths of light[20]. In particular, structures such as the stylets and claws, as well as algae and birefringent granules in the midgut are significant sources of autofluorescence (Figure 9). We urge comparing unstained animals with stained ones before interpreting fluorescent images of tardigrades to ensure that patterns of autofluorescence are not confused with signal from live stains.

Troubleshooting

Problem 1:

The fluorescence signal from stains is weak or non-visible (steps 6-7, 17).

Potential Solution:

Photobleaching is a common challenge in fluorescence microscopy. When establishing imaging conditions, it is important to limit the time and intensity of exposure to laser light. Imaging parameters such as illumination power and exposure time should be optimized to ensure that the fluorescence signal is captured with minimal photobleaching. To improve the quality and longevity of imaging experiments, begin imaging with lower illumination power and shorter exposure times while finding a sufficient signal-to-noise ratio. Minimize the time that samples are exposed to excitation light by acquiring images quickly or implementing techniques such as timelapse imaging with intermittent illumination.

Double check that the stain has not expired or been exposed to light for a prolonged period of time, as this may reduce the fluorescence signal. Re-making working stocks of the stain from fresh freezer aliquots may solve this problem. It is also important while staining and washing the tardigrades to keep them in the dark to limit the loss of fluorescence. If these adjustments are not sufficient to improve fluorescence signal, tardigrades can be kept in the stains for longer periods of time than indicated in Table 1, or the concentrations of dyes can be increased. In

this case, it is important to check the viability of tardigrades at higher concentrations or following prolonged exposure to dyes.

If autofluorescence is limiting the ability to visualize signal from the stains, tardigrades can be kept in starvation conditions, i.e. without algae, before imaging. This can reduce autofluorescence in the midgut and from any algae that may adhere to the cuticle. Note that starvation also impacts the physiology of the tardigrades and may cause changes in the structure or composition of the components being visualized.

Problem 2:

Tardigrades are contracted within their cuticles, impeding fluorescent imaging and potentially compromising long term viability of the animal (Figure 10, step 17).

Potential Solution:

Separation from the cuticle may be indicative of osmotic stress, potentially due to excessively high concentrations of levamisole or other osmolytes in the anesthetizing solution. The concentration of levamisole in the anesthetizing solution can be reduced if this is a persistent problem. In addition, carbonated water can be used as an alternate method to anesthetize tardigrades. Tardigrades can simply be transferred to carbonated water (seltzer water) for ~5 minutes[21]. Note that tardigrades anesthetized in carbonated water typically remain paralyzed for shorter periods of time compared to those anesthetized in levamisole.

Problem 3:

Tardigrades are not completely anesthetized, and the animal's movement is impeding clear imaging (Steps 8-10).

Potential Solution:

Tardigrades may not be fully anesthetized if the concentration of levamisole in the anesthetizing solution is too low or if time of exposure to levamisole is too short. Increase the concentration of levamisole in the anesthetizing solution or exposure time. In addition, carbonated water can be used as a substitution to anesthetize tardigrades. However, the time of animal paralysis in carbonated water should be expected to be less than in the levamisole solution.

Problem 4:

Tardigrades on slides for imaging are ruptured and dead (Figure 11, steps 14-15).

Potential Solution:

It is essential to apply glass beads as spacers when mounting tardigrades on slides for imaging. If the glass beads are not added, or if the size of the glass beads is too small, tardigrades will be crushed during mounting. This problem can also occur if the coverslip is applied with too much pressure. Double check that the glass beads were added and are of the recommended size. Depending on the age and size of the animals, smaller bead sizes can be used to compress animals between the slide and coverslip.

Problem 5:

The staining is lethal to the tardigrades (step 21).

Potential Solution:

Tardigrades from old cultures (with high population density and yellow or brown algae) are generally more prone to lethality. Maintaining healthy cultures of tardigrades is essential to the staining protocol. It is recommended that cultures are split into fresh water and algae every two weeks.

Resource availability

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bob Goldstein (bobg@unc.edu).

Technical contact: Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Clayton Harry (cjharry@unc.edu).

Materials availability: This study did not generate new unique materials or reagents.

Data and code availability: This study did not generate new datasets or code.

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Author contributions

Conceptualization: CJH, JDH, and BG. Funding acquisition: BG. Investigation: CJH, JDH, AD, PD, ME, MF, JL, LL, JL, SBT, BV, and BG. Supervision: JDH and BG. Writing – original draft: CJH. Writing – review and editing: CJH, JDH, and BG. All authors have read and approve of this protocol.

Declaration of interests

The authors declare no competing interests.

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Figures

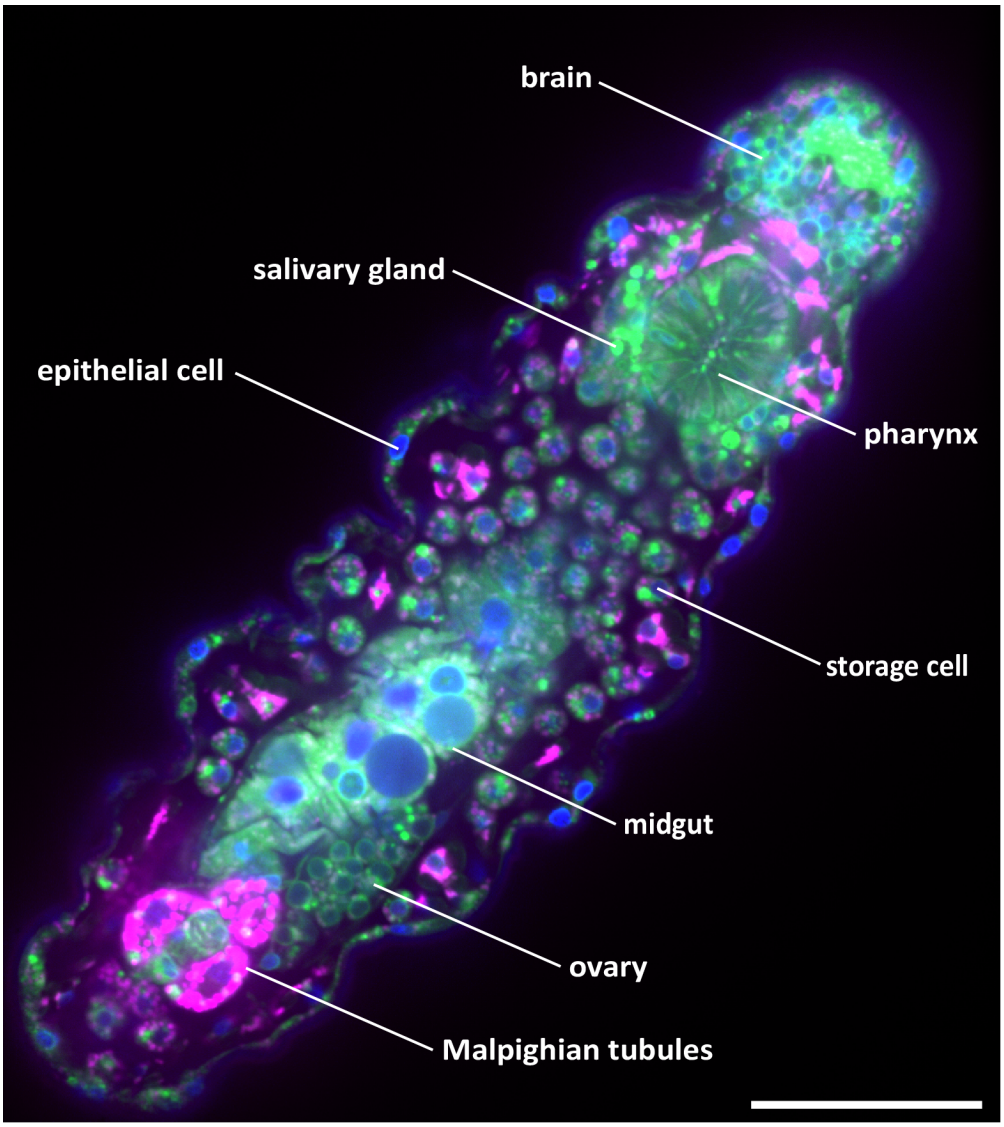

Figure 1. Multiple stains can be used simultaneously. This tardigrade (*H. exemplaris*) has been stained to label lysosomes, mitochondria, and nuclei using LysoTracker Green (green), TMRE (magenta), and NucBlue (blue) respectively. Some anatomical structures are labeled. Scale bar: 30 µm.

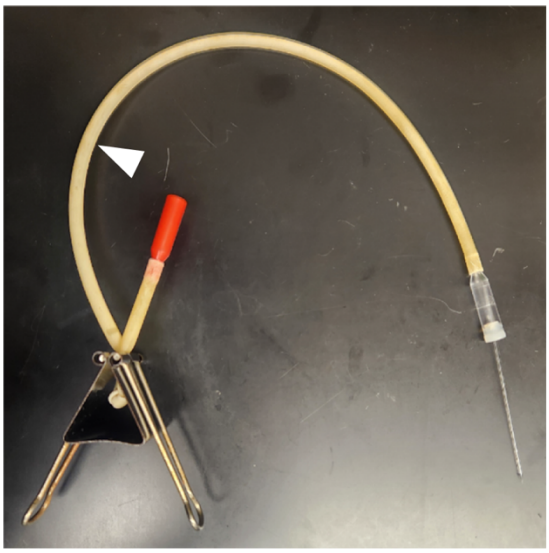

Figure 2. This aspirator pipette can be assembled according to the protocol outlined by McGreevy et al[17]. The white arrowhead indicates the approximate region to squeeze to expel tardigrades.

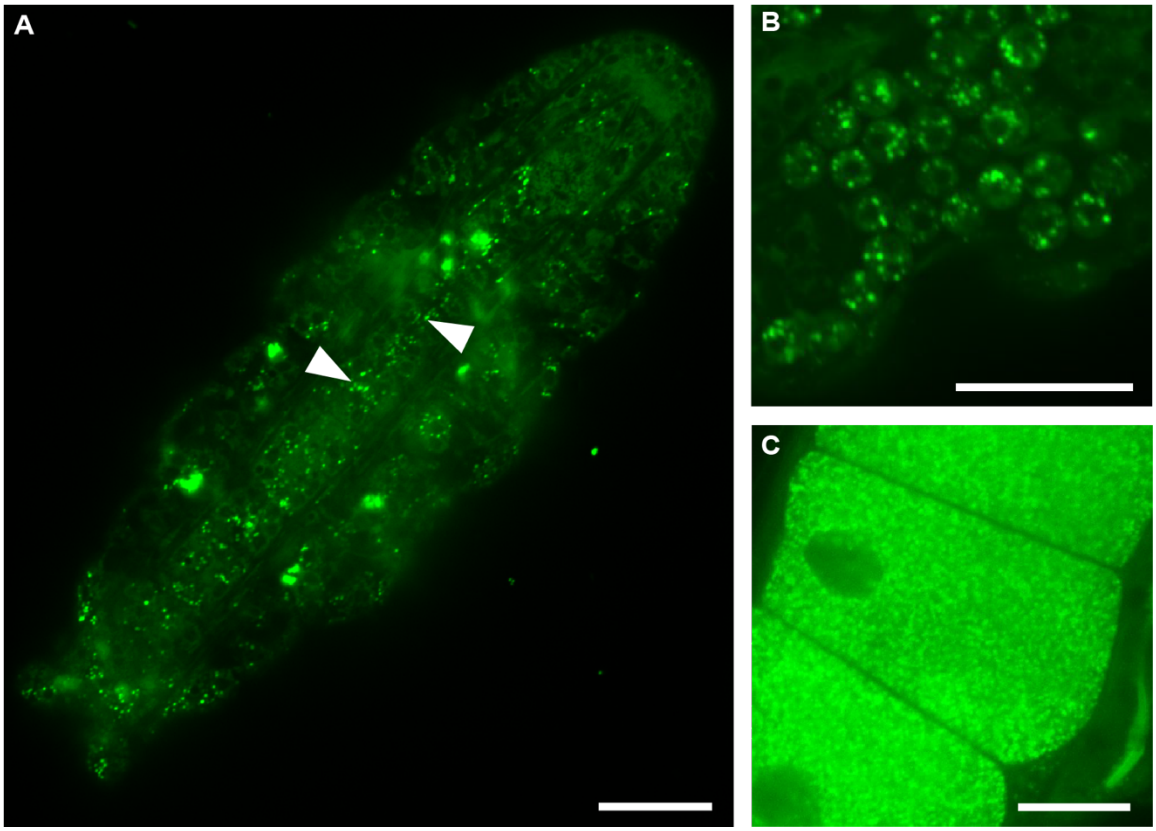

Figure 3. Bodipy effectively stained epithelial cells (A), storage cells (B), and oocytes (C) of *H. exemplaris*. Arrowheads indicate stained lipid droplets. Scale bars: 20 µm.

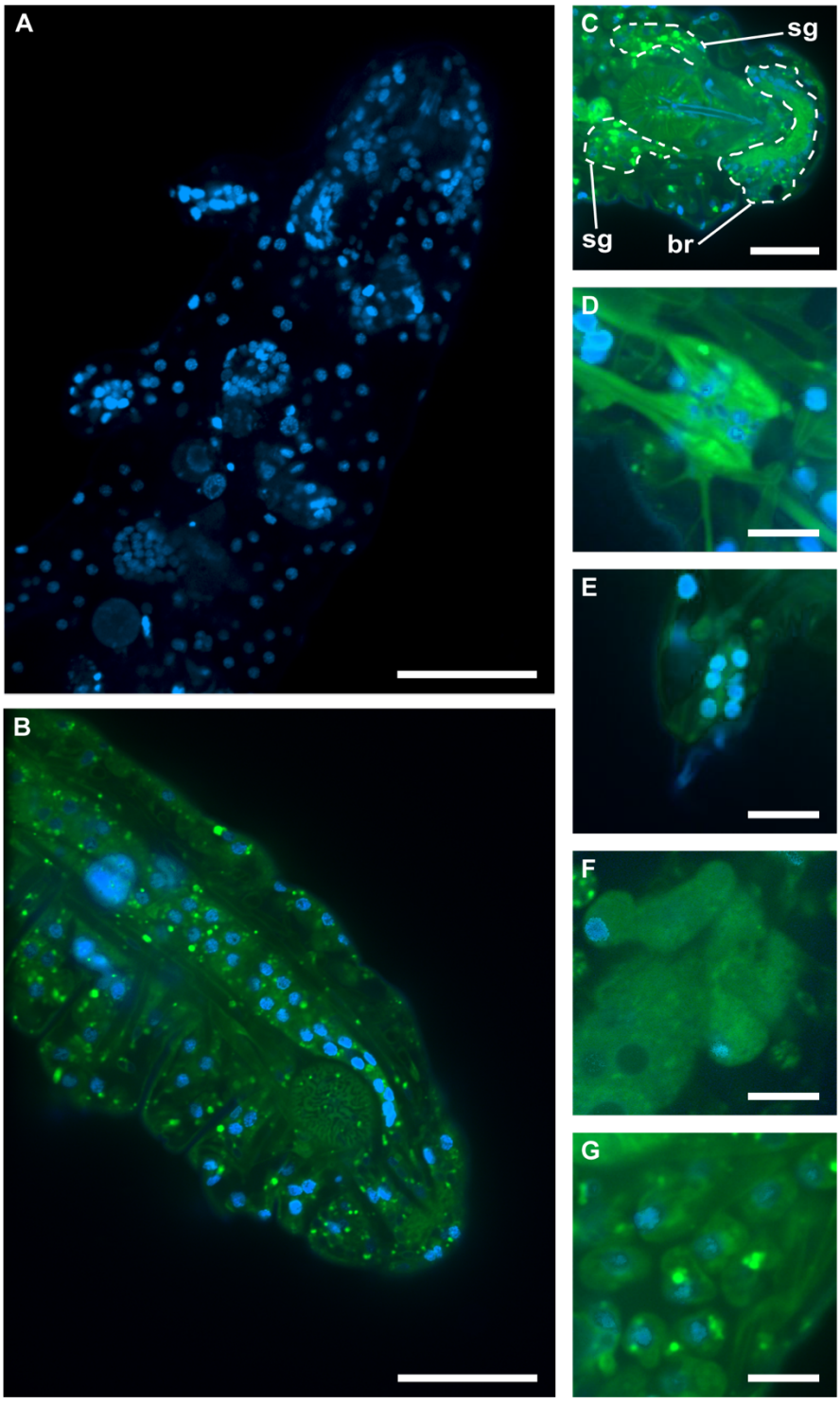

Figure 4. Representative images show NucBlue staining (blue) in an adult tardigrade (*H. exemplaris*). Staining could be seen throughout many tissues in the animal (A), including the epithelial cells (B), salivary glands (C), brain (C), ganglia (D), claw glands (E), Malpighian tubules (F), and storage cells (G). LysoTracker green is shown as a co-stain to highlight particular anatomical features. Note that some of the staining of lysotracker green, such as in the ganglia (D) is interpreted as non-specific, but nonetheless useful for depicting anatomy. Scale bars: A,B, 30 µm; C, 20 µm; D-G, 10 µm. Labels indicate salivary glands (sg) and brain (br).

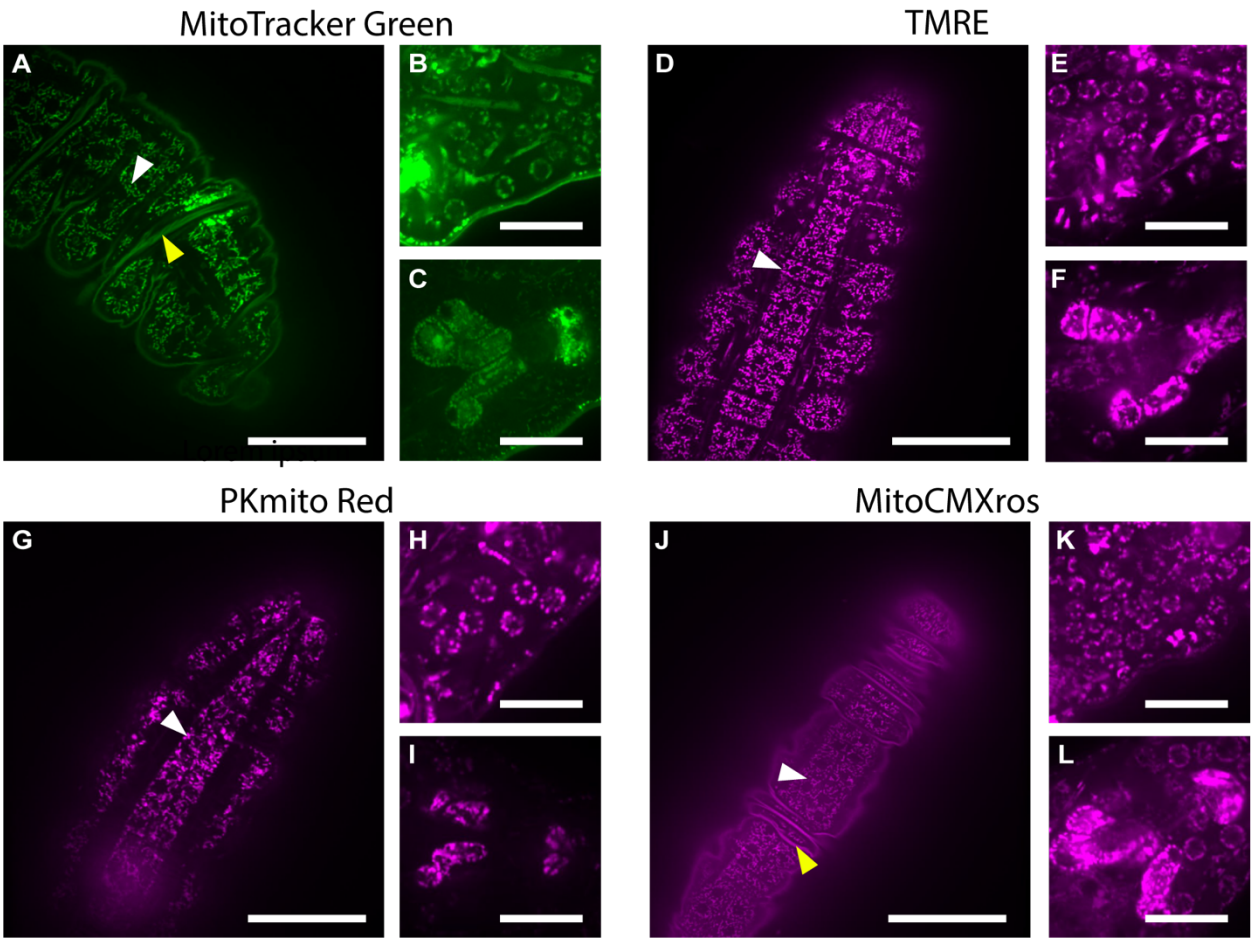

Figure 5. MitoTracker Green (A-C), TMRE (D-F), PKmito Red (G-I), and MitoCMXros (J-L) effectively stained epithelial cells (A, D, G, J), storage cells (B, E, H, K), and Malpighian tubules (C, F, I, L). It should be noted that MitoTracker Green and MitoCMXros staining also produced fluorescence in the cuticle (A, J). White arrowheads indicate signal in epithelial cells. Yellow arrowheads indicate fluorescence in the cuticle. Scale bars: A, D, G, J, 40 µm; B, C, E, F, H, I, K, L, 20 µm.

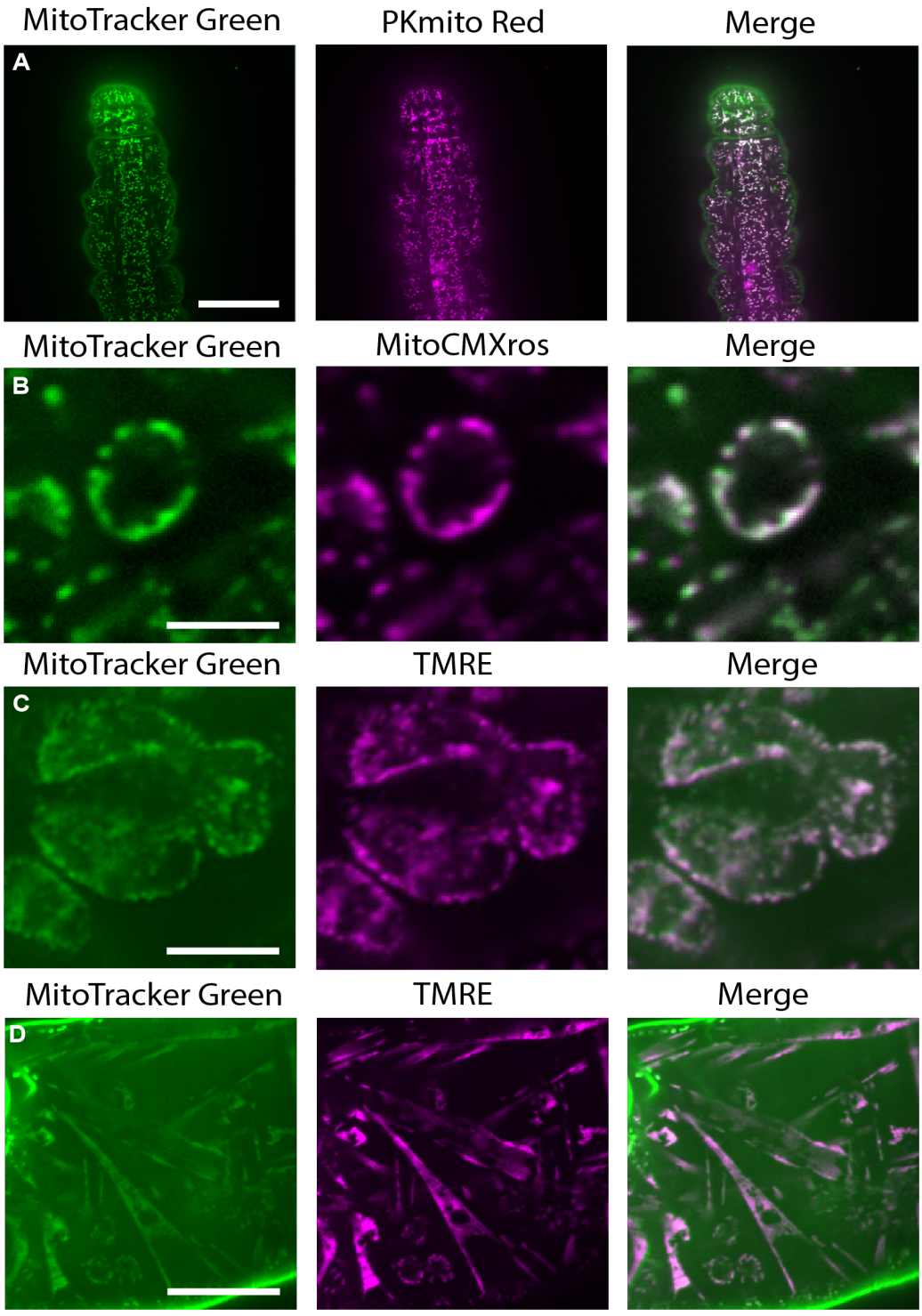

Figure 6. MitoTracker Green was observed to colocalize with each of the three red mitochondrial stains. Representative images depict colocalization in the epithelial cells (A), storage cells (B), Malpighian tubules (C) and muscles (D). Scale bars: A, 30 µm B, 5 µm; C, 10 µm; D, 20 µm.

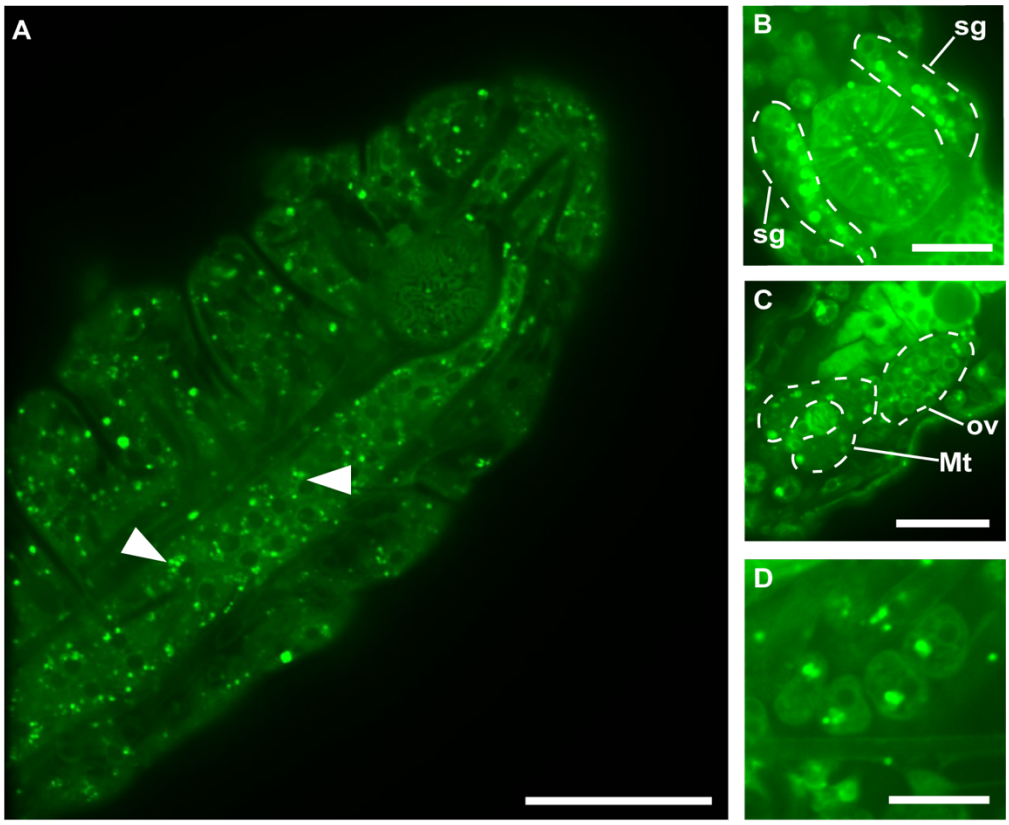

Figure 7. LysoTracker green effectively stained multiple cell types including epithelial cells (indicated by arrowheads) (A), salivary glands (sg) (B), Malpighian tubules (Mt) (C), ovary (ov) (C), and storage cells (D). Scale bars: A, 30 µm; B-D, 15 µm.

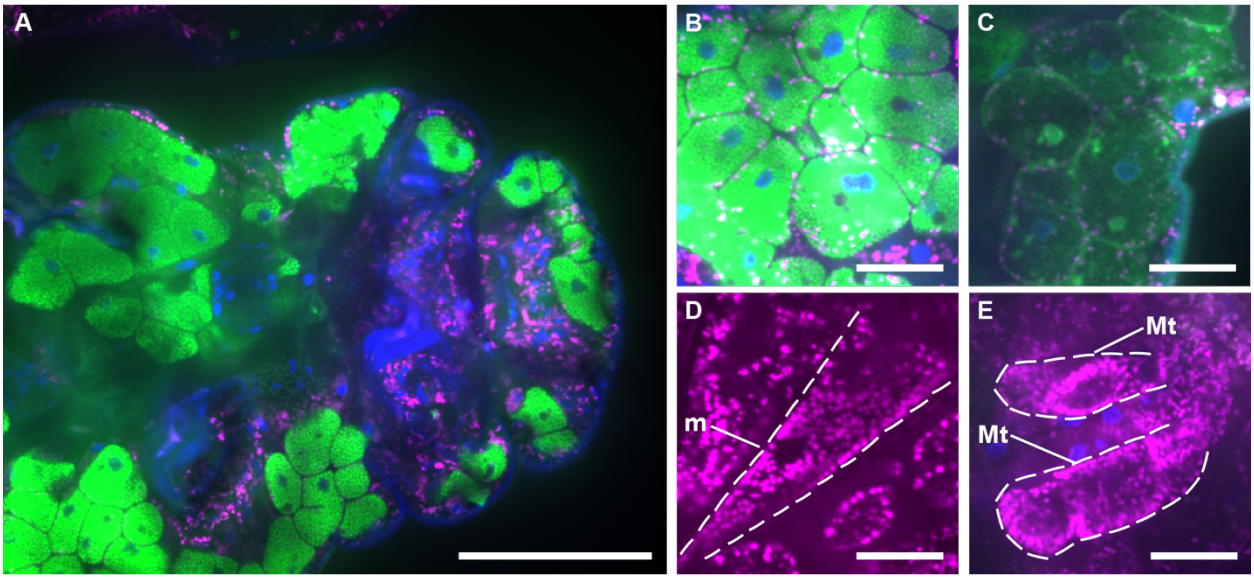

Figure 8. This protocol was also effective in staining *Ramazzottius varieornatus* (YOKOZUNA-1). NucBlue stained nuclei of multiple tissues (blue in A,B,C,E); BODIPY stained lipid droplets, which were particularly prominent in storage cells (green in A,B); mitochondrial stains were seen in storage cells (magenta TMRE in A,B; PkMito in C,D); and lysotracker green could be seen in storage cells (green in C). Mitochondria could also be visualized in other cells and tissues including muscles (m) (magenta CMXRos in D) and Malpighian tubules (Mt) (magenta CMXRos in E). Scale bars: A, 30 µm; B-E, 10 µm.

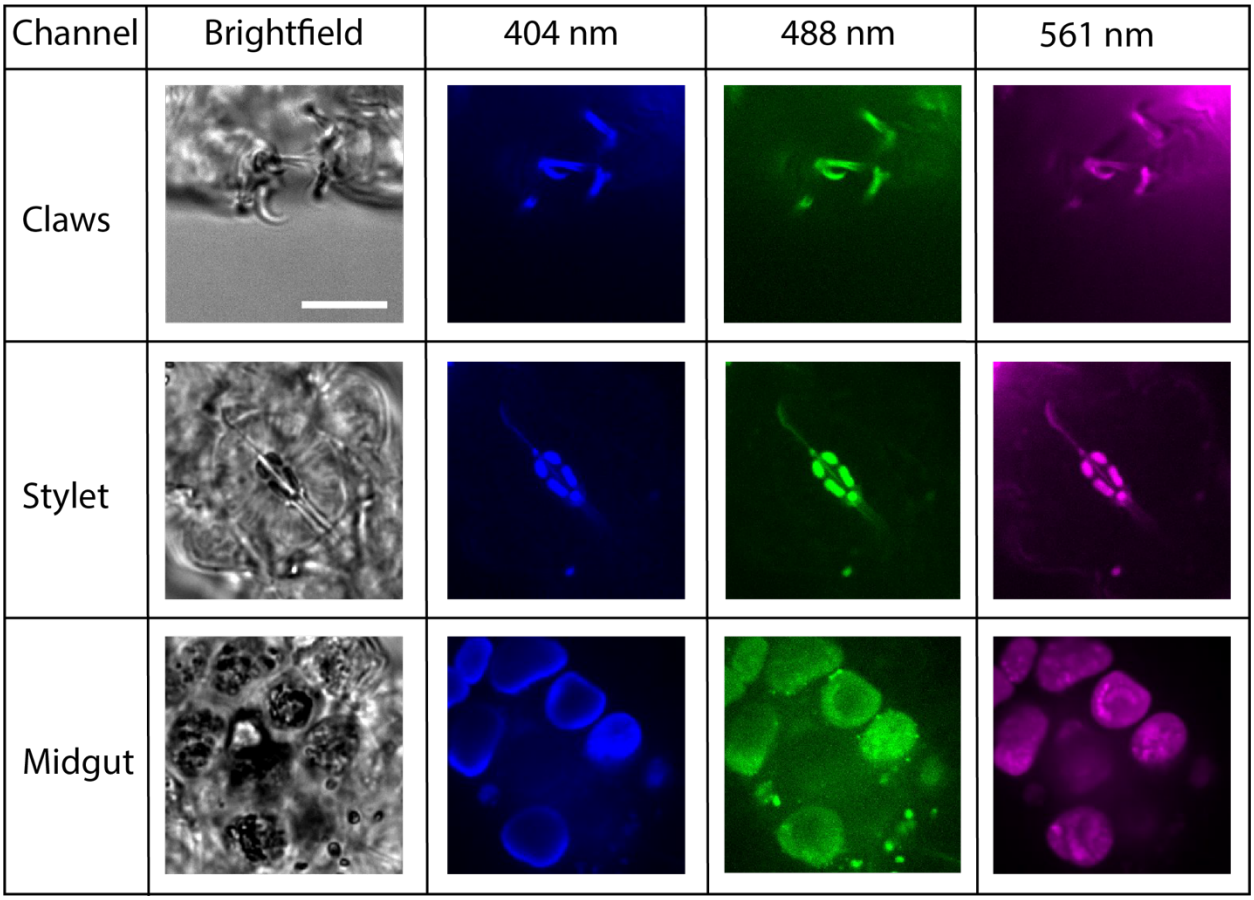

Figure 9. Autofluorescence is present in the claws, stylet, and midgut of unstained tardigrades (*H. exemplaris*) for each of the illumination wavelengths used for the stains used in the protocol. The heading of each column indicates the wavelength of the laser used for excitation. Scale bar: 10 µm.

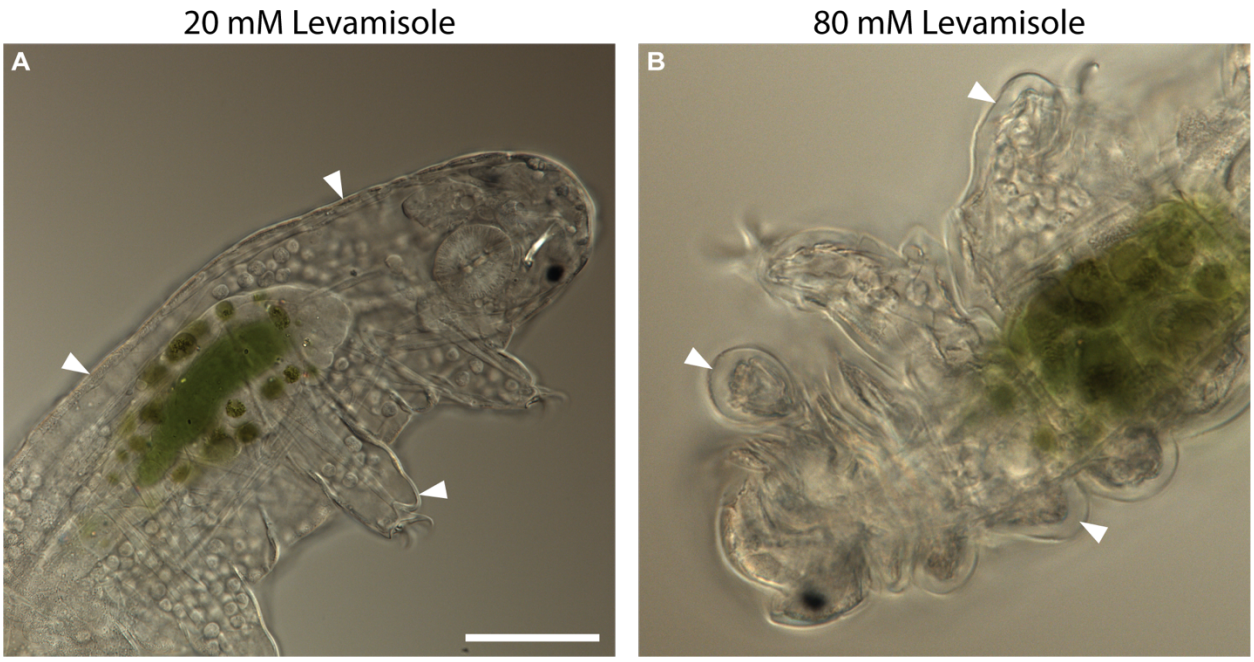

Figure 10. Tardigrades (*H. exemplaris*) anesthetized in 20 mM levamisole retain normal morphology and should not retract from the cuticle (A). Excessive concentrations or extended times of exposure in levamisole can lead to contraction of the animal within its cuticle (B). Arrowheads indicate the cuticle of the tardigrades. Scale bar: 30 µm.

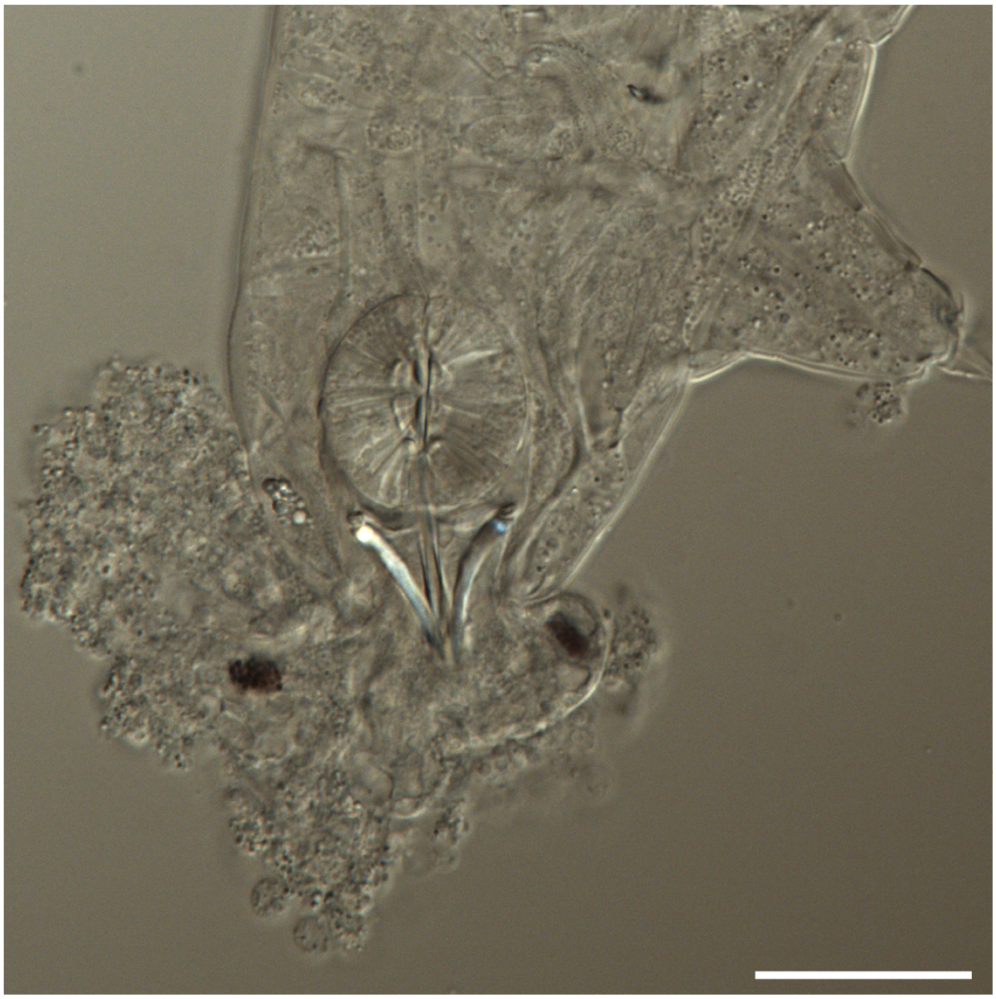

Figure 11. While mounting, using excessively small glass beads, forgetting to use beads, or applying the cover slip with too much pressure can cause tardigrades to rupture. This tardigrade (*H. exemplaris*) was mounted using 5 µm beads. Scale bar: 30 µm.