

METHODS MANUSCRIPT

Live imaging of intracellular pH in planarians using the ratiometric fluorescent dye SNARF-5F-AM

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Abstract

Physiological parameters such as resting potential and pH are increasingly recognized as important regulators of cell activity and tissue-level events in regeneration, development, and cancer. The availability of fluorescent reporter dyes has greatly increased the ability to track these properties *in vivo*. The planarian flatworm is an important and highly tractable model system for regeneration, stem cell biology, and neuroscience; however, no protocols have been published for investigating pH in this system. Here, we report a simple and effective protocol for imaging pH gradients in living planaria suitable for intact and regenerating flatworms.

Keywords: physiology; pH; fluorescence; planaria; imaging

Introduction

Planarian flatworms are an increasingly popular model system for regenerative and stem cell research, as much for their ease of use and inexpensive upkeep as for their ability to regenerate a complete worm from the smallest of fragments [1–4]. Recent technical advances, such as a sequenced genome, *in situ* hybridization methods, and the identification of tissue-specific markers, have greatly increased the accessibility of this model organism [5]. However, the development of tools for investigating real-time physiology *in vivo* has not progressed at the same pace, despite growing evidence that ionic regulation plays an important instructive role during regeneration [6–15]. This protocol describes how to use the fluorescent pH indicator seminaphthorhodafluor (SNARF)-5F 5-(and-6)-carboxylic acid, acetoxymethyl ester,

acetate (SNARF-5F-AM) to measure *in vivo* intracellular pH (pH_i) in live planarians. SNARF-5F (and its cell-permeant acetoxymethyl ester) is a ratiometric dye with dual emission properties. Excitation of the dye at 543 nm produces emissions at both pH sensitive (640 nm) and pH insensitive (580 nm, isosbestic point) wavelengths, enabling researchers to correct for uneven dye uptake and photobleaching for a more accurate measure of pH_i.

Related information

It is assumed the reader is familiar with both the planarian model system and fluorescence microscopy. For more information, see protocols for “Establishing and Maintaining a Colony of Planarians” [16], “Gene Knockdown in Planarians Using RNA Interference” [17],

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and “General Principles for Measuring Resting Membrane Potential and Ion Concentration Using Fluorescent Bioelectricity Reporters” [18]. This protocol was used in “A Chemical Genetics Approach Reveals H₂K-ATPase-Mediated Membrane Voltage Is Required for Planarian Head Regeneration” [8].

Materials and methods

Materials

Cautions: Please see below for cautions on reagents marked with <!.>.

Reagents

Planaria, ≤6 mm in length, immobilized by either PC2-RNA interference (by dsRNA) (RNAi) [16]
or low percent ethanol (EtOH) [19]
See Steps 1–3

Planarian water, species appropriate [16]

SNARF-5F-AM (Molecular Probes S-23923, special packaging of 50 µg aliquots)

Keep at –20°C in the dark.

<!.> Pluronic F-127, 20% in Dimethyl Sulfoxide (DMSO) (Invitrogen P-3000MP)

<!.> Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) protonophore (Sigma C2759)

Keep as 10 mM stock in DMSO at 4°C in dark.

<!.> 2-(Cyclohexylamino)ethanesulfonic acid (CHES) buffer (Sigma C2885)

<!.> Sodium acetate (NaOAc) buffer (Sigma S2889)

Cautions

CCCP

<Danger> Toxic by inhalation, ingestion, or skin absorption. Wear protective gloves, safety glasses, and protective clothing.

CHES

Causes serious eye irritation. May be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Pluronic F-127

Causes serious eye irritation. When in solution in DMSO, components of the product may be absorbed into the body through the skin. May be harmful if swallowed. Wear appropriate gloves and safety glasses.

NaOAc

May be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Equipment

24-Well non-treated (suspension) tissue culture plates

Transfer pipettes

FluoroDishes, 35 mm diameter (World Precision Instruments FD35COL)

Round coverslips, 25 mm diameter

Glass microscope slide

Microscope with fluorescence optics and the following filter set or its equivalent:

Chroma filter set 71005b

[SNARF 540/25; 565 and 610; 580/25 (EM Filter1) and 640/25 (Em Filter 2)]

Image analysis software with background correction, segmentation, and pixel quantification Functions

Method

Preparation

1. Immobilize worms. Choose Procedure A (Step 2) or Procedure B (Step 3) and continue with Step 4.

2. Procedure A: Immobilize planaria by injecting with PC2-RNAi, selecting animals exhibiting a strong immobilization phenotype [16]].

Inject and wait 14 days for phenotype to come to full effect before selecting animals. Then treat and/or cut worms as your experimental assay dictates, prior to staining with SNARF.

3. Procedure B: Immobilize planaria with a low percent EtOH [19].

Treat and/or cut worms as your experimental assay dictates. Two hours before SNARF imaging, place worms into 3–5% EtOH (in worm water) for 1 h (3% for *Schmidtea mediterranea* and 5% for *Dugesia japonica*). Wash 3× with regular worm water before staining with SNARF.

Staining

4. Add 17 µl of 20% Pluronic F-127 solution to one 50 µg aliquot of SNARF-5F-AM.

This equals 5 mM stock. “Keep in dark.” Do not reuse this stock. Alternatively can make your own 20% w/v solution from powder (Sigma P2443). Heat at ~40°C for half an hour with vortexing to get into solution in DMSO.

5. Place worms, grouped by condition (e.g. control vs. treated) into a 24-well plate.

6. Load dye by soaking worms in 5 µM SNARF-5F-AM, made by diluting 5 mM Stock 1: 1000 in worm water. “Keep in dark.”

Remove worm water from wells with a transfer pipette and replace with enough 1000-fold diluted SNARF to cover worms completely in each well.

7. Incubate, covered, for 1 h at room temperature.

8. Wash out SNARF 3× with plain worm water, keeping covered as much as possible.

9. Using transfer pipette, place worms into 35 mm FluoroDishes in a pool of plain worm water, grouped by condition (one dish per condition).

Typically, do not put more than 3–5 worm fragments or 1–2 whole worms per dish. “Keep in dark.”

10. Place a round coverslip slowly over the well, trying to avoid air bubbles. “Keep in dark”.

Remove excess liquid with transfer pipette so that water is contained within the well under the coverslip. Make sure worms are not near edges of coverslip, and that the well is completely filled but not overflowing from the coverslip.

Imaging

11. Place FluoroDish on top of a glass slide (so stage controls can be used during imaging).

12. Select the condition expected to have the highest pH/brightest emission (most alkaline) first to set exposure times.

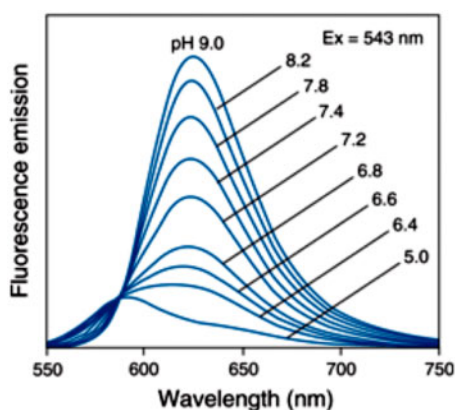


Figure 1: Graph of SNARF-5F fluorescence as value of pH_i . Reprinted from Liu et al., 2001 [20].

13. Use bright-field light to find and focus on a worm.

14. Exciting at 543 nm and capturing images at both 580 nm and 640 nm, set exposure times for each wavelength individually.

580 nm is the isosbestic point (or pH insensitive) wavelength, whereas 640 nm is the pH sensitive wavelength. Keep these exposure times constant throughout for all worms and between all conditions (Figure 1).

15. Take picture sets (one picture at each wavelength, 580 nm and 640 nm) for each animal.

16. "Worms must be completely still between 580 nm and 640 nm image captures," or the picture set must be discarded and not used (see Step 20).

After taking each picture set, look by eye at both images. If you have any doubts about whether the animal has moved or not, always take another picture set. Getting an in-register picture set may be facilitated by using time lapse to make many picture sets from which to choose.

17. Repeat for all animals and conditions.

When done imaging, carefully remove coverslip (recovering any worms from it) and move worms into fresh worm water (if needed). FluoroDishes (although they come sterile) do not have to be sterile and can be reused. After use, wash carefully with a kimwipe and plain water and allow to air dry on bench top.

Analyses

18. Use image software to open one picture set.

19. Use the function in your imaging software that divides the signal from one image by the signal from another to obtain a ratio image (640/580).

Place the 640 nm (pH sensitive) image in the numerator and the 580 nm (pH insensitive) image in the denominator. This ratio image represents the fluorescent signal intensity resulting from pH_i levels only, rather than from uneven dye loading or photobleaching. Note: your software may offer the choice of scaling the resulting image (multiplying the numerator by a constant). Metamorph, e.g. has a default scaling of 100. This can be helpful, since many of the pixels will have values between 1 and 10. Choose whatever value is most appropriate, but be sure to use the same scaling for all images (Figure 2).

20. Examine your ratio image closely to ensure the animal was in perfect register (did not move) between captures.

While animals that moved significantly will be obvious at the time of imaging (Figure 3A), subtle changes are not often apparent until after ratio-ing (or merely overlaying) the two images (Figure 3B). Ratio images subtly out of register will often be evident as a black "halo" around one or more edges of the animal. "DISCARD" these ratio images (and their associated picture sets), and instead use a different picture set of that animal (repeat Steps 18–20). Using images out of register leads to artifacts that skew your quantification data, resulting in incorrect conclusions about pH_i changes (Figure 3).

21. Save this ratio image as a new file, with a file name that links it to its original picture set.

For instance, if your picture set was comprised of Control-1a-580 and Control-1a-640, save the ratio image as Control-1a-ratio.

22. To display photos (of ratio images), change ratio images from monochrome to a Red–Green–Blue (RGB) color gradient (based on signal intensity, with Red high and Blue low) and save as a new image.

Often results are not obvious to the eye until you switch from monochrome to RGB. This is best done in your imaging software, where you get more accurate gradations, rather than secondarily (such as with Photoshop).

23. Quantify relative changes in pH_i (based on signal intensity), using the function in your imaging software that allows for pixels to be selected (or masked, or painted) to highlight (or mask, or paint) the region of interest on the "RATIO-IMAGE" only.

Make sure to use anatomical markers (e.g. borders of the blastema and anterior edge of the pharynx) to select your desired region, and use the same markers every time to keep your region of interest consistent between animals.

24. Once your region of interest is highlighted, use the function in your imaging software that quantifies pixel intensities to generate a new file of the statistics for the highlighted region.

Most imaging software programs can measure the signal intensity for selected pixels. Make sure your software can generate mean intensity (average signal intensity for your entire highlighted region) and intensity standard deviation. Use these intensity statistics for comparisons of relative pH_i . Typically, your imaging software will save multiple quantifications to a single file, with each line item listed by the original ratio image file name. Save all quantifications of the same region for all animals and all conditions to the same file.

25. Repeat for all regions of interest (e.g. anterior blastema and posterior blastema), highlighting and quantifying each region individually.

If quantifying multiple regions, it is best to save the quantification for each region to its own, separate file. (Make separate files for each region of interest.)

26. Repeat for all ratio images.

It is best to first create all your ratio images, saving them to a separate subdirectory. Then open your ratio images and do all your quantifications at one time.

27. If your imaging software has not automatically created a data sheet, export the quantification data to Excel (or another statistics package) for analysis.

28. Compare the mean signal intensity values from different regions and/or treatments using a T-test (two-tailed distribution).

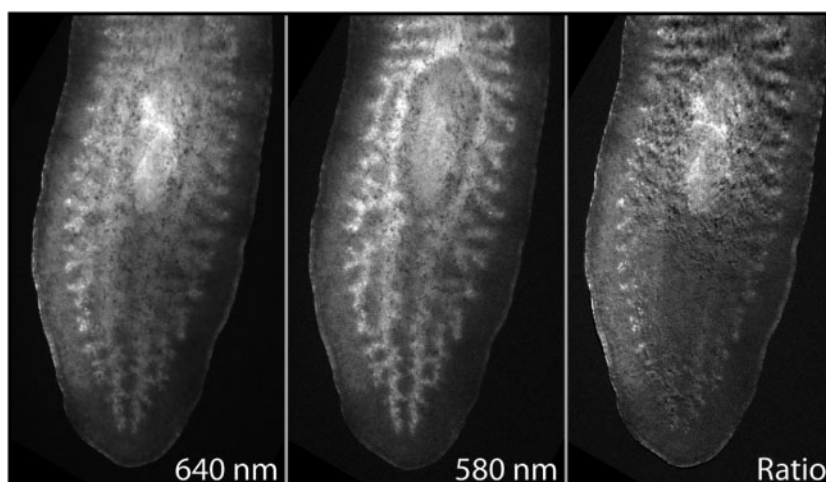


Figure 2: Creating ratio images with SNARF-5F-AM. Picture set of original 640 nm and 580 nm images, and the resultant ratio image (640/580), of the tail of an uncut *S. mediterranea* worm (EtOH-immobilized). Ventral view, anterior is up.

Controls

29. Alter pH_i in control worms to verify the protocol is working.

Use a protonophore in buffered worm water with both higher and lower pH than control water. Higher pH (alkalinization) should result in a brighter signal, whereas lower pH (acidification) should result in dimmer signal than seen in untreated worms.

Suggested controls: 250 nM CCCP in worm water with 10 mM CHES buffered to pH 9; and 250 nM CCCP in worm water with 10 mM NaOAc buffered to pH 5.5.

30. Image unstained worms (without SNARF-5F-AM) capturing at both 580 nm and 640 nm using identical parameters to those used to image experimental (stained) animals.

This will confirm that autofluorescence is not contributing to the signal. Any autofluorescence should be significantly dimmer and present in a different pattern than SNARF fluorescence.

31. If appropriate: recover imaged worms into fresh worm water and place into normal culture dishes, then allow worms to complete regeneration.

See Step 17 for worm recovery. This controls for any possible toxicity of the SNARF-5F-AM exposure when combined with your experimental treatment. Regeneration phenotypes should be comparable between SNARF-exposed and non-SNARF-exposed worms.

32. To improve signal-to-noise ratios, at each imaging session take two sets of control pictures (one set for each wavelength captured), a dark-field image (captures electronic noise) and a flat-field image (captures variation in illumination across the field of view), and use these to correct picture set images prior to generating ratio images.

For more details, see Ref. [18]. Using the same exposure times/settings you established in Step 14, close the shutter and capture images at 580 nm and 640 nm. These are your dark-field images. With the same settings, open the shutter, lower the stage until image is out of focus, and capture at 580 nm and 640 nm. These are your flat-field images. Prior to making ratio images (see Step 19), use your imaging software program's arithmetic function to correct each picture set as follows:

- i. $(640 \text{ worm image}) - (\text{Dark-field } 640 \text{ image}) = (\text{Corrected } 640 \text{ worm image})$

- ii. $(\text{Flat-field } 640 \text{ image}) - (\text{Dark-field } 640 \text{ image}) = (\text{Double-corrected } 640 \text{ image})$
- iii. $(\text{Corrected } 640 \text{ worm image}) / (\text{Double-corrected } 640 \text{ image}) = (\text{Final } 640 \text{ image})$
- iv. Repeat (i)–(iii) for 580 worm image

Results

Figure 4 shows a typical image obtained from this method, revealing the endogenous pH gradient in a living planarian, and in particular the fact that dorsal and ventral tissues can have different pH levels.

Troubleshooting

Problem: SNARF does not fluoresce or is very dim.

[Step 14]

Solution:

Make sure you are using the AM ester, and the dispersing agent Pluronic F-127. The first makes the dye cell-permeant and the second increases cell permeability. Make sure to use SNARF-5F (which has a more physiological pK_a of ~ 7.2) rather than SNARF-1 ($pK_a \sim 7.5$). Suspend fresh SNARF-5F-AM each time, do not reuse your stock; for this reason, use of the special package (50 μM aliquots) is recommended. Ensure aliquots, suspensions, and experiments are kept covered (dark).

Problem: Worms will not stay stationary.

[Step 16]

Solution:

- a. If using PC2-RNAi to immobilize, your phenotype may not be strong enough. Either repeat injections or make sure you select only worms with the strongest phenotype.
- b. PC2-RNAi is often not sufficient to prevent the fine movements at the animal's margin (although gross movements like crawling across the slide are prevented). Try EtOH immobilization (see Step 3).
- b. If you used EtOH to immobilize, the worm may be recovering. Planaria 4–7 mm in length typically recover within 3–4 hours after being washed out of EtOH. Make sure to EtOH treat, load with SNARF, and image promptly. In addition, image in small batches (multiple image sessions, but each takes less time) or in timed batches (one long image session, with worms going into EtOH, then SNARF, at multiple times).

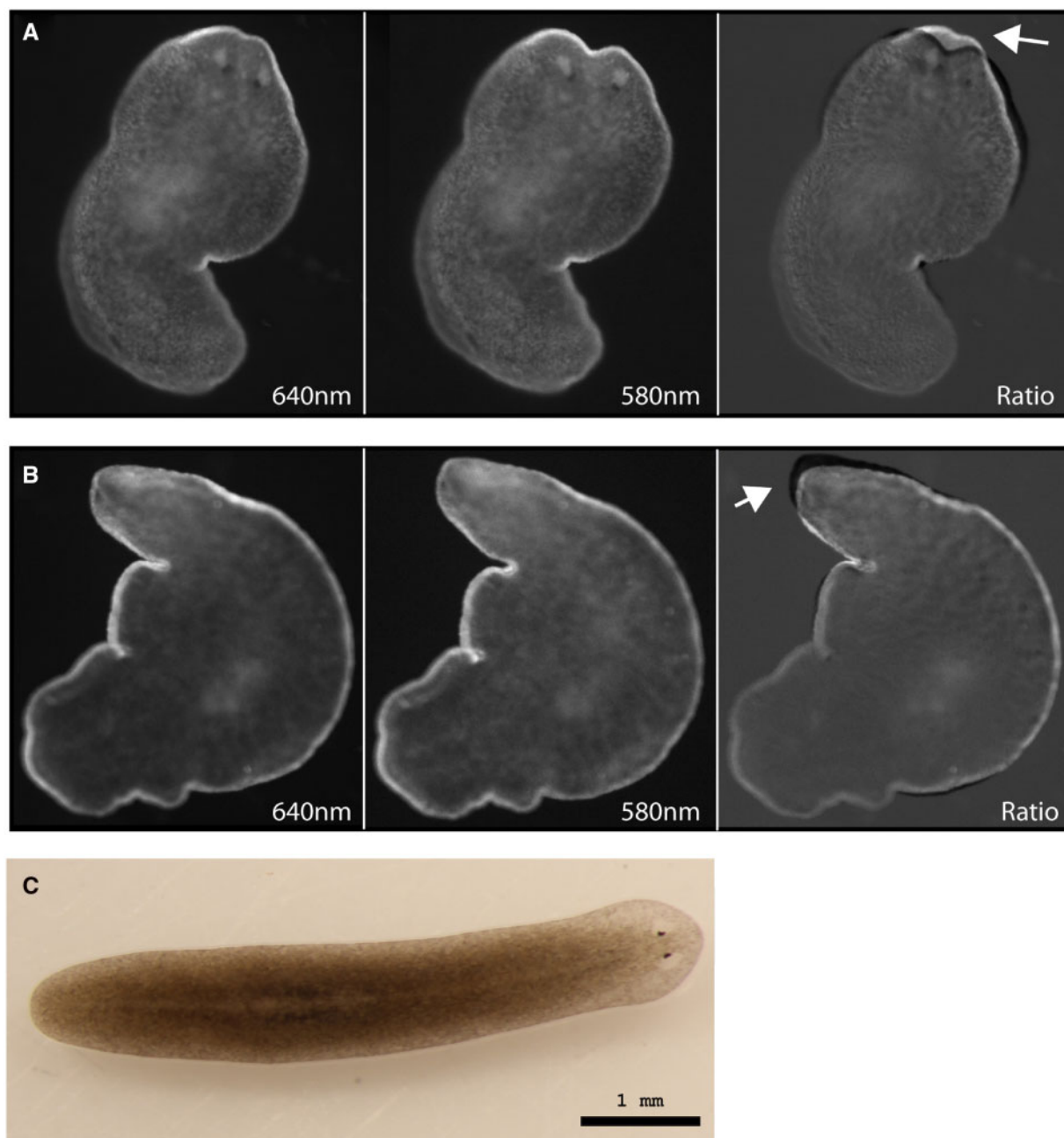


Figure 3: Non-overlapping ratio images should not be used. (A) Picture set of original 640 nm and 580 nm images that are obviously out of register prior to ratio-ing. Dorsal view. (B) Picture set of a worm that subtly moved between captures, most evident only after ratio-ing. Ventral view. Arrows: Black halo effect signifying lack of register, indicating images should be discarded. Uncut *S. mediterranea* worms. Anterior is up. (C) Typical *S. mediterranea* planarian, as used in all other panels, with scale bar showing its size when fully extended. Anterior is to the right.

Problem: Ratio images are not in register.

[Step 20]

Solution:

Your worm did not stay still between 580 nm and 640 nm image captures. See previous problems for solutions. In addition, make sure to capture the wavelength with the longest exposure time (typically 640 nm) second, and/or take several picture sets of each animal, to maximize your chances of ratio images in register.

Problem: Resolution is too low.

[Step 22]

Solution:

Make sure that your imaging software has autoscaling turned off. Set the grayscale image to its full range, staying away from the extremes (i.e. at 8-bits, a value of 0 is bad data whereas saturated pixels are maxed at a value of 255). For an 8-bit image, set the grayscale image from ± 26 to ± 230 . For a 12-bit image, set the scale to ± 41 to ± 4055 . Then, make sure your exposures use as much of the range as possible. For more details, see Ref. [18].

Problem: Too much variability in pH_i patterns or values between worms, even within the same treatment, interfering with obtaining statistical significance.

[Step 28]

Solution:

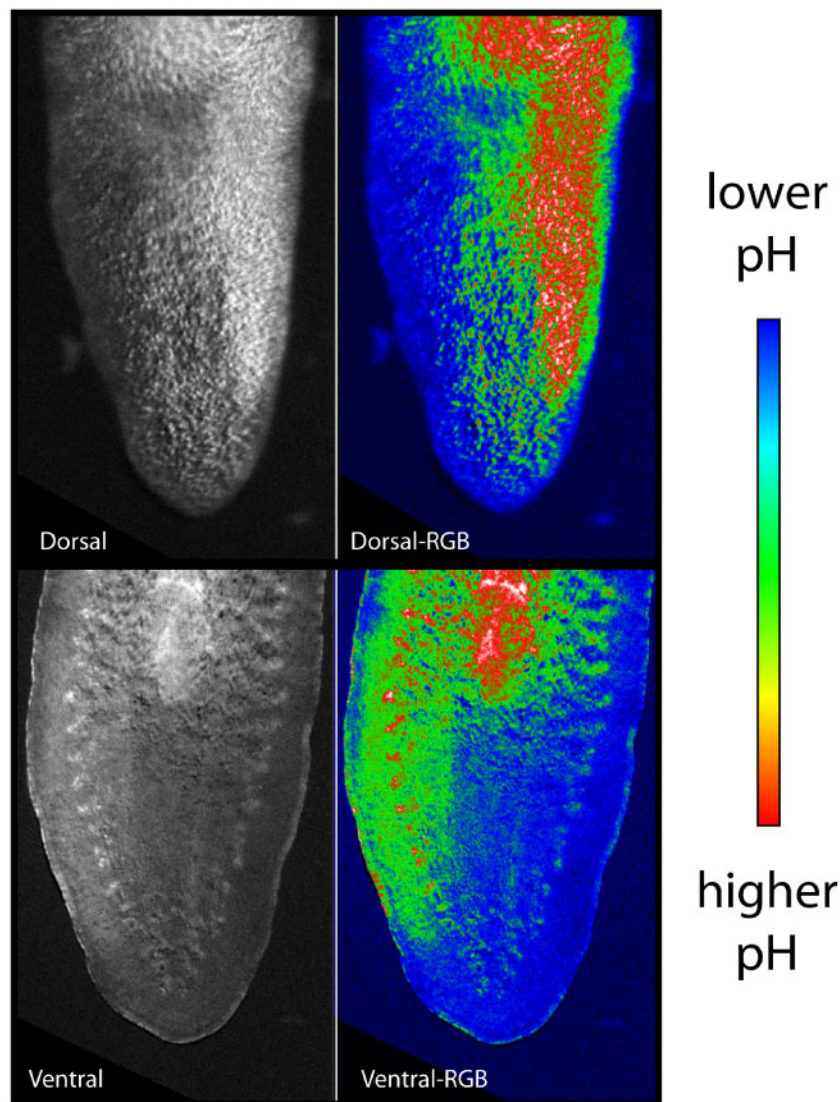


Figure 4: Dorsal and ventral views have different pH_i patterns. Monochrome and RGB ratio images of the tail of a single uncut *S. mediterranea* worm shown from both the dorsal and ventral views. Anterior is up. Blue represents lower pH , whereas red represents higher pH .

- a. Dorsal and ventral sides of the planarian have different pH_i patterns. Make sure to only compare ventral with ventral or dorsal with dorsal images. Ventral images typically give the most consistent readings, since the curve of the animal at the margins from the dorsal side can confuse intensity readings (Figure 4).
- b. Make sure worms are not being compressed between the coverslip and slide. Pressure on the animal can affect the signal intensity/pattern of pH_i . Use of FluoroDishes is recommended, as they eliminate this possibility. If not available, use clay or silicone spacers between traditional coverslips and slides, with constant vigilance not to squeeze worms between them.

Discussion

This protocol outlines a method to measure relative pH_i in live planarians, rather than absolute values of pH_i . Since signal intensity is directly related to pH , it is possible to determine absolute pH_i using this protocol, although calibration using several known levels of pH_i would have to be performed (so that given signal intensities could be related to specific pH levels). If multiple regions from a single animal are to be compared (see Step 25), the entire worm (or worm fragment) should be captured in a single image.

Due to recent advances in immobilizing live planaria (which notoriously fail to respond to most paralytics), *in vivo* imaging using ratiometric dyes is now feasible. Treatment with a low percent EtOH (see Step 3), which is more efficient at keeping worms still during imaging, does result in changes in absolute pH_i ; however, the change is uniform across the entire animal and does not alter relative pH_i quantifications. Despite this advance, preventing worms from moving between image captures can be the most challenging part of the protocol, since a true paralytic has still not been identified. Vigilance is required to ensure that any picture sets not completely in register are not used (see Step 20). But the reward is the ability to use ratiometric fluorescent dyes, such as SNARF-5F-AM [20], which represent a vast improvement over non-ratiometric reporters where data are susceptible to artifact due to uneven cell loading and photobleaching.

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