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Live Imaging of Planarian Membrane Potential Using DiBAC$_4$(3)

Néstor J. Oviedo,

Cindy L. Nicolas,

Dany S. Adams,

Michael Levin[1]

Center for Regenerative and Developmental Biology, Forsyth Institute and Developmental Biology Department, Harvard School of Dental Medicine, Boston, MA 02115, USA

INTRODUCTION

This protocol describes how to use the anionic membrane voltage-reporting dye DiBAC$_4$(3) to generate images of cell membrane potential in live planarians. These images qualitatively reveal variations in time-averaged membrane potential across different regions of the organism. Changes in these images due to experimental treatments reveal how the particular treatment affects this physiological parameter. This method is a great improvement over standard electrophysiological techniques, which cannot be used to gain an understanding of the electrical properties of an entire worm or a regenerating fragment, due to small cell size and large cell number. When the proper controls are performed, this technique is a very powerful and simple way to gather physiologic data.

RELATED INFORMATION

DiBAC$_4$(3) has also been shown to be useful in the study of vertebrate regeneration (Adams et al. 2007) because of the known role of endogenous bioelectric signals in directing cell behavior and morphogenesis (McCaig et al. 2005; Levin 2007).

For an introduction to planarians as a model system, see **Planarians: A Versatile and Powerful Model System for Molecular Studies of Regeneration, Adult Stem Cell Regulation, Aging, and Behavior** (Oviedo et al. 2008a). Protocols for **Establishing and Maintaining a Colony of Planarians** (Oviedo et al. 2008b) and **Gene Knockdown in Planarians Using RNA Interference** (Oviedo et al. 2008c) **are also available.**

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <**R**>.

Reagents

Cationic-membrane-reporting dye (see Step 14.iv)

[1]Corresponding author (mlevin@forsyth.org).

<!>Depolarizing agent (e.g., medium containing a high concentration of potassium gluconate [150 mM] and a potassium ionophore) (see Step 14.ii)

<!>Salinomycin (20 mM) can be used as a potassium ionophore.

<**R**>$DiBAC_4(3)$ (1 mg/mL, prepared in 70% ethanol)

Planarian water (see **Establishing and Maintaining a Colony of Planarians** [Oviedo et al. 2008b])

Worms with a strong *Smed-PC2(RNAi)* phenotype, ~10 d since first injection (see **Gene Knockdown in Planarians Using RNA Interference** [Oviedo et al. 2008c])

Equipment

Camera (digital, attached to the microscope)

Coverslips

Fluorescence filter set suitable for λ_{ex} = 493, λ_{em} = 516 (e.g., fluorescein isothiocyanate [FITC]) Image analysis software with background correction, segmentation, and pixel quantification functions

Microscope with epifluorescence or confocal optics and 4X (or 5X) and 10X lenses

Paper towels

Petri dishes (35-mm) or 24-well plates

Petroleum jelly (e.g., Vaseline) or other temporary sealant

Silicone spacers (Press-to-Seal; each forms one well 20 mm in diameter and 0.5 mm deep)

Slides (25 × 75 × 1 mm)

METHOD

Staining

1. Dilute the $DiBAC_4(3)$ stock solution 1:10 in H_2O, and then dilute that $1:10^3$ in planarian water to a final concentration of ~0.1 ng/µL.
2. Fill a Petri dish or one well of a 24-well plate with the $DiBAC_4(3)$ solution, so that it is at least 2-3 mm deep.
3. Place the immobilized planarians (*Smed-PC2[RNAi]* worms) into the $DiBAC_4(3)$ solution.
4. Incubate the worms in the dark for at least 30 min at room temperature. Keep them in the solution until you are finished imaging.

   The dye has no lasting effect on worm behavior or regeneration.

Mounting

5. Remove the plastic from both sides of the silicone spacer. Place a thin layer of petroleum jelly on one side of the spacer, and lay it, jelly side up, on a paper towel or other clean surface.

6. Position the slide above the spacer, and press down. Watch carefully to make sure that the seal is complete.

7. Flip the slide right-side up. Place a thin layer of jelly on the remaining face of the spacer.

8. Fill the pool created by the spacer with the diluted $DiBAC_4(3)$ solution (from Step 1).

9. Remove a *Smed-PC2(RNAi)* worm from the staining solution (from Step 4), position it on the slide, and cover it with a coverslip (Fig. 1).

10. Add more petroleum jelly along the junctions of the glass and silicone.

This provides extra protection against fluid loss. Moreover, it minimizes changes in the $DiBAC_4(3)$ signal due to pressure on or contact with the worm. The concentration of $DiBAC_4(3)$ must stay constant.

See Troubleshooting.

Imaging

11. Mount the slide on the microscope stage. Using bright-field light and the 4X or 5X lens, locate and focus on the animal.

If the specimen is very small, the 10X lens can be used.

12. Confirm the focus on the $DiBAC_4(3)$ emission using the appropriate filter set.

$DiBAC_4(3)$ will bleach; thus, it is important to minimize the amount of exposure.

See Troubleshooting.

13. Take the picture.

Because the $DiBAC_4(3)$ is constantly moving in and out of the cells, it can be useful to wait 20-30 sec between exposures to give the unbleached dye time to replace the dye that was bleached in the previous exposure.

See Troubleshooting.

14. Perform the following controls:

- **i.** Image unstained animals.

  This confirms that autofluorescence is not contributing to the signal.

- **ii.** Take one image, add a depolarizing agent, such as medium containing a high concentration of potassium gluconate (150 mM), and a potassium ionophore (e.g., 20 mM of salinomycin), and then take another image.

This confirms that the depolarization of cells does cause an increase in emission intensity.

The second image should be brighter than the first. The ionophore concentration must be titrated for each species.

See Troubleshooting.

iii. If possible, include a high-magnification image that illustrates the distribution of dye within individual cells.

This shows whether it is the voltage across the cell membrane or across the membranes of organelles that is reported.

iv. Repeat the imaging with a cationic-membrane-reporting dye such as one of the carbocyanine dyes (e.g., $DiSC_3$[5]).

The intensity distribution should be inverted. Care should be taken with interpretation of cationic dye data because, unlike anionic dyes, cationic dyes will enter mitochondria. In addition, different dyes will have different ranges of potential to which they are most sensitive. Thus, the signal may not be entirely due to cell membrane potential.

See Troubleshooting.

Image Processing and Analysis

15. Use the analysis software to open the image.

16. Correct the background using an appropriate function.

In practice, this step, although required, usually has little effect on the data. The signal-to-noise ratio in $DiBAC_4$(3) images tends to be very strong already; moreover, the result cannot be quantified on an absolute scale, and background noise will not affect the determination regarding which parts of the animal are emitting more intensely than others.

17. Examine the intensity of the pixels. To make areas of different intensity very obvious, pseudo-color the image with a red-green-blue look-up table (LUT).

The pixel intensity indicates the degree of relative depolarization; brighter (more intense) pixels mean that that area of the specimen is depolarized relative to areas with less intense emission.

18. Segment the data using the analysis software.

The intensity of the pixels can be quantified on an arbitrary scale, but it is important to make sure that only pixels with real data are counted.

i. Exclude underexposed or overexposed pixels from the analysis.

Underexposed or overexposed pixels will have values of 0 and 4095, respectively, for 12-bit images (or values of 0 and 255, respectively, for 8-bit images). Because the background correction may have artificially lowered

the values of overexposed pixels to under 4095, it is a good idea to further limit the range of pixel values to be included (e.g., use values of 10-4000).

ii. Use the segmentation function of the software to find pixels within the chosen range.

Drawing regions of interest by hand is not recommended.

iii. To yield additional information about the range of intensities and the sizes of areas showing different intensities, segment into multiple ranges, e.g., use values of 10-1009, 1010-2009, 2010-3009, and 3010-4009.

The number of pixels within each range provides information about the variability and the area of depolarization within the animal.

19. Choose a measure of intensity that will describe your data.

A histogram of pixel-intensity frequencies will work for any data and should be examined first. If the histogram shows that pixel intensities are normally distributed, the mean and standard deviation (or the mean and confidence intervals) are the best measures to report and the simplest to use for comparisons. If intensities are uneven across the region of interest (i.e., blotchy), you may need somewhat more sophisticated statistical tests if you wish to make an inference about the general population; the histogram itself is fine for presentation of data.

See Troubleshooting.

20. If the independent variable is nominal or ordinal (a category, such as treatment, with an arbitrary or unequal scale), compare the means using nonparametric statistics. To compare two means, use a Mann-Whitney test; for three or more means, use a Kruskal-Wallace test. If the independent variable is based on interval or ratio data, use a *t*-test or an ANOVA test.

TROUBLESHOOTING

Problem: Fluid leaks out of well.

[Step 10]

Solution: Return the animal to the original staining dish, and give it at least 30 min to exchange $DiBAC_4(3)$ with the medium before trying again.

Problem: Emission intensity is too high or too low.

[Step 12]

Solution: Vary the concentration of $DiBAC_4(3)$ in the medium, or use neutral density filters.

Problem: $DiBAC_4(3)$ has bleached.

[Step 13]

Solution: Return the animal to the original staining dish, and give it at least 30 min to exchange $DiBAC_4(3)$ with the medium before trying again. The optimal solution is to image using a perfusion system that can completely replace all of the dye solution in the pool before each new image. Chambers for this purpose are available as Press-to-Seal silicone spacers.

Problem: Potassium gluconate plus ionophore has no effect on intensity.

[Step 14.ii]

Solution: Strong buffering, mucous, and other secretions can inhibit movement of ionophores or mute changes in membrane potential. Consider the following:

1. Try a different ionophore or ionophore cocktail.
2. Confirm that the K^+ concentration in the medium is higher than the intracellular concentration.
3. If all else fails, kill the animal by adding a fast-acting toxin (e.g., sodium azide). As the cells die, the membrane potential will go to zero.

Problem: The cationic dye pattern is not the inverse of the anionic $DiBAC_4(3)$ pattern.

[Step 14.iv]

Solution: This may indicate that the two dyes have entered different subcellular compartments or that they act by different mechanisms. There is no general solution to this problem, which makes the other controls even more important.

Problem: A more quantitative measure is required.

[Step 19]

Solution: Molecular Probes (now Invitrogen) has developed voltage sensor probes (VSPs), which are fluorescence resonance energy transfer (FRET)-based ratiometric methods for reporting membrane voltage. Because these are pairs of reporters, these kits tend to be much more expensive than $DiBAC_4(3)$ alone. Nonetheless, because they are ratiometric, they are less affected by artifacts and can be used to generate more quantitative data.

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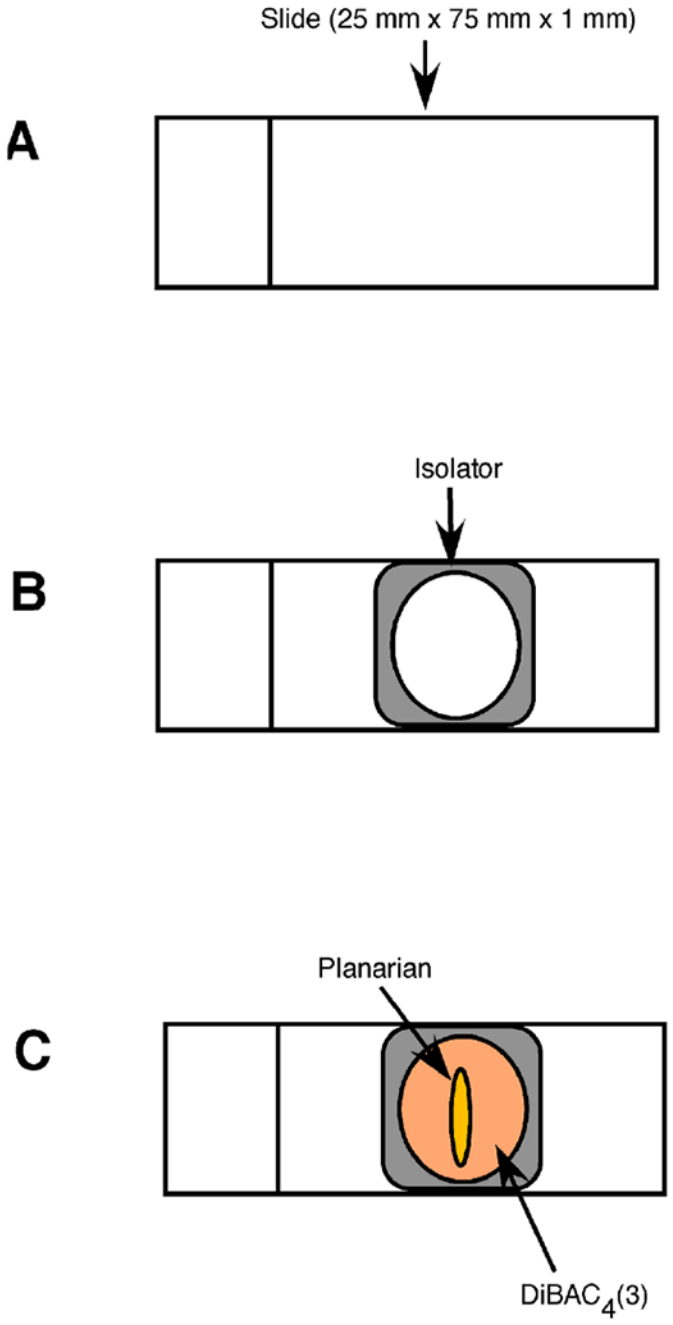

FIGURE 1.
Sequential steps for mounting planarians for imaging with $DiBAC_4(3)$. (*A*) A slide recommended for the procedure. (*B*) Positioning of the spacer after petroleum jelly has been added and attached to the slide. (*C*) Final step involving positioning of the worm in the $DiBAC_4(3)$ solution.