Vital Staining for Protozoa and Related Temporary Mounting Techniques

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Includes links to Micscape articles on some of the protozoa species mentioned.

For the amateur microscopist, the use of vital stains can provide additional information about the cytological details of protozoa. Virtually all stains, even the so-called vital stains, are eventually toxic to the organisms. To achieve the desired effects, the stains must be used in extremely dilute solutions ranging from dilutions of 1:5,000 to 1:500,000. Although vital staining is not much used in modern microscopy, the older microscopists regarded this technique as a source of important information. They experimented with a wide variety of stains;most of which, it is worth remembering were developed for the textile industry. As a consequence of these investigations, a series of stains were identified as being effective as vital stains, and these, certainly are the ones to try out first, but it should not discourage one from trying out other stains which are not on the list. A very helpful list along with dilution and toxicity is provided by Mc Clung and I will include it here:

From *McClung's Handbook of Microscopical Technique*, Paul B. Hoeber, Inc. New York, 1929, p. 395. This chapter on Protozoological Methods was written by D.H. Wenrich.

Dyes	Minimum Concentration That Will Stain Paramecium	Toxicity: Per Cent dead in one hour
Bismarck brown	1 to 150,000	0

Methylene blue	1 to 100,000	5
Methylene green	1 to 37,500	5
Neutral red	1 to 150,000	3
Toluidine blue	1 to 105,000	5
Basic fuchsin	1 to 25,000	30
Safranin	1 to 9,000	30
Aniline yellow	1 to 5,500	0
Methyl violet	1 to 500,000	20
Janus green B	1 to 180,000	40

Wenrich comments on several other dyes that are useful for "intravitam" staining, among them Nile blue (1 to 30,000) and Rhodamine (1 to 20,000).

Some of the vital stains are rather general in their action and provide nice contrast for investigating cytological detail. Others show more specific detail as a consequence of their specific chemical properties. For example, Janus green B is a specific stain for mitochondria and neutral red, while providing general cytological staining, is also a specific stain for "neutral red globules" mentioned by Gravé. [Gravé, Eric V., Discover the Invisible: A Naturalist's Guide to Using the Microscope, Prentice-Hall, 1984.] In addition to being a stain, neutral red is a pH indicator and will stain newly-formed food vacuoles a bright red. As the process of digestion proceeds, these vacuoles will take on a yellowish color indicating a shift to an alkaline pH as the digestion takes place.

While vital staining provides contrast, one should not ignore the possibilities of discovering additional detail by employing additional means of contrast, For example, in a previous note, I discussed the use of powdered carmine as a means of demonstrating food vacuoles in organisms, such as, *Paramecium*. I took some paramecia treated with carmine and looked at them with Nomarski differential interference contrast. The results of observing the effects of carmine with brightfield were very good, but with Nomarski, the results were truly spectacular. Experimenting is imperative! Try brightfield, darkfield, oblique illumination, phase contrast, Rheinberg—in short, every type of optical contrast available to you.

Another helpful technique, which does not actually involved staining, utilizes colored particles that are essentially inert and non-toxic to provide a colored background. For example, India ink can be used to provide a background "field" which can greatly facilitate the observation of the locomotion of certain protozoa. When one achieves a good concentration, the action of cilia, undulating membranes, and flagella become much clearer as the particles are moved around by the activity of these organelles. *Paramecium* and large flagellates are obvious candidates for experimentation, but try this technique also on some of the hypotrichs, such as, *Euplotes* and *Stylonychia*, as well as *Spirostomum*, *Blepharisma*, *Stentor*, and *Vorticella*. In fact, this technique is worth trying on any protozoan which produces strong currents in the surrounding water.

A further technique, which starts out with living organisms, but involves letting them dry up, uses stains which deposit themselves on the pellicle or surface membrane of the organisms, thus revealing the "sculpting" of the surface. This technique only works with protozoa which remain relatively intact during the drying process. The stain most widely used for this purpose is Nigrosin. One can get very nice results with *Paramecium* and *Stentor*. It is important to use samples from rich cultures, for some specimens will show considerable distortion and others will retain their basic features. I have also obtained good results with Toluidine blue. I am certain that will experimentation, one can discover other stains that also give good results.

There are some other techniques not related to "vital" staining, i.e., they involve killing the organisms, but they provide, as temporary preparations, information that can be very helpful in better understanding the morphology of certain types of protozoa. In identifying protozoa, it is often helpful to know the number and form of the nuclei. Slides containing numerous specimens of the organisms being studied should be prepared. Then place a good-sized drop of methyl green-acetic or aceto-carmine at one edge of the cover glass and allow it to gradually be dispersed under the cover glass. However, don't allow the preparation to dry. After a few minutes, the slides are ready to be examined. Methyl green-acetic can be prepared as a 2% solution with 1% concentrated acetic acid added to it. The proportions need not be exact. I have found that the solution works better after it has aged for some months and so, I always keep a stock of aged solution available. Aceto-carmine should be purchased from a supplier and used with caution as it contains 45% acetic acid.

Some organisms do not tolerate this procedure well and they disassociate. However, if one has taken care to include on the slide only specimens of the organisms which you wish to examine, this is not always a complete disadvantage. This is fairly easily accomplished by placing a sample of the culture in a watch glass and then using a finely-drawn micro-pipet, transfer the desired organisms to a small drop of water on a clean slide. By having only one species on the slide, even if the cells disassociate (undergo lysis), one is guaranteed that whatever takes up the nuclear stains, did, indeed, belong to the organisms under investigation. Aceto-carmine stains nuclear material bright red and the Methyl green-acetic stains a vivid bluish-green. In general, the amateur should be content with identification at the genus level. However some organisms are distinctive enough that with careful observation, species determination can be made. A *Stentor* with distinctive bluish-green pigment and a beaded nucleus is assuredly *Stentor coeruleus*. The pigment in this species, which is called stentorin, is dichroic. If one shifts the angle of the light appropriately, the organism has a lovely rose-colored tint. Knowing the form of the nucleus in *Spirostomum* can also help in narrowing the range of possible species, but other morphological characteristics must also be taken into account to get a definitive species determination.

The use of Lugol's solution, an aqueous solution of iodine and potassium iodide, can also be very helpful in determining gross morphology. If one does not have access to Lugol's solution, the standard tincture of iodine sold for first aid kits can be substituted. However, since this is an alcoholic solution, try diluting it down with distilled water to minimize distortion. I have used Lugol's solution to make temporary preparations of *Lacrymaria olor*. A significant number of the specimens remain partially extended and few were killed quickly enough to leave the cytostome open. The cytostome in *Lacrymaria* is controlled by a group of proteinaceous rods called trichites. With luck, one may find a few specimens where the trichites are quite distinctive. Flagellates containing bodies which store starch also stain quite distinctively, since iodine is a specific stain for starch.

Certain fluorescent stains (or flurochromes) can be used to make either vital preparations or temporary fixed mounts. CAUTION: Some of the fluorochromes, in the powdered form or in concentrated solutions, are extremely toxic being both carcinogens and mutagens! Therefore, one must exercise great care in preparing solutions and avoid breathing any of the powder or

allowing it to come in contact with the skin. Fortunately, the solutions are used in very great dilutions and some biological supplies houses will provide widely-used flurochromes already in solution. Nonetheless, as with all potentially toxic materials, care must be exercised.

If one has access to a microscope with fluorescence or epi-fluorescence capabilities, there are two techniques which can provide spectacular results. A particularly useful fluorochrome is Acridine Orange. A few drops of a solution of 0.0001% can be introduced to a rich culture of paramecia. Allow this to sit for several days so that the paramecia slowly absorb a sufficient quantity of the stain. when examined using the techniques of fluorescence microscopy, the macronucleus and the food vacuoles of the paramecia fluoresce brilliant light green, cytoplasmic granules fluoresce orange and the so-called Neutral Red globules fluoresce yellow. As one can imagine, this produces a very striking image. An interesting side effect of the use of very dilute solutions of Acridine Orange is a fascinating phenomenon know as hormesis. Hormesis involves the production of, at least, temporary benefits from a minute amount of a substance know to be toxic at higher levels. The phenomenon is fairly widely recognized, but has certainly not been widely, nor systematically studied in protists. With paramecia, the addition of tiny amounts of Acridine Orange seems to increase their vitality, their resistance to viral infections, and promote reproduction. Hormesis has been recognized for some time in human beings. For example, minute amounts of selenium are highly beneficial to the human diet, but too much can produce toxic reactions.

A second method involves using a somewhat more concentrated solution of Acridine Orange. Here, one can start with a 0.01% solution, but if this proves toxic too quickly, then experiment with dilutions. The object is to get the paramecia (or other protozoa) to absorb the stain fairly fast for a quick examination of nuclei under epi-fluorescence. Blue-violet fluorescence produces very good results with Acridine Orange. I use a 100 watt halogen light source for this purpose. However, Acridine Orange is an intensely photo-active stain and the cells will undergo lysis (disassociation) in a few seconds as the intense light activates the dye. Nonetheless, one can often get important information from such preparations, e.g., the number and form of the nuclei. To obtain preparations which can be observed for longer periods, I have modified this technique by first exposing the organisms to the Acridine Orange solutions for several minutes and then fixing them. The fixation prevents the disassociation of the organisms.

Even if one does not have access to a microscope equipped for fluorescence, once can, nonetheless, sometimes obtain interesting results using fluorochromes as one would use regular stains.

Interesting preparations can also be made using either Delafield's or Ehrlich's hematoxylin. I make up a slide with a cover glass and place a drop of the hematoxylin at the edge of the cover glass and allow capillary action from evaporation to gradually pull the hematoxylin under the cover glass. This method has a couple of nice advantages. As the stain slowly diffuses across the preparation, one gets differentiated staining. Some of the organisms, on the side where the stain is introduced, may be stained too darkly, but others, in the middle or on the opposite side, will achieve an excellent balance. Hematoxylin is an excellent stain for revealing both nuclei and cytoplasmic detail in protozoa. A further advantage is that both Delafield's and Ehrlich's solutions contain glycerine and so the preparations never completely dry out. I have had some slides that were still in good condition many weeks after I first made them.

Some staining techniques are highly sophisticated and complex, but the amateur, using simple techniques and possessing a willingness to experiment, can achieve interesting and informative results.

Comments to the author Richard Howey welcomed.

Editor's notes:
The author's other articles on-line can be found by typing in 'Howey' in the search engine of the Article

Library, link below.

Micscape safety note: As the author reports many stains are extremely toxic and some are both carcinogens and mutagens! The diluted stains are probably the safest form for the hobbyist and these are available commercially. The Material Safety Data Sheets for these chemicals should be provided by the suppliers, consulted and the appropriate precautions taken before use. In any event these chemicals are not suitable for youngsters.

Disclaimer: This article is offered in good faith by the author. Neither the author, Microscopy-UK, Micscape, Onview.net nor any of its administrators or contributors assumes any responsibility for damage to persons or property incurred by using the chemicals described.

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