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A NEW FLUORESCENT TEST FOR CELL VITALITY USING CALCOFLUOR WHITE M2R

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ABSTRACT. The fluorescent fabric-brightener dye, Calcofluor white M2R (CFW), can be used to distinguish between living and dead cells from a variety of animal and plant sources. CFW does not stain living mouse fibroblasts or trout red blood cells and stains only the cell walls in living cells from the epidermis of onion bulb scale, staminal hairs of Tradescantia, and longitudinal sections of broad bean stems and roots. Heat-killed plant or animal cells are recognized by their lightly stained cytoplasm and brightly stained nuclei. The optimum staining concentrations were very low (0.01% to 0.03%) and nontoxic. Using onion scale epidermis in which some cells had been killed by heating as a test system, and the plasmolysis-deplasmolysis reaction as the ultimate test for cell vitality, results from CFW staining correctly predicted cell vitality for about 98% of the cells tested. This success rate was comparable to those for Evans blue, uranin or neutral red in this test system.

Calcofluor white M2R (CFW) is a fabric-brightener of molecular weight 897. This basic dye binds to cellulose and chitin (Hayashibe and Katohda 1973), callose and carboxylated polysaccharides (Hughes and McCully 1975) and a variety of other β-linked polymers (Maeda and Ishida 1967) and as a result of its binding, can be used for a variety of purposes. As a fabric-brightener it binds to the cellulose fibers in textiles, absorbing short wavelengths of light and emitting blue light, thereby increasing the brightness of the fabric (Villaume 1958). It has been used as an inhibitor of cellulose synthesis (Colvin and Witter 1983) and to detect newly synthesized cell walls in fungi (Peberdy and Gibson 1971, Peberdy and Buckley 1973, Gull and Trinci 1974, Benitez et al. 1976), Fucus eggs (Nakazawa et al. 1969), isolated protoplasts of tobacco leaf tissue (Nagata and Takebe 1970, Hahne et al. 1983) and algae (Waaland and Waaland 1975). The porous structure and pattern of primary cell walls can be visualized with CFW (Berliner et al. 1978). It has also been used as an apoplastic tracer with limited mobility (Gunning and Hughes 1976, Weerdenburg and Peterson 1984, Peterson and Perumalla 1984).

Preliminary observations indicated that CFW might be useful in distinguishing between living and dead cells by staining dead cell nuclei. The dependability of CFW as a vital stain was tested against the deplasmolysis system which is a commonly used criterion of vitality in plants (Currier and Van der Zweep 1955, Palta et al. 1977, Taylor and West 1980). The deplasmolysis test has been found to be more dependable than some other vitality tests such as staining with neutral red (Currier 1956, Peterson 1979). The onion scale epidermal tissue system was chosen for the present study since it is easy to obtain, has granular cytoplasm and is in common use by other researchers (Stadelmann and Kinzel 1972, Taylor and West 1980). The stain also was tested on a variety of other specimens.
STAIN TECHNOLOGY

MATERIALS AND METHODS

Preparation of Calcofluor white M2R (CFW) stain. A 1% w/v stock solution of Calcofluor white M2R (4,4'-bis[4-anilino-6-bis(2-ethyl)amino-s-triazin-2-ylamino]-2,2'-disulfonic acid) was prepared in a 0.02 M phosphate buffer (pH 8) and stored in the dark at 5°C. Subsequent tests have shown that the pH may be lowered to 7.6 without diminution of staining intensity. At lower pHs, the stain tends to precipitate at a concentration of 0.03%. The CFW stock solution was diluted with distilled water prior to use on plant material. Diluted stain solutions had a pH of 8 or less. The chemical structure of Calcofluor white M2R (CFW) is shown below.

Plant material. The tissue used for the majority of the studies was the inner epidermis of the bulb scale of Allium cepa L. The inner epidermis of one of the outer fleshy scales was scored with a razor blade in a cross grid pattern. Sections of the scale were vacuum infiltrated with distilled water to decrease the adherence of underlying parenchyma cells to the epidermal cells. Pieces of the epidermis were gently removed with fine forceps and floated in distilled water.

Broad bean (Vicia faba L.) seeds were obtained from the Ontario Seed Company (Waterloo, Ontario, Canada). Before planting, the seeds were agitated in 0.1% Tween 80 for 5 min and then in commercial Javex (6% sodium hypochlorite) for 20 min. The seeds imbibed running tap water overnight and then were planted in vermiculite. Longitudinal sections of stem and root tissue were used. Dead tissue was obtained by immersing the sections in boiling water for 30 min.

Staminal hairs of Tradescantia blossfeldiana (Hort. ex Blossfeld) were obtained from flowers of plants grown in a greenhouse. To kill the hairs, whole stamens were removed and placed in a beaker of water which was placed in boiling water for 10 min.

CFW toxicity to onion scale epidermal cells. Strips of onion epidermis, obtained as described above, were exposed to CFW solutions of 0.005%, 0.01%, 0.03%, 0.10%, 0.30%, and 1.00% for 4 hr. After staining, the tissue was rinsed with distilled water, immersed in 0.5 M mannitol for 30 min, mounted in 0.5 M mannitol and viewed with phase optics. The total number of cells and the number of plasmolyzed cells were recorded. The tissue was flooded with
distilled water and, after 10 min, viewed to determine which cells were still plasmolysed. The cells which did not plasmolyse, as well as the cells which were incapable of deplasmolysis, were assumed to be dead. Those capable of plasmolysis and deplasmolysis were considered to be alive. A total of 300–500 cells on four strips was scored for each CFW concentration.

**Optimum stain concentration of CFW.** The optimum CFW stain concentration was determined for each type of plant cell used. Dead cells, prepared as described above, were exposed to CFW solutions of 0.005%, 0.01%, 0.03%, 0.10%, and 1.00% for 10 min (or 4 hr in the case of *Tradescantia* staminal hairs). The cells then were rinsed, mounted in distilled water and examined using a Nikon apophot microscope with phase contrast optics. The light of an HBO 200 lamp, filtered for excitation wavelengths from 360–440 nm (violet light) was used for dye observation.

**Comparison of CFW to common vital stains.** The accuracy with which CFW distinguishes living cells from dead cells was compared with that of Evan's blue, uranin (disodium fluorescein) and neutral red using onion scale epidermal tissue. The results obtained for each dye were compared to the results given by the deplasmolysis test for vitality. To kill a fraction of the epidermal cells, the tissue was placed in a hot water bath at 55°C for 10 min and then transferred to water at room temperature for 1 hr. Epidermal strips were stained with the desired dye by floating them cuticle-side up on a solution of the dye in a small Petri dish (10 ml capacity) for the required time (Table 1). The strips were removed from the dyes, rinsed, and mounted in the rinsing medium on a slide. The strips were viewed with white light and a drawing of a portion of each strip was prepared using a camera lucida attachment. The tissue was viewed with white or violet light as necessary (Table 1) and the cells which stained were noted on the drawing. The tissue was then bathed in 0.8 M mannitol for 40 min, mounted in the mannitol solution and viewed with phase optics. Plasmolysed cells were noted on the drawing. Finally, the tissue was flooded with distilled water and, after 10 min, deplasmolysed cells were noted on the drawing.

**CFW as a Vital Stain for Various Subjects**

- **Cells in freehand sections of plants**
  - Live and heat-killed longitudinal sections of broad bean were placed in 0.03% CFW for 45 min, rinsed with distilled water, mounted in distilled water and viewed with violet light.
- **Trichome cells**
  - Live and heat-killed *Tradescantia blossfeldiana* staminal hairs were stained as described in a) above except that live hairs were stained for 9 hr and dead hairs were stained for 4 hr.
- **Animal cells**
  - Mouse L cells, A9 (GM0346A) and B82 (GM0347A), were purchased from the Human Genetic Mutant Cell Repository, Camden, NJ. Growth took place in Eagle's minimal essential medium (MEM) with 10% calf serum (CS) at
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (w/v)</td>
<td>0.03%</td>
<td>0.50%</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Solvent</td>
<td>0.0006–0.05M phosphate buffer, pH 7.6–8.0</td>
<td>distilled water</td>
<td>0.067 M KH₂PO₄</td>
<td>0.08 M phosphate buffer, pH 7.2</td>
</tr>
<tr>
<td>Staining Time</td>
<td>10 min</td>
<td>15 min</td>
<td>10 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Rinsing and Mounting Medium</td>
<td>0.0006–0.05M phosphate buffer, pH 7.6–8.0</td>
<td>distilled water</td>
<td>0.067 M KH₂PO₄</td>
<td>0.08 M phosphate buffer, pH 7.2</td>
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<td>white light</td>
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<td>References</td>
<td>—</td>
<td>Taylor and West 1980</td>
<td>Stadelmann and Kinzel 1972</td>
<td>Peterson 1979</td>
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37 °C in an atmosphere of 5% CO₂ and 95% air. For staining, the fibroblasts were removed from the growth surface with 0.25% trypsin and treated with CFW either while the cells were in suspension or after they had settled down onto glass coverslips. CFW was made up in either MEM, or MEM with 10% CS to give a 0.10% (w/v) solution. Red blood cells (RBC) were obtained from the rainbow trout, Salmo gairdneri Richardson, by cutting off the caudal peduncle and collecting blood directly into Alsever’s solution. RBC were collected by centrifugation and resuspended in either MEM or MEM with CS. CFW was applied to cells which were live, fixed in 10% neutral buffered formalin or heat-killed. MEM, CS and Alsever’s solution were purchased from Flow Laboratories (Mississauga, Ontario).

RESULTS

Toxicity tests with Calcofluor white M2R (CFW) proved this stain to be nontoxic to onion epidermal tissue at concentrations up to and including 0.1%. Even at the highest concentration tested (1.0%) CFW killed only 3% of the cells. Higher concentrations of CFW were not tested because of dye insolubility. Optimum stain concentrations were 0.03% for cells from onion scale epidermis, broad bean stems and roots, and staminal hairs of Tradescantia.

When CFW was applied to a strip of onion scale epidermis in which approximately half the cells had been killed by a prior heat treatment, the dye clearly stained the nuclei of some cells but not others (Fig. 1). Stained nuclei showed a bright blue fluorescence with the filter system employed. The results of the plasmolysis-deplasmolysis test for vitality showed that the cells with stained nuclei were dead (marked with an asterisk in Fig. 1) and that the cells with unstained nuclei were alive. The extranuclear cytoplasm of the dead cells also appeared faint blue when stained with CFW. In living cells, CFW stained the cell walls, which fluoresced bright blue. Staining differences between living and dead cells are compared in Figs. 5 and 6, respectively. When Evans blue was applied to similar strips of onion epidermis, the cytoplasm of some cells stained bright blue while the cytoplasm of other cells remained unstained (colorless). As would be expected from earlier reports (Gaff and Okong’O-Ogala 1971, Taylor and West 1980), cells with stained cytoplasm proved to be dead on the basis of the plasmolysis-deplasmolysis test (Fig. 2). Application of uranin to the strips resulted in cytoplasmic staining which manifested itself as a bright yellow fluorescence under violet light. A plasmolysis-deplasmolysis test showed that dead cells were unstained while living cells had been stained (Fig. 3). Neutral red accumulated in the vacuoles of living cells but not dead cells (Fig. 4). The combined results of replicates of the above tests are given in Table 2. In longitudinal sections of broad bean roots and stems, CFW stained only the cell walls of living cells (Fig. 7) but stained the nuclei as well as the walls in dead cells (Fig. 8). Likewise in CFW-treated Tradescantia staminal hairs, living cells had stained cell walls (Fig. 9) while dead cells had brightly stained nuclei as well (Fig. 10).
Figs. 1-4. Drawings of onion scale epidermal cells. Cytoplasmic volume is exaggerated. Cells labeled with an asterisk were judged to be dead on the basis of a plasmolysis-deplasmolysis test.

Fig. 1. Tissue stained with Calcofluor white M2R. Shaded nuclei were stained and fluoresced bright blue under violet light. Nuclei of other cells did not fluoresce.

Fig. 2. Tissue stained with Evan’s blue. Shaded cytoplasm stained blue. Unshaded cytoplasm did not stain.

Fig. 3. Tissue stained with uranin. Shaded cytoplasm stained and fluoresced yellow. Unshaded cytoplasm did not stain.

Fig. 4. Tissue stained with neutral red. Shaded vacuoles accumulated neutral red and were red. Unshaded vacuoles did not accumulate neutral red. None of the cells which accumulated neutral red in their vacuoles were judged to be dead on the basis of a plasmolysis-deplasmolysis test.
TABLE 2. COMPARATIVE EFFICIENCY OF VARIOUS STAINS IN PREDICTING THE VITALITY OF ONION SCALE EPIDERMAL CELLS

<table>
<thead>
<tr>
<th>Stain</th>
<th>Total Number of Cells Examined</th>
<th>Percent Successful Prediction of Cell Vitality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcofluor white M2R</td>
<td>133</td>
<td>98.5%</td>
</tr>
<tr>
<td>Evans blue</td>
<td>109</td>
<td>98.8%</td>
</tr>
<tr>
<td>Uranin</td>
<td>180</td>
<td>97.8%</td>
</tr>
<tr>
<td>Neutral red</td>
<td>109</td>
<td>98.2%</td>
</tr>
</tbody>
</table>

Animal cells in suspension were treated with 0.1% CFW that was made up in either MEM or MEM with 10% calf serum. Mouse fibroblasts and trout RBC did not stain even after a 60 min incubation with the fluorochrome. However, when mouse fibroblasts and trout RBC were fixed for 5 min in 10% neutral buffered formalin and then treated with CFW, strong staining was observed within 5 min. With both cell types total cellular staining was observed but with the RBC the nuclei often stained more intensely than the surrounding cytoplasm. All cells stained when heated for 20 min at 80°C in the presence of CFW.

Mouse fibroblasts on glass coverslips were incubated with 0.1% CFW that was made up in MEM with or without 10% calf serum. Even after a four-day incubation at 37°C in growth medium with 0.1% CFW, the mouse L cells did not stain. However, when they were fixed for 5 min in 10% neutral buffered formalin and then treated with CFW, staining was observed. This was also true when the cells were killed by heating for 10 min at 65°C. In both cases nuclear staining was stronger than cytoplasmic staining and this was more pronounced when the CFW was made up in MEM with serum (Fig. 11).

A short incubation in 0.1% CFW had no effect on the subsequent growth of A9 cells but continuous exposure appeared to have an inhibitory effect. A9 cells that had been treated with 0.1% CFW for 4 hr formed as many colonies as cells that had not been treated. However, if cells were plated and grown in 0.1% CFW, no colonies resulted.

DISCUSSION

Staining with CFW satisfies one of the important requirements of a vitality test: the stain is nontoxic to cells under the conditions employed for the test. Colvin and Witter (1983) have shown that addition of 0.05% CFW to the growth medium of the bacterium Acetobacter xylinum is more inhibitory to cellulose production and cell growth than addition of 0.01% CFW. The effect of lower concentrations was not reported. According to Hahne et al. (1983), CFW concentrations from 0.1% to 1.0% could be nontoxic to isolated tobacco protoplasts which formed cell clusters during a two-week treatment. They further state "These calli could be induced to form flowering plants, demonstrating that prolonged CW treatment did not severely influence the morphogenic capacity of the cell". Peterson and Perumalla (1984) found that the elongation of corn or onion roots grown in 0.01% CFW was not inhibited over a 24 hr test period. In the present study we found that a 4 hr exposure
FIGS. 5–11. Cells stained with Calcofluor white M2R viewed with violet light.

FIG. 5. Living cells of onion scale epidermis. Cell walls (w) fluoresce bright blue. × 88.

FIG. 6. Dead cells of onion scale epidermis. Nuclei (n) fluoresce bright blue; cytoplasm fluoresces faint blue. × 88.

FIG. 7. Living cells in a longitudinal section of broad bean root. Cell walls (w) fluoresce bright blue. × 87.

FIG. 8. Dead cells in a longitudinal section of broad bean stem. Nuclei (n) fluoresce blue. × 86.


FIG. 10. Dead cells in staminal hair of Tradescantia. Nuclei (n) fluoresce bright blue. × 85.

FIG. 11. Mouse fibroblasts killed by heat. Cytoplasm fluoresces blue; nuclei (n) fluoresce brighter blue and may contain very brightly fluorescing regions. × 72.
VITALITY TEST USING CALCOFLUOR WHITE M2R

...to a CFW concentration as high as 0.1% was nontoxic to onion scale epidermal cells. This was an order of magnitude higher than the concentration which provided a good vitality test. Constant incubation of A9 cells in 0.1% CFW inhibited their further growth but applications of the dye for a length of time appropriate for testing cell vitality (4 hr) was nontoxic and did not influence later growth.

CFW was found to be as dependable as three other vital stains in common use (i.e., Evans blue, uranin and neutral red) in distinguishing between living and dead onion scale epidermal cells when tested against the plasmolysis-deplasmolysis system. In our test system, all four stains correctly predicted cell vitality about 98% of the time. CFW also shows promise as a vitality test for diverse types of cells. A lack of nuclear staining in living cells which contrasted with bright nuclear staining in dead cells was observed in higher plant and animal cells. Both uniseriate layers of cells (onion scale epidermal strips and Tradescantia staminal hairs), and sections of multicellular structures (stems and roots of broad bean) were used successfully. Since a cell must be intact to test its vitality, longitudinal rather than cross sections of multicellular organisms with elongated cells are recommended. The sections must be thick enough to include whole cells (Taylor and West 1980).

Like Evans blue, CFW can be classed as a nonpermeating dye (i.e., it cannot penetrate biological membranes) presumably due to its large size and positive charge. For this reason it is excluded from the cytoplasm of living cells. Staining of nuclei in dead cells is a consequence of CFW penetration through a disrupted plasmalemma or cell membrane. Thus, like the majority of stains for cell vitality and the plasmolysis-deplasmolysis test itself, effective CFW staining depends on membrane permeability. The mechanism of CFW staining of intracellular components has not been elucidated but the stain has been reported to bind to proteins (Holt and Milligan 1974).

CFW staining has both advantages and disadvantages as a vitality test. CFW staining does not require the presence of a vacuole in the cell as does neutral red, or that any internal compartment have an appropriate pH as do neutral red, uranin and other stains which are accumulated by the ion trap method (see Stadelmann and Kinzel 1972 for an account of the mechanisms of accumulation of various vital stains). Uranin and neutral red, two permeating stains, sometimes fail to be taken up by living cells (Currier 1956, Peterson 1979, and Peterson, unpublished results). Such difficulties have not been encountered with nonpermeating stains. Before using CFW as a vital stain, tests should be made to establish whether or not satisfactory staining can be achieved when the cells are killed by the toxicant to be employed. Preliminary results have indicated that in onion scale epidermal cells, the intensity of nuclear staining can vary depending on the killing agent. The nuclei of heat-killed cells stain brightly with CFW, but the nuclei of cells killed with paraquat, DNP, CuCl₂ or ZnCl₂ had less brightly stained nuclei. Regardless of the toxicant used, cytoplasmic staining was constant and can always be used to distinguish between viable and nonviable cells.
A nonpermeating fluorescent vital stain such as CFW could be of value in flow cytometry. The proportion of living animal cells or plant protoplasts in a large population could be determined accurately and rapidly. A permeating fluorochrome has recently been used successfully to assess cell viability by flow cytometry (Frankfurt 1983). As pointed out by Gaff and Okong’O-Ogola (1971), since nonpermeating dyes do not affect the protoplasts of living cells, the same cells may then be used for further experiments. Tests for vitality can also be repeated at intervals on the same cells.

ACKNOWLEDGMENTS

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