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Flow Cytometric Identification of Larval Triploid Walleyes

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Abstract.—A simple and rapid flow cytometric method of identifying triploidy in larval fish was developed. Prolarvae (4.6–8.0 mm total length) of walleye (*Stizostedion vitreum*) were mechanically dissociated into single-cell suspensions and stained with the metachromatic fluorescent dye acridine orange. Dissociation of the prolarvae, application of a two-step acridine orange staining procedure, and identification of ploidy level by flow cytometry was accomplished in under 10 min/sample. This procedure could be adapted for other fish species and could be used to identify other levels of polyploidy or mosaicism.

One method of determining ploidy in fishes is by flow-cytometric measurement of the DNA content of large numbers of fluorescent-stained cells (Thorgaard 1983). Ploidy assessment has usually been accomplished by using red blood cells collected from juvenile or older fishes (Thorgaard et al. 1982; Allen 1983; Allen and Stanley 1983; Utter et al. 1983; Solar et al. 1984; Dillon 1988). Determination of ploidy in larval fishes could be a useful technique when larger fishes are not available or when early ploidy determination is advantageous.

The two DNA fluorescent dyes previously used in flow-cytometric research into triploid fish are diamidino-2-phenylindole (DAPI; Thorgaard et al. 1982; Utter et al. 1983; Solar et al. 1984) and propidium iodide (PI; Allen 1983; Allen and Stanley 1983). The acridine orange (AO) staining protocol of Darzynkiewicz et al. (1976) was used in the current study. The purpose of this paper is to describe flow cytometric methods for determining ploidy level of larval walleyes (*Stizostedion vitreum*), with AO as the fluorescent stain.

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Methods

Yolk sacs were first removed from prolarval walleyes (1–2 d posthatch, 4.6–8.0 mm total length) to decrease the amount of debris in the sample. Individual fish were placed into 75 × 15-mm disposable tubes containing 0.50–0.75 mL of Hank's balanced salt solution (HBSS; Gibco Laboratories, Grand Island, New York) at 4°C. Calcium and magnesium were omitted to minimize intercellular binding; absence of cation is especially effective for dissociating embryonic tissues (Waymonth 1982). Each prolarva was mechanically dissociated with a 1-mL syringe and 23-gauge needle until no intact tissue pieces were observed. The syringe and needle were found to be more efficient in dissociating tissues than a Pasteur pipette. The tissue in HBSS was drawn in and expelled from the syringe about five times. The whole-body cell suspension was diluted to 1.0 mL with 4°C HBSS and kept on liquid ice until cells could be stained.

The protocol for flow cytometric measurement was a modification of that used by Darzynkiewicz et al. (1976) and is described by Evenson et al. (1985) and Dillon (1988). The 1.0-mL whole-body cell suspension was diluted to a concentration ($1–2 \times 10^6$ cells/mL) suited for measurement of 100–200 cells/s. A 0.2-mL aliquot of the diluted cell suspension was mixed with 0.4 mL of a low-pH detergent solution (0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100; pH 1.4). After 30 s, 1.2 mL of staining solution (6 μg AO/mL [chromatographically purified, Polysciences, Warrington, Pennsylvania], 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, and 0.1 M citric acid; pH 6.0) were added to the cell suspension. Under these staining conditions, AO intercalates into native DNA and fluoresces orthochromatically green with maximum emission at 530 nm (Lerman 1963), whereas single-stranded RNA stains metachromatically red with maximum emission at 640 nm (Bradley and Wolf 1959). The intensity of green and red fluorescence is proportional to cellular DNA and RNA content, respectively (Darzynkiewicz 1979).

Cell suspensions were analyzed 3 min after AO staining with a Cytofluorograf II model 30-L flow cytometer with a Lexel 100 mW argon ion laser. The flow cytometer was interfaced with a 2150 data-handling system (Ortho Diagnostics, Inc.,

Westwood, Massachusetts). The green (515–530-nm) and red (>600-nm) fluorescence emitted by each cell after laser beam excitation (35 mW, 488 nm) was directed through photomultiplier tubes and quantified.

To serve as a reference marker, 9.93 μm, 25% bright fluorospheres (Coulter Corporation, Hialeah, Florida) were added to the cell suspension immediately after addition to the AO stain. These fluorospheres served as a ploidy reference marker relative to, and in addition to, DNA fluorescence values of known diploid walleye cells.

Mean peak fluorescence values were calculated by the 2150 data-handling system and displayed for each sample. Although both green (DNA) and red (RNA) fluorescence values were recorded, DNA ploidy values were determined from only the green fluorescence values. Mean peak fluorescence values are expressed as relative channel numbers in linear mode (e.g., if a diploid cell had a peak DNA staining value of 200 channels, a tetraploid cell would have a theoretical value of 400 channels). Analysis of single and doublet fluorospheres verified the linearity of the instrumentation.

Results

In all, 161 prolarval walleyes were mechanically dissociated into single-cell suspensions. Yolk-sac removal, mechanical dissociation, and dilution with HBSS were performed in 2–3 min. The AO staining protocol and subsequent flow cytometric analysis were accomplished in about 5 min/sample. Cytograms of dual-parameter red and green fluorescence were recorded for each sample. Figure 1 (A, C) shows an example of raw data demonstrating an obvious difference in the DNA and RNA stainability between samples of different ploidy. Frequency histograms of relative peak green fluorescence (i.e., relative DNA content per cell) readily identified the walleyes as diploid or triploid (Figure 1B). Diploid mean peak fluorescence values ranged from channels 195 to 224; triploid mean peak fluorescence values ranged from channels 290 to 338.

Diploid–tetraploid mosaicism was identified for one sample (Figure 1C, D). Individual fish exhibiting mosaicism have cell populations containing more than one level of ploidy. Two separate peaks of fluorescence were discernable. Mean peak flu-

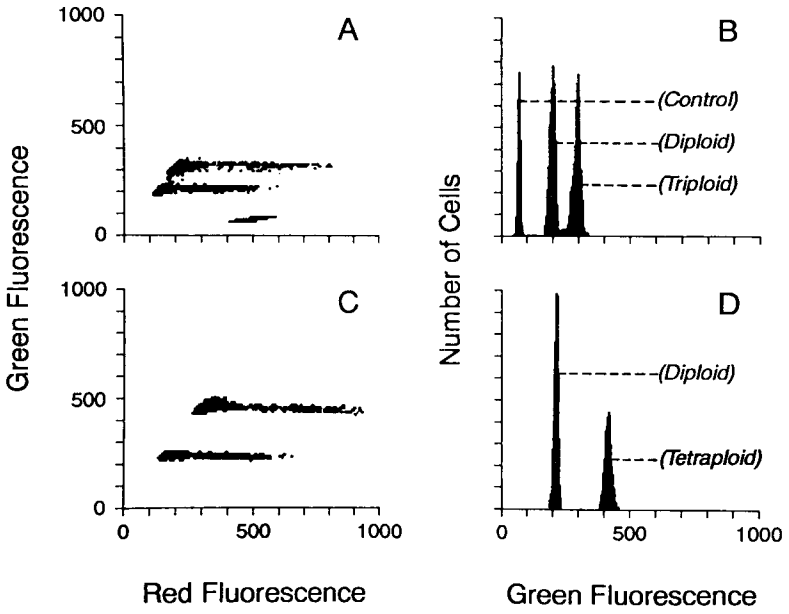

FIGURE 1.—**(A)** Cytogram of acridine orange-stained cells from diploid and triploid prolarval walleyes and fluorescent beads under green and red fluorescence. **(B)** Frequency histogram of cells under green fluorescence for data shown in (A). **(C)** Cytogram of acridine orange-stained cells from a single prolarval walleye showing two populations of ploidy levels under green and red fluorescence. **(D)** Frequency histogram of cells under green fluorescence for data shown in (C). Fluorescence measurements are cytometer channel numbers.

orescence values were channels 219 and 439 for diploid and tetraploid peaks, respectively.

Discussion

Use of larval tissues to identify triploidy allows for determination of triploid rates within days of conducting induction experiments. Early knowledge of triploid induction results is beneficial for numerous reasons. If larval tissues are used, the fish need not be reared to a sufficient size to facilitate blood sampling. The advantage of this is exemplified by walleye fry, which can experience variable and low survival during rearing (Li and Mathias 1982). Time is forfeited and large losses of experimental fish are risked if larvae are not tested for triploidy. Furthermore, by testing larval tissues and then retesting blood cells at later life stages, differential survival between diploid and triploid fish can be estimated.

If improved accuracy of treatment regimes is an objective of triploid induction experiments, testing larval tissues is also advantageous. For example, walleye spawning seasons are variable and often last up to 34 d (Colby et al. 1979). Walleye development rates are dependent upon water temperature, but it is possible to have hatching occur a few days after fertilization (Nickum 1986). Testing for triploidy at early larval stages permits numerous induction experiments during a season; data generated in that same season can be used to adjust induction parameters as needed.

Finally, if the results of triploid induction experiments are obtained from larvae, unwanted groups of experimental fish can be disposed of. Rearing space can be more efficiently used and costs reduced.

One drawback to using larval tissues for flow cytometry is that fish must be sacrificed. However, this method for identifying triploidy is more time-efficient than chromosome counting, which also often requires sacrificing fish when the procedure is done at early life stages.

Flow cytometry is only successful if a single-cell suspension can be obtained, whether it involves teasing cells from fragments of tissue, pipetting small fragments of tissue, or forcing pieces of tissue through various sizes of needles (Brattain 1979). Mechanical dissociation of prolarval walleyes with a 1-mL syringe and 23-gauge needle was time-efficient and caused less cell destruction than other needle sizes. The embryos or larvae of other species may be larger and require initial mincing before dissociation with a needle and syringe. It may be necessary to change from larger-diameter to smaller-diameter needles to complete the dissociation process.

The two-step AO staining protocol allowed samples to be analyzed within minutes of fluorescent dye application. Flow cytometric studies with DAPI and PI as fluorescent stains have required 40–60 min or longer for preparation and staining of the DNA (Allen and Stanley 1983; Solar et al. 1984).

The larval-dissociation method and two-step AO staining protocol described could be adapted to other fish species and could be modified to enable the use of ethanol as a fixative for determination of ploidy levels at later dates (Ewing 1989). Because flow cytometry can be used to analyze large numbers of cells in a matter of seconds, it has provided a rapid and reliable approach for assessing relative DNA contents of various cell types (Crissman et al. 1979). Because diploid, triploid, and diploid–tetraploid mosaic fish were successfully identified in this study, the methods described here should be applicable to identification of tetraploid fishes. There is some interest in tetraploid induction because a cross between a tetraploid and diploid may produce triploid offspring (Thorgaard and Allen 1987).

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