Made in United States of America
Reprinted from THE AUK
Vol. 108, No. 1, January 1991

The Use of Flow Cytometry for Rapid Identification of Sex in Birds

TERRENCE R. TIERSCH,[1] RONALD L. MUMME,[1,2] ROBERT W. CHANDLER,[3] AND DEAN NAKAMURA[4]

Unambiguous identification of the sex of live birds is critical in numerous areas of avian research, including studies of alternative reproductive tactics, sex-ratio manipulation, and conservation biology (e.g. van Rhijn 1973, Snyder and Snyder 1989, Stamps 1990). However, identification of sex can be problematic for researchers dealing with young birds or sexually monomorphic species. Techniques currently available for identification of sex, primarily based on cytogenetics or biochemical genetics, are time-consuming, expensive, or require considerable amounts of tissue. The use of sex-specific DNA probes can overcome some of these shortcomings (Quinn et al. 1990), but this technique is time-consuming also, and probes may not be equally effective with DNA of species from divergent taxonomic groups. Other techniques such as laparotomy do not always work with nestlings or sexually immature birds, are potentially stressful, and may be inadvisable when dealing with threatened or endangered species.

Flow cytometry has been used to measure nuclear DNA content in a wide variety of organisms (e.g. Tiersch et al. 1989). In addition, flow cytometry has been used to identify differences in the DNA content of male and female humans (Deaven 1982, Elias et al. 1988) and other mammals (Kent et al. 1988). Recently, Nakamura et al. (1990) have developed a rapid and inexpensive procedure for sexing live birds through the use of flow cytometry. This procedure allows sex to be assigned on the basis of small but consistently measurable differences in the nuclear DNA content of males and females. Nuclear DNA content is a sexually dimorphic trait in birds because (1) in those species with heteromorphic sex chromosomes, the W chromosome is consistently smaller than the Z chromosome, and (2) males are homogametic (ZZ) and females are heterogametic (ZW). Our purpose in this commentary is to introduce flow cytometry to research ornithologists who might find the technique useful for the identification of sex in live birds.

The flow cytometer measures fluorescence, size, and granularity of cells. Most uses to date have been in areas of medicine (see review by Lovett et al. 1984). Flow cytometry has been applied to the study of cell surface receptors, cell pH, DNA synthesis, characteristics of the cell membrane, DNA base ratios, various cell and nuclear proteins and ions, phagocytosis and oxidative burst, cell RNA content, chromatin structure, and cytoskeletal organization. Other applications include karyotyping, testing for the effects of environmental mutagens (e.g. Deaven 1982, Bickham et al. 1988) and the detection of abnormalities in ploidy level (e.g. Allen 1983). The flow cytometer, also

[1] Ecological Research Center, Department of Biology, Memphis State University, Memphis, Tennessee 38152 USA.

[2] Present address: Department of Biology, Allegheny College, Meadville, Pennsylvania 16335 USA.

[3] Puckett Laboratory, 4200 Mamie Street, Hattiesburg, Mississippi 39402 USA.

[4] The National Law Center, George Washington University; Sughrue, Mion, Zinn, Macpeak & Seas, 2100 Pennsylvania Avenue NW, Suite 800, Washington, D.C. 20037 USA.

called a fluorescence activated cell sorter, can be used to sort cells whether dead or alive from a mixture at a high rate of speed, even when the cell type of interest is present in very low proportion.

The most common application of flow cytometry has been in DNA analysis. Various stains can be used to render nucleic acids fluorescent and allow the detection of DNA aneuploidy and altered cell cycles in cell populations. The characterization of breast cancer cells by this method, for example, has become a standard of medical practice. The DNA content of target cells is usually quantified relative to a standard DNA content in cells from a reference species (Vindeløv et al. 1983, Lee et al. 1984). In brief, blood cells are lysed in buffered detergent, and the nuclei are stained with propidium iodide (Krishan 1975), a fluorochrome that intercalates within the helices of DNA in direct proportion to the mass of DNA present. A laser beam is used to excite the molecules of propidium iodide and the fluorescence emitted by each nucleus is collected and digitized for computer analysis. Differences in the DNA content of cells can be calculated from differences in their level of fluorescence.

When flow cytometry was applied to identification of sex in birds, alternative values of DNA content, attributable to differences in size of the Z and W sex chromosomes, were found in males and females in 29 species representing seven orders (Nakamura et al. 1990). In species that possess heteromorphic sex chromosomes, sex was identified correctly in 119 of 120 birds. The magnitude of the difference in DNA content between males and females appeared to be proportional to the magnitude of the difference in size between the Z and W chromosomes. This is consistent with the finding that in a ratite (*Dromaius novaehollandiae*), which has no or little heteromorphism of the sex chromosomes (Ansari et al. 1988), sex identification was not possible. The W chromosome of the Bald Eagle (*Haliaeetus leucocephalus*) is not much smaller than the Z (Au et al. 1975), and difference between male and female DNA mass, although consistent, was small. It may be that the degree of sex-chromosomal heteromorphism and corresponding DNA content difference found in the Bald Eagle represents a lower limit of sensitivity for the assay.

Sex could be identified within an hour of sample collection in those bird species that possessed heteromorphic sex chromosomes. Only 5–10 min were required for analysis after the flow cytometer had been aligned optically and standardized. Accurate determinations were made in samples that had been refrigerated for up to 2 weeks or had been stored at −20°C. Microliter volumes of whole blood, obtained by clipping a toenail or by brachial puncture, provided material for multiple analyses. The technique can be applied to any type of nucleated cells including those in feather pulp.

Flow cytometry was most effective in species that possessed a large difference in the size of the Z and W chromosomes. However, because the approach is based on the differential quantity of nuclear DNA of males and females, other influences such as chromosomal polymorphism or occurrence of repeated DNA sequences could reduce accuracy. Accordingly, flow cytometry should not be considered in all cases as a substitute for cytogenetic or other techniques (including sex-specific DNA probes) used for the identification of sex. Furthermore, the technique is limited by the need for concurrent male and female control samples, and thus would not be useful for analysis of a single bird. Flow cytometry would be useful in identifying the sex of chicks in relation to their parents, or in studying large groups of birds that can be analyzed simultaneously.

In spite of these potential shortcomings, flow cytometry offers ornithologists a quick and effective means to determine the sex of live birds from small samples of blood or other nucleated tissues. Like the sex-specific DNA probe developed recently for geese by Quinn et al. (1990), flow cytometry may prove to be a potentially useful and powerful tool to ornithologists whose research involves accurate identification of sex in live birds.

Literature Cited

Allen, S. K. 1983. Flow cytometry: assaying experimental polyploid fish and shellfish. Aquaculture 33: 317–328.

Ansari, H. A., N. Takagi, & M. Sasaki. 1988. Morphological differentiation of sex chromosomes in three species of ratite birds. Cytogenet. Cell Genet. 47: 185–188.

Au, G. U., N. S. Fechheimer, & S. Soukup. 1975. Identification of the sex chromosomes in the Bald Eagle. Can. J. Genet. Cytol. 17: 187–191.

Bickham, J. W., B. G. Hanks, M. J. Smolen, T. Lamb, & J. W. Gibbons. 1988. Flow cytometric analysis of the effects of low-level radiation exposure on natural populations of slider turtles (*Pseudemys scripta*). Arch. Environ. Contam. Toxicol. 17: 837–841.

Deaven, L. L. 1982. Application of flow cytometry to cytogenetic testing of environmental mutagens. Pp. 325–351 *in* Cytogenetic assays of environmental mutagens (T. C. Hsu, Ed.). Totowa, New Jersey, Allenheld, Osmun Publ.

Elias, S., R. W. Chandler, & S. Wachtel. 1988. Relative deoxyribonucleic acid content of interphase leukocytes by flow cytometry: a method for indirect diagnosis of chromosomal abnormalities with potential for prenatal diagnosis. Am. J. Obstet. Gynecol. 158: 808–818.

Kent, M., R. Chandler, & S. Wachtel. 1988. DNA analysis by flow cytometry. Cytogenet. Cell Genet. 47: 88–89.

Krishan, A. 1975. Rapid flow cytofluorometric anal-

ysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. 66: 188–193.

LEE, G. M., J. T. THORNTHWAITE, & E. M. RASCH. 1984. Picogram per cell determination of DNA by flow cytofluorometry. Anal. Biochem. 137: 221–226.

LOVETT, E. J., III, B. SCHNITZER, D. F. KEREN, A. FLINT, J. L. HUDSON, & K. D. MCCLATCHEY. 1984. Application of flow cytometry to diagnostic pathology. Lab. Invest. 50: 115–140.

NAKAMURA, D., T. R. TIERSCH, M. DOUGLASS, & R. W. CHANDLER. 1990. Rapid identification of sex in birds by flow cytometry. Cytogenet. Cell Genet. 53: 201–205.

QUINN, T. W., F. COOKE, & B. N. WHITE. 1990. Molecular sexing of geese using a cloned Z chromosomal sequence with homology to the W chromosome. Auk 107: 199–202.

SNYDER, N. F. R., & H. A. SNYDER. 1989. Biology and conservation of the California Condor. Curr. Ornithol. 6: 175–267.

STAMPS, J. A. 1990. When should avian parents differentially provision sons and daughters? Am. Nat. 135: 671–685.

TIERSCH, T. R., R. W. CHANDLER, S. S. WACHTEL, & S. ELIAS. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry 10: 706–710.

VAN RHIJN, J. G. 1973. Behavioural dimorphism in male Ruffs, *Philomachus pugnax* (L.). Behaviour 47: 153–229.

VINDELØV, L. L., I. J. CHRISTENSEN, & N. I. NISSEN. 1983. Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. Cytometry 3: 317–322.

Received 22 August 1990, accepted 22 August 1990.