



TECHNICAL REPORT

Analysis of Three Methods to Determine Ploidy in Sturgeon: Blood Smear, Flow Cytometry and Coulter® Counter

WESTERN REGIONAL AQUACULTURE CENTER

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Introduction

White sturgeon (*Acipenser transmontanus*) have been commercially produced in California and Idaho since the early 1980s, with the original broodstock collected from the Sacramento Bay-Delta and the Columbia River.^{1,2}

These early successes re-stimulated interest in conservation hatchery production of white sturgeon for river enhancement programs.³ Early culture efforts were not concerned with sturgeon ploidy, and ploidy studies were not initiated until the 1990s.⁴

Aquaculture has benefited from the manipulation of ploidy in both fish and shellfish, and direct induction of triploidy, using thermal, pressure, chemical, or electrical shock, has been used in numerous fish and shellfish species. In some triploid animals, there are performance improvements, such as faster growth (since they are functionally sterile), and because of this, triploids can also be used in genetic containment programs.⁵

During artificial spawning, white sturgeon may exhibit spontaneous autopolyploidy, whereby spontaneous triploids (12N) are produced when the normal ejection of the second polar body during meiosis is disrupted.⁶ Hatchery personnel were not intentionally inducing triploidy, so the discovery of spontaneous triploids was unexpected.

White sturgeon ploidy was found to be more complex than for other triploid farmed fish. White sturgeon are considered to be evolutionary octoploids (8N), with 8 copies of each chromosome, and a total of approximately 240 chromosomes.⁷ White sturgeon 12N triploids have a genome that is 1.5 times larger (~360 chromosomes) than 8N fish and have been identified in both commercial aquaculture and conservation hatcheries.^{7,8} In addition,

female 12N white sturgeon, which are not sterile, have been unintentionally crossed with 8N males to produce progenies of intermediate ploidy (10N) with ~300 chromosomes.

Although 10N and 12N males mature normally at age 4–5 years, the females exhibit delayed or impaired maturation.⁹ This phenomenon of spontaneous autopolyploidy prompted interest in the identification of sturgeon ploidy as it affects both the commercial sturgeon industry and conservation hatcheries. In general, California white sturgeon females mature between 7–10 years of age, while Idaho stocks reared in cooler temperatures take 12–16 years to mature.

In commercial sturgeon aquaculture, meat and caviar are the terminal products. The first return on investment is when the fish are sexed at body size 4.5–9.0 kg (age 3–4 years, in California), whereupon males and a percentage of slow-maturing females are sold for meat. The remaining females are retained for an additional 4–6 years, when the ova mature, and they are harvested for both meat and caviar production, or used to replenish broodstocks.¹⁰ There are considerable costs in terms of both maintenance and resource commitment to culture females for caviar, and therefore feeding late or non-maturing female fish for several additional years reduces the profitability of the commercial caviar industry.

Conservation managers also want methods of determining ploidy of broodstock fish before spawning induction and incidence of spontaneous autopolyploidy in the subsequent families produced in the hatchery to maintain the prominence of the 8N population in the natural environment.⁸

Overall Project Background and Objectives

After the first 12N sturgeon triploids were identified at a commercial hatchery, a multi-state research project, “Determining causes, costs and benefits of triploidization to improve sturgeon caviar production,” was awarded by the Western Regional Aquaculture Center (WRAC).

Major Objectives

The project’s major objectives were to:

- Validate blood-smear analysis to assign sturgeon ploidy.
- Identify variables associated with 12N production, such as oocyte ageing.
- Evaluate differences in sex ratio, growth, and physiological performance of 12N triploids and 10N intermediate ploidy white sturgeon relative to 8N individuals.
- Examine ploidy levels of existing broodstock and non-maturing females and evaluate the effect of triploidization on caviar farming.
- Develop outreach materials to disseminate project results and discuss the implications for sturgeon farms.

These objectives were also relevant to sturgeon conservation aquaculture programs seeking to cease production of any 12N individuals that might be released into wild populations.

In this report, we refer to the normal ploidy of white sturgeon as 8N, triploids as 12N, and intermediate ploidy sturgeon as 10N. We discuss the evaluation of technologies used to determine sturgeon ploidy in a commercial or conservation aquaculture facility. We also present the findings of the blood-smear validation study to assign ploidy of white sturgeon broodstock from farms in California and from the Kootenai Tribe of Idaho’s conservation hatchery.

A critical component of ploidy determination in hatcheries is an accurate, consistent, and relatively fast method of ploidy analysis. Although karyotyping (a test to identify and evaluate the size, shape, and number of chromosomes in a sample of body cells), allows for the actual counting of chromosomes and determining ploidy,

it is very time consuming and requires specialized techniques under laboratory conditions. Due to these challenges for use on farms, we did not consider karyotyping to be a practical approach for determining sturgeon ploidy.

The overall goal of this project was to evaluate and compare available methods used for ploidy analysis and to determine which method would be most suitable for use in commercial and conservation fish hatcheries based on speed, accuracy, and accessibility. We present our findings for three common approaches: 1) blood smear analysis, 2) flow cytometry, and 3) Coulter® Counter to evaluate sturgeon ploidy. We also include the pros and cons for each method (summarized in Table 1, page 8).



Photos: Andrea Schreier



Techniques for Evaluating Ploidy

Flow Cytometry

Flow cytometry instruments rapidly analyze single cells stained with one or more fluorescent reagents that flow in a buffered salt-based solution past single or multiple lasers. The fluorescence signal detected as the reagents are excited by the laser is used to determine size and complexity of the cells or cellular structures. Flow cytometry analysis for measuring DNA content in red blood cell (RBC) nuclei is considered the highest standard of technology to validate other indirect techniques, like measuring RBC size in blood smear analysis or RBC nuclei volumes in Coulter® Counter analysis.

One advantage of flow cytometry is that it measures actual DNA content in the RBC nuclei, and the analysis and subsequent results of blood samples using flow cytometry are relatively rapid once the samples are prepared.⁸

There are a number of disadvantages to using flow cytometers on a farm setting: First, there is the capital and operating cost to maintain a flow cytometer. A small flow cytometer can cost up to \$20,000 USD, and blood sample preparation requires trained staff with technical expertise. Furthermore, the solutions employed in flow cytometry are toxic and carcinogenic chemicals, requiring additional hazardous waste disposal costs, which limits its potential application in fish hatcheries. We believe that flow cytometry is also not very practical for either commercial or conservation hatcheries.

Methodology: In our analyses, RBCs are prepared for flow cytometry by diluting them to a concentration of 10⁶ cells/mL and staining them with the fluorescent dye propidium iodide (PI).⁷ Propidium iodide binds to DNA so the amount of PI fluorescence detected by the flow cytometer is proportional to the amount of DNA in a cell (Figure 1).

Blood Smear Analysis

Although blood smear analysis is relatively inexpensive, the total process is both slow and time consuming. After blood smear preparation, 15–20 minutes are required to image and analyze a single smear.¹¹ However, its major drawback is lack of accuracy in separating ploidy groups, and it would also require validation with a second method such as a flow cytometer or a Coulter® Counter before use. It should not be used as a stand-alone method.

Results of the blood smear studies showed a high degree of overlap in red blood cell (RBC) long-axis

length between 8N and 10N white sturgeon from both California and Idaho. In the California fish, there was also some overlap between 10N and 12N individuals, and among Idaho fish there was even a high degree of overlap between 8N and 12N individuals in some families.¹¹ We do not recommend blood smear analysis as a viable methodology to accurately distinguish between 8N, 10N, and 12N white sturgeon for either commercial or enhancement facilities.

Methodology: To create blood smears, blood was drawn using a 1 cc syringe with a 22-gauge needle from the caudal vasculature, then 2–3 µL samples were placed onto two glass slides for each individual fish. The blood was gently smeared across the surface of the slides using a third, clean glass slide held at a 45° angle. After drying, blood smears were fixed in methanol for 2 minutes. Slides were then stained in Wright Giemsa (Sigma 5Aldrich®, St. Louis, Missouri; 80% methanol, 19% glycerin, 1% Giemsa's stain) for 30 seconds, and then soaked in deionized water for 5 minutes, and followed by 20 dips in fresh deionized water.

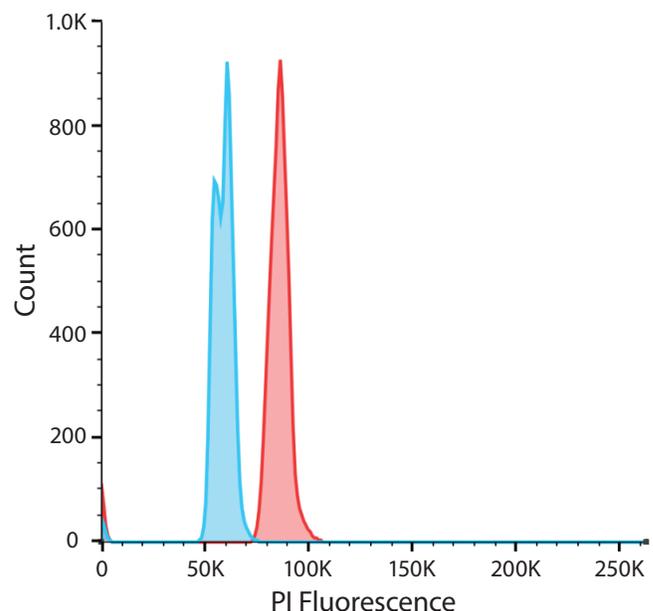
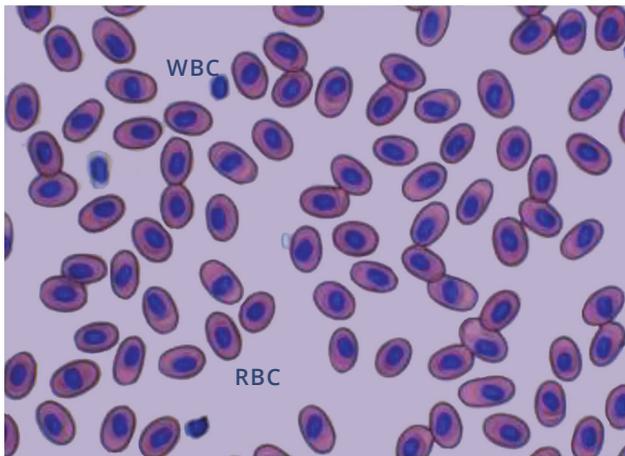


Figure 1. Flow cytometry plot showing differences in fluorescence between 8N (blue) and 12N (pink) white sturgeon red blood cells stained with propidium iodide (or PI). Count refers to the number of cells detected per sample.

After drying overnight, blood smears were imaged using a Lumenera Corp Infinity 2 microscope camera with 40X magnification, with enough images taken per slide to obtain 40 intact RBCs for measurement (Figure 2). Images were analyzed using the software program ImageJ, and then evaluated by Linear Discriminant Analysis (LDA). The analyses examined both length and width measurements of RBCs, based on the fact that increased DNA content is reflected in a larger RBCs and larger RBC nuclei size.^{12, 13}

a.



b.

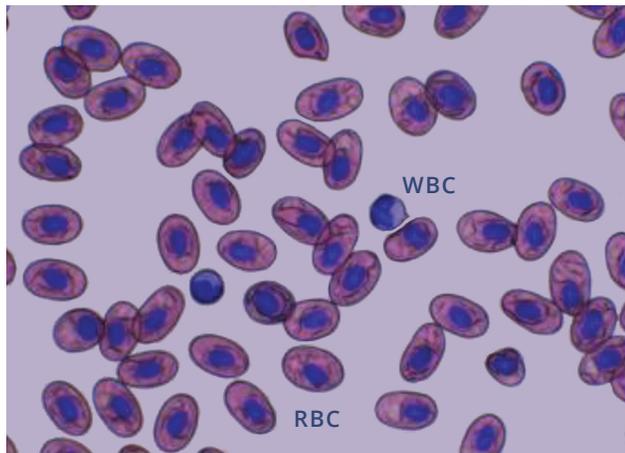


Figure 2. Example of Wright Giesma stained blood smears from an 8N (a) and 12N (b) white sturgeon imaged at 40X magnification. RBC indicates example red blood cells and WBC indicates example white blood cells.

Adapted from Schreier et al. (2021).

Coulter® Counter

Coulter® Counter instruments can count and measure the volume of RBC nuclei (if the cells are lysed, as is the case for ploidy analysis) or other particles suspended in a conductive fluid at low concentration. Particle size and density (nuclei ploidy and concentration) are determined by collecting a series of pulsed fluid movement through an aperture over time and knowing the total volume of fluid drawn through the aperture.^{14, 15}

We found that this technology had advantages over both blood smear and flow cytometry technologies in sturgeon ploidy applications. White sturgeon ploidy results were 100% accurate when compared to flow cytometry results.¹¹ Our research demonstrated that white sturgeon of different ploidies had significantly different nuclear volumes¹¹ and simply utilizing the mode value of any given blood sample would clearly distinguish between an 8N and 12N individual (Figures 3, 4), and it can also distinguish a 10N individual, falling in-between the 8N and 12N mode distributions (Figures 3, 4).

Other advantages in support of the Coulter® Counter over the other methods include: 1) toxic chemicals are not needed for the analysis, 2) preparation of samples is not complex and only requires a small volume of blood (1–2 µL sample) from each sturgeon (Figure 5), and 3) results from the analysis (running time) for each sample can be obtained in only 30–40 seconds, making it possible to process 400 to 600 samples per day. In addition, blood samples do not need to be processed immediately. We found that the 1 µL blood samples, when placed into the cell counting vials, can be stored in a refrigerator for up to 6 days and still give accurate results,¹¹ and for field collected blood samples, when placed in a heparin vacutainer or heparin microtainer, can be stored for 30 days in a refrigerator and then processed with 100% accuracy.⁹

The cost of a Coulter® Counter is the greatest disadvantage over the other methods, but not beyond the operational budgets of large white sturgeon meat and caviar producers and most conservation hatchery programs. The current price for a new Multisizer 4e Coulter® Counter is about \$45,000 (Nov 2021); however, there may be used or refurbished units available, including older, discontinued models such as the Z2 and the Multisizer 3. The use of a Coulter® Counter is applicable for hatchery programs requiring analysis of hundreds to thousands of juvenile sturgeon each year, such as conservation programs needing to validate ploidy prior

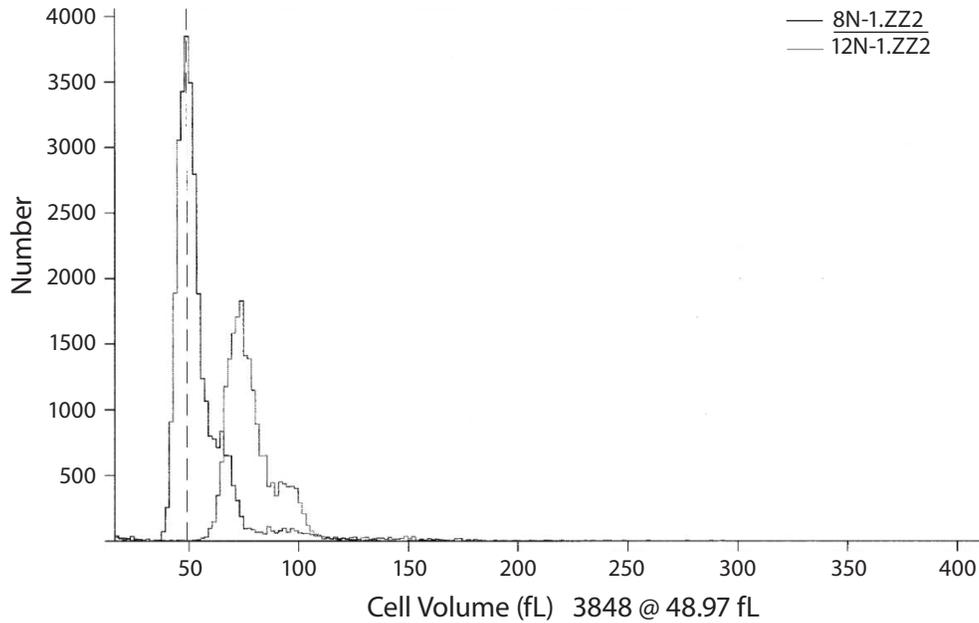


Figure 3. Frequency distributions of red blood cell nuclei volume for an 8N (left) and 12N (right) white sturgeon, as analyzed by a Z2 Coulter® Particle Count and Size Analyzer. Mode nuclei volumes were 48.97 fL and 73.89 fL, for the 8N and 12N, respectively. Number of nuclei measured at each volume is on the Y-axis. Femtoliter (fL) is a metric unit of volume that is equal to one cubic micrometer (μm^3).

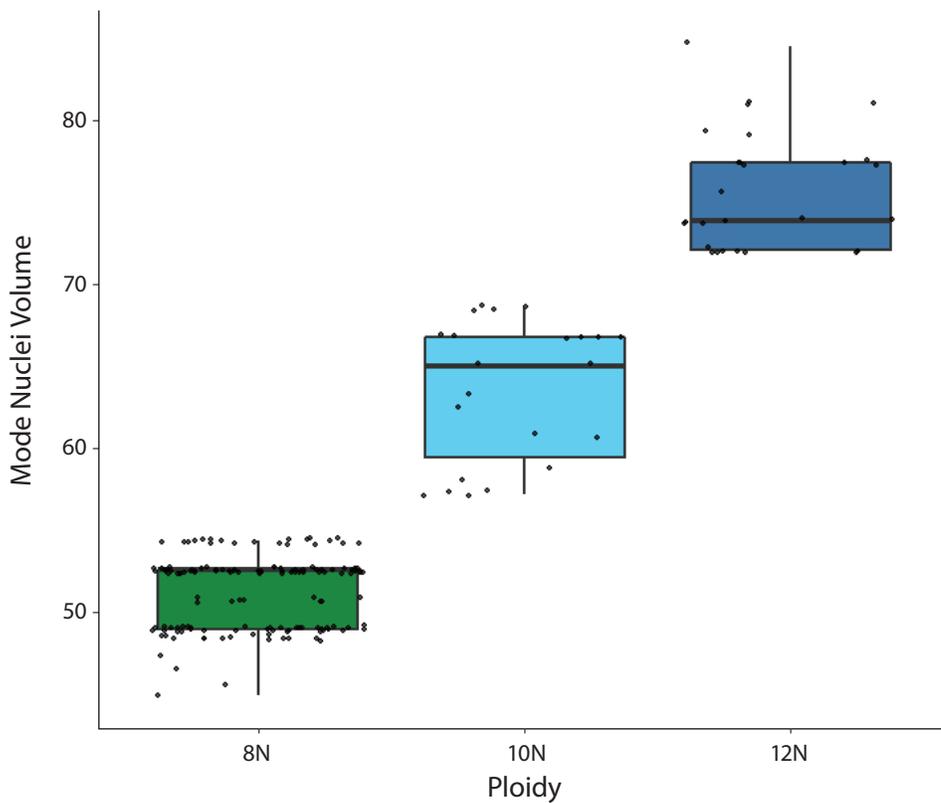


Figure 4. Boxplots illustrating mode red blood cell nuclei volume data for 8N, 10N, and 12N California captive reared female white sturgeon, 26–105 kg in body weight and 10–16 years old. Lines in box plots indicate median values.

to releasing hatchery-reared sturgeon into various river systems.

Other options for smaller producers or those looking to defray the expense of an instrument include a cooperative or contracting with an established laboratory to conduct the blood analyses, which is likely the best option for commercial aquaculture facilities needing to verify their broodstock are all 8N, so as not to inadvertently cross any 10N or 12N broodfish. Typically, their broodstock numbers are < 100 animals, and after the initial broodstock are evaluated, there is only the need to analyze new incoming broodstock every few years, as they are added to the population.^{8, 11}

Methodology: The collection of blood from large broodstock is typically done with a 5–10 mL heparin vacutainer (an equivalent of 5000–10,000 μ L blood sample for comparison) and a 1½" long, 21–22 gauge needle (https://www.youtube.com/watch?v=_gBuUHt11hw).

The vacutainers are kept cool with gel packs and when back in the lab, the required 1 μ L of blood is pipetted from each vacutainer into the pre-prepared blood cell counting vials that are utilized by the Coulter® Counter instrument.



Photo: Fred Conte

Figure 5. Non-lethal collection of blood from a fingerling white sturgeon. The fish are anesthetized in buffered tricaine methane sulfonate, and blood is collected from the caudal vasculature with an insulin syringe, with a 31-gauge needle. As soon as blood is seen entering the barrel of the syringe, collection is stopped, as the amount of blood in the bore of the needle itself is enough for analysis. Thousands of small sturgeon have been processed using this technique, with no mortalities.

The fluid used by the Coulter® Counter to mix with the blood sample is usually an electrolyte solution in water or in an organic liquid. The Counter measures the nuclei of lysed RBCs as the nuclei are passed through a small aperture in the wall of an electrical insulator, called the aperture tube (Figure 6). An electrical current is also passed across the aperture, which creates an electrical sensing zone in and around the aperture tube. As each nucleus enters the aperture, it displaces a volume of the fluid equal to its own volume, and this results in a short duration electrical pulse created by each particle, which is proportional to the volume displaced by the nuclei. The mode nuclei volume, rather than mean or median nuclei volumes, was found to be preferable because it minimizes the influence of non-target particles (detritus, whole RBCs) in the sample.

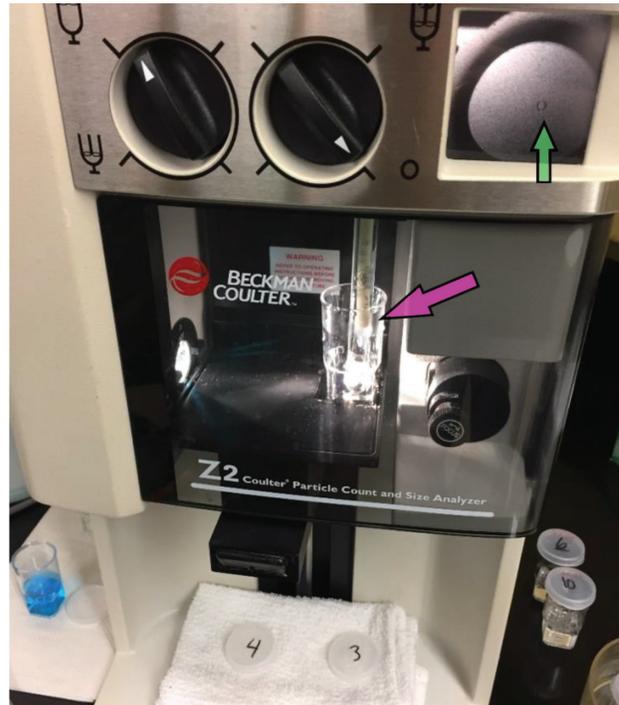


Photo: Courtesy of Andrea Schreier

Figure 6. The Z2 Coulter® Particle Count and Size Analyzer. The pink arrow indicates the glass aperture tube immersed into a blood cell counting vial containing 10 mL of Isoton II (saline) plus three drops of Zap-o-globin (lysing reagent) and 1 μ L of white sturgeon blood. The green arrow directs you to the magnified 100-micron opening of the aperture tube. While samples are running, this window is observed for any aperture blockages that occasionally occur.

Conclusion and Project Impacts

We found that Coulter® Counter analysis is an accurate and accessible method for ploidy analysis in both commercial and conservation white sturgeon facilities.

As a result of the findings from this USDA WRAC-funded project, several commercial aquaculture facilities are working with laboratories, such as the Genomic Variation Lab (Davis, California) and the Vancouver Island Trout Hatchery (Duncan, British Columbia, Canada), for Coulter® Counter ploidy analysis of white sturgeon broodstock.

Another important outcome of the research is that technology transfer and method adoption have occurred at several white sturgeon conservation facilities in the Columbia-Snake River drainage and also on the Kootenai River.

Following this project, other research on the ploidy of cultured shortnose and Atlantic sturgeon using a Multi-sizer 3 Coulter® Counter is now occurring outside of the US at the Marine Science Centre, in St. Andrews, New Brunswick, Canada.

Table 1. Summary of the pros and cons of flow cytometry, blood smear analysis, and Coulter® counter analysis for sturgeon ploidy determination. Adapted from Schreier et al. (2021).

METHOD	PROS	CONS
Flow cytometry	<ul style="list-style-type: none"> • Gold standard • Highly accurate • Requires no validation 	<ul style="list-style-type: none"> • Expensive instrumentation required • Requires significant expertise • Requires standard • DNA stains are toxic to humans • Time consuming • Not ideal for remote locations • Can only screen juveniles >25 g non-lethally
Blood smear	<ul style="list-style-type: none"> • Requires least amount of equipment and expertise • Only 2–3 µl of blood required • No standard required 	<ul style="list-style-type: none"> • Least accurate method • Sample prep and analysis time longer than Coulter® Counter • Requires validation with flow cytometry or Coulter® Counter • Immature red blood cells can confound analysis
Coulter® Counter	<ul style="list-style-type: none"> • Highly accurate • Required chemicals less toxic • Simple sample transport and storage • Simple sample preparation • Only 1–2 µl of blood required • Rapid throughput 	<ul style="list-style-type: none"> • Expensive instrumentation required • Must standardize sample storage and analysis conditions

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Photo: Barbara Rasco



The Western Regional Aquaculture Center (WRAC) is one of five centers in the United States. Developed to take advantage of the best aquaculture science, educational skills, and facilities within a twelve-state area, WRAC works to enhance viable and profitable commercial aquaculture production in the U.S. for the benefit of producers, consumers, and the American economy.

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