**New Chapter**

# *Flow Cytometry for the Assessment of Sperm Quality in Aquatic Species*

**Jonathan Daly and Terrence R. Tiersch**

## Introduction

The use of fluorescent staining in conjunction with flow cytometry for the assessment of sperm quality in aquatic species has increased over the past decade. However, in contrast to the common use of flow cytometric assessment of sperm quality in mammalian species (i.e., hundreds of published studies), the use of this technique in aquatic species is relatively unexploited but becoming increasingly important. The increase in usage has been largely driven by the adaptation and adoption of sperm quality assays developed for use in mammalian species, which are vast in number compared to the range currently applied in aquatic species (Martinez-Pastor et al. 2010). Most assays developed in mammalian species are designed to target specific sperm structures or processes that are often sufficiently conserved across species to allow application in aquatic species. In addition to the assays adapted from mammalian species, more specialized sperm quality assays have been optimized in aquatic species for biomonitoring applications (e.g., Jenkins et al. 2010) and these have great potential for use in sperm quality assessments for cryopreservation.

Among the sperm quality assays that are currently used in aquatic species, assays for sperm membrane integrity and mitochondrial membrane potential have had the greatest application. One of the most commonly used in aquatic species is the SYBR 14/ propidium iodide (PI) assay for plasma membrane integrity, often referred to as a "sperm viability" assay. Several studies have used this assay to assess sperm quality in fresh and post-thaw samples to evaluate the success of cryopreservation protocols (e.g., Cabrita et al. 2005, Paniagua-Chávez et al. 2006). This assay has also been used in environmental toxicity studies to determine the effect of exposing sperm to antibiotics (Segovia et al. 2000) and herbicides (Favret and Lynn 2010) in a range of vertebrate and invertebrate aquatic species. Another commonly tested parameter in sperm from aquatic species is mitochondrial function, which is assessed using mitochondrion-specific fluorescent dyes that accumulate in proportion to membrane potential. The most commonly used mitochondrial dye in aquatic species is rhodamine 123, which is often combined with PI to enable concurrent assessment of membrane integrity (e.g., Ogier de Baulny et al. 1997, Liu et al. 2007), but a few studies have used other mitochondrial stains such as MitoTracker Red (Favret and Lynn 2010) and JC-1 (Guthrie et al. 2008).

In addition to the often kit-based assays adapted from mammalian species, assays that are currently used in aquatic species for biomonitoring applications also have potential utility in sperm quality assessments for cryopreservation, including assessment of DNA integrity (Jenkins et al. 2010) and ploidy analysis (Psenicka et al. 2009). These assays use fluorescent dyes that label DNA, such as PI or 4',6-diamidino-2-phenylindole (DAPI), and are typically used to assess the effect of environmental toxins on spermatogenesis and subsequent sperm quality. Although they require a greater level of flow cytometric expertise than the kit-based assays and require research for validation studies, they offer additional means of assessing and predicting the effect of cryopreservation on sperm survival and fertilizing ability.

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## Considerations for Flow Cytometry in Aquatic Species

As the use of flow cytometry for sperm quality assessment in aquatic species continues to increase, it is important to ensure that a consistent approach is taken to the application of new and existing assays. The main factors that should be considered and reported when analyzing sperm quality by flow cytometry are outlined in Table 1, and discussed briefly below.

**Table 1. Suggested minimum reporting requirements for sample preparation and flow cytometric analysis of sperm from aquatic species.**

| Parameter | Information required |
|---|---|
| Sample storage | Temperature and duration of sample storage after collection |
| Sperm concentration | The number of cells per unit volume (e.g., sperm cells/mL) |
| Staining conditions | Final concentrations of each dye to which sperm were exposed, expressed in molar units (e.g., nM, µM) |
| | Staining volume (i.e., the volume of sample to which stains were added) |
| | Staining temperature (e.g., room temperature, on ice, 4 ºC) |
| Staining time | Total staining time for each dye used |
| | Time between staining and analysis (i.e., how long after the staining time samples were analyzed) |
| Collection parameters | Number of events (or volume) collected |
| | Basis for event counts (i.e., based on FSC/SSC, gated, or fluorescent counts) |
| | Flow rate |
| Gating parameters | Identification of the sperm population |
| | Method for exclusion of debris from analyses |
| Reporting of results | Concentrations where possible (e.g., concentration of intact cells) |
| | Basis for calculation of percentage data (e.g., percentage of the total or gated, event count) |

Although only a few flow cytometric assays are used regularly for sperm quality assessment in aquatic species, direct comparison of results among studies is problematic, if not impossible, due to the large amount of variability in sample preparation, staining, and flow cytometry protocols. For example, among 15 studies that reported membrane integrity data using the SYBR 14/ PI assay in aquatic species in the past 10 yr, there was considerable variation in, or failure to report, sperm concentration, dye concentration, collection parameters (e.g., the number of events collected, flow rate, and population gating), and temporal components such as the time between collection or thawing and assessment, duration of treatments, and time between staining and flow cytometric assessment. This variability is in part due to limited access to flow cytometers for aquatic species research, with researchers often having to analyze samples at facilities focused on the analysis of humans or livestock. This has meant that researchers working with aquatic species often have limited opportunity to gain significant flow cytometry experience, and therefore must rely on the expertise of mammalian researchers who usually have minimal experience working with non-mammalian samples. While the past problems and variations in the use of this technology are understandable, the same approach cannot be used going forward. A standardized approach to sperm quality assessment for aquatic species is urgently required for research and eventual industrial application (Leibo 2000). There are many factors that can affect the quality and effectiveness of flow cytometric analyses, and all must be taken into account to allow accuracy and reproducibility of results.

Perhaps the most important factor in sperm quality assessment is sperm concentration, which aside from being a measure of quality in itself, is also relevant to all other measures of quality. The measurement and control of sperm concentration is extremely important for artificial fertilization and cryopreservation in aquatic species (Dong et al. 2007), but is usually not recognized, nor is it considered when assessing sperm quality. As such, flow cytometry results are typically reported as percentage data with no indication of the actual number of competent cells within a sample. It is important to note that percentage data are only useful in the context of concentration, i.e., proportions must be considered relative to the whole (put simply, 50% of $10^8$ cells is much different from 50% of $10^6$ cells). This is particularly important to consider when assessing thawed sperm samples. Cryopreservation and related processes (e.g., cryoprotectant exposure) can damage or destroy sperm, affecting concentration but not necessarily the proportion of intact cells, and this would not be detected with percentage data alone. Although traditional flow cytometry systems cannot calculate sperm concentration directly, concentration can be calculated by the addition of a known number of fluorescent counting beads to a sample, or separately by hemocytometer counts or spectrophotometry. Newer flow cytometry systems, such as the Accuri C6 (*www.accuricytometers.com*), are able to measure the sample volume collected, enabling direct calculation of concentration, and it is likely that this feature will be increasingly incorporated into future flow cytometry.

When percentage data are reported, it is important to define how the percentages are calculated as this can greatly affect the outcome and accuracy of results. For example, the proportion of intact cells is likely to be much lower when expressed as a percentage of the total event count (i.e., all forward scatter (FSC) vs. side scatter (SSC) events, including debris and other non-sperm events) than as a percentage of the gated event count (i.e., the events identified as the sperm population). This is particularly important for species that require dissection and crushing of the testes for sperm collection, as is the case for several commercially important aquatic species, including channel catfish *Ictalurus punctatus* (Christensen and Tiersch 2005) and Pacific oysters *Crassostrea gigas* (Dong et al. 2005), and small-bodied biomedical model species such as zebrafish *Danio rerio* and swordtails (*Xiphophorus* species) (Yang and Tiersch 2009). Sperm samples collected by dissection and crushing of the testes typically contain significant contamination with blood and other somatic cells, which will affect total event counts and present difficulties in gating and subsequent percentage calculations. It is therefore important to provide details on how the sperm population was identified from within the overall accumulation of events, and report gating parameters that were used to exclude non-sperm events from analysis (e.g., Figure 1). This information is essential to ensure accurate interpretation and reproducibility of the reported procedures.

In addition to sperm sample considerations, consistent control and reporting of staining and flow cytometry settings is required to enable comparison among studies. At present, there is a great deal of variability in staining and flow cytometry methodology among studies. For example, of the 15 studies in the past decade that reported flow cytometry data using the SYBR 14/ PI assay, 11 reported various combinations of 5 concentrations of SYBR 14 (10 – 400 nM) and 3 concentrations of PI (10 – 14 µM), while the other 4 studies only reported dilution ratios or volumes of stain added to the samples. When this is combined with the inconsistencies in the amount of sperm to which the dyes were added (variously reported as concentration ranging from $1 \times 10^6$–$1 \times 10^9$ sperm/mL, or dilution ratio ranging from 1:6-1:99) this leads to immense variation in the sperm:dye ratios among these studies. Although some amount of variability in methodology among different species or laboratories is to be expected, such large differences

make direct comparison of results or methodologies among studies virtually impossible, especially when dilution ratios or volumes are used with no indication of starting or final sperm or dye concentration.

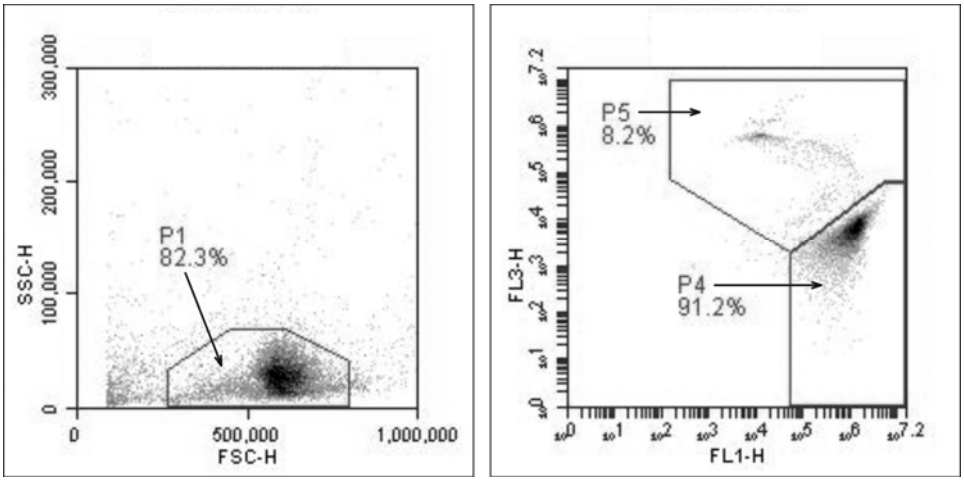


**Figure 1. Forward scatter vs. side scatter (FSC vs. SSC, left panel) and FL1 vs. FL3 (SYBR 14 vs. PI, right panel) scatter plots of zebrafish sperm in 300 mOsmol/Kg HBSS. The region designated as "P1" in the FSC vs. SSC plot is the gated sperm population, and events falling outside this region are debris. The region designated as "P4" in the SYBR 14 vs. PI plot is the intact sperm population (i.e., sperm with intact plasma membranes), and those falling in the "P5" region have damaged plasma membranes.**

One of the main potential sources of error in flow cytometric analysis of sperm samples arises from how the samples are analyzed. Although reports often state how many events (discrete electronic signals corresponding to target cells or other materials depending on instrument settings) were analyzed from a particular sample (typically 10,000 or more), information on how these events were counted or the flow rate at which the events were collected is often omitted. For example, 10,000 events based on FSC vs. SSC will be different to 10,000 events based on the gated sperm population, and this difference will be magnified in samples that were collected by dissection and crushing of the testes. In addition, the accuracy of these counts will be affected by the flow rate. If the flow rate is too fast or the sample is too concentrated, the sensitivity and precision of the flow cytometer are affected (Shapiro 2003) and the ability of the software to distinguish between individual events and between target cells and debris is greatly reduced. Consequently, the ability to distinguish and gate around target cells to exclude debris in the resulting scatter plots is reduced, affecting the accuracy of data analysis. This once again emphasizes the importance of measuring and controlling sperm concentration when analyzing sperm quality by flow cytometry, and highlights the need to consider and report data collection settings when reporting flow cytometric methodology.

Another major factor to consider when analyzing sperm quality by flow cytometry is temporal variability, which is relevant to all activities ranging from sperm collection to quality assessment. For flow cytometry, it is important to report how long after collection or thawing the samples were analyzed, and the total duration of exposure to fluorescent stains prior to analysis. Although minor variations in the time between collection and quality assessment may not significantly affect the results obtained for fresh samples, temporal effects are likely to have a

greater impact on aged, thawed, or poor quality samples. Sperm that have been thawed after cryopreservation are generally of lower quality than fresh, unfrozen sperm, and therefore may degrade faster than fresh sperm and make accurate comparisons between treatments or individuals difficult.

While the factors mentioned should be considered for all sperm samples, they are particularly important when analyzing thawed samples. As mentioned above, cryopreservation and related processes can weaken or destroy sperm. This can affect the flow cytometric data by decreasing the concentration of sperm in the sample, and by increasing the amount of debris present. This can in turn affect the accuracy of collection parameters (if collecting a set number of events) and the calculation of percentage values. In addition, thawed sperm may be more susceptible to temporal effects, such as variations in the time between thawing and analysis, which could further affect the results. It is for these reasons that cryopreserved sperm samples cannot be considered to be the same as fresh sperm samples, and care must be taken to account for these factors when collecting and comparing flow cytometric data from fresh and thawed sperm for assessment of cryopreservation effects.

## Future Applications of Flow Cytometry in Aquatic Species

As sperm cryopreservation in aquatic species moves towards high-throughput applications for aquaculture and repository storage of genetic material (NIH-NCRR 2007), standardization of sperm quality assays and minimum reporting standards will become even more important. For aquaculture, it will be important to provide accurate assessments of sperm quality of the males selected for genetic storage and to allow end users (e.g., hatchery producers or farmers) to have confidence in the final cryopreserved product and enable efficient usage of genetic material. Information on the quality of samples from endangered species stored in genetic repositories will be important to ensure that high quality samples are preserved for future use, and for accurate calculation of sperm-to-egg ratios to ensure that valuable gametes are not wasted by using poor quality sperm for fertilization or by using more sperm than are required for a particular batch of eggs.

A standardized approach will be required for future assessments of sperm quality using existing assays, such as membrane integrity and mitochondrial membrane potential, and for the development of new assays specifically for aquatic species. Over the past few years there has been an increase in biochemical research, such as proteomic analysis, in sperm from aquatic species (e.g., Li et al. 2010) and it is possible that work such as this will lead to development of new assays for sperm quality. It is essential that minimum standards for reporting of flow cytometry data be applied to all studies on sperm quality. By controlling and reporting these factors, researchers can ensure the accuracy and reproducibility of their data, allow others to replicate or build on that work, and ensure that flow cytometry can be utilized and interpreted correctly for gamete quality in aquatic species as it is currently used in mammalian species.

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