

Final Report
Undergraduate Research Opportunity Program

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by
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Abstract

Measurement of cell concentration and quality are critical components of sperm handling and artificial fertilization, which are techniques used for propagation of aquatic species worldwide. Control of sperm concentration is required to ensure consistent and reproducible results for cryopreservation and fertilization protocols, and accurate measures of sperm quality are required to assess the success of protocols and for selection of samples for fertilization. Flow cytometry offers a range of sperm quality assays to complement motility assessment and is increasingly being used to assess sperm quality in aquatic species, but because of traditional design constraints cannot be used to directly determine sperm concentrations. Using an Accuri C6 flow cytometer, which uses a novel platform that does not volumetric counting beads, work was undertaken to determine the optimal flow cytometry settings (Accuri C6 flow cytometer[®], Accuri Cytometers, Ann Arbor, MI) required to determine sperm concentration simultaneously with membrane integrity assessment (using the SYBR 14/propidium iodide assay) in three important aquatic species: the zebrafish (*Danio rerio*), channel catfish (*Ictalurus punctatus*) and the eastern oyster (*Crassostrea virginica*). Serial dilution of sperm samples was used to determine the maximum sperm concentration that can be measured accurately using flow cytometry. Results were compared to sperm concentration measurements determined using hemacytometer counts and micro-spectrophotometer absorbance measurements to determine the accuracy and reproducibility of the flow cytometry technique for measuring sperm concentration. Development of an efficient means of determining sperm concentration using flow cytometry for simultaneous performance of assays for sperm quality would maximize the efficiency of sperm usage and reduce the amount of variability within and among studies. The ability to conserve sperm samples is particularly important when dealing with small-bodied biomedical model fishes, such as zebrafish, or endangered species where gametes are scarce or valuable.

Introduction

Measurement of sperm concentration and quality are critical components of sperm cryopreservation and artificial fertilization. Control of sperm concentration is required to ensure consistent and reproducible results for cryopreservation and fertilization protocols. Accurate measurement of sperm quality is required for assessment of the success of protocols and for selection of samples for fertilization (Tiersch 2001). Significant uncontrolled variation from failure to control sperm concentration has been proposed to be a main reason for inconsistencies in results among studies in aquatic species (Dong et al. 2007, Tiersch et al. 2007). In particular, there is a large degree of variability in the studies conducted on selection of cryoprotectants and their relationship to the concentration of the collected sample. The variability is caused by the differences in the sperm population and cryoprotectant dilution ratios. This can lead to poor protocols for sperm samples, which, in turn, leads to toxicity effects or an insufficiency of cryoprotectants present. In either case, the cells are not preserved (Tiersch, 2006).

There are several methods that can be used to determine sperm concentration. The most common is the use of a hemacytometer (Prathalingam et al. 2006). Samples are loaded into a counting chamber, and individual cells are counted to obtain an approximate concentration based on the dilution of the sample. This method is time consuming and requires a relatively large sample volume, creating an issue of sample size for small aquarium fish. The accuracy can be affected by a number of factors, including the experience of the person counting, the design of the hemacytometer being used, and protocol, which requires that a certain numbers of cells are counted to be considered accurate (Christensen et al. 2005).

Sample concentration can also be determined using a spectrophotometer, which measures absorption of the sample at specific wavelengths. The final concentration is calculated using a predetermined, species-specific, standard curve that has been developed through the use of hemacytometer counts. The use of a micro-spectrophotometer, such as the NanoDrop® (Thermo Fisher Scientific, Wilmington, Delaware) can be useful when dealing with small sample sizes, as they only require a 1-2 uL sample volume. Microspectrophotometers, however, do not produce qualitative information about the sample and cannot take into account sample debris.

It is possible to determine sperm concentration in a sample by using a flow cytometer. Although flow cytometers typically do not yield an actual concentration values, there are newer instruments that can (such as the Accuri C6 flow cytometer Accuri Cytometers, Ann Arbor, Michigan), which uses a novel platform that is able to estimate a known volume of sample. This allows for the direct calculation of concentration to be without the addition of volumetric counting beads.

In addition to determining sample concentration, a double-stained assay, using the nuclear dyes SYBR 14 and propidium iodide (PI), allows for assaying of membrane integrity of sperm (Paniagua-Chavez et al. 2006, Daly et al. 2008). If the plasma membrane were damaged or altered in composition, PI would enter the cell passively and

intercalate with the DNA in the nucleus. The sperm cells are discriminated based on their fluorescence at a particular wavelength, which is determined the presence of the hydrophilic dye within the cell (Petrunina et al., 2010). The relative sizes of the sperm populations with stained DNA can be determined using flow cytometry methods (Ogier de Baulny et al. 1999).

The samples are estimated on the flow cytometer using a predetermined collection volume and the numbers of events are recorded. Events are particles that are able to scatter light or fluoresce at the given wavelengths. The total concentration of the sample can be calculated using the fluorescent population per microliter of sample. The ability to gate the population removes possible contaminants that could lead to an overestimation of the sperm concentration. The overall goal of this project was to develop an efficient means of determining, sperm concentration by the use of flow cytometry and to develop a single assay that would simultaneously evaluate multiple parameters of sperm quality.

Methods

Sample collection: Male zebrafish, *Danio rerio*, were obtained from a commercial supplier (Segrest Farms, Gibsonton, Florida). Males were measured for body length and weight. The males were killed and their testes collected by dissection. The testes were weighed and crushed in 300 mOsmol/Kg Hanks' balanced salt solution (HBSS) at a ratio of 40 μ L HBSS/mg of testes. Sperm samples were filtered through 35- μ m mesh to remove cellular debris.

Flow cytometry analysis: Sperm samples were diluted at ratios of 1:9 and 1:99 with HBSS. An Invitrogen live/dead sperm kit (L701 Invitrogen live/ Dead Sperm Viability Kit, Eugene, Oregon) was used to determine membrane integrity of the sperm cells. By measuring membrane potential, samples were tested for quality and quantity. Samples were stained with 100 nM SYBR 14 and 12 μ M PI and incubated for 10 minutes. Each sample was analyzed with an Accuri C6 flow cytometer by collecting 10 μ L of sample at a flow rate of 35 μ L/min (medium) and 66 μ L/min (fast). Samples were gated using forward and side (90°) light scatter to exclude debris and somatic cell contamination. The number of gated events detected was used to calculate the sperm concentration for each collection speed and volume, which was used to determine the concentrations of the original samples.

Measurement of sperm concentration: Actual sperm concentration from the 1:9 and 1:99 dilutions was measured with a microspectrophotometer (NanoDrop[®]) and hemacytometer (Hausser Scientific Hemacytometer, Horsham, Pennsylvania). For hemacytometry, a 10- μ L sample of sperm suspension was loaded, and the sperm number was counted at 200-x magnification. Five squares were counted and used for concentration calculation. For microphotometry, the protocol established by Tan et al. (2010) was used following the standard equation of:

$$y = (3 \times 10^8) (x) - 3 \times 10^7$$

with “x” being defined as the absorbance measured at the wavelength of 400 nm. Briefly, a 2- μ L sample of sperm suspension from each male was loaded onto the lower pedestal of the NanoDrop, and absorbance was measured at a wavelength of 400 nm. The concentration was calculated from the mean of 3 replicates using the previously developed concentration curve equation (Tan et al. 2010).

Results and Discussion

Sperm concentration measurement: Hemacytometer counts showed the final concentration of the samples to be 1.49×10^8 . This was slightly higher than the concentration determined by the NanoDrop, which was 1.10×10^8 . These concentrations were compared to the various flow rates and dilution factors.

Sperm concentration measured by flow cytometry: The dilution ratio affected the staining intensity of the intact sperm population, resulting in the population shifting closer to the origin in the 1:9 dilution samples. The decrease noted in the staining intensity was likely due to an imbalance in the sperm-to-dye ratio at the higher sperm concentration. The samples measured at medium speed were found to have higher sperm concentrations compared to those measured at the fast speeds. This apparent difference in concentration is likely due to individual event signals being more readily detected at the medium speed. By increasing the measurement rate, the flow cytometer was not able to distinguish individual events at higher concentrations. This was reflected in the decreased concentration measurement for the fast setting when compared to the other rates.

These results suggest that the flow cytometry system has difficulty processing signals from individual events at a high sperm concentrations and flow rates. When comparing all results, the hemacytometer gave higher readings for all samples. This apparent increase in concentration could be caused by human error, as well as cellular debris contaminates. The sperm concentrations calculated from the NanoDrop absorbance values were similar to those calculated by flow cytometry in the 1:99 fast, and the 1:9 and 1:99 medium samples. The results of this study show that sperm dilution ratio and flow rate can have a considerable effect on the accuracy of flow cytometric analysis of sperm samples (Figure 1).

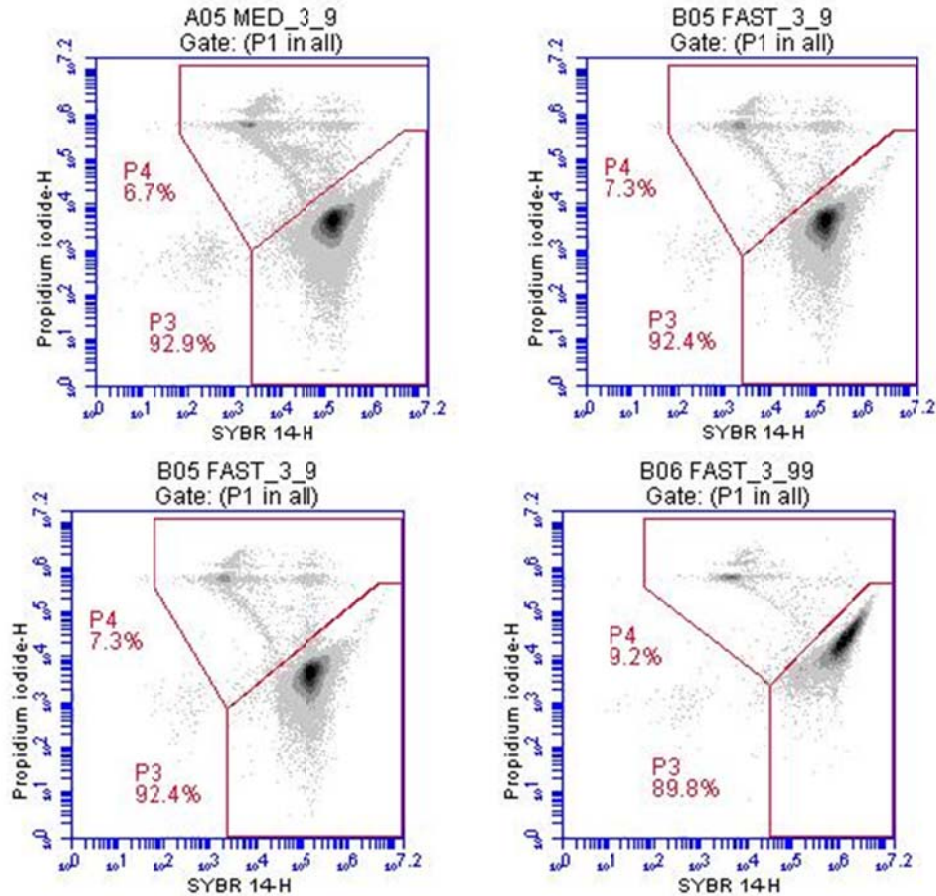


Figure 1: SYBR 14 vs. PI scatter plots of zebrafish sperm diluted 1:9 (left panels) and 1:99 (right panels). Samples were analyzed at medium (top panels) and fast (bottom panels) flow rates. The region designated P3 is the intact sperm population, and the region designated P4 is the damaged sperm population. Events falling outside of the gates were considered to be debris and are not included in the final concentration calculation. The 1:99 dilutions contained a higher concentration than those diluted to 1:9. This reflects the variation that can occur if concentration and flow rate are not controlled.

The final concentration determined by flow cytometry varied depending on the flow rate selected and the sample dilution factor. The final concentration determined for the 1:9 dilution measured on medium flow rate 1.07×10^8 . The 1:9 dilution at the fast rate had a lower concentration (5.15×10^7). The 1:99 dilution measured at the medium rate and was intermediate (1.27×10^8). This was slightly higher than the 1:99 dilution measured at the fast rate (1.16×10^8) (Figure 2).

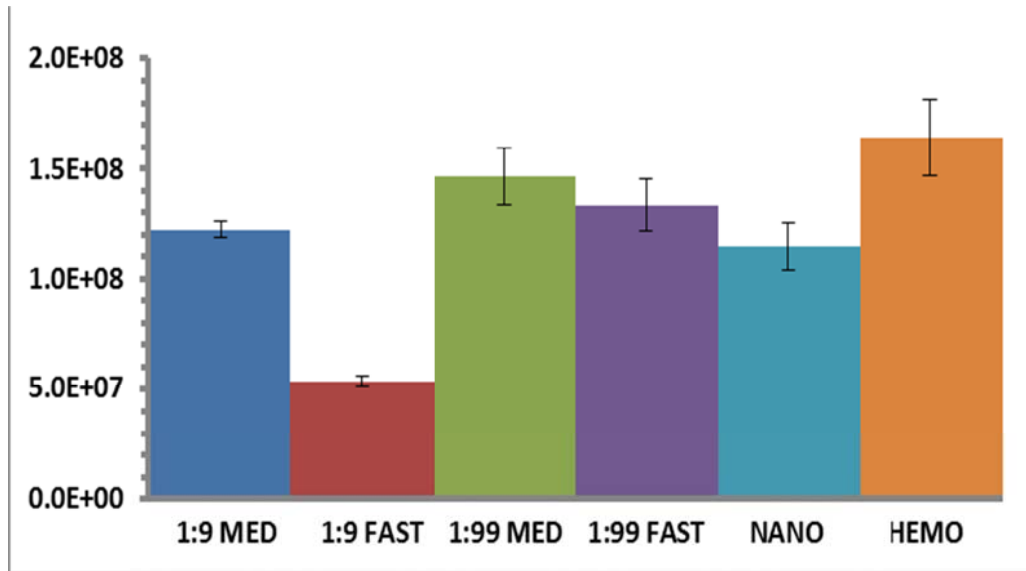


Figure 2. Sperm concentration measured using flow cytometry at two sperm dilution ratios (1:9 and 1:99) and two flow rates (medium and fast), and by NanoDrop (nano) and hemacytometer (hemo). Columns are the mean \pm S.E.M. of four samples.

Further Continuation of This Project

We would like to publish this work. However, due to the limited spawning seasons of the channel catfish *Ictalurus punctatus*, and eastern oyster *Crossostrea virginica*, and the delayed start of the project (March 2010), I was not able to finish collecting all of the necessary samples to have a complete publishable data set. During the next Spring spawning season, I will finish collecting samples to complete the data and finish the necessary experimental work.

Because of the stripping and crushing method used to collect the catfish sperm, there was a large amount of tissue debris in the samples. Samples were filtered through 200- μ m and 100- μ m filters. Catfish data followed a similar trend seen with the zebrafish dilution rates. 1:9 dilutions measured on the flow cytometer had a lower sperm population when compared to the 1:99 dilutions. The NanoDrop and hemacytometer readings closely matched the 1:99 calculated concentrations.

In the coming semester, I plan to continue my research on developing flow cytometry protocols using zebrafish sperm. Using several samples of controlled concentrations that have been determined by the NanoDrop, the concentration range of each flow rate will be measured. The samples will be tested on flow rates slow, medium, and fast to determine the accuracy and range of each rate. A concentration within the determined range will be tested at various volumes to determine volumetric range of the flow cytometer.

The research completed during this UROP project will be presented as a poster at the annual meeting of the World Aquaculture Society, to be held on March 1, 2011 in New Orleans, Louisiana. We will send a copy of this poster to the Sea Grant office.

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