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Determination of Sperm Concentration Using Flow Cytometry with Simultaneous Analysis of Sperm Plasma Membrane Integrity in Zebrafish *Danio rerio*

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Abstract

Control of sperm concentration is required to ensure consistent and reproducible results for cryopreservation and *in vitro* fertilization protocols. Determination of sperm concentration is traditionally performed with a counting chamber (e.g., hemocytometer), or more recently with a spectrophotometer. For small-sized biomedical model fishes, the availability of sperm sample is limited to microliters, so it is desirable to develop fast and accurate approaches for concentration determination that also minimize sample use. In this study, a new approach was developed for sperm concentration determination using a flow cytometer (Accuri C6, BD Biosciences, San Jose, CA) with simultaneous measurement of sperm membrane integrity after fluorescent staining with SYBR®−14 and propidium iodide (PI) in sperm from Zebrafish *Danio rerio*. The goal was to develop a protocol for simultaneous determination of sperm quality and quantity by flow cytometry. The objectives were to (1) determine the effects of sample volume (250 and 500 μl) and analysis volume (10 and 50 μl) on the accuracy of particle counting using standard volumetric validation beads; (2) identify the effective range of sperm concentrations that flow cytometry can measure; (3) test the precision and reproducibility of the sperm concentration measurements; and (4) verify the flow cytometry approach by comparison with measurement with a hemocytometer and a microspectrophotometer. Sample volumes of 250 and 500 μl and analysis volumes of 10 and 50 μl did not affect bead count with the factory-set flow rates of "medium" or "fast," and the precision and accuracy was retained across a concentration range of $1 \times 10^3 - 1 \times 10^7$ cells/ml. The approach developed in this study was comparable to traditional methodologies such as hemocytometer or microspectrophotometer. This study provides an efficient, accurate, and rapid method for determination of sperm concentration using flow cytometry while providing simultaneous assessment of sperm membrane integrity. Such approaches can reduce the time needed for quantity assessment and maximize the use of valuable sperm samples.

Key terms

zebrafish; flow cytometry; sperm concentration; membrane integrity

THE control of sperm concentration is essential for cryopreservation and artificial insemination in aquatic species to ensure consistent and reproducible results in research and in hatchery settings. Studies on cryopreservation of sperm from aquatic species have shown inconsistency because of a lack of standardization of sperm concentration (1,2). For small-bodied research model fishes, the availability of sperm from individuals is usually limited to only 1–5 μl (3); therefore, it is important to control sperm concentration to ensure that these miniscule volumes are utilized as efficiently as possible.

Generally, the traditional method for determining cell concentration is to count cells by use of a hemocytometer. This method has been widely applied for many years and is considered an accurate means of estimating cell concentration (4–6). With

some modifications, specific counting chambers, such as the Makler® counting chamber (Irvine Scientific, Santa Ana, CA) (7) and fixed-depth slides for computer-assisted sperm analysis (8) (CASA, www.hamiltonthorne.com), are also used for cell counting. Meanwhile, spectrophotometry has been used for determination of cell concentration by identifying the linear relationship between particle concentration and light absorbance (9–12), which provides a more rapid estimation of concentration than actual counting methods. In addition, considering the sperm volume required, microspectrophotometry with 1–2 μl sample sizes has been developed specifically for concentration determination of sperm from small-sized biomedical research model fishes (13).

Flow cytometry is a laser-based biophysical technology for analysis of suspended cells in a stream of fluid passing by an electronic detection apparatus. It has been employed for research applications including cell sorting and biomarker detection, and can rapidly analyze samples with thousands of events per second. For sperm biology, flow cytometry has been used for assessment of sperm quality parameters such as plasma membrane integrity, mitochondrial activity, and acrosome integrity (14–17). Flow cytometry has been used for cell concentration measurement by addition of a known concentration of fluorescent beads to a sample to enable indirect calculation (18). This approach was shown to be a repeatable method for measuring cell concentration with the coefficient of variation (2.3%) when compared to image analysis, fluorescent plate reader, hemocytometry, spectrophotometry, and microcell analysis (11). In another study, flow cytometry was found to be 2–4 times more accurate than a spectrophotometer for determining cell concentration (19). Flow cytometry has proven to be an accurate and repeatable method for sperm concentration measurement when gate settings are based on a combination of cell size, granularity, and DNA staining (18). However, one report showed that cell samples containing latex counting beads can lead to overestimation of sperm concentration by as much as 109% when compared to manual counting (20). The reason for this overestimation was probably the different fluorescence patterns affected by the presence of alien particles, and thus a mathematical equation was proposed for correction of values for estimation of sperm concentration (21).

Recently, an alternative type of flow cytometer (Accuri C6, BD Biosciences, San Jose, CA) has become commercially available with a nonpressurized peristaltic pump to drive the fluidics system, which can directly derive sample volume. Therefore, cell concentration can be calculated from cell counts and volume measurement, and no counting beads are needed for reference. Theoretically, any analyses with cells directly stained (or labeled) with fluorescence can be used for concentration calculation with this type of flow cytometer. The ability to accurately determine sperm concentration simultaneously with other sperm indicators would provide a rapid and efficient means of assessing multiple parameters in a relatively small volume of sample. This could be especially useful for limited and valuable sperm samples such as those from the small-sized biomedical model fishes.

In this study, flow cytometry was used for the simultaneous measurement of sperm concentration and sperm membrane integrity by staining sperm cells from zebrafish with SYBR-14 and propidium iodide (PI) (LIVE/DEAD® sperm viability kit, Molecular Probes, Inc., Eugene, OR) and analysis with an Accuri C6 flow cytometer. The goal was to develop a protocol for determination of sperm concentration by flow cytometry. The objectives were to 1) determine the effects of sample volume (250 and 500 μl) and analysis volume (10 and 50 μl) on the accuracy of bead counting using standard volumetric validation beads; 2) identify the effective range for sperm concentration measureable by flow cytometry; 3) test the precision and reproducibility of the concentration measurements; and 4) verify the flow cytometry approach with hemocytometry and microspectrophotometry. This study showed that flow cytometry can be used for determination of sperm concentration while simultaneously analyzing sperm membrane integrity. This is an accurate, rapid, and efficient methodology, and would be useful in sperm biology especially for small, valuable samples.

Materials and Methods

Experimental Fishes

Male zebrafish used in this study were obtained from two sources: 1) AB wild-type from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA) with ages of more than 6 months and 2) *Danio* zebrafish *Danio rerio* from Segrest farms (www.segrestfarms.com, Gibsonton, Florida). Fishes were maintained at a density of two fish per liter at 26°C in a recirculated system for zebrafish (Aquatic Habitats, Apopka, FL, USA) at the Aquaculture Research Station of the Louisiana State University Agricultural Center. Photoperiod was set at 14 h light:10 h dark and fish were fed twice daily with commercial pellets (Aquatic Eco-system, Apopka, FL, USA) and Artemia larvae grown from cysts (INVE Group; Grantsville, UT, USA). The filter system was back-flushed weekly. Guidelines from the Institutional Animal Care and Use Committees of Louisiana State University were followed for animal care and use in this study.

Sperm Collection

Sperm samples were collected by crushing of dissected testes. Before dissection, body length and weight were measured after the fish were anesthetized by placement on crushed ice for 1 min. Testes were collected by dissection at 10× magnification, and were transferred to a tared 1.5-mL centrifuge tube for weighing. Sperm suspension was obtained by crushing the testes in Hanks' balanced salt solution (HBSS) at an osmolality of 300 mOsmol kg^{-1} (HBSS300) with a volume (μl) of 40 times the testis weight (mg). The recipe for HBSS was 0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.2, and the final osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT, USA), and pH was determined (AB15, Fisher Scientific). Sperm samples were

filtered through 20-μm screen to remove cellular debris for analysis with flow cytometry.

Flow Cytometry

Samples were analyzed using an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm, 50 mW solid-state laser. Flow cytometer performance was validated each day using fluorescent validation beads (Spherotech 6-peak and 8-peak beads, BD Biosciences, Ann Arbor, MI, USA) to ensure that coefficient of variation (CV) values for the fluorescence detectors were <3.0% (based on full peak height). Flow cytometry was performed using the CFlow plus® software (BD Biosciences, Ann Arbor, MI, version 1.0.202.1).

Staining of Sperm Cells with SYBR-14 and Propidium Iodide

Sperm cells were stained with SYBR-14 and PI from a commercial live/dead sperm kit (Invitrogen, L7011, Eugene, OR) for evaluation of plasma membrane integrity. Filtered sperm samples were mixed with 100 nM SYBR-14 and 12 μM PI for 10 min in the dark. SYBR 14 is a membrane-permeant nucleic acid stain and PI is a conventional dead cell stain, and thus live sperm cells with intact cell membranes can fluoresce bright green of SYBR-14 detected with a 530 ± 15 nm band-pass filter, while cells with damaged cell membranes fluoresce red of PI detected with a >670-nm long-pass filter. Sperm population gating to exclude debris was based on forward scatter (FSC) vs side scatter (SSC) plots. These gated events were considered to be sperm cells if they retained cell structure (not debris) and stained with SYBR-14 or PI. Cells meeting these criteria were used for calculation of sperm concentration. Furthermore, these gated cells were viewed on a scatter plot showing FL1 (SYBR 14) vs FL3 (PI) with fluorescence compensation to reduce spectral overlap. Sperm stained with SYBR-14 alone were classified as "membrane-intact cells." For this analysis, the sperm membrane integrity is usually calculated as the percentage of membrane-intact cells out of the total cells stained with PI and SYBR-14.

Measurement of Sperm Concentration by Microspectrophotometry and Hemocytometer

Sperm concentration was estimated with a microspectrophotometer (NanoDrop®, Thermo Scientific, Wilmington, DE) and a hemocytometer (Hausser Scientific Hemocytometer, Horsham, PA). For microspectrophotometry, the protocol established by a previous study (13) 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 400 nm. Briefly, a 2 μl sample of sperm suspension from each male was loaded onto the lower pedestal of the NanoDrop, and absorbance was measured at 400 nm. For hemocytometry, a 10 μl sample of sperm suspension was loaded, and the sperm number was counted at 200× magnification. Five squares were counted and used for concentration calculation. Three replicates were made for each sample and the mean was used for concentration calculation.

Experiment I. Effects of Sample Volume, Analysis Volume, and Flow Rate

Based on the specification for this flow cytometer, most accurate volume measurements are made at flow rates of medium (35 μl min^{-1}) or fast (66 μl min^{-1}), and can be influenced by sample volume and the type of sample tubes used. In addition, the analyzed sample volume is directly related to the number of events collected, which is also related to the accuracy of concentration calculation. Therefore, sample volumes (250 and 500 μl) and analysis volumes (10 and 50 μl) at medium (35 μl min^{-1}) and fast (66 μl min^{-1}) flow rates were tested by using the standard Sphero™ AccuCount fluorescent beads with a stated concentration of approximately 1×10^6 beads mL^{-1} (51,011 particles per 50 μl, Spherotech Inc., Lake Forest, IL). These counting beads were diluted in 0.22-μm-filtered, deionized water at 20 times into a master solution by following the manufacturer's instructions, and were divided into eight tubes for each combination of running conditions for counting. Three replicates were performed for each treatment and the concentrations of beads measured by flow cytometry were compared with the stated concentration (51 particles μl^{-1}) specified by the manufacturer.

Experiment II. Determination of the Concentration Range for Flow Cytometry

Testes (~10 mg) from 3–5 males were pooled and suspended in HBSS300. The concentration of sperm was first estimated by microspectrophotometer and adjusted to 1×10^8 cells ml^{-1}. From this initial concentration, serial dilutions (50 μl of sperm sample plus 450 μl HBSS) were performed by using the same pipette and tip to yield sperm concentrations at 1×10^7, 1×10^6, 1×10^5, 1×10^4, and 1×10^3 cells ml^{-1}. Samples at each concentration were analyzed with flow cytometry at the medium flow rate after staining with SYBR-14 and PI. Analysis volume was 10 μl for samples at 1×10^8, 1×10^7, and 1×10^6 sperm ml^{-1}, and 50 μl for samples at 1×10^5, 1×10^4, and 1×10^3 cells ml^{-1} to collect sufficient events for analysis. At the same time, each sample was counted by using a hemocytometer for concentration calculation. The sperm concentrations measured by flow cytometry and hemocytometry for each sample were compared and analyzed. Three replicates were produced by using the testes from different males.

Experiment III. Precision and Reproducibility of Concentration Measurement by Flow Cytometry

To test the precision of the concentration measurements, sperm samples were prepared and separated into four aliquots after the concentration was adjusted to 1×10^6 cells ml^{-1} based on estimation by microspectrophotometer. Before analysis of sperm samples, flow cytometry was verified with AccuCount beads to assure the variance of bead counts obtained was within 10% of the expected concentration. Each aliquot was analyzed after staining with SYBR14/PI at a medium flow rate and a 10-μl analysis volume, and was repeated 5 times for each aliquot. Four replicates were produced using sperm from 4–6 males for each.

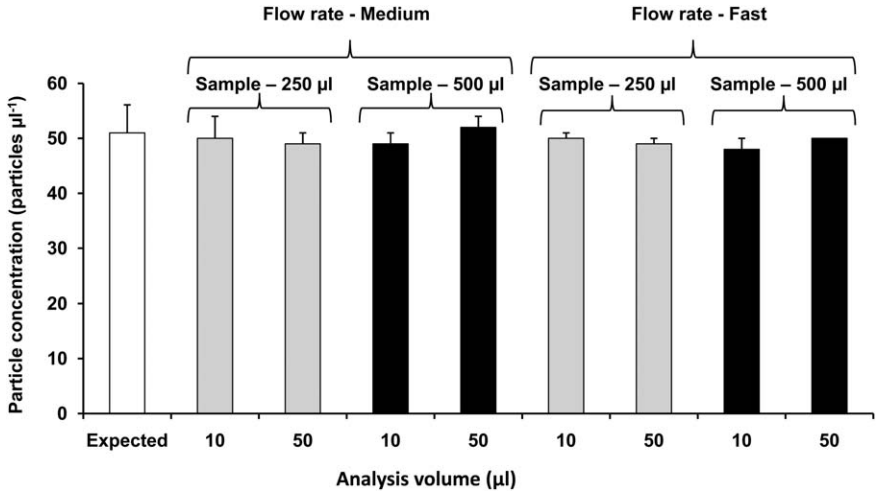

Figure 1. Concentration measurement of standard florescent particles (51 particles per µl) analyzed by flow cytometry (Accuri C6) at analysis volumes of 10 and 50 µl, sample volumes of 250 and 500 µl, and at flow rates of medium (35 µl min^{-1}) and fast (66 µl min^{-1}).

Experiment IV. Verification of the Approach with Sperm Samples of Unknown Concentrations

To confirm the technique for concentration measurement with flow cytometry, sperm samples with unknown concentrations were measured with flow cytometry, microspectrophotometer, and hemocytometer. Sperm samples were prepared by suspending testes pooled from 3 to 5 males in HBSS300 at a volume (µl) of 40 times testis weight (mg), and separated into three parts: one was for concentration measurement with NanoDrop; the other two were diluted with HBSS300 at a ratio of 1:9 or 1:99 to create two samples for concentration measurement with hemocytometer (for the 1:99 diluted sample) and flow cytometer (for 1:9 and 1:99 diluted samples) at fast and medium flow rates. Four replicates were produced by using 3–5 males for each.

Data Analysis

Data were analyzed by using SYSTAT 13 (SYSTAT Inc., Chicago, IL). Equality of variance, *t* test, and ANOVA were performed after transforming of data. Significance level was set at $P \leq 0.050$.

Results

Experiment I. Effects of Sample Volume, Analysis Volume, and Flow Rate

Concentrations of fluorescent particles analyzed by flow cytometry at analysis volumes of 10 and 50 µl, sample volume of 250 and 500 µl, at flow rates of medium and fast were between 48 and 52 particles per µl (Fig. 1). Compared with the expected concentration (51 particles per microliter), no significant differences were found among the measurements ($P \geq 0.200$).

Experiment II. Determination of Concentration Range for Flow Cytometry

Flow cytometry measurements did not show significant differences compared to the measurement by hemocytometry at concentration levels from 1×10^3 to 1×10^7 cells ml^{-1} ($P \geq 0.054$), but at a sperm concentration level of 1×10^8 cells ml^{-1}, the measurement by flow cytometry was significantly lower than that measured by hemocytometer ($P = 0.006$) (Fig. 2).

Experiment III. Precision of Sperm Concentration Measurement by Flow Cytometry

Variance and coefficient of variation (CV, the standard deviation in relation to the mean value) were used for precision analysis in this experiment. The analysis of equality of variance among the four aliquots in each of the four replicates did not show significant differences ($P \geq 0.199$), and the CV of replicates 1–4 were 3.4%, 7.3%, 4.6%, and 4.8% (Fig. 3), all lower than 10% which is considered the standard level of accuracy required for counting beads.

Experiment IV. Verification of the Approach with Unknown Sperm Concentration

The average concentration of sperm suspensions determined by microspectrophotometry was $1.14 \pm 0.21 \times 10^8$ cells ml^{-1} for the four replicates (Fig. 4). The concentration of

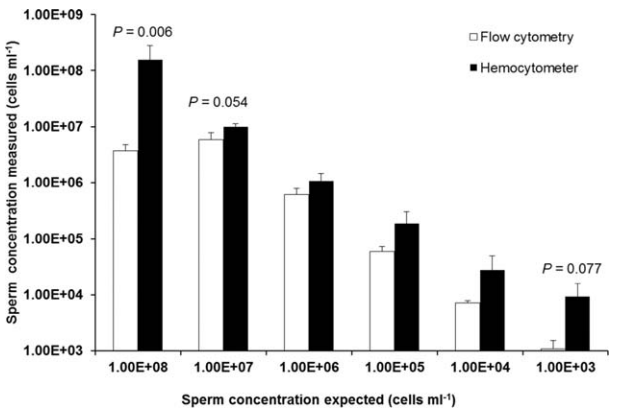

Figure 2. Estimation of concentration range using flow cytometry and hemocytometer counts of zebrafish sperm from 1×10^3 to 1×10^8 cells ml^{-1}.

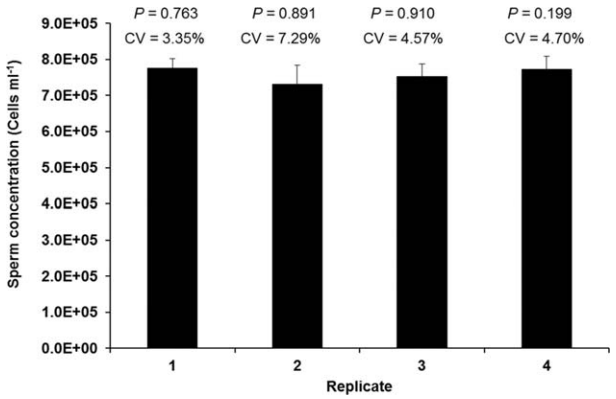

Figure 3. Precision of zebrafish sperm concentration measurements by flow cytometry. Variance and coefficient of variation (CV, the standard deviation in relation to the mean value) were used for precision analysis.

sperm suspension determined by hemocytometer of the 1:99 dilution sample averaged $1.64 \pm 0.35 \times 10^8$ sperm ml^{-1} for the four replicates (Fig. 4). The concentration of sperm suspension determined by flow cytometry for the 1:99 diluted sample was $1.47 \pm 0.26 \times 10^8$ cells ml^{-1} at medium flow rate, and $1.33 \pm 0.24 \times 10^8$ cells ml^{-1} with fast flow rate (Fig. 4). Flow cytometric analysis of samples with 1:9 dilution showed the concentration of sperm suspension was $1.22 \pm 0.08 \times 10^8$ cells ml^{-1} at medium flow rate, and $0.53 \pm 0.44 \times 10^8$ cells ml^{-1} with fast flow rate (Fig. 4). There were no significant differences ($P \geq 0.069$) in the concentrations determined by these methods except the flow cytometry measurement of the 1:9 diluted sample with fast flow rate, which was significantly lower than all others ($P \leq 0.002$).

The sperm membrane integrity values measured simultaneously with sperm concentration by flow cytometer were $91 \pm 2\%$ (medium flow rate) and $91 \pm 1\%$ (fast flow rate) for the 1:99 diluted samples, and $93 \pm 2\%$ (medium flow rate) and $93 \pm 1\%$ (fast flow rate) for the 1:9 diluted samples. There were no differences among these measurements ($P \geq 0.075$).

Discussion

Considerations and Requirements for Concentration Determination by Flow Cytometry

Although it was demonstrated that this form of flow cytometer (Accuri C6) can be used for sperm concentration, some precautions are necessary to guarantee the accuracy of measurements: 1) the peristaltic pump tubing should be <2 months old; 2) the fluidics of the flow cytometer should be calibrated for precise volume measurements by using the same volume and type of sample tube; 3) the correct collection of fluorescent signals by the flow cytometer should be validated by use of standard 8-peak and 6-peak beads (from BD Biosciences); and 4) the accuracy of event counting should be confirmed by using standard counting beads of a known concentration. These precautions were met for the experiments performed in this study.

According to the operation manual for the Accuri flow cytometer, the minimum sample volume in tubes for analysis should not fall below 150 µl. To meet this requirement, and to consider sample concentration and the total event number for collection, sample volumes of 250 and 500 µl and analysis volumes of 10 and 50 µl were chosen in this study. The standard (within ±10% of the expected count) used for precision analysis was based on the manual of the AccuBead counting beads that were used in this study.

The results from *Experiment I* indicated that sample volumes of 250 and 500 µl, analysis volumes of 10 and 50 µl, and flow rates of medium (35 µl min^{-1}) and fast (66 µl min^{-1}) did not produce significant differences in cell counting. These results were therefore used to define the instrument settings needed for subsequent experiments.

Effective Concentration Range for Flow Cytometric Analysis

Currently, the most commonly used methods for determination of sperm concentration include hemocytometry, Makler counting chamber, and spectrophotometer, although there are many other methods that have been reported (9). Each of those methods has their own effective concentration range for measurement based on their underlying counting mechanism. For example, concentration determination by hemocytometer comes from the direct counting of cells in each observation square using a microscope. Thus, it is critical that cells be visually distinguishable (not too crowded to count), and yet they should be plentiful enough for counting to avoid extrapolation when calculating the concentration. For the spectrophotometer, the effective range is limited by the ability to accurately measure absorbance so varies depending on the instrument type used. For the microspectrophotometer, previous studies have shown that it can be only used reliably for measuring sperm concentrations ranging from 1×10^7 to 3×10^8 cells ml^{-1} (13).

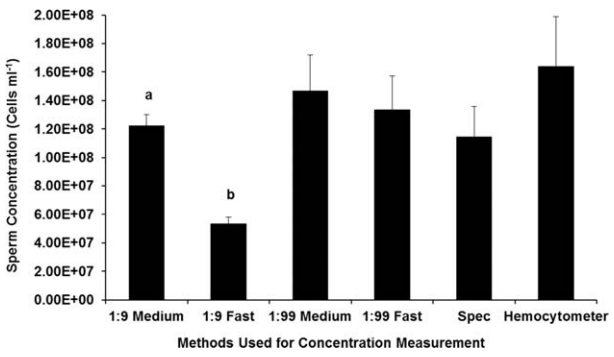

Figure 4. Verification of the approach for sperm concentration measurement using flow cytometry to measure unknown sperm concentrations of zebrafish, compared to measurement by hemocytometer counting and microspectrophotometer (Spec). Notes: 1:9 Medium, sperm sample was diluted at 1:9 ratio and analyzed at medium flow rate; 1:9 Fast, sperm sample was diluted at 1:9 ratio and analyzed at fast flow rate; 1:99 Medium, sperm sample was diluted at 1:99 ratio and analyzed at medium flow rate; 1:99 Fast, sperm sample was diluted at 1:99 ratio and analyzed at fast flow rate.

For flow cytometry, cell counting is based on collection of signals from each cell in the passing stream of suspended particles. Therefore, it is critical that individual events be distinguishable, especially for samples with high concentrations because individual cells could be grouped as clusters and recognized as a single event. The results from *Experiment II* indicated that within a concentration range of $1 \times 10^3 - 1 \times 10^7$ cells ml^{-1} flow cytometry could count cells accurately. Thus compared to the other common methods, flow cytometry provided a wider range of cell concentrations that could be accurately measured.

In practice, samples can be diluted before concentration determination. However, considering the volumes needed for measuring concentration, especially for valuable small samples, flow cytometry would require fewer samples compared to other methodologies because it has a wider effective range for concentration measurement. For example, if the sperm sample to be used is 1×10^8 cells ml^{-1} (which is the most commonly used sperm concentration for cryopreservation or artificial fertilization), the total sperm sample needed for analysis would be calculated equally around 0.0025 μl for flow cytometry, 0.1 μl for microspectrophotometer, and 0.25 μl for hemocytometer at this concentration to ensure reliable counting for sperm concentration calculation. This becomes meaningful when dealing with small sample sized of biomedical fishes that are often 1–2 μl total before dilution.

Concentration Determination with Flow Cytometry and Comparison with Other Methods

The approach to determine sperm concentration by using flow cytometry was verified in *Experiment IV*. Among the methods tested, hemocytometer yielded apparently higher readings for all samples, especially when compared with these from the microspectrophotometer, but there was no significant difference.

For flow cytometry, the sperm concentration calculated from samples with a 1:9 dilution at a fast flow rate was significantly lower than that from samples with a 1:99 dilution (*Experiment VI*). This could be because in the samples with a 1:9 dilution, sperm cells were aggregates passed through the detecting platform, especially at the fast flow rate, and thus signals from individual cells could not be distinguished and captured because these cells were excluded from counts due to setting on the instrument. In fact, based on the flow cytometry analysis of SSC-H/SSC-W, FSC-H/FSC-W, SSC-H/SSC-A, and FSC-H/FSC-A, signals that suggested clumping or doublets did occur in the 1:9 dilution samples with fast flow-rate in which cell count by flow cytometer was lower than hemocytometer.

To avoid counting doublets or clusters of cells, in *Experiment II*, the effective range of concentration was determined to be between 1×10^3 and 1×10^7 cells ml^{-1}, for the sample with 1:9 dilution, the actual concentration at analysis was 1.4×10^7 cells ml^{-1} which was just beyond the effective range. These results emphasize that measurement of cell concentration can only be performed accurately within effective ranges, which is true for flow cytometry, and for spectrophotometry and hemocytometer.

Conclusions

This study demonstrated that flow cytometry can be used for determination of sperm concentration while simultaneously analyzing membrane integrity. This approach is comparable to counting results obtained from other methodologies such as hemocytometer or absorbance measurement by microspectrophotometer, and the precision and accuracy were retained across a concentration range of 1×10^3 cells ml^{-1} to 1×10^7 cells ml^{-1}. The approach developed can be used for determination of sperm concentration and quality of valuable small samples.

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