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Andrology laboratory review: evaluation of sperm concentration

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Abstract

This article is the result of the work of the andrology task-force of the Association of Applied Animal Andrology, American College of Theriogenologists, European College of Animal Reproduction, Society for Theriogenology, and National Association of Animal Breeders. It is intended to serve as a comprehensive reference on methods to evaluate sperm concentration and to contribute to the adoption of best practices in veterinary andrology laboratories. The information covered in the article includes sample preparation and the use of manual counts, spectrophotometers, computer-assisted semen analysis, NucleoCounter, and flow cytometry. Emphasis is given to the principles of the methods and equipment, performing the evaluation, and common mistakes and/or pitfalls. In addition, the precision and accuracy of the different methods are also discussed.

1. Introduction

Evaluation of sperm concentration is an essential component of semen analysis and results are used, among others, for breeding soundness certification, diagnosis/prognosis of reproductive disorders, study of minimum insemination dose, characterization of semen samples for trade, and assessment of treatment effects on sperm production (e.g. toxicology and nutrition studies). Despite these very significant implications, evaluation of sperm concentration is sometimes viewed as a trivial test and results are taken for granted without proper validation.

A different reality exists however, as demonstrated by several multi-center studies involving human andrology laboratories. Reported inter-laboratory coefficients of variation for sperm concentration results range from 23 to 73% [1], 53 to 80% [2], and 21 to 34% [3] for individual samples, underscoring the difficulty to compare results among laboratories and to generalize the findings of scientific studies. Although similar studies have not been reported in the veterinarian literature, the predicament of animal andrology laboratories is likely not very different.

The reasons for the large variations in sperm concentration results among laboratories likely involve variations in methods and techniques, virtual absence of comprehensive reference technical material, improper training and proficiency testing of technicians performing the analysis, and lack of quality assurance/control programs. It is the responsibility of clinicians, researchers, and industry to follow best practices in order to provide meaningful information to animal owners, academic community, semen customers, and regulators. The objective of this manuscript is to serve as a reference source to these professionals and contribute with the adoption of best semen evaluation practices in veterinary andrology laboratories.

2. Sample preparation

2.1. Species-specific considerations

Differences in the reproductive biology, including testicular size, sperm production capacity per testicular mass, epididymal sperm storage capacity, and ejaculate volume dictate the physiological differences in sperm concentration observed in the ejaculate among species. Different methods of semen collection, sexual stimulation, and the environment can also affect quantitative ejaculate parameters. In addition, the ejaculate of some species consists of distinct fractions that differ in number of sperm and might also differ in other physical characteristics that might affect sperm concentration evaluation, like viscosity, opacity, and presence of particles. In the latter case, the sample might have to be processed before sperm concentration can be determined (e.g. removing the gel from boar and stallion semen).

Since no existing method allows all sperm in a semen sample to be counted, a subsample is counted to represent the whole sample. The objective is to obtain a representative sample that contains a sufficiently small number of sperm so that counts can be performed efficiently; the optimal number of sperm to be counted varies according to the counting method. Therefore, the technician must take into account the method to be used and the expected sperm concentration in the sample in order to dilute the sample appropriately prior to evaluation. Dilution rates ranging from 1:1000 for highly concentrated samples (e.g. ram semen) to 1:5 for less concentrated samples (e.g. boar semen) are used.

2.2. Diluents

The basic property required of any diluent used for sperm concentration evaluation is the ability to disperse sperm and not interfere with the counting method. Therefore, diluents are usually

translucent solutions that prevent sperm from agglutinating. Simple salt solutions (e.g. sodium chloride or sodium citrate), buffered solutions (e.g. sodium bicarbonate or phosphate), more complex media (e.g. TALP and Hepes), semen extender, and even distilled water can be used as diluents for sperm concentration evaluation depending on the counting method. Other required properties are specific to the counting method and/or application and might include sperm immobilization, disruption of the plasmalemma, and prevention of auto-fluorescence.

Immobilization of sperm is essential when performing manual counts and might also increase the precision and accuracy of computer-assisted semen analysis (CASA) results [4]. Distilled water can be used to immobilize sperm since it results in hypotonic shock. Sperm diluted in water have altered morphology (coiled tails) but can be easily counted otherwise. Dilution of bovine semen in diluent containing 30 mM sodium fluoride immobilizes sperm in a characteristically rigid form [5]. Sperm can also be immobilized by adding 0.35% formalin to the solution [6,7], but the solution must be tested before use because formalin may cause sperm agglutination when combined with certain salts/buffers. The NucleoCounter requires the use of a non-permeable dye to stain sperm for evaluation of concentration. Therefore, a special diluent containing detergent is used to disrupt the plasmalemma and allow penetration of the dye into all sperm in the sample [8]. It has also been demonstrated that different media have different effects on sperm autofluorescence; therefore, these should be evaluated when employing methods that rely on detection of fluorescence like flow cytometry [9].

2.3. Preparing dilutions

Since semen samples invariably have to be diluted prior to evaluation, obtaining precise results requires very precise dilution. Because diluent and semen sample volumes are usually small and dilution ratios are relatively large, even minor sampling errors can significantly affect the results.

Manual micropipettes are the most common instrument used for sampling and diluting semen for concentration analysis. Like all precision instruments, pipettes produce more reproducible results when operated with attention to detail and proper technique. Unfortunately, training on proper pipetting technique is often neglected and calibration of instruments and evaluation of technician performance are afterthoughts at best. In order to ensure consistency, andrology laboratories should adopt standard operating procedures for pipetting techniques and ensure that all instruments and operators are periodically evaluated.

Pipettes are classified as “air” or “positive displacement” according to the mode of operation. Air displacement pipettes have a piston in a cylinder that moves to the appropriate position once the volume is set. The volume of liquid aspirated or expelled is the same as the volume of air contained in the cylinder. Positive displacement pipettes also have a piston in a cylinder or capillary tube that moves to the appropriate position once the volume is set. However, the piston is in direct contact with the liquid in this type of pipette and the volume of liquid aspirated or expelled is also dependent on the dimensions of the tip containing the piston (Figure 1). Direct contact of the piston with the sample enhances accuracy and precision for liquids which are too viscous to be displaced by air.

In addition to the type of pipette, the pipetting technique should also be considered. The operation button in air pipettes has two positions: the first stop and the second ("blow out") stop. When using forward pipetting technique, the operating button is pressed to the first stop, the tip immersed into the sample and the desired volume is aspirated by slowly releasing the button. When the operating button is depressed to the first stop again, the air dispenses the liquid. In order to empty the tip completely, the operating button is pressed to the second stop. When using reverse pipetting technique, the operating button is pressed to the second stop for aspirating the sample and is only pressed to the first stop when dispensing the liquid; therefore some liquid is left in the pipette tip when using this technique. Only forward technique can be used with positive displacement pipettes.

Semen diluents are usually simple aqueous salt solutions that can be properly pipetted using the most common combination of air pipette and forward technique. Reverse pipetting technique is actually not recommended for aqueous fluids, since the pipette tends to deliver more than the calibrated volume. Semen on the other hand, whether raw or extended, is a viscous solution that requires special consideration when pipetting. Use of forward pipetting technique with air pipettes usually produces inaccurate results due to the formation of a film inside the pipette tip and the inability to dispense the entire semen volume. Use of positive displacement pipettes or reverse pipetting technique with air pipettes is recommended when working with semen. Alternatively, the pipette tip can be rinsed by repeatedly aspirating and dispensing the diluent to remove the semen that lines the tip; properly wiping the pipette tip after semen sampling is essential when using this technique.

Regardless of the type of sample, pipette, or pipetting technique, following these good practice recommendations will help increase the accuracy and precision of sperm concentration results:

1. Maintain temperature and relative humidity (RH) within optimal operational range: air pipettes are calibrated at ambient temperatures around 20 ºC and substantial deviation in ambient temperature when preparing samples might affect the accuracy of measurements. It is also difficult to ensure accurate pipetting when operating in environments with RH < 30% due to rapid evaporation rates. Conversely, RH > 75% can cause inaccuracy due to condensation. Therefore, samples should be preferably prepared in environments with RH between 45 and 75%.
2. Select appropriate pipette: accuracy and precision are maximal when pipettes are used to measure 35 to 100% of the designated capacity. Working with volumes between 10 and 35% of the capacity requires excellent technique, whereas working with volumes <10% is not recommended.
3. Select appropriate pipette tip: for accurate volume delivery, choose a tip that is designed for use with the type of pipette being employed. Mismatching a tip and pipette or using poor quality tips can result in an inadequate seal between the pipette and tip. Quality tips are flexible and have thin walls, providing airtight seals and dependable sample delivery. Tips are usually designed for single use; they should not be cleaned for reuse as their metrological characteristics will no longer be reliable.
4. Make sure pipettes, tips, and solutions are at the same temperature: the volume of sample delivered by air displacement pipettes varies with air pressure, RH, and vapor pressure. Working with all components at a constant temperature minimizes variation.

5. Set the micrometer consistently: approach each volume in the same direction each time. Turn the micrometer 1/3 revolution above the desired volume, look straight to the dial with one eye closed, and then dial down to the desired volume.
6. Hold the pipette vertically during the aspiration of the sample: the hydrostatic pressure of the liquid column in the pipette tip falls as the angle of inclination of the pipette increases resulting in increased aspirating volume; holding a pipette 30^{o} off vertical can cause as much as 0.7% more liquid to be aspirated.
7. Press and release the operating button slowly and with consistent pressure: releasing the button abruptly can cause liquid to be "bumped" inside the pipette and reduce accuracy.
8. Immerse the pipette tip to appropriate depth: the pipette tip should be immersed 2-5 mm below the meniscus of the sample and well clear of the container walls and bottom. If the tip is immersed too deep, the results could be erroneously high due to the adhesion of liquid to the tip and transfer along with the aliquot in the tip. If the tip is not immersed deep enough then air could be drawn into the tip which could yield results that are incorrectly low. Pressing or resting the tip against the container walls or bottom restricts entry of the sample.
9. Pre-rinse tip with the same liquid that is being sampled: aspirate sample into tip, and then dispense back into reservoir or to waste. Pre-rinsing provides identical contact surfaces for all aliquots and increases accuracy.
10. Pause consistently after aspiration: pause with the tip in the liquid for about 1-2 seconds after aspirating the sample. It takes a moment for the liquid in the tip to finish moving after the plunger stops, so failure to pause will cause the volume to be too low.

11. Pull the pipette straight out of the container after aspirating a sample: do not touch the pipette tip to the sides of the container. This technique is especially important when pipetting small volumes. Surface tension effects cause the sample volumes to vary if the exit angles vary; touching the tip against the container walls results in loss of sample.
12. Remove any remaining liquid by wiping the pipette tip: examine the tip before dispensing the sample and wipe the tip if there is liquid on the outside. Be extra careful not to touch the tip orifice since absorbent material rapidly carries sample from the tip if it contacts the tip opening and unnecessary tip wiping increases the possibility of sample loss. Preferably, the outside of the tip should be covered and the wipe moved away from the opening.
13. Place the filled tip at an angle of 30 to 45º against the inside of the vessel for dispensing: this helps all of the liquid in the tip to be dispensed.
14. Check calibration regularly, depending on the frequency of use and on the application, but at least once a year. Instructions for evaluating pipette accuracy and precision, as well as instructions for recalibration, can usually be found in the manufacturer's instruction manual [10-12].

Other methods for preparing dilutions include autodiluters, autodispensers, bottle-top dispensers, glass blood diluting pipettes (e.g. Thoma and Potain), and disposable blood diluting capillary systems (e.g. Unopette®) (Figure 1). Autodiluters are equipment that use two syringes connected through tubes to the sampling tip in a closed system primed with diluent, making it a positive displacement system. The first syringe is used to sample the diluent, whereas the second is used to sample the semen sample; both diluent and semen are dispensed at once. Autodiluters are

excellent options for preparing samples for sperm concentration evaluation. In one study, the precision and accuracy of auto-diluters were compared to that of air pipettes using forward technique. Results from this study demonstrated that volumes of water obtained by experienced technicians using air pipettes and forward technique were as precise and accurate as those obtained using an automatic diluter, but volumes for semen extender were two times more precise and ten times more accurate when using the auto-diluter (Table 1). Autodiluters, as any other type of precision equipment, should be evaluated and calibrated periodically [13,14].

Autodispensers are equipment that use one syringe connected through tubes to the sampling tip. Autodispensers are good options for dispensing diluent, but have the same limitations of air displacement pipettes when used for dispensing semen and their use should be avoided for this purpose. Bottle-top dispensers are designed to be screwed directly to the bottle containing the diluent and might be good options when relatively large volumes (> 5 mL) of diluent are required. The accuracy and precision of bottle top dispensers are generally very good.

Glass blood diluting pipettes contain a mixing chamber with a mixing bead. These pipettes are usually marked at 0.5 and 1 µl volumes below the mixing chamber, and at 11 and 101 volumes above the mixing chamber (white blood cell and red blood cell models, respectively). Therefore, dilutions ranging from 1:10 to 1:201 can be obtained with these pipettes. Disposable blood diluting capillary systems consist in a capillary tube used to sample the semen and a plastic reservoir containing a specific volume of diluent into which the contents of the capillary are emptied. Systems with different diluent volume are available to produce different dilution ratios. These systems also allow the capillary tube to be used to dispense the diluted sample and the

diluent contains preservatives that inhibit sperm motility (e.g. ammonium oxalate). It is important to note that blood pipettes and capillary systems have been developed specifically for blood and that no data validating the accuracy of these methods for semen seem to be available; therefore, their use should be avoided when very accurate results are required.

In a study designed to investigate the effect of the type of material of the tubes used for processing and diluting frozen-thawed bovine semen no difference in sperm concentration results were observed among borosilicate glass, polypropylene, or polystyrene tubes. However, the method of dispensing the semen from the straw significantly affected the results. When samples were obtained by draining the contents of the straws by gravity after cutting both sealed ends, sperm concentration was 13.0% lower for 0.5 mL straws and 9.4% lower for 0.25 mL straws when compared to samples obtained by cutting the ultrasound-sealed end and pushing the cotton plug along inside the straw using a metal stylet [15]. Therefore, frozen-thawed semen straws should be emptied by pushing the cotton plug through the straw and not by gravity draining.

3. Manual sperm counts

Manual sperm count using especially designed chambers is the oldest method of sperm concentration evaluation. It is relatively simple, very inexpensive and, contrary to some other methods, allows direct visualization of sperm during evaluation. For these reasons, manual sperm count is widely used in andrology laboratories.

3.1. Principles of the method and equipment

Clinical pathology methods to manually count cells have been adapted for counting sperm. These methods require the use of chambers into which cells can be observed and counted within a known area, then allowing the calculation of the number of sperm per unit of volume.

3.1.1. Hemocytometers

Hemocytometers are the most common type of chamber used for manual sperm count. These are thick glass slides with a rectangular indentation that creates an 'H' shaped area at the centre that defines two separate counting chambers. The glass coverslip is held at a specific height above the surface of the counting areas by glass ridges on either side of the vertical grooves of the 'H' shape. Although some manufactures produce specialty hemocytometers, the standard depth of the counting chambers is 100 μm. Hemocytometer are prepared by placing the coverslip over the chambers and filling the space with the semen sample. Approximately 10 μL of the sample is loaded through a V-shaped notch at either end of the chambers and the fluid is drawn into the chamber by capillary action (Figure 2).

A counting grid is etched on each of the counting chambers. Different grid patterns are available and hemocytometers are usually referred to by the name of the grid pattern (Figure 2). The most common grid pattern used for evaluation of sperm concentration is the Improved Neubauer. This grid is divided into nine 1 mm^2 large squares. The squares located on the four corners of the grid are divided into 16 smaller squares (0.25 mm^2), whereas the central square is divided in 25

smaller squares (0.2 mm^2). The Bürker grid is also divided into nine 1 mm^2 squares delimited by three continuous lines (Q squares). Each Q square is divided into 16 smaller squares with an internal area of 0.2 mm^2. The Thoma grid has a central grid of 1 mm^2, composed by 16 smaller squares (0.04 mm^2).

3.1.2. Makler chamber

The Makler® chamber has been designed specifically for evaluation of sperm concentration [16]. It has a unique design with a glass piece mounted in the center of a metal disc. Four, 10 μm quartz pins define the depth of the chamber (i.e. 1/10 of the depth of hemocytometers) (Figure 3). According to the manufacturer's instruction, 5 μl of the semen sample should be placed in the center of the chamber and then covered immediately with the cover glass, avoiding the formation of bubbles. The 1 mm^2 Makler grid is divided into 100 smaller squares (0.1 mm^2); differently from hemocytometers the grid is imprinted on the coverslip.

3.1.3. Disposable slide chambers with grid

Disposable slide chambers have been developed to eliminate time-consuming and non-productive handling of reusable chambers, and to minimize the risk of contact with potentially infectious material. These chambers usually have the same format of common slides and contain two separate counting chambers, but use different designs and grid placement (Figure 4). For example, the CellVision® slide has a fixed coverslip with specific chamber depth. The grid pattern is imprinted on the slide and the chamber is filled by capillary action. The

Cell-Vu® slide on the other hand has a patterned printed inert surface that supports a coverslip that contains a laser etched grid [17]. The coverslip is applied after the sample has been placed onto the slide. Disposable slide chambers are available in a variety of depths (10, 20 or 100 μm) and grid patterns.

3.1.4. Disposable slide chambers without grid

Other types of disposable slide chambers are also available without a counting grid and manual counts can be performed using an eyepiece grid. These slides have a fixed coverslip with specific chamber depth and are filled by capillary action. Examples of these include Leja®, MicroCell®, and CellVision® slides, which are available with different numbers of chambers (2, 4 or 8) and a variety of depths (10, 12, 20, 50 or 100 μm).

To adapt the eyepiece for counting, a small circular disk-shaped glass reticle with a grid is added at the plane of the field diaphragm. Because the reticle lies in the same plane as the field diaphragm, it appears in sharp focus superimposed over the image of the semen. Eyepieces using reticles must contain a focusing mechanism that allows the image of the reticle to be brought into focus. Different types of counting grids are available, but the most commonly used for evaluation of sperm concentration are 5 or 10 mm^2 divided into 5 x 5 or 10 x 10 squares. In addition to the eyepiece grid, a stage micrometer is also required for calibration and calculation of sperm concentration.

3.2. Performing the evaluation

3.2.1. General considerations

The precision of sperm concentration estimates depend on the total number of sperm counted. Sampling errors can be calculated as a percentage of the counted number (n) of sperm using the formula $\sqrt{n} * 100/n$. Accordingly, to maintain sampling error below 5% it is recommended to count a minimum of 400 sperm. In addition, the total sperm count should be obtained in at least two replicates using separate chambers and results validated in order to maintain adequate precision. The confidence interval for the difference between two independent counts can be calculated and reference tables with acceptable count differences based on the sum are available [7]. Alternatively, it is recommended at the very minimum to discard the results if the difference between two counts is greater than 10% of the mean. In these cases, it is recommended to perform two additional independent counts with freshly prepared samples.

Given the recommendation to count a minimum of 400 sperm in at least two replicates (e.g. 200 sperm per replicate), the sample dilution and/or the area evaluated should be adjusted accordingly. If an initial evaluation reveals that the minimum number of sperm cannot be counted, either a smaller dilution factor should be used for preparation of a new sample or the area evaluated should be increased. Alternatively, if the number of sperm in the area to be evaluated is too great, making counting difficult and more time consuming, either a greater dilution factor should be used for preparation of a new sample or sperm could be counted in a smaller area.

Different schemes of counting sperm can be adopted according to the type of grid used. The most common is to count all sperm in one 1 mm^2. Alternatively, sperm are counted in smaller areas within a 1 mm^2 square, like for example counting five of the small squares in the center square of the Improve Neubauer grid (Figure 5) or 10 of the small squares of the Makler grid. Although the pattern itself is not important, a standard pattern (e.g. diagonal lines, straight lines or corners/center) for selecting the area to be counted should be used within the laboratory for consistency.

Only sperm heads are counted and tails should be disregarded. It is extremely important to understand which line(s) of the grid delimit the area that should be evaluated in order to avoid under or overestimation associated with erroneous characterization of the area. In order to avoid counting the same spermatozoon in adjacent squares twice, sperm which heads touch the left or lower square boundary lines should be counted, whereas those that touch the right or upper boundary lines should not be counted (Figure 5). Sperm counts should be conducted under 200 or 400 X magnification using phase-contrast microscopy. Alternatively, stains (Bengal rose, trypan blue, or gentian violet) can be added to the diluent to allow visualization of sperm using bright-light microscopy and adjusting the condenser. Hemocytometers with mirrored chambers provide better cell contrast and facilitate counts.

Although relatively simple and inexpensive, manual sperm count is fairly time consuming. The following are step-by step recommendations for performing manual sperm counts:

1. Determine the dilution factor to be used and prepare the semen sample; sperm should be immobilized before counting.
2. Verify that chambers and coverslips are clean and dry. For hemocytometers, position the appropriate coverslip (0.4 mm-thick) on the chamber ridges (pillars).
3. Sample the diluted semen immediately after thoroughly mixing to avoid sperm settling out of suspension.
4. Slowly load an appropriate volume of the semen sample into two separate chambers using the manufacturer's recommendations.
5. Allow the sample to settle for 5 minutes, preferably inside a humidity chamber to avoid evaporation, so that sperm heads lay flat on the surface of the grid.
6. Tally the number of sperm in the pre-determined area in each chamber using a cell counter.
7. Verify that the minimum number of sperm was counted and validate the replicate results (see above).
8. For reusable chambers, clean the surface and coverslip with 70% ethanol prior to next use.

3.2.2. Calculation of sperm concentration using chambers with grid

Formulas described in the literature and in technical materials for calculation of sperm concentration based on sperm counts can be presented in different manners, for different types of chambers, depending on how the counts are made. It is important to understand the underlying assumptions behind the calculation to avoid confusion and minimize clerical errors. The most

important relationship to remember is that 1 mm^3 corresponds to 0.001 mL. The variables used for the calculation include: (1) dilution factor, (2) averaged sperm count from two or more replicates, (3) area evaluated, and (4) chamber depth.

Example 1: Using a dilution of 1:200 and an Improved Neubauer hemocytometer, an average of 250 sperm are counted in one of the 1 mm^2 squares of the grid (see Figure 5). Since the depth of the hemocytometer chamber is 0.1 mm (100 μm), the total area evaluated is 0.1 mm^3 or 0.0001 mL. Therefore, 250 x 10000 sperm are present in 1 mL. Multiplying this number by the dilution factor (200), reveals that the concentration in the original sample is 500 x 10^6 sperm/mL.

Example 2: Using a dilution of 1:10 and an Improved Neubauer hemocytometer, an average of 200 sperm are counted in five of the 0.2 mm^2 squares in the middle square of the grid (see Figure 5). In order to obtain the number of sperm in 1 mm^2, the result is multiplied by 25, which total 5000. Since the depth of the hemocytometer chamber is 0.1 mm (100 μm), the total area evaluated is 0.1 mm^3 or 0.0001 mL. Therefore, 5000 x 10000 sperm are present in 1 mL. Multiplying this number by the dilution factor (10), reveals that the concentration in the original sample is 500 x 10^6 sperm/mL.

Example 3: Using a dilution of 1:200 and a disposable slide with Improved Neubauer grid, an average of 500 sperm are counted in one of the 1 mm^2 squares of the grid. Since the depth of the slide is 0.02 mm (20 μm), the total area evaluated is 0.02 mm^3 or 0.00002 mL. Therefore, 250 x 100000 sperm are present in 1 mL. Multiplying this number by the dilution factor (200), reveals that the concentration in the original sample is 500 x 10^6 sperm/mL.

Example 4: Using a dilution of 1:10 and a Makler chamber, 50 sperm are counted in 10 of the 0.1 mm^2 squares in the grid (see Figure 3). In order to obtain the number of sperm in 1 mm^2, the result is multiplied by 10, which total 500. Since the depth of the Makler chamber is 0.01 mm (100 μm), the total area evaluated is 0.01 mm^3 or 0.00001 mL. Therefore, 500 x 100000 sperm are present in 1 mL. Multiplying this number by the dilution factor (10), reveals that the concentration in the original sample is 500 x 10^6 sperm/mL.

3.2.3. Calculation of sperm concentration using chambers without grid

In addition to the variables used for the calculation of sperm concentration when using chambers with grid, a calibration factor (F) is also required when using eyepiece grids to compensate for the optical variation that is experienced from microscope to microscope, even those of the same model and manufacturer. The calibration factor is calculated using the formula *F = 1000000/chamber depth x distance across a single box of the reticule*^{2}. The distance across a single box of the reticule is obtained using a stage micrometer. Sperm concentration (C; in millions/mL) is then calculated using the formula *C = F x average number of sperm in a single box*. It is important to note that a different F must be determined for each magnification and all microscopes must be calibrated separately. Re-calibration is also necessary every time the reticule is changed or replaced.

Example: Using a dilution of 1:10 and a 20 μm chamber, 50 sperm are counted in 10 boxes (mean 5 sperm per box). Since the distance across a single box is 50 μm, F = 1000000/20 x 50^2 =

20. Multiplying this value by the dilution factor (10), reveals that the concentration in the original sample is 1000 x 10^6 sperm/mL.

3.2.4. The Segre-Silberberg effect

The physical characteristics of the slide chamber and the viscosity of the semen sample dictate the dynamics of the sample flow when these slides are filled by capillary action. The velocity of sample flow varies among different locations within the chamber and this velocity gradient produces a transverse lifting force on suspended particles that drives particles toward two stable, fast-moving layers situated a short distance from the chamber's walls, a phenomenon known as the Segre-Silberberg (SS) effect. Due to transport into faster moving layers, sperm accumulate at the meniscus causing a reduction in concentration per unit of area behind the meniscus [18]. The concentrated sperm wave is located approximately 3 mm closest to the meniscus when using Leja 20 μm four, chamber slides. The wave normally disappears into the exit chamber port when a sample is completely loaded, leaving a high sperm concentration region in the inaccessible and invisible exit port and a lower concentration region in the rest of the chamber behind the wave. Therefore, a correction factor must be used to compensate for the SS effect when calculating sperm concentration. The viscosity of the semen sample determines the correction factor to be used and the slide manufacturer recommends evaluating the chamber fill time to determine the correction factor. In general, a correction factor of 1.3 is adequate for diluted, 'watery' samples [19,20]. The impact of the SS effect on sperm concentration and a description of correction factors are not readily available for other types of slide chambers with different depth and geometry.

3.3. Common mistakes and pitfalls

Errors introduced in the preparation of dilutions are by far the most significant source of imprecision and inaccuracy and must be minimized. In addition, failing to thoroughly mix the sample just prior to evaluation may result in non-uniform distribution of sperm in the suspension and biased results.

Although chambers are designed to allow overflow, it is advisable to avoid excessive overfill. Chamber underfill or evaporation of the sample inside the chamber affect the volume of the sample in the chamber and/or sperm distribution and render counts meaningless. Standard coverslips should not be used with hemocytometers because they result in altered chamber volume from bending due to increased surface tension when the chamber is filled. Air bubbles will also alter the sample volume in the chamber and care must be taken to avoid them, especially when using chambers with which the coverslip is applied onto the sample. Delaying covering the sample with the coverslip has been demonstrated to be a significant source of error when using the Makler chamber [21].

Factors that affect enumeration of sperm can be sources of errors. As previously described in this section, dilution should be adjusted so that a minimum number of sperm can be counted to minimize sampling error. However, dilutions that result in 'overcrowding' make it more difficult to visualize individual sperm and keep track of counts. Use of magnification less than 200 X makes it more difficult to observe sperm, whereas more than 400X might make it more difficult

to ‘track’ the grid lines and follow the desired counting pattern. Counts might also be affected if sperm cannot be properly visualized as when not enough time is given for samples to settle before analysis or when bright-field microscopy is used instead of phase-contrast.

Mischaracterization of the counting area (i.e. counting sperm over an area smaller or greater than intended) and failure to follow counting patterns (i.e. counting sperm that touch all boundary lines) are significant sources of errors that can be avoided with appropriate training and use of reference materials (e.g. grid illustrations). Special attention should be paid to how the final results are computed, including calculations of means, validation of replicate results, and formulas used to convert numbers related to area into volume in order to minimize clerical errors. In addition, the SS effect must be considered and corrected when appropriate.

4. Spectrophotometers

Spectrophotometers (or photometers or colorimeters) have been adapted for the measurement of sperm concentration as an alternative to the more technically involved and laborious use of hemocytometers for evaluation of semen. Although the method does not involve direct enumeration of sperm, precise and accurate results can be obtained when the equipment is adequately calibrated and properly used. Semen analysis using spectrophotometers is rapid and requires a small amount of sample, equipment and consumables are relatively inexpensive, and laboratory technicians can be easily trained to perform the evaluation. For these reasons,

spectrophotometry is the most common method used for evaluation of sperm concentration in semen processing centers.

4.1. Principles of the method and equipment

Spectrophotometers are specialized equipment used for measuring the intensity of light. In a strict sense, spectrophotometry is generally used to refer to the quantitative measurement of light transmission trough solutions, whereas turbidimetry is the analytical technique involving measurement of light transmission through particle suspensions; both of these techniques make use of spectrophotometers [22,23]. Semen samples used for determining sperm concentration not only contain sperm, but also soluble organic and inorganic compounds and in some cases extenders with additional soluble and insoluble components. These samples are therefore complex suspensions which light transmission characteristics are not determined solely by the number of sperm. This is an important concept for understanding the principles and limitations of the use of spectrophotometers for semen analysis.

Light is a form of electromagnetic radiation that might be transmitted when passed through a suspension or dissipated by absorption, reflection or scattering. The amount of transmitted light is related to the characteristics of the suspension and forms the basis of the analysis of sperm concentration using spectrophotometers. Transmittance (*T*) is defined as the fraction of light in the original beam that passes through the sample and reaches the spectrophotometer detector. It is calculated using the formula $T = I/Io$ or $T\% = I/Io*100$, where *Io* is the intensity of the light at the origin and *I* is the intensity of the light at the detector [22-25].

Absorption refers to the transformation of radiant energy into a different form of energy (usually heat) upon interaction with matter, whereas reflection and scattering refer to changes in radiation trajectory. Absorbance is the predominant mode of energy dissipation when solutions are analyzed, whereas reflection and scattering are predominant when studying particle suspensions. Although absorption, reflection and scattering are distinct phenomena, all result in reduction of the light reaching the spectrophotometer detector. Therefore, in sperm concentration analysis (as in turbidimetry) it is common to refer to absorption as the difference between the intensity of light at the origin and the transmitted fraction, as opposed to the definition used in physics Absorbance (A) might be defined in terms of transmittance and is calculated using the formula $A = -logT$ or $A = 2 - logT\%$ and is also be referred to as optical density (OD) [22,23].

Although absorbance can be calculated based on transmittance, the former is preferred for calculation of sperm concentration since it follows the principles of the Beer-Lambert's law, which states that the quantity of light absorbed by a suspension is linearly proportional to the concentration of the particles in the suspension and the path length of the light. Thus, the plot of sperm concentration according to absorbance results in a straight line. The relationship of transmittance with sperm concentration is exponential and estimates of concentrations are less accurate than those obtained using absorbance [23,25].

Light transmittance and absorbance of a given suspension is strongly dependent upon the wavelength of light. For this reason, analysis is performed using light with a singular wavelength (monochromatic light). In order to obtain the highest sensitivity and to minimize deviations from

Beer-Lambert's law, it is important to conduct the analysis using light with a wavelength at which the absorbance is the greatest [24]. The most appropriate wavelength is selected by plotting the sample's absorbance in relation to wavelength. Wavelengths between 500 and 650 nm have been used for evaluation of sperm concentration.

Spectrophotometers used for evaluation of sperm concentration are single beam devices that operate over the wavelength range of visible light with air in the light path. Although instruments may differ with respect to design, versatility, and quality of components, the basic spectrophotometer is composed of a light source, a monochromator, a cell (sample) compartment, a radiation detector, and a readout device. Connected to each are the appropriate electrical and mechanical systems used to control the equipment. A power supply is required for operation of the spectrophotometer (Figure 6).

Light sources should produce high intensity radiation that is stable overtime and over the spectral distribution suitable for the application. Traditionally, the most common radiation source for visible spectrophotometry is the tungsten lamp. Light from the source lamp is passed through a monochromator, the component that functions to isolate the specific wavelenght to be used in the analysis. The size of the light bean radiating through the sample is termed bandwith and is determined by the exit slit of the monochromator. Some spectrophotometers designed for evaluation of sperm concentration use luminescent electro diode (LED) lamps with especific wavelength, thus dispensing the use of monchromators. In addition, some of these instruments use optical fiber to conduct the light signal to the detector and the bandwidth is determined by the dimensions of the fiber.

The cell (sample) compartment is positioned between the light source and the detector. The most common type of cell used for spectrophotometry is the rectangular cuvette, but other unique cell types are used by some equipment. The cell should be of a material that does not absorb radiation in the spectral region being used. Cuvettes for evaluation of sperm concentration might be made of quartz or glass, but disposable, inexpensive plastic cuvettes are more commonly used. The dimensions of the cell are important with respect to the amount of sample required for analysis and the light path length. Cells should also have flat faces where the entering and exiting light beams are to be passed in order to minimize reflection and scattering from the cell walls; walls through which light beams do not pass are usually opaque to minimize stray radiation.

The most common radiation detector is the photomultiplier tube (PMT). A PMT is a sealed, evacutaed transparent quartz or glass envelope containing a photoemissive cathode that emits electrons when struck by photons. This component is extremely sensitive to radiation and care must be taken not to expose it to bright light to avoid damage. The energy received by the PMT is converted into voltage fluctuation and displayed in the readout device. Digital displays are now the most common readout device and several spectrophotometers can be directly connected to a computer for display of the results.

Technical specifications and examples of spectrophotometers specifically developed for evaluation of animal sperm concentration are shown in Table 2 and Figure 7.

4.2. Calibration

Since spectrophotometers do not directly quantify sperm, a calibration curve is used to establish the relationship between absorbance and sperm concentration. This relationship is determined empirically through analysis of a series of samples of know concentration (standards) determined using methods that directly quantify sperm (i.e. hemocytometer, Nucleocounter or Flow-cytometer; [26]). Use of spectrophotometers also require semen samples to be diluted into an optically clear solution (e.g. saline, sodium citrate, PBS, formalin) prior to the analysis.

It is crucial that standards are prepared using the same conditions (dilution ratio, diluent, mixing, time allowed before analysis, etc) that will be used for future analyses. The optimal dilution ratio, i.e. the ratio that would provide optimal readings for most samples within the limits of the spectrophotometer, must be considered for each species and application. The optimal dilution ratio should result in approximately 0.3 absorbance units (50% transmittance), so that most samples would fall within the optimal limits. Ideally, standards should cover the sperm concentration range expected for the samples to be analyzed.

Initially, a sample that contains diluent without semen is used to determine the blank absorbance value; this value is subtracted from the values obtained from standards before analysis of the data. Adjusted absorbance values obtained with the standards are then used to determine the best fit equation using absorbance as the independent variable and sperm concentration as the dependent variable. If the data follows the Beer-Lambert's law, the best fit should be obtained with a linear regression equation (Figure 8). Sperm suspensions with unknown concentration can

then have their concentrations determined by measuring absorbance and using the linear regression equation to calculate the corresponding concentration.

Non-linear calibration curves are generally observed when dilution of the sample results in absorbance values that are outsize the optimal operational range. Measurements at intermediate transmittance values tend to have lower error (greater relative precision) than measurements made at either very high or very low tramsmittance. The optimal range for absorbance measurements on simple spectrophotometers is from 0.2 to 0.8 absorbance units (15 to 65% transmittance) [27,28]. The nonlinear calibration curve reflects the fact that that the calibration sensitivity, defined as a change in absorbance per unit change in sperm concentration, is not constant.

4.3. Performing the evaluation

The following are step-by step recommendations for using spectrophotometers. The most important thing to remember is that results will only be accurate when the analyses are performed using the same conditions (wavelength, dilution ratio, diluent, mixing, time allowed before analysis, etc) used for calibration or when strictly following the recommendation of the manufacturer when using commercially available sperm concentration spectrophotometers.

1. Turn on spectrophotometer and allow the lamp to warm up for 15-30 minutes before starting evaluations.
2. Set the wavelength to the desired value.

3. Do not touch the side walls of the cuvette, as fingerprints interfere with light transmission.
4. If a cuvette needs to be cleaned, use only lens paper in order to avoid scratches.
5. Add diluent to the cuvette and place it in the spectrophotometer making sure that the transparent walls are positioned in the light path.
6. “Zero” (or “blank”) the instrument and remove the cuvette.
7. Add diluent and semen to another cuvette, cap it and mix the sample by inversion five times.
8. Do not shake the cuvette, as it will result in the formation of bubbles that interfere with light transmission.
9. Let the cuvette sit for at least 10 seconds until small bubbles rise and sperm become randomly oriented.
10. Estimate absorbance promptly after preparing the sample.
11. Place the cuvette in the spectrophotometer making sure that the transparent walls are positioned in the light path.
12. Close the cover and read instantly.
13. Re-dilute samples that fall outside the optimal range of absorbance; increase the dilution for samples that are too concentrated and decrease the dilution for samples that too diluted.
14. Use absorbance reading to calculate sperm concentration based on calibration line. Make sure to factor the dilution ratio if that was adjusted.
15. Turn off the instrument and cover when finished.

4.4. Common mistakes and pitfalls

As with any automated method of semen analysis, the most common pitfall when using spectrophotometers to evaluate sperm concentration is to blindly accepted the results as precise and accurate without carefully considering the technical aspects of the method and without any periodical validation.

Minimizing errors during the preparation of dilutions is essential to improve precision and accuracy. In addition, since spectrophotometry does not specifically identify sperm but rather measures the transmittance of light through the sperm suspension, non-sperm material will also limit light transmittance and artificially elevate sperm concentration values. Therefore, results obtained with spectrophotometers are not accurate for samples that contain excessive debris or particulate matter, or are contaminated with cells other than sperm (e.g. RBC, WBC). Pigmentation of the seminal fluid does not significantly interfere with the results, neither does the normal numbers of bacteria present in the semen of healthy animals using proper collection techniques [29].

Other potential sources of errors associated with the sample include mixing, time allowed before analysis, and quality of the sample cell. The period between dilution and evaluation should be standardized because absorbance might change overtime [30]. Just prior to evaluation, samples should be thoroughly mixed to ensure uniform distribution of the sperm suspension and avoid sedimentation, especially when using diluents that immobilize sperm. Cuvettes and other sample

cell types should always be inspected prior to use for scratches, stains, or dirty that might interfere with the readings.

Equipment performance might also be a source of error. In addition to routine checks of wavelength calibration, the stability of the spectrophotometer readings should be evaluated daily. If the value displayed in the readout device is fluctuating or is highly variable when multiple readings are attempted without any cuvette inserted into the equipment, the power source and/or the lamp must be checked. It is also good practice to "zero" the spectrophotometer after every 5-10 samples; the process should also be repeated every time a new batch of cuvettes or diluent is used.

It is important to recognize that calibration lines need to be derived for each species to account for differences in sperm shape and size. In addition, calibration lines are specific for the type of equipment used to derive them and are not transferable to different types of equipment. Interchangeable use of calibration lines across species and spectrophotometers, as for example using equations reported in the literature, will generally result in inaccurate results. These are also the reasons why it is important to select the proper species program in commercially available sperm concentration spectrophotometers. Attention must also be paid to clerical errors when calculating sperm concentration based on absorbance with special consideration to any adjustments necessary to the dilution ratio.

Lastly, it is of uttermost importance to periodically evaluate the precision of the spectrophotometer results by evaluation of replicates of the same sample and to compare sperm

concentration values with those obtained using methods that directly quantify sperm like hemocytometer, Nucleocounter or flow-cytometer to ensure the accuracy of the results [26].

5. Computer-assisted semen analysis (CASA)

Computer-assisted semen analysis systems were developed for automated analysis of sperm images. Use of CASA allows quick, inexpensive, and fairly precise estimation of sperm concentration, but accuracy is marred by several technical issues and variations. Although use of CASA for evaluation of sperm motility has gained enormous popularity in research and clinical labs, its use for evaluation of sperm concentration for clinical or commercial purposes has not been recommended by the WHO [7] or the National Association of Animal Breeders [26], respectively.

5.1. Principles of the method and equipment

The principle of CASA involves visualization and digitization of successive images of sperm using a microscopy setup (hardware) followed by image processing and analysis to identify and count sperm (software). Since the area of the images is know, the volume evaluated and the sperm concentration can be calculated.

There are more than 12 CASA systems marketed for use with animal sperm and, although these systems are based on similar principles, there are several differences in the hardware and

software among systems [31,32]. Some systems use a 'stand-alone' microscope, whereas the microscopy setup is built-in in some specially designed equipment. A manual or automated mechanical stage is used to position the sample at the desired X/Y coordinate and to adjust the focus in the Z axis. Most systems use broad-band illumination within the visible spectrum with 10X or 20X negative (or positive) phase-contrast objectives combined with matching condensers, whereas other systems are also equipped with other type of illumination (ultraviolet light is the more common) and special filters for detection of fluorescence. Images are captured by a digital camera usually during a period of 0.5 sec using a pre-determined frame acquisition rate (e.g. 60 MHz) controlled via a camera shutter or the pulse-duration of strobe illumination.

Most CASA systems use proprietary software to detect sperm heads and establish a centroid (i.e. central point on the head) used to track sperm trajectories **(Figure X)**. Sperm head detection is based on user-defined parameters such as brightness and number of pixels; therefore, modifying these parameters might significantly impact concentration estimates. In addition, different algorithms are used to discern sperm with crossing trajectories or sperm that collide during the evaluation period and to determine how sperm that either leave or enter the evaluation area during the evaluation period are handled. Formulas for calculation of sperm concentration are part of the software package and different levels of user input might be required and/or allowed. The area of evaluation is determined by the magnification used, whereas the volume evaluated is also determined by the depth of the chamber used for analysis. The dilution factor and the SS factor must be taken into account for the calculation.

Disposable (Leja®, MicroCell®, CellVision®, Cell-Vu®) or re-usable (Makler) measurement chambers 10 or 20 μm deep are required for estimating sperm concentration using CASA. Chamber depth must be sufficient for unconstrained sperm motion and will vary depending on the species, but should not be excessive in order to have sperm within the useful depth of field of the microscope (focus). Analysis is preferably conducted at standard, pre-defined locations within the chamber and several fields should be evaluated. Evaluation of at least 400 is advisable to improve precision. Therefore, due to the SS effect on capillary-filled chambers, validation of CASA systems must include chamber type and number and location of measured fields. Such validation needs to be species-specific since differences in viscosity of the semen, size and shape of the sperm head and motility characteristics all can influence sperm flow and distribution in the counting chamber.

5.2. Performing the evaluation

The following are step-by step recommendations for using CASA:

1. Make sure that all necessary equipment is prepared and available. All elements that contact the semen should be pre-warmed, including the stage, utensils, extender, pipette tips, vials, and chambers.
2. Determine the dilution factor to be used according to the manufacturer's recommendation and prepare the semen sample; accuracy of all measurements (concentration and motility) depends on a specifically defined number of cells in each measured field.
3. Check and adjust the microscope optics.

4. Check the calibration of the optics. Any change in the microscope optics requires a new calibration.
5. Check the calibration of the automated scan stage (if used) to assure measurements at the correct spots within the chamber. Check the number and location of measured fields.
6. Check and adjust the species-specific settings.
7. Check (or adjust) settings for sperm concentration calculation including dilution factor, chamber depth, and SS factor.
8. Load an aliquot of semen into the chamber according to the manufacturer's recommendation.
9. Adjust the light intensity according to the manufacturer's recommendations.
10. Focus the image before measurements are started; this is essential for adequate sperm recognition.
11. Select number and localization of measured fields according to the manufacturer's recommendation.
12. When using the Leja chamber, measurements must be completed within one minute after the chamber has been loaded. Other chamber types may require different time frames.
13. Once the measurement is finished, visually check the recognition of sperm using the replay function, if available.

5.3. Common mistakes and pitfalls

A primary factor affecting the performance of CASA systems is the technical competence of their users. It is important that technicians are trained to understand the theory behind CASA, as

well as the influence that the initial settings can have on the data produced [33,34]. As with all methods used for measuring sperm concentration, correct pipetting, dilution and thoroughly mixing of the sample are essential for obtaining reliable results. In addition, semen extender, sperm concentration, frame acquisition rate, and type of chamber have all been shown to affect CASA results [35,36].

Presence of foreign particles that are identified as sperm and sperm agglutination that render sperm "too large" to be identified are common problems when CASA is used. Visual observation of the samples is essential for detecting these problems and making necessary adjustments. Semen in certain extenders (e.g. milk) require staining of sperm with fluorescent dyes in order allow proper distinction between sperm and other particles for CASA. Sperm concentration also affects estimates; high concentration increases the number of crossed trajectories and sperm collisions that difficult detection and tracking of individual sperm, whereas the effects of foreign particles is amplified in samples with low concentration. The ability of the system to track individual sperm is also related to the frame acquisition rate and lower rates might produce erroneous results.

Using a CASA/measuring chamber system which has not been validated for sperm concentration measurements is another obvious pitfall. The impact of the SS effect on sperm concentration results has not been described for all available chambers and variations in chamber geometry might have additional effects on estimates. In addition, toxic effects of the chamber's glue have been reported to influence results [37] and it might be recommended to test every new batch of chambers for accuracy and accurate sperm recognition. A consistent sample depth cannot be

obtained using simple wet mounts, even when a standard sample volume and coverslip size are used; therefore, sperm concentration cannot be evaluated using this preparation.

When publishing data it is important to include: instrument and software model and version numbers, microscope optics and magnification, camera type and resolution, image acquisition rate, track sampling time, number of sperm sampled, and type of chamber (including depth) [33]. Information on image acquisition such as head area and formulas used to calculate standard data should also be included in the report. Information on semen handling, semen extenders, concentration of sperm loaded into the chamber, and volume loaded into the chamber are elements that should be reported. Currently, there is no information that allows direct comparisons of data collected from one manufacturer's machine to that of another manufacturer. Care must even be taken comparing data obtained in different labs using the same manufacturer's machine, as machine settings can vary.

6. NucleoCounter

The NucleoCounter is an instrument designed for automated evaluation of sperm concentration. Evaluation is quick and requires only a small volume of sample (10 to 100 μL depending on the anticipated concentration). No calibration is required, operation is easy, and precise and accurate results can be obtained. Since sperm identification is relatively specific, there is no interference from seminal plasma composition and gel, lubricants, extenders or debri, thus allowing

evaluation of a wide range of sample types. For these reasons, use of the NucleoCounter is becoming more common in clinical settings and semen processing centers.

6.1. Principles of the method and equipment

The NucleoCounter®SP-100™ is an instrument manufactured by ChemoMetec A/S, Denmark to measure sperm concentration. The equipment allows automatic quantification of fluorescently-labeled sperm within a known volume and calculation of sperm concentration. Operation of the instrument also requires the use of a specific diluent (SP-100 Diluent™) and disposable sampling cassettes (SP1-Cassette™). According to the manufacturer, the equipment has been validated for use with semen from boars, bulls, stallions, dogs, rams, buck goats, and buck rabbits (http://chemometec.com/product/nucleocounter-sp-100/).

When using the NucleoCounter, sperm samples are diluted in SP-100 Diluent™, which contains a detergent that immobilizes sperm and permeabilizes the sperm membrane. Approximately 60 µL of this diluted sample is then aspirated into the SP1-Cassette™, the lumen of which is lined with a fluorescent dye (propidium iodide; PI) that penetrates the sperm and binds to the DNA. The cassette is then inserted into the equipment, wherein approximately 1 µL of the sample is further aspirated into the measurement chamber (Figure 10) [8]. The depth of the measurement chamber is determined at the factory, the information is embedded in the cassettes, and the value is read by the instrument at the time of the analysis; therefore, precise manufacturing is less critical and accuracy and precision are improved.

The NucleoCounter is equipped with light emitting diodes (LED) as excitation light source, excitation and emission filters, optical lenses, and charged coupled device (CCD) camera, in addition to software for image processing. A green light is used to excite PI and the red fluorescence light emitted from sperm nuclei is captured by the camera. Algorithms are used to differentiate sperm nuclei from other those of other cells and quantify the number of sperm (Figure 10). Since the area of the image captured by the camera is fixed, the depth of the chamber is used to calculate the volume of the sample analyzed. This information, together with the dilution ratio inputted by the user, is used to calculate the sperm concentration in the original sample. Analysis time is approximately 30 seconds and results are presented in the equipment's digital display. Documentation of the results can be done by connecting the instrument to a printer or to a computer when using propriety software (SemenView™).

6.2. Performing the evaluation

Step-by step recommendations for using the NucleoCounter include:

1. Turn on the machine and the computer (optional); no calibration is required.
2. Select the appropriate program for the species being evaluated.
3. Determine the approximate dilution ratio using manufacturer supplied chart. The evaluator must estimate the expected final concentration so that the appropriate dilution factor (semen/diluent) can be used. The concentration range that can be measured is broad and the manufacturer claims that it can measure any value greater than 1×10^6 sperm/mL.

4. Enter the dilution factor (e.g. 11, 101, 201) to be used for calculation of the final sperm concentration.
5. Prepare dilution using a clean tube or container. The size of the tube should be such as to allow the tip of the cassette to be immersed into the sample.
6. Mix the sample well and immediately aspirate into the cassette.
7. Immediately place cassette into the NucleoCounter and press the start button.

6.4. Common mistakes and pitfalls

Using a dilution rate outside the optimal limits described by the manufacturer results in reduced accuracy and the instrument might display an error message if the number of counted sperm is too small or too large. Samples should be loaded into cassettes promptly after dilution and cassettes should be evaluated promptly after loading; long time lags might affect the readings. Although this method is highly specific, since PI is a DNA-specific dye, there is the potential, if enough debris containing fragments of DNA or other nucleated cells are present, to create non-specific fluorescence that will be interpreted as sperm.

Although no calibration is necessary, the equipment must be properly setup. Since there are differences in sperm nuclear size among species, the correct program must be used in order for the equipment to correctly analyze the images obtained during the analysis. Keying the incorrect dilution rate will also result in erroneous estimates. The equipment lenses should be inspected regularly using SemenView™ and cleaned when necessary, since debris in the lenses can affect sperm counts.

7. Flow cytometry

Flow cytometry allows rapid (minutes), automated counts of large numbers of sperm (tens of thousands). This ability, combined with the ability to exclude other semen components (e.g. gel, extender, debri) and cell types makes flow cytometry a very precise and accurate method for evaluation of sperm concentration. However, routine use of flow cytometry has been limited by expensive instrumentation, need for a skilled operator, and somewhat complex methods of sample preparation and data evaluation. This method has been used primarily for research purposes, validation of other methods, and calibration of different instruments.

7.1. Principles of the method and equipment

This article does not the intention to review all technical features of flow cytometry. It rather captures some aspects that are especially important for assessing sperm concentration. Readers with additional interest in basic aspects of fluorescent probes and flow cytometry should refer to specialized publications.

Flow cytometry uses detection of light scatter and fluoresce of individual cells to determine sperm concentration. Methods to evaluate sperm concentration include those based on the quantification of a defined number of events (e.g. 10000) and those based on true volume. The former method involves mixing the semen sample with pre-defined concentration of reference

beads. The ratio of detected beads in relation to sperm is used for the calculation of sperm concentration [38,39]. Flow cytometry with true volume count is straightforward and quantification of sperm does not require the use of reference beads. This also eliminates variations and errors related to inappropriate mixing and pipetting of bead solutions. Instruments with true volume option either have electrodes placed at a fixed distance within a sample tube to sense the lowering of the fluid level or are equipped with high precision syringes to inject pre-defined volumes of samples into the system.

Flow cytometers utilize hydrodynamic focusing to order the randomly distributed sperm present in a sample into a stream of single sperm that passes through an interrogation chamber where they are exposed to a laser beam. As each spermatozoa passes through the beam, it scatters light and emits fluorescent light. These light signals are collected by the optics system and reach the photodetectors. Light that is scattered in the forward direction is collected by the forward scatter channel (FSC) and can be used to determine particle size and distinguish sperm from other particles and debris. Light scattered approximately at a 90^{o} angle to the excitation line is collected by the side scatter channel (SCC) and provides information about the granular content within a particle. Separate fluorescence channels equipped with specific filters are used to detect emitted and provide data about fluorochrome-labeled cells or organelles (Figure 11) [40]. With the rapid technical development in the field of flow cytometry the trend is for instruments with multiple excitation wavelengths. Either multiple lasers or lasers combined with LEDs are used. The most common for new bench top instruments is the combination of a traditional blue laser (488 nm) with a violet laser (405 nm). The range of available excitation lines and emission filters defines the combination of dyes that might be used to label sperm for concentration estimates.

The signals received by the photodetectors are converted into electrical signals and then into numerical values by the electronics system. Data can be displayed, analyzed, and saved using specific analytical software packages that accompany flow cytometers. Single-parameter histograms display a single measurement parameter (e.g. green fluorescence) on the x-axis and the number of events (i.e. sperm, other cells, debris) on the y-axis. Two-parameter histograms or dot plots display two measurement parameters (e.g. FSC and green fluorescence), one on the x-axis and one on the y-axis; individual events are represented by individual dots on the plot. Using these histograms, the events of interest can be selectively visualized to eliminate unwanted particles, a procedure called gating. Selected events can be easily quantified by selecting populations directly on histograms.

7.2. Performing the evaluation

There is no standard protocol described in literature to estimate the concentration of semen samples. Instead, a variety of protocols exists that have often been adapted to a specific setup of a machine or software. However, basic aspects of the sample preparation, measuring procedures and gating strategy, i.e. how spermatozoa were identified, can be compared and are discussed with respect to their potential and limitations.

1. Sample preparation
    a. Undiluted
    b. Diluted [41]

c. Diluted and stained [8,39,42-47]

d. Diluted, permeabilized, and stained [38,48,49]

2. Measuring mode

a. True volume count [41]

b. With simultaneous assessment of reference beads [38,39,43,44,46-48] [8]

3. Sperm identification (gating strategy)

a. Only FSC

b. FSC and SSC characteristics [41,48]

c. FSC + positive DNA stain of permeabilized cells [49]

d. FSC + viability with DNA marker + cytoplasma marker

e. FSC + viability with two DNA marker [8,39,43,44,46]

f. FSC + other stains [42]

g. FSC + SSC + positive DNA stain of permeabilized cells [48]

h. FSC + SSC + viability with DNA marker + cytoplasma marker

i. FSC + SSC + viability with two DNA marker

The multiplicity of estimation methods requires strict guidelines to report assay protocols in publications, guidelines, and elsewhere to be followed. Guidelines on the minimum information that should be recorded and reported about the experiment overview, samples, instrumentation, and data analysis have been developed by the International Society for Advancement of Cytometry [50,51]. These guidelines promote consistent annotation of clinical, biological, and technical issues surrounding a flow cytometry experiment by specifying the requirements for

data content and by providing a structured framework for capturing information. Adopting these guidelines to flow cytometry in andrology may help to improve quality of assays used.

7.2.1. Sample preparation

The type of sperm treatment is closely connected to the gating strategy. Sperm concentration in the measurement vial must be adapted so that the flow rate (events/second) is within the optimum range for the type of flow cytometer used. Typically, semen samples are diluted before flow cytometric assessments to achieve optimum flow rates during analysis. Whether dilution is combined with permeabilization or not depends on the aim of the assessment and the gating strategy. Permeabilized samples may be used solely for estimates of concentration whereas unpermeabilized samples may be used for a combined assessment of sperm concentration and viability (either plasma membrane integrity alone or a combined assessment of plasma and acrosomal membrane integrity). More important than the decision of permeabilizing samples or not is the choice of dyes used and the gating strategy.

For estimates of concentration, all spermatozoa (permeabilized or not) should be tagged with a DNA-binding stain to differentiate them from non-DNA containing particles. Other cell markers (cytoplasmic or membrane bound) are inappropriate due to the fact of dye leakage from the cell or the risk of inhomogeneous staining. In the case of permeabilized cells one DNA-binding stain (e.g. propidium iodide) is suitable. In the case of non-permeabilized sperm a combination of two DNA-binding stains should be used (e.g. propidium iodide and SYBR-14) to assess membrane-intact and -defect sperm. This allows a simultaneous assessment of viability in these samples. In

addition to DNA-binding dyes, other markers may be included in the staining procedure to identify specific contaminants of the ejaculate (see species-specific aspects).

7.2.2. Measuring mode

Systems without true volume count require careful and reliable sampling as the calculation of sperm concentration depends on the detection of a pre-set reference bead count. True volume count offers the benefit of calculating sperm concentration directly based on the number of detected events. The user has to rely on the accuracy of the devices used for determining the measured sample volume and accuracy must be checked periodically using control solutions with pre-defined bead concentrations.

7.2.3. Sperm identification (gating strategy)

The gating strategy for identifying sperm cells is a crucial step in estimating sperm concentration. Light scatter (FSC and SCC) are features identified without the need for fluorescent probes and are commonly used to discern the cell of interest from other particles in the solution, like for example extender components and cellular debris such as epithelial cell fragments. The main limitation of flow cytometry is that it is an indirect method of cell identification since cells are identified based either on light scatter or fluorescent characteristics, but not by direct visualization such as would occur under a microscope. There might be no perfect strategy yet available, but a combination (logical) gate on DNA-positive events with light scatter characteristics typical for sperm seems to be the most valid approach [48]. Whether light

scatter criteria should be based on gating on FSC alone or on FSC and SSC combined is debatable. In both variants, a simple rule applies: the tighter the gate is set, the more likely an underestimation of sperm concentration is possible (see also comments on "focusing" and "mistakes & pitfalls").

7.2.4. Example protocol using true volume count

The evaluation relies on measuring a known volume of the diluted sample. Sperm are identified using defined gating strategies by their light scatter and fluorescence properties. The total number of sperm per volume can be determined using available computer software. This is a step-by-step example of a protocol:

1. Instrument: DAKO Galaxy; excitation: 488 nm laser (20 mW); emission: 630 LP
2. Mix semen (raw or extended) with distilled water (supplemented with 0.5 mg/mL PVA and PVP each) and propidium iodide stock solution (1 mg/mL in 10 mM HEPES, 150 mM NaCl, pH 7,5 at 20°C) to an approximate concentration of 20 x 10^6 sperm/mL. The final volume of the sample for counting should be adapted to the type of flow-cytometer used.
3. Mix diluted sample well and check under the microscope to verify staining and that there is no sperm agglutination.
4. Incubate for 5 min at room temperature.
5. Set up equipment with at flow rate of 400- 500 events per second which corresponds to a measuring speed of 3 to 5 µL/second.

6. The equipment automatically enters concentration measurement mode when the first electrode is free of solution and ends when the second electrode is also free.
7. Data are collected in three histograms (Figure 12):
    a. one parameter plot of FSC
    b. one parameter plot of PI fluorescence intensity
    c. dot plot of FSC vs. PI fluorescence intensity
8. In the dot plot, events that are PI-positive and above a certain threshold are considered to be sperm; the number of sperm within the chosen subpopulation is quantified by the software.
9. Multiple the number of sperm by the dilution factor used during sample preparation to obtain the sperm concentration in the original sample.

7.2.4. Example protocol using reference beads

The evaluation relies on combining a known concentration of fluorescent beads with the semen sample. Both sperm and beads are quantified and the ratio used for the calculation of sperm concentration. This is a step-by-step example of a protocol:

1. Mix semen (raw or extended) with distilled water (supplemented with 0.5 mg/mL PVA and PVP each) to an approximate concentration of 5 million sperm/mL to standardize the sperm concentration for analysis and minimize the error commonly associated with very low or high sperm concentrations.

2. Add 800 µL of 1%Triton-X in PBS (v/v), 100 µL of the pre-diluted semen, 100 µL of fluorescent microspheres (with a known concentration).
3. Add propidium iodide (PI) to a final concentration of 12.5 µg/mL.
4. Mix diluted sample well and check under the microscope to verify sperm and bead staining, and that there is no sperm agglutination.
5. On a histogram displaying both the PI-stained sperm population and the fluorescent beads, created a gate around the beads. Set up the flow cytometers to count a predetermined number of beads (e.g. 2000) and record the number the number of sperm enumerated during the process.
6. Calculate sperm concentration per milliliter using the formula: *(number of sperm/number of beads) x bead concentration in the sample x semen dilution rate*. For example, if the dilution used is 1:40, the concentration of beads in the sample is 1×10^6, and the number of counted sperm is 10000, then $(10000/2000) \times 1000000 \times 40 = 200 \times 10^6$ sperm/mL

7.3. Common mistakes and pitfalls

The most common pitfall when using flow cytometry is failure to realize that this is a powerful, but complex technology. Accurate and precise results cannot be expected without proper training, knowledge of technical considerations and variations, and adequate equipment maintenance/calibration. The following is not intended to be a comprehensive review of factors that can interfere with results and readers with especial interest in flow cytometry are encouraged to also consult other specialized publications.

Since preparation of samples for flow cytometry might include pipetting several different solutions (i.e. semen sample, diluent, stains, reference beads), dilution errors can quickly multiply. Training, appropriate equipment, and sampling best practices are crucial for obtaining accurate results. When using reference beads it is also important to realize that a relatively high variability of pre-defined concentrations might be accepted by the manufacturers and that bead solutions should be thoroughly mixed (sonicated and vortexed) before use to minimize bead clumping [38].

Flow cytometry does not involve direct visualization of sperm, but rather relies on the inference that sperm can be identified and enumerated based on specific light scatter or fluorescent characteristics. Therefore, evaluation of samples under the microscope is important to check samples for presence of debris, non-sperm particles, or sperm agglutination. Diluents might also be filtered prior to use (e.g. PES membrane with 0.22 μm pores) to reduce "noise" during flow cytometry assessments. In addition, the specificity and intensity of fluorescent stains should be verified in order to make sure that assumptions used for gating and data analysis are correct.

Different sperm identification strategies should be used for samples with debris and non-sperm particles to increase the accuracy of estimates. For example, using light scatter alone to identify sperm might result in overestimation (5 to15%) of sperm number due to the inclusion of debris in the "sperm" population, but use of light scatter plus DNA-binding stains helps reduce the error rate [52]. On the other hand, the use of DNA-binding stains must be considered carefully in semen of species which erythrocytes have a DNA-containing core (fish, amphibian, reptiles,

birds) as overestimation of sperm concentration might occur if these cells cannot be properly excluded from analysis.

Other example involves the evaluation of semen samples contaminated with other cell types. Human semen usually contains considerable numbers of leukocytes. Leucocytes have a nucleus and stain positive with DNA-stains. In addition to careful gating on DNA-containing events with light scatter characteristics (FSC and SSC) of spermatozoa, a positive marker for leucocytes like the fluorescent-labeled anti-human CD45 antibody, that does not cross-react with spermatozoa may be used for the analysis of such samples. Leucocytes may be identified as positive for the antibody and be excluded from the analysis [45].

Agglutinated sperm are registered as a single event during analysis and lead to underestimation of sperm concentration in the sample. Samples with considerable agglutination will not provide reliable results and should not be used for analysis. Gating on non-agglutinated sperm is not a solution and fresh samples should be prepared, perhaps using different types of diluent to avoid agglutination.

Some considerations regarding the equipment proper include adjusting the flow rate (counts/second) according to the manufactures recommendations as inadequate flow rates affect quantification of events. Although the carryover rate (number of sperm from a previous sample contaminating the next sample) was found to be negligible (< 0.2%) when human sperm samples with concentrations of 14.4, 25.0, and 60.9 x 10^6/ml, were analyzed [42], it is not clear whether this rate would be higher when animal samples with higher sperm concentrations are analyzed.

Low amounts of macromolecules might be included in the diluent to minimize sperm sticking to surfaces and reduce carryover.

8. Precision and accuracy

The precision and accuracy of sperm concentration estimates are determined primarily by the technician skills and limitations inherent to the method used, including equipment specifications and setup in case of automated methods. Although other statistical methods are sometimes used, precision (or repeatability) is usually reported as the coefficient of variation (CV) for a number of replicates. Several factors might affect the CV observed in a study, like for example the number of samples, the range of concentration of the samples, the number of technicians performing the evaluations, the number of replicates, and if replicates involve just the 'counting' procedure or also preparation of separate diluted samples. Reported CV's for sperm concentration estimates according to method are summarized in Tables 3 and 4.

Even though there is considerable variation among studies, some generalizations might be made. The precision obtained with hemocytometers was relatively good and most studies reported CV's between 7 and 11%. Although similar CV's are reported with manual counts using 20 μm disposable slides and eyepiece grid, the precision observed using the Makler chamber was considerably poorer (i.e. CV's around 20%). In general, higher precision can be obtained using automated methods. The CV's obtained with CASA in most studies were between 6 and 8%, whereas those for spectrophotometers were between 3 and 6%. The CV's obtained with the

NucleoCounter or flow cytometry were consistently between 3 and 4%. Although the precision obtained with hemocytometers can be considered adequate, it is important to note that the NucleoCounter and flow cytometry can produce estimates that are approximately three times more precise.

Determining the accuracy of sperm concentration estimates is more complex as it requires a method to be defined as the gold standard against which estimates obtained using other methods are compared. Therefore, the assessment of accuracy is greatly dependent on the 'quality' of the results obtained using the gold standard and how close these truly are to the 'real' sperm concentration. Since the optimal dilution might vary according to method, comparisons of different methods usually also require the preparation of multiple diluted samples making it difficult to isolate the differences attributed to variations in method from the variations attributed to dilution. Furthermore, for most methods the estimate is predicated on the fact that the evaluation is performed on known volume, which accuracy is determined by the manufacturing quality of the equipment, whether hemocytometers, slide chambers, NucleoCounter cassettes, or flow cytometer sample flow apparatus. Accuracy is described using a variety of statistical methods, including simple correlation, regression, comparison of means, and estimation of percentage differences. The Improved Neubauer hemocytometer is considered the gold standard for evaluation of human sperm concentration [7] and virtually all studies include estimates using hemocytometers for determining the accuracy of other methods.

Although hemocytometers are considered to be the gold standard method for evaluation of sperm concentration, differences among different types of hemocytometers have been reported. In one

study with human semen, use of the Bürker hemocytometer resulted in overestimated sperm concentration when compared to the Improved Neubauer hemocytometer [53], whereas in another study with bovine semen, use of the Thoma hemocytometer resulted in underestimation of sperm concentration when compared to the Bürker-Türk hemocytometer [54]. Since the chamber dimensions in these hemocytometers are the same and the only difference is the grid pattern, these observations are difficult to explain and might reflect differences in training and degree of difficulty keeping track of the area evaluated and visualizing sperm. Estimates obtained using the Makler chamber for evaluation of human semen were 32% greater than those obtained using the Improved Neubauer hemocytometer [4]. However, in different experiments with bovine and porcine semen estimates obtained with the Makler chamber were either comparable or 20% less than those obtained with the Bürker-Türk hemocytometer [54]. Considering these observations (and the low precision of the Makler chamber), use of the Improved Neubauer hemocytometer in lieu of other reusable chambers is recommended.

The literature describing the accuracy of disposable chambers for evaluation of sperm concentration is relatively sparse, especially considering the variety of manufacturers and models currently available in the market. In addition, applying the SS correction factor when disposable counting chambers filled by capillarity are used is recommended and results describing accuracy when the correction is not performed might not be valid. Results of manual counts performed on unfixed human semen using the Leja 20 μm or Microcell slides were significantly lower than those obtained with the hemocytometer; however, results were comparable when fixed semen (i.e. immotile sperm) was evaluated instead [4,55]. High correlation and similar means were also between estimates obtained with the hemocytometer and Microcell slides for bovine semen [41].

Reports on the accuracy of CASA have been fairly inconsistent. In one report with human semen, the percentage difference between results obtained with CASA and hemocytometers ranged from -15 to 471%. Although comparable results were obtained in the middle range of sample concentration and errors were randomly distributed, often only one-third or less of cases actually agree within 10% [56]. In addition to the system proper (hardware and software), considerations about the type of chamber and sperm preparation, also affect the accuracy of concentration estimates obtained using CASA. Correlations of equine sperm concentration estimates obtained with the NucleoCounter and those obtained with CASA using the Leja 10, 12, and 20 μm slides were low to moderate (r = 0.34 to 0.74), but means were not significantly different; however, use of the Makler chamber resulted in overestimation of sperm concentration [57]. When CASA results for porcine semen obtained using the Leja 20 μm were corrected with the SS factor, the regression coefficient with estimates obtained using the hemocytometer was high (r^2 = 0.94) and the regression slope was 0.98 [19]. When the same counting chamber (Makler) was used for evaluation of human and dog semen, CASA results were either significant greater or lower than those obtained with manual counts [35,58].

The reason for the discrepancies in CASA results might also be related to inability to properly gate to exclude non-sperm particles, lack of recognition of static sperm that appears too small and not bright enough to be recognized by the system, and sperm clumping. User intervention (i.e. manual deletion of particles and inclusion of sperm) post auto analysis is a strategy that might be used to address these limitations and have allowed similar means to be obtained with CASA in comparison with hemocytometer [59], but use of DNA fluorescent stains is a more

practical alternative. In one study using human semen, the percentage difference between estimates obtained with the hemocytometer and CASA was reduced from 12.1% to -0.4% when a DNA fluorescent stain was used [60]. When all of this information is taken together, it is apparent that very strict technical considerations must be followed for obtaining accurate results with disposable chambers either when performing manual counts or using CASA. These include use of validated chambers, immobilization of the cells, use of the SS correction factor, and use of DNA fluorescent probes (for CASA).

Comparisons between sperm concentration estimates obtained using hemocytometers and those obtained using spectrophotometers revealed high correlation and regression coefficients (r and r^2 > 0.9) [41,61,62] and comparison of the means obtained using hemocytometers and a general purpose spectrophotometer for evaluation of raw bull semen revealed no significant differences between the methods [41]. In another study, the mean difference of paired results obtained using a Makler chamber and a commercial sperm concentration spectrophotometer for evaluation of boar semen was -0.6% with all results between -15 and 15% [63]. When different models of commercial sperm concentration spectrophotometers were used for evaluation of stallion semen, the results were consistently greater than that obtained with the hemocytometer for one model (range 9 to 17% depending on the concentration), consistently lower for two models (range -2% to -25%), and variable for another model (range -25% at high concentration to 26% at low concentration) [64].

The use of semen visual scores based on the presence of particles coupled with the use different calibration lines based on the score have been described as an alternative to increase the

precision and accuracy of spectrophotometer results [30]. This is especially important for species that normally produce seminal fluid with various quantities of particles. In one study, rabbit ejaculates were either evaluated without any corrections based on the amount of particles in the seminal fluid or were allocated into three groups with particle/sperm ratios of 0-1.9, 2-3.9 or ≥4 prior to evaluation. The correlation coefficients of duplicate readings increased from 0.75 to 0.97 when calibration lines specifically constructed for semen containing the estimated ratio of particles were used. In addition, the correlation coefficients between the results obtained with the spectrophotometer and results obtained with the hemocytometer increased from 0.76 to 0.94 [65]. These observations indicate that accurate sperm concentration estimates can be obtained using spectrophotometers when good quality assurance process are in place to calibrate and periodically validated the equipment.

Recent studies have also used the NucleoCounter and flow cytometry as gold standards for accuracy assessment. Comparisons between sperm concentration estimates obtained using hemocytometers and those obtained using either the Nucleocounter or flow cytometry revealed high regression coefficients ($r^2 > 0.9$). Similarly high regression coefficients have also been reported when the hemocytometer was compared with flow cytometry [8,39,41,46,66,67]. The percentage difference between means obtained with hemocytometers and NucleoCounter ranged from 10 to 18% in equine semen [66], whereas the difference between means obtained with hemocytometers and flow cytometry ranged from 12 to 36% in human semen depending on the sperm concentration [48]. For bovine semen, the error of the estimate obtained with flow cytometry as compared to the hemocytometer was 23 x 10^6 sperm/mL over a wide range of concentrations (approximately 500 to 2000 x 10^6 sperm/mL) [39]. In one study with bovine

semen, comparison of means did not reveal any differences in results obtained with hemocytometers or flow cytometry [41], whereas in another study results obtained with hemocytometers were significantly lower than those obtained with the NucleoCounter or flow cytometry [8].

Despite the small inconsistencies among studies, authors have generally concluded that the NucleoCounter and flow cytometry are accurate methods for estimating sperm concentration. Considering that the results obtained with these automated methods are much more precise than those obtained using hemocytometers, some authors have concluded that differences in results might actually be attributed to 'erroneous' estimates obtained with hemocytometers instead of the other way around [8], leading others to suggest that these might be more appropriate methods to be used as gold standards [15]. The NucleoCounter has been the method recommended for evaluation of porcine sperm concentration by the Danish National Committee for Pig Production [68] and seems to be the method of choice for evaluation of frozen bovine semen in North American processing centers. The NucleoCounter and flow cytometry, along with the hemocytometer, are all considered reference methods and are recommended by the National Association of Animal Breeders to be used for calibration and quality assurance of spectrophotometers [26].

9. References

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Table 1. Coefficients of variation (CV) and accuracy for manual and automatic pipetting of water and semen extender (egg yolk + glycerol). Four technicians participated in the study and each measurement was replicated 20 times by each technician. Forward manual pipetting was performed using Pipetman pipettes (Gilson, Middleton, WI, USA) serviced and calibrated prior to the study. A P-1000 was used to pipette 500 and 1000 µL of water and a P-250 was used to pipette 50, 100, and 200 µL of extender. Automatic pipetting was performed using an auto-diluter (Microlab 500 Series; Hamilton) equipped with 5000 and 100 µL syringes.

	Manual	Automatic
Water		
500 µL intra-technician CV (%)	0.22 to 1.71	0.04 to 0.1
1000 µL intra-technician CV (%)	0.1 to 0.37	0.05 to 0.08
500 µL inter-technician CV (%)	0.92	0.11
1000 µL inter-technician CV (%)	0.40	0.07
500 µL intra-technician accuracy (%)	-1.01 to -0.1	-0.16 to 0.05
1000 µL intra-technician accuracy (%)	-0.86 to -0.1	-0.01 to 0.08
500 µL inter-technician accuracy (%)	-0.51	-0.03
1000 µL inter-technician accuracy (%)	-0.49	0.03
Extender		
50 µL intra-technician CV (%)	0.73 to 2.73	0.68 to 0.96
100 µL intra-technician CV (%)	0.54 to 3.61	0.29 to 0.73
200 µL intra-technician CV (%)	0.4 to 1.03	--
50 µL inter-technician CV (%)	2.45	0.9
100 µL inter-technician CV (%)	2.96	0.5
200 µL inter-technician CV (%)	1.46	--
50 µL intra-technician accuracy (%)	-7.19 to -3.46	-0.76 to -0.07
100 µL intra-technician accuracy (%)	-8.18 to -2.84	-0.67 to -0.42
200 µL intra-technician accuracy (%)	-5.54 to -2.37	--
50 µL inter-technician accuracy (%)	-5.51	-0.43
100 µL inter-technician accuracy (%)	-5.03	-0.52
200 µL inter-technician accuracy (%)	-3.57	--

Table 2. Technical specifications of some spectrophotometers specifically developed for evaluation of animal sperm concentration.

	IMV Accuread	IMV Accucell	Minitube SDM 1	Minitube SDM6
Length x width x height	6 x 18 x 15 cm	15.5 x 27 x 23.5 cm	25 x 13 x 6.5 cm	28 x 38 x 18 cm
Weight	0.5 kg	2.2 kg	1 kg	2.5 kg
Battery operation	Yes	No	Yes (4 AA batteries)	No
Light source	LED	Halogen	LED	Halogen
Adjustable wavelength	No	Yes	No	No
Wavelength	496 nm	300 to 600 nm	546 nm	546 nm
Analysis cell type	Cuvette	Cuvette	Microcuvette	Cuvette
Recommended diluent	NaCl 0.9% or sodium citrate 2.9 % or clear media	NaCl 0.9% or sodium citrate 2.9 % or clear media	None	NaCl 0.9%
Semen sample volume	10 to 100 µl	10 to 100 µl	1 drop (approx. 50 µL)	8 to 120 µL
Output	Optical density, transmission, sperm concentration	Optical density, transmission, sperm concentration, extender volume, number of doses	Sperm concentration	Optical density, sperm concentration, extender volume, number of doses
User input regression?	No	Yes; polynomial	No	No
Species and recommended dilution	Boar - 1:25 Bull - 1:100 Stallion - 1:20 Ram/buck - 1:400 Duck/turkey - 1:200	Boar - 1:25 Bull - 1:100 Stallion - 1:20 Ram/buck - 1:400 Duck/turkey - 1:200	Boar, bull, stallion, ram, dog; no dilution for samples < 1.5×10^9 sperm/mL	Boar - 1:50 Bull - 1:100 Stallion - 1:25 Ram - 1:500 Fish - 1:400

Table 3. Precision (coefficient of variation; CV) of sperm concentration estimates using manual count methods.

Method	Species	n	CV (%)	Reference
Hemocytometers				
Improved Neubauer hemocytometer	Human	10	8.5	[4]
Improved Neubauer hemocytometer	Human	50	7.4	[55]
Improved Neubauer hemocytometer	Bovine	107	4.5	[67]
Improved Neubauer hemocytometer	Porcine	161	7.1	[46]
Bürker hemocytometer	Porcine	50	11.1	[69]
Bürker-Türk hemocytometer	Bovine	50	9.2	[39]
Bürker-Türk hemocytometer	Bovine	15	7.7 to 12.0[a]	[54]
Bürker-Türk hemocytometer	Bovine	50	11.5	[54]
Bürker-Türk hemocytometer	Porcine	50	10.2	[44]
Thoma-50 hemocytometer (50 μm)	Bovine	15	6.6 to 14.1[a]	[54]
Thoma hemocytometer	Bovine	50	10.6	[54]
Thoma hemocytometer	Bovine	15	7.8	[41]
Thoma hemocytometer	Porcine	34	7.2	[70]
Unspecified hemocytometer	Bovine	40	6.8	[15]
Unspecified hemocytometer	Equine	120	6.7	[66]
Unspecified hemocytometer	Equine	100	9.6	[64]
Makler chamber				
Makler chamber	Human	10	13.1 to 17.9[a]	[4]
Makler chamber	Bovine	15	15.9 to 24.2[a]	[54]
Makler chamber	Bovine	50	22.1	[54]
Disposable slides				
Leja slide (20 μm) w/ eyepiece grid	Human	10	9.8 to 10.2[a]	[4]
Leja slide (20 μm) w/ eyepiece grid	Human	50	10.6	[55]
Microcell slide (20 μm) w/ eyepiece grid	Human	50	10.2	[55]
Microcell slide (20 μm) w/ eyepiece grid	Bovine	15	10.7	[41]

[a]Range according to dilution

Table 4. Precision (coefficient of variation; CV) of sperm concentration estimates using automated methods.

Method	Species	*n*	CV (%)	Reference
Spectrophotometer				
Prototype spectrophotometer	Bovine	13	4.1	[61]
L'Aiglon	Bovine	50	5.0	[39]
Genesys 20	Bovine	15	4.1	[41]
ARS Densimeter 534B, HR Sperm Counter 10, Micro-Reader I, SpermaCue	Equine	100	3 to 6.2[a]	[64]
Corning 254	Porcine	50	6.3	[44]
Corning 254	Porcine	161	10.4	[46]
Sherwood 252 and Ciba-Corning	Porcine	50	3.7	[69]
AccuCell and AccuRead	Porcine	34	1.8 to 3.2[a]	[70]
CASA				
Prototype CASA w/ Leja slide (20 μm)	Human	5	1.0 to 5.0[c]	[59]
HTM IVOS™ w/ Leja slide (20 μm)	Equine	100	5.3 to 5.7[b]	[64]
HTR UltiMate™ w/ Leja slide (20 μm)	Porcine	161	5.4	[46]
SpermVision™ w/ Leja slide (20 μm)	Porcine	161	8.1	[46]
HTR Ceros™ w/ Leja slide (20 μm)	Porcine	50	12.4	[69]
HTR UltiMate™ w/ Leja slide (20 μm)	Porcine	34	5.3	[70]
SpermVision™ w/ Leja slide (20 μm)	Porcine	10	11 to 26[d]	[34]
Nucleocounter				
NucleoCounter SP-100	Bovine	107	2.9	[67]
NucleoCounter SP-100	Bovine	284	2.6 to 3.9[e]	[15]
NucleoCounter SP-100	Equine	120	3.2	[66]
NucleoCounter SP-100	Porcine	161	3.1	[46]
NucleoCounter SP-100	Porcine	34	4.2	[70]
Flow cytometry				
Partec PAS	Human	5	3.9 to 7.6[f]	[45]
BD FACSCount	Bovine	50	3.3	[39]
Partec Cyflow Space	Bovine	15	2.3	[41]
BD FACSCount	Bovine	288	2.4 to 3.5[g]	[47]
BD FACSCount	Bovine	50	3.3	[39]
BD FACSCount	Equine	120	2.9	[66]
BD FACSCount	Porcine	50	2.7	[44]
BD FACSCount	Porcine	161	2.7	[46]

[a]Range for different equipment; [b]range for dark-field phase-contrast and fluorescence microscopy; [c]range for individual samples; [d]range for before and after e-learning; [e]range for duplicates and triplicates; [f]range for intra- and inter-technician; [g]range for raw and frozen-thawed semen.

Fig.1. Hemocytometers are reusable cell counting apparatus with two separate counting chambers. A glass coverslip held at a specific height above the surface of the counting area defines the chamber depth (top middle). Although some manufactures produce specialty hemocytometers, the standard depth of the counting chambers is 100 µm. The hemocytometer's chambers are filled by capillary action by loading the sample through a V-shaped notch at either end of the chambers (top right). Different types of counting grids are etched on each of the counting chambers, and hemocytometers are usually referred to by the name of the grid pattern (bottom).

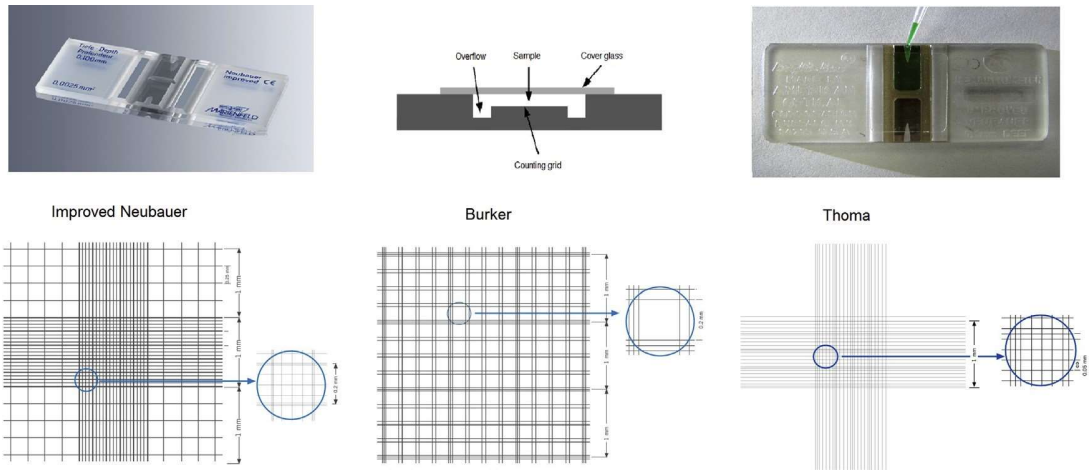


Fig. 2. The Makler counting chamber used for evaluation of sperm concentration has a unique design. A glass piece with four 10-µm quartz pins is mounted in the center of a metal disc, and a 1-mm2 counting grid subdivided into 100 squares is on the cover glass. Pictures courtesy of SefiMedical Instruments.

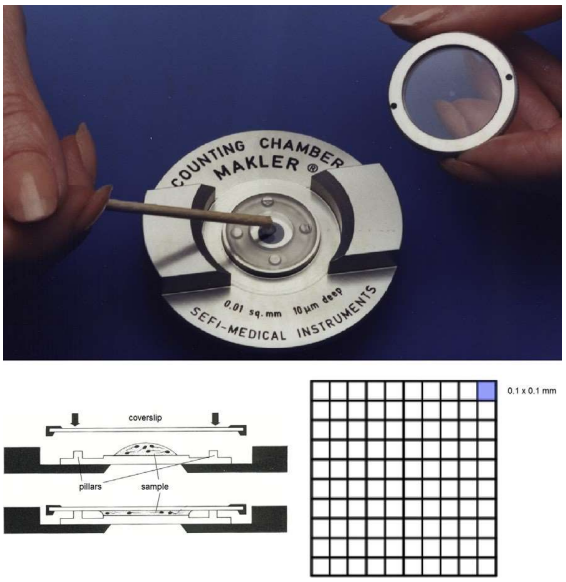


Fig. 3. Examples of disposable slide chambers with grid. CellVision (top) are ‘one-piece’ slides (i.e., mounted coverslip) that are filled by capillarity, whereas CellVu (bottom) slides have separate coverslips with laser etched grids. This type of slides contain two independent chambers and are available in a variety of depths (10, 20, or 100 µm) and grid patterns. From: http://www.cellvision.nl and http://cellvu.com (with permission).

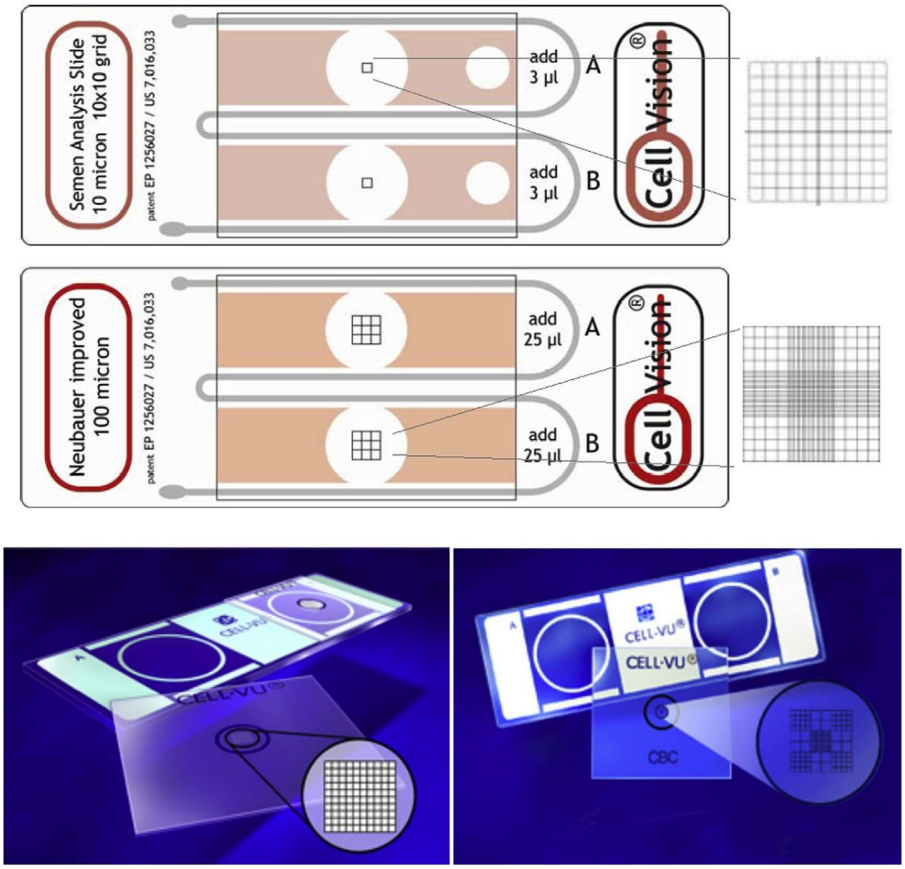


Fig. 4. Examples of disposable slide chambers without grid. Leja (left) and CellVision (right) are ‘one-piece’ slides (i.e., mounted coverslip) that are filled by capillarity. This type of slides are avaibale with different numbers of chambers (2, 4, or 8), chamber geometry, and depths (10, 12, 20, 50, or 100 µm).

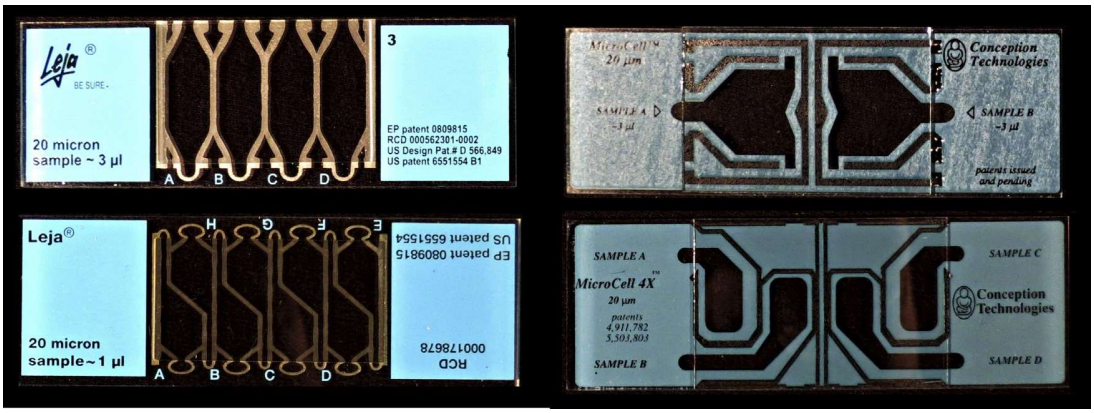


Fig. 5. Example of counting areas using the Improved Neubauer hemocytometer. Counts can be performed on any of the 1-mm2 areas defined in the grid (green) or on smaller areas within a 1-mm2 area (yellow). Regardless of the defined counting area, sperm which heads touch the left or lower square boundary lines should be counted (black ovals), whereas sperm that touch the right or upper boundary lines (open ovals) should not be counted (or vice versa) to avoid counting the same spermatozoon in adjacent squares twice. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

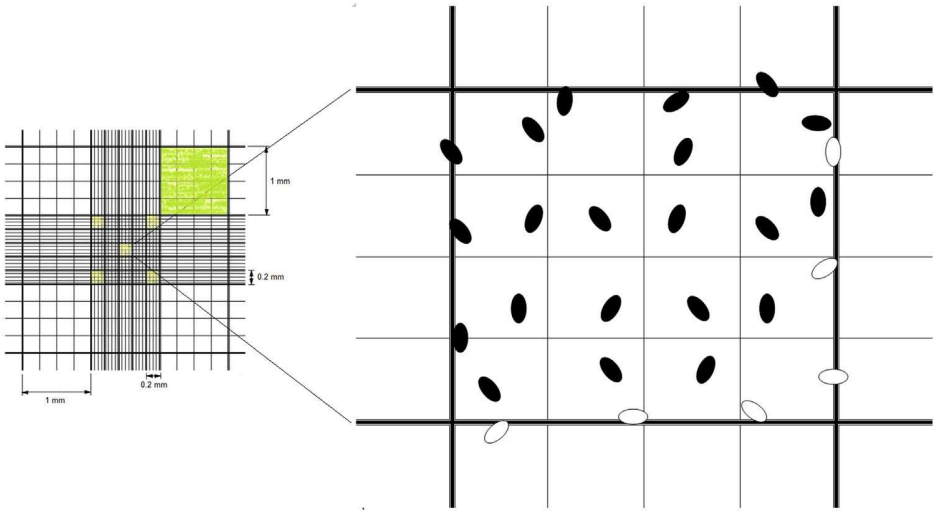


Fig. 6. Typical optical configuration of a spectrophotometer. A specific light wavelength to be used in the analysis is isolated after passing through a monochromator. After passing through the sample, the light intensity received by the detector is converted into voltage fluctuation. The difference of intensity at the source (I0) and at the detector (I) is displayed in the readout device. From: UC Davis ChemWiki, http://chemwiki.ucdavis.edu (with permission).

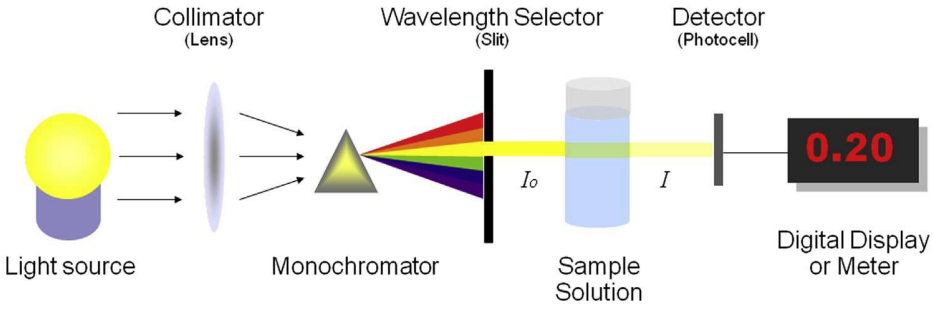


Fig. 7. Example of data used to calibrate a spectrophotometer for evaluation of sperm concentration. Regression analysis was used to calculate sperm concentration according to the sample optical density (OD; independent variable) using results obtained with a NucleoCounter as standard (dependent variable). It is important to note that calibration is only valid when standards are prepared using the same conditions (dilution ratio, diluent, mixing, time allowed before analysis, and so forth) used for analyses.

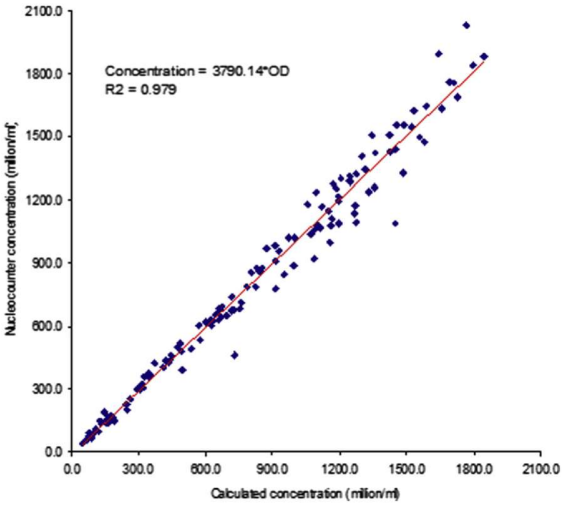


Fig. 8. Computer-assisted semen analysis (CASA) systems use software to detect sperm heads. In this example screenshot of the analysis, note that all sperm identified by the system are indicated by a colored dot (immotile sperm) or line (motile sperm). The number of sperm is automatically quantified (42 in this example), and the sperm concentration can be calculated.

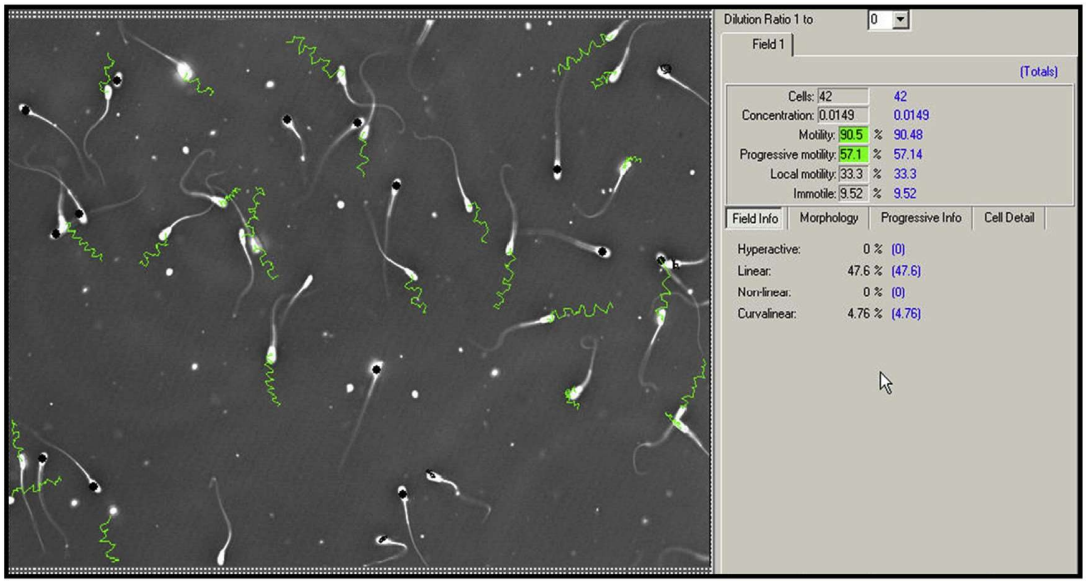
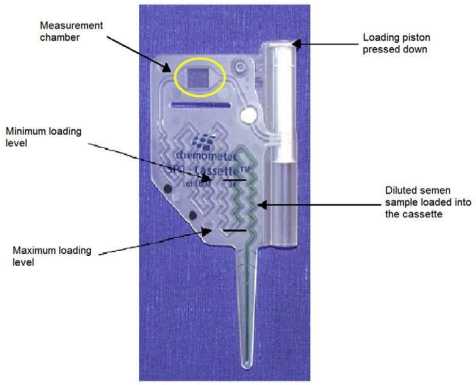
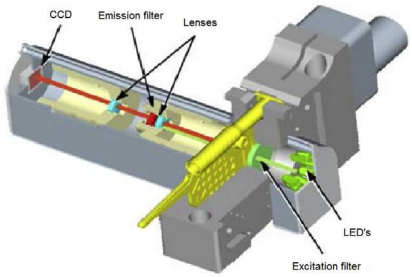


Fig. 9. The NucleoCounterSP-100 is an instrument specifically designed for evaluation of sperm concentration. The diluted semen sample is loaded into a disposable sampling cassette, which channels are impregnated with propidium iodide, and the cassette is inserted into the instrument (middle). An integrated fluorescent microscope with a charged coupled device (CCD) camera is used to image the sample, and stained sperm nuclei are quantified using image analysis software (bottom). Pictures courtesy of Chemometec A/S.





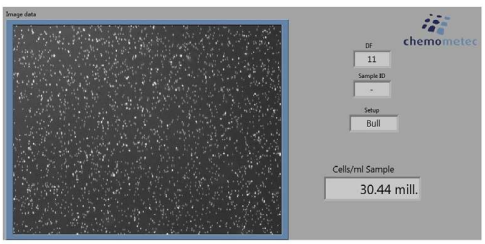


Fig. 10. Example of a flow cytometer optical configuration. As the fluidics system transports sperm to the interrogation chamber, individual cells pass through a laser beam so that their light-scattering properties and fluorescence can be measured. In this example, the instrument is equipped with 405, 488, and 642 nm lasers. Forward light scatter (FSC) measured in the plane of the beam gives information on cell size, whereas side light scatter (SSC) measured at 90 to the laser beam gives information on cell granularity and internal features. Additional detectors combined with specific optical filters (BLU, GRN, YEL, RED, RED2, and NIR) are used to detect emitted fluorescent light. From: guava easyCyte Flow Cytometry Systems datasheet, http://www.emdmillipore.com (with permission). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)