Semen sample analysis is routinely performed by microscopical evaluation and manual techniques by laboratory operators; the analysis is affected by a wide imprecision related to variability among observers, influencing its clinical validity. Our aim was to automate sperm analysis with the use of flow cytometry for evaluation of cell counts and typing and with the use of a new membrane-permeant nucleic acid stain for evaluation of sperm viability. Statistical analysis of the comparison between manual and automated methods for sperm counts was performed by the Bland and Altman method; the mean difference was 0.243 $\times 10^6$ sperms/mL. The precision of the flow cytometric analysis was evaluated with whole sperm; the between-run CV was 7.5% and the within-run CV was 2.5%. Data observed suggest that flow cytometric sperm analysis, with high precision and accuracy and low costs, can be proposed for routine use in clinical laboratories.

INDEXING TERMS: vital staining • semen analysis

Infertility is an emerging medical and social problem in Western countries: One of five couples is estimated to have problems with conception. The role of the clinical laboratory is crucial in the clinical evaluation and monitoring of the infertile couple. Hormonological investigations are generally performed for both partners; semen analysis is necessary for studying male infertility. Semen analysis, including spermatozoa count, evaluation of ejaculate volume, sperm motility, viability and morphology, immature sperm cells, and leukocyte evaluation is a common request for the clinical laboratory. The average sperm count in healthy fertile males is 70–80 $\times 10^6$ sperms/mL, but this finding is not indicative of the minimal requirements for fertilization [1]. Traditionally, oligospermia corresponds to a sperm concentration of $<20 \times 10^6$ sperms/mL [2]. In semen analysis, motility is defined as the percentage of sperm that demonstrate movement. A normal semen sample contains $>60\%$ motile sperm [3]. Sperm morphology as well as immature sperm cell evaluation are important in spermatogenesis evaluation. Although traditional methods define categories of sperm appearance as oval, pinhead, roundhead, and tapered, the WHO recommended a more detailed evaluation of abnormality in sperm morphology [2]. A normal semen sample contains at least $60\%$ morphologically normal sperms, and immature sperm cells (also called "round cells") do not exceed $5 \times 10^6$ cells/mL [2]. A high leukocyte count in semen is a sign of genital tract infection, and is associated with poor semen quality [4]. A normal ejaculate should not contain $>1 \times 10^6$ leukocytes/mL [2].

Semen analysis is usually performed by use of manual techniques and microscopical evaluation by laboratory operators. The analysis is tedious, expensive, and not standardized; it is affected by a wide imprecision related to high variability of microscopical evaluation, influencing its clinical validity. In an attempt to standardize manual semen analysis, computer-assisted sperm analysis (CASA) systems have been developed. In the CASA system, a series of videotaped microscopic images are digitalized, and computer software programs are used to evaluate sperm count, motility, and morphology. Current CASA systems are not suitable for evaluating semen samples for many patients. Sperm concentration reportedly was overestimated by 30% when only 11 to 60 cells per high-power field were present, and spurious counts of $3.6 \times 10^6$ sperms/mL were reported in azoospermic donors [5]. In a male fertility study, the range below $20 \times 10^6$ sperms/mL is of particular interest: Only lower sperm concentrations seem to reduce the chance for pregnancy, with a crucial limit in the range $\sim 5 \times 10^6$ sperms/mL [2]. At this range the CASA system presented the poorest performance; the value of its routine use for the determination of sperm concentration in an infertility laboratory

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is limited [6]. Moreover, CASA systems are dedicated only to sperm analysis and are very expensive and not suitable for general laboratories with small batches for semen analysis.

Our aim was the full automation of sperm analysis at low cost and reduced manpower, permitting, in addition, standardization, quality control, and interlaboratory comparisons, as for typical clinical laboratory assays.

Flow cytometric studies of semen samples have been advanced by the need for rapid, sensitive, objective, and multiparameter measurement of fertility potential of human sperm [7, 8]. In the past 3 years, flow cytometric technology has reached a mature level that allows its inclusion in the list of available and routine methods for study of reproductive capacity [9]. We used flow cytometry to perform sperm analysis in man. This technique, allowing rapid analysis of the biophysical (light scatter) and biochemical (via fluorescence probes) characteristics of particles, could represent an ideal tool for such purposes. Unfortunately, the aspherical shape of human sperm increases variability in light scatter and fluorescence signals as a result of the inconsistency of particle orientation with respect to the excitation beam and sensing device [10, 11]. In addition, the densely packed chromatin makes stoichiometric staining of DNA difficult. These factors broaden the CV and thereby confound the detection of subtle differences between normal and abnormal particles. In this study, we report our experience using flow cytometry to evaluate sperm viability, immature cell and leukocyte count, and typing (only motility is still performed manually). Problems related to spermatozoon shape can be consistently solved by using a flow cytometer equipped with a closed flow chamber to limit the variability due to the orientation of particles, which causes pitfalls in two-dimensional light-scatter patterns detection. We also describe an assay that involves light-scatter cytograms to perform cell count and cell typing, and fluorescence signal with the new membrane-permeant nucleic acid stain, SYBR 14®, to evaluate sperm viability. When human semen is incubated briefly with this stain, live sperm cells with intact membranes fluoresce bright green.

Materials and Methods

SEmen SAMPLE COllECTION AND TREATMENT
One hundred semen samples were collected by masturbation from men referred to our Sterility Center after at least 72 h of sexual abstinence. The collection procedures were established with our Ethical Committee. The age of the patients ranged from 18 to 59 years (mean = 38.5). Sample volume ranged from 3 to 7 mL (mean = 3.8). The samples were analyzed within 2 h of collection.

The clinical status of these patients ranged from azoospermic to normal; we included 15 patients with seminal or urethral infection. To obtain semen samples without proteic aggregates, 50 μL of bromelain were added to 450 μL of sperm after liquefaction; after 30 min of incubation at 37 °C, each bromelain-treated sample was diluted 1:8, 1:4, and 1:2 with PBS (0.01 mol/L, pH 7.4) to determine the optimal dilution in relation to the high density of the seminal fluid.

Preparation of Isolated Sperm Samples
An aliquot of 1.5 mL of each semen specimen was subjected to standard Percoll (Pharmacia, Bromma, Sweden) gradient separation [12] to obtain samples with isolated sperm. To each of these aliquots we added 1 mL of Percoll with a concentration of 900 mL/L, 700 mL/L, and 400 mL/L (total volume 3 mL) and centrifuged at 150 g for 20 min. After centrifugation, the sample was washed twice with 3 mL of PBS, pH 7.4, and further centrifuged at 300 g for 10 min. The pellet was reuspended in 1 mL of PBS (pH 7.4).

Indirect Immunofluorescence Assay for Panleukocyte Antigen on Isolated Leukocytes
To detect the leukocyte plot, a dual-parameter analysis (CD53 fluorescence vs forward-angle light scatter and forward- vs large-angle light scatter) was performed on isolated leukocyte samples by using monoclonal mouse anti-human panleukocyte antigen CD53 (Bio-Rad, Segrate, Italy). We used this monoclonal antibody because CD45, traditionally used for recognizing leukocytes, showed cross-reactivity with sperm and immature sperm cells as well as urothelial cells [12, 13].

Blood samples for leukocyte studies were obtained from volunteers between 0900 and 1100. Samples were collected into 3-mL test tubes containing K3 EDTA (Vacutainer Tube; Becton Dickinson, Rutherford, NJ), and leukocytes were separated by gradient sedimentation over Ficoll-Paque (Pharmacia). Five-hundred microliters of the solution containing isolated leukocytes were added to 500 μL of PBS, pH 7.4, and centrifuged at 300 g for 10 min. The pellet was reuspended in 100 μL of PBS, and 10 μL of CD53, previously diluted fivefold with PBS, were added. All the test tubes were incubated for 30 min at room temperature in the dark. Samples were then washed three times with PBS and the pellet was reuspended in 100 μL of PBS. Ten microliters of fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG secondary antibody (Bio-Rad) were added to each tube and the samples were gently mixed for 10 s. After an incubation of 30 min at room temperature in the dark, samples were washed three times with PBS. The pellet was finally reuspended in 200 μL of PBS.

Indirect Immunofluorescence Assay for Panleukocyte Antigen on Semen Samples
Indirect immunofluorescence assay for panleukocyte antigen was performed on semen samples by using monoclonal mouse anti-human CD53 (Bio-Rad). Semen sample aliquots of 500 μL were added to 500 μL of PBS, pH 7.4, and centrifuged at 300 g for 10 min. The staining method was the same as described above.
FLOW CYTOMETRY
Flow cytometric analysis was performed with a Bryte HS 228 flow cytometer (Bio-Rad) equipped with a 75-W xenon lamp (Hamamatsu Photonics, Shimokanzo, Japan). The flow cytometer was daily calibrated with FITC-conjugated microbeads (Bio-Rad) to verify the light scatter and the fluorescence signal reproducibility. The regions of cytometer plot where the different cell subpopulations were identified are defined “windows.”

To define the region of interest related to the light-scattering characteristics of sperm, a “sperm window” was identified by analyzing samples containing isolated sperms. A “leukocyte window” was identified by using dual-parameter analysis (CD53 fluorescence vs forward-angle light scatter and forward- vs large-angle light scatter) on isolated leukocyte samples. Subsequently, semen samples with high numbers of both sperm and leukocytes, or azoospermic samples with high numbers of inflammatory cells were analyzed. Immature sperm cell subset setting (also called “round cell window”) was performed by analyzing semen samples with total absence of mature sperm cells (after Percoll gradient separation) and large amounts of immature cell types (at least $1 \times 10^6$ cells/mL). For each sample, light microscopy was performed to verify cell numbers and preservation. Before data reduction, unstained samples were analyzed to evaluate the fluorescence background, to establish a reproducible cutoff concentration.

CD53 expression was considered positive only when 20% of the cell population showed a fluorescence signal, because of the high fluorescence background of the sperm. Analysis was performed by evaluating at least 10 000 cells for each sample. Each region of interest (sperm, round cell, and leukocyte windows) was memorized by the computer, permitting us to analyze each semen sample every time with the same parameters: The voltage of the light-scatter photomultiplier was selected at 360 eV through linear amplifier. Fluorescence signals were detected with a 525-nm filter by the selection of the photomultiplier at 325 eV through logarithmic amplifier with a dynamic range of four decades of amplification. Cell populations having a fluorescence peak in the third logarithmic decade or more were considered viable. Data from flow cytometric analysis were compared with optical microscopy data obtained by a standard routine sperm viability test (eosin–nigrosine).

VIABILITY DETECTION BY EOSIN–NIGROSINE STAINING
Evaluation of sperm viability is usually performed by microscopic examination of a prepared slide stained with eosin–nigrosine as recommended by the WHO [2]. A solution was prepared by addition of 8.4 g of NaCl and 1.37 g of NaH$_2$PO$_4$ to 1 L of distilled water; the final solution was adjusted to pH 7.4 with NaOH. Two grams of nigrosine (Merck, Darmstadt, Germany) and 0.2 g of

SPERM VIABILITY DETECTION BY FLOW CYTOMETRY
WITH FLUORESCENT STAIN SYBR 14
Semen samples were diluted 1:5 with PBS pH 7.4. Five microliters of SYBR 14 (Molecular Probes, Eugene, OR) diluted 1:10 with anhydrous dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, WI) were added to 500 μL of diluted sample, and test tubes were incubated for 20 min at room temperature in the dark. An aliquot was left untreated with fluorescent stain to evaluate the intensity of background fluorescence signal. Green fluorescence was collected through a 525-nm filter, analyzing only events acquired in the sperm window. Fluorescence signals were detected with a 525-nm filter by the selection of the photomultiplier at 325 eV through logarithmic amplifier with a dynamic range of four decades of amplification. Cell populations having a fluorescence peak in the third logarithmic decade or more were considered viable. Data from flow cytometric analysis were compared with optical microscopy data obtained by a standard routine sperm viability test (eosin–nigrosine).

![Fig. 1. Forward-angle light scatter (LS2) histogram (a) and forward- vs large-angle light scatter (LS1) cytogram (b) obtained by flow cytometric analysis of an isolated sperm sample.](image-url)
eosin (Merck) were added to 20 mL of this solution and mixed for 2 h at room temperature. Finally, the solution was filtered to obtain the staining solution. Two hundred microliters of each semen sample were added to the same volume of staining solution and then incubated for 1 h at room temperature before smearing on slides. Eosin penetrates nonviable cells, which appear red. Nigrosine offers a dark background to facilitate the detection of viable, nonstained cells. Two hundred cells were counted for each slide, identifying viable and nonviable sperm cells, and the percentage of viable cells was calculated.

**STATISTICAL ANALYSIS**
Comparison between flow cytometry and optical microscopy was performed by linear regression analysis. Within-run CV of the flow cytometric analysis was evaluated for 10 consecutive days on 10 replicates of the same semen sample for each day. Between-run precision was evaluated by using a semen sample stored at 4 °C; an aliquot of this was analyzed in duplicate for 5 days consecutively. We performed the between-run precision test for only 5 days because of the problem of cell viability.

Statistical analysis of comparison between the manual and automated methods for sperm counts was performed according to the Bland and Altman method [14].

**Results**
Analysis of isolated sperm samples at different dilutions showed the following results: Dilution 1:2 showed a poor agreement between flow cytometry and optical microscopy ($r = 0.33$); dilution 1:4 showed a higher correlation coefficient ($r = 0.81$); the correlation was better ($r = 0.96$) with 1:8 diluted samples, and a sperm window was fixed (Fig. 1). Statistical analysis of the comparison between the manual and automated methods for sperm counts was done according to the Bland and Altman method [14] (Fig. 2): At a 1:8 dilution, the standard error of the mean difference was 0.084 and the mean difference was $0.243 \times 10^6$ sperms/mL. The mean value of automated sperm counts was $67.5 \times 10^6$ sperms/mL (range $0.5–159.9 \times 10^6$ sperms/mL).

The leukocyte plot was detected by dual-parameter analysis (CD53 fluorescence vs forward-angle light scatter and forward- vs large-angle light scatter) performed on samples of isolated leukocytes obtained as described above. Forward-angle light scatter permitted us to identify leukocytes by using size parameters, whereas large-angle light scatter supplied morphological classification. Cells with dimensional and morphological characteristics of leukocytes showed a strong fluorescent signal allowing for CD53 positivity. No overlap signal was detected in the sperm population. Therefore the analysis of semen samples with high numbers of leukocytes marked with CD53 showed a good correlation with data obtained from the previously performed analysis, and this observation permitted us to define a leukocyte window (Fig. 3). Finally, analysis of semen samples with high numbers of nonstained leukocytes showed good agreement between flow cytometric analysis and optical microscopy count at a 1:8 dilution ($r = 0.94$).

Round cell subset setting, performed with the above-mentioned criteria, showed a contour plot apparently continuous with that observed for sperm cells. A round cell window was defined, in agreement with data obtained from optical microscopy concerning round cell count and typing ($r = 0.93$), analyzing samples with low numbers of sperm cells (maximum $1 \times 10^6$ cells/mL) and high numbers of round cells (at least $2 \times 10^6$ cells/mL) at 1:8 dilution (Fig. 4).

Flow cytometric semen analysis performed with only light-scatter parameters on a whole-sperm sample treated with bromelain and diluted 1:8 with PBS pH 7.4 showed...
a good correlation with data obtained with standard microscopic analysis on the same samples ($r = 0.92$) (Fig. 5). Flow cytometric evaluation of samples from azoospermic donors showed in all cases the presence of low-intensity light scatter in the sperm window.

Flow cytometric sperm viability was evaluated in whole-semen samples and showed good correlation with data obtained with routine eosin–nigrosine staining ($r = 0.97$). The fluorescence signal obtained with SYBR 14 in viable cells showed in all cases three significant peaks of increasing intensity.

The precision of the flow cytometric analysis of cell type and count was evaluated by using a whole-sperm sample. The between-run CV was 7.5% and the within-run CV was 2.5%. These results are better than the difference observed with optical microscopy performed twice on the same sample slide by the same operator (29%). When the same sample slide was evaluated by optical microscopy by two different well-experienced physicians, the difference (34.4%) was higher than that observed when the two operators used flow cytometry (2%).

**Discussion**

The application of rapid, automated, and precise laboratory analysis techniques has proceeded more easily in some areas of medicine than others. Automated instru-
mentation for sperm analysis, based on image analysis, is only now entering the laboratory, but the costs are very high and these systems can be acquired only from specialized centers [15]. We have a strong motivation to automate semen analysis for the general clinical laboratory, owing to CVs >25% on the basis of high variability among operators.

The use of flow cytometry for sperm analysis is an attempt to address the long-standing problem of the subjective nature of the manual method commonly used for semen analysis. An additional source of laboratory variation is the low number of sperms analyzed with manual techniques. Because of time and cost restraints, most laboratories analyze only 50 to 100 sperm to compute the percentage of each cell population and the viability rate. This small sample from a population of millions probably results in a statistical sampling error [16]. Flow cytometry permitted us to analyze many millions of cells in few seconds.

In this study, we demonstrated the feasibility and reproducibility of an automated method to evaluate sperm cell type, count, and viability in human semen samples. The precision of the flow cytometric analysis was satisfactory, and the observed CVs were significantly better than those reported for the manual method. The use of a closed flow chamber-equipped flow cytometer allowed us to reach precision higher than that obtained with a traditional laser lamp-equipped flow cytometer [17]. In fact, all flow cytometers have some type of nozzle with hydrodynamic focusing to induce a laminar flow; the cells follow the narrowest possible path through the measuring region. The water flowing through the nozzle is usually supplied from a pressurized container, while the sample is introduced through a thin tube along the center of the stream in the nozzle. To maintain a laminar flow in air, the flow velocity of the jet in an open-air flow chamber used in laser-based flow cytometers cannot be reduced below a critical limit, which is ~3 m/s. The closed flow chamber used in our arc lamp-equipped flow cytometer does not have this limit and may thus be operated at much lower flow velocities. It therefore facilitates higher sensitivity than the open flow chamber [18]. We have never observed multiple signals related to the different orientation of the sperm through the light beam.

The low-intensity light-scatter signal observed in the sperm window during evaluation of semen samples from azoospermic patients is partially due to the presence of some proteic aggregates. In fact, azoospermia is associated with altered pH values and other biochemical abnormalities in seminal fluid, easily inducing protein aggregation [8]. This problem can be solved by using higher concentrations of bromelain added to azoospermic semen samples. We have observed that 100 μL of bromelain added to 1:8 diluted semen samples are sufficient to eliminate proteic aggregates. Unfortunately, in a few cases this abnormal light-scatter signal is related to the presence of presumable aggregates of inorganic elements: Phosphate salts of calcium, magnesium, or potassium can be observed in semen samples of azoospermic patients affected by some prostatic diseases [19]. They are localized just in the lower left corner of the sperm window; mature sperm cells are in all cases represented in the central area or upper right corner of the same window, in agreement with the complex, asymmetrical head shape when compared with the amorphous structure of the inorganic element aggregates [20].

Considerable information has accumulated on the use of fluorescent staining protocols for assessing sperm function [21]. The SYBR 14 staining of nucleic acids, especially in the sperm head, was very bright in living sperm. Good agreement was observed between the fluorescent staining method and the standard eosin–nigrosine viability test;
the flow cytometric method showed a precision level higher than that of the manual method.

The presence of three distinct peaks of increasing fluorescence reflects the distribution between dead, moribund, and live sperm cells, as observed in experiments on bull sperm cells [22]. The dual stain combination of SYBR 14 and propidium iodide induced a relatively rapid transition from living sperm to dead cells [22]. In our study, we did not use the propidium iodide staining technique because of its demonstrated toxic effect on viable cells [23]. In our preliminary study [24], the use of propidium iodide as a counterstain in association with SYBR 14 caused an increase of the percentage of dead sperm cells when compared with data obtained from the standard eosin–nigrosine viability test. Thus, we recommend the use of SYBR 14 for studying human sperm viability.

Finally, we point out that the routine use of flow cytometry to evaluate semen samples at 1:8 dilution in 1 mL permitted us to perform this analysis on samples that were considered not feasible by optical microscopy. In fact, the manual method required a minimum volume of 1.3 mL, whereas instrumental analysis can be performed on samples of 0.7 mL. We now use the described method routinely, analyzing 12 samples per day against 5 previous samples, reducing manpower (3 h of a technician also performing manual motility analysis vs 6 h of a graduate) and increasing analytical quality.

We still evaluate sperm motility by the use of optical microscopy [2]. We are testing some fluorescent dyes that have the capacity to evaluate the mitochondrial function of sperm cells. This is of particular interest because of the strong correlation between mitochondrial activity, ATP production, and motility. Our preliminary results are encouraging, but further study is needed to improve the clinical validity of this correlation.

The data observed with flow cytometric evaluation of cell typing, count, and sperm viability in semen samples suggest that this method, characterized by high precision and accuracy and low costs, can be proposed for routine use in the clinical laboratory.

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