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Quantification of Temporary and Permanent Subpopulations of Bull Sperm by an Optimized SYBR-14/Propidium Iodide Assay

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Background: The quality of bull sperm is a key factor in the field of controlled reproduction. Viability-testing is an important aspect of sperm quality definition, especially after cryopreservation where multiple factors such as handling, freeze-thaw cycle, and preservation media, have an impact on the metabolic and functional state of sperm cells.
Methods: We investigated the commonly used SYBR-14/propidium iodide (PI) assay to obtain functional information about sperm-dye and dye-dye interactions. After optimizing filter settings, dye concentrations and incubation times we used these dyes for an interruption free flow cytometric kinetic analysis of a mixture of viable and dead bovine sperm.
Results: For the sensitivity of this method and the separation of the different cellular subpopulations fluorescence quenching of SYBR-14 by PI is mainly responsible. Together with a spectral overlap of the two emission spectra of about 5%, even for a wavelength greater than 700 nm, this quenching effect has to be taken into account for a quantitative understanding of the observed fluorescence intensity signals. The fraction of a temporary "intermediate" population to be observed between the viable and dead cells in an SYBR-14/PI-dot-plot diagram becomes greater after stress on the sperm cells caused by cryopreservation. The temporary fraction of "intermediate" cells is maximal at about 6 min after staining and disappears after about 15 min by shifting towards the dead sperm population. The estimation of this "intermediate" population may be a good indicator for handling and storage induced detrimental effects on bovine sperm cells.
Conclusion: The SYBR-14/PI assay is a fast, reliable and sensitive method to assess the membrane integrity of bull sperm and to separate viable, dead, and "intermediate" sperm subpopulations. © 2004 Wiley-Liss, Inc.

Key terms: sperm viability; SYBR-14/propidium-iodide assay; time-resolved flow cytometry; fluorescence quenching

The quality of bull sperm is extremely important in the field of bovine reproduction. Handling, storage, and especially cryopreservation together with the necessary freeze-thaw cycle (1) have a distinct detrimental impact on sperm function. Many assays have been introduced to assess sperm function by morphological changes. A predominant factor is the plasma membrane integrity (i.e., viability) of sperm, followed by more functional parameters such as motility (2), mitochondrial function (3), acrosome reaction (4,5), or calcium influx (6). The many methods employed to evaluate the quality of bull sperm are based primarily on light microscopy, as well as on flow cytometric techniques, although recently a fluorometric assay has been published (7). The flow cytometric approach to assess bull sperm viability uses fluorescence signals from different dyes such as rhodamine 123 (8), carboxyfluorescein diacetate and propidium iodide (PI) (9), or a combination of PI, rhodamine 123, and pisum sativum agglutinin (10). Dual DNA staining of bull sperm with PI and SYBR-14 (11) was introduced to achieve better results in separating viable and dead sperm cells by high SYBR-14 and low PI fluorescence intensities for vital cells and vice versa signals for dead cells. This technique has been extended to a triple-stain cytometric method to assess plasma- and acrosome-membrane integrity (12).

The aim of this study was mainly to address various open questions regarding the SYBR-14/PI assay. We observed unexpected signal intensities of dead cells in the SYBR-14 fluorescence channel as well as in the PI channel. According to the manufacturer's information, dead cells

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should not emit SYBR-14 fluorescence, whereas the intensity observed is higher by more than a factor of 30 than cell autofluorescence. In the PI channel, however, the signal intensities are about 10-fold lower than measured usually in other inactivated cell types compared with their vital fraction. Finally, the occurrence of a transient subpopulation sometimes observed between the vital and dead bull sperm cells (11,12) should be characterized. Therefore, kinetic staining experiments with living and dead cells should be carried out to provide a quantitative answer to these open questions.

MATERIALS AND METHODS

Semen Collection and Handling

Freshly collected bovine semen was filtered and suspended in Biociphos plus® extender (IMV technologies, L'Aigle Cedex, France). Sperm were counted with a hemocytometer chamber before further handling and diluted to 20×10^6 sperm/ml. The storage of cryopreserved samples was performed at 196°C in liquid nitrogen. Before all experiments, freshly collected or cyropreserved sperm were suspended in buffer according to Garner and Johnson (13) (0.760 g NaCl, 0.030 g KCl, 0.252 g fructose, 0.238 g Hepes, 0.015 g $CaCl_2 \times 2H_2O$, 0.010 g $MgCl_2 \times 6H_2O$, 0.100 g bovine serum albumin (BSA), and 100 ml H_2O (18 MΩ), pH 7.4) and diluted to a final sperm concentration of 0.5×10^6/ml. In these kinetic studies a buffer medium was used for avoiding disturbances by egg yolk particles.

Cell Staining

Sperm were stained with the components of the live/dead semen viability kit L-7011 from Molecular Probes (Eugene, Oregon). The final staining concentrations were 100 nM for SYBR-14 and 24 μM for propidium iodide (PI). These dye concentrations were used during the time-dependent measurements as well as for cell prestaining by one dye (SYBR-14: 100 nM, 10 min, or PI: 24 μm, 6 min) immediately before data registration.

Spectrofluorometry

The emission spectra for SYBR-14 and PI were measured by spectrofluorometry (luminescence spectrometer LS 50B; Perkin-Elmer, Neuried, Germany). Both dyes diluted in phosphate-buffered saline (PBS) were excited by an excitation bandwidth of 488 ± 5 nm, where an absorption maximum (data not shown) could be found for SYBR-14 as well as for PI. The emission spectra were registered between 500 nm and 800 nm, using a slit width of 5 nm.

Application of Dyes for Staining Kinetics in Isothermal Condition

An optimized registration of kinetic cellular parameters by flow cytometry needs an interruption-free time and volume controlled injection of stimulating or modifying chemicals into the sample solution. These demands are fulfilled by the adequate use of Hamilton microliter syringes (needle length 120 mm) and a modified sample tube head developed by us (14).

This setup allowed an interruption-free data registration, whereas the time delay between stimulation and registration of its response remains smaller than 3 s due to a short transport time from tube to nozzle enabled by an experimentally standardized stirring and boost procedure (14). To run quantitative kinetic experiments with living cells temperature regulation and stabilization are basic requirements to be achieved for the sample tube solution within small temperature limits (≤ 0.1°C) over long periods of time. The thermostatted sample tube holder (stabilizing the temperature at 37 ± 0.1°C) is well suited for this prerequisites (14,15). In practice, two of these holders were used: one for the actual measurements, the other one for prestaining or prewarming of the next sample tube solution.

Flow Cytometry: Fluorescence Measurements and Data Registration

Laser cytometric measurements of two cellular scatter parameters and two fluorescence signals, simultaneously registered in time mode operation over 1,024 s, were performed with a FACSTARPLUS flow cytometer (Becton Dickinson, Sunnyvale, CA). The laser (argon ion laser, Innova 90, Coherent Radiation) was adjusted to the 488-nm line (1,000 mW) and used for the excitation of the PI- and SYBR-14 fluorescence as well as for the generation of the two scatter signals. Pulse heights of both forward light scatter (FSC) and 90-degree side scatter (SSC) were collected using 488-nm bandpass filters (Becton Dickinson, 488 BP 10). The SYBR-14- and PI-emission signals were recorded with the use of a 530-nm bandpass filter (Becton Dickinson, 530 BP 30) and a 700-nm long-pass filter (Schott, Mainz, Germany), respectively. The signal separation was performed by a dichroic mirror (Becton Dickinson, DM 560) as a beam splitter. The registration of pulse height signals delivers, in a dot-plot diagram of both fluorescences, a better discrimination of the different sperm subpopulations than pulse area registration.

Data recording was done with a HP 9000 Series 300 Computer (Hewlett Packard, Corvallis, OR) in list mode using the FACSTARPLUS research software (Becton Dickinson, Lysis II). The electronic pulses were triggered on the FSC signal set to a threshold level excluding debris related signals and registrated on logarithmic scales with a flowrate of 150–200 cells/s.

Data Analysis

All data analysis and display was carried out with the Data Analysis Software (DAS) Version 4.40 (16). Using the DAS macro programming, two macros have been written to analyze the individual data sets.

First, in the FSC/SSC scatter diagram, a gate is set interactively to define all cellular events, excluding cell debris. For the following steps, only cells in this particular gate are used. In the dot-plot diagram of the two fluorescences, SYBR versus PI, the gates for different sperm subpopulations were defined (live cells: high SYBR-14, low PI; dead cells: low SYBR-14, high PI; intermediate cells: between

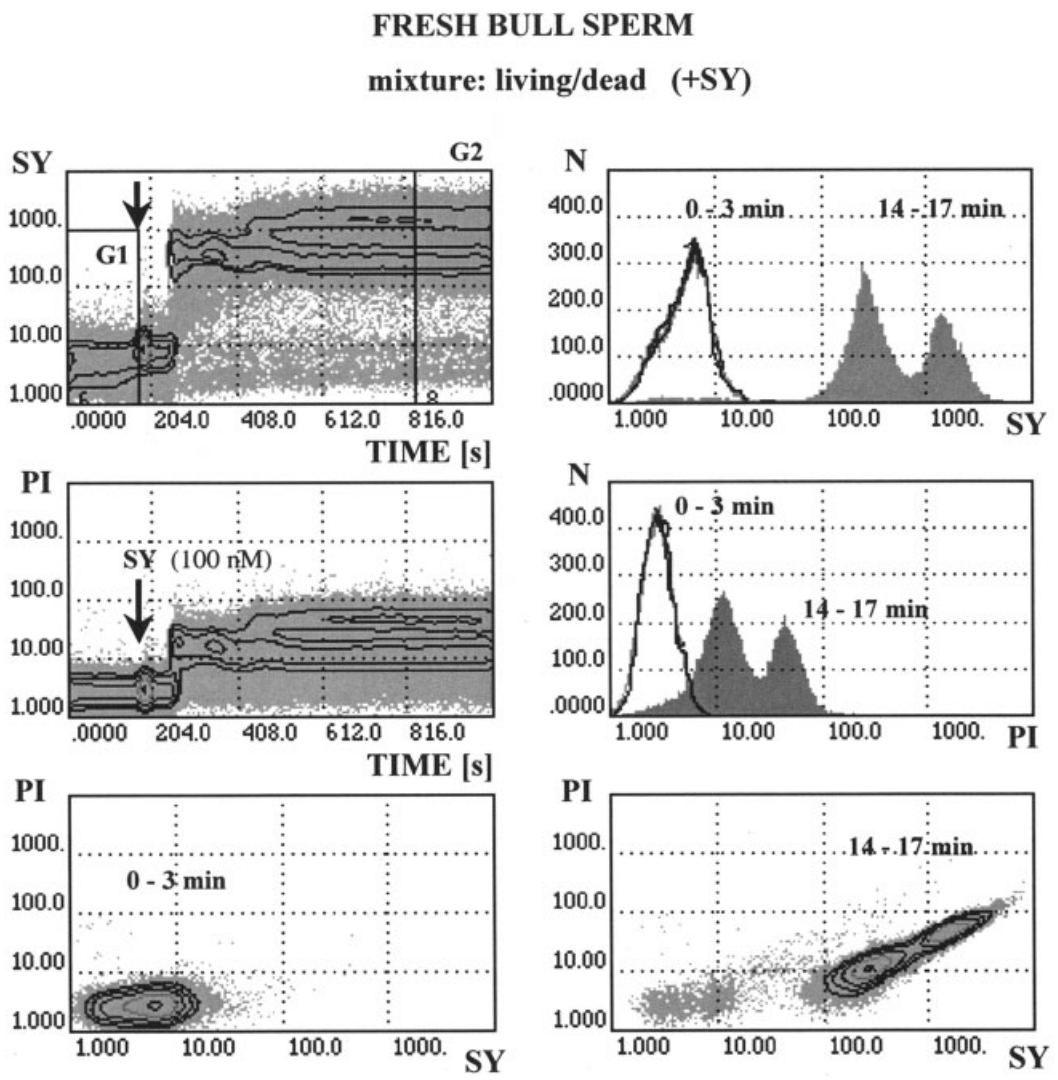

FIG. 1. Staining kinetics of SYBR-14 in a mixture of viable and dead cells of fresh bull sperm. At 10 min after addition of SYBR-14 (arrow), viable sperm cells are five times more intensively labeled than are dead cells (upper right). A similar fluorescence histogram is observed in the propidium iodide (PI) fluorescence channel due to a 5% overlap of the emission spectra even by using a long-pass filter LP700 (see Fig. 2). Time-gates G1 and G2 are defined in the Time/SY diagram (upper row, left) and relate to 0–3 min and 14–17 min, respectively.

the two former ones). Second, a macro has been written to provide an automatic quantification of the experimental data by calculating the statistics of the previously defined subpopulations.

RESULTS

To investigate the dye-cell and dye-dye interactions we used a sample of fresh bull sperm containing a mixture of viable and dead cells (25% and 75%, respectively). In this context, cells that were registered with high green and low red signals are considered as viable. Dead cells show the inverse signal strength of the two registration channels. The subsequent steps of the SYBR-14/PI staining method were analyzed separately for dependence on time with a resolution of 1 s during a time period of 17 min.

Fresh Sperm: Staining Kinetics and Sperm Subpopulations

The addition of 100 nM SYBR-14 to a suspension of unstained fresh bull sperm (Fig. 1, upper left) results, after a lagtime of ~100 s, in a steplike homogeneous dye uptake of all cells. After 200 s this homogeneous intensity level is subdivided indicating the existence of two distinct cell populations in the SYBR-14 channel (7 min after the beginning of the staining procedure). These two stained subpopulations show a 37-fold, respectively, 185-fold increase of SYBR-14 fluorescence in comparison with their autofluorescence level (Fig. 1, upper right). A similar time course as well as similar histograms of the fluorescence simultaneously recorded in the PI channel could also be observed (Fig. 1, middle row). The corresponding SYBR-14/PI dot-plots for unstained and SYBR-14-stained sperm

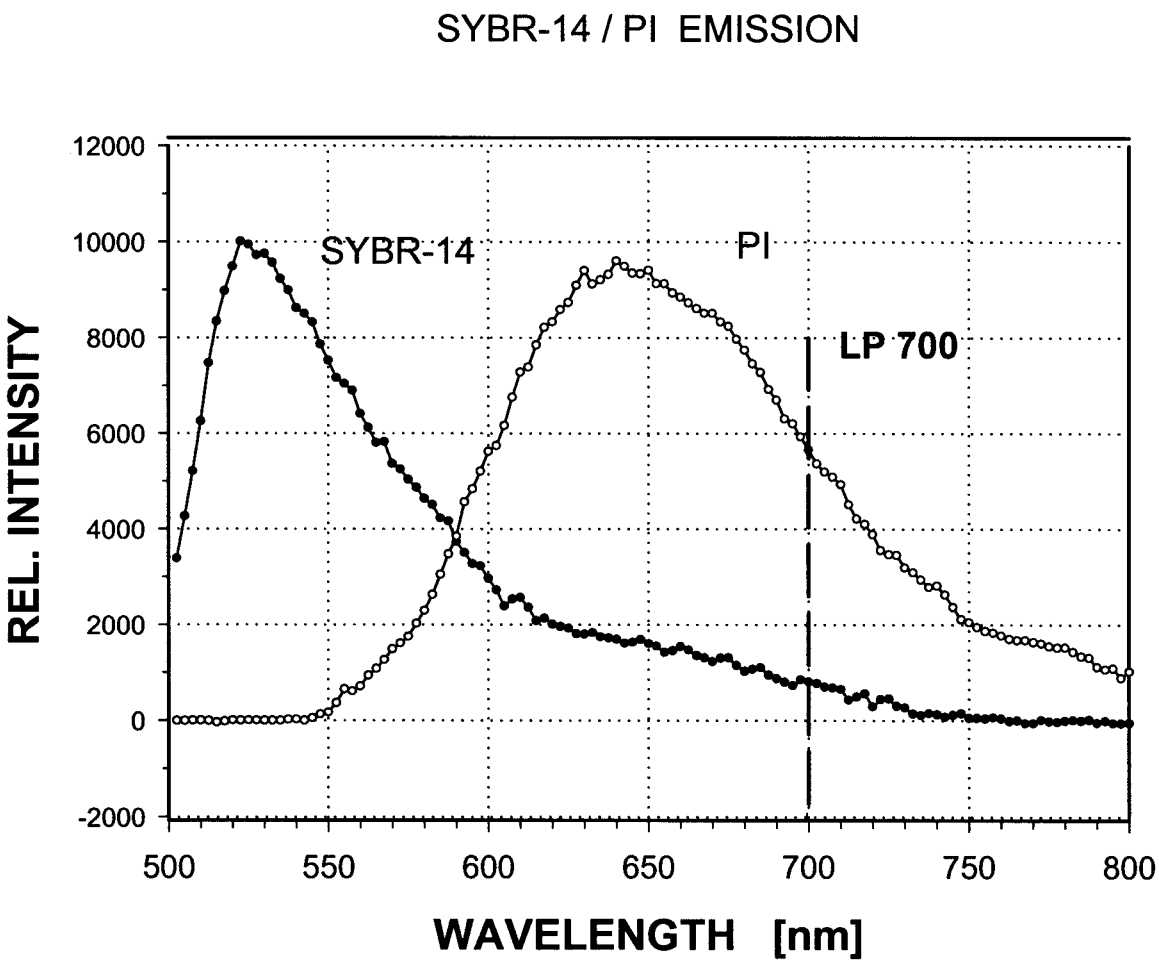

FIG. 2. Emission spectra for SYBR-14 and propidium iodide (PI) show a spectral overlap of about 5% when using a LP700 long-pass filter for recording the PI emission.

cells are shown in the lower row of Figure 1. To explain the fluorescence emission of only SYBR-14-stained cells, recorded in the PI channel, the emission spectra of SYBR-14 and PI were recorded spectrofluorometrically using 488 nm as the excitation wavelength (Fig. 2).

The observed red channel recording of SYBR-14 signals can be explained by the overlap of the fluorescence emission spectra of SYBR-14 and PI by 5% even for wavelengths longer than 700 nm. The spectral overlap is determined as the SYBR-14 emission intensity that is >700 nm corresponding to 5% of that PI emission intensity also registered for wavelength >700 nm and set to 100%.

To determine which peak of the fluorescence histograms belongs to the viable or to the dead population, dead cells only were prepared from fresh bull sperm. This was achieved by storing sperm at −20°C for 1 h without the cyroprotectant glycerol. Staining of dead cells with SYBR-14 results for the SYBR-14 and PI channel (Fig. 3, upper and middle right) in a fluorescence intensity comparable with the lower peak of the stained population of viable cells (Fig. 1, upper and middle right). In the time-dependent dot-plot of the SYBR-14 fluorescence (Fig. 3, upper left) an enhanced SYBR-14 uptake can be observed which leads after ~600 s to a constant intensity value.

Then, the influence of the second dye (24 μM PI) on fresh bull sperm prestained with 100 nM SYBR-14 was investigated. At the beginning of the experimental run (0–3 min), the SYBR-14 prestained cells show SYBR-14 and PI channel fluorescence histograms (Fig. 4, upper right and middle right) comparable with those of the 14–17-min time period (Fig. 1). Addition of PI causes the following effects: In the SYBR-14 channel, the histogram peak for the viable population remains unchanged, whereas the fluorescence emission of the dead cell population is drastically reduced (Fig. 4, upper right). This fluorescence reduction is the main effect for discriminating viable and dead cells by this assay. Regarding the red fluorescence emissions (Fig. 4, middle right) the viable cell population shows nearly no increase in fluorescence intensity, whereas the fluorescence intensity of the dead population is increased by a factor of ~20. The two-parameter dot-plots (Fig. 4, lower row) represent this behavior clearly by exhibiting a shift of the dead population only when comparing the dot-plots before (Fig. 4,

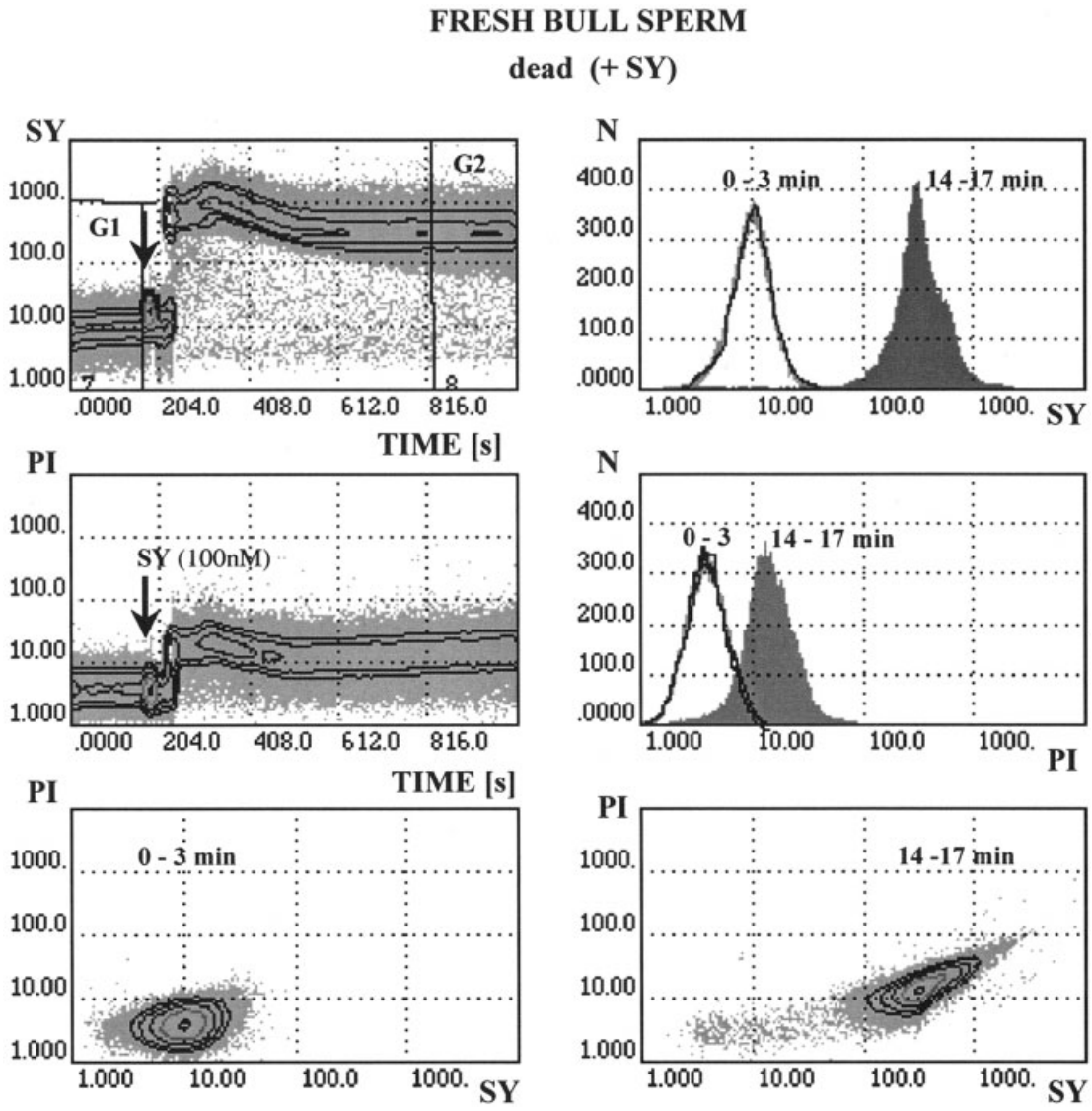

FIG. 3. Staining kinetics of SYBR-14 in dead cells of fresh bull sperm. Addition of SYBR-14 (after 3 min, arrow) results in the peaks of lower intensity observed in the two emission channels compared with Figure 2 (upper and middle right). The time kinetic (upper left) reveals also an enhanced SYBR-14 influx into dead cells equilibrated after 600 s. The time-gates are defined as in Figure 1.

lower left) and after (Fig. 4, lower right) PI addition, whereas the live cell population remains nearly unchanged. The portion of "intermediate" cells is small as seen in the relevant histograms of the fluorescence intensities. The observed reduction of the SYBR-14 intensity after addition of PI may be due to fluorescence quenching. To test this assumption, the relative fluorescence intensities of SYBR-14-stained dead cells were measured at different PI concentrations. Data were normalized to unity with respect to cells without any PI. As shown in Figure 5, increasing PI concentrations cause a significant decrease in the relative SYBR-14 fluorescence emissions. Additionally, these results suggest the use of a PI concentration of 24 μM for enhancing the SYBR-14 signal separation between viable and dead cells.

Cryopreserved Sperm: Staining Kinetics and Occurrence of a Transient Subpopulation

To investigate the effect of cryopreservation by this optimized assay, an aliquot of the same fresh bull sperm suspension was used and stored in Biociphos plus for 2 days at –196°C before thawing at 37°C. The relation of viable to dead cells was 25% to 75% respectively (Fig. 6, upper right) exhibiting no difference when compared to fresh sperm cells, 6 h after semen collection and staining (Fig. 4, upper right: dead 76%, viable 24%). In this context, the definition of "dead" is based on the PI influx criterion without further proof of the sperm fertilization quality.

Figure 6 summarizes the time dependence of the green- and the red-channel signals registered simultaneously

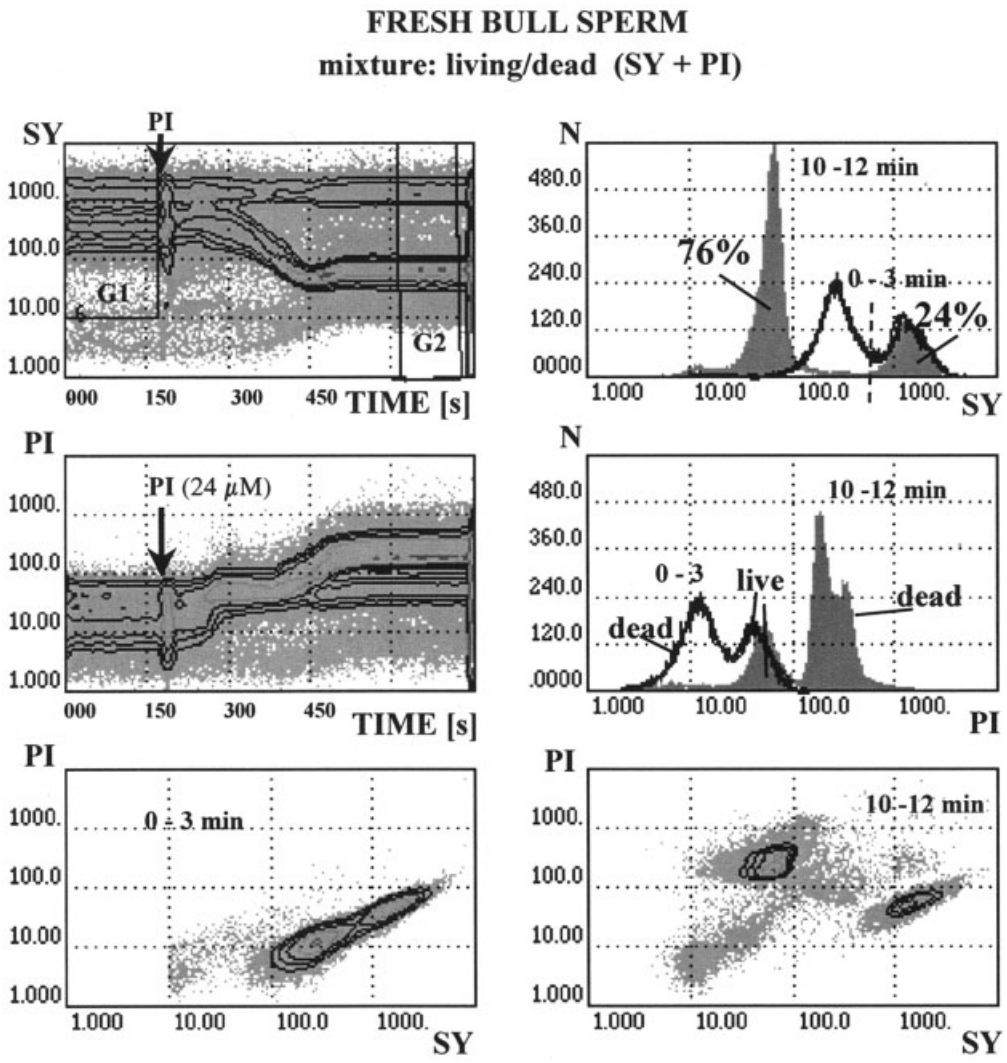

FIG. 4. Staining kinetics of propidium iodide (PI) in a SYBR-14 prestained mixture of viable and dead cells of fresh bull sperm. PI addition to a stationary SYBR-14-stained sperm population (0–3 min) results in dead cells with as well a reduced SYBR-14 fluorescence intensity as an enhanced PI intensity, whereas in viable cells both fluorescence intensities are nearly unchanged. The reduction of the SYBR-14 fluorescence intensity in dead cells is essential for the discrimination between viable and dead cells (lower row). The portion of "intermediate" cells (lower right) is small as can be recognized when analyzing the relevant histograms (upper right). Time-gates G1 and G2 relate to 0–3 min and 10–12 min, respectively.

from a sample consisting of a mixture of living/dead cryopreserved bull sperm.

Three time windows defined by the gates G1, G2, and G3 (Fig. 6, upper left) describe the intensity distributions emitted by SYBR-14 prestained cells before, just after, and 15 min after PI addition.

The SYBR-14 histogram of the prestained cells (Fig. 6, upper middle, 0–3 min, G1) separates weakly between viable (corresponding to the low asymmetric part at the right of the 0–3-min histogram) and dead cells compared to the fresh sperm situation (Fig. 4, upper right, 0–3 min). Within the second time window (6–9 min, G2) two distinct observations are important.

Once again, SYBR-14 quenching by PI causes the discrimination of the three different subpopulations viable, dead, and "intermediate" (IM). The occurrence of this third subpopulation is the second important result seen in this Figure 6, where its existence is clearly documented by the histograms and the dot plot diagram of the middle column. The time dependence of this IM population, however, can be distinctly recognized by the density lines in Figure 6 (upper left), where the upper band (high SYBR-14 intensity) belongs to viable cells, the lower one to dead cells and finally, the one connecting both to the IM subpopulation.

The dot-plot diagrams of the lower row (Fig. 6) normally used in the assay for cell discrimination clearly documents the clustered distribution of the IM cells for the three time-gates in use. The percentage values of the four gates (in the relevant SY/PI-dot-plot diagrams) help quantify the shifting process of the IM subpopulation.

Since the energy transfer to PI molecules is obviously the decisive process for the indication of non viable cells, we investigated the influence of this dye alone on the appearance of the different subpopulations (Fig. 7, upper

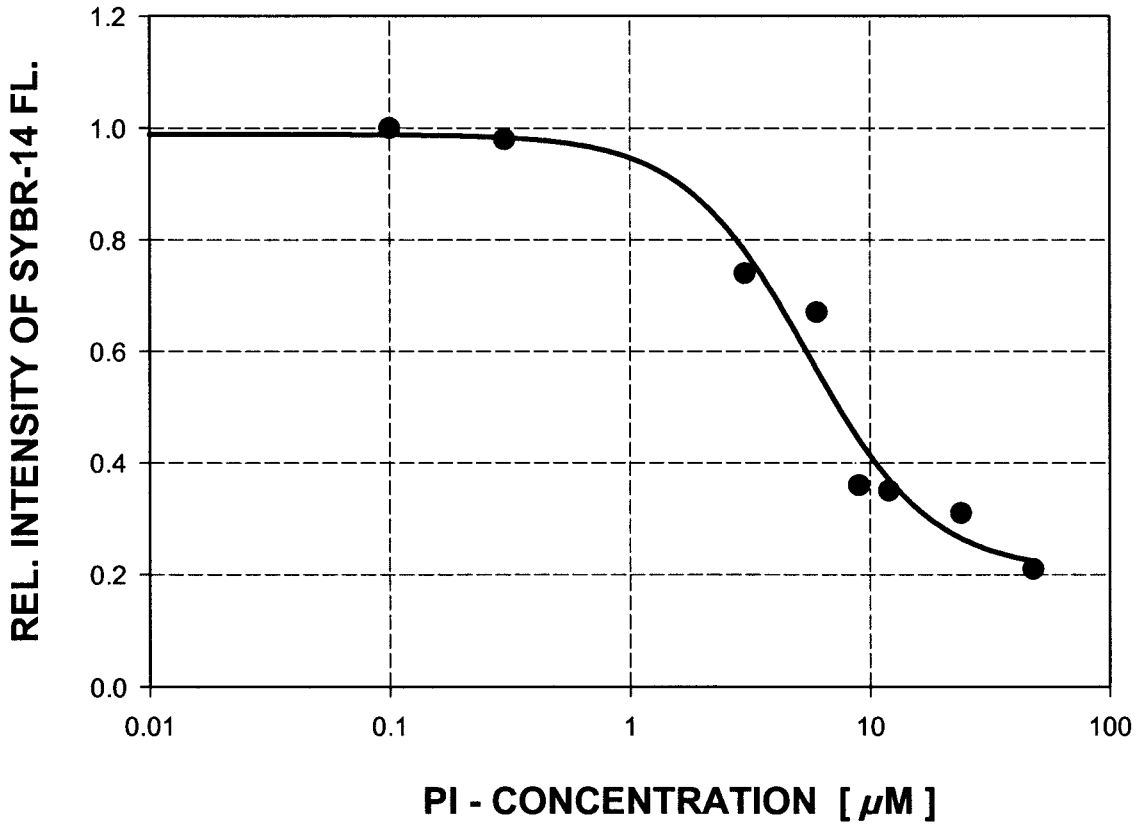

FIG. 5. SYBR-14 quenching by propidium iodide (PI). The relative fluorescence intensity of SYBR-14 from dead sperm cells is reduced with increasing PI concentrations due to fluorescence quenching.

row) followed by SYBR-14 addition for completing the reserved staining sequence on cryopreserved bull sperm. The addition of 24 μM PI (after 180 s) to unstained cells, immediately, leads to the appearance of the three distinct populations. One population (dead cells: d) with bright PI fluorescence, one with immediate (IM) and one population (live cells: l) with very dim PI fluorescence, only 5 times brighter than the autofluorescence (upper row middle). Addition of 100 nM SYBR-14 to these PI-prestained cells (Fig. 7, middle and lower row) is followed by a remarkable uptake of this dye into the viable subpopulation (enhancement by a factor of 100), whereas the 530 nm fluorescence of the dead subpopulation is characterized by a small increase. The analysis of the red channel signals after addition of SYBR-14 reveals the following findings: The PI fluorescence signals show a small right shift (due to the spillover effect), and the disappearance of the IM subpopulation is observable already after 14–17 min (Fig. 7, lower row).

DISCUSSION

Mammal sperm cells have to be in an adequate functional and morphological state during a defined time frame to fertilize the female gamete (17). For bull sperm it is widely known that the individual status of the bull as well as the handling of the sperm samples, especially the process of cyropreservation, have remarkable influences on the quality of semen samples (1,18). To assess the quality of this bull sperm, a variety of morphological and functional assays are in use (2,5,7,19–22). All these assays have to prove their validity against the statistical evaluation of fertility, especially the non-return rates (4,23). It is therefore mandatory to optimize the assays in use in respect to their ability of fertility testing.

The SYBR-14/PI assay analyzed in this report is widely used not only for investigating the status of bull sperm (11), but also for the examination of sperm from humans, ram, boar, rabbit, or mouse (13). Therefore, the main features found by us and explaining the cellular distribution of the signals registered in this assay, are discussed below.

Analyzing the time-dependent staining properties of SYBR-14 as the first dye, it becomes evident that SYBR-14 stains viable as well as dead bull sperm but with an approximately five times lower intensity (Fig. 1, upper right). This observation is in accordance with fluorescence microscopic findings by other investigators (11,13). This reduced SYBR-14 loading of dead cells may be due to a change in DNA-structure or/and in ionic content of the cell plasma. Both changes could cause a reduced retention of the membrane permeable SYBR-14 molecule. Additionally, the spectral overlap of the emission spectra of about 5%, even using a long-pass filter for 700 nm (Fig. 2), explains the spillover signals in the red PI channel after

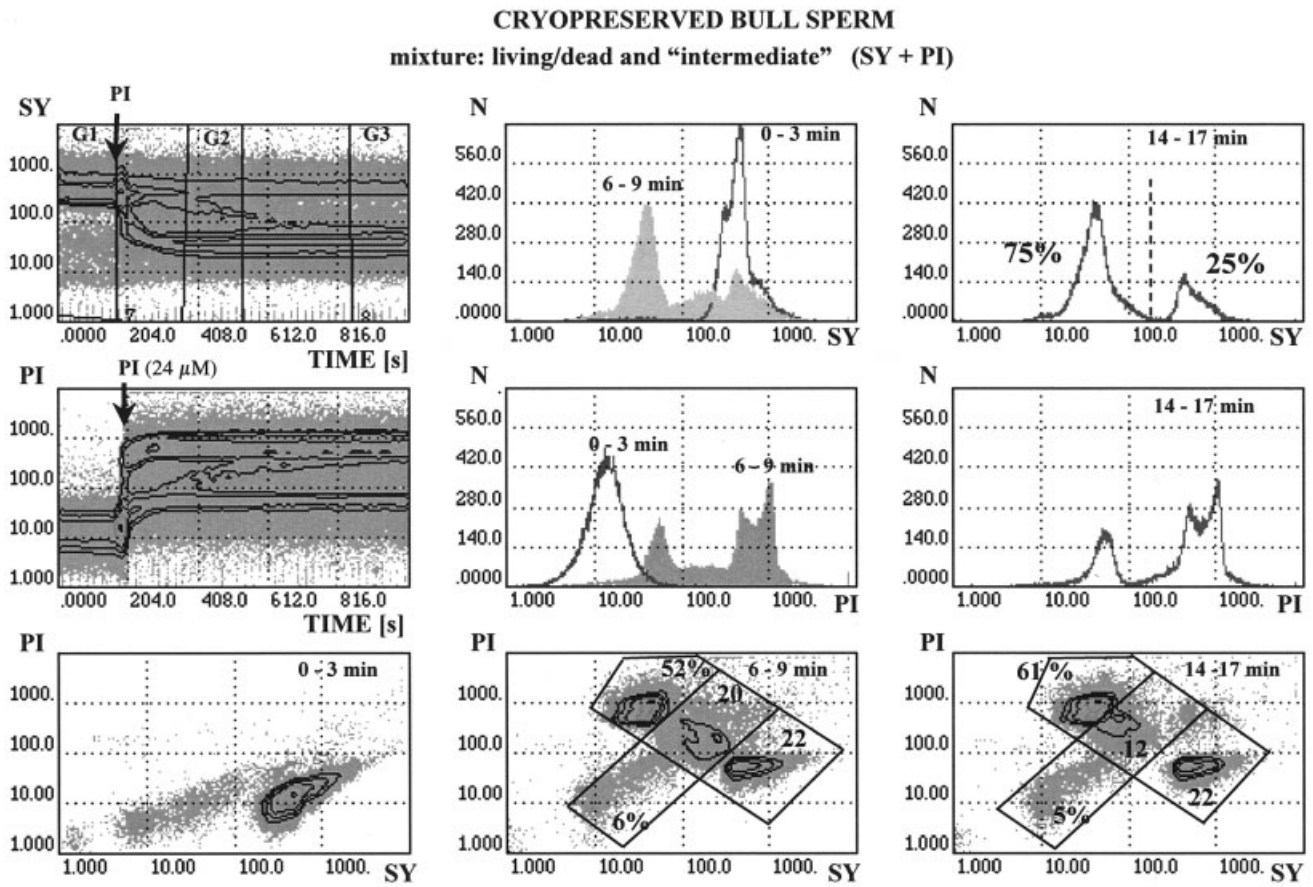

FIG. 6. Staining kinetics of propidium iodide (PI) in a SYBR-14 prestained mixture of viable and dead cells of cyropreserved bull sperm. (The time-gates G1, G2, and G3 defined in the Time/SY-diagram (upper row, left) belong to the time windows 0–3 min, 6–9 min, and 14–17 min, respectively.) After addition of PI, an additional temporary "intermediate" cell population is observable which reaches its maximum portion within 6–9 min (lower row, middle) and begins to disappear or disintegrate after 14–17 min (lower row, right).

SYBR-14 staining of live and dead sperm cells (Figs. 1 and 4, middle right). After addition of SYBR-14 to dead sperm, Figure 3 (upper left) documents an enhanced influx of this membrane permeating dye into the cells. After diffusion to the nucleus and intercalation into the DNA the equilibration process between free and bound dye may be finished 7 min after addition (24). For an understanding of the mechanisms of this assay the effect of SYBR-14 quenching by PI is extremely important (Fig. 5).

The addition of PI to a SYBR-14 prestained mixture of viable and dead fresh bull sperm results after 10 min in a viable population with constant SYBR-14 fluorescence as well as in a dead population with drastically reduced SYBR-14 fluorescence (a factor of ~25; Fig. 4 upper right). This fluorescence quenching is the main reason for discriminating viable and dead cells by this assay. Quenching may be achieved by fluorescence resonance energy transfer, since the absorption spectrum of the absorber (PI: maximum of F_{ex} = 535 nm) overlaps the emission spectrum of the donor (SYBR-14: maximum of F_{em} = 525 nm). Although behavior and molecular structure of SYBR-14 are unknown, SYBR-14 must be a DNA-intercalating molecule such as PI, since a spatial distance as small as 10 nm between SYBR-14 and the PI molecule is necessary to achieve this effect of energy transfer. Obviously, this effect resembles the fluorescence quenching process of TO-PRO-3 by PI (25). (A second fluorescence quenching process due to the absoption of SYBR-14 emitted photons by PI is also possible.) The power of fluorescence quenching is impressively seen in the two parameter dot-plots of Figures 4 and 6 (lower row), where the addition of PI to SYBR-14 prestained cells causes the dead population to shift from the lower left to the upper left position.

The differences between fresh bull sperm and cryopreserved bull sperm after thawing recognized by the time-dependent use of the SYBR-14 PI assay can be documented by comparing the results of the Figures 4 and 6.

The first difference is the intensity distribution of viable and dead cells after SYBR-14 staining alone. The double peak of fresh cells (Fig. 4, upper right) for the time-gate 0–3 min is reduced to one asymmetric peak (Fig. 6, upper middle) indicating a reduction in SYBR-14 binding ability by the viable cells of the cryopreserved sperm. This observation may point to a reduction of both the intercalating capability of the dye into the DNA and the probability that the dye will be retained by the cell membrane.

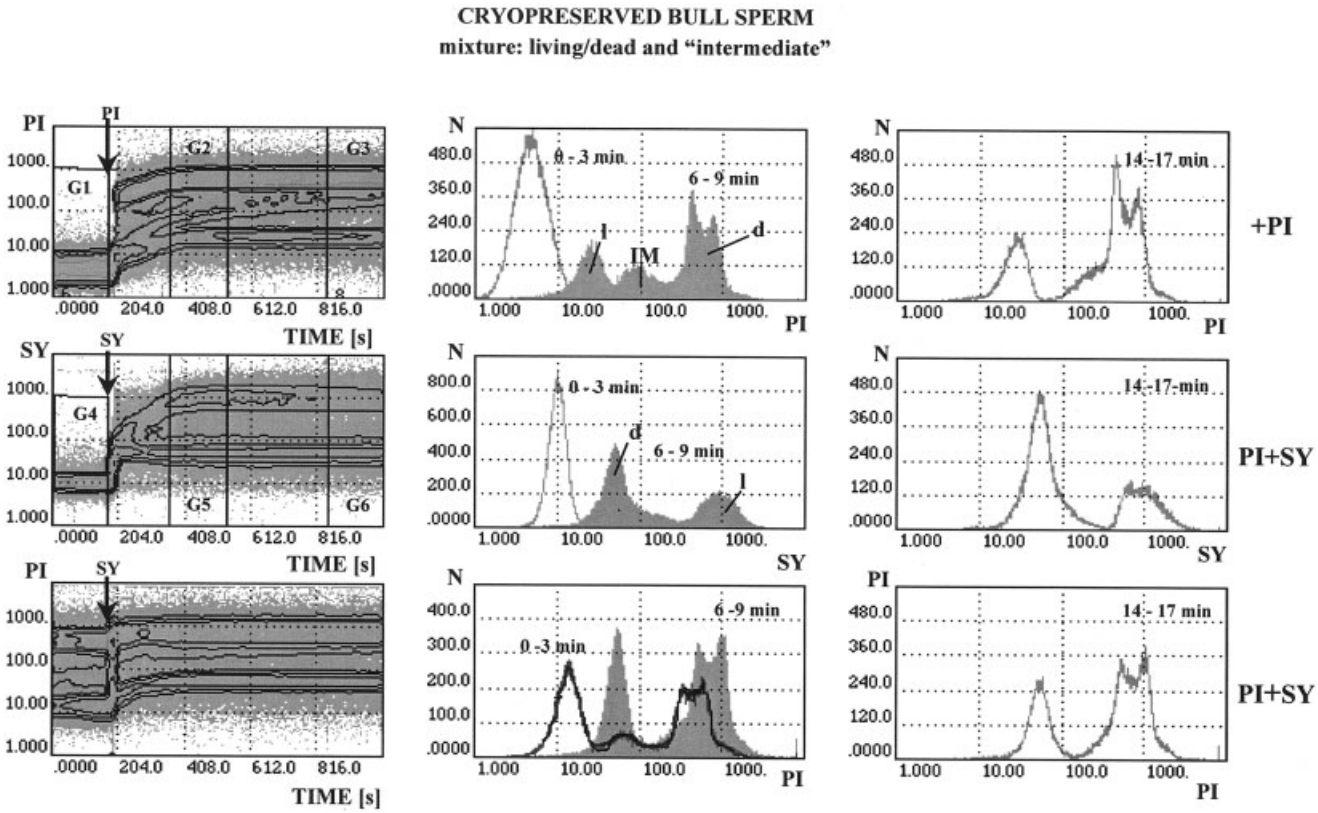

FIG. 7. Upper row: Cryopreserved sperm staining with propidium iodide (PI) alone results in an immediate appearance of all three subpopulations. Middle and lower row: Reversed staining kinetics by addition of SYBR-14 to a PI prestained mixture of viable and dead cells of cyropreserved bull sperm. Addition of SYBR-14 causes an intense staining of viable cells (middle row, middle) and a small right shift of fluorescence signals in the red channel (lower row, middle). During the reversed staining procedure, the disappearance of the "intermediate" cell population is also observable also after 14–17 min (diagrams at the right). Time-gates are as defined in Figure 6.

The second difference is documented by the fast time-dependent uptake of PI molecules through the cell membrane followed by SYBR-14 quenching in the DNA of the dead cryopreserved subpopulation (upper and middle row, left of Figs. 4 and 6). Obviously, the freeze-thaw process induces an enhanced membrane labilization in dead cells, causing a very rapid PI influx (<10 s) compared with the PI uptake period of 200 s in dead cells of fresh sperm.

The third difference is the distinct appearance of a transient dual-stained IM population after addition of PI to a cryopreserved sperm suspension (Fig. 6 upper and middle row; SY- and PI histograms of the 6–9-min time period). The existence of this IM population was also observed by Garner et al. (11,13) and termed "moribund" sperm. Recently, Nagy et al. (12) succeeded in quantifying this type of sperm subpopulation, which is shifted from SYBR-14-positive to PI-positive staining. Probably the freeze-thaw cycle induces this IM population in viable cells of cryopreserved sperms. At ~200 s after PI addition, these IM cells become evident (Fig. 6 upper and middle row, left) starting their transition from the viable cell intensity level and reaching the one of dead cells after 600 s. In comparison with Figure 4 (time dependent runs at the left) one can see that this population has the same kinetics as the dead cell population of fresh bull sperm. The appearance of these IM-stained sperm cells becomes evident between 3 and 6 min after PI uptake (Fig. 6, lower row middle). They shift to the dead population and become indistinguishable from that population about 10 min later (Fig. 6 lower row, right). Quantitatively, this is documented by comparing the percentage values of four different population-gates in these two dot-plot diagrams that are related to the time-gates 6–9 min and 14–17 min. Whereas the fraction of the viable cells (22%) as well as cell debris (6% and 5%) remain constant, the fraction of the IM population is reduced (from 20% to 12%) by nearly the same amount as the dead-cell fraction is increased (from 52% to 61%).

The staining with PI is obviously the key factor for the appearance of the IM population as shown in Figure 6 by the temporal kinetics of the different subpopulations. Confirmation of this assumption was obtained by applying the reverse staining procedure.

Staining with PI alone (Fig. 7, upper row) results immediately in the appearance of the three populations. The addition of SYBR-14 as the second dye leads to a fast and intense uptake of this dye into cells of the viable population, whereas the dead population shows only weak SYBR-14 emission due to instant fluorescence quenching

by already intercalated PI (Fig. 7, middle row). During this reversed staining procedure, the simultaneously recorded signals in the red-channel (Fig. 7, lower row) show a small increase in fluorescence intensity due to the spillover of the SYBR-14 emission effect. Since PI prestaining takes 6 min, the 0–3-min histogram of the lower row equals the 6–9-min histogram of the upper row showing the small amount of uncertainty of this kind of experiments. The disappearance of the IM population is already documented by the corresponding histograms in Figure 7 (middle and lower row, right).

The precision of this method permits observation of a peculiar feature. Bull sperm cells from the dead population are characterized by a PI fluorescence histogram with two distinct peaks separated by a factor of two. These peaks are more pronounced in cryopreserved cells (Fig. 7, upper and lower row, right) than in fresh sperm cells (Fig. 4, middle row, right) and become evident within 3 min after staining with PI. An additional analysis of these double peak populations was performed with the DAS software (16). It could be shown that sperm cells with the higher PI fluorescence have generally both a higher forward scatter signal and a higher SYBR-14 fluorescence intensity. These cells are consequently dead cells with increased cell volume. They may have a perturbed DNA condensation that allows the fixation of a higher amount of dye molecules.

We conclude that the SYBR-14/PI assay is a fast, reliable and sensitive method to assess the viability and the membrane integrity of bull sperm. Fluorescence quenching is mainly responsible for the sensitivity of this assay and has to be taken into account together with the spectral overlap of both dyes for a fully understanding the observed effects. The appearance of an intermediate population is a time-dependent process of cryopreserved cells shifting from the viable to the dead cells population occurring between about 3 and 15 min after staining. The portion of IM cells becomes greater after cryopreservation inducing stress on the cellular membrane integrity and functions. The estimation of this IM population may be a good indicator for handling and storage induced detrimental effects on bull sperm cells.

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LITERATURE CITED

1. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–891.
2. Januskauskas A, Rodriguez-Martinez H. Assessment of sperm viability by measurement of ATP, membrane integrity and motility in frozen/thawed bull semen. Acta Vet Scand 1995;36:571–574.
3. Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57:1401–1406.
4. Januskauskas A, Johannisson A, Soderquist L, Rodriguez-Martinez H. Assessment of sperm characteristics post-thaw and response to calcium ionophore in relation to fertility in Swedish dairy AI bulls. Theriogenology 2000;53:859–875.
5. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;56:991–998.
6. Parrish JJ, Susko-Parrish JL, Graham JK. In vitro capacitation of bovine spermatozoa: role of intracellular calcium. Theriogenology 1999;51:461–472.
7. Alm K, Taponen J, Dahlbom M, Tuunainen E, Koskinen E, Andersson M. A novel automated fluorometric assay to evaluate sperm viability and fertility in dairy bulls. Theriogenology 2001;56:677–684.
8. Evenson DP, Darzynkiewicz Z, Melamed MR. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 1982;30:279–280.
9. Garner DL, Pinkel D, Johnson LA, Pace MM. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol Reprod 1986;34:127–138.
10. Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod 1990;43:55–64.
11. Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 1994;15:620–629.
12. Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68:1828–1835.
13. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide: Biol Reprod 1995;53:276–284.
14. Grundler W, Dirscherl P, Beisker W, Marx K, Stampfl A, Maier K, Zimmermann I, Nüsse M. Early functional apoptotic responses of thymocytes induced by tri-n-butyltin. Cytometry 2001;44:45–56.
15. Grundler W, Dirscherl P, Beck-Speier I, Beisker W, Stampfl A, Zimmermann I, Maier K. Simultaneous recording of calcium transients and reactive oxygen intermediates of human polymorphonuclear granulocytes in response to Formyl-Met-Leu-Phe and the environmental agent sulfite. Cytometry 2000;40:219–229
16. Beisker W. A new combined integral-light and slit-scan data analysis system (DAS) for flow cytometry. Comput Methods Programs Biomed 1994;42:15–26.
17. Harrison RA. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev 1996;8:581–594.
18. Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology 2002;57:327–344.
19. Ericsson SA, Garner DL, Redelman D, Ahmad K. Assessment of the viability and fertilizing potential of cryopreserved bovine spermatozoa using dual fluorescent staining and two-flow cytometric systems. Gamete Res 1989;22:355–368.
20. Garner DL, Thomas CA, Allen CH. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. J Androl 1997;18:324–331.
21. Graham JK. Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 2001;68:239–247.
22. Ostermeier GC, Sargeant GA, Yandell BS, Evenson DP, Parrish JJ. Relationship of bull fertility to sperm nuclear shape. J Androl 2001;22:595–603.
23. Januskauskas A, Gil J, Soderquist L, Haard MG, Haard MC, Johannisson A, Rodriguez-Martinez H. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. Theriogenology 1999;52:641–658.
24. Strange K, editor. Cellular and molecular physiology of cell volume regulation. Boca Raton, FL: CRC Press; 1994.
25. Beisker W, Weller-Mewe EM, Nüsse M. Fluorescence enhancement of DNA-bound TO-PRO-3 by incorporation of bromodeoxyuridine to monitor cell cycle kinetics. Cytometry 1999;37:221–229.