Chapter 12

Sperm DNA Fragmentation Analysis Using the TUNEL Assay

Rakesh Sharma, Jayson Masaki, and Ashok Agarwal

Abstract

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling or the TUNEL assay is an important technique in the assessment of DNA damage. Semen samples are routinely assessed microscopically to assess their fertilization ability. In addition to routine semen analysis, the use of the TUNEL assay can provide information on the level of DNA damage present within a sample. This chapter provides a practical walk-through guide aimed at directing a researcher or a clinical facility interested in setting up and using TUNEL and flow cytometry or fluorescence microscopy for sperm DNA analysis.

Key words: TUNEL, DNA damage, Flow cytometry, Chromatin

1. Introduction

This chapter showcases the application of the TUNEL assay in the measurement of sperm DNA damage. The protocol of the TUNEL assay is provided as a model guide for any lab interested in establishing a similar protocol for measuring the DNA damage in ejaculated semen samples with a detailed explanation of the sperm preparation, staining, and inclusion of appropriate controls, instrument specifications, and troubleshooting tips.

1.1. Sperm DNA Damage

The single goal of the spermatozoon is to deliver the paternal counterpart of genetic material to the oocyte. Routine semen analysis is still the cornerstone in the laboratory workup of the infertile male, and the microscopically observable characteristics of the spermatozoa such as concentration, motility, and morphology are important. However, the inclusion of an additional parameter that assesses DNA damage can provide for a more comprehensive description of semen quality. Expanding the parameters of routine semen analysis to include an assessment of genetic integrity is essential in the complete characterization of a semen sample. After

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all, determining the extent of sperm DNA damage may be of primary importance in predicting the ultimate health of the resulting progeny.

DNA damage may involve single-stranded breaks or "nicks," double-stranded breaks or "fragments," deletions/additions, and base modifications. The term DNA fragmentation is technically associated with endonuclease-mediated double-stranded DNA cleavage as a result of apoptotic programmed cell death. However it has also become interchangeable with the general term "DNA damage" when in the context of TUNEL assay results. This is because TUNEL staining was initially (and still is) used as a marker of DNA fragmentation in distinguishing between apoptosis and necrosis.

The TUNEL staining technique can be used to directly assess DNA damage of a cell in the form of both single- and double-stranded breaks. When coupled with flow-cytometry technology, a quantitative assessment of the proportion of genetically compromised cells within a semen sample can be provided. Understanding the level of DNA integrity of a semen sample can be of diagnostic and predictive value in fertility clinics, as well as in the optimization of experimental methods to aid in treating infertility.

1.2. Etiology of DNA Damage

The causes of sperm DNA damage have been studied extensively. However because of its multifactorial basis and indistinct relationship with infertility, the comprehensive origins of and preventative treatment for DNA damage still remain controversial. Associations of cause and effect are not consistently observed across all studies, and, even more importantly, are confused by various methods of detection (1). In general, the main sources of seminal DNA fragmentation detectable by TUNEL appear to be from free radical damage via reactive oxygen species (ROS) and abortive apoptosis (2, 3). Other factors include radiation, aberrant spermatozoa maturation, or DNA packaging, although these are not specifically attributable to fragmentation as measured by the TUNEL assay.

Oxidative stress occurs when an imbalance of ROS exceeds the locally available antioxidant capacity to prevent oxidative cellular damage. Sperm DNA is especially susceptible to oxidative damage. Spermatozoa have a low cytoplasmic volume, wherein preventive mechanisms against ROS normally exist within a cell. The highly condensed structure of sperm nuclear material rules out enzymatic repair of damaged DNA. Sperm motility is crucial and energy requirements are provided by extremely active mitochondria that generate ROS. Furthermore, processing of semen for various assisted reproductive techniques such as swim-up or density gradient centrifugation removes the seminal plasma that protects the spermatozoa against ROS through its antioxidant properties (4).

Nature provides for a functional deterrent to inhibit spermatozoa exposed to high levels of ROS to reach and fertilize an egg. The sperm plasma membrane contains polyunsaturated fatty acids,

which are extremely susceptible to ROS attack (5). Cells exposed to oxidative stress sufficient to cause DNA damage would likely have a compromised plasma membrane and therefore be incapable of natural fertilization. However, this natural process of functional selection is bypassed when techniques such as Intracytoplasmic Sperm Injection (ICSI) are used.

Sources of increased ROS affecting sperm function can be intrinsic (from within the spermatozoa themselves) or extrinsic. The main significant sources of intracellular ROS are superoxide anions produced during oxidative phosphorylation in spermatozoon mitochondria and plasma membrane NAD(P)H oxidase, and nitric oxide produced by nitric oxide synthase within the plasma membrane (6). Extrinsic sources include largely the leukocytes (granulocytes), unknown chemicals/toxins, and environmental pollution, nutritional deficiency, laboratory processing of samples, advanced age, psychological and physical stress, smoking, alcohol, caffeine, cancer and anticancer drugs, or inflammatory processes/cells. Intrinsic sources of ROS appear to be more damaging to sperm DNA than extrinsic ROS. For example, although leukocyte and inflammatory processes can contribute to increased ROS in the ejaculate on the order of 1,000 times that produced intrinsically (7), DNA fragmentation has a stronger correlation with intrinsic ROS than for leukocyte ROS production (8). This may be due to the fact that the antioxidant capacity of the seminal fluid in which spermatozoa are bathed negates the ROS effects from exogenous sources. However in ART procedures where the seminal plasma is removed, the leukocytes come in close proximity to the normal and abnormal spermatozoa. An inadequate antioxidant capacity would therefore greatly contribute to DNA damage by means of oxidative stress. Dietary and in vitro antioxidant supplementation has been shown to be beneficial in reducing some exogenous ROS-induced DNA damage (9).

In the treatment of ROS-mediated DNA damage, it is important to note that eliminating all sources of ROS is not only impossible but also not advisable, as ROS are normal products of metabolism, and their complete elimination may adversely affect sperm function. Certain levels of endogenous ROS produced at specific times are crucial for the normal physiologic maturation, capacitation, and hyperactivation of sperm (10, 11). Oxidative stress has been shown to correlate with apoptosis (12, 13); however, the relationship is not entirely causal; oxidative stress can induce apoptosis, and is also a product resulting from the process of apoptosis.

1.3. How TUNEL Works

TUNEL utilizes a template-independent DNA polymerase called Terminal Deoxynucleotidyl Transferase (TdT) which non-preferentially adds deoxyribonucleotides to 3′ hydroxyl (OH) single- and double-stranded DNA (14). Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT

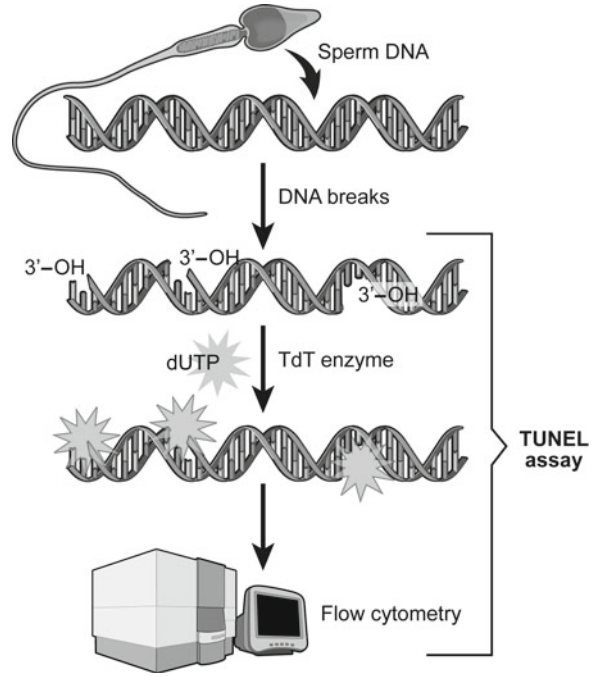

Fig. 1. Schematic of the TUNEL assay.

enzyme to the free 3′-OH break-ends of DNA (see Fig. 1). The added dUTP can be directly labeled and therefore acts as a direct marker of DNA breaks, or the signal can be amplified by the use of a modified dUTP to which labeled anti-dUTP antibody can be adsorbed. The more DNA strand break sites present, the more label is incorporated within a cell. The TUNEL-stained cells can be visualized microscopically; however, for the high-throughput assessment of clinical semen samples it is more practical to use a flow cytometer that detects the intracellular fluorescence of fluorochrome-labeled dUTP within a population of cells. The flow cytometer analyzes one cell at a time, and counts the number of individual events (cells) that it recognizes to have a specific intensity of fluorescence. Fluorescein isothiocyanate (FITC) is the fluorophore label most commonly used in TUNEL staining. Propidium Iodide (PI) is used as a fluorescent counterstain that labels all DNA so that every cell can be counted, and the resultant proportion of those showing DNA damage can be calculated. Because the TdT will bind and incorporate dUTP at the terminal 3′ ends of each DNA strand with the same propensity as interior 3′ ends (15) irrespective of damage, there is a threshold of labeled dUTP-induced fluorescence which should be considered in order to distinguish between viable, relatively undamaged cells and those with severe, irreparable DNA damage. The parameters used for this threshold are

included in the procedure portion of this guide, and are also discussed in Subheading 4.

TUNEL staining kits are readily available in the commercial marketplace. These kits are very cost effective, highly standardized by the manufacturers, and often provide some technical support. Most TUNEL staining protocols are very similar, with only minor variations in centrifugation force, incubation times, temperatures, and reagent concentrations, but all follow the same general principles. The TUNEL kit utilized in the following procedure is the *APO-DIRECT*™ kit (BD Biosciences Pharmingen, San Diego, CA). This kit is designed for use with flow cytometry for the general detection of DNA fragmented apoptotic cells and also provides positive and negative control cells (diploid cell lines) for standardization purposes. When measuring sperm DNA damage it is prudent to include sperm controls for each assay as a precautionary indicator of problems in order to rule out reagent variability between kits, and also because gating is necessary in flow cytometry and must be done on the same cell type.

1.4. Other Assays of DNA Integrity

There are three main groups of assays that are used in the evaluation of cellular genetic integrity and are divided based on what each assay measures. The most obvious and direct method is to assess the actual molecular DNA strand breaks present (in a number of cells in the sample). This snapshot-approach is where the TUNEL assay is categorized, along with COMET using neutral conditions, and in situ nick translation. The next assay group measures the potential susceptibility of the DNA to exogenous damage rather than assessing actual DNA damage directly since these techniques first induce DNA damage by exposure to denaturing conditions. Assays of this type include the Sperm Chromatic Structure Assay (SCSA) and COMET using alkaline conditions. Such assays shed light on associated abnormalities that may affect the genetic integrity such as aberrations in condensation structure/packing, or lack of DNA stabilizing and protective mechanisms. Interestingly, within these two groups of assays there is considerably high correlation between both the measure of DNA damage and the outcome of fertilization using assisted reproductive techniques. The choice of the assay would be based on the ease of the assay, robust nature of the assay (sensitivity and specificity) and if it is cost effective practicality in training, staffing, time, and resources will be the primary determining factors. The third group of assays examines DNA base modification as a means of linking markers of oxidative DNA damage (such as 8-OH-guanosine and 8-OH-2′-deoxyguanosine) with a general loss of DNA integrity. While it is has been shown that these tests can be used to predict male infertility and pregnancy rates (16, 17), the oocyte has the ability to repair such damage, and as a result the focus on the assessment of DNA damage in the clinical

Table 1
Advantages and disadvantages of TUNEL, Comet, and SCSA

Assay	Main advantages	Main disadvantages
TUNEL 1. Adds labeled nucleotides to free DNA ends 2. Template independent 3. Labels SS and DS breaks 4. Measures percent cells with labeled DNA	1. Direct objective 2. Inexpensive 3. Can be performed on few sperm 4. High repeatability 5. Quick and simple (fluorescence microscopy) 6. Objective. High sensitivity (flow cytometry) 7. Fresh or frozen samples 8. Indicative of apoptosis 9. Correlates with semen parameters 10. Associated with fertility 11. Available in commercial kits	1. Thresholds not standardized 2. Variable assay protocols 3. Not designed specifically for spermatozoa 4. Not specific to oxidative damage 5. Need for special equipment (flow cytometer)
Comet For single- and double-stranded DNA 1. Electrophoresis of single sperm cells 2. DNA fragments form tail 3. Intact DNA stays in head Alkaline COMET 1. Alkaline conditions, denatures all DNA 2. Identifies both DS and SS breaks Neutral COMET 1. Does not denature DNA 2. Identifies DS breaks, maybe some SS breaks	1. Indirect assay, subjective 2. Inexpensive 3. Poor repeatability 4. High sensitivity 5. Fresh samples only 6. Correlates with seminal parameters 7. Small number of cells required 8. Versatile (alkaline or neutral)	1. Variable protocols 2. Unclear thresholds 3. Not available in commercial kits 4. Time and labor intensive 5. Small number of cells assayed 6. Subjective 7. Not specific to oxidative damage 8. Lacks correlation with fertility 9. Requires special imaging software
SCSA For single-stranded DNA 1. Mild acid treatment denatures DNA with SS or DS breaks 2. Acridine orange binds to DNA 3. Double-stranded DNA (nondenatured) fluoresces green, single-stranded DNA (denatured) fluoresces red 4. Flow cytometry counts thousands of cells. DNA fragmentation index (DFI)—the percentage of sperm with a ratio of red to (red + green) fluorescence greater than the main cell population	1. Direct objective 2. Inexpensive 3. Established clinical thresholds 4. Many cells rapidly examined 5. High repeatability 6. Fresh or frozen samples 7. Most published studies reproducible	1. Proprietary method 2. Not available in commercial kits 3. Expensive equipment 4. Acid-induced denaturation 5. Small variations in lab conditions affect results 6. Calculations involve qualitative decisions 7. Very few labs conduct this assay

TUNEL terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; *SCSA* sperm chromatin structure assay

setting has been mainly on those tests that evaluate double-stranded DNA breaks (fragmentation)—the most genotoxic type of damage (18, 19).

Some of the major advantages and disadvantages of the TUNEL, Comet, and SCSA assays are outlined (see Table 1). The TUNEL assay is by no means the end-all solution, but it is certainly one of the more simple, reliable, objective, and cost-effective methods available for assessing DNA damage. Multiple approaches can be collaboratively used for better evidence of genetic integrity as well as newer, modified, and improved TUNEL methods that also incorporate sperm vitality assessment (20).

2. Materials

2.1. Semen Collection and Handling

1. Sterile open wide mouth specimen cups.
2. Incubator (37°C).
3. Cell counting chambers.
4. Microscope.
5. Centrifuge tubes—12 × 75 mm with caps (polystyrene recommended to limit cell loss).
6. Micropipettes and tips.
7. Phosphate-buffered saline (PBS, pH 7.4).
8. DNase I—1 mg/ml.

2.2. Sperm Fixation

1. Centrifuge—300 × *g*.
2. Serological pipettes.
3. Transfer/Pasteur pipettes.
4. 3.7% paraformaldehyde.
5. Ice.
6. 70% (v/v) ethanol.

2.3. Staining

1. APO-DIRECT Kit components (store the various components as directed by manufacturer): Reaction buffer (contains coenzyme factors and cacodylic acid), PI/RNase staining buffer (5 μg/ml PI, 200 μg/ml RNase), Rinsing buffer, Wash buffer, TdT enzyme, FITC-dUTP, Positive and Negative control cells.
2. Distilled water.
3. Aluminum foil.

2.4. Flow Cytometry and Data Analysis

1. Flow cytometer FacScan (Becton Dickinson, San Jose, CA).
2. Computer software (FlowJo, Mac Version 8.2.4, LLC, Ashland, OR).

3. Methods

3.1. Semen Collection and Concentration Assessment

1. Collect semen following an abstinence period of 48–72 h. The sample should be collected by means of masturbation into a sterile specimen cup without lubricant. Specimen must be maintained at body temperature and processed within 1 h of collection. Record the sample ID, collection date and time, period of abstinence, and any remarkable conditions. Keep the sample in a 37°C incubator until complete liquefaction has occurred.
2. Following liquefaction perform a sperm count (see Note 1). Approximately $2–3 \times 10^6$ total cells are sufficient to run the assay. To optimize the stains, the sperm concentration should be no more than 5×10^6 (see Note 2). For efficiency samples can be stored at −20°C and batched for TUNEL analysis. Alternatively, the cells can be individually fixed and stored in ethanol until the time of analysis (see fixation step).

3.2. Preparation of Assay Controls (See Note 3)

1. For the positive sperm control, DNA damage is induced by digestion with DNase I. Incubate a sample from a healthy donor after the cell counting step with 100 μl of DNase I (1 mg/ml) for 1 h at 37°C.
2. Similarly, a sample from a healthy donor is used for the negative sperm control, wherein the TDT enzyme is omitted from the staining step as described below.

3.3. Fixation of Sperm

1. Prepare the fixation buffer by adding 10 ml of 37% formaldehyde (100% formalin) to 90 ml of PBS (pH 7.4) to give a 3.7% (v/v) paraformaldehyde solution (see Note 4).
2. Centrifuge the sperm sample at $300 \times g$ for 7 min to pellet and separate the cells from the seminal plasma (see Note 5). All subsequent centrifugation steps are to be done at $300 \times g$ for 5 min.
3. Discard the supernatant by gently aspirating with a transfer or Pasteur pipette (see Note 6).
4. Suspend the cells in the 3.7% paraformaldehyde fixation buffer. Place the suspension on ice for 30–60 min or alternatively refrigerate at 4°C overnight.
5. Discard the supernatant and suspend the pellet in 1 ml of ice-cold 70% (v/v) ethanol at −20°C. At this point the cells can be stored in ethanol at −20°C (see Notes 7 and 8).

3.4. Preparation of Samples for Staining

1. Resuspend all samples (test and controls) by vortexing the tubes since the cells will have settled after prolonged storage in ethanol.

2. For the kit controls, a positive and negative control is provided with the *APO-DIRECT* kit. Remove 2-ml of each of the control cell suspensions (1×10^6 cells/ml, 2×10^6 cells total) and place in 12×75 mm centrifuge tubes.
3. Centrifuge both test and control tubes at $300 \times g$ for 5 min and discard the ethanol supernatant.
4. Resuspend the cells in 1 ml of Wash Buffer (see Note 9), centrifuge at $300 \times g$ for 5 min, and remove the supernatant.
5. Repeat the Wash Buffer treatment a second time.

3.5. Staining

1. Prepare an appropriate volume of the Staining Solution based on the number of samples to be assayed (see Note 10). For each sample to be analyzed, combine 10 µl Reaction Buffer, 0.75 µl TdT Enzyme, 32.25 µl distilled water, and 8 µl FITC-dUTP. The FITC-dUTP reagent should be added last as it is light sensitive (see Note 11).
2. Resuspend the cell pellets in 50 µl of the Staining Solution. For negative semen controls, add staining solution without the TdT enzyme.
3. Vortex to disperse the cells and to allow the staining solution to permeate homogeneously into every cell.
4. Incubate for 60 min at 37°C.
5. After incubation, directly add 1.0 ml of Rinse Buffer to each sample, centrifuge at $300 \times g$ for 5 min, and remove the supernatant by aspiration.
6. Repeat rinse step with an additional 1.0 ml of Rinse Buffer, centrifuge, and again remove the supernatant of each tube.
7. Resuspend in 0.5 ml of the PI/RNase Staining Buffer and incubate tubes at room temperature for 30 min.
8. Analyze the cells in the PI/RNase solution by flow cytometry within 3 h of completing the staining procedure (see Note 12).

3.6. Flow Cytometry, Data Acquisition, and Storage

Care should be taken to use the protocol provided here as only a guide when adjusting the settings of any particular flow cytometry setup. Each machine will be inherently different, but should present similar data when adjusted/calibrated to the same parameters. In our facility an automated flow cytometer is used to analyze the TUNEL-stained sperm. The machine is equipped with a 15 mW argon laser that supplies a filtered 488 nm excitation beam. Green (FITC) fluorescence is measured in the FL-1 channel (480–530 nm) and red (PI) fluorescence is set to be detected in the FL-2 channel (580–630 nm). The steps for setup and data acquisition that we employ for the FacScan flow cytometer is detailed in Subheading 4 (see Note 13).

3.7. Analysis of Data

Data analysis can be performed using the operator's software of choice. The workflow that we employ for the analysis is provided below (see Note 14).

3.8. Clinical Threshold of Infertility

The suggested threshold of infertility used by our lab is 19% DNA fragmentation. This is the cutoff value we have been using to discriminate between a healthy semen sample and one that has positive DNA damage. Every lab should establish its own reference values utilizing appropriate controls (see Note 15).

3.9. Measuring DNA Damage by Fluorescence Microscopy

If flow cytometry is not accessible, the same stained cells can be scored with a fluorescence microscope.

1. Load an aliquot of the stained sample on a slide and cover with a coverslip.
2. Score a minimum of 500 spermatozoa per sample under 40× objective with an epifluorescence microscope (excitation between 460 and 490 nm and an emission >515 nm).
3. First count the number of spermatozoa per field stained with PI (red).
4. Count the number of cells emitting green fluorescence (TUNEL positive) in the same filed.
5. Calculate the percent of TUNEL-positive cells.

4. Notes

1. We recommend that disposable counting chambers specifically made for counting sperm cells (e.g., MicroCell 20 μm 2-chamber) be used for accuracy and operator.
2. The APO-DIRECT kit instructions suggest the use of $1–2 \times 10^6$ cells/ml cell concentration. We suggest that no more than 5×10^6 cells/ml be used, as with each washing and centrifugation step some cells are lost.
3. Rather than making new positive and negative sperm controls every time, previously analyzed samples may be used as standardized/reference control samples with which the flow cytometer analysis software can be calibrated in order to maintain consistent DNA damage percent between batches. Stored aliquots of extra cells from two fixed samples in which DNA fragmentation percentage was previously determined (known positive and negative) should be processed alongside the current batch beginning with the staining procedure. The FC analysis software settings used to gate the new batch of cells can then be adjusted to reflect the previous results of the same samples within ±1%.

While the positive and negative controls provided with commercially available TUNEL kits are adequate for calibration during flow cytometer analysis of somatic cells, they are not suitable as controls for haploid cells such as spermatozoa and will have different nuclear size and level of DNA condensation. Therefore it is important to prepare positive and negative sperm control samples.

4. 3.7% (w/v) paraformaldehyde is used instead of the 1% recommended by the APO-DIRECT kit instructions to ensure that the DNA is completely fixed. We have chosen this concentration out of the convenience of a 1/10 dilution and believe it is justified by the fact that sperm nuclear DNA is the most highly compacted of eukaryotic DNA (21). However, any concentration between 1 and 4% should be sufficient for fixation purposes. Higher concentrations should be avoided as it may inhibit thorough washing of the unwanted formaldehyde from the sample and cause problems in staining. Formaldehyde is carcinogenic and proper handling precaution must be observed.
5. Centrifugation at 300 × *g* for 7 min instead of 5 min allows for cells to be pelleted more fully in the case of increased seminal plasma viscosity.
6. 50–100 μl of supernatant may be left during aspiration to avoid losing cells after each centrifugation step. It is much more convenient to leave a small amount of fluid rather than to have to recentrifuge the sample due to a disturbed pellet. The repeated washing/rinsing steps should negate any residual supernatant.
7. While the fixed cells can be kept in paraformaldehyde for several days or weeks at 4°C, do not freeze the samples in the paraformaldehyde solution. It is recommended that suspension in 70% ethanol be used for long-term storage of samples at –20°C and that samples not to be processed on the same day. The manufacturer (BD Pharmingen™) also suggests that incubation in ethanol for at least 12–18 h yields better staining results.
8. Always ensure that the quantity of ice is sufficient to last 2–3 days in case of an unexpected delay in delivery.

   When shipping samples (individual or batched) from outside labs, cryovials are labeled with the sample information (i.e., date, name, type of sample, volume, etc.). The sample can be either frozen in multiple aliquots soon after liquefaction or processed by suspending the sperm cells ($1–2 \times 10^6$ cells/ml) in 3.7% (w/v) paraformaldehyde prepared in PBS (pH 7.4). The sample is placed on ice for 30–60 min and centrifuged for 5 min at 300 × *g*. The supernatant is discarded and the cells suspended in 70% (v/v) ice-cold ethanol. The cells are stored in 70% (v/v)

ethanol at –20°C. At the time of overnight shipping, cryovials are placed in a cryobox and packed with adequate amount of dry ice.

9. Because volume accuracy is not important in the fixation, washing, and rinsing steps, it is easier to use a serological pipette to add the paraformaldehyde, ethanol, wash, and rinse buffers.
10. A special staining solution without the TdT enzyme should also be prepared for the negative semen control. Because the staining solution is only active for approximately 24 h at 4°C after being prepared, mix the reagents together only when required and prepare no more than the amount necessary. The TdT enzyme volume is the only limiting reagent in the kit, as all other reagents are generously supplied. Briefly centrifuge the TdT enzyme tube to make sure that the reagent is at the bottom.
11. All subsequent steps should be conducted in a low-lighted room and the solutions and samples should be covered by a sheet of aluminum foil when exposure to light is expected.
12. The cells should be analyzed as quickly as possible. Prolonged delay >1 h will result in overstaining and degradation of the cells.
13. *CellQuest*™ software is used to adjust the electronics and setup conditions for data acquisition and storage. To optimize the sample, a *CellQuest* template is set up. The forward scatter and side scatter detectors are adjusted, the forward scatter threshold is adjusted, and the population is gated. The FL1 and FL2 channels are adjusted accordingly. Under the '*Acquire*' menu, select '*Connect to cytometer*'. Again under the '*Acquire'* menu, select '*Parameter Description*'. Here you can define your file storage by selecting the storage location. From the '*Parameter Description*', select the folder. Open your assigned folder, create a new folder (enter name of the folder, date created), and select the newly created folder. From the '*Parameter Description*' select '*File*' and under '*File name prefix*' (custom prefix) enter an appropriate file name. Change 'File count' to 1. Click 'OK' under '*Sample ID*' and '*Patient ID*' insert tube descriptions. Under '*Cytometer*' menu, select '*Instrument Setting*', open the dialogue box and in your folder go to '*Kit*' setting, and hit '*set*' and '*done*'. Now the instrument setting is in place for the assay kit. Go to '*Detectors/Amps*' and only the settings for P4 (FL2 channel) should be changed. Click '*Threshold*'. The threshold for '*FL2-H*' should be fixed at 40 and should not be changed. The first time that the assay kit is run, the settings must be saved. This is saved in a separate folder named "*KIT*". For actual test samples, in the '*Instrument*

setting', open the dialogue box, double click on the 'TUNEL' folder, and then click on '*set*' and '*Done'*. Set the '*FSC-H*' threshold. The threshold for '*FSC-H*' should be fixed at 52 and should not be changed. Similarly, when running the test sample for the first time, once the settings are optimized they can be saved into a folder named "*TUNEL*". When ready to run the sample, change the instrument knob to '*RUN*'. The default is in '*SETUP*' mode where you can preview your cells, adjust instrument setting, and create or modify gates without collecting data. To collect gated data, while in the '*SETUP*', create a gate using the toolbar (Creating Gate/Regions). Under the '*Acquire'* menu, select '*Acquisition & Storage*'. Again select '*Counters*'. This allows monitoring the sample flow rate and event count (see Fig. 2). When ready to collect cells, deselect the '*SETUP*' check box on '*Acquisition Controls*'. A data file will generate when the signal sounds or you can manually save. Once the collecting event is begun, do not alter instrument settings or gates. When finished acquiring the data, exit *CellQuest* and copy/back up your data files to a flash drive.

14. Raw data from the flow cytometer is imported into the *FlowJo* software (Mac version 8.2.4, FlowJo, LLC, Ashland, OR). The '*Workspace*' window is composed of '*Group panel*' and '*Sample*' panel. Transfer the folder into '*Group panel*'. Create subgroups for "*Kit/control*" samples and "*TUNEL/test*" samples. Drag the kit (controls) sample data to the '*Kit*' group and the actual '*Test*' samples to the "*TUNEL*" folder. Next open the sample of interest by double clicking on the sample panel and click on the sample of interest. The *FlowJo* opens a graph window displaying a bivariate dot plot of the cells. The initial graph the *Flow Jo* displays is always a '*Forward vs. Side Scatter plot'* because this view is generally used to gate out dead cells. Select the '*Dot plot*' format. The next step is to create '*Gates*'. To create a polygon gate, click the mouse and add the polygon gate. Once you have closed the gate, give the file name "*Singlet*" to this subpopulation of cells. Next Double click on "*Singlet*" and open a new graph. Display the *X*-axis options by clicking on the axis label and choose "*FL-W*" from the pull-down menu. Click on the *Y*-axis label and select "*FL2-A*" (PI channel). To see the gated area without the gate, double click on the plot. Next click on the *X*-axis and choose "*FL1-H*" (FITC Green channel) and on the *Y*-axis, click "*Histogram*" from the pull-down menu. Create the '*Negative*' gate and give the file name "*Negative*". Similarly, create the '*Positive*' gate and give the file name "*Positive*". Apply all analysis nodes (gated subpopulations) to other samples. Gating parameters are applied to every sample simultaneously by dragging the gating tree onto the "Kit" and "TUNEL" subgroups. To do this, highlight

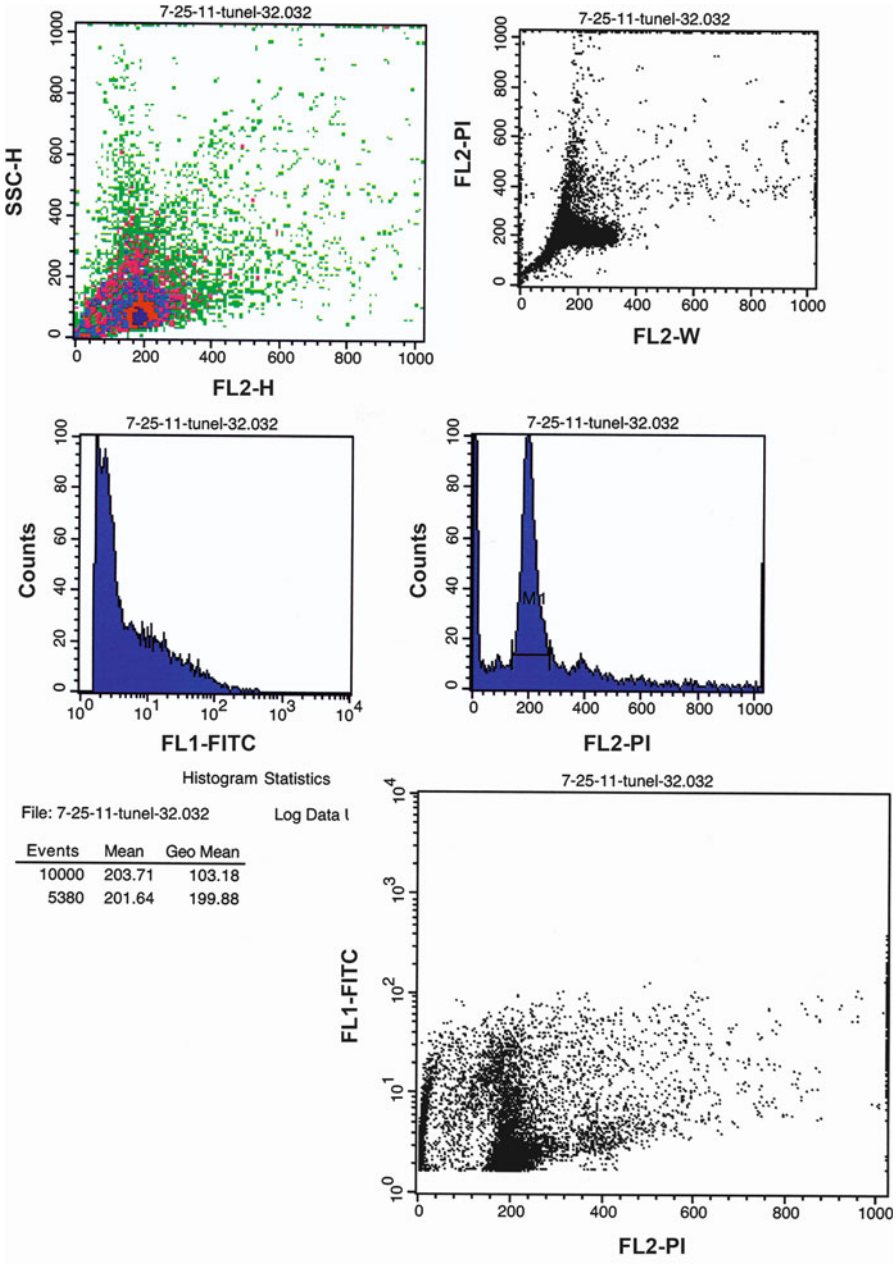

Fig. 2. Representative setup of the data acquisition for a single sample by FACScan before it is analyzed by FlowJo software. FL1 = Green channel; FL2 = Red channel; PI = Propidium iodide; FITC = Fluorescence isothiocynate; SSC = Side scatter count.

the "Singlet", "Negative", and "Positive" subfolders and drag-and-drop to the "KIT" group. Similarly apply the same to the 'TUNEL' group. Note: If the gate needs to be changed/adjusted after the desired gate has been obtained, highlight the "Singlet", "Positive", and "Negative" subfolders and press the

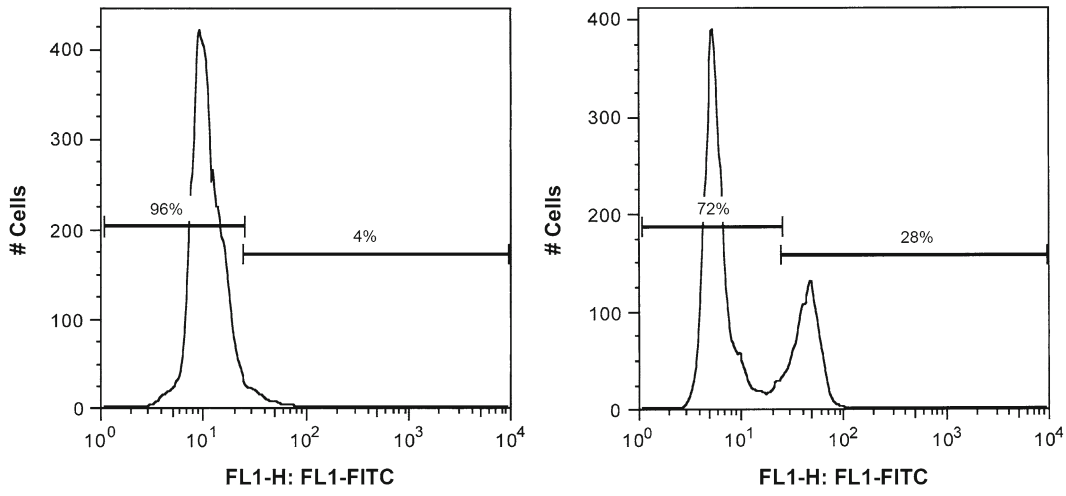

Fig. 3. Representative histogram showing (**a**) TUNEL-negative and (**b**) TUNEL-positive sample.

'Shift' and 'Delete' keys. Redraw the gates and save again. Always save the Workspace frequently.

To prepare graphical reports and generate tables, open the '*Layout Editor*' window by clicking the rightmost function in the Workspace window. The left panel shows the names of the layouts and the right panel is a drawing board. In the drawing board, a graphical display for a single sample can be created. *FlowJo* is able to create a batch report. To do this, click on "*Singlet*" from either the "*Kit*" or the "*TUNEL*" subgroups and drag it to the drawing board. Double click on 'batch' at the upper corner of the 'Batch Report'. Place the report panel according to the number of plots in each row or column (each block represents one printed page). Drag and adjust and print the report accordingly. A representative curve for a sample that is negative and positive for DNA damage is illustrated in Fig. 3. Similarly a table can also be printed with the data statistics.

15. The DNA fragmentation threshold of 19.25% was determined to be the cutoff value which maximized the sum of sensitivity and specificity by receiver operating characteristic (ROC) curve analysis in a recent 2010 study of 194 infertile and 25 healthy male subjects using an identical protocol (22). At this threshold value the study had 100% specificity, 64.9% sensitivity, 97.7% positive and 37% negative predictive values, and an accuracy of 70%. This is very close to the 20% threshold calculated by ROC curve analysis in a 2005 study using a different protocol (yielding 89.4% specificity, 96.9% sensitivity, and 92.8% positive and 95.5% negative predictive values). Several studies have shown a threshold/cutoff range of DNA damage of about 10–40% (23–26).

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