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Telomere Length Measurement by Fluorescence In Situ Hybridization and Flow Cytometry: Tips and Pitfalls

Gabriela M. Baerlocher,[1] Jennifer Mak,[1] Teri Tien,[1] and Peter M. Lansdorp[1,2]*

[1]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada
[2]Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

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Background: Telomeres containing noncoding DNA repeats at the end of the chromosomes are essential for chromosomal stability and are implicated in regulating the replication and senescence of cells. The gradual loss of telomere repeats in cells has been linked to aging and tumor development and methods to measure telomere length are of increasing interest. At least three methods for measuring the length of telomere repeats have been described: Southern blot analysis and quantitative fluorescence in situ hybridization using either digital fluorescence microscopy (Q-FISH) or flow cytometry (flow-FISH). Both Southern blot analysis and Q-FISH have specific limitations and are time-consuming, whereas the flow-FISH technique requires relatively few cells (10^5) and can be completed in a single day. A further advantage of the flow-FISH method is that data on the telomere length from individual cells and subsets of cells (lymphocytes and granulocytes) can be acquired from the same sample. In order to obtain accurate and reproducible results using the flow-FISH technique, we systematically explored the influence of various steps in the protocol on telomere length values and established an acceptable range for the most critical parameters.

Methods: Isolated leukocytes from whole blood are denatured by heat and 70%/75% formamide, then hybridized with or without a telomere-specific fluorescein isothiocyanate (FITC)-conjugated peptide nucleic acid probe (PNA). Unbound telomere PNA is washed away, the DNA is counterstained, and telomere fluorescence is measured on a flow cytometer using an argon ion laser (488 nm) to excite FITC. For each sample, duplicates of telomere PNA-stained and unstained tubes are analyzed.

Results: Cell counts and flow-FISH telomere length measurements were performed on leukocytes and thymocytes of humans and other species. Leukocyte suspensions were prepared by two red blood cell lysis steps with ammonium chloride. Optimal denaturation of DNA was achieved by heating at 85–87°C for 15 min in a solution containing 70%/75% formamide. Hybridization was performed at room temperature with a 0.3 μg/ml telomere-PNA probe for at least 60–90 min. Unbound telomere-PNA probe was diluted at least 4,000–40,000 times with wash steps containing 70%/75% formamide at room temperature. LDS 751 and DAPI were suitable as DNA counterstains as they did not show significant interference with telomere length measurement.

Conclusions: The use of flow-FISH for telomere length measurements in nucleated blood cells requires tight adherence to an optimized protocol. The method described here can be used to determine rapidly the telomere length in subsets of nucleated blood cells. Cytometry 47:89–99, 2002. © 2002 Wiley-Liss, Inc.

Key terms: fluorescence in situ hybridization; flow-FISH; telomeres; telomere length; peptide nucleic acid probes; flow cytometry; DNA staining

Telomeres in all vertebrate cells consist of noncoding G-rich hexanucleotide $(TTAGGG)_n$ repeats and associated proteins (1). Over the past several years, the function and length regulation of telomere repeats have become of major interest in cell biology. The linkage between telomere shortening and replicative senescence of cells and the decrease of telomere length with age first suggested a significant role of telomeres (2–4). The subsequent demonstration that telomerase can maintain or elongate telomeres in cells and immortalize cells with a normal karyotype (5,6) and the evidence of an involvement of this mechanism in tumorigenesis (7) and reproduction (8) have increased greatly the interest in the telomere field. Many studies have contributed to elucidate structural features of telomeres (9,10), telomere-associated proteins, and their interactions (11–13). However, many questions related to the role of telomeres in cell biology and the

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*Correspondence to: Dr. Peter M. Lansdorp, Terry Fox Laboratory, 601 West 10th Avenue, Vancouver, BC, V5Z 1L3 Canada.
E-mail: plansdor@bccancer.bc.ca

mechanisms involved in telomere length regulation remain to be answered.

The length of telomeres in a given cell at a given point in time is influenced by multiple factors. Telomeres are lost because DNA polymerases can only act in the 5′ to 3′ direction and are unable to replicate completely the 3′ end of chromosomes, resulting in a loss of terminal nucleotides with each cell division (14–17). Telomerase, a ribonucleoprotein enzyme with reverse transcriptase activity, counteracts the end replication problem by synthesizing new telomeric repeats onto the 3′ end of telomeres (18,19). Other factors involved in telomere length regulation, such as the processing of chromosome ends following replication (20) and telomerase-independent pathways of telomere maintenance (21,22), are less well defined. The exploration of telomere biology and telomere length dynamics clearly depends on the methods that are available to measure the length of telomere repeats. So far, at least three techniques have been described for telomere length measurement: Southern blot analysis, Q-FISH (23), and flow-FISH (24).

Our laboratory developed the flow-FISH technique (24) in which a fluorescein isothiocyanate (FITC)-labeled telomere-specific peptide nucleic acid (PNA) probe is hybridized in a quantitative way to telomere repeats, followed by telomere fluorescence measurements on individual cells by flow cytometry. Compared with the telomere length measurement by Southern blot analysis, flow-FISH offers the advantages of looking at telomere fluorescence in different subpopulations of cells (lymphocytes and granulocytes) in the same sample and many samples with relatively low cell counts (10^5) can be processed at the same time. Flow-FISH is less time-consuming compared with Q-FISH, where telomere length measurements are performed on individual metaphase chromosomes. Studies using the flow-FISH method to measure the telomere length in human lymphocyte subpopulations (25), in myeloid cells, and in granulocytes (26) have demonstrated the usefulness and power of this technique. However, as with every new method, modifications for improvements in accuracy and reproducibility need to be explored and are necessary to ensure high-quality measurements. Although such work on method development can be tedious and unrewarding, it is sometimes of fundamental importance to advance science in a field.

We describe relevant parameters for each of the six basic steps in the flow-FISH protocol and we outline their influence on telomere length measurements. We also point out where possible pitfalls can arise and we provide recommendations for a standardized protocol for the flow-FISH technique. The results obtained with this protocol are reliable and accurate and should facilitate further studies of telomere biology.

MATERIALS AND METHODS

Cell Separation/Preparation

Blood from healthy human volunteer donors or animals was collected in sodium heparin Vacutainer tubes (Becton Dickinson, Franklin Lakes, NY) or in citrate/EDTA tubes. All collection and research protocols were approved by local ethical and animal care review boards. The blood samples were kept at room temperature (RT) and processed within 24–48 h. Single-cell suspensions of cow thymocytes were prepared by straining/sieving small pieces of thymus and resuspending the collected cells in tissue culture medium (Dulbecco's minimum essential medium [DMEM] catalogue number 36250, StemCell Technologies, Vancouver, Canada) with 0.1 μg/ml Dnase (catalogue number D-4513, Sigma-Aldrich Canada, Oakville, Ontario, Canada). White blood cells (WBCs) were obtained by osmotic lysis of red blood cells (RBCs) with NH_4Cl (catalogue number 07850, StemCell Technologies). However, we also explored removal of red cells by lysis in water, in 3% acetic acid in water (adding 4–100× the volume of the blood sample), by adding the lytic reagent Zap-oglobin II (Beckman Coulter, Fullterton, CA) or by magnetic selection of CD45+ cells (27). For the studies of purified granulocyte and lymphocyte cell suspensions, mononuclear cells were isolated by removing the mononuclear layer after centrifugation of whole blood over Ficoll-Hypaque (catalogue number 17-1440-03, Amersham Pharmacia Biotech, Quebec, Canada) and granulocytes were obtained by subjecting the pellet to an RBC lysis with NH_4Cl. Purified WBCs, granulocyte or lymphocyte cell suspensions, or isolated cow thymocytes were washed either with phosphate-buffered saline (PBS; catalogue number 37350, StemCell Technologies) containing 0.1% bovine serum albumin (BSA; catalogue number 126609, Calbiochem-Novabiochem, San Diego, CA) or with Dextrose 5% in water, pH 4.0, 252 mOsmol/l (catalogue number JB0064, Baxter, Toronto, Ontario, Canada) with 10 mM HEPES (catalogue number H-9897, Sigma-Aldrich) and 0.1% BSA. Cells were either counted in a Neubauer chamber or in a Cell Counter (Beckman Coulter).

In Situ Hybridization

Cells were denatured and hybridized in 1.5-ml V-bottom tubes (catalogue number L-510N, Rose Scientific, Edmonton, Alberta, Canada). Unless indicated otherwise, 3×10^5 cells were resuspended in 300 μl of hybridization mixture containing 70% deionized formamide (catalogue number B 10326-80, BDH, Toronto, Ontario, Canada), 20 mM Tris, pH 7.1, 1% (w/v) BSA or 75% deionized formamide, 20 mM Tris, pH 7.1, 1% BSA, and 20 mM NaCl with either no probe (unstained control) or with a 0.3 μg/ml telomere-specific FITC-conjugated $(C_3TA_2)_3$ PNA probe (kindly provided by Boston Probes, Bedford, MA) for stained samples. DNA denaturation at indicated temperatures and at indicated time intervals was either done in a Thermomixer 5436 (Eppendorf, Netheler, Germany) or in a circulating waterbath (WB; catalogue number 1120A-1, Lindberg/Blue, Ashville, NC). The telomere-PNA probe was, if not otherwise indicated, hybridized for 60–90 min at RT in the dark. To remove excess and nonspecifically bound telomere-PNA probe, washes were performed either at RT with a wash solution containing 70%/75% formamide, 10 mM Tris, 0.1% BSA, and 0.1% Tween 20 (catalogue num-

ber R06435, BDH) or at different temperatures with a wash solution containing PBS, 0.1% BSA, and 0.1% Tween 20. The last wash step for either wash protocol was performed at RT and with a wash solution containing PBS or dextrose 5%/HEPES 10 mM. After each wash step, cells were spun down in a Beckman TJ-6 centrifuge at 16°C for 7 min at 1,500*g* for the formamide washes and at 900*g* for the PBS washes.

DNA Counterstains

After the last wash step, cells were resuspended in a solution containing PBS or FACSFlow (catalogue number 342003, Becton Dickinson), 0.1% BSA, RNAse A at 10 μg/ml (catalogue number 109 207, Boehringer Mannheim, Laval, CA), and the DNA counterstain. Concentrations of DNA dyes used were 0.03–0.3 μg/ml for propidium iodide (PI; p-5264; Sigma-Aldrich), 0.05–0.5 μg/ml for LDS 751 (Exciton Chemical, Dayton, OH), 0.01–1 mM for Syto Orange Dyes (Molecular Probes, Eugene, OR), 0.2–10 mM for Syto Red Dyes (Molecular Probes), 2–100 μg/ml for 7-AAD (Molecular Probes), 3–30 μM for Hoechst H 33342 (Molecular Probes), 0.01–1 μg/ml for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes), and 0.1–1 μM for DRAQ5 (28). Cells were incubated with the DNA solution for at least 20 min prior to analysis.

Flow Cytometric Analysis

The acquisition of the telomere fluorescence for all the samples was performed on a FACSCalibur (Becton Dickinson). The 488-nm argon ion laser was used to excite the FITC-labeled telomere probe and telomere fluorescence was measured in the FL1 channel. A mixture of four populations of FITC-labeled beads (catalogue number 824p-C, Flow Cytometry Standards, San Juan, Puerto Rico), each having a fluorescence corresponding to different known amounts of molecular equivalents of soluble fluorochrome (MESF), was run in each experiment. The mean fluorescence for each of the four different fluorescence peaks was used to establish a calibration curve and to convert telomere fluorescence values into MESF values, allowing comparison among experiments. The analysis of telomere fluorescence by gating on single cells, excluding doublets, and gating on the lymphocyte or granulocyte population was either done using Cell Quest (Becton Dickinson) or WinMDI Version 2.8 (Windows Multiple Document Interface Flow Cytometry Application by Dr. Trotter, La Jolla, CA). For each cell sample, at least duplicate measurements were performed. To correct for the autofluorescence of the cells in FL1, cells hybridized without the FITC-labeled telomere-PNA probe were used. The telomere fluorescence of cells was calculated by subtraction of the (auto) fluorescence of unstained controls from the telomere fluorescence measured in cells hybridized with the FITC-labeled telomere-PNA probe.

RESULTS AND DISCUSSION

The six basic steps of the flow-FISH procedure are outlined in Figure 1. In the following sections, experiments to validate and optimize each step of the flow-FISH procedure are discussed.

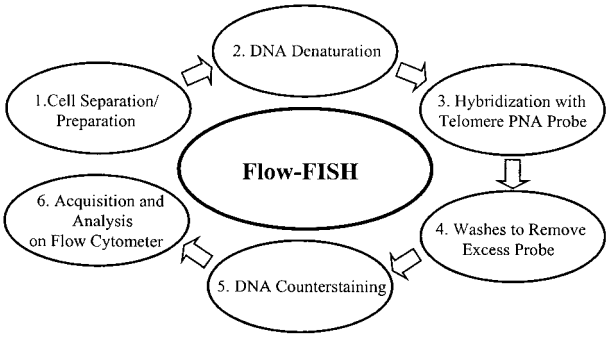

FIG. 1. The six basic steps of the flow-FISH protocol

Purification of WBCs

In order to measure the telomere length in nucleated cells from peripheral blood or bone marrow by flow-FISH, the cells need to be isolated from the bulk of the RBCs. A high concentration of RBCs or hemoglobin (corresponding to a RBC suspension with a hematocrit ≥2%) in the test sample interferes with telomere fluorescence measurements, possibly due to the quenching of the relatively weak FITC fluorescence of the telomere probe by hemoglobin. Unfortunately, there are no good, easy, and fast methods that allow (near) complete RBC lysis without loss or destruction of WBCs or subsets of WBCs. In order to isolate leukocytes from a moderate volume of whole blood (3–7 ml), we tested different RBC lysing reagents (NH_4Cl, water, 3% acetic acid in water, Zap-oglobin) and magnetic removal of RBCs using StemSep. RBC lysis with NH_4Cl was the fastest way to isolate leukocytes from whole blood with a decent recovery. Over 80% of viable WBCs (by trypan blue exclusion) were recovered after two lysing steps and, on average, only 0.05% of unlysed RBCs (from the total RBC count) were still present. The high permeability of NH_4Cl on the RBC membrane with the consecutive exchange of OH^- or HCO_3^- with external Cl^- ions leads to swelling and hemolysis of RBCs within 6–10 min (29,30) without significant loss of WBCs, or even subsets of WBCs, in contrast to hypoosmotic lysing reagents (31). However, as soon as the equilibrium of this process is reached, the potential of RBC lysis disappears (32). In addition, we observed an exhaustion of RBC lysis with NH_4Cl after two rounds. Lysis was furthermore only effective if the dilution of whole blood to NH_4Cl was at least 20 times and most of the plasma had been removed previously. RBC lysis with water or Zap-oglobin resulted in complete RBClysis. However, it also rapidly destroyed WBCs, which complicates handling of multiple samples at the same time. 3% acetic acid in water was very effective for RBC lysis, but resulted in poor recovery of viable WBCs (≤30% of the initial white cell count). Purification of WBCs from 3–7 ml of whole blood with StemSep (by positive selection, i.e., retention of immunomagnetically

labeled cells in the magnetic column) resulted in a 30–50% recovery of viable WBCs and in the contamination of 0.1–0.2% RBCs. However, this method was much more time-consuming than the methods using RBC lysis.

Interestingly, the loss and fragmentation of cells during subsequent steps in the flow-FISH process were lower if, following NH_4Cl lysis, cells were resuspended in dextrose 5% with 10 mM HEPES and 0.1% BSA rather than in PBS with 0.1% BSA. This might be due to a protective effect of dextrose against hypotonicity or another beneficial effect of the dextrose solution on cell metabolism and viability.

DNA Denaturation

The most common technique for denaturing double-strand DNA into single-strand molecules is the application of heat and formamide (33). Typically, denaturation is performed on isolated DNA, where temperature and denaturation time can be completely estimated on the basis of the DNA structure (34). For the flow-FISH technique, DNA needs to be completely denatured to single strands within the cell and the optimal denaturation temperature and time should not result in disintegration of the cell or its DNA. Fixation of cells by formaldehyde (35) does not solve this dilemma because crosslinking reagents are likely to decrease denaturation efficiency. The optimal time and temperature for denaturation of the DNA in fixed metaphase chromosomes on slides following pepsin digestion to remove proteins was 3 min at 80°C for in situ hybridization experiments with fluorescent telomere-PNA probes (36). At higher temperatures or longer time periods, the fluorescence signals decreased due to DNA loss, whereas the fluorescence signal was compromised at lower temperatures or shorter time periods. Figure 2 shows the effect of heat and formamide treatment on the light scatter properties of previously unfixed nucleated blood cells. Three different cell populations (lymphocytes, monocytes, and granulocytes) can be distinguished prior to treatment. After heat and formamide treatment, cells change their light scatter properties and the forward scatter (FSC) versus side scatter (SSC) plot shows only two distinguishable cell populations. These are lymphocytes with a lower FSC and orthogonal scattering and granulocytes with a higher FSC and orthogonal scattering; monocytes most likely fall in the region of the granulocyte population (results based on studies done with purified cell suspensions; data not shown). The plots in Figure 3A show the relationship between telomere fluorescence and the denaturation temperature and time used for human WBCs and cow thymocytes. At higher temperatures, peak values for telomere fluorescence were reached within a shorter time period. Furthermore, the telomere fluorescence values following these peak values in each plot decreased more rapidly with increasing temperatures, most likely reflecting cell and DNA loss with higher temperatures and longer time periods of denaturation. Between 85 and 87°C, the values tended to reach a plateau around 15 min for human WBCs, as well as for cow thymocytes. Similar findings were also seen with leukocytes from different species with marked differences in

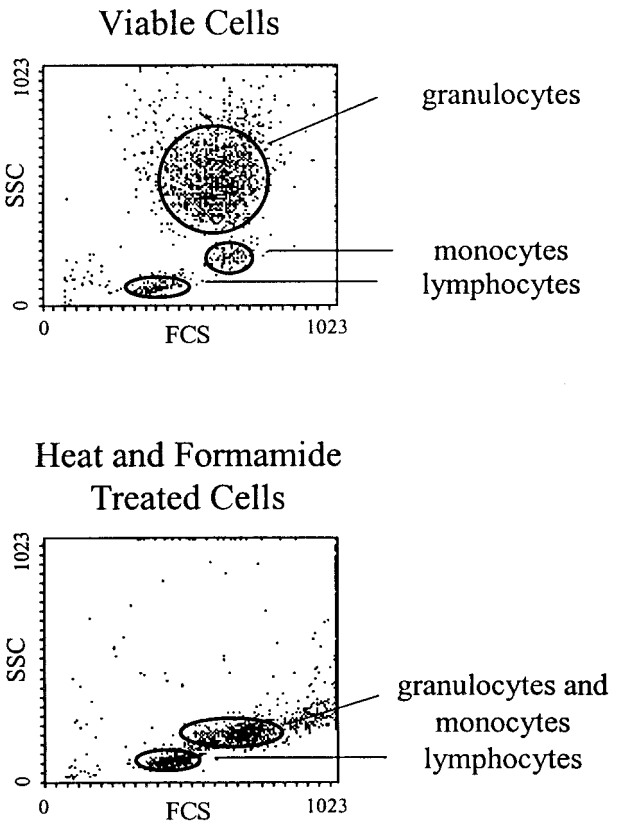

FIG. 2. Influence of heat and formamide (70%) treatment on flow cytometric properties of human leukocytes. Dot plots were acquired with the same settings on the flow cytometer. Using viable human leukocytes (top graph), three subpopulations of cells (granulocytes, monocytes, and lymphocytes) can be distinguished based on FSC and orthogonal light scatter. After heat and formamide treatment, human leukocytes mainly change their orthogonal light scatter and only two subpopulations corresponding to granulocytes including monocytes and lymphocytes can be distinguished (bottom graph).

telomere length (mouse, rabbit, sheep, and pig) as shown in Figure 3B. To explore differences in the denaturation between different cell types, we analyzed the telomere fluorescence in purified human lymphocytes and granulocytes. The variation in telomere fluorescence values with variation of temperatures at a fixed denaturation time was lower for lymphocytes than for granulocytes (Fig. 3C). Granulocytes needed a slightly higher temperature (87°C) to reach the highest fluorescence values (at 15 min) whereas lymphocytes seemed to reach their highest telomere fluorescence value already between 85 and 87°C. This could be due to the condensed DNA in the nucleus of polymorphic granulocytes (37). Above a temperature of 89°C, telomere fluorescence values and the recovery of purified granulocytes or lymphocytes decreased, most likely reflecting disintegration of cells and DNA loss. These results indicate that relative to fixed chromosomes on a slide, higher temperatures and longer time periods are required to denature the DNA within previously unfixed cells in suspension, in agreement with a previous report (38). Importantly, we found that the optimal time

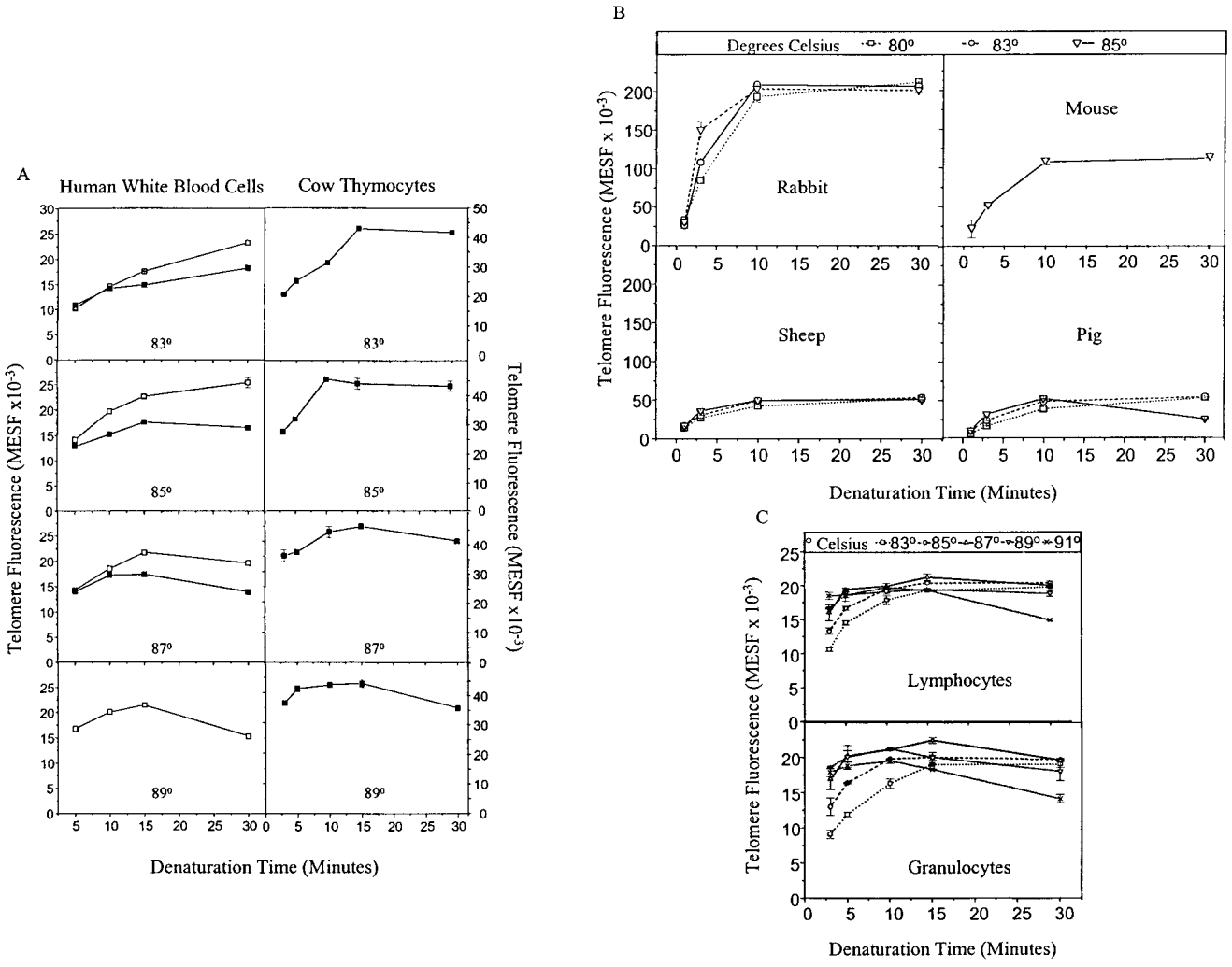

FIG. 3. Influence of denaturation time (x-axis) and denaturation temperature on flow-FISH telomere fluorescence measurements (y-axis) performed with leukocytes of humans, rabbits, mice, sheep, and pigs, and with cow thymocytes. **A:** The left column represents graphs for human WBCs where telomere fluorescence measurements were performed for two individuals (open and closed boxes; mean and SD of triplicate measurements). The right column represents graphs for cow thymocytes (mean and SD of duplicate measurements). Graphs from the top to the bottom are with increasing denaturation temperatures (83–89°C). **B:** Different graphs showing the mean and SD of duplicate measurements of telomere fluorescence in WBCs of four species (rabbits, mice, sheep, pigs). The different temperatures are indicated with different symbols and lines. **C:** The top graph represents telomere fluorescence measurements (mean and SD of duplicates) in purified lymphocytes and the bottom graph represents purified granulocytes. The different temperatures are indicated as different symbols and lines.

period for denaturation also depends on the heating device, the tubes, and the volume of hybridization solution. Briefly, approximately 3–4 min are needed to reach the desired temperature of denaturation (87°C) in 300 μl hybridization solution in 1.5-ml V-bottom tubes on a heating block. Based on our results, another 11–12 min of denaturation appear to be necessary to fully denature telomeres before losses of cells and DNA occur. In our hands an optimal balance between denaturation of telomeric DNA and loss of leukocytes was reached by treatment for 15 min at a temperature between 85 and 87°C. We observed a greater variability in temperature in tubes at different positions in a heating block (mostly lower temperature values on outside positions) compared with the variation in temperature seen in tubes placed in a circulating WB (data not shown). Furthermore, we tested the effect of the concentration of formamide used for the denaturation and hybridization on the light scatter properties and telomere fluorescence values of cells as shown in Figure 4A,B. We observed that with low formamide concentrations, high values for the FSC and orthogonal light scatter properties were obtained as well as high autofluorescence values (FL1) for granulocytes. In contrast, lymphocytes displayed no change in autofluorescence over the range of 50–80% formamide. Surprisingly, the raw telomere fluorescence values (without subtraction of autofluorescence) of lymphocytes and granulocytes appeared to be unaffected by the variation in formamide concentration in the hybridization mixture. The higher autofluorescence of granulocytes with lower form-

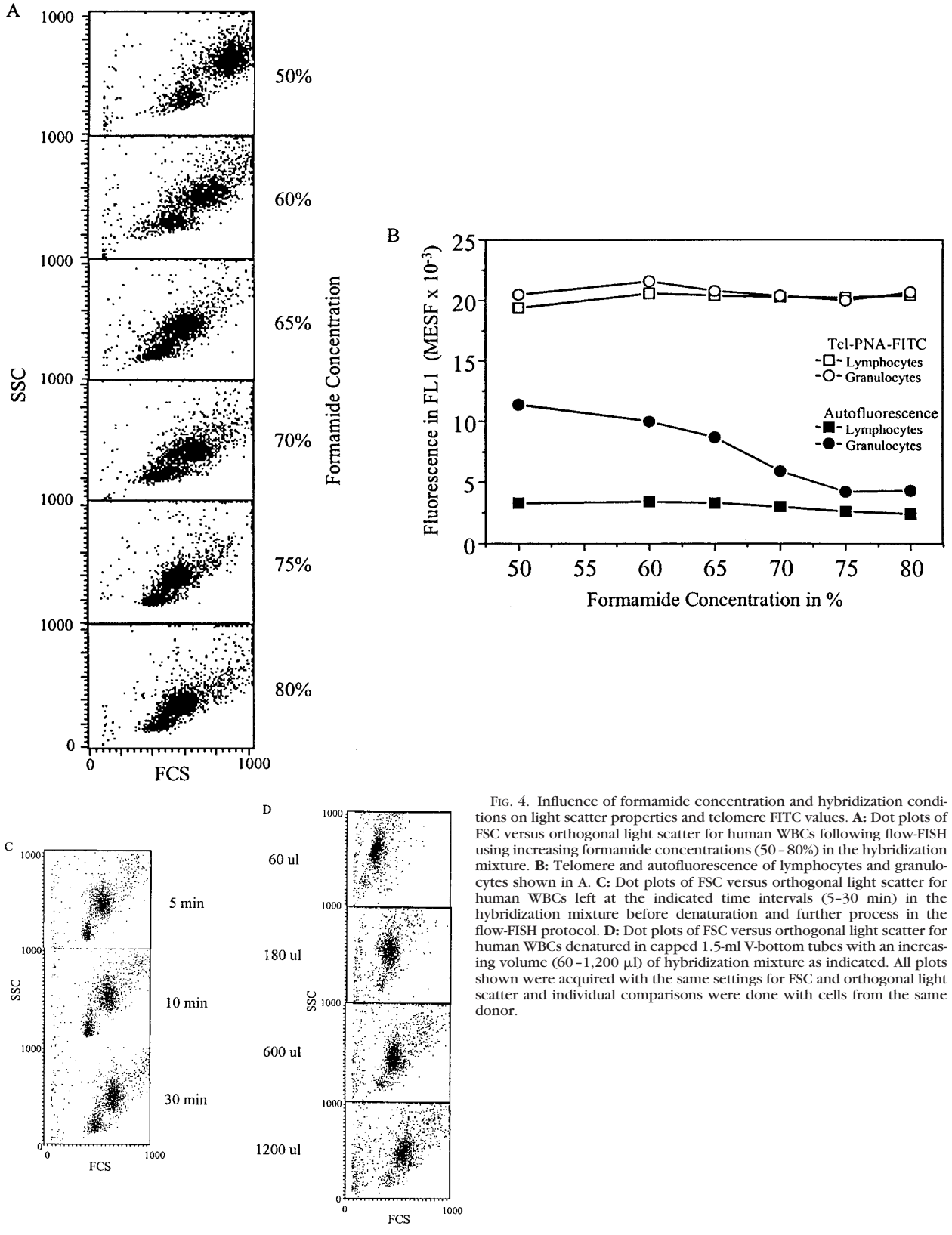

FIG. 4. Influence of formamide concentration and hybridization conditions on light scatter properties and telomere FITC values. **A:** Dot plots of FSC versus orthogonal light scatter for human WBCs following flow-FISH using increasing formamide concentrations (50–80%) in the hybridization mixture. **B:** Telomere and autofluorescence of lymphocytes and granulocytes shown in A. **C:** Dot plots of FSC versus orthogonal light scatter for human WBCs left at the indicated time intervals (5–30 min) in the hybridization mixture before denaturation and further process in the flow-FISH protocol. **D:** Dot plots of FSC versus orthogonal light scatter for human WBCs denatured in capped 1.5-ml V-bottom tubes with an increasing volume (60–1,200 μl) of hybridization mixture as indicated. All plots shown were acquired with the same settings for FSC and orthogonal light scatter and individual comparisons were done with cells from the same donor.

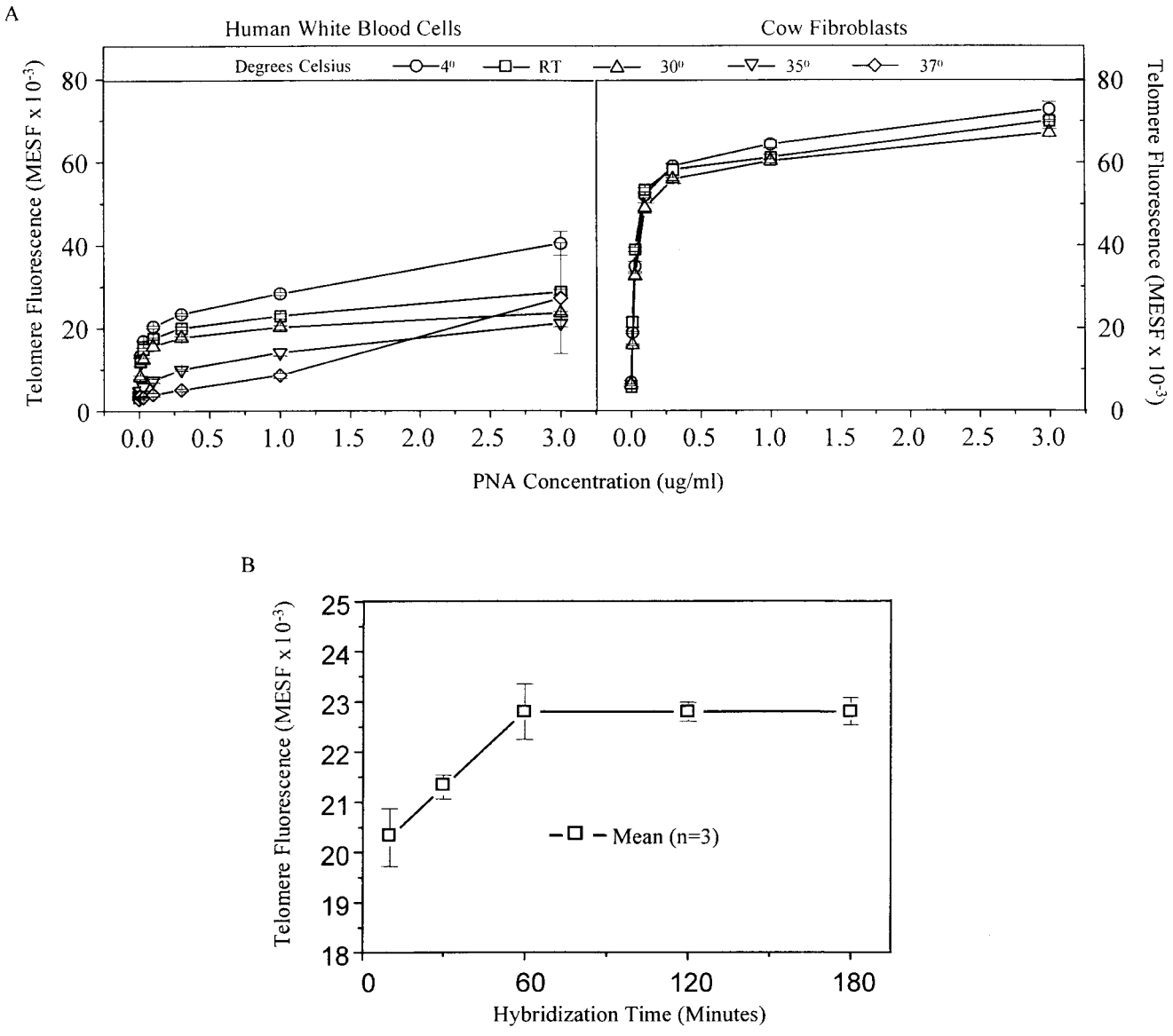

FIG. 5. **A:** Influence of the telomere-PNA concentration (0–3.0 μg/ml; x-axis) and the hybridization temperature (4–37°C) on the telomere fluorescence (y-axis; mean and SD of duplicate measurements) measured by flow-FISH in human WBCs (left graph) or in cow fibrobasts (right graph). The lines with different symbols represent the different temperatures for hybridization. **B:** Effect of the hybridization time (0–180 min; x-axis) on the telomere fluorescence (y-axis) shown for WBCs of a representative individual (mean and SD of triplicate measurements)

amide concentrations, therefore, leads to lower values for the specific telomere fluorescence (telomere fluorescence minus autofluorescence). Most likely this autofluorescence results from fluorescent molecules in granulocytes (39), which seem capable of quenching some of the telomere fluorescence. In addition, the length of time cells were exposed to the hybridization solution (containing formamide) before the denaturation process also influenced the FSC and orthogonal scatter properties of the cells. Cells increased in volume upon prolonged exposure to the hybridization mixture before heat treatment (Fig. 4C). This swelling of cells in the hybridization mixture was more pronounced for cells that were resuspended previously in glucose 5%/10 mM HEPES/BSA 0.1% than for cells that were resuspended previously in PBS/BSA 0.1%. A similar swelling of cells in the hybridization mixture was observed when 20 mM sodium chloride (NaCl) was added to the hybridization mixture for the cells that were resuspended previously in glucose 5%/10 mM HEPES/BSA 0.1%. This addition had no effect on telomere length measurements. Therefore, in order to achieve reproducible scatter plots and reproducible telomere length measurements, the time period in the hybridization mixture before denaturation was kept constant at 10 min and 20 mM NaCl was added to the hybridization mixture for cells that were resuspended in glucose 5%/10 mM HEPES/BSA 0.1% after

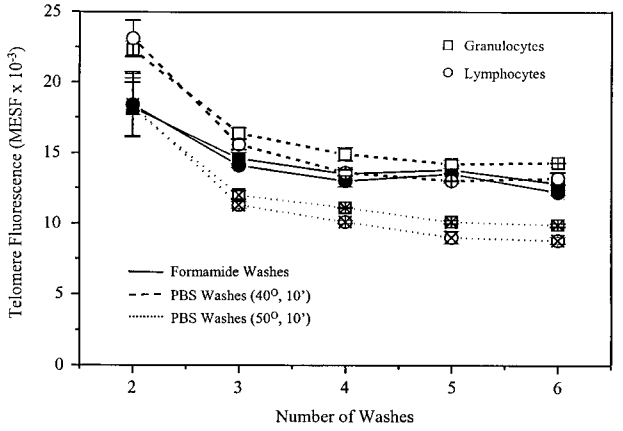

FIG. 6. Influence of the number of wash steps (x-axis) and the type of wash solution (indicated by different lines) on the telomere fluorescence (y-axis) measured by flow-FISH for granulocytes (squares) or lymphocytes (circles) from a normal donor (mean and SD of duplicate measurements).

NH_4Cl lysis. Similarly, we found that the ratio of the hybridization mixture to the air volume in the tube affected the FSC and orthogonal scatter of cells. Most likely, the volume of air space present in tubes above the cell suspension determines the degree of water evaporation from the hybridization mixture, resulting in a decreased water content of the hybridization mixture. This could explain the shrinkage of cells, which were denatured in a tube with a higher air-to-liquid ratio shown in Figure 4D.

Hybridization of the Telomere-PNA Probe

Quantitative hybridization became feasible with the use of synthetic single-strand PNA telomere probes and from findings that such PNA probes maintain a high specificity and affinity for telomere repeats at low salt concentrations that do not favor the renaturation of the complementary DNA strand (40). A low salt concentration might represent a problem when using whole cells. The lowest possible

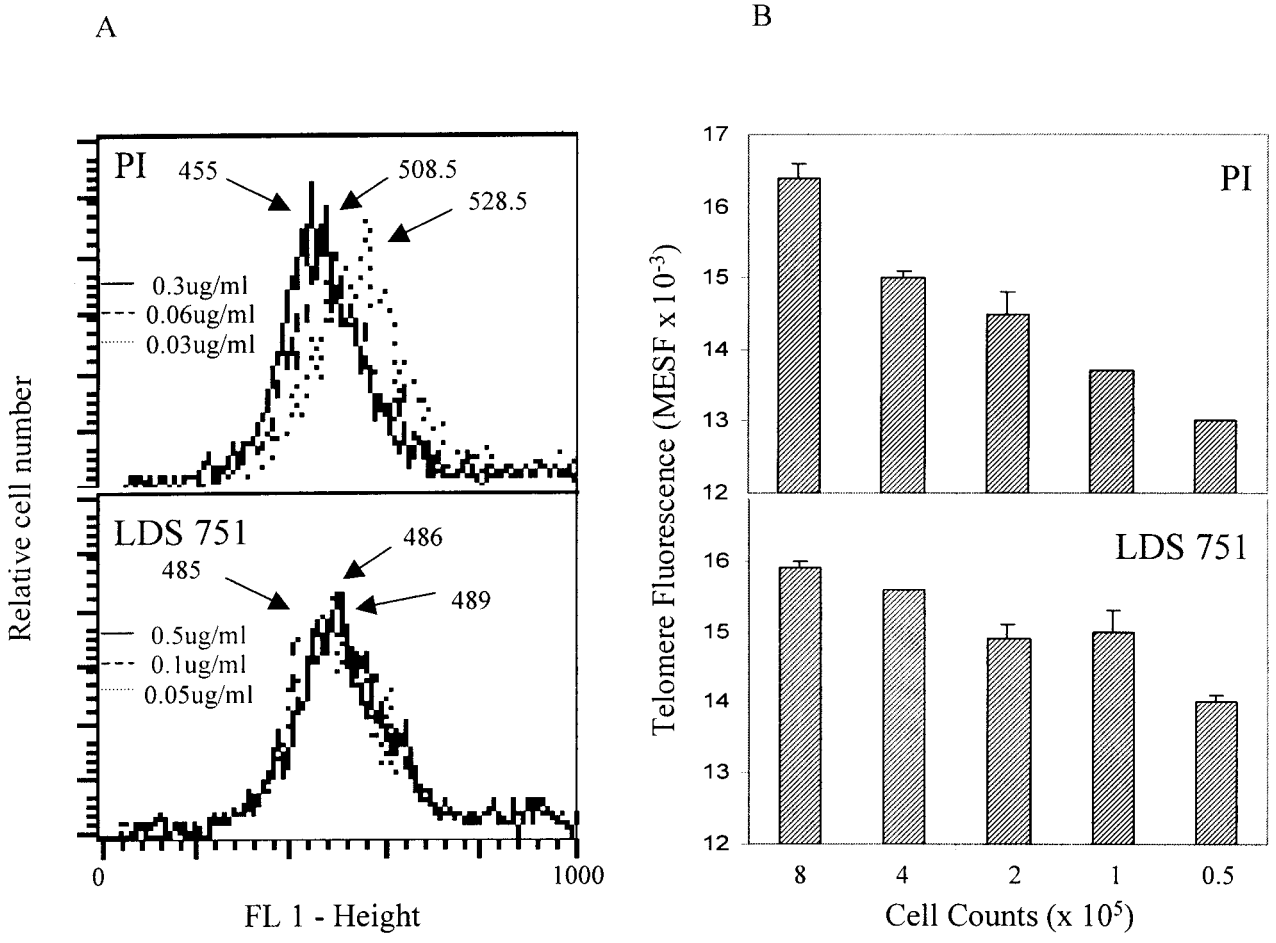

FIG. 7. Influence of the DNA counterstain (PI or LDS 751) on the telomere fluorescence measured by flow-FISH in the FL1 channel. **A:** Histograms of telomere fluorescence. The top histogram shows telomere fluorescence peaks (numbers correspond to peak values) for human WBCs counterstained with PI at different concentrations (0.03–0.3 μg/ml) represented as different types of lines. The bottom histogram shows telomere fluorescence peaks (numbers correspond to peak values) for cells counterstained with LDS 751 at different concentrations (0.05–0.5 μg/ml) represented as different types of lines. **B:** Telomere fluorescence values (y-axis) of human WBCs (mean and SD of duplicate measurements) that were suspended at different cell concentrations (x-axis; 8×10^5–0.5×10^5 cells) per tube prior to flow-FISH and either counterstained with PI (0.06 μg/ml, top graph) or with LDS 751 (0.1 μg/ml, bottom graph).

Table 1
*Recommended Protocol for Telomere Length Measurement in Peripheral Blood Leucocytes by Flow-FISH**

Steps of the protocol	Recommended parameters
Purification/resuspension of WBC	Removal of plasma; 2 × lysis of RBC with NH_4Cl for 10 min on ice with a blood:NH_4Cl dilution at least 1:20; resuspension of WBC following NH_4Cl lysis in glucose 5%/HEPES 10 mM/BSA 0.1%.
DNA denaturation	Resuspension of cells in 300 μl hybmix in a 1.5-ml V-bottom tube for 10 min followed by denaturation at 87°C for 15 min in a circulating waterbath.
Telomere PNA hybridization	Hybridization with 0.3 μg/ml telomere PNA probe at RT and in the dark for 60–90 min.
Washes	Wash I performed at RT and up to a 1:4,000–1:40,000 dilution of the telomere PNA probe; centrifugation of cells at 1,500*g* at RT for 5 min. Wash II performed at RT with an additional dilution of 1:10; centrifugation of cells at 900*g* at RT for 5 min.
DNA counterstaining	Resuspension of cells in LDS 751 at a concentration of 0.01 μg/ml for at least 20 min before acquisition on the flow cytometer.
Acquisition/analysis on flow cytometer	Run beads at the beginning and end of each experiment and take the mean of the slopes to convert fluorescence values into kMESF.

*Hybmix: hybridization mixture containing 75% formamide, 20 mM Tris, 1% BSA, 0.3 μg/ml telomere PNA probe, 20 mM sodium chloride, water. Wash I: 75% formamide, 10 mM Tris, 1% BSA, 1% Tween 20, water. Wash II: Glucose 5%, 10 mM HEPES, 1% BSA, 1% Tween 20.

ionic strength that cells could withstand was 10–20 mM Tris in the hybridization solution containing 70/75% formamide, 1% BSA, 0.3 μg/ml PNA probe, and water. Interestingly, the heat and formamide treatment allows the PNA probes, which are present during the denaturation step, to get into the cell without prior permeabilization of the cells. Hybridization of the telomere-PNA probes to the telomeres, however, only starts (in the presence of 70/75% formamide) below a temperature of 37°C (Figure 5A) and requires between 60 and 90 min to reach a plateau (Fig. 5B). Optimal hybridization was reached with a concentration of 0.3 μg/ml telomere-PNA probe, which represents an x-fold excess (approximately 1,000× for human, 500× for cow, and 100×-fold for rabbit cells calculated on the basis of a molecular weight of 6,000 for the PNA probe, 0.09 μg PNA probe/300 μl hybridization mixture/tube and 460 kb telomere/human cell, 1,200 kb telomere/cow cell, and 2,200 kb telomere/rabbit cell) relative to the calculated number of target sites available for hybridization in human, cow, and rabbit cells. In both human WBCs and cow fibroblasts (with 2–3 times longer telomeres), telomere fluorescence values reach a plateau at a concentration of 0.3 μg/ml telomere-PNA probe (Fig. 5A). The slight increase of the telomere fluorescence values at higher telomere-PNA probe concentrations in these plots most likely is due to a proportionally lower dilution and higher nonspecific binding of the telomere-PNA probe, because the same volume of wash solution was used in spite of the higher telomere-PNA concentration. This implies that for optimal hybridization, a certain concentration of telomere-PNA probe must be present to allow optimal interaction between these molecules and their molecular targets. Above a concentration of 0.3 μg/ml telomere-PNA probe, the likelihood of nonspecific binding increases, which can only be partially remedied using additional steps (not shown).

Washes of Excess Probe

In order to wash away excess unbound telomere-PNA probe, 1 ml of wash solution (the volume is limited by the tube size of 1.5 ml) was added to 300 μl cell suspension containing 70%/75% formamide for the first step and 1 ml of wash solution was added to 100 μl cell suspension for the wash steps thereafter. The first wash step, therefore, dilutes the excess telomere-PNA probe approximately four times and the wash steps thereafter dilute the excess telomere-PNA probe 10 times. Importantly, washes in formamide solution require a higher gravity force to sediment cells than washes in aqueous solution. With this protocol four to five wash steps (4,000 – 40,000 × dilution of the excess and nonspecific bound telomere-PNA probe) are needed with either wash solution (containing formamide at RT or containing PBS at 40 or 50°C) until the telomere fluorescence values stabilize at a plateau (Fig. 6). Additional wash steps do not decrease the telomere fluorescence values in agreement with a very stable nature of the telomere-PNA/DNA duplexes. Washes with PBS for 10 min at 50°C or higher temperatures lead to lower telomere fluorescence values, most likely because specifically bound telomere-PNA probes can dissociate under these conditions. Unfortunately, wash steps with PBS for 10 min at 35°C or lower temperatures resulted in significantly higher fluorescence values, most likely the hydrophobic PNA probes show more nonspecific binding under aqueous conditions. Fluorescence values were comparable if either four to five PBS washes were performed for 10 min at 40°C or if four to five formamide washes were performed at RT. Due to the lower temperature dependence, we prefer to perform the wash steps with formamide solutions at RT despite the toxicity of formamide and the resulting necessity to work in a fumehood.

DNA Counterstains

PI at a concentration of 1–10 μM is used widely for the distinction of single cells from doublets as well as for the distinction of cells in different phases of the cell cycle (41). Due to its broad emission spectrum between 550 and 750 nm, PI can be detected in FL2, FL3, and to some extent in FL1. The overlap of the PI emission spectrum in different detectors and the interference with FITC measurements can be reduced by using lower concentrations of the dye as well as by compensating for fluorescence signals between different detectors. However, with the combination of PI as a DNA counterstain and FITC bound to telomeres, there is an additional problem besides spectral overlap in that both fluorochromes bind to DNA. As a result, some of the FITC fluorescence is absorbed by PI (Fig. 7A). With LDS 751 at subsaturating concentrations, similar to the ones used for PI, we did not observe such energy transfer. For both counterstains, the logical solution is to reduce the amount of DNA dye. However, a limiting concentration of DNA dye results in a decreased resolution of the DNA profile and an undesirable cell dose effect (Fig. 7B). The lower the cell count, and therefore the DNA content in the suspension, the more DNA counterstain can bind to the DNA (or per cell) and the more energy transfer or quenching between the two fluorochromes can take place to reduce the telomere fluorescence. This effect on FITC telomere fluorescence was much more pronounced with PI than with LDS 751. We tested many additional DNA counterstains (Syto Orange Dyes, Syto Red Dyes, 7-AAD, Hoechst H 33342, DAPI, DRAQ5) for the use of flow-FISH, where telomere fluorescence is measured quantitatively and no spectral overlap/energy transfer or quenching can be tolerated. Unfortunately, all of these dyes except LDS 751 at low concentrations and DAPI interfered in various ways with telomere length measurements.

Flow Cytometric Analysis

The variation of the fluorescence values obtained with the calibration beads that were used to convert the telomere fluorescence measurements into MESF was very low on our flow cytometers (FACSCalibur). Changes in the autofluorescence of cells are taken into account by expressing telomere fluorescence values as the difference between a stained (hybridized with telomere-PNA probe) and an unstained sample of similar cell suspensions.

CONCLUSION

When steps are taken to reduce the loss of cells and DNA and to prevent incomplete denaturation of telomeric DNA and nonspecific binding of the telomere-PNA probe, and when possible interactions of fluorochromes in cells are taken into account, the flow-FISH technique becomes a very accurate and reproducible method. Table 1 summarizes our recommendations for the most important steps in the flow-FISH protocol. The protocol has the potential to become an indispensable tool for the measurement of telomere length, especially where experiments are designed with many samples collected at the same time or over a period of time and telomere length differences from sample to sample are expected to be small, as well as where telomere length differences of different subtypes of cells are of interest. Ideally, some of the steps in the protocol should be automated. Such modifications are being explored.

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