Flow cytometry and FISH to measure the average length of telomeres (flow FISH)

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Telomeres have emerged as crucial cellular elements in aging and various diseases including cancer. To measure the average length of telomere repeats in cells, we describe our protocols that use fluorescent *in situ* hybridization (FISH) with labeled peptide nucleic acid (PNA) probes specific for telomere repeats in combination with fluorescence measurements by flow cytometry (flow FISH). Flow FISH analysis can be performed using commercially available flow cytometers, and has the unique advantage over other methods for measuring telomere length of providing multi-parameter information on the length of telomere repeats in thousands of individual cells. The accuracy and reproducibility of the measurements is augmented by the automation of most pipetting (aspiration and dispensing) steps, and by including an internal standard (control cells) with a known telomere length in every tube. The basic protocol for the analysis of nucleated blood cells from 22 different individuals takes about 12 h spread over 2–3 days.

INTRODUCTION

Telomeres in all vertebrates consist of tandem DNA repeats of the sequence d(TTAGGG) and associated proteins[1,2]. A minimum number of telomere repeats must 'cap' each chromosome end to prevent the activation of DNA-damage responses and genome instability[3,4]. In most human cells, the gradual loss of telomere repeats contributes to replicative senescence[5], apoptosis or neoplastic transformation[6], and short telomeres are a risk factor in many age-related diseases[7]. Recent studies have shown that haploinsufficiency for either the telomerase RNA template (*TERC*) gene or the telomerase reverse transcriptase (*TERT*) gene, can cause premature death from complications of aplastic anemia, immune deficiency or cancer[8–11]. Other studies have shown that tumor cells and immortal cell lines typically express high levels of telomerase[12], and are required to do so to sustain their proliferative activity (for a review see ref. 13). The realization that the length of telomere repeats at individual chromosome ends influences biological functions ranging from aging to carcinogenesis[14] has highlighted the need for techniques that can provide accurate information on telomere length in different cell types.

Measuring telomere length

Several methods are available for measuring the length of telomere repeats in cells, each with distinctive advantages and disadvantages. The gold standard remains the analysis of terminal restriction fragment (TRF) length by Southern blot analysis[5]. Although reproducible measurements of average telomere length can be obtained by TRF analysis, this method has disadvantages in terms of the amount of time and DNA that are needed for analysis. Furthermore, because the distance between terminal restriction sites in genomic DNA and the start of actual telomere repeats is known to vary between chromosomes, the results reported with this method overestimate the average telomere length by several kb[15]. Recently, two PCR methods have been described for measurements of average[16] and chromosome-specific telomere length[17]. The first method provides a measure of telomeric DNA relative to genomic DNA (typically a single-copy gene) as a single ratio value in a sample of genomic DNA, whereas the second provides information on the actual length of telomere repeats at a specific chromosome arm, such as the Xp/Yp telomere for up to several hundred individual chromosomes. Both methods have advantages and disadvantages in terms of the information that is obtained, the amount of material needed, the time needed to complete the test, the amount of labor involved and the accuracy of results. A detailed discussion of these issues is beyond the scope of this paper. We have developed quantitative fluorescence *in situ* hybridization (Q-FISH) for measurements of telomere length on individual metaphase chromosomes by image microscopy[18] using directly labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probes[19]. This method is based on the principle that, at low ionic strength, PNA (but not single-stranded DNA) can anneal to complementary single stranded DNA sequences. Quantitative hybridization to telomere repeats is achieved using conditions that only allow labeled $(CCCTAA)_3$ PNA to hybridize to $(TTAGGG)_n$ target sequences. The Q-FISH method and software for Q-FISH image analysis (freely available from the Flintbox Network at www.flintbox.com) has been, and continues to be, informative in the telomere field[20–22].

Telomere length measurements using flow FISH

Q-FISH was adapted for the analysis of cells in suspension by flow cytometry in 1998 (ref. 23). This method was used to show that the loss of telomeric DNA in human nucleated blood cells is most pronounced in the first few years of life, and that the loss of telomeric DNA in lymphocytes with age is more pronounced than in granulocytes[24]. Flow FISH was also used to study the decline of telomere length with age in leukocytes from baboons[25], and to study the role of the regulator of telomere length (*Rtel*) gene in the mouse[26]. Using flow FISH it was found that the telomere length in granulocytes from patients with aplastic anemia correlates with the response to immunosuppressive therapy[27] and, in patients with chronic myeloid leukemia, with time to disease progression[28]. The loss of telomere repeats in hematopoietic cells was found to be restricted to the first year following allogeneic bone-marrow

TABLE 1 | Advantages of a 96-well microdispenser for flow FISH.

Features	Improved
Gentle and even pipetting	Recovery of cells
Aspiration to defined volume levels	Reproducibility of results
High precision of pipetting	Accuracy and reproducibility of results
	Less cross-contamination between tubes
Simultaneous processing of many tubes	Increased number of samples, decreased worker fatigue, increased accuracy
Processing of small volumes of solutions	Economy, efficiency

transplantation[29], and very short telomeres were described in a few cases of late failure of allogeneic bone-marrow grafts[30]. Subsequent significant improvements in flow FISH methods have included the partial automation of the procedure, the inclusion of control cells with known telomere length in every tube and limited immuno-phenotyping to measure the telomere length in sub-populations of nucleated blood cells[31]. Automated multicolor flow FISH is currently the fastest and most sensitive method available to measure the average or median telomere length in granulocytes, naive T cells, memory T cells, B cells and natural killer (NK) cells in human blood[31,32]. More recently, the automated flow FISH technique has been instrumental in the identification of patients with various disorders resulting from inherited abnormalities in telomerase genes[9,11,27,33,34]. In addition to the analysis of the telomere lengths of human leukocytes, nucleated cells from other species such as baboons[25], or cell line cells from different species such as mice[26], can also be analyzed using previously frozen cells as described for human nucleated blood cells. However, the use of antibodies will vary with the species and the objective of the experiment.

Limitations of flow FISH

The flow FISH technique is less than 10 years old. As with most new techniques, several modifications to the original protocol have been made, which have been described in a series of papers[23,24,31,32,35]. In the meantime, the technique has been adopted by a number of different laboratories for various applications[36–46]. For example, to measure the average telomere length in whole kidney epithelial cells[36], it was proposed to use nuclei rather than whole cells in order to detect the weak fluorescence signals of PNA bound to telomere target sequences above the background resulting from cytoplasmic auto-fluorescence and the non-specific binding of the probe. Not all proposed modifications are discussed here. Some make good sense whereas others are puzzling or not recommended. For example, the expression of telomere fluorescence on a logarithmic scale is surprising, and the use of various fixatives (e.g., to preserve epitopes or labeled antibodies at the surface of cells) seems problematic in view of the unpredictable effect of fixation on hybridization efficiency. In our hands, the use of even very low concentrations of cross-linking fixatives such as formaldehyde results in the reduced and more variable fluorescence of cells after flow FISH[31]. A dilemma here is that flow FISH results obtained with (partially) fixed cells might still correlate (to an extent) with actual telomere length. Most primary human cells typically show differences in telomere length within a very small linear range (from 2–10 kb). Because the ability to detect small biologically relevant differences relies largely on method accuracy and reproducibility, the effect of any fixation procedure should be carefully validated. Compared with the immuno-phenotyping of cells (in which absolute expression levels are seldom of interest), the flow FISH technique requires accurate measurements of relatively weak fluorescence signals on a linear scale. The required calibrations and controls make the whole procedure quite time consuming and technically demanding.

Crucial parameters in flow FISH

The aim of this paper is to describe our current protocol for measuring telomere length using flow FISH in sufficient detail to enable individuals with access to a two-laser flow cytometer, such as a FACSCalibur, and a good understanding of multi-parameter flow cytometry to perform accurate and reproducible measurements. We recommend the use of a dedicated 96-well automated dispenser, such as the Hydra system, to facilitate the many pipetting steps in the procedure (see **Table 1** for a list of the advantages of the Hydra system). However, this piece of equipment is not an absolute requirement. The careful set-up of all the parameters that will be measured on the flow cytometer in a flow FISH experiment, together with appropriate controls, are key to successful experiments.

The use of the instrument for sensitive measurements of fluorochromes of interest should be validated using calibration beads. Sensitive detection and calibration of the channel used for the detection of the hybridized PNA probe (typically fluorescein or Cy5) is particularly important to distinguish low levels of specific fluorescence from background or auto-fluorescence.

The use of calibration beads enables instrument problems to be rapidly identified, indicates instrument sensitivity and provides a record of instrument settings and day-to-day variations in instrument performance. We have observed marked differences in the ability of different FACSCalibur instruments to distinguish beads with low levels of fluorescence (corresponding to less than 10,000 MESF units) from unlabeled beads. If cleaning the instrument (following the procedures recommended by the manufacturer) does not result in increased sensitivity, the instrument might require optical realignment, a new flow cell or replacement of a photomultiplier tube.

We include bovine thymocytes as internal control cells because single-cell suspensions of small, viable non-dividing cells are easily obtained from this tissue (typically available fresh from the local butcher), and because the telomere length in bovine thymocytes ($\sim$15–20 kb) is about 2–3 times longer than is typically measured in human cells. These control cells are easily distinguished from the human test cells, and provide a convenient reference point for telomere fluorescence measurements. Only a few g of thymus tissue will give enormous amounts of cells that, following fixation, can be frozen in many small aliquots to serve as internal controls in many experiments. To further improve their usefulness, the bovine thymocytes are mildly fixed with formaldehyde. This results in decreased staining with the LDS751 dye (and decreased PNA

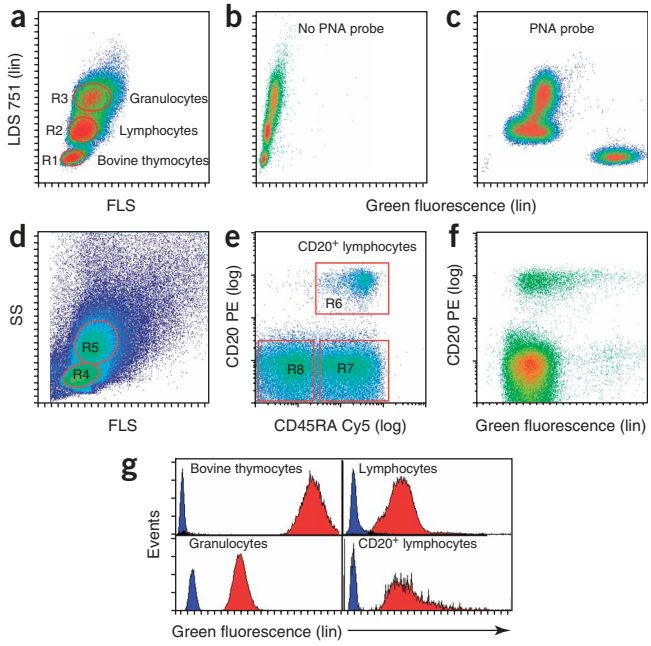

Figure 1 | Example of flow FISH data analysis of nucleated blood cells from a normal human donor (83 years old). For each nucleated blood sample two samples are analyzed: one in which the cells were hybridized to the peptide nucleic acid (PNA) probe (**c**) and one that was treated identically but without the PNA probe (**b**). The latter is required to measure the level of autofluorescence in cells of interest and to enable telomere length to be calculated from specific PNA hybridization (**g**). Cells are counterstained with non-saturating concentrations of the DNA dye LDS751 and various antibodies (CD45RA–Cy5 and CD20– phycoerythrin (PE) in this case) before the acquisition of listmode data. The first step in the subsequent analysis is to identify cells using forward light and side scatter in a bivariate dot plot (**d**). Within gates R4 and R5 three cell populations can be observed in a bivariate plot of forward light scatter signal versus LDS751 fluorescence (**a**). The mild formaldehyde fixation of the bovine thymocytes limits their staining by LDS751, which is useful to distinguish these small cells from human lymphocytes with largely overlapping forward and side light scatter properties. Granulocytes are labeled more brightly by LDS751, and can be distinguished from lymphocytes. The green fluorescence of cells gated as in (**a**) hybridized in the presence or absence of fluorescein-labeled PNA is shown relative to LDS751 fluorescence in the contour plots shown in (**c**) and (**b**), respectively. By combining the gates shown in (**a**) and (**d**), fluorescence histograms (**g**) of the indicated cell populations are obtained, which are used for subsequent calculations of telomere length. Antibodies specific for CD45RA and CD20 cells are used (**e**) to perform telomere length analysis of specific populations within the lymphocyte gate (R2 + R4). Note that the fluorescence histogram of granulocytes is more symmetrical than that of lymphocytes, and that cells with relatively long telomeres are readily identified among $CD20^+$ B lymphocytes.

fluorescence) but increased stability of the cells during the many steps in the flow FISH protocol. In the following protocol all the relevant steps regarding the preparation of bovine thymocytes are included in **Box 1**.

Flow FISH step by step

Each flow FISH experiment begins with the acquisition of the premixed calibration (MESF) beads. Several thousand events are collected, and the mean fluorescence and coefficient of variation (CV) of each of the five peaks is recorded and plotted against the MESF content provided by the manufacturer to control for the linearity of the instrument. CV is defined as the standard deviation (σ) of the fluorescent intensity of a population of beads expressed as a proportion or percentage of the mean (μ) intensity ($CV=\sigma/\mu$). An example of this calibration is provided in **Supplementary Figure 1** online. The next steps are related to the selection of the optimal values for the detectors, amplifiers, fluorescence compensation setting and threshold values for analysis of flow FISH samples. Once appropriate instrument settings have been selected, these can be saved and recalled for future experiments, although minor day-to-day adjustments are typically required between experiments and between samples. The instrument settings are further adjusted to provide a good separation of the events of interest over the entire range in the selected channels. Various compensation settings

BOX 1 | PREPARATION OF BOVINE THYMOCYTES

1. Arrange for fresh bovine thymus through a local meat packer. Request the collection of 2–3 small (e.g., cubic inch) pieces in a 1,000 ml container filled with digest solution. Arrange for pickup and process immediately.
2. Using a Petri dish lid as a cutting plate, place several pieces of thymus from the container into the lid. Pour about 20 ml of digest solution into the other half of the Petri dish containing the strainer. Using a scalpel, remove all white meat (fat) from the thymus. Cut the thymus pieces into smaller pieces about 2–5 mm in size. Put the smaller pieces in the strainer and gently force the tissue using the syringe top through the strainer (do not press too hard). Every 15 seconds, wash the strainer and tissue with digest solution.
3. Transfer the cell suspension from the Petri dish to 50 ml falcon tubes, using a funnel. Collect about 24 tubes. Observe cells through a microscope. Most of the cells should be single, with very little clumping. If too many clumps are present, repeat the strainer step (2).
4. Spin for 10 min at 450 *g*, aspirate supernatant and gently resuspend some of the cells in 10–20 ml PBS with DNase. Do not resuspend the entire pellet, as the bottom will contain most of the clumped cells. Top up the tube with regular PBS up to 50 ml. If there is still a pellet on the bottom but you resuspended lots of cells, decant the suspension into a beaker. As clumps sink rapidly to the bottom, single cells can be enriched by collecting the supernatant after 1–2 min of sedimentation.
5. Pool 400 ml cell solution into a 1,000 ml beaker and add 400 ml of 0.2 % formaldehyde (prepare just before use by adding 20 ml 37–40% formaldehyde to 380 ml PBS). Mix gently. Put on the shaker for 10 min. Aliquot into 50 ml falcon tubes. Spin for 5 min at 450 *g* at 4 °C. Aspirate the supernatant and resuspend the pellets in up to 50 ml PBS. Spin for 5 min at 450*g*. Aspirate the supernatant and resuspend the pellet in 10 ml PBS per tube. Pool the cells in a beaker. Remove an aliquot for cell counting.
6. Dilute with PBS so that you have 5×10^7 cells per ml. Take the same volume of ice-cold 80% FCS/20% DMSO and mix. Aliquot into cryovials so that there is 1 ml per vial.
7. Freeze at -70 °C or lower. Include some unfixed cells to examine the effect of fixation on the hybridization efficiency.

■ **PAUSE POINT** Fixed and frozen bovine thymocytes can be stored for up to 2 years.

are selected for the analysis of cells simultaneously labeled with fluorescein, phycoerythrin (PE), LDS751 and Cy-5. Except for the compensation setting for fluorescence 2 channel (Fl2; PE) fluorescence detected in the Fl1 (green fluorescence) channel, the setting for green fluorescence detection is typically not readjusted after the acquisition of the MESF bead data because the range of telomere fluorescence in test cells is typically known.

Following the hybridization of nucleated human blood cells mixed with bovine thymocytes, three populations of cells can typically be distinguished (**Fig. 1a**) based on two parameters: forward light scatter (providing a measure of cell size) and LDS751 fluorescence (providing a measure of DNA content/accessibility). The three populations are the bovine thymocytes (R1), which are small and only dimly labeled with LDS 751, human lymphocytes (R2), which have intermediate forward scatter and LDS751 fluorescence, and granulocytes (R3), which are most brightly stained with LDS751. Lymphocytes are further separated from granulocytes by combining the gates shown in **Figure 1a** with the gates set in dot plots of side scatter versus forward light scatter shown in **Figure 1d**. Cells within the lymphocyte gate (R2 + R4) are further subdivided on the basis of antibody labeling with Cy5 or Allophycocyanin and PE (**Fig. 1e**). The PE signal is derived either from directly labeled CD20 antibody (**Fig. 1e,f**), or indirectly from biotinylated CD57 antibodies followed by Streptavidin-PE (not shown). After the identification of these various gates the following populations can be distinguished (**Fig. 1**): bovine control cells (R1 + R4), lymphocytes (R2 + R4), granulocytes (R3 + R5), PE-positive lymphocytes (R2 + R4 + R6), PE-negative, CD45RA-positive lymphocytes (R2 + R4 + R7) and PE-negative, CD45RA-negative lymphocytes (R2 + R4 + R8). Each of these populations of cells is analyzed for their autofluorescence by analysing green fluorescence in tubes that were processed as for flow FISH in the absence of a PNA probe (**Fig. 1b** and blue peaks in **Fig. 1g**) and for the fluorescence obtained with the telomere PNA probe (**Fig. 1c** and red peaks in **Fig. 1g**). At least 10,000 events of human cells are typically acquired for each analysis.

MATERIALS

REAGENTS

- FCS (Hyclone or other)
- DNase (Type II-S ~11 mg per bottle, ≥2,000 units per mg; SIGMA)
- PBS, without $Ca2^+$ and $Mg2^+$ (Stem Cell Technologies)
- Heparin (5 ml per bottle, 10,000 USP units per ml, Organon Teknika)
- DMEM (StemCell Technologies)
- Formaldehyde solution (36.5–38%) (VWR) ! CAUTION Formaldehyde is poisonous, and contact with eyes and skin should be prevented. Work in a fume hood to avoid inhalation
- Dimethylsulfoxide (DMSO, ≥99.7%, Hybri-Max) (SIGMA)
- Ammonium Chloride solution (0.8% NH_4Cl with 0.1 mM EDTA) (Stem Cell Technologies)
- BSA (fraction V, fatty acid-free, nuclease- and protease-free, ≥98%) (Calbiochem)
- Hepes hemisodium salt, dry powder H9897(Sigma)
- Dextrose, 5%, pH 4.0, 252 mOsmol per l (Baxter)
- PNA probe (in house synthesis, DAKO or custom order, e.g., from Panagene)
- Sheath fluid (BD Biosciences)
- Formamide (≥99.5%, EMD for wash steps, or ultra-pure formamide ≥99% from Invitrogen for use in hybridization mixture following deionization) ! CAUTION Formamide is a poison and a mutagen, and contact with eyes and skin should be prevented. Work in a fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to local rules and regulations
- Resin AG 501-X8(D) (Bio-Rad)
- Hydrochloric acid (HCl) 37–38% (EMD) ! CAUTION HCl is a poison that will cause severe eye or skin burns. Avoid inhalation
- LDS751 (Exciton)
- Methanol, ≥99.8% (EMD) ! CAUTION Methanol is a poison and a highly flammable liquid
- RNase T1 500,000 U (Sigma)
- Sodium Azide, ≥98% (EMD) ! CAUTION Sodium Azide is a poison. Avoid exposure to the eyes and skin
- N,N Dimethyl-Formamide (DMF; Sigma) ! CAUTION DMF is a poison and an irritant for the eyes and skin. Work in a fume hood and avoid plastics such as polystyrene, which will dissolve in this strong solvent
- Tween 20 (VWR)
- MESF Beads (Quantum FITC MESF Premix Low Level; Bangs Laboratories)

EQUIPMENT

- Hydra microdispenser (Matrix Technologies)
- FACSCalibur flow cytometer (BD Biosciences)
- EDTA blood collection tubes (BD Biosciences) or other tubes for collection of ACD or heparin blood
- Automatic pipette aid (Drummond Pipet Aid or equivalent)
- Repeater pipette plus tips
- Filter 0.2 μm (Fisher)
- Metal rack, custom made, used in 87 °C water bath
- PCR foil (VWR)
- Water bath circulating 87 °C (Lindberg/Blue M)
- Benchtop Centrifuge (Eppendorf) with Deep Well Swing-Bucket Rotor

REAGENT SETUP

PNA probes PNA probes (Cy5 or fluorescein labeled CCC TAA CCC TAA CCC TAA; C terminus with or without one or two spacer moieties in between the fluorescent label and the first nucleotide) can be synthesized manually or with dedicated equipment by chemists familiar with peptide and nucleic acid synthesis. Alternatively, fluorescein-labeled or Cy5-labeled PNA can be ordered from DAKO as a kit (K5327 Telomere PNA FISH) or from other suppliers such as Panagene as a custom order. The latter is typically more economical, as many hundreds of experiments can be done with just a few OD260 units of labeled PNA. PNA, the use of PNA for measurements of repetitive DNA sequences in chromosomes and cells and measurements of telomere length are subject to several patents in and outside the United States.

Antibodies Very few antibodies recognize epitopes that survive the harsh conditions required for DNA used in flow FISH. We have identified a few useful reagents. Monoclonal antibody 8d2 (anti-CD45RA) was made in our laboratory. The antibody is directly labeled with Cy5 using a Cy5 protein-labeling kit from Amersham. An alternative is APC anti-human CD45RA from eBioscience. CD20 antibody clone L26 is available from Beckman Coulter and can be used either directly labeled with phycoerythrin or indirectly after labeling with a R-Phycoerythrin Mouse IgG2a Labeling Kit (Zenon). Biotinylated anti-CD57 is obtained from BD Biosciences and used indirectly with Streptavidin-PE (Molecular Probes).

Digest solution Add 500 ml RPMI, 25 ml FCS, 5 ml Heparin and one bottle (~11 mg) DNase to a 1,000 ml bottle. Work aseptically in a biohazard hood. The solution can be stored at 4 °C for 2 days.

PBS containing DNAse Add one bottle (~11 mg) of DNase to 500 ml of PBS. The solution can be stored at 4 °C for 2 days.

Freezing solution (80% FCS/20% DMSO) Mix 160 ml FCS and 40 ml DMSO and put on ice ▲ CRITICAL Prepare just before use. Discard any unused solution.

Red cell lysis solution (ammonium chloride) As an alternative to commercially available ammonium chloride solution, this solution can be prepared fresh on the day of use. Prepare 0.155 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM EDTA from powder (except EDTA), adjust pH to 7.4 on ice. The addition of $KHCO_3$ to the NH_4Cl solution accelerates red blood cell lysis and improves the reproducibility of red blood cell lysis time. The pH of the red cell lysis solution should be adjusted at 4 °C as the pH of this solution varies with temperature. The lysis solution must be kept at 4 °C and used within a day. Sterilization (not essential for flow FISH) is only possible by filtration, as it decomposes upon autoclave treatment. The recommended procedure for the lysis of red blood cells was developed by Roos and Loos[47].

100 mM Hepes solution Pour one pack of Hepes into a 1 l graduated cylinder. Add H_2O up to 1 l. Cover with parafilm and mix by inversion. Transfer to a 1 l glass bottle. Store at room temperature (between 20–24 °C) for up to 3 months.
10% BSA (sterile) In a clean glass bottle, add 450 mL of H_20 and 50 g of BSA. Add a magnetic stir bar and stir on a stir plate for 1 h. Leave the solution at 4 °C overnight. Transfer the solution to a clean graduated cylinder and bring the volume to 500 ml with H_2O. Filter the solution with a Whatman filter into a clean flask. Filter again with a 0.2 µm filter using a suction system. Aliquot the solution into clean, sterile 50 ml tubes and label with solution identity, the date prepared and the initials of the preparer. Store at –20 °C.
PBS with 0.2% BSA In a clean graduated cylinder, add 25 ml of PBS. Add 1 ml of 10% BSA. Bring the volume to 50 ml with PBS. Cover with parafilm and mix by gentle inversion. Transfer to a 50 ml centrifuge tube ▲ CRITICAL Prepare and use fresh.
Hybridization buffer In a clean graduated cylinder, add 15 ml of 5% dextrose, 2 ml of 100 mM Hepes solution, and 200 µL of 10% BSA. Bring the volume to 20 mL with 5% dextrose ▲ CRITICAL Keep on ice until use. Discard any unused solution.
Freezing solution (80% FCS/20% DMSO) Mix 4 ml FCS and 1 ml DMSO ▲ CRITICAL Put on ice and prepare just before use. Discard any unused solution.
Flow FISH PNA probe and control blank preparation Use polypropylene tubes and pipettes only. Add 2 ml H_2O and 2 ml DMF to a clean 15 ml tube with an adjustable pipette and pipette tips. Mix by retropipetting gently. In the vial with the probe, add 1 µl DMF/H_2O per µg of probe to give 1 mg per ml probe. If the probe does not readily dissolve, warm it up for a few minutes at 50–80 °C. The unused DMF/H_2O mixture should be used to prepare a control blank solution. Label vials and keep stock solution frozen at -70 °C or lower.
1 M Tris pH 7.1 Add 24.2 g Tris (Trizma base, minimum 99.9%, Sigma) to 150 ml H_2O in a clean 250 ml flask. Add a magnetic stir bar and stir on a stir plate for 10 min. Slowly add HCl until the pH reaches 7.1. Transfer the solution to a clean graduated cylinder and bring the volume to 200 ml with H_2O. Transfer to a glass bottle and label with the solution identity, date prepared and the initials of the preparer. Store at 4 °C for up to 1 year.
1 M NaCl pH 7.4 In a clean 100 ml graduated cylinder, add 5.84 g NaCl (≥99%, EMD) and H_2O up to 100 ml. Cover with parafilm and mix by inversion. Transfer to a 100 ml glass bottle and label with the solution identity, date prepared and the initials of the preparer. Store at 4 °C for up to 1 year.
Formamide (de-ionized) In a clean 1,000 ml glass bottle, add approximately 500 ml Formamide. Wash 25 g Resin AG 501-X8(D) briefly with 25 ml of Formamide, discard the Formamide and add the washed Resin to the 500 ml Formamide. Add a magnetic stir bar and stir on a stir plate for approximately 2 h. Leave at room temperature overnight. Filter twice with a Whatman filter. Aliquot in 50 ml tubes and label with the solution identity, date prepared and the initials of the preparer. Store at –20 °C for up to 1 year.
Prepare hybmix stock In a clean 50 ml tube add 40.5 ml deionized formamide, 1.08 ml 1M Tris pH 7.1 and 7.92 ml H_2O. Mix by retropipetting gently. Add 24.25 ml of this mixture to two clean 50 ml tubes. In one tube add 750 µl of 1 mg per ml PNA probe and mix by retropipetting gently; this gives a probe stock with a concentration of 30 µg per ml. In the other tube add 750 µl blank and mix by retropipetting gently, this yields the unstained stock. For both solutions aliquot 500–1,000 µl into microcentrifuge tubes. Label with the solution identity, date prepared and the initials of the preparer. Store at –20 °C.
10% Tween 20 In a clean flask add 45 ml H_2O and 5 ml Tween 20. Mix by retropipetting gently. Label with the solution identity, date prepared and the initials of the preparer ▲ CRITICAL Prepare just before use. Discard any unused solution.
RNase T1 (100,000 U per ml) Add sterile PBS to make a final volume of 5 ml RNase T1 500,000 U. Transfer the solution into microcentrifuge tubes. Heat deactivate by incubating for about 20 min at 80 °C in a heat block. Wrap parafilm around the lids of the microcentrifuge tubes. Label with the solution identity, date prepared and the initials of the preparer. Store at 4 °C for up to 1 year.
LDS751 (200 µg per ml) In a clean 50 ml tube add 2 mg LDS751 to 10 ml methanol. Mix by retropipetting gently. Label with the solution identity, date prepared and the initials of the preparer. Seal cap with parafilm. Store at room temperature in the dark for up to 2 years.
20% sodium azide In a clean bottle add 10 g sodium azide. Then add PBS up to 50 ml, cover with parafilm and mix by inversion. Label with the solution identity, date prepared and the initials of the preparer. Store at 4 °C for up to 1 year.
DNA counterstaining solution In a clean 250 ml flask, add 121.5 ml sheath fluid, 1.24 ml 10% BSA, 1.24 ml Rnase T1 (100,000 U per ml), and 6.2 µl LDS751 (200 µg per ml). Mix well by gently shaking the flask, this gives DNA counterstaining solution. Fill a clean V-tray (Matrix Technologies) with the DNA counterstaining solution. Transfer 320 µl DNA counterstaining solution from the V-tray to a rack with clean 1.4 ml tubes (Micronic Systems) using the Hydra microdispenser. Repeat the last step three times to fill four racks sufficient for 4 × 96-well plates. Cover the tubes with parafilm and put the lid on the rack. Label with the solution identity, date prepared and the initials of the preparer, and store at 4 °C for up to 1 month. This solution is aliquoted using the Hydra 96 microdispenser. After use the Hydra system should be cleaned following the recommendations of the manufacturer.
Antibody wash solution In a clean 500 ml flask add 388.5 ml sheath fluid, 21 ml FCS, and 10.5 ml 20% sodium azide. Mix well by gently shaking the flask, this gives antibody wash solution. Fill a flat-bottom tray (Matrix Technologies) with antibody wash solution. Transfer 1,040 µl of antibody wash solution from the flat-bottom tray to a rack containing clean 1.4 ml tubes or a clean Sigma-deep-well plate (Sigma) using the Hydra microdispenser. Repeat the last step three times to fill four racks sufficient for 4 × 96-well plates. Cover the tubes with parafilm and put the lid on the rack (if using the Sigma-deep-well plate put the lid on the plate). Label with the solution identity, date prepared and the initials of the preparer, and store at 4 °C for up to 4 months. This solution is aliquoted using the Hydra 96 microdispenser.
Wash 1 solution In a clean 2 l flask, add 341.3 ml H_2O, 32.5 ml 1M Tris pH 7.1, 16.25 ml 10% BSA, 16.25 ml 10% Tween 20, and 1218.8 ml formamide. Mix well by gently shaking the flask, this gives wash 1. Fill a clean flat-bottom tray with wash 1. Transfer 1,040 µl of wash 1 from the flat-bottom tray to a rack containing clean 1.4 ml tubes or a clean Sigma-deep-well plate using the Hydra microdispenser. Repeat the last step 11 times to generate enough wash solution for 4 × 96-well plates. Cover the tubes with parafilm and put the lid on the rack (if using the Sigma-deep-well plate put the lid on the plate). Fill a flat-bottom tray with wash 1. Transfer 900 µl of wash 1 from the flat-bottom tray to a rack containing clean 1.4 ml tubes using the Hydra microdispenser. Repeat the last step three times to fill four racks sufficient for 4 × 96-well plates. Cover the tubes with parafilm and put the lid on the rack. Label with the solution identity, date prepared and the initials of the preparer, and store at −20 °C for up to 2 months. This solution is best aliquoted using the Hydra 96 microdispenser. After use the Hydra system should be cleaned following the recommendations of the manufacturer.
Wash 2 solution In a clean 2 l flask, add 374 ml 5% dextrose, 42.5 ml 100 mM Hepes, 4.25 ml 10% BSA and 4.25 ml 10% Tween 20. Mix well by gently shaking the flask, this gives wash 2. Fill a flat-bottom tray with wash 2. Transfer 1,040 µl wash 2 from a flat-bottom tray to a rack containing clean 1.4 ml tubes or a clean Sigma-deep-well plate using the Hydra microdispenser. Repeat the last step three times to fill four racks sufficient for 4 × 96-well plates. Cover the tubes with parafilm and put the lid on the rack (if using the Sigma-deep-well plate put the lid on the plate). Label with the solution identity, date prepared QJ;and the initials of the preparer, and store at 4 °C for up to 2 months. This solution is aliquoted using the Hydra 96 microdispenser. After use the Hydra system should be cleaned following the recommendations of the manufacturer.
Hybridization mixture In a clean 250 ml flask, add 3.72 ml H_2O, 1.67 ml 1M Tris pH 7.1, 1.67 ml 1M NaCl, 8.37 ml 10% BSA, and 62.7 ml deionized formamide. Mix well by gently shaking the flask. From this solution, add 38.6 ml each to two clean 100 ml flasks. Mark one flask 'unst' and the other flask 'tel'. To the flask marked 'unst' add 413 µl hybridization blank stock, this gives the unstained hybridization mixture. To the flask marked 'tel' add 413 µl hybridization PNA probe stock, this gives the telomere hybridization mixture. Mix well by gently shaking both flasks. Fill a clean V-tray with the unstained hybridization mixture (always start with unstained hybridization mixture so that no PNA probe is transferred into unstained hybridization mixture during the filling process). Transfer 190 µl of unstained hybridization mixture from the V-tray to a rack with containing 1.4 ml tubes using the Hydra microdispenser. Repeat the last step once. Fill a clean V-tray with the telomere hybridization mixture. Transfer 190 µl of telomere hybridization mixture from the V-tray to a rack containing clean 1.4 ml tubes using the Hydra microdispenser. Repeat the last step once. Rearrange the tubes with unstained and telomere hybridization mixtures in the racks to the optimal order for flow FISH; for example, alternating unstained and telomere rows. Cover the tubes with parafilm, put the lid on the rack, Label with the solution identity, date prepared and the initials of the preparer, and store at -20 °C for up to 2 months. These solutions are best aliquoted using the Hydra 96 microdispenser. After use the Hydra system should be

cleaned following the recommendations of the manufacturer. The above volume of hybridization mixture is sufficient for 4 × 96-well plates.

EQUIPMENT SETUP

Microdispenser The Hydra microdispenser is a bench-top instrument that provides high-speed, repetitive dispensing of µl and nl volumes into microplates, 96 or 384 samples at a time. The Hydra microdispenser features 96 (or 384) glass syringes held in a fixed array. The array forms a horizontal X–Y grid that aligns each needle in the exact center of a well in a microplate. A precision motor assembly moves the plungers within the syringes up and down under microprocessor control, dispensing or aspirating liquids to or from microplates and other receptacles. Equipment required to use the Hydra microdispenser include: Flat-bottom tray (Matrix Technologies, cat no. 1064-05-4), V-tray (Matrix Technologies, cat no. 1064-05-5), micro tubes (1.4 ml with rack, Micronic Systems, cat no. M226RP), deep-well multiwell plate & lid (Sigma, cat no. 239, 927-1; lid, cat no. 237-929-8) and PCR tubes with rack (Nunc, cat no. 248161). Values for all parameters of the microdispenser have to be set for each microdispenser and every type of equipment.

Flow cytometer A FACSCalibur flow cytometer equipped with 488nm and 1635 nm lasers has been successfully used for flow FISH. All data analysis can be performed using Cellquest Pro software (BD Biosciences). Data should be stored either locally on the computer attached to the flow cytometers or from remote analysis stations connected to a computer network that is also used to store flow cytometry data.

PROCEDURE

Preparation of cells for flow FISH

1| Transfer 1,000 µl of whole blood to 50 ml falcon tubes pre-filled with 10 ml PBS/0.2% BSA. EDTA blood samples are preferred but heparin or ACD (citrate) blood samples can also be used. The volume needed for flow FISH varies, but typically a few ml of blood gives enough cells for telomere length measurements. Blood samples should be processed within 24 h of collection, as there is poor recovery of granulocytes after that period.

2| Spin down for 5 min at about 450*g* at room temperature in a bench-top centrifuge.

3| Aspirate most of the supernatant with a suction system. Do not disturb the white blood cells (WBC) on the red blood cell (RBC) pellet, be careful not to aspirate the buffy coat.

4| Working quickly, add ice-cold NH_4Cl up to 50 ml and mix by gentle inversion. The temperature of the lysis solution should be kept at 0 °C throughout the red cell lysis procedure to avoid damage to the nucleated cells.

5| Incubate on ice for 10 min.

6| Spin down for 10 min at 290*g* at 4 °C in a bench-top centrifuge.

7| Aspirate the supernatant with a suction system. Remove the red cell lysate by careful aspiration. Gently tap the pellet.

8| Working quickly, add 1 ml of NH_4Cl, mix by gently retropipetting and transfer the suspension to a 1.5 ml microcentrifuge tube. Incubate on ice for 10 min. Resuspend the cell pellet consisting of granulocytes, monocytes, lymphocytes, platelets and remaining red cells.

9| Spin for 20 s at about 16,000*g* at room temperature in a bench-top microcentrifuge. Carefully aspirate the supernatant and gently tap the pellet.

10| Resuspend the cells in 200 µl of hybridization buffer (5% dextrose/10 mM Hepes/0.1% BSA), and mix by gently retropipetting. Count the cells, if needed, using a hemocytometer or a V-cell counter. The cells can now be used either directly in flow FISH experiments or, more typically, frozen for future flow FISH analysis.

? TROUBLESHOOTING

11| In a well-marked cryogenic vial, add about 5 × 10^6 cells per 200 µl of hybridization buffer (5% dextrose/10 mM Hepes/0.1% BSA). Prepare for freezing by adding 200 µL ice-cold 80% FCS/20% DMSO. Mix by gently retropipetting, transfer to a Styrofoam box and immediately freeze at -80 °C. After 2–5 days, transfer to a –135 °C freezer or a liquid nitrogen freezer for storage.

12| If samples require shipment, ship on dry ice.

■ **PAUSE POINT** Samples can be stored for up to 10 years.

Hybridization and antibody labelling of cells

13| Turn on the 87 °C water bath about 2 hours before use. Check water level and temperature before use.

14| Remove tubes with unstained and telomere hybridization mixture from the freezer and keep in the dark at room temperature.

? TROUBLESHOOTING

15| Remove frozen nucleated blood cells and fixed bovine thymocytes (see **Box 1**) from the freezer and keep them on dry ice.

? TROUBLESHOOTING

16| Thaw bovine thymocytes in a 37 °C water bath, and mix the cell suspension by gently pipetting.

17| Fill a clean V-Tray with 11.5 ml hybridization buffer.

18| Transfer 1 ml of the bovine thymocytes to the V-Tray and mix the suspension by gently shaking the V-tray.

19| Transfer 100 µl bovine thymocytes from the V-tray to PCR test tubes in a rack using the Hydra. This will give approximately 2×10^5 cells per tube.

20| Thaw human test cells in a 37 °C water bath (for about 1 min) and mix the cells by gently pipetting.
? TROUBLESHOOTING

21| Add 1–9 $\times$ 10^5 nucleated blood cells to PCR tubes, 4 tubes for each sample (samples that need to be stained with two antibodies labelled with the same fluorochrome are treated as two individual samples). The inclusion of a human reference sample (nucleated blood cells from one donor, one bleeding; multiple aliquots frozen at −135 °C) in each experiment is recommended.

22| Repeat Step 20 and Step 21 for each sample, recording the position of each sample in the 96-well PCR tube rack.

23| Spin the PCR tubes for 5 min at 290 *g* at 4 °C in a bench-top centrifuge.

24| Aspirate the supernatant until 10 µl remains using the Hydra. This step is illustrated in **Supplementary Video 1** online.

25| Mix cells using the Hydra.

26| Transfer 170 µl of hybridization mixture from the 1.4 ml tubes in the 96 well rack to the cells (for each sample there should be two unstained and two that contain the telomere probe) using the Hydra.
? TROUBLESHOOTING

27| Mix using the Hydra.

28| Incubate for exactly 10 min at room temperature. Start the clock as soon as the Hydra stops mixing. Put self adhesive PCR foil on top of the tubes and press it well to seal the tubes. Transfer the PCR tubes to a metal rack, mark the front of the rack and put a metal lid on top. Place the rack in the dark for the remainder of the 10 min.

29| Place the metal rack in an 87 °C water bath and incubate for 15 min, checking the temperature.

30| Take the metal rack out of the water bath and transfer the tubes back to the plastic rack.

31| Hybridize for 90–120 min at room temperature in the dark. Remove the tubes or plates containing wash 1 solution from the –20 °C freezer and keep them at room temperature until use.

32| Carefully remove foil from the PCR tubes.

33| Transfer the cell suspension from PCR tubes to 1.4 ml tubes pre-filled with 900 µl wash 1 using the Hydra.

34| Centrifuge cells for 5 min at 1,500*g* at 16 °C in a bench-top centrifuge.

35| Aspirate supernatant using the Hydra until 100 µl remains.
! CAUTION Aspirating to less than 100 µl will result in the increased loss of cells.

36| Mix the cell pellet using the Hydra.

37| Add 1,000 µl wash 1 solution to the cells using the Hydra.

38| Centrifuge cells for 5 min at 1,500 *g* at 16 °C in a bench-top centrifuge.

39| Repeat previous 4 steps (Steps 35–38) twice.

40| Aspirate supernatant using the Hydra until 100 µl remains.

41| Mix using the Hydra.

42| Add 1,000 µl wash 2 solution to the cells using the Hydra.

43| Centrifuge cells for 5 min at 900*g* at 16 °C in a bench-top centrifuge.

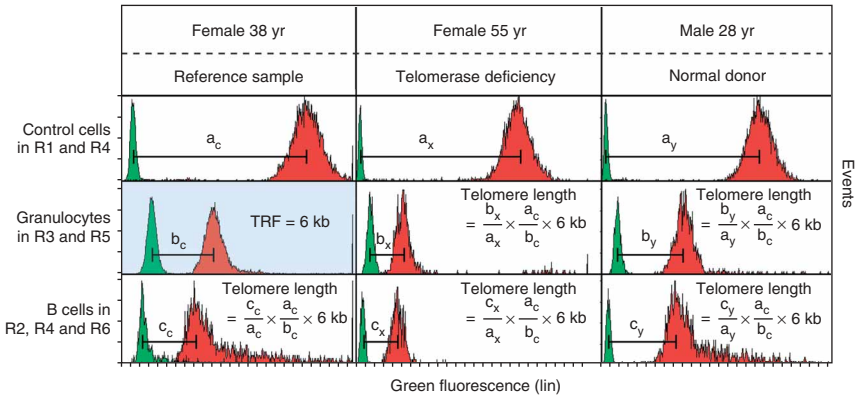

Figure 2 | Calculation of telomere length from flow FISH data. Fluorescence histograms (green = no peptide nucleic acid (PNA) probe; red = telomere PNA probe) are shown for 3 different cell types (gated as shown in **Fig. 1**) from 3 different individuals. Two controls are included for each flow FISH experiment; internal control cells (fixed bovine thymocytes, top panels of histograms) and a human reference sample (left panels). The telomere length in purified granulocytes from the reference sample is measured by Southern blot analysis. The average terminal restriction fragment (TRF) length in the reference granulocytes (blue panel) serves to calculate the telomere length in each of the gated cell types as follows: the specific telomere fluorescence for each cell type within the selected gate is calculated by subtracting the median autofluorescence (green histograms) from the median fluorescence signal obtained in the presence of the probe (red histograms). The resulting specific telomere fluorescence (horizontal bars in histogram plots) is used to calculate the telomere length in the cells of interest using the known TRF value of the reference granulocytes in the formula shown in the panels. The error in telomere length estimates is reduced by taking into account the specific telomere fluorescence of the control cells in each tube, as well as the specific telomere fluorescence of the reference granulocytes in each individual experiment. Note the heterogeneous telomere fluorescence in B cells (with some memory B cells having brighter fluorescence than the control bovine thymocytes) and the short telomeres and absence of B cells with long telomeres in leukocytes from a patient with telomerase deficiency resulting from a mutation in the *TERT* gene (middle panels).

44| Aspirate the supernatant to 20 µl volume using the Hydra.
? TROUBLESHOOTING

45| For counterstaining use option A, unless samples need to be stained with antibodies, in which case use option B.
(A) Counterstain in the absence of antibody staining.
(i) Resuspend the cell pellet (in the 20 µl volume) using the Hydra.
(B) Antibody staining.
(i) Prepare antibody dilutions and calculate the amount of antibody solution needed (number of tubes plus 2 × 30 µl).
(ii) Add 30 µl of antibody solution to the tubes that contain cell pellets in wash solution 2.
(iii) Centrifuge the tubes at 900*g* at 16°C in a bench-top centrifuge and stop the centrifuge when the speed reaches about 200*g* to ensure antibodies are mixed in the next step.
(iv) Mix the cells and antibodies using the Hydra.
(v) Incubate on ice or at 4 °C for 20–60 min.
(vi) Add 1,000 µl antibody wash solution to the cells using the Hydra.
(vii) Centrifuge the cells for 5 min at 900*g* at 16 °C in a bench-top centrifuge.
(viii) Aspirate supernatant using the Hydra until 20 µl remains.
(ix) Repeat Step 45B(i)–Step 45B(viii) if a second antibody is used.
(x) Resuspend the cell pellet (in the 20 µl volume using the Hydra).
? TROUBLESHOOTING

46| Add 300 µl of DNA counterstain solution to the cells and mix using the Hydra. Cover the tubes with parafilm.

47| Incubate the samples on ice for 20-60 min.
■ **PAUSE POINT** The samples can be stored for up to 7 days at 4 °C.

Data acquisition by flow cytometry
48| Turn on the flow cytometer and computer and wait at least 15 min for the lasers to warm up. Remove samples from the fridge and keep cells on ice e.g., in a styrofoam box with a lid, keep the lid on the box as much as possible.

49| Launch the acquisition software and flow FISH template if available (**Supplementary Figure 2** online). From the cytometer menu follow the pathway 'Open Detectors/Amps/Threshold/Compensation'. Open a folder to store the files.

50| Run a calibration bead sample: suspend two drops of beads in 300–1000 µl sheath fluid in a FACS tube (mix well before use) and acquire about 5,000 events corresponding to single beads in a FCS/SSC dot plot using FL1 settings suitable for human test cells and bovine thymocytes. Set markers in the FL1 Histogram over the five peaks and record the mean and CV for all five peaks. Calculate the slope of the beads from the MESF values versus the FL1-fluorescence values of the five bead

populations. Record the slope value (see **Supplementary Figure 1**). Continue only when FACSCalibur is linear, or choose a different instrument.

51| Adjust the detectors/amps, compensation and threshold instrument settings on the flow cytometer menu for the acquisition of cells. Open the data acquisition settings file if available (**Supplementary Figure 2**).

52| Mix cells using a vortex at medium speed and run samples on the flow cytometer. Start data acquisition in setup mode, adjust the regions in the FSC/SSC dot plot (**Fig. 1d**), the FSC/Fl3 dot plot (**Fig. 1a**) and the FL2/SSC dot plot and adjust the markers in the FL1 histograms over the peaks. Adjust the amp gain for FL3 so lymphocytes and granulocytes are in the middle of FL3/FSC dot plot, other detectors should not need to be adjusted, unless significant maintenance work is done on the instrument (such as the lasers being re-aligned or flow cell replaced) Adjust the compensation settings (FL1–%FL2, FL4–%FL3 and FL2–%FL3) as follows: using the 'unst' sample, adjust compensation FL4–%FL3 to separate Cy-5 negative and positive cells, this should be about the same for all samples; using the 'unst' sample adjust compensation FL2–%FL3 to separate PE negative and positive cells, this should be about the same for all samples. 'tel'/PE-negative cells are usually higher in FL2 than 'unst'/PE-negative cells: using the 'tel' sample adjust compensation FL2–%FL1 to separate PE-negative and positive cells, this should be about the same for all samples. Using the 'unst' sample adjust compensation FL1–%FL2, set for each sample: in 'unst' samples (count at least 10–30 PE-positive cells) adjust compensation so that the median (auto)fluorescence of the PE-positive and PE-negative cells are similar (median fluorescence within 5 fluorescence channels from each other). CD57biotin-streptavidin-PE results in cell populations with low, intermediate and high fluorescence staining. For compensation use low and bright cells.
? TROUBLESHOOTING

53| Acquire 10,000 events of cells without the PNA probe. Aim to collect at least 1,000 cells in each of the selected gates.

54| Acquire 10,000 events of human cells hybridized with the PNA probe. Adjust the regions if necessary. Fixed bovine thymocytes should be on the right side of the Fl1 scale. Adjust regions and compensation settings if needed. This step is illustrated in **Supplementary Video 2** online.

55| Repeat Steps 53 and 54 with the next samples until data on all samples has been acquired. Run distilled water on the flow cytometer between samples. Adjust regions between samples if necessary.

56| Run beads: repeat Step 50. Clean the machine with bleach and water, leave it on standby or shut down.
■ **PAUSE POINT** The data analysis can be performed any time after acquisition has been finished.

Flow FISH data analysis
57| From the flow cytometry data-analysis software program open the flow FISH analysis template document or create a window with dot plots and histograms as is shown in **Figure 1**. Open the file to be analyzed.

58| Drag the regions over the correct populations in the FL3/FSC dot plot: R1 over the bovine thymocytes; R2 over the lymphocytes; and R3 over the granulocytes. In the SSC/FSC dot plot: R4 over the bovine thymocytes and lymphocytes and R5 over the granulocytes. In the FL4/FL2 dot plot: R6 over the PE-positive/Cy5-positive and intermediate; R7 over the PE-negative/Cy5-positive and R8 over the PE-negative/Cy5-negative.

59| Check if all FL1 (tel-FITC) histograms are compatible with single populations of cells, if needed (with a small number of cells and/or noise events) adjust gates or place markers around the peaks.

60| Select all plots and histograms and copy them to a Microsoft Word document.

61| From the Word document, transfer median fluorescence values for unst a, tel a, unst b and tel b for all cell types analyzed to cells in Microsoft Excel for calculations.

● **TIMING**
Preparation of cells; Step 1– Step 12, 2 h.
Hybridization, antibody labeling and DNA counterstaining of cells; Step 13–Step 30, about 2 h. Step 31, 1.5–2 h. Step 32–Step 45A, 1-2 h. Step 32–Step 45B: 3–4 h. Step 46–Step 47, 0.5 h.
Data acquisition by flow cytometry; Step 48–Step 56, depends on the amount of samples, 3–4 h for about 48 tubes.
Flow FISH data analysis: Step 57–Step 61, depends on the amount of samples, 1–2 h for about 48 tubes.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1–10	Not all red cells are lysed	Lysis solution, lysis time	Check the pH of the NH_4CL solution, compare old and fresh NH_4CL solution, check the time and temperature for the lysing steps
	Loss of white blood cells	Cell death owing to the toxicity of the lysis step	
1–10	Too much haemoglobin in the cell suspension before hybridization — haemoglobin interferes with the detection of peptide nucleic acid (PNA) fluorescence	Inefficient red blood cell lysis (see above) or insufficient wash steps after red cell lysis	See above and/or add an additional wash step to remove the haemoglobin
15, 20	Loss of leukocytes after thawing, too many dead cells or clumped cells	Damaged cells were frozen	Check viability of cells prior to freezing, reduce time interval prior to freezing, replace buffers; thaw and resuspend cells quickly
		Thawing procedure takes too long (long exposure to DMSO is toxic to cells), Free DNA (from dead cells) is sticky and can lead to the clumping of white blood cells	Try adding DNAse (1 μg per ml) to avoid cell clumping
14, 26	No fluorescence signal	PNA probe not added	Check hybridization solution and make sure that PNA probe was added according to the protocol
		The denaturation temperature for the DNA was not 87 °C, or the time allowed for denaturation was not 15 min	Check the temperature and time during denaturation
14, 26	High fluorescence signal in all samples	Hybridization solution without PNA also contains PNA telomere probe, or hybridization solution with PNA probe was also added to tubes for unstained samples Hybridization solution with PNA probe spilled into tubes which should be unstained samples	Make sure that hybridization solutions with and without PNA probe are made according to the protocol and that they are pipetted into the appropriate tubes Be careful when pipetting: cross contamination with PNA probe can result in high "autofluorescence"
29–44	Badly duplicated fluorescence signals	Denaturation conditions were not uniform for all tubes	Make sure that the temperature and the time of denaturation are according to the protocol; in our hands a circulating waterbath gives more uniform heating conditions for many tubes than a 96-well heating block
		Wash steps were not uniform for all tubes	Make sure that wash steps are carefully performed according to the protocol – small differences in the dilution of the excess PNA probe can result in unequal fluorescence
33–44	Background fluorescence is too high	Insufficient washing of unbound probe	Increase wash steps following hybridization, make sure that the dilution of the excess PNA probe is more than 10^4 – fold for human blood cells
		Insufficient dilution of excess PNA probe; note, fibroblasts and some other cells have a very high intrinsic autofluorescence, and might bind PNA to cytoplasmic components	Analyze cells by fluorescence microscopy: only the nuclei should show punctuate fluorescence from the PNA telomere probe. If the cytoplasm is fluorescent, compare cells with and without PNA to distinguish autofluorescence from any non-specific binding of the probe; consider isolating nuclei or use

TABLE 2 | Continued.

Step	Problem	Possible reason	Solution
			alternate methods to measure telomere length
33–44	Loss of cells during washes	Gravity force during wash steps too low to sediment cells in formamide-containing solutions	Make sure that gravity force for washes with formamide-containing solutions is at least 1,500*g*
33–44	Loss of cells during washes	Gravity force too high during wash steps, resulting in cell aggregates that are difficult to resuspend, fragmentation and loss of cells	Reduce time or gravity force during wash steps. Use the recommended optimum time and gravity force in the protocol. We leave 100 μl of solution above partially sedimented cells (loose cell pellet) and wash four times to avoid the aggregation and fragmentation of the cells, which are fragile after DNA denaturation
45B	Failure of antibody staining	Epitopes recognized by antibodies or labels are not heat- and formamide-resistant	Some CD45RA, CD20, CD57 epitopes areknown to resist the harsh steps in the protocol – (most) epitopes (recognized by other antibodies) do not
46–52	Subpopulations of cells can not be recognized on the flow cytometer	LDS751 concentration is too high	Make sure that the LDS concentration is according to the protocol
		Inadequate compensation between FL4 and FL3	Adjust compensation using cells with and without individual fluorochromes

ANTICIPATED RESULTS

The type of results that are generated by flow FISH analysis of human nucleated blood cells are shown in **Figure 2**. Note that the histograms shown in this figure are derived from cells that were gated exactly as is shown in **Figure 1**. By including a human reference sample in every experiment and an internal reference in every tube very accurate results can be obtained, and telomere values of duplicate samples that are measured on different days are typically within 0.5 kb or less. The telomere length in human reference cells — which can be granulocytes, as is shown, lymphocytes or another cell population — within unfixed human blood is measured by TRF analysis as described[5,15]. This human reference is used to calculate telomere length as is described in **Figure 2** and its legend. Note that according to this calculation the average telomere length by flow FISH is expressed relative to a measured TRF value. Because some subtelomeric DNA is inevitably included in TRF values, the average telomere length values measured by flow FISH will exceed the actual average length of telomere repeats by around 2–4 kb.

Note: Supplementary information is available via the HTML version of this article.

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COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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