
Original Articles

Telomere Length Measurements in Leukocyte Subsets by Automated Multicolor Flow-FISH

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Background: Telomeres are essential protein-DNA structures at the end of chromosomes which are implicated in genome stability and cell replication. The average length of telomere repeats can be measured by in situ hybridization and flow cytometry [flow-FISH]. Such telomere length values reflect telomere shortening (resulting from cell divisions, oxidative damage and other causes) and telomere elongation (mainly resulting from telomerase activity) of the chromosome-specific telomere length inherited in the gametes. Here we report improvements in flow-FISH methodology that enable measurements of telomere length in subsets of human nucleated blood cells.

Methods and Results: In order to measure the telomere length in granulocytes, naive T cells, memory T cells, B cells and natural killer (NK)/NKT cells within a blood sample, we combined flow-FISH with antibody-staining

(Multicolor flow-FISH). Most steps in the staining protocol were automated using a 96-well microdispenser device. The minimum detectable difference in telomere length and the reproducibility of the method are in the range of 0.2–0.5 kb and measurements can be made with as few as a thousand cells.

Conclusions: Automated multicolor flow-FISH will greatly facilitate studies of telomere length regulation in subsets of nucleated blood cells, especially when only few cells are available and when differences in telomere length are small. Cytometry Part A 55A:1–6, 2003.

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Key terms: telomere; telomere length; multicolor flow fluorescence in situ hybridization; automation

Telomeres are distinctive protein-DNA structures at the end of the chromosomes that are essential for maintaining the integrity and stability of the genome (1–3). To counteract the loss of telomere repeats with each cell division, germline cells, highly proliferating cells from the skin, the gut and hematopoietic tissues as well as tumor cells express telomerase, an enzyme complex with reverse transcriptase activity, which adds new telomere repeats onto chromosome ends (4,5). Despite expression of telomerase, the telomere length in highly proliferating cells from long-lived species decreases during development (6) and with replication in vitro and with age in vivo (7,8). Most likely, telomerase levels in such cells are tightly regulated and are insufficient to make up for the net loss of telomere repeats with cell division. Limiting levels of telomerase in highly proliferating cells from long-lived species may have evolved as a tumor suppressor mechanism (9). That telomerase is critically involved in regulating cellular proliferative capacity is illustrated by the immortalization of somatic cells by ectopic expression of the telomerase

reverse transcriptase gene (10) and, strikingly, by the recent finding that a partial telomerase deficiency in patients with an autosomal dominant form of dyskeratosis congenita results in serious illness from bone marrow failure, immunodeficiency and cancer (11). Paradoxically, loss of telomere length also appears to promote tumor development: critically short telomeres can lead to chromosome fusions and genomic instability (12,13) and have been implicated in human colon and pancreatic cancer (14–

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16). Taken together, these findings highlight the importance of telomeres in human biology and explain the continued interest in methods that can be used to measure telomere length.

A few years ago, we introduced a flow cytometry based method (flow-FISH) using fluorescence in situ hybridization (FISH) with fluorescein isothiocyanate (FITC) labeled peptide nucleic acid probes (PNA) to measure the telomere length in interphase cells (17). We have been seeking additional improvements in the flow-FISH technique with the goals: (1) to measure the telomere length with improved sensitivity, accuracy and reproducibility simultaneously in granulocytes, naive T cells, memory T cells, B cells, and natural killer (NK)/NKT cells within one blood sample even when cell counts are low and (2) to provide the basis to perform this analysis on a larger scale and potentially adopt the method for routine testing.

Here, we describe automation of most flow-FISH steps by a robotic microdispenser device (Hydra by Robbins Scientific), the inclusion of an internal standard and the combination of antibody-staining with quantitative hybridization.

MATERIALS AND METHODS

Preparation and Telomere Length Measurement of Control Cells

Cow thymus was cut in small pieces and gently strained to obtain a single cell suspension. The cells were collected in tissue culture medium (Dulbecco's Minimum Eagle's Medium; cat. no. 36250, StemCell Technologies Inc., Vancouver, BC, Canada) with 0.1 µg/ml DNase (cat. no. D-4513, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), fixed once with 1% formaldehyde (cat. no. B 1011203-76, BDH Inc., Toronto, ON, Canada) for 10 min and washed twice with Dulbecco's phosphate buffered saline (PBS; cat. no. 37350, StemCell Technologies Inc.). Aliquots of cells were frozen at -135°C in PBS containing 10% (v/v) dimethyl sulfate (cat. no. D 2650, Sigma-Aldrich Canada Ltd.) and 40% fetal calf serum (FCS; Hyclone FBC, Logan, UT). The telomere length was determined in unfixed cow thymocytes by Southern blot as described previously (18) and the telomere fluorescence was measured in unfixed and fixed cow thymocytes by flow-FISH as outlined below.

Telomere Length Measurement by Automated Flow-FISH

Details of the basic protocol are described in Baerlocher et al. (19). Cell concentrations of 8×10^2 to 5×10^6 of each sample were pipetted manually into four 200 µl V-bottom polypropylene GeNunc tubes (cat. no. 248151, Nalge Nunc International, Rochester, NY) containing 100 µl of glucose 5% (cat. no. JB0064, Baxter Corp., Toronto, ON, Canada), 20 mM Hepes (H-9897, Sigma-Aldrich), 0.1% (w/v) bovine serum albumin (BSA; cat. no. 126609, Calbiochem-Novabiochem, San Diego, CA) and 2×10^5 control (cow thymocytes) cells. After centrifugation at 500g for 5 min at 4°C , the supernatant was aspirated with a high precision microdispenser device (Hydra, Robbins Scientific, Sunnyvale, CA). In the following steps, the aspiration, the dispensing and mixing was performed with



FIG. 1. Hydra⁹⁶ microdispenser used for automation of various steps in the flow-FISH protocol (high resolution image kindly provided by Robbins Scientific, Sunnyvale, CA).

the Hydra: the cell pellet, recovered in 10 µl 5% glucose/20 mM hepes/0.1% BSA, was gently resuspended with 170 µl of hybridization solution containing 75% deionized formamide (cat. no. 10326, BDH Inc.), 20 mM Tris, pH 7.1, 1% (w/v) BSA and 20 mM NaCl with either no probe (unstained control) or 0.3 µg/ml telomere specific FITC conjugated $(\text{C}_3\text{TA}_2)_3$ PNA probe (kindly provided by Applied Biosystems, Bedford, MA). The cell suspension was kept at room temperature for 10 min prior to denaturation in a circulating waterbath (WB 1120A-1, Lindberg/Blue, Ashville, NC) at 87°C for 15 min. Hybridization was performed for 90 min at room temperature in the dark. Four washes were performed at room temperature by adding 1 ml of 75% formamide, 10mM Tris, 0.1% BSA, 0.1% Tween 20 (cat. no. RO 6435, BDH Inc.) followed by centrifugation of the tubes at 1,500g for 5 min at 16°C and aspiration of the supernatant leaving 100 µl in which the cell pellet was gently resuspended before addition and

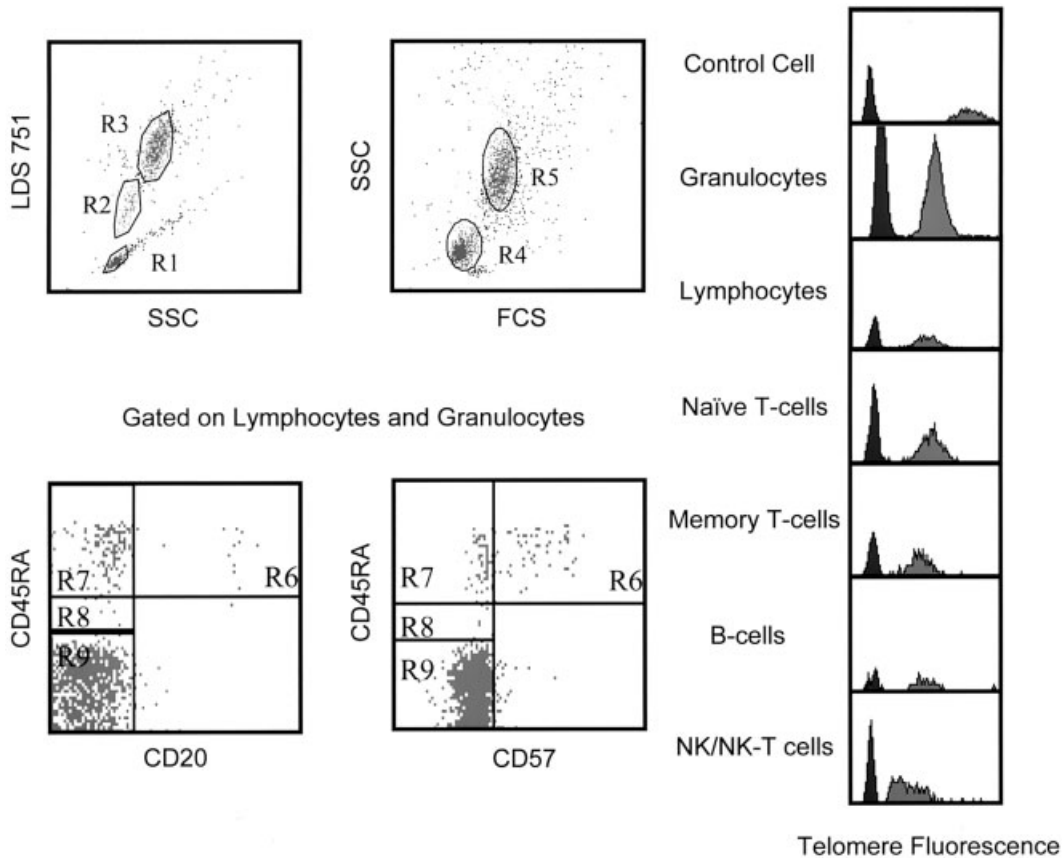


FIG. 2. Telomere length analysis of subsets of human leukocytes from the peripheral blood of one individual by multicolor flow-FISH. To obtain the histograms for the (auto-)fluorescence and telomere fluorescence of each subtype of cell, we used the following gating strategy. (a) control cells (R1 + R4), lymphocytes (R2 + R4) and granulocytes (R3 + R5) can be distinguished based on their staining with LDS 751 and on their forward and light scatter properties. (b) Naïve T cells (R2 + R4 + R7), memory T cells (R2 + R4 + R9) and B or NK/NKT cells (R2 + R4 + R6) can be separated by additional gating based on their staining with anti-human CD45RA, anti-human CD20 and anti-human CD57. Note the differences in median telomere fluorescence and distribution pattern of telomere fluorescence for each cell type from one individual.

mixing with 1 ml of fresh wash fluid. The last wash step was performed with 1 ml of 5% Glucose containing 10 mM Hepes, 0.1% BSA and 0.1% Tween 20 (BDH Inc.) and centrifugation was at 900g for 5 min at 16°C. The cell pellet was mixed and the cells were counterstained with FACSFlow (cat. no. 342003, Becton Dickinson, San Jose, CA) containing 0.1% (w/v) BSA, RNase A at 10 µg/ml (cat. no. 109207, Boehringer Mannheim, Laval, CA) and LDS 751 (Exciton Chemical Co. Inc., Dayton, OH) at 0.01 µg/ml for 20 min. The acquisition of light scatter and fluorescence signals was performed on a FACSCalibur (Becton Dickinson) and the analysis was performed either with CellQuestPro (Becton Dickinson) or FlowJo (Tree Star Inc., San Carlos, CA). A mixture of 4 populations of FITC labeled beads (cat. no. 824p-C, Bangs Laboratories, Fishers, IN) was run for each experiment to convert fluorescence into molecular equivalents of soluble fluorochrome (MESF). The specific fluorescence of each cell type per sample was calculated as the median telomere fluorescence measured in cells hybridized with the FITC labeled telomere PNA probe minus the median (auto-)fluorescence of unstained controls and for each sample the

mean of duplicates was calculated. The specific fluorescence of the control cells served as an internal standard which was used to convert the MESF values of cells of interest within a tube into an average telomere length in kilobases (kb) in those cells by the formula:

$$\frac{(\text{MESF value test cells} - \text{MESF value unstained test cells}) : 92^a}{(\text{MESF value control cells} - \text{MESF value unstained control cells}) \times 1.22^b : 120^c} \times 15.0 \text{ kb}^{d,e}$$

where a = number of chromosome ends in a normal diploid human cell, b = correction factor to compensate for the measured difference in median telomere fluorescence between fixed and unfixed control cells (fluorescence fixed cells was 82% of that in unfixed control cells in two separate experiments), c = number of chromosome ends in each control cell (bovine thymocyte), d = measured telomere restriction fragment size in DNA from control bovine thymocytes, and e = assuming the length of subtelomeric DNA in terminal restriction fragments is

similar in human and bovine DNA and that internal T₂AG₃ repeats in human and bovine chromosomes do not contribute significantly to the telomere fluorescence.

Flow-FISH Combined With Immunostaining

After the washes, cells were spun down and incubated for at least 20 min with 30 μ l antibody solution containing anti-human CD45RA-Cy5 and anti-human CD20-PE, or with anti-human CD45RA-Cy5 and anti-human CD57-biotin (Becton Dickinson) and in a second step with streptavidin-PE (cat. no. S-21389, Molecular Probes). One wash with FACSFlow (Becton-Dickinson) containing 5% FCS and 0.1% sodium azide (cat. no. B30111, BDH Inc.) was performed after each incubation with the antibody solution. Cells were then counterstained with LDS 751 (Exciton Chemical) as mentioned above. Compensation was performed individually for each sample between FL1 and FL2 since any fluorescence from the PE-labeled antibodies could interfere with the quantitation of the telomere-PNA-FITC fluorescence. With the concentrations used for the telomere-PNA-FITC probe (0.3 μ g/ml) and the PE-labeled antibodies (0.1 μ g/ml CD20-PE, 2 μ l/test CD57-Biotin and 2 μ g/ml streptavidin-PE) compensation between FL1 and FL2 was in the range of 5-15%.

RESULTS AND DISCUSSION

Automation and Internal Standard

Figure 1 illustrates the Hydra⁹⁶ microdispenser device. This bench top instrument features 96 syringes arranged in a standard array format. Hydra microdispensers can be programmed for precise microliter dispensing, aspiration and mixing of reagents into 96-well plates or tubes and are used extensively for genome analysis and drug discovery programs (20). To our knowledge, application of this instrument has not been described for work with cell suspensions. After confirming that we could use this instrument to process viable and heat and formaldehyde treated cells, we adapted our standard flow-FISH protocol (19) to use the Hydra for most of the steps required in our protocol. As a result, the variability in cell recovery for flow-FISH processed cells and the variation in telomere fluorescence decreased significantly. In addition, the volumes of solutions and reagents used in most steps of the protocol could be reduced by about 50% and, compared to the manual procedure, 2-6 times more samples could be processed in a single experiment.

To increase the accuracy of measurements, we tested the addition of control cells as an internal standard as was suggested by Hultdin et al. (21) We chose diploid cow thymocytes as an internal control, which are abundantly available, have about two times longer telomeres than human cells and which proved to have very little variation in telomere length between cells. After mild fixation with 1% formaldehyde, such fixed cow thymocytes can be easily distinguished from unfixed, flow-FISH processed cells based on their weaker staining with the DNA dye LDS 751 as seen in Figure 2.

After optimization and standardization of the flow-FISH protocol and automation of most steps, the coefficient of

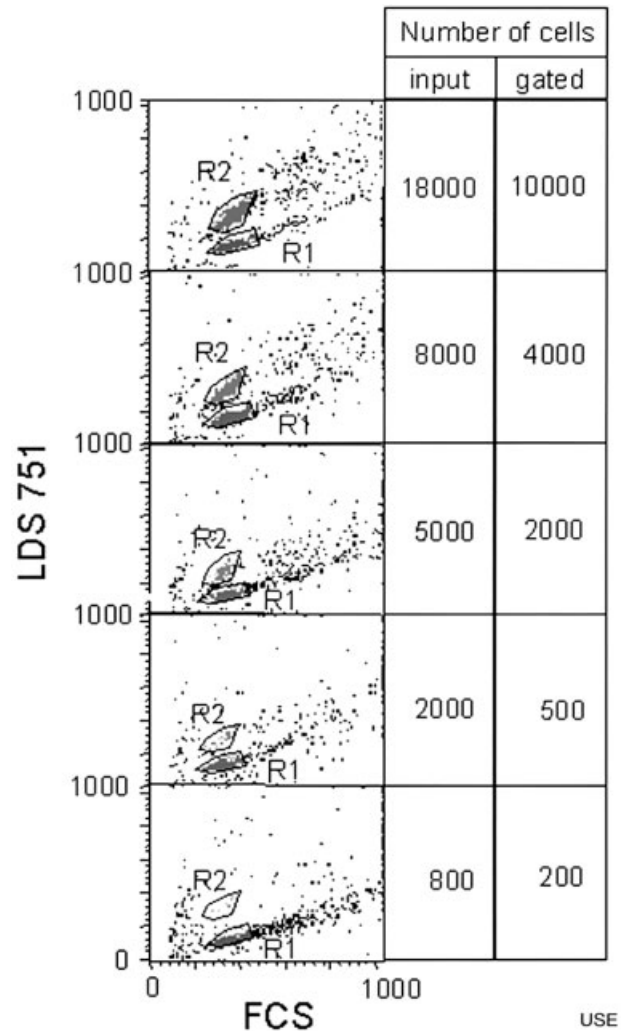


FIG. 3. Titration of lymphocyte cell count and recovery of lymphocytes after flow-FISH. Gating on control cells (R1) versus lymphocytes (R2) based on their LDS 751 staining and forward scatter properties becomes more difficult with a decreasing number of lymphocytes (input 18,000-800 cells). Furthermore, the lymphocyte cell recovery (cell number gated in relation to cells put in the tube before the flow-FISH process) decreases with a decreasing initial cell number.

variation in telomere fluorescence (in MESF $\times 10^{-3}$) of our internal standard measured from tube to tube and from experiment to experiment ranged from 2.6-6.3% and was on average around 4% (which corresponds to approximately 0.6 kb). Using the cow thymocytes as internal controls (stored in frozen aliquots and added to each tube) when measuring telomere length in other cell types, we were able to reduce the variation in calculated telomere fluorescence in human hematopoietic cells from 5-10% (17) to 2-5% within one experiment and from 20-30% (17) to 5-10% from experiment to experiment. As a result, the minimum detectable difference in telomere length for human, baboon, cow and murine hematopoietic cells is now in the order of 0.5 kb. Comparison of telomere length measurements by Southern blot with telomere

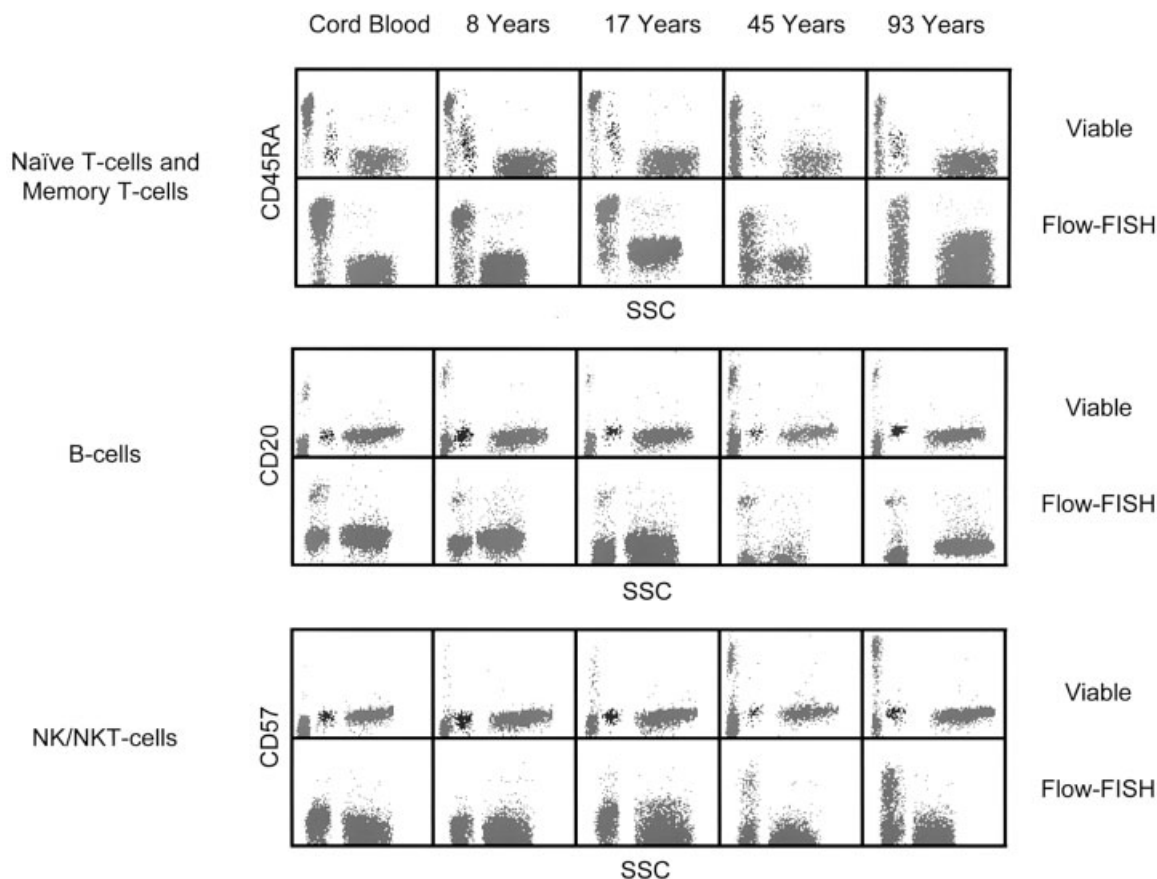


FIG. 4. Antibody-staining with anti-human CD45RA, anti-human CD20 and anti-human CD57 on viable and flow-FISH processed hematopoietic cells from the peripheral blood of 5 different human donors aged 0-93 years. Note the excellent correlation between the number of cells stained with the different antibodies before (viable cells) and after flow-FISH. Also note that the percentage of CD45RA positive cells decreases with age whereas the percentage of CD57 positive cells increases with age.

length measurements by the improved and automated flow-FISH method showed a high correlation ($R = 0.87$, results not shown). Importantly, differences in telomere length in cells with very short or very long telomeres were easily detectable by flow-FISH, where accurate measurements by Southern blot failed.

Automation for large scale analysis is only useful when multiple samples can be measured at the same time. For this purpose, we tested out whether we could use previously frozen nucleated blood cells to perform telomere length analysis by flow-FISH. Indeed, telomere length measurements on fresh or frozen cells yielded highly reproducible results (± 0.5 kb), whereas much higher variations were observed when measurements were performed by Southern blot. In addition, we could assess the telomere length in samples of previously frozen human lymphocytes containing only a few thousand cells as is shown in Figure 3.

Many of the conditions and processing steps during the flow-FISH protocol lead to cell fragmentation and cell loss. Previously, the recovery of flow-FISH processed cells was in the range of 1-10%, which therefore required cells in the range of 10^5 per tube. Most likely, adaptations in the

basic flow-FISH protocol (19), the prior freezing of cells and the gentle mixing of the cell suspensions by the Hydra microdispenser helped to improve the cell recovery (up to 25-50%), as is shown in Figure 3.

An important caveat of the flow-FISH approach to measure the average telomere length is that the cells of interest must be diploid as is typically the case for circulating nucleated cells in normal individuals. However, telomere fluorescence values of aneuploid cells or cells that are in cycle (e.g. from pathological specimens or normal bone marrow) will give rise to significant errors in calculated telomere length values. Ideally, telomere fluorescence measurements should be combined with more accurate DNA measurements than can be obtained with LDS 751.

In Situ Hybridization and Immunostaining

Telomere length measurements in subsets of cells within one sample are of major interest to determine the different replicative histories of cells and to look at telomere length regulation in subsets of cells in vitro and in vivo. Prior cell sorting can be quite costly and time consuming and high cell numbers have typically been necessary to measure telomere length. Therefore, the combina-

tion of immunostaining with in situ hybridization would be a major advance in this technique. One of the big challenges here is the combination of immunostaining with quantitative in situ hybridization. In our hands, any linker or fixative reagent (even in low concentrations) such as Bis (sulfosuccinimidyl)suberate (BS3) or formaldehyde to attach antibodies labeled with heat stable fluorochromes to the cell surface prior to in situ hybridization or to preserve epitopes during exposure to heat and formamide as suggested by others (22,23), resulted in a variable decrease in hybridization efficiency. However, we found that some epitopes (typically recognized by antibodies used for immunohistochemistry), still recognize their epitopes after formamide and heat treatment following the flow-FISH protocol (24). We used such antibodies (anti-human CD45RA, anti-human CD20, anti-human CD57) to stain both viable cells and flow-FISH processed leukocytes from the same human donor (Fig. 4). The percentage of positive cells for all three antibodies correlated well between viable cells and flow-FISH processed cells. Note that the percentage CD45RA positive cells and CD20 positive cells decreased with age whereas the percentage CD57 positive cells increased with age as was previously described (25). The results of Multicolor flow-FISH using these antibodies to measure the telomere length in diverse leukocyte subsets within one sample are shown in Figure 2. The mean or median telomere fluorescence as well as the heterogeneity in telomere fluorescence within the population of each cell type can be assessed. As shown in the right panel of Figure 2, the telomere fluorescence, the autofluorescence, as well as the distribution in telomere fluorescence varies from cell type to cell type within a single individual.

In summary, we improved the accuracy and versatility of the flow-FISH technique to measure the telomere length in interphase cells and we demonstrate the feasibility to measure hexanucleotide repeats in rare but biologically relevant subsets of human nucleated blood cells.

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