Telomere length measurement by FISH and flow cytometry

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Abstract

Telomere length is an important measure of cellular differentiation and progression to senescence. Flow cytometric assays for measuring telomere length have become an important adjunct to more laborious Southern blotting methods; telomere length can be estimated with considerable accuracy in small numbers of individual cells by flow cytometry, and can be measured in cell population subsets with simultaneous fluorescent immunophenotyping. In this chapter, we describe the standard flow cytometric assay for measuring telomere length, including the incorporation of fluorochrome-conjugated antibody immunolabeling for measurement in cell subsets.

1. Introduction

Telomere length has been actively investigated in the latest decade for its relationship to the telomerase activity and its involvement in several major diseases and in the aging process ***(1,2)***. Telomerase, the enzyme responsible for maintaining telomere length, is essential and strictly regulated for the synthesis of telomeres in normal cells during development ***(2)***. Telomeres are GT-rich sequences present in DNA as chromosomal end-caps that interact with telomeric binding proteins providing various genetic and cellular functions such as genomic integrity and stability ***(1,3)***. For normal human somatic cells presenting minimal or no telomerase activity, the telomere length decreases at each step of the cell division ***(4,5)***. It has been concluded that telomere length acts as a possible internal cellular clock or feed-back checkpoint, providing a marker for the number of accumulated cell divisions and controlling the onset of cellular senescence ***(5-6)***.

Telomerase activity appears to be highly correlated to the onset of various diseases. An increase in telomerase activity leads to telomere synthesis, resulting in higher stability of the genome with greater telomere length ***(1)***. On the other hand, an accelerated reduction of the telomere length reaching critical limits provides a senescent signal to stop cell division. Such excessive conditions can alter the homeostasis of normal cellular aging, and can result in a number of age-related diseases ***(7-9)***.

Indeed, the number of cell replication for normal cells cannot exceed the Hayflick limit, the number of cell divisions that can occur prior to the onset of senescence. Above this

number, the critical telomere length will trigger the senescent signal to stop growth ***(10)***. For other cells, by selective inactivation of cell cycle check points and by massive selective death, cellular immortilization is observed by maintaining or stabilizing the telomere length above the critical limit with the presence of telomerase activity such in tumor or cancer cells ***(11,12)***. Controlling the telomerase activity to stabilize or to push the telomere length towards defined ranges has been a goal for various therapeutic purposes ***(13-15)***. Before being able to modify the telomerase activity, it is essential to have an accurate assessment of its biomarker, the telomere length.

It is therefore necessary to develop a reliable, rapid and sensitive biomedical technology for the determination of the telomere length as an important physiological marker for diseases and treatments. Traditional methods for determining telomere length have generally required whole genomic DNA extraction and Southern blotting for the telomere repeat, a labor-intensitve procedure that does not allow estimation of telomere length in individual cells. A fluorescence-based *in situ* telemere-length assay would have significant advantages over the traditional approach, including the integration of fluorescent immunophenotyping for identification of telomere length in specific cell subsets. Flow-FISH, an *in situ* flow cytometric assay utlilizing fluorochrome-tagged telomere-complementary oligo probes to estimate telomere length, has become widely used for this purpose ***(16-23)***. This chapter will focus on the general principles for the determination of the telomere length by flow cytometry. It will describe techniques to standardize the assay, calibrate the flow cytometer, and quantify telomere length. An

additional protocol for combining phenotyping and telomere length quantification will also be discussed.

2. Materials

2.1. General Supplies

- 1X Phosphate-buffered saline (PBS) without Ca++ and Mg++
- Centrifuge
- Vortex mixer
- Water bath or heating block for 40^0C and 82^0C
- 1.5 ml Eppendorf tubes
- disposable 12 X 75 mm Falcon tubes
- Cell counter or Hemocytometer
- Flow cytometer

2.2. Reagents

2.2.1. Calibration of flow cytometer

- *Quantum^{TM}24 premixed FITC MESF beads* (Flow Cytometry Standards Corp, San Juan PR).

2.2.2. Fluorescence in situ hybridization

- *PNA (peptide nucleic acid) fluorescence probe* for the telomere repeat sequence.

Fluorescein–conjugated PNA probes can either be specifically synthesized (PerSeptive Biosystems, Framingham, MA ***(17)*** or Boston probes, Bedford, MA ***(22)***)) or purchased as a kit (Telomere PNA Kit/FITC) from DAKO, Viareal Carpinteria, CA)**.** *See* **Note 1.**

- *Hybridization buffer* (70% formamide; 20mM Tris-HCl, pH 7.0; 1% BSA with/without 0.3µg/mL PNA probe)
- *Washing buffer* (70% formamide; 10m*M* Tris; 0.1% BSA and 0.1% Tween 20)
- *Resuspension buffer with DNA stain* (PBS with 0.1% BSA) *with DNA stain* (either *propidium iodide* (PI) at 0.06 µg/mL with DNase-free RNaseA at 10 µg/mL; or 7-aminoactinomycin D at 0.01 µg/mL with no RNase). *See* **Note 2.**

3. Methods

3.1. Standardization and Calibration

The fluorescence *in situ* hybridization technique presently used for labeling telomeres relies on the introduction of specific synthetic peptides that mimic the DNA sequences complementary to the telomere sequence. These synthetic peptides are labeled with low molecular weight fluorochromes allowing a quantitative measurement by flow cytometry of the number of probes non-covalently bound to the telomeric sites. The peptide nucleic acid (PNA) probe specific for the telomere repeat sequence ($(CCCTAA)_3$-PNA) has proven to be very reliable for FISH analysis for telomere length measurements ***(16-25)***. PNA probes have significant advantages over traditional cDNA oligo probes, including

reduced non-specific binding to DNA, resistance to nuclease activity and strong binding stability to the telomere sequence. The telomere PNA probe is specific for the repetitive end sequences of the X chromosome; therefore, only the telomere lengths of this chromosome will be determined. The mathematical association between PNA probe binding and fluorescence and the approximate number of telomere repeats has been previously determined empirically by comparison to Southern blotting data and can be applied to a variety of cell types ***(16);*** however, the quantification of the number of non-covalently bound PNA probes per telomere repeat needs to be calibrated and standardized for each flow cytometer and directly linked to some internal fluorescence value. These arbitrary units are then converted to the telomere length in kbs.

3.1.1. Assay standardization And MESF calibration

To compensate the differences existing between flow cytometers and their daily characteristics (laser intensity responses, change in alignment, etc.), a standardization/calibration procedure is necessary to accurately quantify telomere length ***(16,18)***. "Molecules of equivalent soluble fluorochrome" (MESF) Quantum^{TM}24 fluorescent beads (Bangs Laboratories, Fishers, IN, formerly Flow Cytometry Standards Corporation) are used to both calibrate the individual instrument and to establish a fluorochrome-based standard curve for the assay. These beads have known numbers of fluorochrome molecules on their surfaces, allowing for the calibration and linearity determination of the flow cytometer. Thus, a particular fluorescence intensity value on a flow cytometer can be correlated with an actual number of fluorochrome molecules; if the number of fluorochrome molecules attached to a PNA probe is known, a standard

fluorescence curve can be established that will correlate relative fluorescence signal with the number of PNA probes bound (and the number of telomere repeats) per cell. An example of this is shown in **Figures 1** and **2**. A "cocktail of unlabeled and labeled MESF beads with progressively larger numbers of bound fluorochrome molecules (such as fluorescein (FITC) or phycoerythrin (PE)) is analyzed by flow cytometry, and the population of unlabeled beads adjusted between channel numbers 1 to 10 (the first log decade of a four-log scale). A mixture of beads with different MESF values are then analyzed at the same instrument setting (**Figure 1**). Ideally, the resulting profile should give a linear relationship between different MESF beads, assuming the flow cytometer detector gives a linear response over its entire dynamic range (**Figure 2**). The linear relationship between the MESF beads and the fluorescence channel is then calculated using linear regression.

$$FLchannel\#-FLchannel\#(blank) = Slope * MESF\# \qquad [1]$$

Detector linearity for the fluorochrome in question (such as PE) can thereby be evaluated for individual flow cytometers, a necessary requirement for telomere length measurement (*see* **Note 3**). The slope of the regression line as shown in **Fig.2** will also be subsequently used for the determination of the telomere length of the samples. Ideally, a MESF bead calibration should be incorporated into every flow-FISH assay to account for day-to-day instrument variations.

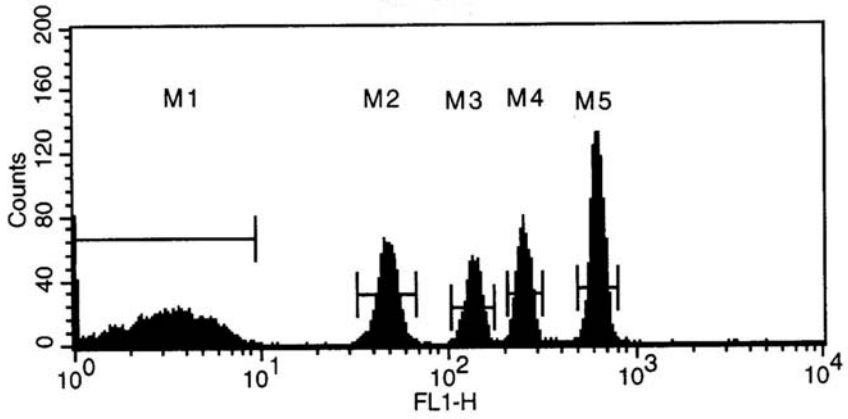


Figure 1. FL-1 Histograms of MESF beads mixtures (Quantum^{TM} 24 Premixed from Bangs Laboratories / FCSC). Region M1 corresponds to the blank beads; regions M2 to M5 correspond to the labelled fluorochrome beads with varying MESF values

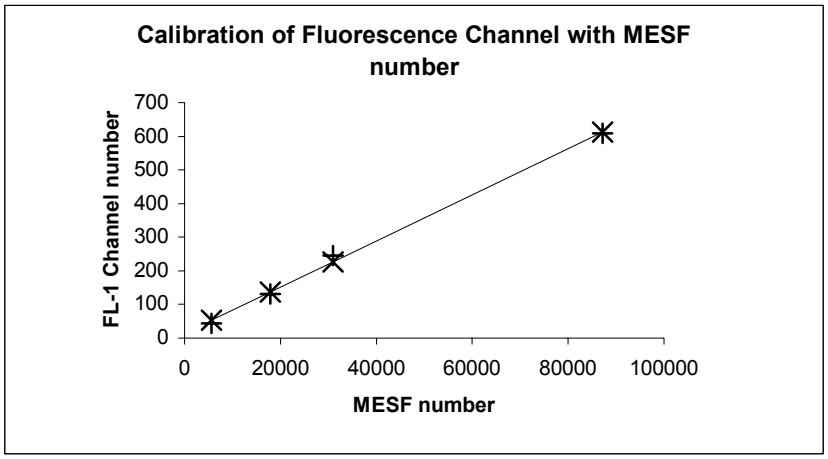


Figure 2. Typical calibration of the FL1 channel of flow cytometer using molecules of Equivalent Soluble Fluorochrome (MESF). The MESF numbers are lot-specific and are determined by the bead manufacturer. (+) ticks corresponds to the recorded FL-1 channel number and (-x-) ticks to the adjusted value of the detector channel number using a linear regression method.

3.1.2. Flow Cytometer Calibration For Telomere Length Using A Conversion Line

Rufer et al. ***(16)*** has previously calculated the correlation between telomere fluorescence measured by flow-FISH using a FITC-conjugated PNA probe, and the telomere length determine by Southern blots for different subpopulations of lymphocytes. This correlation can be generally applied for many cell types. In this system, FITC fluorescence was arbitrarily quantified in terms of a flow cytometer channel number by the equation ***(14)***:

$$TelomereLength(kb) = 0.019 * (\text{FLchannel\#-FLchannel\#(blank)})_{\text{Rufer et al.}} \quad [2]$$

A later study from Rufer et al. ***(16)*** used a more quantitative system using FITC-conjugated MESF beads to calibrate the FITC channel values as described above. Taking the MESF value as a standard unit instead of the arbitrary channel number for FITC, the telomere length from Southern blot is therefore expressed in MESF units using the following equation ***(16)***:

$$(FLchannel\# - FLchannel\#(blank))_{Ruferetal.} = MESF * 0.02604 \quad [3]$$

Eliminating the arbitrary units from equations 2 and 3 provides the final expression of the telomere length in standard MESF units ***(16,18)***:

$$TelomereLength(kb) = MESF * 0.019 * 0.02604 \quad [4]$$

This equation is independent of the flow cytometer used for the assay if it is calibrated with the same standardized MESF beads. Eliminating MESF units from equations 1 and 4 provide a direct calibration of the flow cytometer FITC channel for the determination of the telomere length by flow-FISH using the following equation:

$$TelomereLength(kb) = (FLchannel\# - FLchannel\#(blank)) * 0.019 * 0.02604 / Slope \quad [5]$$

3.2. Experimental Protocol for Quantifying Telomere Length

Cells should be reduced to single cell suspensions prior to labeling. Each cell sample should be divided, one fraction for incubation with the PNA probe, and one without as a background control. Duplicate or triplicate samples are highly recommended if cell sample numbers allows it (*see* **Note 4**). It is of critical importance to run control cells with each assay to check the hybridization process. Ideally, cell controls for both short- and long-telomere lengths should be run simultaneously with actual samples. Hultdin et al. ***(17)*** have used 1301 cell line with in each sample as internal control. These immortalized cells have very long telomeres and are easily distinguishable from the other cells. Other cell lines are also available for control. Our laboratory has used CCRF-CEM cells as long telomere length controls and Jurkat cells as short telomere length controls. These controls are run with each experiment as separate samples to allow comparisons between multiple experiments run on different days. Once control cell lines were identified, cells with the same passage numbers were aliquoted in large numbers and frozen before being used giving a reliable source of internal controls (*see* **Note 4**).

3.2.1. Sample Preparation

- Wash cells in PBS (w/o Ca^{2+}/Mg^{2+}) containing 0.1% BSA.
- Count cells and add $0.5x10^6$ cells to 1.5 mL Eppendorf tubes. Centrifuge cells at 500 x g to pellet, and decant supernatant.
- Resuspend the pellet in 300 µL of hybridization buffer with PNA probe at 0.3µg/mL (90 ng/sample final concentration). A corresponding tube without PNA probe should be included with each sample as a negative control. Some titration of PNA concentration may be necessary for different probe preparations and cell types.
- Incubate the tubes in an 82^0C heat block or water bath for 10 minutes. *See* **Note 5.**
- Mix the samples by vortexing and incubate them for 2 hours ***(16,24)*** or overnight ***(17)*** at room temperature in the dark for hybridization.
- Resuspend the cells in 1 ml wash buffer, mix and incubate in a 40^0C water bath or heating block for 10 minutes.
- Centrifuge the cells at 500 x g for 7min. Decant the supernatant and repeat the above step.
- Resuspend the cells in 500 µL of DNA staining buffer (PBS, 0.1%BSA, 10 µg/mL RNase A with PI at 0.06 µg/mL, or 7-AAD at 0.01 µg/mL without RNase). Transfer the cells to standard 12 x 75 mm polystyrene flow cytometry tubes and incubate at room temperature for 2-4 hrs.
- Analyze the cells in the flow cytometer.

3.2.2. Flow Cytometry

The calibration and linearity of the flow cytometer should be checked at the beginning and at the end of the experiment.

- Run the fluorescent MESF beads prior to cell samples to establish the standard fluorescence curve, as described in Section 2.
- Analyze the cell samples, leaving the FITC detector (usually denoted arbitrarily as FL1) at the same voltage setting as that used for the MESF beads. Initially visualize the cells for forward scatter (FSC) versus DNA dye fluorescence, both with linear scaling (**Figure 3A**). For both PI and 7-AAD, this will usually be done through the instrument's long red detector. The detector voltage settings the forward scatter and DNA dye fluorescence can be changed from the MESF settings. All fluorescence compensation settings should be set to zero. *See* **Note 6.**
- Draw a gate around the G0/G1 cell cycle phase, and display a histogram for FITC fluorescence gated on this population (**Figure 3B**). ***(17)***.
- Record the data as a listmode file including forward scatter (linear), DNA dye (linear) and FITC PNA probe fluorescence (log) as saved parameters.

3.2.3. Data analysis

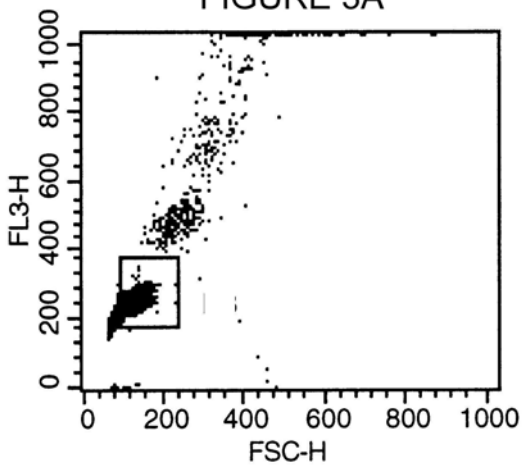
- As described in **Section 3.1.1.**, linear regression between the MESF quantum beads and the FITC channels is calculated for the flow cytometer used for the experiment using equation 1:

$$FLchannel\#-FLchannel\#(blank) = Slope * MESF\# \quad [1]$$

- Gate the single G0/G1 cell population using FSC vs. PI or 7-AAD as described above (**Figure 3A**), and gate into a FITC histogram displaying the FITC PNA probe fluorescence (**Figure 3B**). Record the mean fluorescence intensity (MFI) for both PNA probe and control samples. Subtract the MFI value of the control cells from the PNA probe labeled cells. The telomere length can then be calculated from the MSEF standard curve using equation 5:

$$TelomereLength(kb) = (FLchannel\#-FLchannel\#(blank)) * 0.019 * 0.02604 / Slope \quad [5]$$

FIGURE 3A



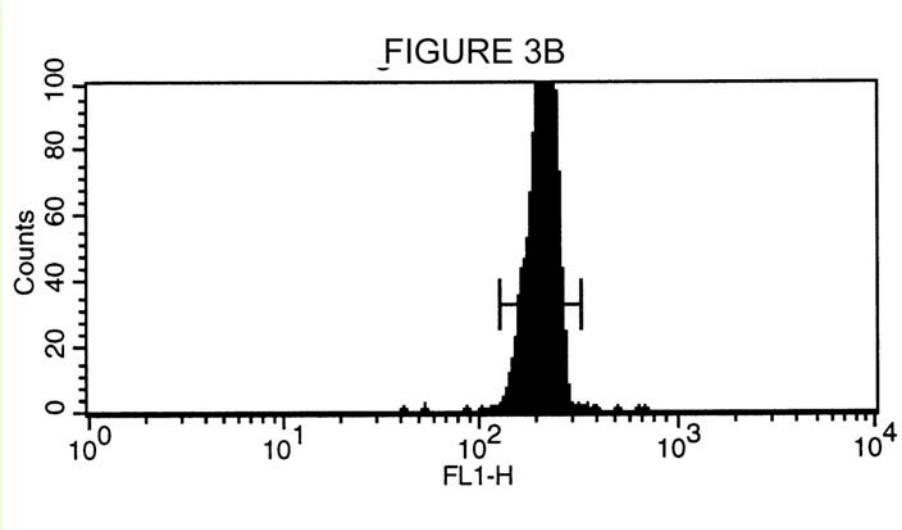


Figure 3. **(A)** Dot plot of forward scatter (FSC) vs. PI fluorescence (FL3). Boxed or gated cells are in the G0/G1 phase cell cycle phase. **(B)** Histogram of FITC intensity for gated cells from **Fig. 3A**.

3.2.4. Data example using human CD4 naïve and memory T cells

A useful test of this assay is to measure the telomere lengths of naïve and memory T cells isolated from normal human PBMCs; memory T cells would be expected to have shorter telomere lengths than naïve. The results of this experiment are shown in **Figure 4**. Jurkat and CCRF-CEM cell lines were simultaneously used as short and long telomere controls, respectively. CD4-positive naïve and memory cells were obtained from the same donors by fluorescence-activated cell sorting, based on their expression of CD4 and presence or absence of the memory marker CD45RO. The results are shown Figure 4 for five independent assays using the same cell populations. Reproducibility between replicates was excellent based on the standard deviation, and sensitivity between naïve and memory telomere length was easily detectable based on the T-test analysis, which gave a p-value of 0.018 (n=5).

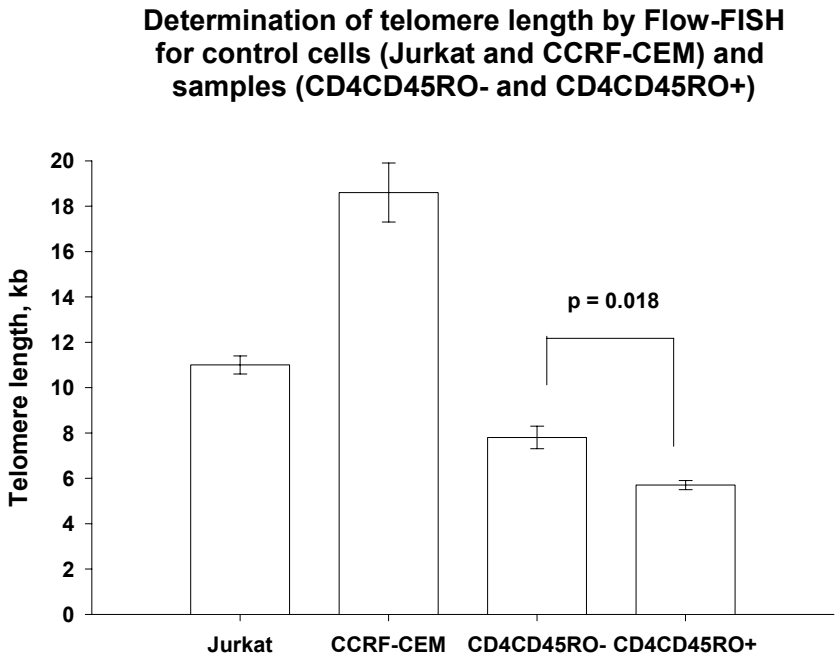


Figure 4. Telomere length determination using flow-FISH for CD45RO-negative naïve and CD45RO-positive memory CD4+ T cells.

3.3. Experimental protocol for phenotyping and telomere length quantification

A key advantage of using flow cytometry to measure telomere length or any cell characteristic is the ability to measure multiple fluorescent parameters simultaneously in the same cell; telomere length measurements can therefore be made in cells simultaneously labeled for cell surface markers, a valuable method for characterizing telomere length in diverse populations of immune cells. In order to use the above method for complex blood cells or tissues, samples have to be physically sorted prior to telomere length for the different populations (as was done for the naïve and memory T cell subsets in **Figure 4**). Incorporation of fluorescent immunophenotyping using fluorochrome-conjugated antibodies would eliminate the need for subset isolation; however, to measure the telomere length, cells have to be heated to 82^0C, and many fluorochrome nor antibody-antigen complexes cannot withstand these conditions ***(20)***. Recently Batliwalla et.al. ***(24)*** published a

procedure using one color cell surface marker in conjunction with the measurement of telomere length. The low molecular weight monomeric cyanin probe Cy5 flurochrome was used as a secondary label since it is stable at high temperatures ***(24)***. In addition, the antibody-surface antigen complex was stabilized with a covalent crosslinking reagent, protecting it from heat treatment.

3.3.1. Additional Reagents

- *Bis(sulfosuccinimidyl) substrate (BS3)* (Pierce, Rockford, IL)

  This reagent is used for crosslinking phenotyping antibodies to the cells surface prior to heat denaturation. The powdered stock should be stored desiccated at –20°C. Solutions of BS3 should be used promptly and the remainder discarded.

- *Stop buffer* (100m*M* Tris-HCl, pH 7.0 and 150 m*M* NaCl)

- Cy5-conjugated antibody against the marker of interest

  Cy5-conjugated secondary antibodies and streptavidin can be obtained from Caltag (Burlingame, CA) or Jackson ImmunoResearch (West Grove, PA). Kits for direct conjugation of Cy5 to most antibodies can be obtained from Amersham Biosciences (Piscataway, NJ). *See* **Note 7.**

- Flow cytometer equipped with two laser, a 488nm argon-ion and a 633nm red HeNe or 635 nm red diode

  Cy5 requires a red laser for excitation, usually a HeNe 633 nm or red diode 635 nm source. Most cell sorters and several commercial bench top flow cytometers offer this option. *See* **Note 8.**

3.3.2. Cell surface labeling

- Count $1x10^6$ cells and label with either the directly conjugated Cy5 antibody, or a biotinylated antibody against the surface marker of interest for 25 minutes at 4^0C.
- If using directly conjugated antibodies, centrifuge wash the labeled cells with 4 mL PBS containing 0.1% BSA and resuspend the cell pellet to 100 µLof PBS.

3.3.3. Cross-linking of Antibody

Prior to the 82^0C denaturation step, the Cy5 label complex is stabilized by crosslinking with bis(sulfosuccinimidyl) substrate (BS3) (Pierce, Rockford, IL). BS3 is water-soluble and acts by cross-linking primary amines. It covalently bridges the antibody-fluorochrome complex to the cell surface.

- Prepare BS3 at 2 mM stock concentration in PBS. BS3 should be freshly prepared for each experiment, since it rapidly hydrolyzes in solution. To cross-link cells, add an equal volume of BS3 solution to the resuspended cell pellet (usually about 100 µl) and incubate for 30 min. at 4^0C.
- Quench the excess BS3 by adding 1 ml of stop buffer (100 m*M* Tri-HCl, pH 7.0 and 150 m*M* NaCl) for 20 min.
- Centrifuge the cells and proceed with protocol in Section 3.2.1.

3.3.4. Analysis

- Analyze the sample for forward scatter and DNA dye fluorescence and gate on single G0/G1 cell population as described in **Section 3.2.2.**
- Analyze this gated G0/G1 cell population for Cy5 fluorescence using a histogram set to the appropriate fluorescence channel, and gate on the Cy5 positive cells.
- Analyze this gated G0/G1 Cy-5^+ cell population in the FITC PNA probe histogram as described in Section 3.2.3. (*see* **Note 8**).
- Proceed with the measurement of telomere length as described in **Section 3.2.3.**

3.4. Recent Developments

It is theoretically possible to perform immunophenotyping for multiple surface markers in combination with flow-FISH for characterization of multiple subpopulations in a complex sample. However, analysis is limited to fluorochromes that are sufficiently heat-stable. The ever-increasing variety of low molecular weight fluorochromes available for flow cytometry (including the Cy dyes from Amersham and the Alexa Fluor series from Molecular Probes, Eugene, OR) are providing a number of likely candidates. Phycobiliproteins, extremely bright protein fluorochromes commonly used in flow cytometry, are unstable at high temperatures even with covalant cross-linking and are not recommended for flow-FISH. Recently Schmidt et al. ***(25)*** has published positive results using Alexa Fluor 488 and Alexa Fluor 546 for simultaneous immunophenotyping with a Cy5-conjugated PNA probe and Hoechst 33342 for DNA analysis; a multiple-laser flow cytometer with red and UV excitation sources was necessary for this combination.

4. Notes

1. **Kits.** The components of the flow-FISH can be assembled separately, or the system can purchased in kit form (such as the FITC-PNA flow-FISH system from DAKO). When using a kit, it is recommended to follow the manufacturers directions. Cy5 immunophenotyping can be easily incorporated into these kits.
2. **DNA binding dyes.** Propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) can both be used for flow-FISH assays. Propidium iodide can be obtained from many suppliers; it is well-excited by 488 nm argon-ion lasers and emits in the 570 – 620 nm range. 7-aminoactinomycin D can be obtained from Sigma (St. Louis, MO) or Molecular Probes (Eugene, OR). It is also well-excited by blue-green lasers and emits farther in the red, with an emission maxima of 650 nm. Both can be analyzed in the far red detector (often designated "FL3") on most commercial flow cytometers. While both dyes work well for flow-FISH, 7-AAD might be preferable in some systems due to its better spectral separation from FITC.
3. **Instrument linearity.** Flow cytometer photomultiplier tube linearity can depend on a number of factors, including the detector itself and the log amplifer circuits of the cytometer. Generally detector linearity does not extend to all four log decades of most commercial flow cytometers; linearity usually starts to fall off in the first and fourth log decades. Most flow-FISH samples will fall within these boundaries. If a detector appears to be non-linear throughout its entire dynamic range, both the PMT and the log amp circuits can be replaced to detect this problem. While this problem will affect all flow cytometric analysis, it is

particularly acute in quantitative flow techniques like flow-FISH. The recent advent of fully digital flow cytometers (rather than the hybrid analog-digital systems available earlier) will reduce the errors introduced by electronic log conversion and should improve apparent detector linearity considerably.

4. **Controls.** A common problem in designing flow-FISH assays is the identification of good long-telomere controls. Cell lines derived from fetal tissue or pediatric tumors would be expected to have long telomeres; however, these cell lines are often only available in isolates that have undergone multiple passages, resulting in eventual telomere shortening. The 1301 cell lines has been used previously as a control; however, any cell lines (particularly ones with a long passage history) should be scrutinized carefully prior to use. Cord blood lymphocytes may make a useful long-telomere control if they can be obtained in sufficient quantity.
5. **Denaturation/hybridization temperature.** The denaturation and hybridization temperatures are critical parameters; they cannot vary be more than +/- 2^{o}C.
6. **Instrumentation.** Most commercial flow cytometers are equipped with a 488 nm argon-ion laser source and are therefore applicable for flow-FISH using a FITC PNA probe and PI or 7-AAD. The BD Biosciences instruments (including the FACScan, FACSort, FACSCalibur, FACStar and FACSVantage) and Beckman Coulter (XL, Epics, Altra and FC500) are the most common and are all capable of this analysis. Instruments from other manufacturers (such as Partec and Cytomation) should be equally useful. Benchtop instruments (such as the FACSCalibur and the XL) are particularly useful for flow-FISH, since their fixed

alignments and semi-automated quality control allow for good reproducibility in quantitative flow assays. For both the BD and Beckman Coulter instruments, the detector designation for FITC is usually "FL1"; for PI and 7-AAD, the designation is usually "FL3".

7. **Cy5.** The monomeric cyanin dye Cy5 (Amersham Biosciences) excites with most red laser sources and emits at a peak of 670 nm. It is spectrally well-separated from most other fluorochromes (including FITC, PI and 7-AAD) and is poorly excited by 488 nm laser light, avoiding any crossbeam compensation issues with blue-green excited fluorochromes.
8. **Dual laser instruments** (equipped with 488 nm and a second red laser) are now quite common. The BD Biosciences FACSCalibur is one such instrument, equipped with a second red diode laser emitting at 635 nm. The Beckman Coulter FC500 uses a red HeNe laser emitting at 633 nm. More complex cell sorters (such as the BD FACSVantage or the Beckman Coulter Altra) usually have multiple lasers (including red); however, their adjustable alignments will make them more complex for analyzing flow-FISH, unless a larger number of included fluorochromes mandates their use.

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