Comparison of Different Protocols for Telomere Length Estimation by Combination of Quantitative Fluorescence In Situ Hybridization (Q-FISH) and Flow Cytometry in Human Cancer Cell Lines

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Abstract. Background: The end of eukaryotic chromosomes terminates with nucleoprotein structures called telomeres. They insure several functions including capping the end of the chromosomes, ensuring their stability and protecting them from end-to-end fusion and preventing the activation of the DNA damage checkpoints. Materials and Methods: A flow-FISH methodology, i.e. quantitative fluorescence in situ hybridization (Q-FISH) in combination with flow cytometry, has been developed in our laboratory in order to estimate telomere length in three human cancer cell lines: K-562 (chronic myelogenous leukaemia), IM-9 (multiple myeloma) and 1301 (T cell lymphoblastic leukaemia). Telomeres were visualised after hybridisation with FITC-labelled PNA (Peptide Nucleic Acid) probes. We evaluated the most critical steps of the flow-FISH protocol to ensure reproducibility. Different methodological set ups were compared. Three fixation procedures (ethanol 80%, methanol 80% and formaldehyde 4%) were tested besides different fixation times (15 min and 60 min) as well as hybridization times (2 h and overnight). For each of these protocols the following parameters were compared: forward scatter (related to the cell size), side scatter (related to the cell granularity), DNA (FL3 and FL4 fluorescence) and PNA content (FL1 fluorescence) using an EPICS XL flow cytometer. Results: Regarding the fixation procedures, methanol proved to be the best, followed by ethanol and formaldehyde, with respect to the efficiency to measure the different parameters cited above. Indeed, fixation using methanol gave the optimal PNA signal compared to using ethanol and formaldehyde in two of the studied cell lines (K-562 and 1301); the difference observed was highly significant in the 1301 cell line. The duration of fixation did not show significant interference in the reproducibility of the results for the three cell lines studied. An overnight hybridization appeared to be more effective when compared to the 2-h hybridization in the case of the K-562 cell line. Conclusion: The most important steps of the flow-FISH technique, namely the fixative procedure, as well as the hybridization and the fixation times, were investigated. Considering the latter, suitable protocols were set up for routine and fast telomere length estimation in the cancer cell lines.

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Telomeres are the nucleoprotein structures present at the ends of chromosomes. They consist of short tandem repeated DNA sequences which, in vertebrates, are 5’-TTAGGG-3’ associated to telomere binding proteins (TBP). Several functions are attributed to telomeres (reviewed in 1, 2) including capping the end of the chromosomes, ensuring their stability and protecting them from end-to-end fusion and from being recognized as double strand breaks in the genome.

The inability of the ordinary DNA polymerases and primases to replicate the very tips of linear DNA molecules is known as the "end replication problem" (3-6). As a consequence, telomeres shorten with each round of cell division. Normal somatic cells can only undergo a limited number of cell divisions, known as the Hayflick limit. They are interrupted in their proliferation irreversibly when critically short telomeres length is reached. Thus, telomeres are thought to function as a molecular clock that controls the replicative capacity of human cells and their entry into senescence, as well as the onset of cancer. The enzyme that overcomes the "end replication" problem is called "telomerase". It was first identified in the ciliate...
Tetrahymena (7). This enzyme is a ribonucleoprotein complex, which has the ability to specifically catalyze the addition of telomeric repeats onto the ends of the chromosomes, using its inner RNA component as a template for telomere synthesis. Telomerase activity is repressed or inactivated in the majority of human somatic tissues. Yet, more than 80% of tumour cells are telomerase-positive (8, 9) and exhibit short but stable telomeres which permit a prolonged life-span. The introduction of the catalytic subunit of telomerase (hTERT) to human fibroblasts (10, 11), which normally do not express it, restores the telomerase activity, maintains a stable telomere length and extends the proliferation capacity of these somatic cells. From these observations, telomerase has been proposed to play an important role in tumorigenesis. It defines one of the telomere maintenance mechanisms and compensates for the telomeric loss arising from the "end replication" problem and other telomere attrition mechanisms. Understanding the molecular pathways underlying the regulation mechanisms that switch telomerase on and off is important in the fight against cancer.

Besides telomerase, there are some lines of evidence for the existence of additional mechanisms for telomere length maintenance based on homologous recombination (12). Indeed, some human cell lines, immortalized in vitro and negative for the telomerase, have long and heterogeneous telomeres. This phenomenon was termed ALT, for Alternative Lengthening of Telomeres (13). A study in yeast reported the existence of an alternative backup pathway which restored telomere function (14). It was demonstrated that a minor subpopulation of yeast, lacking one essential gene (Est1) for telomerase, continued to grow ultimately and that the stability of these cells was dependent on the function of Rad52p, a key component of the homologous recombination pathway. Therefore, this alternative mechanism for telomere extension seems to be conserved from yeast to humans.

Because telomeres are involved in many important cellular functions like replication, replicative ageing and the onset of cancer, several methods have been developed for telomere length assessment within cells (15-20). Among the commonly used techniques are Telomere Restriction Fragment (TRF) analysis (i.e. Southern blot-based assessment of average telomere length), quantitative fluorescence in situ hybridization (Q-FISH) and its modification, primed in situ hybridization (PRINS) labelling. The first gives information about the average telomere length within a population of cells. Since Southern blot protocols rely on the use of restriction enzymes, that fragment the genomic DNA close to the telomere, subtelomeric regions are included in the telomeric fragments. These subtelomeric DNA sequences are the major drawback associated with this technique.

Quantitative fluorescence in situ hybridization (Q-FISH), applying fluorescently-labelled (21) telomeric peptide nucleic acid (PNA) probes that bind quantitatively to the telomeric sequences, is an alternative for the quantitative evaluation of telomere length. An outline of the fluorescence signals within each individual cell is given. The major advantage of this method is the possibility of measuring individual telomere lengths from individual chromosomes. However, when dealing with routine analysis or many samples, this latter becomes elaborate and time consuming.

Flow cytometry is a well established method and its combination with the Q-FISH gives rise to a flow-FISH technique, a procedure reviewed in (22) and previously described in (23, 24). Since then, several studies have been conducted on a wide range of cell types and subsets in order to determine telomere length using the flow-FISH approach (25-33). Among the above-mentioned approaches for telomere length measurement, to date none of them has been standardized across laboratories and a standard protocol is still needed to facilitate comparison.

In the present study, the flow-FISH procedure was used to estimate the telomere length in three different human cancer cell lines (IM-9, K-562, 1301) and to optimize the methodology in order to use those cell lines as internal standard for future studies. Similarly to other studies (23, 24, 30), we used fluorescein-labelled PNA probe for telomere hybridization but, unlike the study from Rufer et al. (23), we preferred to perform our flow-FISH protocol including a fixation step as it is believed that fixation preserves the morphological scatter characteristics of the cells. Furthermore, it inactivates biohazardous agents and allows prolonged storage of the prepared samples, thus allowing a more flexible scheduling of the flow-FISH experiments.

The various fixatives (ethanol, methanol and formaldehyde) act differently on the cellular structure (34), therefore, we studied the effect of the different fixations and duration of the fixation step on the cell cycle quality and telomere fluorescence intensities. To guarantee optimal conditions for telomere length assessment, the duration of the hybridization (2h/overnight) was tested as well.

Materials and Methods

Cell lines. K-562 (derived from chronic myelogenous leukemia) and IM-9 (derived from multiple myeloma) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) (35). The 1301 (derived from T-lymphoblastic leukemia) cell line was a kind gift from Professor G. Roos (Umeå University, Umeå, Sweden). Cells were maintained in 1640 RPMI medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). They were grown at 37°C in a humidified incubator containing 5% CO2/air and sub-cultured at 80% confluency.
Flow-FISH. The protocol used for telomere length estimation was inspired by the flow-FISH technique previously established and described in (24). Different methodological set-ups were compared in order to obtain the most stable telomere fluorescence signal above the autofluorescence level combined with the best cell cycle pattern.

Each Q-FISH experiment started with 2x10^6 fresh cells. These were washed in phosphate-buffered saline (PBS) and centrifuged at 3000 rpm for 7 min. The pellets were resuspended in 1 ml of one of the following fixatives: 80% ethanol, 80% methanol or 4% formaldehyde. To avoid cellular aggregation, we added the fixatives drop by drop under continuous shaking. Thereafter, the cells were incubated for either 15 min or 1 h at room temperature, then washed three times with 1 ml of PBS. All the centrifugations between the washing steps were performed at 3000 rpm for 7 min. One ml of PBS was added, the cells were counted and at least 250,000 cells were collected for the remaining steps of the protocol. The cells were then centrifuged and the pellets were resuspended in 500 μl of hybridization solution (70% formamide, 1% BSA, 10mM Tris-HCl pH 7.2, 0.3μg/ml PNA probe) and incubated for 10 min at room temperature in the dark. Denaturation was performed at 87°C for 10 min. Finally the cells were kept for either 2 h or overnight in the dark at room temperature.

After the hybridization step, the pellets were centrifuged and incubated twice at room temperature for 10 min in 500 μl of buffer containing 70% formamide, 0.1% BSA, 0.1% Tween 20 and 10 mM Tris-HCl pH 7.2. The cells were then centrifuged in 1 ml of buffer B containing 0.15 M NaCl, 0.1% BSA, 0.1% Tween 20 and 50 mM Tris-HCl pH 7.5 and then centrifuged at 3000 rpm for 7 min. Thereafter, pellets were resuspended in 1 ml of buffer B and transferred to flow cytometry tubes, filled in advance with 3 ml of buffer B. After an incubation of 10 min at room temperature, the tubes were centrifuged at 3000 rpm for 5 min. Then the pellets were resuspended 500 μl of a PBS solution containing 0.1% BSA, 10 μg/ml of RNase A and 0.1 μg/ml of propidium iodide (PI). The tubes were then stored in the dark at 4°C overnight prior to flow cytometry analysis.

Flow cytometry analysis. The acquisition and the analysis of the data were performed on an EPICS XL flow cytometer (Beckman Coulter, San Diego, CA, USA). The green fluorescence emitted by the PNA-FITC-labelled probe hybridized to the telomeres was measured in the FL1 channel, whilst the red fluorescence emitted by the DNA counterstained with PI was detected in both the FL3 (linear scale) and FL4 (logarithmic scale) channels. For a better visualization of the double labelling, the PNA-FITC signal intensity values were plotted against the PI signals.

A threshold on the forward scatter was set in order not to take into account cellular debris. Considering the debris alone is not sufficient to fit the data, the possibility of cell aggregation introduced by the preparative procedures for flow cytometry (i.e. fixation step) must also be taken into account. In this perspective, the "doublets" and the aggregates were identified and discriminated on the basis of a pulse peak vs. pulse area analysis.

The PNA values reported in this work correspond to the PNA values of the cells present at the different phases of the cell cycle (G1, S, and G2) as detected by the flow cytometer. For each experiment, at least 10,000 cells were analyzed per condition.

Calibration. At the beginning of each experiment, Flow-Check fluorospheres beads (Coulter Corporation, Miami, USA) were used to calibrate and to verify the optical alignment of the laser system as well as the fluids system of the flow cytometer. Those ensure accuracy and reliability of the machine. After the calibration step, another assortment of FITC-labelled fluorescent beads (Spherotech, IL, USA; Molecular Probes, OR, USA) was used for monitoring the variations in the laser linearity. Each of this group of beads contains a known amount of molecules of equivalent fluorescein (MEFL) and emits a green fluorescence upon excitation by an argon laser of 488nm. The different fluorescence intensities emitted by the beads were related to the appropriate mean channels giving rise to a standard calibration curve. This latter was used to convert the telomere fluorescence values into MEFL values. Each sample was...
prepared in triplicate. To correct for the autofluorescence signals in FL1 channels, parallel experiments (in which no PNA probe was used) were run simultaneously with the experiments using the PNA probe. Taking into account these two procedures (with and without PNA probe), the telomere autofluorescence values measured for each cell line were subtracted from the corresponding telomere fluorescence signals.

TRF analysis. For evaluation of performance of our flow-FISH technique, the mean telomere length of the cell lines under study was determined via classical TRF analysis. To this purpose, high quality DNA was isolated from K-562, IM-9 and 1301 cells using the Puregene® DNA Purification kit (Gentra, Minneapolis, USA). Genomic DNA (5 µg) was digested with RsaI and HinfI for 2 h at 37°C and fragmented by Field inversion Gel Electrophoresis between 5 and 100 kb. After, Southern blotting blots were hybridized overnight with a P32 radiolabelled 5-mer synthetic oligonucleotide telomeric probe. Blots were analysed via phosphoimaging and mean TRF length was determined as previously described (19, 20) using the formula TRF=iOD(MW-OD(i)). With OD(i)=OD at a given position and MW(i)=molecular weight at that position (calibration to DNA markers).

Statistical analysis. Results were expressed as the means±standard error of the mean (SEM). In each experiment, at least 10,000 cells were analyzed per condition and all the experiments were performed in triplicate. A paired t-test was performed to assess significant differences between means (p<0.05).

Results

Fixative procedures. The K-562, 1301 and IM-9 cell lines were analyzed using Q-FISH in combination with flow cytometry. A gate on the forward scatter (FS) and side scatter (SS) was first set to avoid the debris. Then, a second gate based on PI staining (FL3 in linear) was used to discriminate and thus exclude doublets as well as aggregates.

The remaining gate on the cell population, therefore, only included G1, S and G2 cells. To correct for the autofluorescence, cell suspensions were divided into two: one half was stained with the PNA probe, whilst the other half was not. The telomere fluorescence signal (PNA values collected in FL1) of each cell suspension was then calculated by subtracting the autofluorescence, (i.e. fluorescence emitted by the unstained cells (-PNA)) from the fluorescence intensity of the PNA-stained cells (+ PNA) (Figure 1).

To determine the optimal conditions for telomere fluorescence signal (PNA values), three fixation procedures (80% methanol, 80% ethanol, 4% formaldehyde) were compared on the 1301, K-562 and IM-9 cells (Figures 2a and 2b). For the 1301 cell line, the telomere fluorescence intensity was shown to be optimal (the highest PNA signal once the autofluorescence subtracted) when methanol was used as fixative, whereas ethanol showed intermediate fluorescence. Formaldehyde did not show an optimal fluorescence intensity. The differences which were dependent on the fixative were significant as indicated by the p values (Figures 2a).

Within the K-562 cell line, differences in telomere fluorescence signal intensity were also obtained when using the three fixatives. Although statistically not significant, the PNA signal after methanol fixation was higher than the PNA signals obtained with ethanol and formaldehyde as fixatives. The IM-9 cells reacted differently from the other two cell lines. The intensity of the telomere fluorescence
signal was higher when using formaldehyde in comparison with methanol or ethanol fixatives.

For the following experiments, the cells were fixed with methanol. Since methanol gave an optimal telomere fluorescence signal as well as a good cell cycle quality (which permits deeper analysis of the G1- and G2- phases), we decided to use methanol and to compare it with ethanol for optimization of the duration of fixation. Formaldehyde was not taken into consideration for the next part of the work.

Duration of the fixation. In order to investigate whether the duration of the fixation step interfered with the access of the PNA probe to the target DNA, for the intensity of the telomere fluorescence signal, 15 min and 60 min fixation times were compared. In this case, we compared methanol and/or ethanol as fixatives. As shown in Figure 3 a,b and c, no significant differences were obtained according to the fixation time in the three cell lines used.
Hybridization procedure. To determine the conditions which allow optimal hybridization of the telomere PNA probe to the telomeres, 2 h and overnight hybridization times were compared using methanol fixative (Figure 4 a and b).

For the 1301 and K-562 cell lines, differences in PNA fluorescence signal intensity were observed between the two procedures. The overnight hybridization showed a higher PNA fluorescence signal than did the 2-h hybridization procedure. This difference was highly significant in the case of the K-562 cell line. However, for the IM-9 cell line, there was no significant difference between the two hybridization procedures.

Telomere fluorescence during the cell cycle. The ability to measure the telomere fluorescence intensity of each phase of the cell cycle constitutes one of the strong points of the flow-FISH technique. Indeed, Hultdin et al. (24) showed that, in the 1301 cell line, telomere fluorescence signal had already increased by 80% in the middle of the S-phase. In this work, we used the general profile of the PNA and DNA fluorescence intensities as a control to check the reliability of the method. In all the samples, the distribution of PNA and DNA fluorescence were similar in their general profile, i.e. telomere fluorescence was always lower in G1 cells, intermediary in S-phase and statistically higher in G2 cells. The telomere fluorescence distribution over the different phases of the cell cycle in the three cell lines is shown in Figure 5.

Telomere length estimation by Q-flow-FISH and Southern blotting followed by densitometry analysis. The genomic DNA extracted from the three cell lines was subjected to telomere restriction fragments (TRF) analysis. The mean TRF length from the three cell lines was correlated to the telomere PNA fluorescence signals obtained by Q-flow-FISH. The correlation between the values obtained with the two techniques (Q-flow-FISH and TRF analysis) is shown in Table I. A good correlation is observed when using methanol and ethanol as fixatives, $r=0.989$ and 0.987, respectively.

When using formaldehyde as fixative, the correlation between the values obtained with the Q-flow-FISH did not match those obtained with densitometry analysis. Indeed, the K-562 cell line is known to be triploid (20) and, therefore, a higher telomere fluorescence signal over the IM-9 cell line, which is diploid, was expected. Despite the good correlation coefficient when using formaldehyde ($r=0.908$), the PNA fluorescence signal from the IM-9 cell line was higher than the one from K-562, which is not consistent with the values from the densitometry analysis (3.15 and 6.65 Kbp, respectively) and with the reported chromosomal content of those cell lines.

Discussion

In this paper, the flow-FISH technique was used to estimate the telomere fluorescence signal in three cell lines. Traditionally, telomere length is measured by TRF analysis, the major disadvantage associated with this technique being the contribution of the sub-telomeric DNA sequences, resulting in less accurate telomere length estimation. An alternative to the TRF analysis is the Q-FISH (quantitative fluorescence in situ hybridization). This latter is elaborate and time-consuming and, thus, not suitable for routine purposes. In this work, the protocol we used was inspired by the previously described protocols (23, 24), thereafter improved and optimized for cancer cells growing in suspension. Flow cytometry was shown to be accurate and fast in the concomitant detection of size, granularity, telomere length and cell cycle on thousands of cancer cells.

Two main features specific to the flow-FISH are worth mentioning: 1) the use of fluorescent beads to check the linearity and the stability of the laser, as well as the use of beads with known fluorescein molecules to convert the fluorescence intensity into Molecules of Equivalent Fluorescein; 2) the advantage of using a PNA instead of a DNA probe. Regarding the latter, it has been shown that DNA/PNA complexes are more stable over the DNA/RNA or DNA/DNA complexes (36). Indeed, PNA binds to its complementary nucleic acids with high affinity and specificity. Moreover, it can hybridize to its target over a broad range of salt concentrations that destabilize native nucleic acid structures and avoid the reannealing of the target strand. These characteristics suggest that PNA should be superior to DNA for use as a hybrid probe.

During the flow-FISH protocol, the cells are subjected to harsh conditions (fixation, heat 87°C in 70% formamide solution). Our main purpose was to find the best conditions allowing optimal hybridization results, in combination with a good cell cycle quality. Three standard fixatives were compared to determine their suitability for the measurement of telomere fluorescence intensity. In the case of the 1301 and K-562 cell lines, the PNA fluorescence intensity was optimal when cells were fixed with methanol, in comparison to ethanol or formaldehyde. However, with the IM-9 cell line, formaldehyde gave better results regarding detection of the PNA fluorescence intensity.

To ensure the accessibility of the PNA probe to its DNA target, a good fixation had to ensure the preservation of cellular and subcellular architecture and allow free access of the PNA probe to all cells and to all their nuclear compartments. The fixatives used in this study belong to two different reagent groups: alcohols (ethanol, methanol) and aldehydes (formaldehyde, glutaraldehyde). Their mode of action on biological samples is different and this is translated by the differences observed in the PNA fluorescence...
Figure 3a. Fixation time. The three cell lines were fixed for 15 minutes or 60 minutes with methanol fixative. Results are expressed as mean of molecules equivalent of fluorescence (MEFL) ± SEM of at least 3 independent experiments.

Figure 3b. Fixation time. The three cell lines were fixed for 15 minutes or 60 minutes with ethanol fixative. Results are expressed as mean of molecules equivalent of fluorescence (MEFL) ± SEM of at least 3 independent experiments.
intensity. Indeed, alcohols permeabilize the cells and act by precipitating the proteins and removing lipids (37, 38). To circumvent this problem, aldehydes, which are known to preserve cell structure (39), are generally used to cross-link the cell surface proteins prior to alcohol fixation. However, cross-linking reagents such as formaldehyde form intermolecular bridges and bonds with the amino, imino and peptide groups proteins, which in turn react with active hydrogen atoms of other groups, forming cross-links between peptide chains. It also induces DNA-protein cross-links (DPC), limiting the binding of the probes to its target and, thus, lowering the resulting intensity signal (40). Whilst
Figure 4a. Hybridization procedure. Two hours and overnight hybridizations were evaluated and compared on the three cell lines using the PNA-specific telomere probe and methanol fixation. Results are expressed as mean MEFL ± SEM of at least 3 independent experiments.

Figure 4b. Hybridization time. Dot plots showing 2 hours and overnight hybridization in the 1301, K-562 and IM-9 stained with PNA telomere probe and fixed with methanol.
alcohols permeabilize cells and avoid covalent modifications of DNA, RNA, proteins or carbohydrate epitopes, the cross-linking reagents require the addition of a permeabilization step. Our experiments were performed without this step, relying on the denaturation conditions as did Rufer et al., allowing the access of the PNA probe to the internal environment of the cell. The PNA probe penetration worked without permeabilization, which is probably due to its small molecular weight and to the high temperature used during the DNA denaturation step, which increases the fluidity of the cell membrane. Nevertheless, the resulting PNA fluorescence signal was not optimal when fixing the cells with formaldehyde instead of methanol or ethanol and this in two (1301 and K-562/IM-9) out of the three cell lines used. This is probably due to the DNA-protein cross-links effect induced by the cross-linking reagents (formaldehyde), which hinder the access of the PNA probe to its telomeric DNA target.

The two hybridization times tested (2 h vs. overnight) indicated that, in the case of the K-562 and 1301 cell lines, the overnight hybridization allowed a better access of the PNA probe to its DNA target, enabling a better telomere fluorescence intensity. This observation was not verified for the IM-9 cell line. Regarding the duration of the fixation, the two times tested (15 min and 60 min) did not show any change in the quality of the PNA fluorescence intensity.

From our results, presented here, it is clear that an appropriate treatment exists for each individual cell line we studied. Therefore, testing the experimental conditions before adapting the technique for routine purposes is advisable. The correct choice of the fixative is dependent on the cell type, on the target being examined (i.e. DNA sequences, proteins) and on the properties of the probe used. Thus, when studying cellular morphology and cell surface proteins, cross-linking reagents are advisable. When looking at DNA and nuclear factors, alcohol solvents are preferable.

Although most of the chemical fixatives gave a PNA fluorescence signal, methanol was chosen for its good cell cycle quality in the three cell lines and for its better PNA fluorescence intensity when compared to ethanol and

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>TRF (Kbp)</th>
<th>MEFL Values</th>
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<tbody>
<tr>
<td></td>
<td>Methanol</td>
<td>Ethanol</td>
</tr>
<tr>
<td>1301</td>
<td>23.48</td>
<td>204308</td>
</tr>
<tr>
<td>K-562</td>
<td>6.65</td>
<td>11138.14</td>
</tr>
<tr>
<td>IM-9</td>
<td>3.15</td>
<td>7801.23</td>
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| r (Correlation coefficient) | 0.989 | 0.987 | 0.908 |

![Figure 5. Telomere fluorescence in function of the phases of the cell cycle. Telomere fluorescence in the three cell lines according to the phases of the cell cycle (methanol fixation). Results are expressed as mean of molecules equivalent of fluorescence (MEFL) ± SEM of at least 3 independent experiments.](image-url)
formaldehyde in the case of the 1301 and K-562 cell lines. Ethanol was discarded because of its excessive clump effect on cells and formaldehyde eliminated because of the low intensity of the PNA signal and the bad cell cycle quality for the 1301 and K-562 cell lines. Moreover, when comparing the telomere values obtained with Q-flow-FISH and TRF analysis, we did not observe a good correlation between the two sets of data when using formaldehyde. In future studies, if one of these cell lines is used as an internal standard with each sample to be measured for its telomere content (as advised by Hultdin et al. (24)), it is important to check whether the fixative used is suitable for the two cellular types (the standard and the sample to be tested).

Regarding the hybridization time, the PNA intensity after an overnight hybridization was significantly higher when compared to the 2-h hybridization in the K-562 cell line. The duration of the fixation (15 min and 60 min) did not interfere with the quality of the results, being the case for the three cell lines tested. Thus, methanol fixative (15 minutes) and an overnight hybridization were chosen for the telomere estimation in the 1301 and K-562 cell lines. However, when taking into account the IM-9 cell line alone, it is better to use formaldehyde as fixative (15 min) and overnight hybridization.

Improvements in the flow-FISH protocol are still under development and new applications are available. The combination of flow-FISH and immunophenotyping is one of these recent improvements (41). It enables the determination of telomere length in different cell subsets without prior cell separation using heat-stable fluorochromes as Cy5. To reduce the fluorescence variations linked to sample preparation and instrumentation, the automation of most steps in the staining protocol using a microdispenser device (42) now ensures accuracy and reproducibility and has been shown to be an efficient way to avoid the reproducibility problem that is usually associated with fluorescence techniques.

In conclusion, the flow-FISH analyses are quite cell type-dependent and require specific attention to protocollar differences that take into account this specificity. Nevertheless, we successfully tested some important parameters for quantitative fluorescence in situ hybridization in combination with flow cytometry in three human cancer cell lines and optimized the protocol for routine telomere length estimation.

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