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Review

Telomere length measurement—Caveats and a critical assessment of the available technologies and tools

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ABSTRACT

Studies of telomeres and telomere biology often critically rely on the detection of telomeric DNA and measurements of the length of telomere repeats in either single cells or populations of cells. Several methods are available that provide this type of information and it is often not clear what method is most appropriate to address a specific research question. The major variables that need to be considered are the material that is or can be made available and the accuracy of measurements that is required. The goal of this review is to provide a comprehensive summary of the most commonly used methods and discuss the advantages and disadvantages of each. Methods that start with genomic DNA include telomere restriction fragment (TRF) length analysis, PCR amplification of telomere repeats relative to a single copy gene by Q-PCR or MMQPCR and single telomere length analysis (STELA), a PCR-based approach that accurately measures the full spectrum of telomere lengths from individual chromosomes. A different set of methods relies on fluorescent *in situ* hybridization (FISH) to detect telomere repeats in individual cells or chromosomes. By including essential calibration steps and appropriate controls these methods can be used to measure telomere repeat length or content in chromosomes and cells. Such methods include quantitative FISH (Q-FISH) and flow FISH which are based on digital microscopy and flow cytometry, respectively. Here the basic principles of various telomere length measurement methods are described and their strengths and weaknesses are highlighted. Some recent developments in telomere length analysis are also discussed. The information in this review should facilitate the selection of the most suitable method to address specific research question about telomeres in either model organisms or human subjects.

Contents

Abbreviations: FISH, Fluorescence *in situ* hybridization; TRF, Telomere Restriction Fragment; STELA, Single Telomere Length Analysis; Q, Quantitative; MMQ, monochrome multiplex quantitative.

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1. Introduction

In almost all species that have cells with linear chromosomes, telomeres consist of G-rich repeats and associated proteins. Each of the 92 telomeres in a diploid human cell contain between less than 0.5 kb to more than 20 kb of $(TTAGGG)_n$ repeats [1] which are in dynamic equilibrium with a specific set of proteins [2]. The G-rich strand is invariably orientated 5′–3′ and the very 3′ end terminates in a 100–200 bp single stranded overhang [3] which is believed to be important in forming a t-loop structure by infiltrating telomere arrays in *cis* [4]. Telomere length in human cells is strikingly heterogeneous [5,6], but at least a few hundred nucleotides of telomere repeats must "cap" each chromosome end in order to avoid activation of a DNA damage response and DNA repair pathways [7–9]. Variant telomere repeats are interspersed with pure telomeric repeats in the initial 1 kb of the array; whether this region should be defined as telomeric or subtelomeric, and whether it retains any/all telomere function [10] is still under debate (Fig. 1). Telomeres have been intensely studied since the observation that telomeres in the germline are longer than in somatic cells [11] and the proposal that telomere loss could cause cell senescence [12]. That gradual loss of telomere repeats contributes to replicative senescence or apoptosis in human cells was confirmed [12] and loss of telomeres has been implicated in genomic instability and neoplastic transformation [13] as well as many age-related diseases [14]. Several recent studies have shown that haplo-insufficiency for either the telomerase RNA template (*hTERC*) gene or the telomerase reverse transcriptase (*hTERT*) gene can cause fatal conditions including aplastic anemia, pulmonary fibrosis or cancer [15–19]. Other studies have demonstrated that tumor cells and immortal cell lines typically express high levels of telomerase [20] and are required to do so in order to sustain their proliferative activity [21]. The realization that the length of telomere repeats at individual chromosome ends is a critical variable in cell fate decisions and biological functions ranging from aging to carcinogenesis [22] has highlighted the need for techniques that can provide accurate information on the length of telomere repeats in different cell types. Here we review the methods that are most commonly used to measure the telomere length in chromosomes, cells or genomic DNA. These methods have been used in studies of telomere length in cells from humans as well as various model organisms. In some cases, such as studies involving the laboratory mouse, telomere length measurements pose a special challenge since telomeres are very heterogeneous in length and on average outside the range for techniques that utilize genomic DNA. Some techniques are also unsuitable for studies of cells from species with large arrays of intrachromosomal $(TTAGGG)_n$ repeat arrays such as hamsters [23] or require significant re-optimization in organisms with different telomere repeat sequences such as $(TTTAGGG)_n$ in certain plants and $(TTAGGC)_n$ in nematodes.

Each of the published methods to measure the length of telomere repeats has distinct advantages and disadvantages. It is important to realize what is actually being measured by each technique, as portions of the subtelomeric region and/or the variant repeat region of the telomere are present in some methods but not others (Fig. 1). It is also important to consider the accuracy of each technique, the quantity and quality of starting material required, and the speed, number and ease of processing samples. Here, we discuss the advantages and potential flaws of the main techniques used in measuring telomere length, highlighting applications unique to particular methods and related technical variations that have been explored.

2. Terminal restriction fragment (TRF) analysis

The first technique used to measure telomere length was Terminal Restriction Fragment (TRF) length analysis [1] by Southern blot [24]. TRF analysis yields an estimate of the average telomere length within a population of cells [12,25]. In part because it was for many years the sole quantitative method available to the telomere research field and because it has been used as a reference for methods developed thereafter, it is considered as the gold standard and little has changed in the procedure since the first reports. It exploits the specific and redundant nature of the telomeric sequence by fully digesting genomic DNA with frequent cutting restriction enzymes which specifically exclude telomere repeats, resulting in short genomic fragments and longer uncut telomeres. The DNA digest is resolved according to size by agarose gel electrophoresis and telomere fragments are detected through either Southern blotting or in-gel hybridization using a labeled probe specific for telomeric DNA. The resulting labeled DNA smear is used to estimate average genomic telomere length by comparison to a DNA ladder size standard and normalization to a reference sample to correct for "gel effects" between experiments. Variation in telomere length between cells and between chromosome ends results in a very heterogeneous smear from which to estimate the

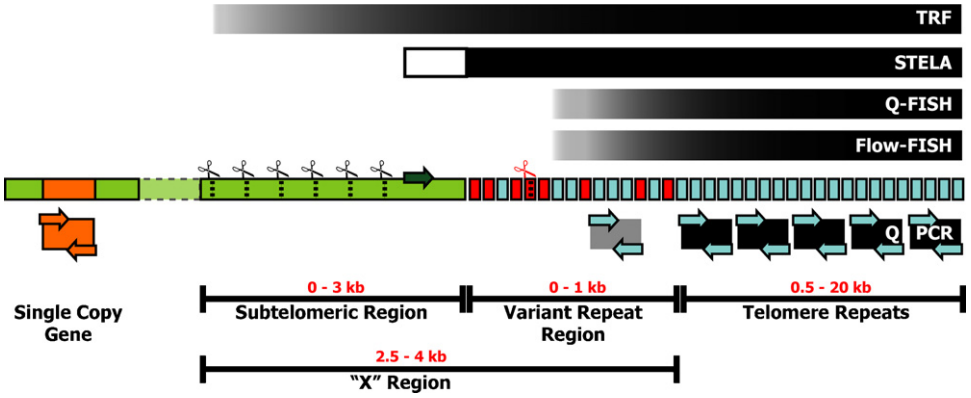

Fig. 1. Extra-telomeric regions detected using different telomere measurement techniques. Telomere length measured by TRF includes subtelomeric DNA (green bar) and telomere variant repeats (red bars) which can occupy up to 2 kb of the start of the telomere. This "X" region overestimates the length of pure telomeric repeats (blue bars), and is variable based on the choice and location of restriction enzymes (represented as scissors). Some selected restriction enzymes will cut a particular common variant repeat (red scissor), and restriction sites will differ between chromosomes and individuals. STELA encompasses the variant and pure telomere repeats, but since the primer site is known (green arrow), the subtelomeric length can be subtracted (white box). Both Q-FISH and Flow-FISH use probes that are specific for pure telomeric repeats, although it is unknown whether arrays of pure telomere repeats in the variant region affect measurements. Q-PCR compares amplification of pure telomere reads using telomeric primers (blue arrows) to a single copy locus (orange) amplified with a second primer set (orange arrows). N.B. Techniques calibrated to TRF results will include the "X" region in the calculated telomere length, even if "X" is not detected in the method itself.

average telomere length. The length of this smear as well as the intensity are important factors in calculating an accurate average telomere length, and since longer telomeres have the ability to hybridize to more probe, the signal strength should be corrected for in the analysis (see [26]). It is important to note therefore, that while TRF is a well established technique, telomere length data generated over time may not be readily comparable across studies because techniques were not standardized with respect to restriction enzyme selection, starting DNA quantity and quality, and blot analysis. Various restriction enzyme pairs or in some cases single cutting enzymes have been used over time and in different studies. This results in further variability of "X", defined as the average distance between the first available restriction site(s) and the start of true telomeric repeats (Fig. 1). The specific distance to the terminal-most restriction site is unknown, and differs in location depending on the enzyme pairs used. Further, since this distance as well as the composition and length of variant telomere repeats is likely to differ between chromosome ends as well as between individuals, "X" and the size of the fragments resolved by TRF will always remain a variable. Estimates of "X" by TRF analysis range from 2.5 kb to 4 kb [27,28] and may depend on subtelomeric modifications that protect against restriction digestion [29]. Other techniques such as Flow-FISH (discussed later) were originally calibrated upon TRF and as such the "X" region is incorporated in these measurements. Subtracting the average length of this region from Flow-FISH measured telomeres lengths in the NK/NKT cells of older healthy individuals and in telomerase deficient individuals, suggest that telomeres with on average 2 kb or less telomere repeats at each chromosome end are sufficient to sustain cell viability.

The most prominent drawback of the TRF technique is the requirement for substantial amounts of DNA. Genomic DNA from at least 10^5 cells is needed, which restricts studies to situations when large amounts of cells or genetic material is or can be made available. Another disadvantage is the relative insensitivity of the TRF technique to very short telomeres, which contain fewer telomere repeats and hybridize to fewer probe molecules. Since the shortest telomeres are probably most important in triggering senescence [30], the insensitivity to detect short ends diminishes the versatility of the TRF technique.

A number of variations to the TRF technique have been developed over the years. A marginal increase in sensitivity was achieved by adapting TRF to examining particular chromosome ends, such as using restriction enzymes that cut centromeric from specific subtelomeric probes to detect telomeres from a single chromosome [31]. Alternatively, chromosome-specific telomere length information can be obtained by TRF analysis of DNA from chromosomes sorted by flow cytometry [6], although both of these methodologies are technically challenging and require a significant amount of starting material. In order to increase sample throughput while reducing the amount of input DNA, a slot blot variant of TRF was developed, in which DNA is deposited directly onto a membrane using a vacuum manifold and probed with labeled telomeric sequence [32]. This strategy requires no in-gel resolution of fragments, allowing less starting material (as little as 9 ng of DNA or 800 cells [33]), but does not provide a direct measure, instead relying on a ratio of telomere signal to centromere signal. Further this technique is relatively insensitive (±1 kb), and has been applied seldomly.

In summary, TRF analysis is the oldest and most commonly used method to measure telomere length, and has been used to calibrate some of the other techniques discussed below. Despite inevitable limitations in sensitivity and accuracy of calculated results and the technical and analytical differences between research groups, the errors of this method are relatively small. With this in mind, and combined with the simple protocol, it is unsurprising that TRF is the standard technique to validate and calibrate most new methodologies.

3. STELA

Single TElomere Length Analysis or STELA involves the application of single molecule PCR to generate highly accurate telomere measurements from limited starting material [34]. By exploiting the fact that all telomeres end with a single stranded 3′ G-rich overhang, STELA involves annealing and ligating an oligonucleotide linker to the 5′ end of the telomere using the overhang as a specific template. A linker-specific primer is then used in conjunction with a primer specific for a unique subtelomeric sequence in a small-pool PCR reaction to generate an individual amplicon for each single telomere. The main caveat of this method is that not all chromosome ends have suitable sequence for the design of unique chromosome arm primers, and thus STELA is usually restricted to several well characterized ends; XpYp, 2p, 11q, 12q and 17p [9]. To minimize PCR artifacts, sub-visible amplification is conducted, and STELA products are resolved by agarose gel electrophoresis, Southern blotted and probed with the specific subtelomeric sequence. The resultant blots display intricate banding patterns, with each discrete band representing a single telomere from a single chromosome end. The size of each discrete product is measured either individually or binned based on size and the mean calculated in comparison with a DNA ladder standard. The number of atypically truncated telomeres can also be calculated statistically, either through standard deviations from the mean [34] or median absolute deviations [35].

While STELA requires no specialized equipment, it does require a significant degree of initial optimization and experience in single molecule PCR techniques. The biggest advantage with STELA is the ability to generate highly accurate telomere length measurements with limiting starting material. Indeed DNA in the picogram range [34] or as few as 50 cells [35] are sufficient to provide reliable length measurements and in this regard, STELA is well suited to analysis of rare cell types.

While the restriction site in TRF analysis is an unknown distance from the start of the telomere, the exact base of the subtelomeric primer used in STELA is known and stable between cells, samples and individuals and so telomere length is measured most accurately. Since accuracy is paramount in methods that measure telomere length, and telomeres are highly heterogeneous between chromosome ends, analysis of a single end offers a distinct advantage in the detection of even subtle changes in telomere length. It is clear however that while advantageous in comparison of minor telomere length changes, analysis of a single chromosome end is also restrictive as a specific chromosome end may not be representative of the telomere length status within the entire cell. Progress has been made on increasing the pool of chromosome ends that can be amplified [9], but analysis of each is labor intensive, and the complexity of telomere-adjacent DNA [36] prohibits the use of most ends in STELA. STELA on the XpYp telomere is most commonly used as there is over 3 kb of unique telomere-adjacent sequence [37] and many single nucleotide polymorphisms that can be exploited for allele-specific STELA [34]. However it has been proposed that there is a difference in telomere length decline between active and inactive X chromosome in females, with the latter exhibiting elevated shortening [38], and as such the use of the XpYp telomere is not ideal.

STELA is also limited in the analysis of very long telomeres. The resolution limits of the technique are dependent on the degree of optimization, but the typical detection limit of 20 kb [34] will potentially skew data in samples with long telomere subsets. This limitation also curtails STELA in the analysis of samples from model

organisms with long telomeres such as *Mus musculus*. STELA is however ideally suited to the specific analysis of abruptly shortened telomeres (those that fall outside the normal range of telomere length), and has shown that these are subject to fusions [39,40], which may occur at different rates on different chromosomes [9] and are fewer in primitive blood cells than differentiated cells [35]. Acute loss of telomeric DNA is potentially important in triggering senescence and generating fusions, and thus far only STELA and Q-FISH (discussed later) have the ability to detect these statistical outliers from the mean telomere length.

In addition to mean telomere length information, STELA has uncovered other important telomere features. By annealing and ligating different permutations of the linker oligonucleotide, STELA was adapted to determine that the majority of telomeres end in CCAATC on the 5′ strand, a finding that suggests that the 5′ "underhang" processing is tightly regulated both on the leading and lagging strand telomeres [41]. Further variations have allowed the G-rich strand to be amplified, which includes both double stranded telomere and the overhang, to estimate the replication timing of G-strand elongation by telomerase [42], and using STELA as a means of amplifying telomeres to assess the composition and mutation frequency of variant repeats [43].

An attempt to simultaneously amplify all telomere ends by STELA has also been undertaken, but this "universal STELA" (U-STELA) functions poorly in measuring mean telomere length. It can be used to count absolute numbers of short telomeres, although statistically shorter ends cannot be calculated as the mean length is unknown, and the number of input molecules cannot be normalized, which is vital when picogram changes affect the number of amplicons [44].

Studies have compared results from STELA against TRF [34] and XpYp-specific Q-FISH [35], and in both cases mean telomere lengths were equivalent. While being highly accurate and requiring very few cells, STELA has limited large-scale clinical applications due to its technically challenging, labor intensive and low-throughput nature. However, it is a powerful research tool allowing high-resolution analysis of telomere dynamics, both from natural erosion and abrupt shortening by mutation processes, combined with the ability to study rare sorted subsets and non-dividing senescent cells.

4. Q-PCR and MMQPCR

More recent molecular strategies to assess telomere length have been reported, and have been rapidly adopted for clinical as well as some epidemiological studies: Q-PCR [45] and its improved version monochrome multiplex Q-PCR or MMQ-PCR [46]. In view of the widespread use of PCR techniques and PCR machines these methods have found rapid and widespread application. In both cases telomeres (T) are PCR-amplified using primers that anneal to both the C- and G-rich strands of the telomere but have mismatches across their length. These mismatches reduce primer dimer formation, and adaptations in the MMQ-PCR method ensures amplification only occurs on the telomere templates at a low annealing temperature used in the first two PCR cycles. The remaining PCR cycles are conducted at a higher annealing temperature, preventing further template priming, specifically targeting only those molecules amplified in the initial cycles and generating a fixed length product. The amplification is measured quantitatively and compared to that of a single copy gene (S) performed either in a separate tube containing an equivalent sample (Q-PCR) or in the same tube (MMQ-PCR) generating a ratio between telomeric and specific amplification called a *T*/*S* ratio. The advance of MMQ-PCR over the earlier Q-PCR technique is particularly important since pipetting volume discrepancies between telomere and single copy control reactions result in significant errors, amplified by PCR, in telomere length ratio measurements as illustrated by the respective R^2 coefficients for each of the PCR methods compared to TRF (see Table 1).

While each sample is measured in triplicate and MMQ-PCR has improved the reproducibility of measurements, an important assumption in this assay is that the results from DNA samples of different qualities are comparable. Furthermore, while the inherent differences in efficiencies between amplification of telomeric and single copy DNA are controlled using a ratio, it is unknown whether results obtained from separate research centers that may not use identical reagents or single copy loci can be directly compared. In addition, while it is assumed that the amplified control gene is unique in the genome, it is important to consider that copy number variation or chromosomal duplications can potentially alter the gene copy number, significantly altering the *T*/*S* ratio. This fact reduces the applicability of telomere length measurements by PCR to cells and samples that are diploid and karyotypically stable, at the exclusion of many models that take advantage of immortalized transformed cell lines as well as tumor tissue samples.

Some of these assumptions are difficult to validate in individual experiments even if appropriate controls are included and for this reasons, it is of paramount importance when setting up telomere length measurements by PCR to carefully and systematically perform quality controls and calibrations in comparison to another established method. It is also important to include calibration testing as well as a panel of standard samples in every experiment to ensure reproducibility between experiments. Without controls on every plate, it is also unclear how comparable results between different experiments are, both between and within groups. As an example, the MMQ-PCR generated *T*/*S* ratios of between 0.6 and 1.9 in 95 normal whole bloods [46], but in bone marrow failure patients, an average *T*/*S* of 0.99 was considered short compared to fibroblasts and buccal cells [47]. Indeed one buccal sample gave a *T*/*S* of 6.44 [47], equivalent to 25.2 kb calculated from the linear correlation to TRF [46]. It is often difficult to evaluate from a publication which control steps have been undertaken, or what variability may exist between laboratories performing an identical basic protocol, which may in part explain the publication of contradictory reports on similar questions [48,49]. For these reasons, the popularity and rapid adoption of the Q-PCR method has raised some concerns [50] and although the Q-PCR methods are very attractive for their short timeline and cost, variability within and between samples remains relatively high. Each laboratory adopting a Q-PCR method should therefore conduct an initial calibration to a non-PCR based telomere length measurement, optimize the technique until a high R^2 coefficient is reached, and include internal size standards within each experiment to ensure reliability of results and facilitate comparison between publications.

5. Q-FISH

Quantitative fluorescence *in situ* hybridization of telomere repeats or Q-FISH using image cytometry and metaphase chromosomes [6,51] uses directly fluorescently labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probes as a high affinity alternative to DNA oligonucleotide probes that specifically hybridize to denatured telomere DNA repeat arrays. The fluorescent signal can then be detected and measured relative to standards of known telomere length in metaphase spreads with specific software for Q-FISH image analysis [51] (freely available at www.flintbox.com). Q-FISH is the method of choice for high resolution telomere length measurements at specific chromosome ends (and for all chromosomes) because it allows for simultaneous karyotyping. The analysis of 15–20 metaphases per samples is typically required to obtain reli-

Table 1
Summary of telomere length measurement methods.

Method	TRF	STELA	*T/S* Q-PCR and MMQPCR[a]	Q-FISH	Flow FISH[a]
Cell number required	1–3 × 10^6 (0.5–10 μg DNA)	1–1 × 10^5	Blood sample or DNA sample (20 ng DNA/reaction)	Actively dividing cells for chromosome spread (cell type dependent) 15–20 metaphases analysed	0.5–2 × 10^6 freshly isolated or frozen white blood cells Alternate processing of nuclei for other cell types
Cell viability required at time of processing	No	No	No	Yes	Yes
Estimate of telomere length	Mean length for total cell population	Single chromosome end specific length	Amplification of Telomere to Standard single copy gene ratio	Cell average length	Cell specific average length
Replicate Sample testing	No duplicate test for QTRF	1 reaction ran in 10–20 gel lanes	3 replicate DNA extraction?	No	2
Additional information	Optional overhang measurement	Allele specific average length and distribution – **Critically short telomeres**	NA	**Chromosome end specific distribution – Telomere-free ends – telomere fusion events**	**Antibody staining – mitosis tracking – comparison between cell subpopulations**
Reported Statistics					
Resolution	1 kb	0.1 kb	ND	0.3 kb	0.2–0.3 kb
Correlation to TRF[b]	**"Standard"** 0.876 (*Hph*I/*Mnl*I vs. *Hinf*I/*Rsa*I)	NA	0.6671 (QPCR) 0.844 (MMQPCR)	0.9	0.87
Inter-assay mean CV[b]	0.9–12%	ND	3.13% (MMQPCR-geometric mean of CV)	ND	3.3% (lymphocytes)
Intra-assay mean CV[b]	ND	ND	5.8% (QPCR) 5.22% (MMQPCR-geometric mean of CV)	11.2% (same slide)	1.6% (lymphocytes)
References	[1,12,25,26]	[32,39]	[42,43]	[5,6,48]	[54,55]

NA, not applicable. ND, not done.

[a] Established methods that have been adapted for high throughput analysis.

[b] Values reported by investigators who have used these methods in their own research (see References). Note that to date, no independent, impartial assessment of various methods has been undertaken.

able telomere length measurements. Q-FISH has also been used to detect ends without detectable repeats (<0.5 kb) as well as chromosome fusion events. The technique is well established to study telomere biology in many settings [52,53] and may be a good choice for measuring telomere length in rare cells [54]; the main characteristics of the assay are summarized in Table 1. The main drawback of performing metaphase Q-FISH is that it is not possible to measure telomeres in terminally senescent cells, highly aberrant cells or generally in cells that are unable to divide. Further, it may be challenging to test cell types with very low mitotic indices. To circumvent this, higher throughput Q-FISH options, allowing for the rapid acquisition of many cells therefore significantly increasing the typical low statistical power of the technique, have been developed. These methods measure the telomere length of either interphase blood cells or cells embedded within tissue sections: HT Q-FISH [55] and tissue Q-FISH [56], respectively. The two main assumptions for these methods are that the hybridization efficiency of the PNA probes to telomeric target sequences following fixation is invariable between cells and that all cells are considered to have 2*n* DNA content. Both methods measure telomere length by normalizing the fluorescent signal from telomeres to that of a centromere probe. While Q-FISH on tissue sections adds informative pathological parameters within a specific tissue, it also introduces new technological challenges for the hybridization protocol within sections as well as in the analysis of sections that likely do not contain complete nuclei. The HT Q-FISH method has been adapted to cells in a multi-well culture set-up so as to maximize the number of cells (adherent or attached from suspension) that may be analysed and aims to improve on the statistical power of metaphase Q-FISH. It likely has best resolution when used with centromere normalization. Although it has been compared to other established methods, the use of a very long telomere length "high" calibration standard with very broad distribution likely skews correlations and makes it difficult to conclusively assess the measurement precision as well as inter and intra-assay reproducibility [55].

6. Flow FISH

Similar to Q-FISH, flow FISH uses directly fluorescently labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probes albeit hybridized to cells in suspension where telomere fluorescence is then analysed by flow cytometry [57,58]. Flow FISH accurately measures the median telomere length in individual cells in suspension and can be used to measure the telomere length in distinct cell populations within a single sample by antibody staining (for a very limited set of cell surface markers that are retained after the hybridization procedure – alternatively, specific cell populations can be cell sorted prior to flow FISH). Using flow FISH it was found that the telomere length in granulocytes from patients with aplastic anemia correlates with the response to immunosuppressive therapy [59] and the loss of telomere repeats in hematopoietic cells was found to be restricted to the first year following allogeneic bone marrow transplantation [60]. Since these earlier studies, flow FISH has been adapted for higher throughput and enhanced reproducibility by using a semi-automated 96 well format with a robotic microdispenser to perform some of the steps in the procedure [57]. Flow FISH now offers the most extensive quantitative reference data related to telomere length currently available and is the first of the telomere length methods to have been validated for clinical diagnostic purposes [15]. 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)/NKT-cells in human blood [61,62]. Data on telomere length relative to age are particularly useful in the context of the cells of the immune system as well as in cases of suspected telomerase deficiencies [15,17,18]. Flow FISH can also be adapted to the study of species other than humans and was used to study the decline of telomere length with age in leukocytes from baboons [63] and to study the role of the *R*egulator of telomere *l*ength (*Rtel*) gene in the mouse [64]. The great advantage of studying telomere length in nucleated blood cells is that this tissue is readily available. A caveat is that ongoing immune responses may lead to skewing in telomere length measurements [65–67]. Also, measuring telomere length in the blood of patients with leukemia and a high blood tumor burden will result in measuring the tumor telomere length and not necessarily reflect that of other somatic tissue types in that individual.

While the flow FISH technique has been adopted by a number of different laboratories the technique has several limitations. Since unfixed cells are very fragile following FISH, conditions for washing are selected that avoid clumping of cells in pellets following centrifugation. This is achieved using multiple wash steps and relative small wash volumes. However, as a result there are many cell wash steps in the optimized flow FISH protocol which are tedious without some degree of automation using e.g. a 96 well liquid dispenser. Second, the method was optimized for nucleated blood cells which typically have a high nucleus to cytoplasm ratio. Since PNA probes may non-specifically bind to components in cytoplasm, it was found that nuclei rather than whole cells should be used in order to measure the average telomere length in large cells such as whole kidney epithelial cells [68]. By avoiding the use of various fixatives (e.g. to preserve epitopes or labeled antibodies at the surface of cells) flow FISH is limited to fresh blood samples. This limitation was imposed to avoid unpredictable effects of fixation on hybridization efficiency. A dilemma here as well as with many telomere length measurement techniques is that results following fixation of cells may still correlate (somewhat) with actual telomere length. Because the ability to detect small biologically relevant differences often relies largely on method accuracy and reproducibility, the effect of any fixation procedure should be carefully validated. Compared to immuno-phenotyping of cells (where 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 rather time consuming and technically demanding.

7. Telomere length measurement methods: further considerations

Factors that influence telomere length are commonly studied using *in vitro* cellular models as they offer experimental settings with the least amount of variability. Variability can be further reduced when the cell population tested is clonal in origin. In these studies, minimizing variation in telomere length measurements allows small differences to be detected between control and test cells. As well as offering the best experimental set up for identifying changes in telomere length, these cells types also make the best calibration controls for the technical set-up of telomere length measurement methods. Furthermore, the best control cells have a telomere length that remains stable over time. Since cells in culture will typically show genetic drift and karyotypic as well as telomere length variations over time, the minimal requirements for optimizing and validating telomere length measurement techniques are to include a "low" and a "high" telomere length standard that fit the above criteria and is identical between experiments.

Whole cells or extracted DNA derived from biological tissue samples (blood or solid tissue types) have inherently more vari-

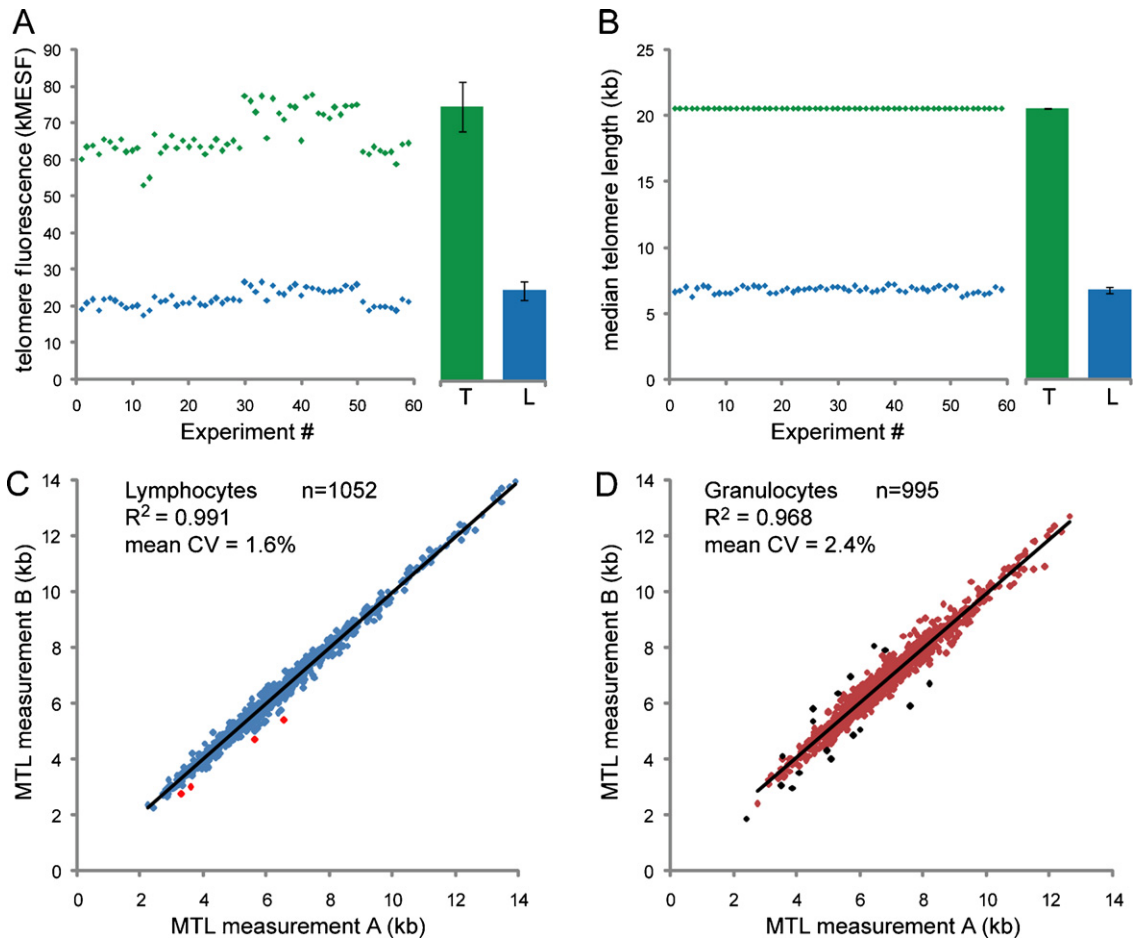

Fig. 2. Inter-assay variability in flow FISH (A and B). Each flow FISH telomere length measurement includes duplicate assays of telomere-PNA probe stained versus unstained conditions (or 4 tubes per test). Average median telomere length fluorescence from duplicate measurements of the same blood sample in over 59 independent experiments are plotted; measurements for gated lymphocytes (L, blue) are shown (panel A). To control for differences in hybridization, each condition (tube) includes an internal hybridization control consisting of diploid primary cells with a stable karyotype, bovine thymocytes (T, green), which are identical between each experimental plate set up and are used to normalize the fluorescence data (panel B). The average median telomere length calculated for this sample over the 59 independent experiments is 6.8 kb with a standard deviation of 0.2 kb (average CV = 3.3%). Intra-assay variability on flow FISH (C and D). Comparison between ~1000 consecutive replicate telomere length measurement tests were compared for lymphocytes (C) and granulocytes (D). Regression analysis showed correlation between replicates was 0.991 and 0.968 for lymphocytes and granulocytes respectively. Outlier sample replicates with CV > 10%, 4/1052 (0.38%) for lymphocytes (red), and 18/995 (1.8%) for granulocytes (black) were excluded from analysis.

able telomere lengths due to the presence of a mixture of cell types. This is due to the cellular complexity within the sample as a source of variability beyond the natural wide range of telomere lengths seen between individuals, set genetically. The variability is thought to in part reflect the different biological and replicative histories of different cells. It is important to consider this variation when measuring telomere lengths in biological specimens from animal models (where variability due to genetic diversity is usually limited) or from individuals. Although the relative synchrony of telomere length between different cell types of the same individual has been reported [69], more data are required to define what the limits and implications of these findings are in relation to the research questions that are being asked.

8. Conclusion

It is clear that at present there is no single technique that can accurately, easily and rapidly measure telomere length. Selection of a method to measure telomere length must therefore be made based on the specific scientific questions that need to be addressed. In situations where hundreds or thousands of samples require testing, the Q-PCR methods are currently the only high-throughput strategy available. Despite the comparative inaccuracy of the technique, general trends between population groups can be made as long as errors are assumed to be similar between groups. This makes the PCR based methods perhaps an attractive choice for larger consortiums, epidemiology studies and some clinical screening applications, but less appealing for studies with few samples or in which accurate and absolute measurements need to be made. Between 20 and 50 samples can be easily processed for flow FISH (and multiple blood cell types can be simultaneously analysed) and up to 130 with TRF [26]. As such these techniques lend themselves well to analysis of smaller populations and accurate clinical screening programs. Q-FISH and STELA are only applicable to small number of samples, approximately 5–10 and 1–5 samples, respectively, and are therefore better suited to detailed laboratory studies than screening of populations or patients. These two techniques are however highly accurate and can measure abruptly shortened telomeres, giving them significant advantages in answering fundamental biological questions with respect to the other methods. Flow FISH is also very accurate, and has proven to be useful in both population [70,71] and longitudinal [72] telomere length studies. Subtle changes in telomere length can be hard to detect with TRF,

and may be even harder to detect with Q-PCR, so these are the least ideal methods if precise measurements are required. Finally, experiments must be designed based on the quantity and type of material obtained from biological samples. Both STELA and Q-FISH require low amounts of DNA (<100 pg) and cells (<300), respectively, allowing analysis on very rare samples. Q-PCR and flow-FISH require much more material, and TRF requires even more, limiting these applications to samples with more abundant material.

Telomere length is of great interest in the contexts of aging and disease research, and has already proven to be a useful biomarker in many research and clinical settings. However, no single measurement technique is ideally suited to the screening of telomere length in populations or dissecting telomere dynamics at the molecular level. The advantages and disadvantages of telomere measurement techniques outlined in this review are therefore intended to aid in the selection of the most optimal method for answering specific questions in telomere research.

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Conflict of interest disclosure

PML is a founding shareholder in Repeat Diagnostics Inc., a company specializing in leukocyte telomere length measurements using flow FISH. All other authors declare no competing financial interests.

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