Telomere Length

A Review of Methods for Measurement

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Background: The exciting discovery that telomere shortening is associated with many health conditions and that telomere lengths can be altered in response to social and environmental exposures has underscored the need for methods to accurately and consistently quantify telomere length.

Objectives: The purpose of this article is to provide a comprehensive summary that compares and contrasts the current technologies used to assess telomere length.

Discussion: Multiple methods have been developed for the study of telomeres. These techniques include quantification of telomere length by terminal restriction fragmentation—which was one of the earliest tools used for length assessment—making it the gold standard in telomere biology. Quantitative polymerase chain reaction provides the advantage of being able to use smaller amounts of DNA, thereby making it amenable to epidemiology studies involving large numbers of people. An alternative method uses fluorescent probes to quantify not only mean telomere lengths but also chromosome-specific telomere lengths; however, the downside of this approach is that it can only be used on mitotically active cells. Additional methods that permit assessment of the length of a subset of chromosome-specific telomeres or the subset of telomeres that demonstrate shortening are also reviewed.

Conclusion: Given the increased utility for telomere assessments as a biomarker in physiological, psychological, and biobehavioral research, it is important that investigators become familiar with the methodological nuances of the various procedures used for measuring telomere length. This will ensure that they are empowered to select an optimal assessment approach to meet the needs of their study designs. Gaining a better understanding of the benefits and drawbacks of various measurement techniques is important not only in individual studies, but also to further establish the science of telomere associations with biobehavioral phenomena.

Key Words: nursing research • telomere • telomere length • telomere measurement

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The paradigm-shifting study of Epel et al. (2004), which showed an association between chronic stress and telomere length, has resulted in the recognition by several investigators of an association between adverse social and environmental influences and telomere length (Shalev et al., 2013). In a previous issue of *Nursing Research*, we reported the results of an integrative review of factors associated with telomere length and the implications for biobehavioral research (Starkweather et al., 2014).

Telomeres are caps (repetitive nucleotide sequences) at the end of the linear chromosomes that play a critical role in facilitating complete chromosome replication. The structure of the telomere was first recognized by Hermann Muller and Barbara McClintock through their studies in *Drosophila* (Muller, 1938) and *maize* (McClintock, 1941), respectively. Muller concluded that a special structure at the end of the chromosome was required for its integrity and first coined the term ''telomere.'' Three years later, McClintock (1941) proposed that telomeres stabilize chromosome ends and prevent them from being recognized as DNA double-strand breaks. In 2009, the Nobel Prize in Physiology or Medicine was jointly awarded to Elizabeth Blackburn, Carol Greider, and Jack Szostak ''for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase.''

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As a result of intensive research that has been completed since these pioneering studies, much is currently known about telomeres. Telomeres can now be more precisely described as noncoding tandem arrays of a TTAGGG DNA sequence that are located at the terminal ends of all vertebrate chromosomes, including those of humans (Moyzis et al., 1988). A G-rich single-stranded 3′ (read as ''3 prime'') overhang is present at the end of human telomeres and is thought to be important for telomere function (Makarov, Hirose, & Langmore, 1997; Stewart et al., 2003; Wright, Tesmer, Huffman, Levene, & Shay, 1997). This single-stranded 3′ overhang folds back on itself, forming a large loop structure called a telomere loop or T-loop that has a shape similar to that of a paper clip. The telomere is stabilized by a six-protein complex called ''shelterin,'' which includes telomeric repeat binding factors 1 and 2 (TRF1 andTRF2), protection of telomeres 1 (POT1), TRF1 and TRF2 interacting nuclear protein 2 (TIN2), the human ortholog of the yeast repressor/activator protein 1 (Rap1), and TPP1. Shelterin components specifically localize to the telomere due to the recognition of TTAGGG repeats by three of its components: TRF1 and TRF2 recognize the duplex part of telomeres and bind to it, whereas POT1 recognizes the single-stranded repeat sequence in the 3′overhang localized within the T-loop structure (specifically within the ''displacement'' or D-loop). TIN2, TPP1, Rap1, and POT1 are recruited to the telomere by TRF1 and TRF2 (de Lange, 2005; Palm & de Lange, 2008).

By combining the knowledge that the properties of DNA replication prevent cells from fully replicating the ends of linear chromosomes (Watson, 1972) with the observation that normal cells have a limited capability to replicate, Olovnikov (1973) proposed his theory of marginotomy. It has been reported that he developed this hypothesis while waiting for a subway train in Moscow. As he heard the train coming, he imagined the train, specifically the engine, being the DNA polymerase and the track being the DNA. The engine (DNA polymerase) would not be able to replicate the first segment of DNA (the track) because it lay exactly underneath the engine. It seemed unlikely that with each cell division a DNA segment containing important genes was lost. Therefore, Olovnikov reasoned that the repeated noncoding telomeric nucleotide

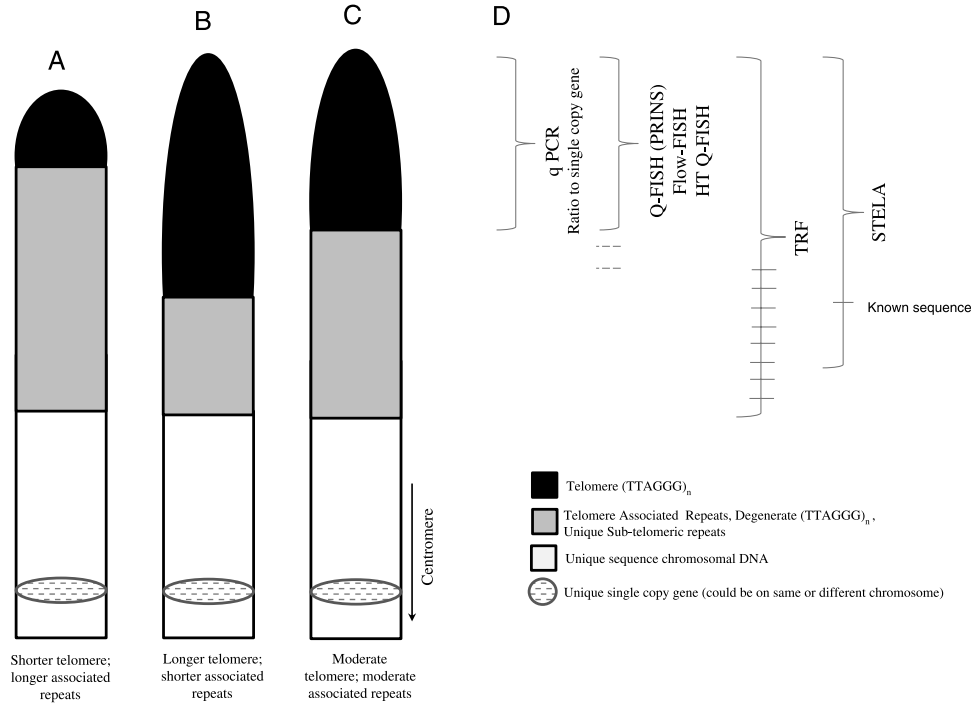

FIGURE 1. Schematic showing telomeric and subtelomeric regions targeted in telomere length estimation methods. (A–C) Human telomeric and subtelomeric regions are heteromorphic and vary between chromosomes (both within a person and between individuals). Telomeres (shown in black) demonstrate a continuous range of size from shorter (A), to moderate (C), to longer (B). The regions that juxtapose the telomere (shown in gray) include telomere-associated repeats, degenerate (TTAGGG)n repeats, and unique subtelomeric repeats. This area also shows variation between chromosomes (both within and between people), as illustrated here with chromosomes having long (A), short (B), or moderate (C) juxtaposed repeat regions. The TRF method results in an assessment of both the juxtaposed (subtelomeric) and true telomeric regions (indicated by brackets) with the localization of the subtelomeric region included in the measurement being variable (based primarily on the restriction enzymes used; shown by series of solid horizontal lines). The STELA assay also includes sequences from the juxtaposed region, but the area included is specific (sequence based). Q-FISH methodologies (which include PRINS, Flow-FISH, and HT Q-FISH) use a probe specific for the telomeric region to estimate length (shown by brackets). Although the probe tends to be specific for the telomeric region, it is possible that the probe could bind to a portion of the juxtaposed region (especially the degenerate repeat region). The uncertainty of inclusion of the degenerate repeats in the length estimates obtained with this methodology is indicated by a dotted line. The qPCR technique uses primers for the telomere region and a single copy gene (may be on the same chromosome as illustrated for simplicity here or on a different chromosome).

TABLE 1. Methods Used to Assess Telomere Length

Method	Analyte	Measures		Resolution (kb)	Optimally suited for large studies
		Average	Chromosome-specific		
TRF	DNA	Yes	No	1.0[a]	No
qPCR, MMqPCR, aTLqPCR	DNA	Yes	No	?[b,c,d]	Yes
STELA	DNA	No	Yes	0.1[a]	No
Q-FISH	Metaphase chromosomes	Yes	Yes	0.15–0.3[a,b]	No
	Interphase nuclei (telomere)	Yes	No	0.15–0.3[a,b]	No
PRINS	Metaphase chromosomes	Yes	Yes	0.3[a]	No
	Interphase nuclei (telomere)	Yes	No	0.3[a]	No
Flow-FISH	Interphase nuclei	Yes	No	0.2–0.3[a]	No
HT Q-FISH	Interphase nuclei	Yes	No	0.2–0.3[b]	Yes

Note. aTL = absolute telomere length; DNA = deoxyribonucleic acid; HT Q-FISH = high-throughput quantitative fluorescence in situ hybridization; kb = kilobase; MMqPCR = monochrome multiplex quantitative polymerase chain reaction; qPCR = quantitative polymerase chain reaction; STELA = single telomere length analysis, Universal STELA; TRF = terminal restriction fragment; PRINS = primed in situ subtype of Q-FISH; Q-FISH = quantitative fluorescence in situ hybridization. [a]Aubert et al. (2012). [b]Vera and Blasco (2012). [c]The resolution has not been clearly defined. [d]O'Callaghan and Fenech (2011).

sequences act as a buffer to protect gene coding sequences. He correctly speculated that, with each round of cell division, a portion of the telomere ''buffer'' would be lost and that the length of the telomeric ''buffer'' could be important for determining a cell's ability to proliferate (Greider, 1998; Hayflick, 1998).

Telomere attrition is now among the well-known, cell-intrinsic events associated with normal cellular aging (Mayer et al., 2006). More importantly, telomere attrition and dysfunction have been shown to be causal factors in the acquisition of many age-related diseases, including, but not limited to, atherosclerosis (Bentos et al., 2004), myocardial infarction (Brouilette, Singh, Thompson, Goodall, & Samani, 2003), Alzheimer's dementia (Panossian et al., 2003), and heart failure (Oeseburg, de Boer, van Gilst, & van der Harst, 2010). Several lifestyle factors have also been associated with telomere shortening (Shammas, 2011), with speculations emerging that *biological* age may be important for recognizing individuals who are at risk for developing health conditions that have historically been associated with *chronological* age.

Regardless of whether telomere length has a direct or indirect association with biobehavioral traits and/or health conditions, its assessment has been shown to hold promise as a biomarker to allow for improvements in risk assessments of diverse health outcomes. However, the utility of the measure depends on valid and reliable techniques to quantify telomere length. Therefore, understanding the measurement strategies and issues related to telomere length is necessary for comparing the results published by different investigative teams, as well as designing experiments for future research. A summary of the primary methods used for telomere length assessments follows, including methodology attributes of each technique shown in Figure 1 and Table 1 and strengths and weaknesses listed in Table 2. Telomere measurement approaches have also been the focus of recent reviews by investigators in the field (Aubert, Hills, & Lansdorp, 2012; Lin & Yan, 2005; Samassekou, Gadji, Drouin, & Yan, 2010; Vera & Blasco, 2012).

METHODS FOR QUANTIFYING TELOMERE LENGTH

Terminal Restriction Fragmentation

Terminal restriction fragment (TRF) analysis is the original technique that was developed for determining telomere length and, hence, is often described as the ''gold standard'' method. In this procedure, genomic DNA is exhaustively digested using a cocktail of frequent cutting restriction enzymes that lack recognition sites in the telomeric and subtelomeric regions (and hence do not ''cut'' telomeric DNA). The intact telomeres from all chromosomes are then resolved, based on size, using agarose gel electrophoresis, with the telomeric fragments being visualized by either southern blotting or in-gel hybridization using a probe specific for telomeric DNA. The varying lengths of telomeres will present as a smear, with the size and intensity of the smear being assessed by comparison to a DNA ladder comprising known fragment sizes (Allshire, Dempster, & Hastie, 1989; Harley, Futcher, & Greider, 1990; Kimura et al., 2010).

The integrity of the extracted genomic DNA is crucial for the application of this technique as well as all the other methods used to quantify telomere length. Clearly, DNA degradation—a process by which the DNA breaks down into smaller fragments—could lead to inaccuracies in telomere length assessments, producing a bias toward shorter lengths. DNA degradation may be due to a number of different causes, including, but not limited to, repeated thawing and freezing of the DNA, leaving the DNA at room temperature for a long time, and the presence of residual nucleases due to improper purification. Therefore, precautionary measures should be taken when handling and extracting genomic DNA to prevent it from being degraded.

TABLE 2. Comparison of Advantages/Limitations of Methods Used to Assess Telomere Length

Method	Advantages	Limitations
TRF	• "Gold standard"[a] • Numerous studies for comparisons • Does not require specialized equipment	• Requires large (≥1 μg) amount of DNA • Labor intensive • Subtelomeric polymorphisms can impact data • Provides mean length measure, but not recognition of individual short telomeres or ends lacking a telomere
qPCR	• Can use small (ng) amounts of DNA	• Variation between and within "batches"
MMqPCR	• Less labor intensive	• Reference standards lacking
aTLqPCR	• Referenced to standard single copy gene • Multiplex controls for DNA amount added	• Requires qPCR equipment • Does not provide absolute kilobase length estimate unless coupled with standard oligo[b] • Provides mean length measure but does not allow recognition of individual short telomeres or ends lacking a telomere
STELA	• Allows for detection of critically short telomeres • Does not require viable cells • Does not require specialized equipment	• Only provides information for a small subset of specific chromosome ends • Does not provide mean telomere data • Does not recognize ends lacking a telomere • Limited in ability to detect long telomeres • Labor intensive
Q-FISH	• Can identify single telomere changes (higher resolution) • Can assess telomere lengths in specific cell types • When used on metaphase chromosomes, can identify individual telomeres (long or short), signal free ends, end-to-end telomeres, and a mean telomere length measure	• Labor intensive • Requires high skill level for chromosome assessment • Requires microscope (typically fluorescent) • "Length" expressed as relative fluorescence unit (often compared to standard [centromeric] value) • Requires mitotically active cells for metaphase chromosomes, but not for interphase nuclei
PRINS	• Can identify single telomere changes (higher resolution) • Can assess telomere lengths in specific cell types • When used on metaphase chromosomes, can identify individual telomeres (long or short), signal free ends, end-to-end telomeres, and a mean telomere length measure	• Labor intensive • Requires high skill level for chromosome assessment • Requires microscope (typically fluorescent) • "Length" expressed as relative fluorescence unit • PCR efficiency can contribute to variability and can negatively impact accuracy • Requires mitotically active cells for metaphase chromosomes, but not for interphase nuclei
Flow-FISH	• Can determine mean "length" for specific cell populations • When coupled with antibodies can provide cell type specific information • Potential for automation	• Labor intensive • Requires high skill level • Requires flow sorting equipment • "Length" expressed as relative fluorescence unit • Provides mean length measure, but not recognition of chromosome-specific individual short telomeres or ends lacking a telomere
HTQ-FISH	• Allows recognition of short telomeres and mean telomeres • Can provide estimates for specific cell populations	• Does not recognize telomere-free ends or chromosome-specific lengths • Requires confocal microscope; length expressed as relative fluorescence unit

Note. aTL = absolute telomere length; DNA = deoxyribonucleic acid; HT Q-FISH = high-throughput quantitative fluorescence in situ hybridization; kb = kilobase; MMqPCR = monochrome multiplex quantitative polymerase chain reaction; qPCR = quantitative polymerase chain reaction; STELA = single telomere length analysis, Universal STELA; TRF = terminal restriction fragment; PRINS = primed in situ subtype of Q-FISH; Q-FISH = quantitative fluorescence in situ hybridization. [a]This gold standard is used as a reference when comparing advantages and disadvantages of alternative telomere length assays. [b]O'Callaghan and Fenech (2011).

As shown in Table 2, strengths of this method include the ability to compare ones results to those obtained by other investigators and to provide a kilobase size estimate for the telomere length. Also, because this method does not require the use of costly, specialized equipment, it can be a convenient technique for proof-of-concept studies. A limitation of this method is that the restriction enzymes used result in the inclusion of subtelomeric DNA that is contiguous to the telomere, thereby leading to overestimation of the true telomere length (Figure 1). These subtelomeric and telomeric regions can also include polymorphisms that can confound the interpretation of the data. Also, the results can vary from lab to lab if different restriction enzymes are used. Other limitations of the TRF assay include the need for large amounts of DNA (micrograms),

rendering this technique more widely applicable to analyzing telomere length in blood samples than other tissue samples. This methodology is also labor intensive and is unable to detect short telomeres that are present on a small number of chromosomes, with this latter shortcoming reflecting hybridization limitations because very short telomeres may not bind to the probe efficiently. These shortcomings, as well as the fact that the TRF value is expressed as an average of the smear size and does not provide information regarding single telomeres (no clear recognition of the range or values at the extremes of the smear spectrum), are significant limitations for using the TRF method to assess telomere length in studies involving large numbers of participants using epidemiological study design approaches (Aubert et al., 2012; see Tables 1 and 2).

Polymerase Chain Reaction-Based Techniques (qPCR, MMqPCR, and aTL qPCR)

To overcome the hurdle of needing large quantities of DNA to evaluate telomere lengths, polymerase chain reaction (PCR)-based telomere length analysis methods have been developed. These procedures include quantitative (or real-time) PCR (qPCR), monochrome multiplex quantitative PCR (MMqPCR), and absolute telomere length (aTL) quantitation. PCR amplifies a DNA sequence of interest over 20–40 cycles using specifically designed primers, with the quantity of the PCR product (the amplicon) doubling with each cycle. Typically, in qPCR, the amount of the DNA sequence of interest is quantified through the use of a fluorophore (which emits a fluorescent signal) that intercalates with double-stranded DNA (i.e., SYBR green) or a probe with an attached fluorophore that is released when the sequence of interest is amplified (i.e., TaqMan probes). After each cycle, the amount of emitted fluorescence is measured, allowing the quantity of starting material to be inferred (Ding & Cantor, 2004).

In 2002, Cawthon reported the development of a primer set and protocol using qPCR technology to elucidate telomere length. Prior to this development, qPCR had not been successfully used for telomere length estimation, largely because the repeating TTAGGG sequence of the telomere required the use of primers that were complimentary, resulting in the formation of primer dimers, which occurs when two primers bind to one another and amplify the primer sequence, rather than amplifying the target DNA from the patient/cell line. This primer dimerization problem was cleverly overcome by Cawthons use of (a) primers that bind to the C- and G-rich segments but are mismatched at the other bases and (b) lower temperatures during the first two cycles (allowing the primers to bind or pair with the telomeric DNA). The remaining cycles were then completed at higher temperatures to amplify only the specimen-specific products from the first two cycles (patient/cell line DNA rather than primer DNA).

Cawthon's (2002) initial qPCR technique is the method used most frequently by investigators. Telomere length was quantified by comparing the amount of the telomere amplification product (T) to that of a single-copy gene (S), with amplification of the telomere and single gene proceeding in separate wells or tubes. The T/S ratio was then calculated to yield a value that correlates with the average telomere length (but is not a base pair estimate or equivalent measure). However, because of unavoidable limitations in measurement precision (pipetting, etc.), one can have variation in the amount of DNA present between the T and S wells/tubes, thereby compromising the precision of the assay. To eliminate this methodological shortcoming, Cawthon (2009) adapted the original protocol to complete the amplification of both the telomeric and single-copy DNA regions from the same tube—with this revised method being called MMqPCR (Cawthon, 2009).

Another adaptation of the basic qPCR-based technique was developed by OCallaghan and Fenech (2011) and was described by these authors as an aTL qPCR method. Briefly, this procedure is performed using a protocol comparable to that of the initial qPCR assay (T/S ratio based on amplification of telomeric region and single-gene region from separate wells) but has the adaptation of using a standard curve of known telomere lengths. The curve is based on the values of serial dilutions of a synthesized oligomer standard that comprised 14 copies of the TTAGGG telomeric sequence (for a total of 84 base pairs in length) to provide a base pair length estimation for specimen telomere lengths (rather than a relative T/S value; OCallaghan & Fenech, 2011).

Like the TRF assay, PCR-based techniques require high-quality DNA that is not compromised by degradation. However, unlike TRF, the PCR-based methods require smaller amounts of DNA (nanogram, rather than microgram, quantities of the specimen being evaluated). PCR-based techniques have become a popular method for estimating telomere length because of relatively low cost, amenability for high-throughput testing, and relative ease of investigators access to the necessary equipment used in the assay. Whereas the PCR-based techniques are well suited for large epidemiological studies, the results from these studies are limited in the ability to allow for comparisons between studies. This limitation is due to differences in the DNA quality based on the method used for genomic DNA extraction, as well as differences in sample fixation methods in the case of fixed and paraffin-embedded tissue samples (Cunningham et al., 2013; Koppelstaetter et al., 2005) and by their relatively high level of variation among replicate estimates (Table 2). To better assess the coefficient of variation of the PCR-based compared to TRF methodologies, Aviv et al. (2011) completed an impartial, blinded, replicate analysis of leukocyte telomere length estimates from 50 subjects using different aliquots of DNA extracted from a single sample/person, following a 2-month interval. Although both methods showed positive correlations between the replicate measures within a method (at least .92) as well as between methods (.85 assuming a linear model), the coefficient of variation value for the qPCR method was 6.45%, whereas the coefficient of variation for the TRF

measures was estimated to be 1.74%. Thus, even when performed by experts, the PCR-based method results in variation, the latter of which may reflect (but not be limited to) differential amplification efficiency or measurement variation between aliquots (Aviv et al., 2011).

Single Telomere Length Analysis

One shortcoming of both the TRF and PCR-based assays is that the values resulting from these methods only provide measures of the average telomere length of the specimen being evaluated (mean of 92 telomeres, assuming a normal human chromosomal complement) and do not provide insight regarding individual telomere lengths. Given that a single (or small number of) critically short telomere(s) has (have) been suggested to serve as a signal leading to cellular senescence/apoptosis, some study designs may benefit from the use of an assay that can detect the length of specific, individual telomeres (Abdallah et al., 2009; Hemann et al., 2001). To meet this need, Baird, Rowson, Wynford-Thomas, and Kipling (2003) adapted the qPCR-based method to provide single telomere length analysis (STELA) for a subset of chromosomes (Baird et al., 2003). This ligation-based method targets the amplification of telomeric DNA from a single chromosomal end through the use of primers that are specific to the subtelomeric sequences of a single chromosome (Figure 1). Unfortunately, because of the complexity and lack of specificity of individual chromosomal subtelomeric regions, only a small subset of chromosomes (Xp, Xq, 2p, 11q, 12q, and 17p) have met the criteria needed to allow for the design of primers that yield successful chromosome-specific/chromosome arm-specific telomeric DNA amplification (Britt-Compton et al., 2006). If there are differences in the rate at which specific telomeres attain critically short lengths, which seems likely, given the heterogeneity in heritable telomere lengths between individual telomeres (Graakjaer et al., 2003; Leach, Rehder, Jensen, Holt, & Jackson-Cook, 2004), the STELA method may not provide a suitable tool for the recognition of all critically short telomeres. An additional adaptation of the STELA method has been called Universal STELA (Bendix, Horn, Jensen, Rubelj, & Kolvraa, 2010). This procedure allows for the detection of any critically short telomere, regardless of its chromosomal location. The Universal attributes of this method arise from the use of the following:

1. digestion of DNA by restriction enzymes (MseI/NdeI);
2. a ligation-based step that suppresses the amplification of the intragenomic fragment; and
3. sequential ligation/fill in steps that ultimately allow for the telomeric fragment to be amplified for short telomeric regions on any chromosome, followed by detection of the telomeric repeat fragments in a gel.

The STELA and Universal STELA techniques provide a means for recognizing the presence of short telomeres on single chromosomes from specimens yielding small amounts of DNA (even in specimens that also contain telomeres having longer lengths). Thus, STELA approaches are well suited for studies in which the type of cells being evaluated is in low concentrations, and the primary goal is to identify critically short telomeres. However, a significant limitation of STELA and Universal STELA is the inability of this technology to measure telomeres having long lengths (few telomeres having lengths in excess of 8 kb are detected using Universal STELA; Bendix et al., 2010; see Table 2). Other limitations of these methods are that they are labor intensive/technically challenging (Aubert et al., 2012) and are sensitive to the amount of template DNA added, as shown by Bendix et al. (2010). Too much template can result in the presentation of a smear because of uncompleted amplicons serving as primers that create technical artifacts. Also, akin to the TRF method, the telomere length estimates obtained using STELA procedures include sequences from the degenerate repeats and subtelomeric repeats regions of the chromosomes (Figure 1). However, because the subtelomeric region included in the STELA methods is well characterized and the primer step is sequence based and can be corrected for in the telomere length estimates, the inclusion of these juxtaposed regions does not tend to confound the accuracy of measurements derived using STELA.

Quantitative Fluorescence In Situ Hybridization

Quantitative fluorescence in situ hybridization (Q-FISH) of telomeric repeats is performed by assessing metaphase chromosomes or interphase nuclei following hybridization/labeling with a fluorescent $(CCCTAA)_3$ probe. Unlike the TRF and PCR-based assays, the substrate for Q-FISH is cells (rather than DNA). The cells used for assessment with Q-FISH methods can be fresh (required for chromosome-specific analyses); frozen; formalin fixed, paraffin embedded; or permeabilized.

Metaphase Chromosome Q-FISH

The Q-FISH method for telomere length assessment, as developed by Lansdorp et al. (1996), with an early adaptation of this methodology being reported by Krejci and Koch (1998), is a technique in which telomeres are visualized by hybridization using a probe for the telomeric repeat sequence $(CCCTAA)_3$, with the remaining chromatin on the chromosome being visualized by a nonspecific DNA stain (such as 4',6-diamidino-2-phenylindole [DAPI] or propidium idodide; Krejci & Koch, 1998). Typically, the probe used for this assay is a synthetic peptide nucleic acid (PNA) probe. The PNA probe has been shown to provide higher hybridization efficiency for telomeric repeat sequences than DNA probes due to the PNA probe having a neutral (uncharged) backbone (Egholm et al., 1993). An advantage for using the Q-FISH approach is that it allows one to estimate sizes for each of the individual 92 telomeres in humans and is not limited to an average or just small telomeres. Furthermore, this is also the only method of assessment

that will allow for the recognition of telomere-free ends; that is, chromosome ends lacking the presence of a telomere sequence large enough to successfully hybridize and be visualized. Metaphase Q-FISH studies have been essential for providing information about variation in the length of telomeres between different chromosomes and for providing insight as to the frequency of chromosomal instability associated with telomere-free ends (Aubert et al., 2012; Vera & Blasco, 2012; see Figure 2). Q-FISH has been optimized to study telomere biology in many settings (Artandi et al., 2000) and is considered especially valuable for measuring telomere length in rare cells (Goldman et al., 2008).

Although the Q-FISH method is a very strong approach for enabling one to recognize individual, chromosome-specific (and cell-specific) telomeric alterations, this method also has shortcomings. Arguably, the greatest weakness of the metaphase Q-FISH technique may be that it cannot be used to measure telomeres in cells that are not mitotically active (such as terminally senescent cells), and its use is limited for specimens having a very low proliferation rate.

The metaphase Q-FISH approach is also labor intensive, costly, and technically demanding (requires knowledge of chromosomal banding patterns). Thus, this procedure is not well suited for large, epidemiological studies.

Interphase Q-FISH

To overcome some of the limitations of Q-FISH, adaptations of this procedure have been developed, with these adaptations involving the use of interphase cells rather than metaphase chromosomes. Interphase Q-FISH is amenable for assessing telomere lengths in nuclei from multiple specimen types (blood cells, formalin-fixed, paraffin-embedded tissues, frozen tissues). Many investigators using interphase Q-FISH compare the fluorescent signal obtained from a telomere-specific probe to that of a centromeric probe and calculate a ratio of signal intensity between the targeted sequences (Aubert et al., 2012; Vera & Blasco, 2012). A clear advantage for using interphase Q-FISH methodology is that it allows one to concurrently collect information regarding telomere length and histological information, having the potential to be combined with immunostaining techniques to localize specific cells of interest (sometimes referred to as ''telomapping''; Vera & Blasco, 2012). A disadvantage of the interphase Q-FISH method is that it does not allow for the recognition of specific telomeres and does not allow for the detection of telomere-free ends. Also, the data are typically presented as a mean value, because the overlaying of the 92 telomeres can prohibit one from unequivocally recognizing each individual telomere. However, interphase Q-FISH is less labor intensive than metaphase Q-FISH, with recent adaptations of this technique, called high-throughput (HT) Q-FISH, being amenable to automation and use in larger epidemiological studies (Canela, Vera, Klatt, & Blasco, 2007).

Flow-FISH

Another adaptation of the Q-FISH approach is called flow-FISH. As its name suggests, this method combines flow cytometry methodology with the hybridization of a pantelomeric (binds to all telomeres) probe to cells in a suspension (rather than hybridizing to cells fixed to slides, as is done for metaphase and interphase Q-FISH). Flow cytometry is a technology in which cells in solution flow one by one past lasers. This technology can separate populations of cells based on their fluorescent emission/signals. Flow-FISH methodology typically uses the same telomeric $(CCCTAA)_3$ PNA probe used in other Q-FISH approaches to quantify the mean amount of fluorescence present in cells. This value is then used to provide an average telomere length for the cell population being evaluated (Hultdin et al., 1998). A strength of this approach is that it has the capability to sort cells into subpopulations based on size, granulation, and/or antibody labeling. Because of this potential, flow-FISH has been widely used for determining mean telomere length in hematopoietic cell subtypes. Flow-FISH is also the first of the telomere assays to be used as a clinical diagnostic tool, with the method being used to assist with the recognition of patients having dyskeratosis congenitaa condition that is associated with shortened telomere lengths (Alter et al., 2007). Another advantage of this method is that it provides a means for inferring the three-dimensional distribution of telomeric signals within cells (Samassekou et al., 2010).

However, like the other measurement approaches, flow-FISH has limitations that may compromise suitability for use in research/clinical studies. Specifically, unfixed cells can be challenging to process (fragility, clumping, etc.), but the technique is sensitive to fixatives used to preserve cells, with the reliability of the measures reflecting these technical parameters. Also, the PNA probe used in flow-FISH has been shown to demonstrate nonspecific binding to cytoplasmic structures. Thus, it has been suggested that isolated nuclei, rather than intact cells, may be optimal for assessment with this methodology. Because of the above noted technical issues, flow-FISH is not readily adaptable for use in a wide range of cell types, with its application being primarily for use with fresh blood samples (Aubert et al., 2012). Also, like many of the other techniques, this method provides only a mean value of telomere intensity and provides no information regarding individual telomeres or a subset of shortened telomeres.

Primed In Situ

A primed in situ (PRINS) approach can be used—in lieu of a PNA probe—to label telomeres for the Q-FISH methods. Briefly, PRINS labels (using fluorescently tagged nucleotides and PCR techniques with telomeric primers) the telomeric sequences in situ on metaphase chromosomes or interphase nuclei (Therkelsen, Nielsen, Koch, Hindkjaer, & Kølvraa, 1995). The intensity of the FISH signal can then be assessed as described for the probe-based Q-FISH approaches, with

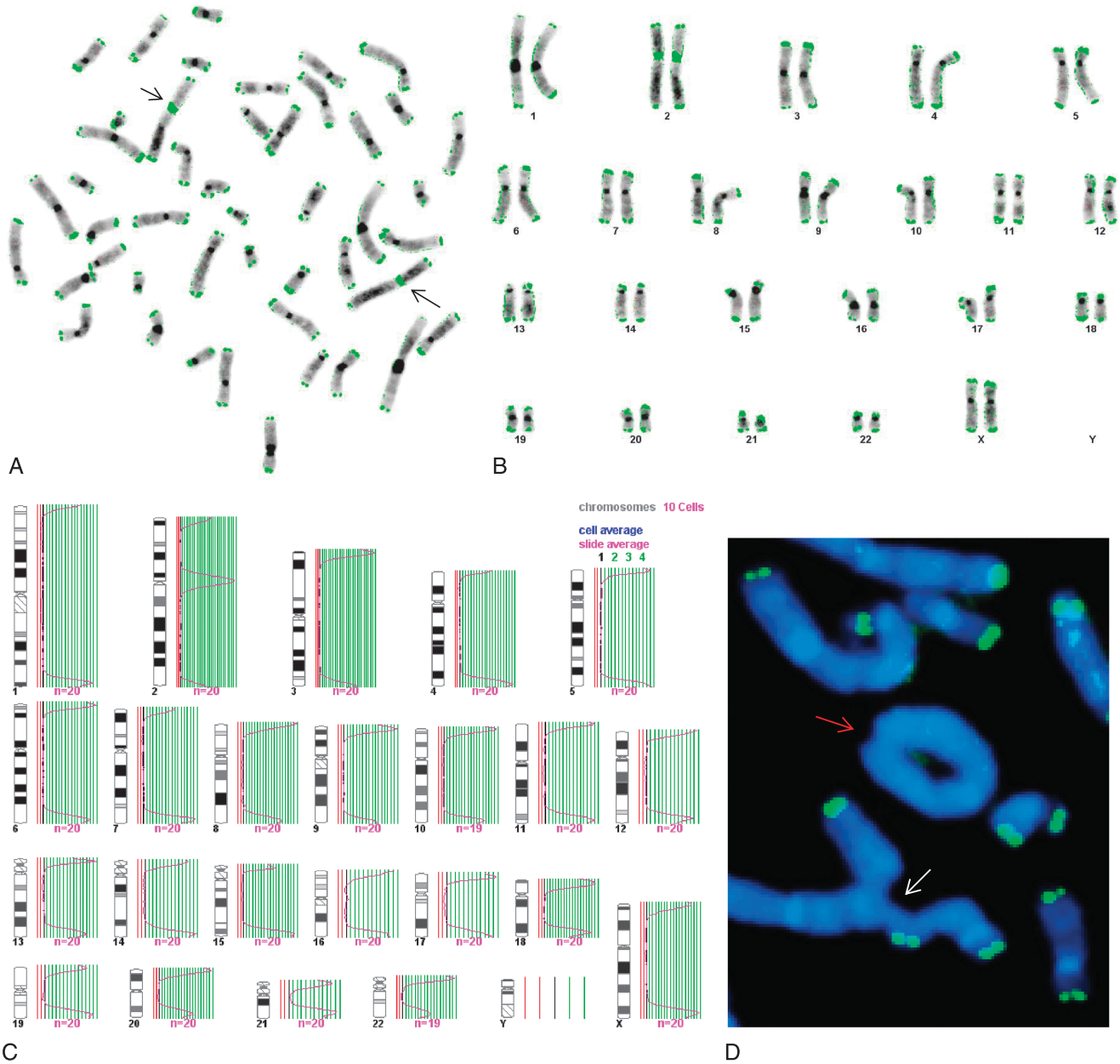

FIGURE 2. Q-FISH using metaphase chromosomes to estimate telomere length. This image shows a metaphase spread (A) that has been hybridized using a PNA probe specific for the telomere (green dots at ends of chromosomes) and a PNA probe specific for the centromeric region of chromosome 2 (control probe; highlighted by arrows). The chromosomes are also stained with DAPI to visualize their banding patterns. On the basis of their reverse DAPI banding patterns, the chromosomes are identified and aligned into a karyogram (shown in B). Following identification of the chromosomes, the average intensity of the telomeric regions is calculated to result in chromosome-specific and arm-specific telomere fluorescent intensity values (C). The Q-FISH method on metaphase chromosomes also allows for the recognition of telomere-free ends (D). Chromosomes lacking a telomere may have an increased frequency of chromosomal rearrangements, such as ring chromosomes (D; red arrow) or fusions between chromatids from different chromosomes (white arrow). This image was prepared by C Jackson-Cook using data collected from her laboratory. Image was developed for this manuscript.

the same strengths and limitations of the Q-FISH methodologies being applicable to this adaptation in approach (Lin & Yan, 2005).

Hybridization Protection Assay

The hybridization protection assay is a DNA-based method that involves a comparison of the ratio of telomeric to Alu repeats present in a specimen (Nakamura et al., 1999). Advantages of this method are that it is relatively quick (approximately 45 minutes), does not require high-quality (unsheared with purity) DNA, and does not require large quantities of DNA. However, there are several weaknesses of the methodology, which have resulted in this approach being infrequently used. These weaknesses include difficulty in interpreting the ratio

values because of variation in the Alu repeat sequences between samples and relating the ratio to a kilobase size. This method is also limited to providing only a mean value of telomere length (no cell- or chromosome-specific data), and by the consistency of the assay results (Lin & Yan, 2005).

Single-Strand 3′ Overhang Measurement

In addition to methods that estimate the full telomere length, there are procedures to quantify the length of the telomeric 3′ overhang. These procedures include, but are not limited to, telomere oligo (oligonucleotide) length assistance, G-tail hybridization protection assay, overhang protection assay, single-strand electron microscopy, primer-extension nick translation, and double-strand specific nuclease (Chai, Du, Shay, & Wright, 2006; Cimino-Reale et al., 2001; Tahara, Kusunoki, Yamanaka, Matsumura, & Ide, 2005; Wright et al., 1997; Zhao, Hoshiyama, Shay, & Wright, 2008). These techniques have been helpful for understanding telomere biology, but tend to have more focused applications than the other telomeric assays.

MATCHING METHODOLOGY TO RESEARCH NEEDS

When incorporating telomere length into a research study, it is important to thoroughly evaluate the research question, population, sample type, timing of analysis, and available resources in order to select the most appropriate telomere length measurement method to use. In addition to methodology, other attributes that warrant consideration when exploring telomere length within the context of biobehavioral research include (but are not limited to) (a) subject cofactors, such as age, gender, body mass index, exercise patterns, diet, smoking, or childhood trauma and (b) biological specimen to be evaluated (i.e., peripheral blood versus specific tissues). For example, regarding the latter point, it is important to recognize that telomere attrition (or shortening) is dependent on the rate at which the cell replicates, so one could anticipate that cells having a higher replicate rate might show more rapid shortening of telomere length (and vice versa). For example, peripheral blood, which is one of the most frequent biological specimens evaluated in telomere research studies, will reflect the lengths of the various blood cells, the latter of which divide at different rates. Specifically, granulocytes (including, neutrophils, eosinophils, and basophils) have a lifespan of hours to days, whereas agranulocytes (including, lymphocytes and monocytes) can have a lifespan of days to years. Therefore, it may be helpful to know which cell type/types are used for measurement to allow one to assess how a differential count of the blood cell constituents might impact the findings of the study.

Several factors should also be considered when selecting a lab to use for providing telomere length quantitation. These factors will include issues related to ease for collaboration, transportation of specimens, and specimen quality. Questions one may wish to discuss with potential collaborators that relate to the quality of their testing include (but are not limited to) the following:

- What validation studies have been completed to ensure the accuracy of their testing?
- What control specimens (cases having short telomeres and cases having long telomeres) are evaluated with each ''batch'' of telomere assessments?
- What is the reproducibility of the assay they use?
- What is their experience in performing telomere length testing?
- Are they a Clinical Laboratory Improvement Amendment-approved lab (Centers for Disease Control, 2013)?

In addition, many of these aforementioned considerations are also applicable when reviewing primary reports of telomere length.

CONCLUSION

For nurse scientists, telomere measures are emerging as a tool that (either singly or in concert with other biological, health, and biobehavioral attributes) may have implications for prevention, disease monitoring, intervention development, and, ultimately, for further refinement of biobehavioral theory. As the body of telomere science continues to be developed, understanding the trajectory of telomere length—beginning in the prenatal period through adult life—may further explain the need for optimally timed interventions. In addition, exploration of the impact that prolonging telomeres or mitigating telomere shortening might have on overall health outcomes will be another important consideration. Studies examining the effects of multiple influences (e.g., neighborhood, stress levels, disease states) and health habits (e.g., nutrition, exercise) may permit better understanding of how to target interventions to mitigate risks while enhancing the salutatory effects of well-timed and targeted interventions for general health promotion and monitoring progression in chronic disease states. Expanding the intervention paradigm to include optimal timing of interventions may be possible with the better identification of periods of higher vulnerability so that potential interventions aimed at telomere lengthening or the mitigation of telomere shortening may be initiated at the most biologically and developmentally indicated time periods.

Biobehavioral theories provide a framework for understanding the psychosocial and behavioral factors that contribute to accelerated telomere attrition and increased vulnerability to chronic diseases. Additional challenges include quantifying the amount of stress, such as thresholds and chronicity, that modulate accelerated telomere attrition, and identifying the extent to which telomere attrition impacts disease progression and survival. Although research has linked many stress-related diseases with decreased telomere length, identifying the mechanistic pathways that link psychosocial and behavioral

factors to the pathogenesis of disease warrants further investigation and is an important area of future nursing research.

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