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Review

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Comparison of telomere length measurement methods

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The strengths and limitations of the major methods developed to measure telomere lengths (TLs) in cells and tissues are presented in this review. These include Q-PCR (Quantitative Polymerase Chain Reaction), TRF (Terminal Restriction Fragment) analysis, a variety of Q-FISH (Quantitative Fluorescence In Situ Hybridization) methods, STELA (Single TElomere Length Analysis) and TeSLA (Telomere Shortest Length Assay). For each method, we will cover information about validation studies, including reproducibility in independent laboratories, accuracy, reliability and sensitivity for measuring not only the average but also the shortest telomeres. There is substantial evidence that it is the shortest telomeres that trigger DNA damage responses leading to replicative senescence in mammals. However, the most commonly used TL measurement methods generally provide information on average or relative TL, but it is the shortest telomeres that leads to telomere dysfunction (identified by TIF, Telomere dysfunction Induced Foci) and limit cell proliferation in the absence of a telomere maintenance mechanism, such as telomerase. As the length of the shortest telomeres is a key biomarker determining cell fate and the onset of senescence, a new technique (TeSLA) that provides quantitative information about all the shortest telomeres will be highlighted.

This article is part of the theme issue 'Understanding diversity in telomere dynamics'.

1. Background

Telomeres are the noncoding tandem repetitive $TTAGGG_n$ arrays at the ends of all vertebrate linear chromosomes (figure 1). Telomeres are important in protecting chromosome ends from being recognized as DNA double-stranded breaks by forming special telomeric structures called T-loops (figure 1) that are bound directly or indirectly by the shelterin proteins [1–4]. It is well established that all dividing somatic cells show progressive telomere shortening (figure 2) [5–8]. Telomerase is the cellular reverse transcriptase that is expressed during human development that becomes silent in most tissues in the adult. Many human somatic cell types express little or no telomerase activity, leading to telomere loss (figure 3*a*). Even in proliferating normal stem cells that express regulated telomerase, it is insufficient to maintain telomeres and gradual telomere erosion also occurs (figure 3*a*). It is also generally accepted that progressive telomere shortening can lead to *in vitro* replicative senescence and, in the presence of other alterations, short telomeres can form bridge-fusion-breakage cycles leading to chromosome instability and the development of cancer [9–13]. Most cancer cells (85–90%) reactivate telomerase to divide indefinitely [14] and the vast majority of these cells maintain stable short telomeres (figure 3*b*).

Understanding the benefits and drawbacks of TL measurement methods is important because short telomeres, not average TL, limit long-term stem cell divisions essential for tissue renewal. In addition, there are genetic disorders affecting telomeres (telomere spectrum disorders or telomeropathies) that lead to earlier onset disease when telomeres are short [15–17]. Thus, robust and reproducible TL measurements that may predict the onset of certain genetic and age-related pathologies in humans and other animal species are important. Finally, there is

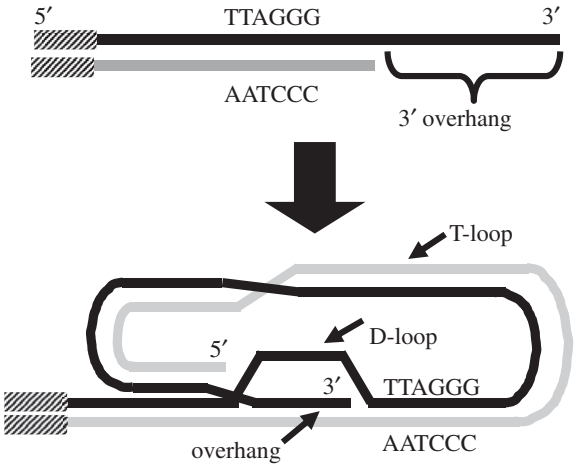

Figure 1. A model of a typical telomere ending with a single-stranded 3′ overhang that loops backward and invades into the duplex DNA forming a T-loop. This results in a strand displacement producing a single-stranded telomeric D-loop.

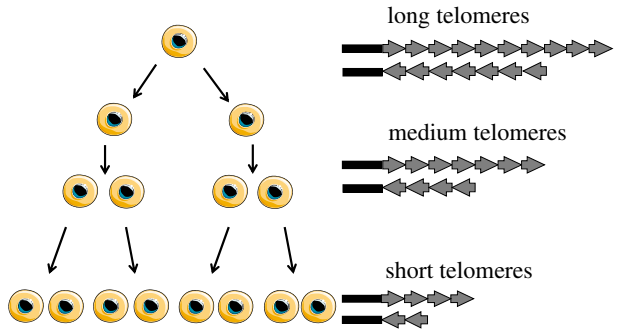

Figure 2. Normal human somatic cells progressively shorten their telomeres with each cell division. Eventually, some of the telomeres become sufficiently short that they can no longer be protected by the shelterin proteins. This can result in a senescence growth arrest and DNA damage at telomeres. (Online version in colour.)

also a growing appreciation that lifestyle factors such as obesity, smoking, lack of exercise and chronic stress can influence TLs in circulating peripheral blood leucocytes (see: life-length/com/telomeres-101/#articles for comprehensive and up-to-date references). Thus, telomere attrition leading to telomere dysfunction correlates with many age-associated diseases such as infertility, arthritis, diabetes, cancer, cardiovascular and neurodegenerative diseases (figure 4) [17–21]. However, essentially all published studies are correlative in nature because in general very small changes in average TL are measured and reported in the literature. Even when large population studies reveal statistical significances, a key question is what do these small changes in average or relative TL really mean biologically?

There are clearly trade-offs between easy-to-do and high-throughput TL measurement methods for epidemiological studies involving large numbers of individuals versus gaining mechanistic cause-and-effect insights using perhaps more precise methods that can provide TL distribution information for all the telomeres including the shortest. There have been several methods developed for the study of telomere biology and all have certain advantages and disadvantages. In the current overview, the utility of each major TL measurement strategy will be briefly discussed providing the strengths and weakness of each method. Information about technical skills required, reliability, robustness and reproducibility is critical if the field is to progress. While there have been many reviews about TL measurement methods in the past [22–27], some recent advances using new methods will also be reviewed.

2. Methods to measure telomere length

(a) Quantitative Polymerase Chain Reaction

The Q-PCR assay is a relatively easy assay not requiring a large amount of starting DNA (approx. 50 ng). By measuring telomere signal (T) to a reference single-copy gene signal (S), this method allows the investigator to calculate the T/S ratios (figure 5) [28,29]. This ratio is proportional to average TL and therefore, can be used to determine relative TLs. As the nature of this technique can be applied to a high-throughput format, this method is widely used in larger population studies. However, as Q-PCR provides only relative quantification, the data are generally not presented in absolute TL values in kilobase (kb) pairs unless compared to a reference cell line with an average TL determined by another method. There is also reported variability within and between samples (intra-assay coefficients of variation may be higher than 10% using this method [26]). Thus, there may be large variations between independent laboratories because different single-copy loci are used and in some instances the amplified control genes may not be unique in the genome affecting the T/S ratio [26]. In addition, Q-PCR does not provide information about the shortest telomeres. Finally, Q-PCR for TL measurements may not be useful for cancer studies where the reference single-copy gene may have been duplicated or lost due to aneuploidy, which is known to occur in almost all cancer cells [30]. Thus, the applicability of Q-PCR is limited to samples that are normal diploid and karyotypically stable.

(b) Terminal Restriction Fragment analysis

The initial determination of a panel of vertebrate telomeres was accomplished by DNA sequencing [31]. Based on the knowledge that the sequence $TTAGGG_n$ was highly conserved, the TRF analysis was developed using a $TTAGGG_n$-labelled probe and is now a widely used method for telomere measurement [32–34]. Regarded as the 'gold standard' for TL measurement, TRF analysis measures the intensity of telomere smears to generally determine an average TL. While a range of telomere sizes can be imaged on Southern blots, the shortest telomeres cannot be visualized. When genomic DNA is digested into small fragments with a combination of common four base-pair restriction enzymes, the repetitive telomere sequences ($TTAGGG_n$) remain intact because they do not contain sites for the used restriction enzymes. Thus, each of the 92 telomere ends in normal human cells will remain undigested (though of variable lengths), while the rest of the genomic DNA is cut into small fragments. Immediately after the canonical telomeric repeats, on each chromosome, there is a region of the subtelomere consisting of non-canonical (degenerate) telomere repeats and the telomere-adjacent sequences without restriction sites to be digested. Thus, depending on where the restriction enzymes digest the sub-telomeric region, each TRF will contain a certain amount of non-telomeric sequences (termed the X

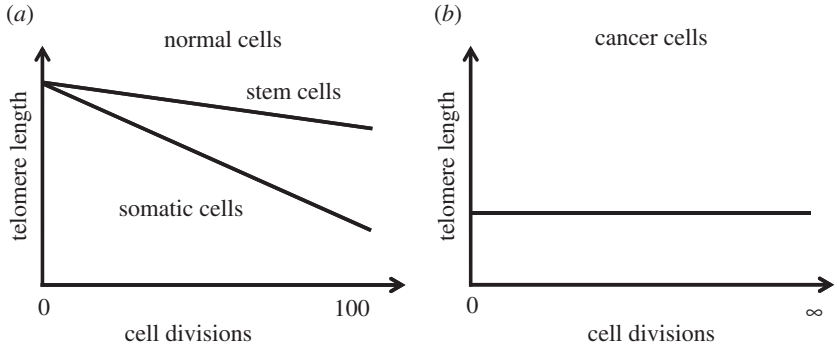

Figure 3. (*a*) Some somatic stem-like cells can transiently activate telomerase, a cellular reverse transcriptase that can partially but not completely maintain telomeres. Most somatic cells do not express any detectable telomerase and have more rapid telomere erosion (*b*). Almost all cancer cells activate or upregulate telomerase and generally maintain stable but short telomeres.

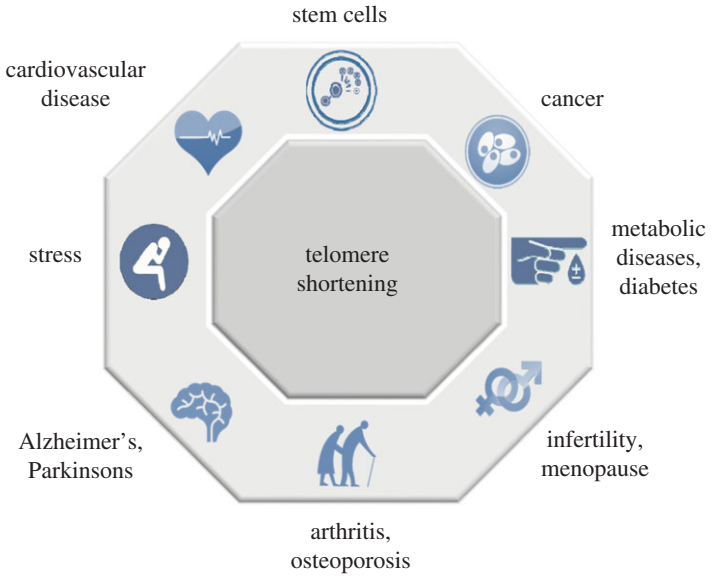

Figure 4. There have been a large number of publications correlating human diseases with progressive telomere shortening. Almost all of these studies are correlative because the assays mostly measure average or relative telomere lengths in cross-sectional studies. (Online version in colour.)

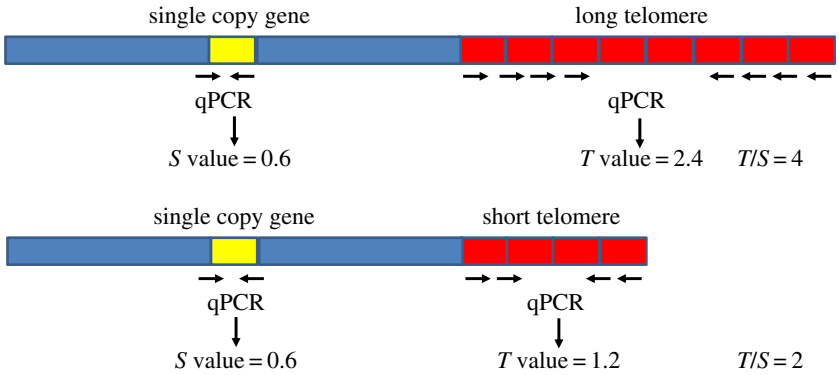

Figure 5. Q-PCR method is commonly used because it is a relatively easy procedure, does not require a lot of input DNA, and can be conducted with high-throughput PCR methods. The method provides a relative telomere length (*T*) compared to a single (*S*) copy gene and results are expressed as a *T*/*S* ratio as depicted. (Online version in colour.)

region). After DNA digestion, gel electrophoresis is performed and the telomeric sequences can be detected by Southern blot analysis (figure 6*a*). A smear of variable lengths of telomeres is detected by radioactive or non-radioactive telomere probe, and the average TL is then determined by quantification of the intensity of labelled DNA smear and comparison to a DNA ladder with known fragment sizes [32–34]. While TRF analysis has been proved to be useful for many studies, the requirement of large amounts of starting DNA (approx. 3 µg) and a relatively long time to perform the assay limits its use for very large population studies except in a few specialized laboratories. However, commercially available

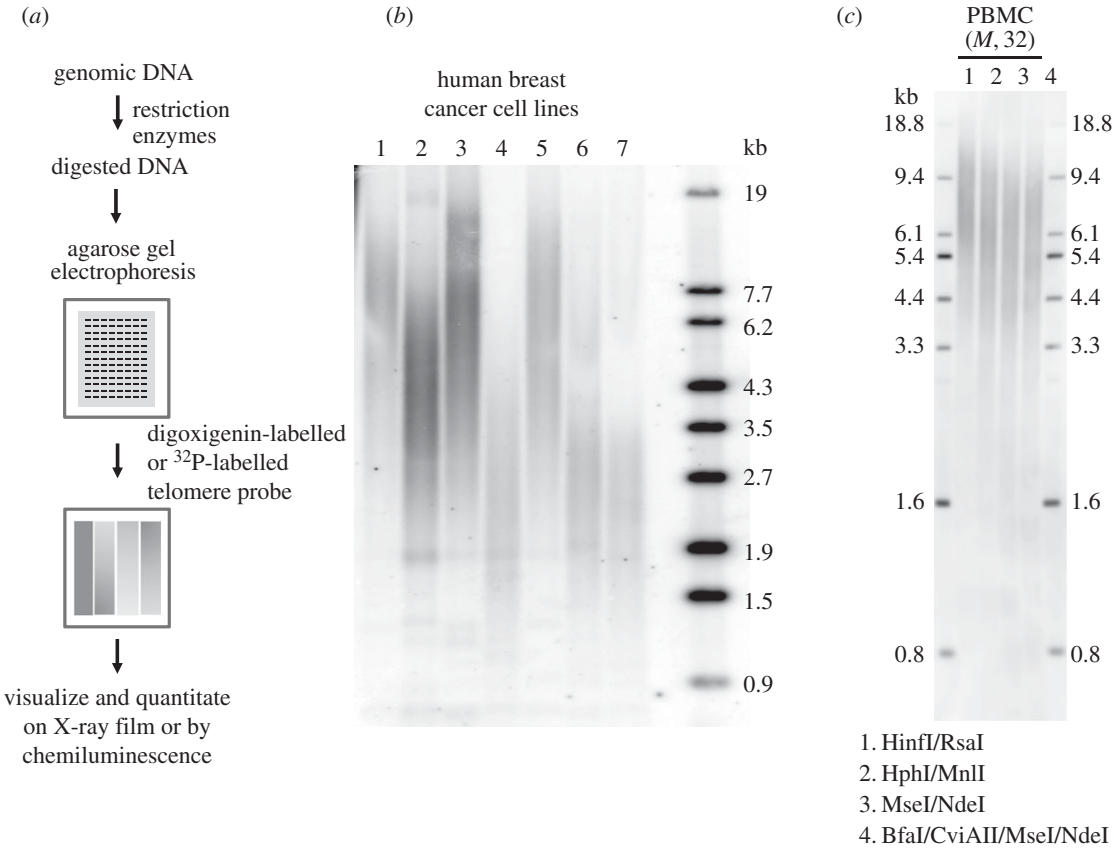

Figure 6. TRF is the 'gold standard' for TL measurements. (*a*) The methodology and (*b*) examples of a panel of human breast cancer cell lines of varying TLs. (*c*) Depending on the panel of restriction enzymes used to digest genomic DNA, the same DNA can vary greatly in TL. It is difficult with this Southern blot method to detect and quantitate the shortest telomeres.

kits are now available for TRF that may help improve inter-laboratory variations. In addition, due to hybridization kinetics, telomeres that are relatively short (approx. 2 kb or less) are difficult to detect and quantitate (figure 6*b*). Finally, depending on the panel of restriction enzymes used, TRF analysis can vary widely. For example, using a panel of four different combinations of restriction enzymes on the same starting material (figure 6*c*), the human peripheral blood mononuclear cells (PBMCs) population of cells can vary, on average, by several kb. Thus, unless there is a consensus going forward in the field of which restriction enzymes to use, averages in TL cannot easily be compared between laboratories and various published studies. Using restriction enzymes that provide the shortest overall telomere spreads would be optimal because it minimizes the inclusion of the X region (e.g. Mse1/Ndel or Bfal/CviAII/MseI/Ndel). Despite these variations among different groups, cross-sectional studies of cells from humans at different ages have revealed a general trend for telomere loss with increased age in mitotically active tissues [35–38].

(c) Quantitative Fluorescence In Situ Hybridization

TLs can also be measured using several Q-FISH methods, which are based on similar principles but with some modifications for various applications. Interphase Q-FISH (figure 7*a*,*b*) uses a microscope to determine telomere fluorescence intensity after hybridization with a fluorescent peptide nucleic acid (PNA) telomeric repeat ($CCCTAA_3$) probe [35]. Similar experiments can be done with metaphase spreads (figure 7*c*). Metaphase Q-FISH can measure the TL at each individual chromosome end with higher accuracy but require proliferating cells [34]. Commercial modification of interphase Q-FISH, termed high-throughput Q-FISH (HT Q-FISH) [39], can use automated procedures on 384-well plates for large-scale studies on fixed lymphocytes. This assay is termed Telomere Analysis Technology and is CLIA (Clinical Laboratory Improvement Amendments) certified (Lifelength.com). This assay has intra-assay coefficients of less than 5%. Flow FISH (similar to Q-FISH) [40] is also a commercially available method (Repeatdx.com) that is CLIA certified as a clinical diagnostic tool that determines telomere fluorescence in individual interphase cells by fluorescence-activated cell sorting analysis (FACS) technology. Flow FISH can be adapted for modestly higher throughput, thus permitting some larger scale studies, primarily on human lymphocytes. However, due to the limits on probe hybridization, Q-FISH methods do not detect fluorescent signals at telomeres at chromosome ends that have telomeric repeats below the threshold for the PNA probe hybridization (so-called telomere-free ends). Another disadvantage of all Q-FISH techniques is that the probe may also bind to some interstitial telomeric sequences (ITSs), which consist of telomeric repeats located away from chromosome ends in vertebrates [41,42], thus generating some false-positive results. There is also a concern that some very bright Q-FISH signals may represent clusters of several telomeres in close proximity and it is not known how these may be reflected in the quantitation. Another application of Q-FISH is conducted on tissue sections, also called 'telomapping' [41]. The advantage of telomapping is that it can be performed on biopsies from any type of tissue and thus may have value in pre-clinical and clinical studies using archival formaldehyde-fixed paraffin-embedded

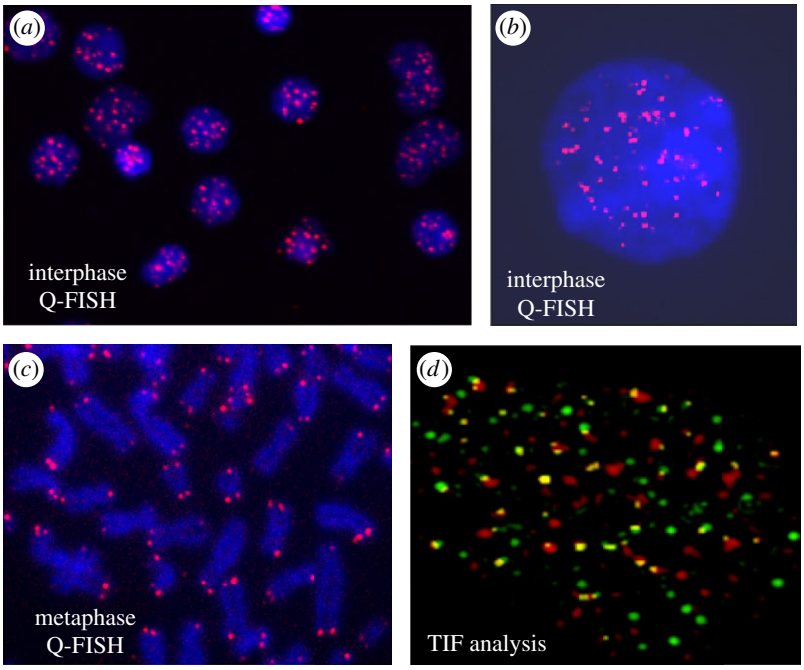

Figure 7. Q-FISH generally uses a TTAGGGn PNA labelled probe to hybridize to interphase cells (*a*,*b*). Alternatively, the same probe can be hybridized to metaphase chromosomes (*c*). With the help of a skilled cytogeneticist, this permits determination of which chromosome ends have longer (stronger signals) or shorter (weaker signals) telomeres. Chromosome ends that do not show fluorescence signals are reported as signal-free ends when they actually still have canonical telomeric repeats that are not sufficiently long to hybridize with probes. When telomeres become critically short, they are recognized as DNA damage. The commonly used technique Telomere dysfunctional Induced Foci (TIFs) indicates this damage by co-localization of a DNA damage recognition antibody (such as gamma H2AX or 53BP1) with a telomere-specific labelled probe or a shelterin antibody such as TRF2 (*d*).

specimens. Many of these assays often refer to intra-assay coefficients as being less than 5% which, on the surface, seems really good. However, there are limits to the usage of intra-assay coefficients as an accurate reflection of repeatability. For example, others have discussed whether the coefficient of variation is an appropriate statistical approach when assessing TL measurements [43–45]. Finally, it is important to point out that while there are limitations to the Q-FISH methods, there have been many key studies using Q-FISH reported. For example, Q-FISH techniques have demonstrated that it is the shortest telomeres, not average TL, that are critical for cell viability and chromosome stability in mice [10,46,47]. Metaphase Q-FISH has also provided valuable information on the shortest telomeres during replicative senescence in human cells [13] and in a variety of species in the mammalian radiation [48,49].

(d) Telomere dysfunctional Induced Foci analysis

A telomere dysfunction assay that is useful for DNA damage studies is TIF analysis (Telomere dysfunctional Induced Foci). This method is generally conducted on interphase cells *in vitro* or in tissue sections and generally involves using two antibodies, one to a shelterin protein such as TRF2, and the other to an antibody that recognizes DNA double-stranded breaks, such as gamma H2AX or 53BP1. One can also use a PNA telomeric repeat probe to co-localize with gamma H2AX or 53BP1 [50]. As telomeres are only approximately 1/6000th of the total genome, any co-localization by chance would be very rare. As illustrated in figure 7*d*, the large number of yellow co-localized signals suggest damage at many telomeres. This assay may be useful as a biomarker for following clinical trials that inhibit telomerase. However, this assay does not provide information about TL, only that some telomeres are so short or uncapped that they appear as damaged DNA. In combination with TRF or combined with Q-FISH, TIF analysis could provide information about the average TL when telomeres are sufficiently short to initiate onset of disease.

(e) Single Telomere Length Analysis

Single TElomere Length Analysis (STELA) was designed to measure telomeres on individual chromosomes [51]. STELA measures the abundance of the shortest telomeres using a combination of ligation, PCR-based methods and Southern blot analysis. There have been many significant advances using this method. For example, STELA has revealed extensive allelic variation and ultrashort telomeres in senescent human cells [51]. In addition, STELA has demonstrated telomere dysfunction and fusion during the progression of chronic lymphocytic leukaemia indicating evidence for telomere crisis [52]. Another advantage of STELA is the ability to accurately measure chromosome-specific TL with limited starting material. STELA has also been adapted to develop telomere-end ligation protocols to determine the terminal nucleotides of both the C-rich and G-rich telomere strands [53] and to determine that most chromosomes ends have telomere extension during steady-state conditions [54]. However, one limitation of STELA is that not all chromosome ends have unique sequences for the design of primers, and this restricts the number of chromosome ends that can be followed. To attempt to resolve this problem, the Universal STELA (U-STELA) method was introduced in 2010 [55] that is able to detect telomeres from every chromosome end, making it possible to monitor changes in the shortest telomere in cells.

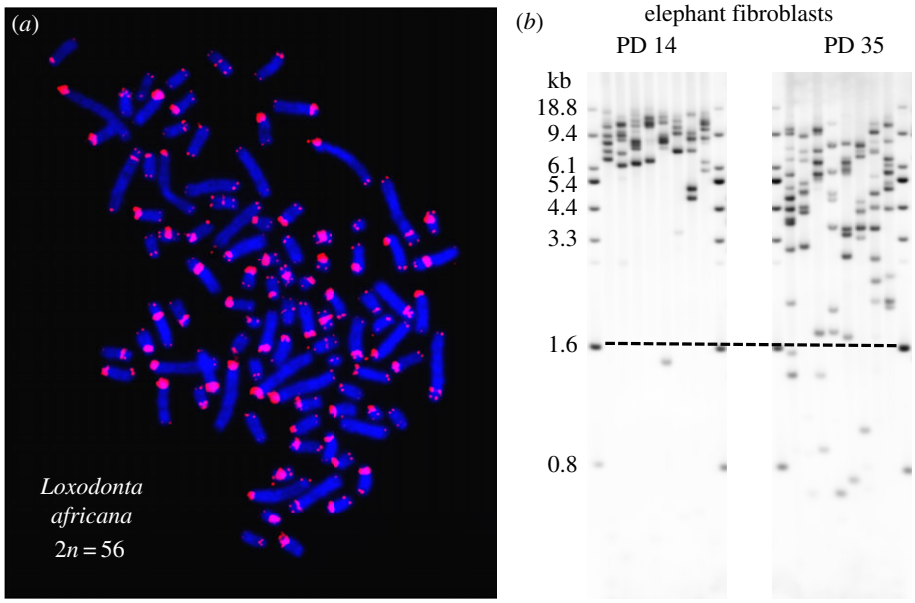

Figure 8. TeSLA (Telomere Shortest Length Assay). (*a*) A metaphase spread of elephant chromosomes after hybridization with a Q-FISH PNA probe. When examined closely, there are many chromosomes that have large tracks of interstitial telomeric sequences (ITSs). Most telomere measurement methods will include these in their analyses. The TeSLA assay was designed to specifically not include ITSs, so only the canonical telomere ends are quantitated and a minimal amount of sub-telomeric region (*b*).

However, U-STELA has several limitations, as previously detailed [26]. For example, it is not efficient at detecting TLs over 8 kb [26,55], which affects the detection and accuracy of TL distribution. It can, however, be used to count absolute numbers of the shortest telomeres, but this requires manual quantitation of the 100s of telomere sizes detected in both STELA and U-STELA.

(f) Telomere Shortest Length Assay

To address the need for a more sensitive, accurate and unbiased method to measure lengths of all the telomeres including the shortest telomeres without detecting ITSs, a new method termed TeSLA was developed. TeSLA requires small amounts (less than one microgram) of starting DNA and employs an improved ligation and digestion strategy, the classic Southern blot analysis with hyper-sensitive digoxigenin-labelled probe [56], and user-friendly image-processing software to automatically measure the distribution of telomeres at different lengths [57]. This allows measuring the abundance and unbiased distribution of telomeres from less than 1 kb to approximately 18 kb. With TeSLA, subtle TL change can be monitored in a shorter period of time and the method provides insights into telomere dynamics during various cellular processes. For example, the length of telomeres was measured longitudinally in PBMCs during human ageing, in tissues during colon cancer progression, in telomere-related diseases such as idiopathic pulmonary fibrosis, as well as in *TERT* knockout mice and other organisms [57]. The disadvantage of TeSLA is that it is low throughput (as is STELA and U-STELA) and does not measure the very longest telomeres, such as those in inbred strains of mice. However, for human studies and most animal studies, TeSLA will be useful for determining longitudinal changes in the shortest telomeres over a variety of time intervals and experimental manipulations. In humans, determining the shortest telomeres could have diagnostic implications for disease development (pathological thresholds) where earlier interventions may result in better patient management. In addition, TeSLA does not amplify ITSs that are very large in some animals [57]. As an example, we compared elephant telomeres by Q-FISH on metaphase chromosomes (figure 8*a*). As seen in the metaphase spread after Q-FISH, there are some very large regions of ITS in elephants, but most telomeres at the chromosome ends have much reduced telomere signals. TeSLA (figure 8*b*) only reveals the canonical telomeric repeats at each chromosome end and thus one can quantitate the shortening of telomeres with passage in cell culture and longitudinally in a variety of animal species, even those with ITSs. Details about the comparisons of TeSLA to TRF, Q-FISH and both STELA and U-STELA can be obtained from the original source [57], including the ability to download the software package that should facilitate quantitation for STELA, U-STELA and a variety of additional assays besides TeSLA.

3. Pros and cons of telomere length measurement methods

Table 1 provides a brief overview of the main advantages and limitations of each of the mentioned TL measurement methods. Gaining a better understanding of the benefits and drawbacks of various measurement techniques is important to further establish the science of telomere associations with human disease. Several factors should also be considered when selecting a university or commercial laboratory for providing TL quantitation. These factors will include issues related to ease of collaboration, transportation of specimens and specimen quality. The type of questions one may wish to discuss with potential collaborators and reference laboratories that relate to the quality of their testing include (but are not limited to) the following:

Table 1. Comparisons of different TL measurement methods.

method	advantages	limitations	references
Q-PCR (research and commercial)	easy to conduct; small amount of starting DNA required; many population-based studies for comparisons	large variations among different laboratories, but reproducibility is better in commercial setting; not useful in cancer studies due to aneuploidy; only average TL is provided as a relative ratio	[28]
TRF (research)	common method for research studies; highly reproducible in some laboratories; many published studies for comparative research	larger amounts of starting DNA required; provides most information on average TL; need to standardize restriction enzymes used to compare studies between laboratories (subtelomeric polymorphisms can alter data obtained); labour intensive	[5]
interphase Q-FISH (research)	can be conducted on fixed tissues and cells	labour intensive; TLs expressed as relative fluorescence units (not actually TLs) but using standards measured by TRF actual TLs can be inferred	[34]
HT Q-FISH (research and commercial)	same as interphase Q-FISH; very reliable and reproducible results; CLIA certified	does not distinguish telomere clustering in interphase cells; does not recognize telomere-free ends	[35]
Flow FISH (research and commercial)	same as interphase Q-FISH; can provide cell- type-specific information on mostly average telomere lengths; reproducible; CLIA certified	requires an expensive FACS instrument; almost universally uses peripheral blood mononuclear cells (PBMCs)	[39,40]
metaphase Q-FISH (research)	can potentially detect telomeres on all chromosomes	does not detect the telomeres that are very short that do not hybridize with probes (appear as telomere-free ends); requires highly skilled cytogeneticist for chromosome-specific analyses	[34]
STELA (research)	can detect the shortest telomeres on specific chromosomes	works on only a small subset of individual human chromosomes; low throughput; labour intensive	[51]
Universal STELA (research)	measures mainly the shortest telomeres	does not detect larger telomeres and can detect ITSs; manual quantitation; low throughput; labour intensive	[55]
TeSLA (research)	measures all the telomeres less than 1 kb and up to 18 kb on all chromosome ends; works on many animal types; automatic quantitation of telomere sizes using user-friendly software	low throughput; labour intensive	[57]

— What is the evidence for reproducibility, accuracy, reliability and sensitivity of the methods used?
— What validation studies have been completed to ensure the accuracy of their testing?
— Are appropriate control standards of short and long telomeres included to control for batch to batch variations?
— How long will it take to obtain results (e.g. is the method high throughput or low throughput)?
— What information (reports) will be provided about the shortest telomeres as well as average or relative TL?
— For clinical specimens, is the laboratory CLIA certified and what is the cost per sample?

4. Concluding remarks

In the last decade, there have been literally thousands of published studies indicating that average or relative TL correlates with human disease onset or progression (figure 4). Although it is well established that progressive telomere shortening occurs with increased human age, small, cross-sectional studies on the association with psychosocial and environmental factors should be interpreted with caution until larger, longitudinal-based studies can be carried out. While there have been some larger population studies conducted in humans (generally using PBMCs or buccal washes), most of these used Q-PCR relative TL measurement approaches. Q-PCR is easy to conduct, but does not provide information about the shortest telomeres, so these studies are at best associations with specific diseases and thus do not prove cause-and-effect relationships. While telomere dynamics may have important implications for disease monitoring, it will be critical to have methods that provide mechanistic information about all the telomeres in a population of cells. Small changes in average TL, even in large studies that are statistically significant, are only correlative. Any reports suggesting cause-and-effect relationships should be interpreted with great caution at the present time.

Following all the telomeres (both long, average and short) longitudinally during the lifetime of various animals including humans is needed. Scientists interested in the effects of nutrition, exercise, stress, smoking, obesity, etc. on human health outcomes should recognize that TL testing is only a test at a particular point in time. One can think about TL testing today as we did about cholesterol testing many decades ago. When cholesterol testing was first initiated, we did not even know about the different types of cholesterol and there were no cholesterol-lowering drugs available. However, if an individual had a high cholesterol count, this was like a tap on a patient's shoulder that perhaps something was not quite right and additional tests would be ordered to see if the first test was correct and, if so, changes in personal behaviour (e.g. diet intake, reducing weight or increasing exercise) were recommended with repeat testing for cholesterol levels on a regular basis. TL testing is at a similar early time point as a potential biomarker of health status.

Perhaps, in the future, we will be able to show cause-and-effect TL relationships when telomerase activators are discovered, especially if they slow down the rate of telomere erosion and result in a decreased onset of clinical disease. Initial evidence suggests that a dietary supplement, TA-65, serves as a telomerase activator [58,59]. One might imagine that some of the telomere spectrum genetic disorders, such as dyskeratosis congenita or idiopathic pulmonary fibrosis, may provide early tests of telomerase activators. These patients have few options and delaying the onset or perhaps even preventing the disease by elongating the shortest telomeres may be an effective interventional strategy. Carefully designed longitudinal studies should be considered in the future. Progress in developing robust methods in the telomere field is critical to move from association/correlative studies to more mechanistically relevant studies. A better understanding of the trajectory of TL changes (especially on the shortest telomeres) may provide important biomarkers for timing interventions not only to ameliorate specific chronic diseases but also to target interventions for general overall healthy ageing. The basic and applied science of how to elongate telomeres without increasing the risk of cancer is clearly an important direction in the future.

5. Added notes in proof

This special theme issue of the *Philosophical Transactions of the Royal Society – Biological Sciences* has additional contributions that should be reviewed when considering future TL studies. These include heritability of telomere variation [60]; experimental manipulation of TLs [61]; associations between TL and behaviour [62]; telomeres and genomic evolution [63]; and the role of telomeres in life-history trade-offs that may influence the relationship between ageing and cancer [64–66].

Data accessibility. This article has no additional data.

Authors' contributions. J.W.S. wrote the original draft. T.P.L. and W.E.W. edited. All authors provided substantial contributions, conception, design, acquisition of data, or data analysis and interpretation. All authors gave final approval of the submitted version.

Competing interests. We have no competing interests.

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