Fluorescence Spectroscopic Detection and Measurement of Single Telomere Molecules

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SUPPLEMENTARY INFORMATION

Supplementary Note 1: Acquisition of fluorescence signal

Detection of the fluorescently labeled telomere is performed in a microfluidic channel as described previously (1). Briefly, a solution containing the labeled telomeres is driven through the microchannel using a pressure source at various settings. An expanded laser (488 nm Argon ion laser) confocal spot spans the entire cross-section of the microchannel, and as each molecule passes through this region, it is excited and the emitted photons are collected by an avalanche photodiode (APD). The fluorescence signals collected from the molecules at 0.1 ms intervals are processed using custom Labview software. ND filters are applied when more than 10% of the peak heights exceed 1,000 photon counts per 0.1 ms interval in order to prevent detector saturation. We define a peak as having been detected when the fluorescence intensity exceeds a pre-determined threshold, \( t = 100 \) and 40 photon counts when no ND and ND 0.5 is applied to the laser source, respectively (Figure 2A). To minimize measurement artifacts arising from fluorescence fluctuation around the threshold, the beginning and end of each peak are signified when the fluorescence drops below the baseline value, \( b = 40 \) and 15 for no ND and ND 0.5). The having different values for \( t \) and \( b \) ensure that signal fluctuating around the threshold will not register as multiple peaks. Peak parameters are calculated as described in the main text.

For a typical sample, plotting Size (\( S \)) vs. Width (\( W \)) yields two separate clusters of peaks, representing the actual sample and the thresholding artifacts (Supplementary Figure S1). Lowering the threshold and baseline values improves the sensitivity of the method, but also increases the number of spurious events arising from background fluctuation. To help us identify the peaks corresponding to actual fluorescent molecules, the peak parameters are modeled as described in the following section.

Supplementary Figure S1. Plotting the peak size and width on a log-log plot yields two distinct clusters. One cluster is due to the thresholding artifacts (gray) and the other is the actual sample we are testing (red), as determined by the model. Each dot represents a single detected fluorescent molecule.

Supplementary Note 2: Modeling peak parameters

The laser excitation and detection volume, referred to as the observation volume (OV) of CICS represents the space in which fluorescent molecules will be excited and detected, and is uniform across the channel.
(x-axis) and along the optical axis (z-axis), and Gaussian along the flow axis (y-axis)(1). As each molecule traverses this Gaussian OV along the flow axis, the number of photons or fluorescence intensity, $I$, detected can be modeled as a convolution of the OV with the distribution of fluorophores along the flow axis ($\Phi(y, L)$), of the general form

$$I(y, L) = \Phi(y, L) \otimes OV(y)$$

Single fluorophores and short DNA sequences hybridized to fluorescent PNA probes can be modeled as point emitters, i.e.

$$\Phi(y, L) \propto \delta_y$$

$$\Phi(y, L) = \kappa \cdot L \cdot \delta_y$$

where $\kappa$ and $L$ represent a constant of proportionality and the length of the DNA, respectively. This assumes that the length of the DNA is directly proportional to the number of bound probes, and hence fluorescence. The resulting intensity function, $I(y, L)$, thus assumes the shape of the OV, i.e. Gaussian, where

$$OV(y) = e^{-\frac{y^2}{c^2}}$$

$$I(y, L) = \kappa \cdot L \cdot e^{-\frac{y^2}{c^2}}$$

where $c$ is the parameter describing the size of the OV, and has been determined to be 0.125 microns previously (1).

On the other hand, longer DNA molecules can be modeled as approximately spherical (if unconfined within the microchannel) or ellipsoidal (if confined) finite-sized random coils with the fluorescence homogeneously distributed in the corresponding volumes. In the first case, the radius of gyration, $R_g$, is estimated as

$$R_g \propto L^\gamma$$

$$R_g = \beta L^\gamma$$

The $\beta$ value (0.0412) is derived from reported hydrodynamic radii for linear DNA (2). The $\gamma$ value (0.6) is based on the well-established Flory approximation, assuming a free polymer chain in a good solvent (3,4). For a channel with a depth, $d$, of 0.5 micron tall channel, the onset of weak confinement on the DNA free coil is around 8-9 kb, corresponding to $R_g \approx 0.3d$, or 150 nm (3). Even 50-60 kb DNA molecules (representing the typical maximum DNA length that can be extracted from cells) experience only moderate confinement ($R_g < d$) (3,5). As such, we will restrict the analysis to spherical approximations of the DNA molecules.

$$V = \frac{4}{3} \pi R_g^3 = \frac{4}{3} \pi \beta^3 L^3$$
where \( V \) is the total volume of DNA molecules. Since the total number of fluorophores is proportional to the length of DNA, we can define total fluorescence of each DNA molecule, \( F \), and its spatial density, \( f_v \),

\[
F(L) = k \cdot L
\]

\[
f_v(L) = \frac{F(L)}{V} = \frac{k \cdot L}{V} = \frac{3kL^{1-3\gamma}}{4\pi\beta^3}
\]

In this model, \( L \) represents the total length of the fluorescent segment, while \( k \) is a scaling parameter to account for both the intrinsic brightness of the fluorophore, as well as the intensity of the excitation source.

The incremental volume, \( dV \), along the flow axis is parabolic for both unconfined and confined DNA random coils, with different coefficients. The distribution of mass along the flow axis can thus be represented by \( \rho(y,L) \), where

\[
\rho(y,L) = \begin{cases} 
\pi(R_g^2 - y^2) = \pi((\beta L')^2 - y^2), & \text{for } -\beta L' < y < \beta L'; \\
0, & \text{otherwise}
\end{cases}
\]

\[
dV = \rho(y,L) \cdot \delta y
\]

Assuming that the fluorophores are homogeneously distributed in this volume, the fluorophore distribution, \( \Phi(y,L) \), is the product of this parabola \( (dV) \) and the fluorophore density \( (f_v(L)) \).

\[
\Phi(y,L) = \rho(y,L) \cdot f_v(L) \cdot \delta y = \frac{3kL^{1-3\gamma}}{4\beta} \left( 1 - \left( \frac{y}{\beta L'} \right)^2 \right) \cdot \delta y, \quad -\beta L' < y < \beta L'
\]

The peak profile, \( I(y, L) \), will then be simply the convolution between the fluorophore distribution, \( \Phi(y, L) \), and the Gaussian OV profile \( OV(y) \) (Supplementary Figure S2).

**Supplementary Figure S2.** The peak profile is the result of the convolution of the fluorophore distribution profile and OV profile. Depending on the relative sizes of these two contributing factors, the resulting profile can be parabolic, Gaussian or intermediate between the two in shape.

As the radius of the DNA increases, the peak profile transitions from Gaussian (when \( R_g << c \)) to parabolic (when \( R_g >> c \)), as the OV and \( \Phi \) functions dominate the peak profile function, respectively. It is worth noting here that while the horizontal axis in Supplementary Figure S2 is in terms of position \( (i.e. y) \).
value), it can be converted into a time axis if the velocity is known since the APD samples the fluorescence at fixed time intervals.

The DNA diameter corresponding to the largest telomeres (~0.3 microns for 10 kb molecules) is comparable to the size of the Gaussian OV, which has a $1/e^2$ diameter of around 0.25 micron along the flow axis (1). Consequently, the peak profile for long telomeres is intermediate between Gaussian and parabolic, for which no simple analytical solution exists. Nevertheless, numerical approximations of the peak profile for different DNA lengths and the corresponding parameters can be calculated using mathematical software (Wolfram Mathematica 9).

$S$-values are obtained by integrating the peak shape over the time interval, $W$, which is determined using the same threshold that is used for the experimental data. To obtain the peak height or $H$ from this model, it is noted that the maximum signal occurs when the $\Phi$ and OV functions are centered, i.e. when the DNA lies at the center of the OV, such that $I(0, L)$. To recapitulate the peak shape using the model, only two variables have to be chosen empirically using our experimental data. The first is the $k$-value, which is a single factor to account for excitation intensity, detector efficiency, DNA labeling density, and dye quantum yield. The second is a scaling factor applied to both $S$ and $W$ to account for velocity.

**Supplementary Figure S3.** Comparison between the experimental and predicted peak heights and widths demonstrates the remarkable agreement between the two.

Comparing the peak heights and widths from the model with experimental results obtained from Hind III digested λ DNA labeled with TOTO-3 indicates a good fit between the two (Supplementary Figure S3). The λ DNA digest system was chosen because the sizes of the fragments are well defined, and the longest fragment is larger than 20 kb, as opposed to only 2.4 kb for our telomere standards. This allows us to examine the accuracy of the model when deviating from the point emitter idealization. Furthermore, since any difference between the Hind III digest and Alexa488-labeled telomere systems can be accounted for by adjusting the $k$ (incident intensity, dye quantum yield, and labeling density) and $c$ (width of the Gaussian OV) factors, there are no explicit reasons to expect significant differences between this and the telomere system. Thus, the model should be able to account for both these systems with minimal change. Derived essentially from first principles, the extensive agreement between the model and
experimental data suggests that the model does indeed describe the physical system, and can be used to inform our gating strategy, and even predict the peak parameters.

**Supplementary Note 3: Detection of Telomere Standards by PHAST**

Previous implementations of CICS utilized peak burst size for quantification. Since peak size changes with flow velocity (slower moving molecules will reside in the OV longer, resulting in larger number of total detected photons), a calibration step is necessary to account for different flow speeds. Although the flow velocity varies linearly with the driving pressure, the exact linear relationship (the slope, x- and y-intercepts) changes from chip-to-chip, depending primarily on the hydrophobicity of the PDMS microchannel (which increases with time after oxygen plasma bonding with glass), and to a lesser extent the variations in the dimensions of the microchannels. Since we can only control the driving pressure directly, there is no way to be certain of the flow velocity without utilizing flow tracers such as fluorescent microspheres (1,6,7).

Flow tracers typically require a separate fluorescence channel (that is, an additional laser excitation source and emission detector) that is coincident with the sample channel. This is non-trivial since the alignment of the optical components has to be extremely close. Since light of different wavelengths focus at slightly different positions along the optical axis, it is necessary to record the two spectral channels sequentially, with an additional focus adjustment step in between. Doing away with the flow tracers greatly simplifies the demands on the instrumentation. For the existing CICS instrument, a tracer-free CICS also frees up an additional spectral channel to permit simultaneous multi-color detection.

In our model, changes in flow velocity merely broaden peaks proportionately, resulting in a simple scaling of the peak sizes and widths without any change to the shape of peaks. This was borne out by experimental data where the slope of $\log_{10}(\text{width})/\log_{10}(\text{size})$ curve for each telomere standard hybridized with Alexa 488-labeled PNA at different driving pressures has a slope of around 1 (Figure 2E). Furthermore, since the $\log_{10}(\text{size})/\log_{10}(\text{width})$ curve for each velocity has the same slope (~0.2) and thus never intersects with another, a characteristic curve corresponding to each flow velocity can be obtained. Consequently, each $\log_{10}(\text{size})$-$\log_{10}(\text{width})$ co-ordinate corresponds to a unique velocity and telomere length (TL), i.e. for a given set of experimental conditions, we expect all the real peaks to lie parallel to the TL axis (Figure 2E).

Peak size (i.e. total photons detected during the transit of a fluorescent molecule through the detection volume) is, in principle, the best measure of the actual TL, once the flow velocity is accounted for. This is because each fluorophore has the same path integral in CICS, and hence the total number of detected photons corresponds to the total fluorophore content, and by extension TL, regardless of the spatial distribution of the fluorophores (i.e. peak shape). However, fluctuations in the flow velocity are inevitable, which introduce additional variation to the peak size. This variation can be observed in Figure 2E, where each cluster of peaks is elliptical, with its long axis approximately parallel to the Flow Velocity axis. A transformation can be performed to convert the flow velocity and TL axes into an orthogonal pair for
simple telomere sizing. Alternatively, given the consistent ellipsoidal molecular shape for all the expected DNA molecules (as discussed in the previous section), peak height can also be utilized for telomere sizing.

The model predicts that in general, the peak height is a good proxy for each TL that is independent of flow velocity, except when the velocity is high enough to render the sampling rate inadequate, or if the DNA molecule is much larger than the OV. The former can be seen in minimal shifts in peak heights (~10%) with an 8-fold change in flow velocity when the driving pressure was increased from 0.25 to 2 psi (Figure 2F). Therefore, by simply flowing the samples through the microchannels at adequately low pressures (~ 0.5 psi), it is possible to obtain good TL estimates without correcting for the exact velocity.

Because most of the DNA molecules are small compared with the Gaussian OV and hence well described by the scaling parameter \( \kappa \) (Supplementary Note 2), peak height also varies linearly with TL for relatively short telomeres (< 10 kb). On the other hand, very long DNA molecules deviate from the point-emitter idealization. In these cases, the fluorophores are much more spatially distributed, and only part of the DNA lies in the detection volume at any point in time, and thus results in a decrease in the maximum instantaneous fluorescence (i.e. the peak height). While the telomere standards are too short for us to observe this drop-off (the longest telomeric segment is only 2.4 kb long), both the initial linear relationship and the subsequent drop-off were observed experimentally for Hind III digested lambda DNA (Supplementary Figure S3). Although this nonlinearity presents some uncertainty in obtaining TL values from peak height by extrapolation, for TLs up to 10 kb in length, a linear approximation is deemed sufficient. Even for telomeres up to 30 kb in length, the linear approximation underestimates TL by only 15-20%. Therefore, the peak height is considered adequate as a general sizing approach. Based on our experiments, the conversion from peak height, \( H \), to TL can then be performed by a simple linear transformation, where

\[
TL \text{ (kb)} = \frac{H - 50}{780}
\]

for Alexa488 labeled probes.

**Supplementary Note 4: Labeling of Telomeres in Total Genomic DNA**

Unlike cloned telomeric standards, total genomic DNA contains a large amount of non-telomeric sequences. In fact, telomeres constitute around just 0.01-0.02% of the total DNA mass. As a result, even though only a small proportion of genomic DNA will hybridize non-specifically to the Alexa 488 labeled PNA (PNA-AF488) probes, the sheer abundance and different sizes of these molecules makes them difficult to remove by size selective methods (dialysis, microspin columns, etc). Because each DNA molecule is expected to bind very few PNA probes in this manner, the presence of these non-specifically labeled molecules manifests as a generally higher background, which increases the chance of erroneous detection. Furthermore, since we define the start and end of peaks as the points at which the photon count crosses the baseline level (Figure 2B), if the background were to approach or exceed the baseline,
it will cause errors in the measurement of all peak parameters, since the demarcation between one peak and the subsequent one(s) may not be possible to ascertain.

4.1 Pull-Down Assay for Removal of Non-Telomeric DNA

To address this, we developed a one-tube purification assay to allow removal of virtually all of the non-telomeric DNA molecules (Supplementary Figure S4). Briefly, telomere-containing DNA molecules are hybridized with biotinylated telomere-specific PNA capture probes (pcTel-Bio) without any prior digestion, then immobilized on streptavidin-coated magnetic beads, allowing the non-telomeric DNA fragments-containing supernatant to be removed (Supplementary Figure S4, Steps (I)-(III), Panels A and B). After rinsing, the captured telomeres are released into a solution of PNA-AF488 probes at an elevated temperature. By using 100 times as much PNA-AF488 probes as biotinylated ones, we ensure that the fluorescent probes will compete favorably against any biotinylated capture probes that remains in solution (Supplementary Figure S4, Steps (IV)-(V), Panels C and D). This whole process is performed in a single tube, and thus further development such as automation and implementation on a microfluidic platform is uncomplicated.

Supplementary Figure S4. Overview of the PHAST purification protocol and performance. This figure shows the one-tube sample preparation for PHAST assay at the sampling handling (Steps (I)-(VI)) and molecular levels (Panels A-D). Telomere-containing DNA is purified by incubation with the pull-down probe, pcTel-Bio, before using streptavidin-coated magnetic beads to remove it from solution (Steps (I)-(II)). The supernatant containing genomic DNA is then removed and the beads are washed (Step (III)). At this point, the telomere-containing DNA is left on the beads (Panel B). Next, the fluorescent PNA-AF488 is introduced at 100-fold excess compared to the pcTel-Bio (Step (IV)). This ensures that even if free pcTel-Bio were present, the PNA-AF488 can successfully label the telomere DNA. Heating the sample up
to 85 °C then allows the DNA to simultaneously detach from the beads, and hybridize to the fluorescent probes (Panels C-D). To minimize competition from the now free pcTel-Bio on the beads, the 2-hour incubation at room temperature is performed on a magnetic holder (Step (V)). The sample can now be column-purified and tested on CICS (Step (VI)). The purified sample yields fluorescent peaks on CICS, which can then be binned and plotted on a histogram (Panels E-F).

To demonstrate the effectiveness of the pull-down protocol, 45 pg of 2.4 kb telomere standard in 1 µg of genomic DNA is processed. The gel image (Supplementary Figure S5A) shows the intense fluorescence due to the genomic DNA, which completely obscures the telomere band. After the pull-down procedure, the telomeric band can be clearly seen (white dashed box). Non-specific pull-down of long genomic DNA fragments invariably occurs. However, due to the specificity of the biotinylated PNA probes, these fragments are not in sufficient concentrations to show up in the CICS trace (Supplementary Figure S5B, C). The pull-down step greatly reduces the background fluorescence, and thus improves the performance of PHAST.

Supplementary Figure S5. Performance of the pull-down assay. (A) Gel electrophoresis of genomic DNA spiked with 2.4 kb telomere standards showed intense fluorescence over a broad range of DNA lengths. After pull-down, the very intense smear at the bottom of the gel is almost completely removed. The telomere standards (white dashed box) can be clearly seen. Long genomic DNA fragments were also recovered via non-specific interaction with the streptavidin-coated beads, though not in sufficient quantities to affect the signal-to-noise ratio of PHAST. (B) Raw signal traces for the sample before and
after the pull-down assay show the effect of non-specific hybridization. Although the specificity of the PNA probes ensure that the increase in the background is manageable, the proximity of the background level to the threshold results in a significant number of peaks (red arrows) that cross the threshold. These correspond to a TL of around 65 bp, and their sheer numbers skew the resulting telomere size distribution. (C) After pull-down, the background returns to a level comparable to the genomic DNA-free standards. (D) Comparison of the pull-down at different concentrations show that at low concentrations comparable to that found naturally, the efficiency is uniform across different TLs (blue). However, when the concentration of telomeres is very high (~100-fold higher), longer telomeres are preferentially extracted.

4.2 Selectivity of PHAST One-Tube Purification Assay

To ensure that the pull-down purification process does not bias the TL distribution, telomere standards at known ratios (total telomere mass of 180 pg) are spiked into heat-fragmented genomic DNA (~100-1000 bp, 1 µg), and purified using our procedure. At this relative telomere concentration, which is comparable to that expected from mammalian genomic DNA, telomeres were recovered in a size-independent manner (~ 50% for all tested TLs, Supplementary Figure S5D, blue bars).

However, when much higher concentrations of telomere standards were used (16 ng in 1 µg), we found that the relative pull-down efficiency of the DNA increases with the length of the telomere (Supplementary Figure S5D, red bars). This observation can be explained by noting that, in general, we expect the number of biotinylated probes bound to the telomeres to be proportional to the TL. Furthermore, assuming that streptavidin/biotin binding is non-cooperative (a reasonable assumption since it is unlikely for adjacent biotinylated PNAs to bind to the same streptavidin molecule), and since a single successful tether is sufficient to capture a DNA, the association rate of the telomeres will scale with the number of bound biotinylated PNA (8). Because streptavidin/biotin conjugation can be considered to be an essentially irreversible process, the relative pull-down efficiency for telomeres of different lengths will depend only on the association rate, though partially mitigated by the differences in diffusivity and steric hindrance. If the number of streptavidin is insufficient to pull down all the telomeres, the faster binding rate of longer telomeres with more biotin hybridized to them ensure that they will occupy a larger portion of the available surface.

Supplementary Note 5: Gating Procedure for Background Removal

Although the pull-down purification step reduces the background to a more manageable level, in order to detect the shortest telomeres, the threshold is lowered to improve the detection limit. This increases the thresholding artifacts, which occur when the background noise drifts above the threshold, tend to hover around the baseline, such that

\[ S_{\text{artifact}} \sim (W_{\text{artifact}} / \text{interval}) \times \text{baseline}. \]
As a result, the log_{10}(width)/log_{10}(size) slope for the artifacts is approximately 1. On the other hand, based on the model described previously, it is determined that the peak width increases fairly slowly with DNA length and hence peak size (log_{10}(width)/log_{10}(size) slope ~ 0.2 for the conditions tested).

These characteristics have been exploited to formulate the gating strategy for the peaks. By clustering the peaks, two subpopulations can be clearly identified (Supplementary Figure S1). As can be seen in Supplementary Figure S6, a simple threshold in any of the parameters (burst Size, Width or Height) will fail to adequately separate the actual and spurious peaks. On the other hand, a two-dimensional (width/size) gate can separate them (Supplementary Figure S6A, red dashed box). Selecting the cluster with the smaller slope allows us to include many more peaks than merely using a higher threshold, while also excluding the spurious ones. All the cell and tissue samples were gated in this manner and compared to the q-FISH results.

Supplementary Figure S6. Gating strategy for PHAST. The burst sizes and widths for the peaks in a sample are plotted. The gating is performed by selecting the cluster of peaks that has a smaller slope (A). The various plots show the relationships between the different parameters (A-C). The final TL distribution can be calculated from the height distribution (D) using a simple linear formula.

Supplementary Note 6: PHAST vs. TRF

TRF followed by Southern blotting is one of the most commonly used methods for telomere sizing. It is an elegant method for measuring the size of telomeres that takes advantage of the persistence of the canonical hexanucleotide (TTAGGG) repeats in the presence of restriction enzymatic activity (9). It is one of very few methods that can yield any actual absolute telomeric size information, and is particularly useful when comparing the changes to the TL in a cell population over time. However, since the length of the final telomere-containing molecule depends on the proximity of the nearest restriction site to the telomere sequence, the length of this non-canonical sub-telomeric region varies from chromosome to chromosome (Supplementary Figure S7A, B). Consequently, a strong signal on a particular position on
the gel may be due to a large number of short telomeres with long non-telomeric regions, or a small number of long telomeres with short non-telomeric regions. TRF/Southern blotting is unable to distinguish between these two cases.

On the other hand, telomere sizing by PHAST utilizes only the fluorescence intensity, which varies proportionately to the TL. Furthermore, since molecules are detected one at a time, PHAST can decouple the abundance and length of the telomere-containing fragments, thus yielding the actual TL distribution.

To illustrate this, 2.4 kb telomeric sequence ligated into a plasmid vector was digested with Rsal/HinfI restriction enzyme mix, commonly used in TRF protocols. Gel electrophoresis shows the large shift in electrophoretic mobility that results from the loss of the non-telomeric region after digestion (Supplementary Figure S7C). When tested with our method, the samples before and after digestion are virtually indistinguishable (Supplementary Figure S7D). Since extracted genomic DNA typically consists of linear fragments, there is no need for any enzymatic digestion when performing PHAST. This also serves to eliminate any chance of encountering the “star activity” of the nucleases, where non-specific cleavage occurs under sub-optimal reaction conditions and yields erroneous TL measurement (10).

Supplementary Figure S7. Advantage of PHAST over TRF analysis by Southern blot. (A) Genomic DNA is digested by restriction enzymes, while telomeric DNA, which do not contain the restriction sites, remain intact. Differing lengths of sub-telomeric DNA remains on the molecule, depending on the proximity of the restriction site closest to the telomeres. (B) As a result, TRF overestimates the TL by varying degrees. In this schematic, telomeric DNA of identical sizes are misidentified as three molecules of different sizes, resulting in inaccurate length estimates. On the other hand, since telomere fluorescence intensity on PHAST is solely determined by the length of the telomeric sequence, the TL measurement is representative of the underlying TL distribution. (C) To simulate the effects of TRF on the telomere sizing, we digested 2.4 kb telomere standards in linearized plasmids with Rsal/HinfI restriction enzyme mix (gray
dotted box). The large band shift can be clearly seen, highlighting the inability of TRF Southern blot to distinguish between the lengths of the telomeric and non-telomeric portions in a DNA molecule. In an actual sample, the shifted band will be far less well-defined, since the undigested sub-telomeric region will vary greatly in length, depending on the sequence of the DNA. When we tested the same samples using PHAST, it is clear that the frequency (y-axis) of telomere fluorescence (x-axis) is essentially unchanged.

**Supplementary Note 7: Cell Numbers Needed for Telomere Sizing**

To determine the number of cells necessary to obtain a good estimate of the telomeric distribution in a sample, we used Q-Q plots to compare the distribution of varying numbers of telomeres – corresponding to around 5, 10, 20 and 50 cells – against that of approximately 100 cells (10,000 telomere-containing DNA molecules, **Supplementary Figure S8**). Since typical q-FISH uses only around 10 metaphase spreads (corresponding to 1840 telomeric ends) to estimate the telomere distribution, we consider 100 cells more than sufficient to represent the TLs in a sample. A linear curve-fit is applied to the Q-Q plot in each case, and the residuals is used to estimate the goodness of fit.

While the linear fit is relatively good in each case, the residuals become smaller (closer to zero) as the number of cell equivalents increased from 5 to 50. We find that at ~1,900 peaks (corresponding to 20 cells, **Supplementary Figure 8C**), the TL distribution is very similar to that of the 100-cell sample. Since this is also close to the typical number of telomeric ends in q-FISH, we collect 1,900 or more peaks for all our samples, unless otherwise stated.

**Supplementary Figure S8.** Determination of the number of telomeres needed to adequately represent the distribution.

In principle, since CICS can detect every fluorescent molecule that passes through the microchannel, very few cells are needed to perform the sizing of the telomeres. However, due to a relatively large handling volume (e.g. 25 µL), a detection volume of only 2.5 fl, and a flow velocity of less than 1 mm/s to
avoid underestimating the TL, an inordinate amount of time will be needed to fully sample the whole volume (~ 30 million seconds). Therefore, in practice, the number of cells is typically larger (~ 2 \times 10^5 to 2 \times 10^6, comparable to a buccal swab) to compensate for the low concentration, and allow detection to be completed within 10 minutes to 1 hour. However, alternative sample preparation procedures, including in situ or on-chip lysis of cells, coupled with a DNA concentrator can overcome much of this limitation.

Supplementary Note 8: Determining Absolute TL Distribution

Since q-FISH is unable to provide absolute size of telomeres, we converted the q-FISH telomere fluorescence intensity units (TFU) to telomere length by scaling the TFU to match the modal values between PHAST and q-FISH. The resulting distributions from the two methods were found to be similar. The mean TL, and fractions of short (< 0.1 times mean TL) and long telomeres (> 3 times mean TL) are tabulated (Supplementary Table S1). For each cell line, the fraction of long telomeres and mean TL were essentially the same for the two methods.

Supplementary Table S1. Relative abundance of long and short telomeres determined by PHAST and q-FISH, for WI-38, R83 and U2OS cell lines.

|          | WI-38 | R83 | U2OS |
|----------|-------|-----|------|
| PHAST, n = 47 | q-FISH, n = 32 | PHAST, n = 20 | q-FISH, n = 19 | PHAST, n = 57 | q-FISH, n = 30 |
| % Short Telomeres | 8.5 | 14.6 (10.6) | 2.0 | 4.2, (3.2) | 5.3 | 9.9 (6.9) |
| % Long Telomeres | 4.8 | 4.7 | 1.7 | 1.5 | 2.0 | 0.9 |
| Mean TL (kb) | 6.0 | 5.8 | 3.2 | 3.6 | 5.4 | 6.0 |

* Genomic equivalents of telomere molecules detected, derived by dividing total peak count by 92, corresponding to p and q arms on each of 46 chromosomes.

* Values in parentheses represent proportions of short telomeres after removal of all data points that correspond to less than 65 bp.

Although PHAST consistently identifies fewer short peaks than q-FISH, the difference is most likely artificial. This is because q-FISH yields TFU values ranging from zero to thousands, with some of these short peaks (<2% of total) corresponding to telomere lengths shorter than the PNA probe (18 nt), based on the scaling used to normalize the distribution to PHAST. The presence of these extremely short telomeres may be due to variable detection efficiency across the imaging field in q-FISH, and suggests caution when interpreting q-FISH results for very short telomeres. It also explains the practical detection limit of 150-300 bp commonly cited for q-FISH (11), and likely accounts for the difference between the two
distributions at the lower end of the range (< 400 bp), where q-FISH may underestimate the length of the short telomeres. Since a cut-off of 65 bp (corresponding to up to 3-4 bound PNA probes) is imposed for PHAST during thresholding to minimize detection of spurious peaks from the data, we placed a similar cut-off on the distribution from q-FISH, and found that the difference between the two methods was reduced (Supplementary Table S1). Given the differences in the sample preparation, signal acquisition, and data handling, the agreement between the two methods is better than one might expect, and validates the PHAST method.

We also compared HeLa telomere lengths determined using PHAST and that measured with TRF (12). TRF and similar methods cannot decouple the abundance of telomeres from their lengths. As a result, longer telomere molecules can show up on the gel as more intense, even if they are less abundant than shorter DNA molecules. Therefore, when calculating the mean telomere length from TRF gels, it is common to use the equation

\[
\text{Mean Telomere Length} = \frac{\sum OD}{\sum (OD/MW)}
\]

where OD is the optical density or intensity on the gel, and MW is the molecular weight of the DNA, as calculated using DNA ladders (9,13,14). However, the presence of a subtelomeric region that is resistant to restriction enzyme digestion, estimated to be around 2 kb (15,16), means that the calculated mean telomere length will underestimate the abundance of short telomeres.

To emulate TRF results, we binned the peaks detected on PHAST, and multiplied the abundance in each bin by the telomere length corresponding to the center of the bin. This yielded the intensity distribution, as might be observed on a gel, with the maximum intensity corresponding to a telomere length of 4.25 kb.

Supplementary Figure S9. The Normalized Abundance (blue) and Intensity (black) of the telomere distribution, as determined by PHAST. The Intensity curve emulates the results from TRF, and yields a maximum at 4.25 kb.
To account for the 2 kb subtelomeric, the histogram is shifted higher by 2 kb, and the mean telomere length is back-calculated using the above equation. This gives an estimated value of 5.0 kb, which is a typically reported value. However, the actual mean telomere length is only 2.4 kb. In other words, the overestimation of average telomere length is even larger than the length of the subtelomeric region. Our estimate is also corroborated by a new electrophoresis-based method called TeSLA,(17) which minimizes the length of the subtelomeric region, and gave an estimate of around 2.5 kb for HeLa cells. These results also highlight the difficulty in traditional methods for telomere measurement, and argue for the need to introduce new and more convenient approaches.

**Supplementary Note 9: Pull-Down Assay for Long Telomeric Sequences**

As discussed in the main text, the size of our telomere standards is limited to 2.4 kb because the cloning vector is unable to accept a larger cassette. In order to simulate long telomeres, we used padlock probe chemistry in conjunction with rolling circle amplification to generate long DNA molecules containing telomeric sequences (**Supplementary Figure S10A**). Like real telomeres, each sample prepared will contain DNA molecules of different lengths.

**Supplementary Figure S10. Testing of pull-down process for long telomeres.**

By subjecting the sample to the pull-down process, and comparing the telomere distribution determined by PHAST before and after the pull-down, we determined that our process did not bias the telomere size distribution, as evidenced by the linearity of the Q-Q plot (**Supplementary Figure S10B**). Furthermore, it is worth noting that some of the telomeres detected here were longer than 10 kb. This is comparable to the telomere lengths found in cells, and the absence of bias confirms the veracity of our length estimates.

**Supplementary Note 10: Testing Clinical Samples on PHAST and qFISH**
Clinical samples (two from cancer patients, one from a control subject) were collected and tested by both PHAST and q-FISH. We found that both methods yielded distributions that were not significantly different between the control and patient samples. This is not unexpected, since there is no simple relationship correlating telomere biology to cancer incidence. However, by analyzing larger patient cohorts, in a further study, and controlling for various parameters, it may be possible elucidate the role of telomere length in cancer.

Supplementary Figure S11. Q-FISH measurements do not show obvious differences in telomere distribution between the cancer patient samples (BA01628 and BA01650) and control sample (CB3319).
Supplementary Figure S12. Similarly, PHAST measurements do not show obvious differences in telomere distribution between the cancer patient samples (BA01628 and BA01650) and control sample (CB3319).

Supplementary Note 11: Application to Huntington CAG Tandem Repeats

Our method is expected to be applicable to other types of tandem repeats. To demonstrate this, we chose to test out the Huntington CAG trinucleotide repeat, one of the most well-known tandem repeats. In Huntington’s Disease (HD) patients, the number of repeats range from 37 to 86, with the age of onset inversely related to the repeat length (18,19). For this experiment, we procured 12-nt PNA probes labeled with Alexa488 dye (PNA Bio). We also ordered an oligonucleotide that contained CAG repeats, that can bind a maximum of 16 probes (192 base long). The average repeat length measured by our method is 195 bases. Although the coefficient of variation is somewhat higher than that achieved for telomeres, this is attributed to the need to optimize the sample preparation process, which lies beyond the scope of this manuscript.

Supplementary Figure S13. Application of the method to CAG trinucleotide repeat associated with Huntington’s Disease yields a good estimate. However, further optimization is needed to reduce the coefficient of variation for this sample/probe combination.
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