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Automated Analysis of Telomere Intensity with Quantitative Fluorescence In-Situ Hybridization Using CellProfiler

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ABSTRACT

Telomeres are noncoding DNA sequences that protect chromosome ends from degradation. Quantitative fluorescence in situ hybridization (Q-FISH) is the favored technique for measuring telomere length. It can be applied to individual chromosomes in metaphase cells. Much of the currently available software to analyze fluorescence signal intensity as a proxy for telomere length is only commercially available. This study sought to develop a pipeline for the automated analysis of telomere fluorescence intensities in metaphase Q-FISH using modules from the open-source, freely available CellProfiler software. Intensities telomere and centromere were stained with fluorophore-conjugated peptide nucleic acid probes then evaluated using our pipeline. Telomere and centromere intensities were measured in a total of 30 images, giving an average relative telomere intensity of 22.17 %. Overall, our study showed that the pipeline can automatically identify and measure fluorescence signals from telomeres and centromeres in approximately 36 sec/image. However, the pipeline requires high-quality metaphase chromosome images with minimal touching and overlapping of chromosomes. Nevertheless, CellProfiler provides a free and flexible platform for the automated analysis of Q-FISH images that can be easily optimized and shared between researchers

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Introduction

Telomeres are noncoding tandem TTAGGG repeats that protect chromosome ends from degradation during DNA damage repair¹⁻³. Telomeres form unique T-loop structures bound directly or indirectly by shelterin proteins, preventing DNA ends from being recognized as double-strand breaks³. After a number of cell divisions, telomeres can reach a critical length that can trigger cellular senescence⁴. There are several methods for measuring telomere length, including quantitative polymerase chain reaction (qPCR) for amplifying telomere sequence repeats, Southern blotting coupled with terminal restriction fragment (TRF) analysis, and quantitative fluorescence in situ hybridization (Q-FISH) for quantifying telomere repeats in individual cells (interphase-FISH and flow-FISH) or on individual arms of the chromosome (metaphase-FISH)⁵. Q-FISH is particularly useful as it can be performed in whole cells and employs fluorophore-labeled peptide nucleic acid (PNA) probes (CCCTAA)₃ that specifically hybridize to denatured telomere repeats, allowing for their quantification via fluorescence signal using software specifically designed for Q-FISH image analysis⁶.

There are several programs available for analyzing telomere length from Q-FISH, including the Telometer plugin for ImageJ, the Telomere module of Isis, MetaSystem, and TFL-Telo⁷⁻¹⁰. Some of this software is open-source and free to download, while others are only commercially available⁷. Although the commercial software is typically easy and convenient to use, particularly those bundled with a fluorescence microscope, their high cost and lack of flexibility limit their use. Moreover, their proprietary code prevents researchers from modifying it as needed. Among the available open-source software, CellProfiler is particularly popular for automated image analysis as it allows users to create customized pipelines. CellProfiler was developed by the Carpenter laboratory at the Broad Institute of Harvard and MIT. The first version was introduced in 2005 and CellProfiler 1.0 was published in 2006¹¹. It has been cited more than 6,000 times at a rate of more than 1,000 citations/year. CellProfiler allows researchers with minimal image processing analysis experience to perform automated image analysis and extract large quantities of information on phenotypic data relatively easily¹². This study sought to develop a reliable, free, and easy-to-use CellProfiler pipeline for quantifying telomere fluorescence intensity in metaphase cells.

Method

Blood cultures

Blood samples were obtained with informed consent from a single 33-year-old, healthy male donor. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples by diluting 3 mL blood into 3 mL medium (Rosewell Park Memorial Institute (RPMI) 1640 and 20% fetal bovine serum (FBS)), layering with 3 mL Histopaque[®]-1077 (Sigma-Aldrich, St. Louis, MO, USA), and centrifuging without brakes for 30 min at 244× *g*. The PBMCs were then washed with 5 mL washing medium (RPMI 1640 and 2% FBS) and cultured by mixing with 4.5 mL RPMI 1640, 0.9 mL FBS, 0.1 mL streptomycin/penicillin, and 0.12 mL phytohemagglutinin (PHA). The PBMC culture was incubated for 48 h at 37°C in 5% CO₂. Colchicine (0.05 µg/mL final concentration) was added to the culture after 45 h. PBMCs were harvested by centrifuging the culture flasks for 5 min at 214× *g*, removing the supernatant, and resuspending the pellet in 0.2 mL pre-warmed (37°C) 0.075 M KCl. The suspension was incubated for 20 min in a 37°C water bath and centrifuged for 5 min at 214× *g*. The cells underwent two rounds of fixation using 30 µL fixative solution (3:1 methanol:acetic acid) and were stored at 4°C overnight. The fixed cells were then spread onto a clean, wet glass slide and stored at -20°C until needed¹³.

Telomere and Centromere Staining

Telomeres and centromeres were stained with sequence-specific PNA probes (provided by Dr. Radhia M'kacher/Cell Environment) as described by M'kacher et al., with minor modifications¹⁴. Briefly, the slides were washed with 1× PBS for 5 min at room temperature and fixed for 2 min at room temperature with 4% formaldehyde in PBS. The slides were then rinsed three times with PBS for 5 min, treated with pepsin (0.5 mg/mL) at 37°C for 7 min, and washed three times with PBS for 5 min. The slides were sequentially dehydrated with 50%, 70%, and 100% ethanol and air-dried for 20 min. The slides were then treated with the telomere/centromere PNA probes, denatured for 3 min at 80°C on a hot plate, and incubated in the dark for 2 h at room temperature. The slides were then washed twice with 70 % formamide/10 mM Tris pH 7.2 for 15 min and three times with 50 mM Tris pH 7.2/150 mM NaCl pH 7.5/0.05% Tween-20 for 5 min. Finally, the slides were washed in PBS, counterstained with DAPI, and mounted with mounting solution.

Slide scanning and metaphase image acquisition

Metaphase cell images were acquired on a Zeiss Axioplan 2 Imaging epifluorescence microscope connected to a Cool Cube digital high resolution CCD camera using the Autocapt module of the Metafer software v3.11.2 (MetaSystems, Altlussheim, Germany). The images were exported as .tif files with a resolution of 1280×1024 pixels. A total of 640 images were collected, 30 of which were used for further analysis.

CellProfiler measurements of telomere and centromere intensities

The latest version (at the time of submission) of CellProfiler (v4.0.7) was downloaded (www.cellprofiler.org) and installed on a laptop with an Intel® Core i3-6100 CPU @ 2.30 GHz processor, 4.09 GB RAM, and Windows 10 Pro 64-bit operating system. CellProfiler uses a pipeline consisting of several modules that can be ordered to allow for automatic identification and quantification of biological objects or cells in images (Figure 1). Each module consists of an advanced algorithm, with the optimal experimental parameters defined by the user^{11,15,16}. The pipeline and sample images from this work are available upon request to the authors. The analysis time for a single image was recorded by the CellProfiler time counter.

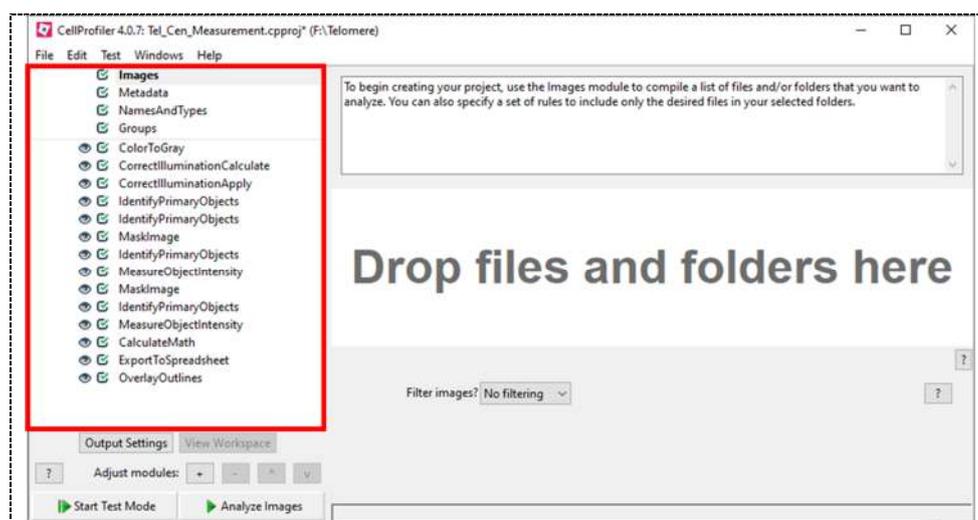


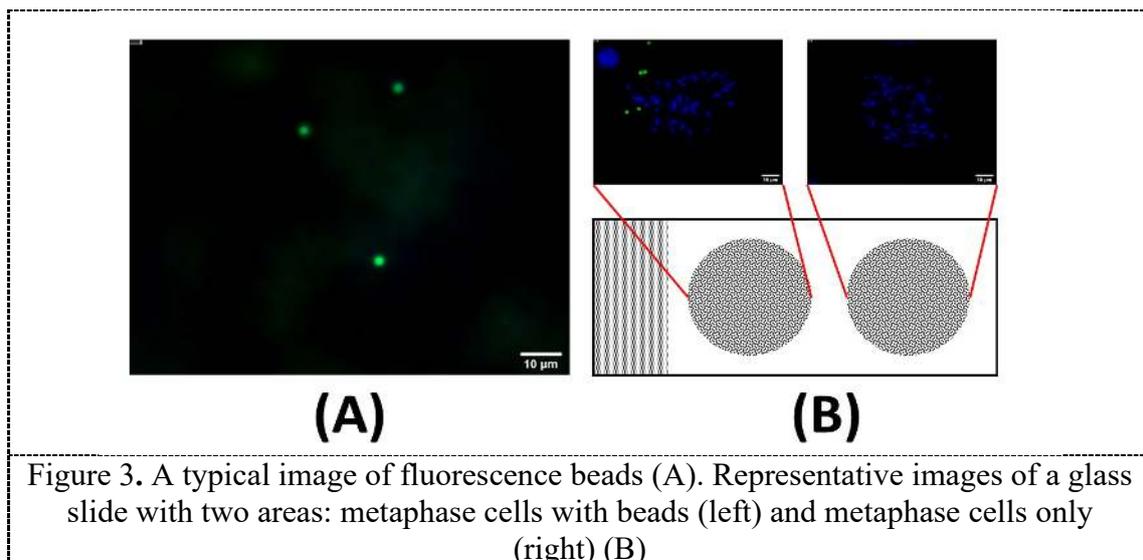
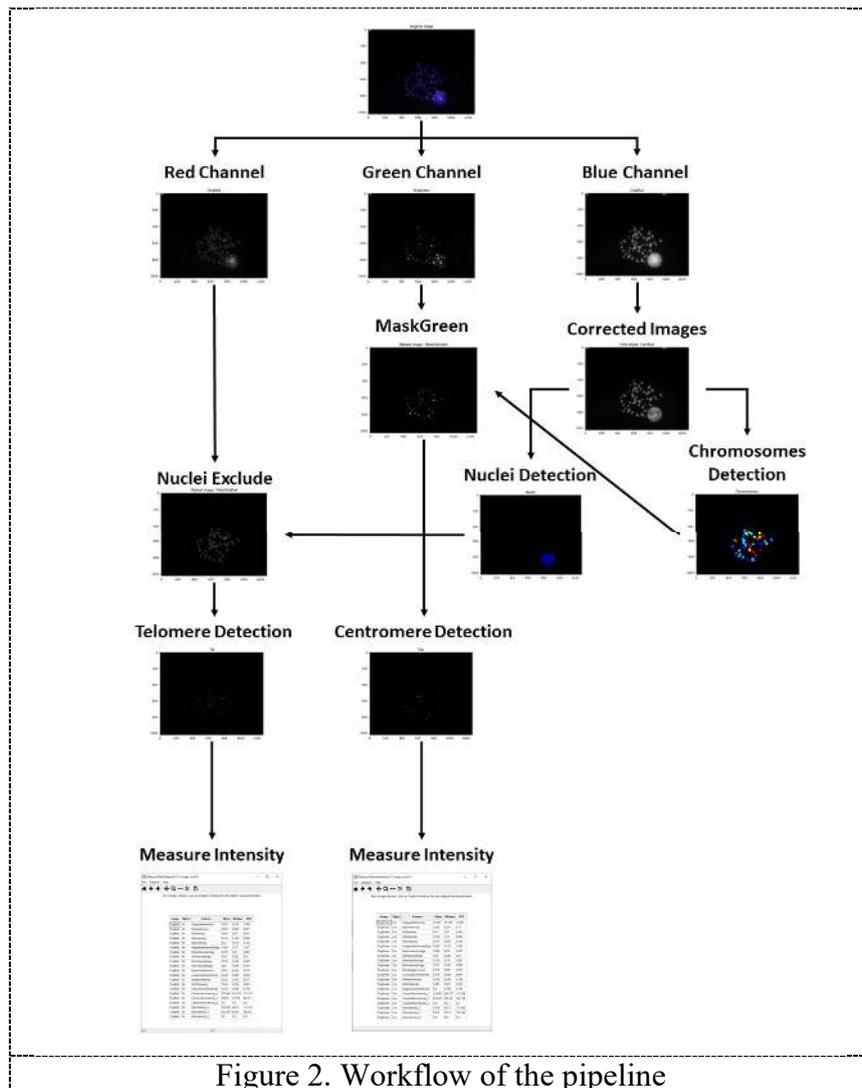
Figure 1. CellProfiler v4.0.7 graphical user interface (GUI). The pipeline panel (red square) consists of several modules for the automatic identification and quantification of biological objects in an image

Results and Discussion

Images are loaded into CellProfiler for the automated detection and measurement of telomere and centromere intensities (Figure 2). Individual or multiple images can be dragged into the “File list” of the “Images module” and metadata can be extracted using the “Metadata module”. The first step of image analysis used the “ColorToGray” module to split the color image into each grayscale channel, with the telomere observed in the red channel, the centromere in the green channel, and the chromosomes and unstimulated/stimulated nuclei in the blue channel. The “CorrectIlluminationCalculate” and “CorrectIlluminationApply” modules were then used to correct uneven illumination and reduce uneven background in the blue channel. The “IdentifyPrimaryObjects” module was used to identify stimulated and unstimulated nuclei around the metaphase stage of the cell cycle, as well as chromosomes. The unstimulated or stimulated nuclei were excluded from downstream analysis using the chromosome object as a mask in the red channel in the “MaskImage” module. The “IdentifyPrimaryObjects” module was used to identify telomeres within the chromosomes, with their intensities measured with the “MeasureObjectIntensity” module. Similar steps were repeated to identify centromeres and measure their intensities. The relative telomere intensities were calculated by subtracting the centromere intensities and multiplying by 100.

The pipeline was used to calculate the relative telomere intensities in 30 images, with an average of 22.17%. The average processing time needed for each image was 36 s. No comparison between automatic and manual analyses was performed as telomere intensity cannot be manually quantified. While many applications of FISH imaging do not require accurate fluorescence intensity measurements, the analysis of telomere length using Q-FISH is based on the correlation between telomere length and integrated fluorescence intensity (IFI) values, making the accurate measurement of these values essential⁸.

It is important to normalize the fluorescence intensity between experiments due to variability in mercury lamp power, for example, by using fluorescence beads of a known size when capturing metaphase images (Figure 3a). Another approach is to use the centromere fluorescence signal for normalization. While we used the centromere signal for normalization, fluorescence beads could be considered for future investigations. Unfortunately, capturing the fluorescence beads using the Autocapt module in Metafer is challenging, as it requires the previous capture of metaphase cells by the MSearch module in MetaSystems. Moreover, the beads cannot be manually captured, as the same system is used for telomere acquisition. Based on our experience, this problem can be overcome by adding beads adjacent to fixed cells on a previously prepared glass slide just prior to the experiment, such that two areas exist on a given slide: one containing both beads and fixed cells and the other containing fixed cells only. With this strategy, there should be at least some images containing both metaphase cells and beads and others containing only metaphase cells (Figure 3b).



Software for analyzing fluorescence intensity from telomeres in metaphase cells have already been reported, with the most powerful free software developed by Peter Landsrops (TFL-Telo)⁸. Unfortunately, this software could not be easily and effectively modified for use with our images and we focused on developing the CellProfiler pipeline for this work. Low-quality metaphase chromosome images, particularly those where artifacts overlap chromosomes and can be misidentified as telomeres, presented problems for our pipeline. Another limitation occurred when multiple metaphase chromosomes were touching or overlapping, which led to failures during the segmentation process. At present, the only way to increase the accuracy of telomere fluorescence intensity measurements using our pipeline is by analyzing high-quality metaphase chromosome images. Despite these limitations, the use of CellProfiler to evaluate telomere fluorescence provides several advantages. First, the optimal parameters for each module can be determined using the CellProfiler “Test Mode” before applying it to larger data sets¹². Second, CellProfiler provides a free and flexible platform accessible to users with varying degrees of experience. Importantly, these pipelines can be easily shared across the research community, allowing for rapid improvement and increased reproducibility¹².

Conclusion

This study shows that our CellProfiler-based pipeline can quantify telomere and centromere fluorescence intensities in metaphase cells stained with selective PNA probes, despite some limitations when using lower quality images. Future work should focus on improving the quality of metaphase chromosome spreads and further optimizing module parameters.

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