

## Introduction

Advancing age is the strongest risk factor for cancer in humans, driven largely by significant increases in the incidence of epithelial cancers particularly breast, colon, skin, and prostate. Continued age-dependent epithelial renewal has been linked to telomere erosion. Telomeres function to maintain chromosomal end integrity and are comprised of higher-order structures that consist of up to 2000 tandem repeats of the hexanucleotide sequence TTAGGG. Telomere dysfunction and resultant chromosomal end-to-end fusions in turn set the stage for the breakage of fused chromosomes at anaphase (bridge-breakage cycles), generating double stranded DNA breaks that provide a nidus for amplification or deletion at the site of breakage. Mounting evidence in multiple tissues indicates that telomere erosion is closely correlated with the emergence of rampant genomic instability that likely fuels tumor progression.

The genomic instability is a hallmark feature of human cancer and a number of model systems have been generated to understand its etiological role in cancer pathogenesis. Genetically engineered mouse models have been instrumental in validating the etiological role of individual genes and various combinations. In contrast to conventional genetically engineered mouse models of breast cancer, the telomerase deficient (*Terc-/-*) *p53-/-* mice have critically short telomeres and impaired *p53*-dependent telomere checkpoints. Approximately 10% of *mTerc-/- p53-/-* females develop spontaneous breast adenocarcinomas with advancing age. Notably, the breast adenocarcinomas of late generation *mTerc-/- p53-/-* mice appear after a long latency (>1 year) and exhibit an attenuated malignant phenotype—findings consistent with the view that, while telomere dysfunction serves to drive the initiation of neoplasia, it also constrains full malignant progression of these initiated neoplasms. Thus, it appears that full malignant progression requires the activation of telomere maintenance mechanisms. With telomere dysfunction as an important initiator of breast adenocarcinoma, we have speculated that reconstitution of telomerase activity in these initiated breast lesions will facilitate full malignant progression. Importantly, only a small number of cells may be residing in developing cancerous lesions proliferate to maintain the final tumor.

Taking into account the role of telomere dynamics in cancer biology, there has been strong interest in a measuring of telomere length in tissues. The gold-standard method is based on Southern blotting. This method requires the isolation of high-molecular-weight genomic DNA digested with two restriction enzymes, followed by Southern blotting and hybridization with the repeat-specific DNA probe. The resulting smear pattern represents a size range of restriction fragments. The telomere length can be compared between two samples based on differences in electrophoretic mobility of corresponding terminal restriction fragments (mean or median of the smear). The major drawback of this technique is the overall lack of information about individual cells and subpopulation of cells within the tissue.

In contrast to Southern blotting, fluorescent *in situ* hybridization (FISH) is a cell-centric approach. FISH with repeat-specific probes as the method was developed more than a decade ago. This technique has since been modified and widely applied to metaphase chromosome spreads. Advantages of this technique consist from high sensitivity ensured by the use of peptide nucleic acid (PNA) hybridization probe. The information is obtained on the analysis of individual chromosomes. Tel-FISH has also been successfully applied to the analysis of isolated interphase nuclei, as well as snap-frozen and paraffin-embedded tissue sections. The late is particularly important since gives the ability to exploit the vast array of archival pathological material available. Unfortunately, the power of telomere-tissue-FISH (TFISH) appears to be underutilized, possibly due to the lack of an objective way for quantification of the specific fluorescent signal across the specimen section.

A desirable technology should allow automated analysis of large tissue sections at high magnifications, preserving the tissue morphological organization while performing flow cytometry-like analysis of cellular markers. To this end, we adopted the FISH method by using a dynamic hybridization device and combined this with laser scanning cytometry for the effective mapping of tissue architecture for cells with different telomere lengths.

The objective of this experiment is to distinguish between two cell populations with known differences in telomere length. Challenges to be overcome include the following: 1) engineer cells with short and long telomeres; 2) make uniform hybridization conditions for samples that should be compared; and 3) image analysis algorithms.

## Technical Setup: The LSC

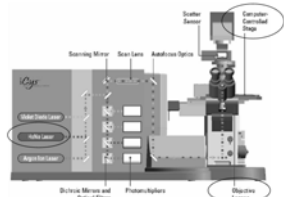


Fig. 1. Block diagram of the iCys. Three lasers are used in a multiplexed fashion to illuminate the specimen from underneath. Fluorescent light is returned along the same pathway and is diverted and directed by dichroic mirrors and filters to four photomultiplier tubes measuring distinct bandwidths. Major modified parts are shown in circles.

LSC is an analytical technology analogous to flow cytometry. In contrast to flow cytometry, the position of each fluorescent, chromatic or scatter event is recorded, and electronic bi-map images of the scan are created. These images of tissue sections reveal the architectural context in which the event has occurred. The iCys Research Imaging Cytometer (CompuCyte) is designed around an inverted-formal Olympus IX71 microscope (Fig. 1). Many of the microscope's functions are left intact, giving the iCys visualization capabilities, including the ability to associate images with any of the events in the population data (see Luther et al., 2004 for details).

We have adopted the system of dynamic mixing, MAUI Mixer, originally designed to perform hybridizations with DNA/RNA microarray chips. In this case, the experimental conditions become virtually identical for collocated samples



## Dye Selection

To accomplish the best match to the fluorochrome combination, a customized iCys instrument was developed, with the 633 nm HeNe red laser replaced by a 532 nm green diode laser (405-488-532). A number of additional enhancements were made that include 60X dry objective and increased spatial resolution from 0.5 to 0.1 μm size of stage steps, as well as redesign of dichroic mirror, blue and yellow filter cubes. The iCys software (CompuCyte) was upgraded to version 3.3.1Bbeta.

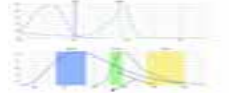


Fig. 2. DAPI Tail: Removed by Two pass Scan. Pass one excites DAPI with a 405 laser, and pass two excited FITC with the 488 laser allowing for pure green emission to be imaged.

## Confocal Analysis

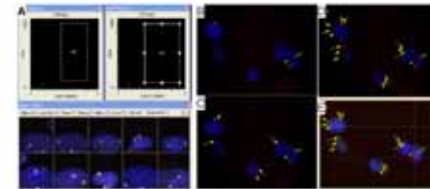


Fig. 3. LSC analysis of a gene-specific FISH. (A) BAC probe spots are easily detectable, with high levels of intensity. Plots showing that the green (FITC) probe rarely exceeds two counts per nucleus, but yellow (Cy3) gives additional signals. This finding was confirmed by a subsequent manual evaluation. (B-C) Three 100X confocal 2 μm step 2-planes. Arrows incrementally show the position of red and green FISH spots. (C) 40X laser scan image. Note: all spots were detected.

## Cellular Data

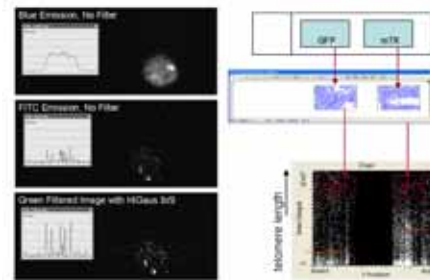


Fig. 4. Details of the telomere spots definition and the high resolution scan setup for tel-FISH with interphase spreads.

## Cellular Contour Results

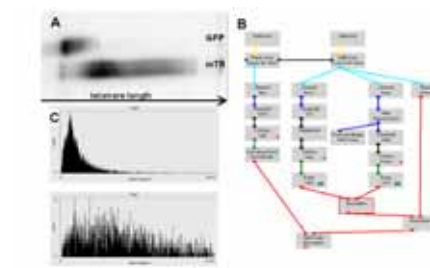


Fig. 5. Comparison of the Southern blotting with iCys analysis. (A) TRF distributions for the mock (top) and the reconstitution (bottom). (B) Block diagram of the Tel-FISH protocol. (C) LSC quantification of telomere length for parental (top) and telomerase-reconstituted (bottom) cell populations.

## Cellular Stereological Results

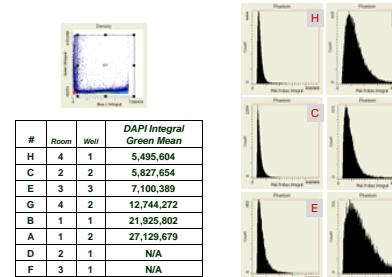


Fig. 6. Comparison of six different cell lines either genotyped as either G4 or wild-type mouse spleen cells. Analysis done using stereological contours.

## Tissue Analysis

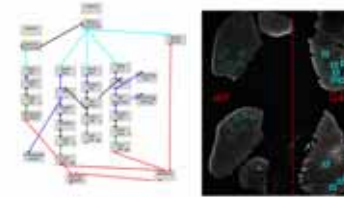


Fig. 7. Block diagram of the TFISH protocol, a low resolution view of tissue and high resolution scan areas.

## Data Acquisition & Processing

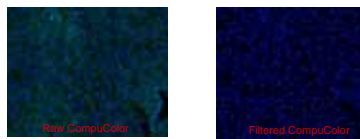
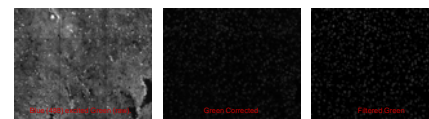


Fig. 8. Example of data acquired during tissue PNA scans

## Tissue Results

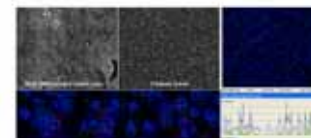


Fig. 9. Visual validation of the corrections, and definition of cells with long (red squares), medium (blue) and short (green) telomeres.

## Tissue Telomere Length Cartesian Map

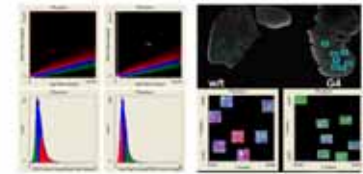


Fig. 10. Galies applied to the densely scatter-gram of DAPI over FITC corrected fluorescence give definitive separation between the wild-type and 4 telomere-deficient cells: long (red squares), medium (blue) and short (green) telomeres.

## Conclusions

In conclusion, our experiments rely on precise measurements of telomere length for the interpretation of the phenotypes elicited by our genetic manipulations of members of the telomere maintenance pathway, particularly with respect to cancer and degenerative disease. In the context of organismal aging, we are investigating the role that telomere dysfunction and DNA damage checkpoints in degenerative disease and organ homeostasis. Our model combines telomerase deficient mice with those lacking some other target genes. These mice exhibit many of the hallmarks of accelerated cellular and organismal aging, particularly affecting tissue stem cell populations. In this setting, we require the ability to accurately measure telomere integrity in intact organs, such as liver, intestine, brain, and others – and these data will be correlated with histological and physiological assessments of organ and cellular dysfunction that we have already noted in these mice. Further, we are embarking on an ambitious project to test the ability of telomerase re-expression to stem the aging process in these mice. We measure telomere DNA by hybridization of telomeres with a fluorescent probe and quantification of the relative fluorescence intensity as a direct measure of telomere length. Regular Telomere-FISH technology is limited to the analysis of metaphases from cultured cells and in its throughput by the laborious microscopy required. [The Laser Scanning Cytometry will greatly increase our experimental interpretations and throughput.](#)

## Acknowledgments

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## Comments