HIGH-THROUGHPUT SCREENING OF ALTERNATIVE LENGTHENING OF TELOMERES (ALT)-ASSOCIATED CANCER GENES

Abstract

There are many proposed methods to inhibit growth of various types of cancer, but none are as universal as abrogating telomere maintenance mechanisms (TMMs). Telomerase has been widely studied for decades as a cancer target and as such telomerase-based anti-cancer strategies have received extensive support from academia and industry. High throughput telomerase-based assays have been extensively used to discover and develop various telomerase inhibitors that are now being tested in clinical trials. However, a significant portion of cancer uses a telomerase-independent mechanism known as ALT, and recent reports suggest that some tumors may activate both TMMs simultaneously and that telomerase inhibition may select for ALT cancer activity. Thus, a combinational inhibition of both TMMs will most likely be required for a successful global anti-cancer strategy. Since the current lack of known ALT inhibitors is, according to leading researchers in the field, due to the absence of a reliable and high-throughput assay for this type of cancer activity, we propose to develop high-throughput versions of the common ALT activity assays to screen for potential genes in cancer cell lines that are involved in the ALT pathway. Our results will potentially generate invaluable genetic targets for the development of anti-cancer therapies that specifically shut down the ALT mechanism.

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Background

Introduction

It is estimated that somewhere between 10% to 15% of all cancer cells have no detectable telomerase activity, and a significant proportion of these tumors rely on the alternative lengthening of telomeres mechanism (ALT)[1,2] to maintain and/or elongate their telomeres through an as of yet poorly-described mechanism(s) involving homologous recombination[3]. The therapeutic importance of this field is further highlighted by several studies reporting that some cancers contain both telomere maintenance mechanisms (TMM),[4–9] and *in vitro* as well as preclinical data indicate that telomerase inhibition may select for cancers that use ALT[10–13]. Additionally, with the exception of gliomas, the presence of TMM in tumors is often associated with poor prognosis and survival[7,8,14]. In fact, detection of ALT in liposarcomas and osteosarcomas is often associated with either the worst or just as poor prognosis and survival for patients as telomerase is[8,15,16]. Nonetheless, the majority of academic and industry resources are currently directed toward telomerase as it is viewed as a nearly universal target for cancer cells[3,17–19], but due to the perceived shortcomings of only targeting telomerase, cancer researchers have proposed that the future of cancer therapies will involve combining inhibitors of both TMM[1,12,20–22]. This futuristic thinking is why the focus of the OncoSENS research team at Mountain View, CA is on finding genes that are specific and essential to the ALT mechanism.

Given the minimal amount of support directed to researchers in the ALT field, it is no surprise that it is far behind anti-telomerase therapy. For instance, as of July 2013, searching the term 'telomerase' on PubMed gives about 12,000 results while searching 'alternative lengthening of telomeres' returns only 286 articles, which is merely about 2.4% of all telomerase-based work published to date (Figure 1). Yearly publication trends for the telomerase and ALT fields (Figure 1) also suggest that telomerase research dramatically expanded after the invention of the TRAP assay, but a similarly robust assay capable of inducing a comparable trend for ALT has not been achieved yet. The gold standard for ALT activity is telomere length maintenance in the absence of telomerase[3], but several ALT-associated hallmarks have been identified. This type of TMM is thought to be dependent upon homologous recombination and DNA damage repair proteins[23–25], and cells maintaining telomeres in this fashion are reported to have heterogeneous telomere lengths, extra-chromosomal telomeric repeats (ECTR), which include partially double-stranded C-rich circular DNA (C-circles), and ALT-associated promyelocytic nuclear bodies (APBs)[26]. It is hypothesized that ALT telomere synthesis is a recombination-based mechanism that may be occurring in an APB (Figure 2)[23]. Inhibition of several APB-associated components or APB assembly in ALT-positive cells has been reported to induce senescence, cell death or telomere shortening and are thus potential anti-cancer targets[3,27–30] (see also Supplementary Table 1).

Telomerase inhibitors are currently in clinical trials and it is widely believed that this is a result of robust assays that allowed researchers and clinicians to reliably and quickly identify genes and compounds that target this TMM[1,19]. The telomerase field has been generating a wealth of data (Figure 1) for decades in the same format of the NCI 60 viability screen of the late 80s[31] with assays such as the TRAP assay that originated in 1994[32] and the subsequent miniaturizations[33], and high-content viability assays that have developed since[19]. Using current ALT assays, several genes have been identified that modulate ALT activity, but virtually all these gene candidates are vital for many non-ALT cell functions and thus are not ideal therapeutic candidates[3,10,25,27–29,34]. Overall, the currently most reliable assays for

ALT activity, namely C-circle (CC) and APB assays, are very low throughput as they require DNA dot blotting[26], advanced qPCR techniques[35], or complex 3-dimensional confocal microscopy imaging[34], which are all very labor and/or data intensive and thus not amenable to high-throughput screening of larger gene databases. In sum, a TRAP assay equivalent for ALT activity has not materialized yet.

The OncoSENS team is working to overcome those limitations. We are using the same type of low-throughput experiments described above to develop high-throughput assays for ALT activity. Our team has had success transitioning the original CC Assay, which relies on radioactive probes, to a safer luminescent reporter (DIG-based) format that does not require treatment of the samples with restriction enzymes and thus yields results much faster. Additionally, we have had some preliminary success in adapting the CC assay to a more high-throughput friendly, ELISA-based format (see below), which eliminates the need for radioactive probes and DNA blotting altogether. As for the APB assay, the advancements we have made in adapting this assay to work in a high-throughput fashion are even more promising (see below). Our version of the APB assay replicates key published data without the need for confocal microscopy or 3-D image reconstruction for quantitative analysis, which yields results faster than the original assay[34].

In summary, we are developing the first truly high-throughput assays for ALT activity in cancer cells. *We hypothesize that by using these assays, we can quickly identify gene targets for ALT cancers by screening large gene datasets by RNA interference*. This would allow us to narrow down gene candidates faster and focus on non-vital genetic targets that are unique to ALT activity and not required by every cell in the body, which unfortunately is the drawback of virtually all of the ALT-associated genes reported so far (Supplementary Table 1). The assays developed by this project could also be applied for screening molecular compounds for drug development targeting ALT-positive cancers.

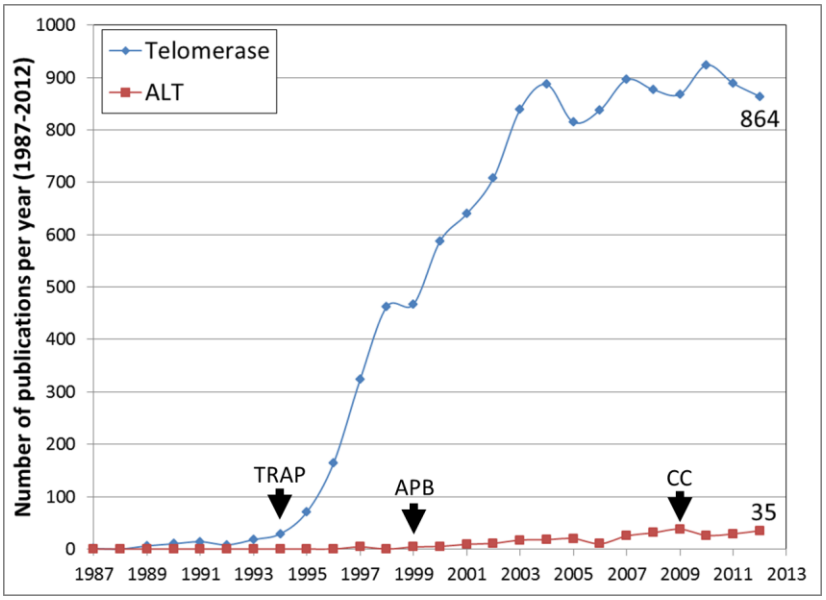

Figure 1. Medline (PubMed) publication trends for telomerase and ALT research. The first telomerase articles appeared in the late 80's while ALT publications started in the mid to late 90's. Arrows indicate the invention of the most relevant assays for each field, namely the TRAP assay in 1994[32], the APB assay in 1999[36] and the CC assay in 2009[26]. Source: Alexandru Dan Corlan. Medline trend: automated yearly

statistics of PubMed results for any query, 2004. Web resource at URL:http://dan.corlan.net/medline-trend.html. Accessed: 2013-07-23. (Archived by WebCite at http://www.webcitation.org/65RkD48SV)

ALT telomere synthesis models

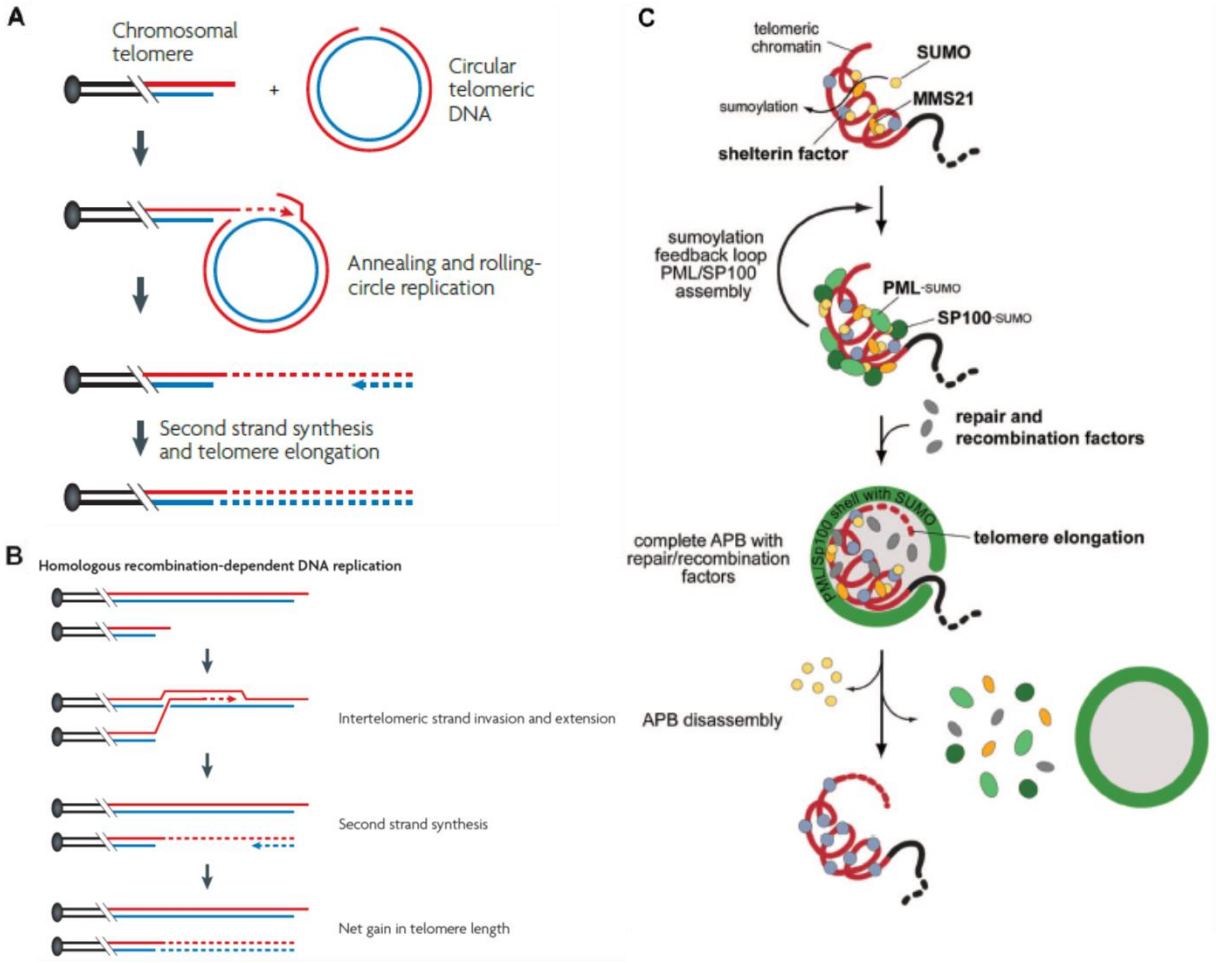

Figure 2. Proposed mechanisms of ALT activity. An RCA-based synthesis model involving circular telomeric DNA may explain the presence of C-Circles (A), but the homologous recombination theory has more support (B)[3]. The synthesis is believed to occur in an APB, which is composed of the assembly of several different proteins (C)[23].

Unlike telomerase, which uses an RNA template and reverse transcriptase activity to modify telomere length, ALT is believed to involve DNA repair and recombination enzymes and a linear or circular DNA template (Figure 2)[1,3]. This telomere synthesis activity is proposed to occur during the S and G2 phases of the cell cycle inside APBs (Figure 2)[23,34,37]. C-circles have been proposed to work as DNA templates during elongation of telomeric repeats by rolling circle amplification (RCA), but it is unclear so far if they are actively contributing to the ALT mechanism or are merely a by-product of recombination-based telomere elongation by ALT[1,3,38]. APBs were the first bona fide molecular markers

of ALT activity identified in cancer cells[36]. However, there are only two ALT-positive, *in vitro* SV40-immortalized cell lines, namely AG11395 and C3-c16, that do not possess APBs[26,39,40]. Also, culture of ALT cells in conditions that cause growth arrest and senescence were found to artificially increase the formation of APBs[25,41].

Current ALT Screening Methods

APB assay

The ALT-associated promyelocytic body (APB) assay was the first alternative lengthening of telomeres (ALT) assay to be developed. Prior to its discovery in 1999[36] (see Figure 1), the only known criteria for determining the presence of ALT activity were long and heterogeneous telomere maintenance and undetectable telomerase activity by the TRAP assay[36]. This assay involves testing for colocalization of telomeric DNA and promyelocytic body protein (PML). Though PML plays a role in a variety of functions from senescence to proliferation[42], it is very rarely found at the telomeres[34]. Mortal and telomerase-positive (TEL+) cells typically have at most two or less APBs per nuclei, while established ALT-positive (ALT+) cell lines and those in the process of immortalization by ALT are reported to have in excess of four[34,36].

This has led to the use of the APB assay in the search for genes involved in ALT[25,27,34] as well as for determining telomere maintenance mechanisms in patient tumor samples[7,43]. Detection of APBs in tumors has been reported to have prognostic significance in various types of cancer[7,43]. For years, different image acquisition setups, counting methods, and culture conditions have led to various numbers of APBs to be reported by different groups[25,36,44–46]. Recently, an improved method for detecting and counting APBs was reported[34] in which confocal imaging and 3-D image reconstruction combined with colocalization techniques were used to determine the impact of various siRNAs on the number of APBs per cell[34]. Though this new 3-D assay has been exciting for the field, image acquisition alone requires about 17-34 hours per slide (384 spots) while image analysis using a multi-node supercomputer takes about 6-8 hours (23-42 hours total) and it is thus very impractical for high-throughput screening. Nonetheless, this 3-D APB assay is one of the most detailed screening analyses to date, and the key data are summarized in Figure 3[34]. U2OS cells (Figure 3A) have on average 4.4 APBs per nuclei with the distribution shown in Figure 3B, with over 90% of U2OS cells having 1 or more APBs, while HeLa cells have only 0.5 APBs per nuclei on average (Figure 3C) with only about 34% of these cells containing colocalizations. Analysis of the size of both telomeres and PML in APBs shows that these are enlarged inside APBs as compared to the ones outside of these bodies (Figure 3D-E). Since the APB assay in its current format has limited investigators to screening a maximum of 9-14 genes in a single study[25,34], we have developed an improved APB assay format optimized for high-throughput screening, which is fully described in more detail below .

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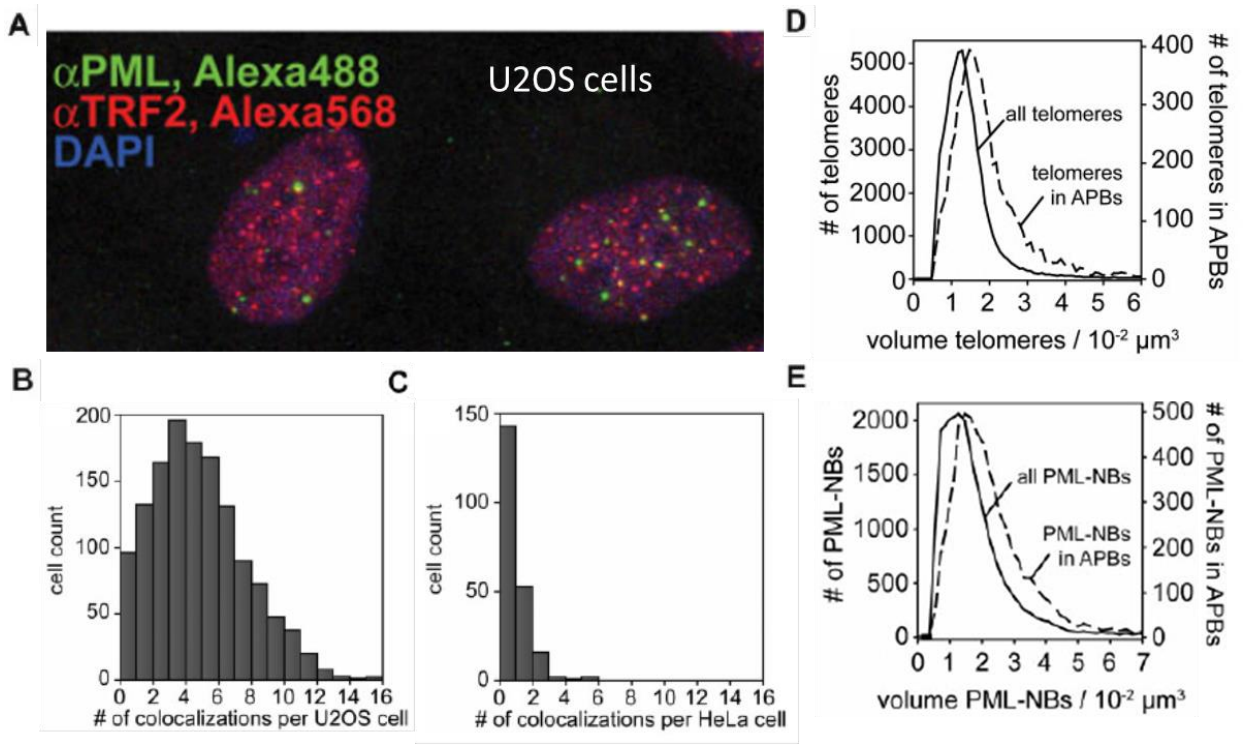

Figure 3. Current methods of detecting and counting APBs in cancer cell lines. Immunofluorescence images of APBs as reported in the literature for ALT+ U2OS cells (A). Note that yellow indicates colocalization of PML and TRF2 in nuclei here. Published methods for automated counting of APBs have yielded statistically significant results in ALT+ U2OS (B) and TEL+ HeLa (C). The size of telomeres (D) and PML (E) in APBs is larger than in foci outside of APBs.

C-circle Assay

Extra-chromosomal telomeric repeats (ECTR) are one of the major phenotypes reported in ALT+ cells[26,47]. The various ECTR types include: G-circles, C-circles (CC), and t-circles[3]. Though there are various differences in the abundance of these circles among mortal, ALT and telomerase cell lines, the presence of CC at high levels is the most tightly correlated phenotype with ALT activity identified so far[26]. The presence of CC in 38 cancer cell lines isolated from various tissues and immortalized through a myriad of different ways is a testament to the strength of the CC assay[26]. CC appear during immortalization at the same time as other markers of ALT activity (e.g., APBs) and they gradually decrease to undetectable levels the more ALT activity is inhibited[26].

This assay has been successfully used to identify ALT as the telomere maintenance mechanism from blood samples of osteosarcoma patients and to confirm that many of the genes previously known to be involved in ALT also affect CC levels[26]. Though its isothermal expansion steps make this assay suitable to use by more labs without a PCR machine, the applicability of the original assay to high-throughput screening is very limited[26]. It requires 3-4 days of very extensive amplification, blotting, labeling and developing steps as well as a radioactive probe[26] (Figure 4A), which is not an option for

many research and diagnostic labs. A quantitative PCR-based version that can be done slightly faster does exist[35], but it is even more labor intensive in both the setup and data analysis, and thus it is not very high-throughput friendly. We have developed a non-radioactive version of the original C-circle assay that takes less time to complete (Figure 6A-B), but it still requires DNA blotting and as such is not suitable for a high-throughput screening format. We are developing a high-throughput, ELISA-based version of the CC assay and it will be further described in the next section (see Figure 6).

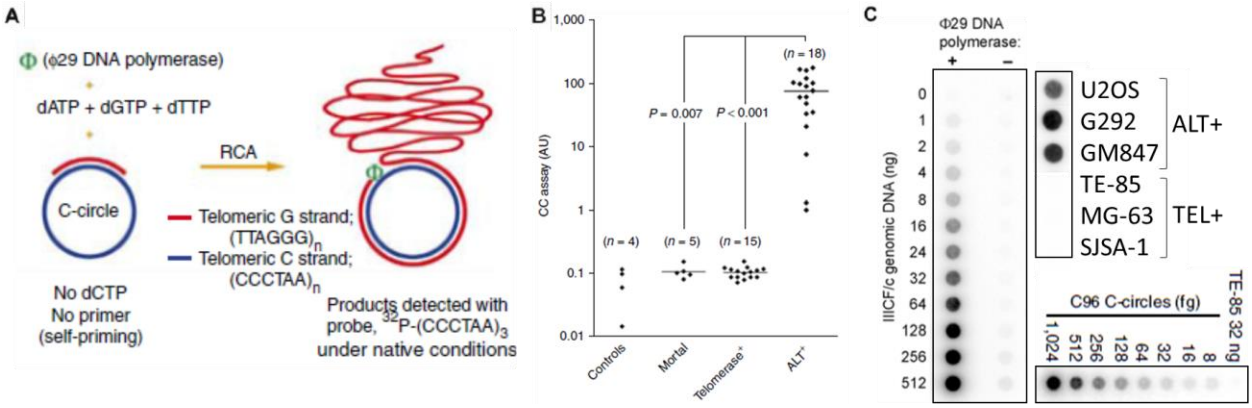

Figure 4. The C-circle assay is tightly correlated with ALT activity[26]. To test for the presence of C-Circles, circular DNA present in samples is used as a template by phi29 DNA polymerase to produce linear sequences which are then probed for the resultant G-rich, telomeric repeat strands (A). This radioactive assay has been reported to accurately measure ALT activity in 38 different cell lines (B). Typical CC assay results for a panel of ALT+ and TEL+ cell lines, as well as artificial CC (C96) samples is shown (C).

Preliminary Data

OncoSENS High-Throughput ALT Screening Methods

2-D APB assay

It has been previously reported that automated APB screenings can take up to 34 hours per slide, but it can hypothetically be set up to only take 17 hours[34]. The total time to screen 3 slides of 384 spots each is about 72 hours (24 hours per slide) in the best case scenario (600-1200 cells total per condition, 11 genes or conditions total). With this limitation in mind we set out to look for ways of making this assay more high-throughput friendly and less data intensive. Using the Molecular Devices IXM high-throughput screening microscope (Supplementary Figure 1) and customized software algorithms for data collection and analysis, we achieved very exciting results (Figure 5). We are currently able to acquire, detect and quantify the APBs in cells seeded on a full 96-well plate in just about 2.5-3.5 hours (Figure 5), with 150-300 cells analyzed per condition. **We can thus complete an APB screen of four full 96-well plates (384 wells total) in about 14 hours (600-1200 total cells per condition, 12 genes or conditions total), which is more than 5 times faster than the hypothetical maximum speed per screen reported in the literature[34].** And this can be accomplished without using a supercomputer for image analysis since we only need to analyze less than 48,000 images per screen in 2-D versus the 780,000 images in 3-D in the current automated method[34]. Our assay also does not rely on artificially

increasing APB formation by creating senescence culture conditions[25]. Our data reproduces key APB parameters reported in the literature,[34] including distribution of APBs per nuclei in ALT+ and TEL+ cells (Figure 5B-C), the average number of APBs per nuclei in these cells (Figure 5D), the percent of cells containing at least 1 APB per nuclei (Figure 5E), and the shift in the size of telomeres and PML (Figure 5F-G). Our assay also revealed new data on the lack of an effect of POT1 gene knockdown on APB formation in 143B (TEL+) cells (Figure 5D).

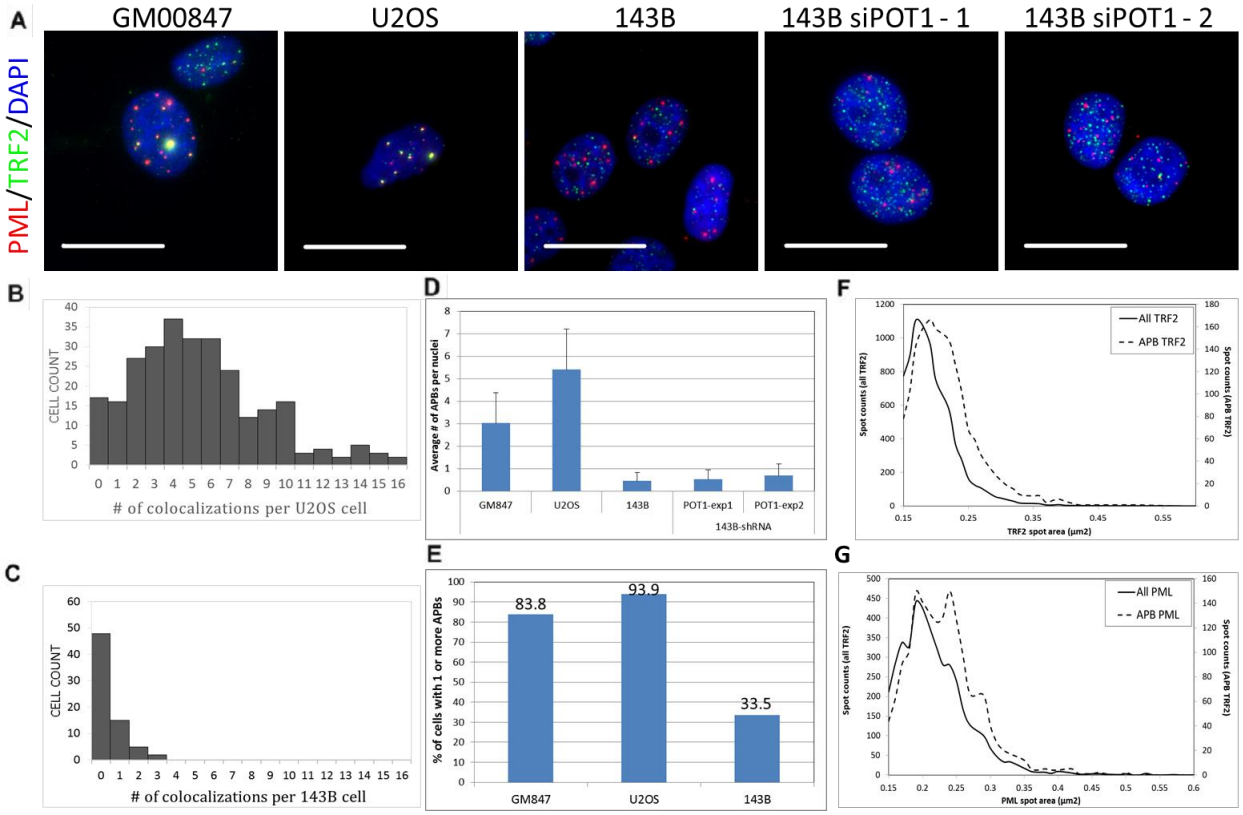

Figure 5. The two-dimensional APB assay is capable of screening multiple samples at once at high speeds.(A) Representative fluorescent images of GM00847, U2OS, 143B, and 143B cells transfected with a POT1 knockdown construct from our high-throughput 2-D APB assay taken with Molecular Devices IXM using customized deconvolution and z-stack projection scripts. Our immunofluorescence-based 2-D APB high-throughput detection method yields colocalization numbers similar to those reported in the literature (B-E)[34]. Telomeres (F) and PML (G) increase in size, as measured by area (μm^2), when present in APBs. See also Figure 3 for comparison with the 3-D APB automated detection method.

ELISA-based CC assay

As described previously, the challenges associated with the CC assay limit its use to mainly low-throughput experiments[26,35]. We are attempting to overcome that hurdle by incorporating detection technologies into the assay that are more amenable to high-throughput screening. We already

succeeded in creating a non-radioactive version of the assay (Figure 6A-B), which reproduces the main results from the original CC assay study[26] (Figure 4C). We are now working to make the CC assay detectable by ELISA using biotin-labeled capture probes and DIG-labeling the phi29-expanded RCA products (Figure 6C-D), which shorten time to detection and greatly simplify data collection and quantification. The goal is to achieve a high-throughput assay for CC like the TRAP assay is for telomerase activity[19]. We had reasonably preliminary success with detecting CC amplification signals from ALT+ cell lines as quickly as less than 24 hours (Figure 6C-D), but significant work still needs to be done to improve the sensitivity and reproducibility of the assay. Once this assay is further developed, it could be used in addition to the 2-D APB assay described above to perform high-throughput genetic screens on ALT-associated genes. Also, **the ELISA-based CC assay could potentially yield results in as little as 16-18 hours from sample DNA isolation to detection as opposed to the 2-4 days currently required for the original CC assay**.

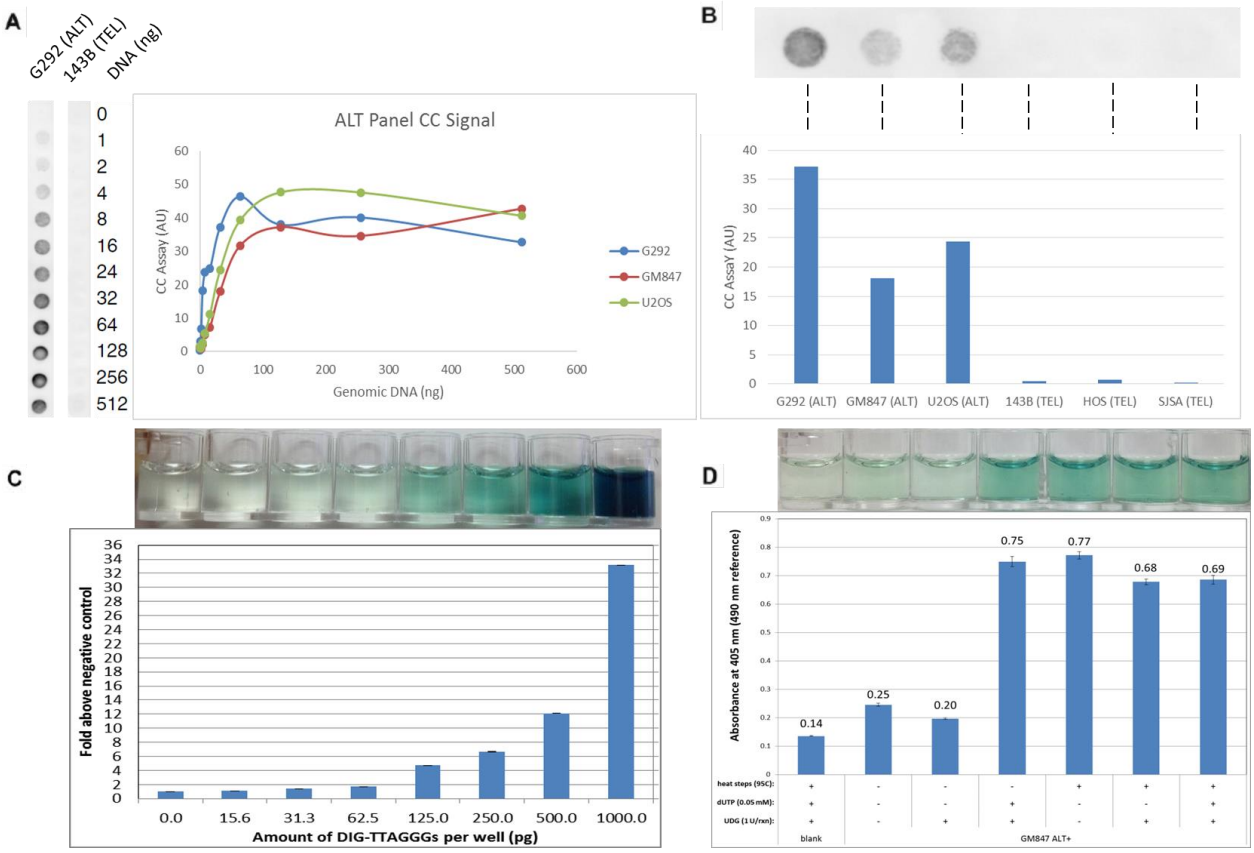

Figure 6. Development of novel C-circle assays by the OncoSENS team. The non-radioactive version of the CC assay shows similar sensitivity to the original assay[26] (A-B).The ELISA-based CC assay attempts to incorporate the successful high-content aspects of the TRAP assay for telomerase activity into a C-circle assay format as shown in (C-D). Though we are still exploring several different sample preparation

methods to increase sensitivity and reproducibility, we can report successful detection of positive controls (C) and ALT+ samples for the assay (D). Heating as well as chemically mediated cleavage (D) of the RCA telomeric strands derived from CC show promising results. The intensity of the green color is directly proportional to the amount of sample detected by the assay.

Research Plan

Rationale and Expected Impact

- The ALT cancer field currently lacks robust assays and funding as compared to telomerase-based cancer research.
- Our goal is to develop and establish functional high-throughput and high-content ALT assays that are useful for testing patient samples, quickly identifying gene targets, screening for small molecule drugs, and accelerating research on ALT mechanisms in general.
- Our group will use these screening methods to identify genes that are essential for ALT activity in cancer cell lines.
- These ALT genes will provide the cancer research field with a wealth of therapeutic candidates and leads in the investigation of how the ALT mechanism works.
- The assays developed in this project ma also grow the scientific interest in the ALT field.

Strategy and Methodology

- We will fully establish our two-dimensional (2-D) APB assay and work on optimizing the ELISA-based, high-throughput version of the CC assay.
- The most comprehensive siRNA 3-D APB screen performed to date will be replicated with our two-dimensional assay and the CC ELISA assay once it is functional.
- Our assays will also be used to screen other genes that have been reported to modulate ALT activity by assays other than APB (see Supplementary Table 1).
- Cell lines representative of a variety of cancer types and telomere maintenance mechanisms will be screened with these high-throughput assays to quickly identify genes involved in ALT (Figure 7).
- These cell lines will then be tested for genes that have been validated as modulators of ALT activity (Supplementary Table 1) as well as several other genes that are reportedly differentially expressed in ALT+ versus TEL+ cells[48] but that have not been functionally tested by any ALT-related assay yet (Figure 8).
- In the long-term, stable cell lines expressing genes of interest or shRNAs to target genes will be created and tested using our *in vitro* high-throughput ALT assays as well as others: C-Circle assay, APB assay, telomere length determination by qPCR and cell viability (Figure 8).

- In vivo studies on the stable cell lines will be performed to establish a correlation between lack of ALT activity and the tumorigenicity of these cells as well as survival of experimental animals (Figure 8).
- Information will be shared with the field through national and international conferences and publications.

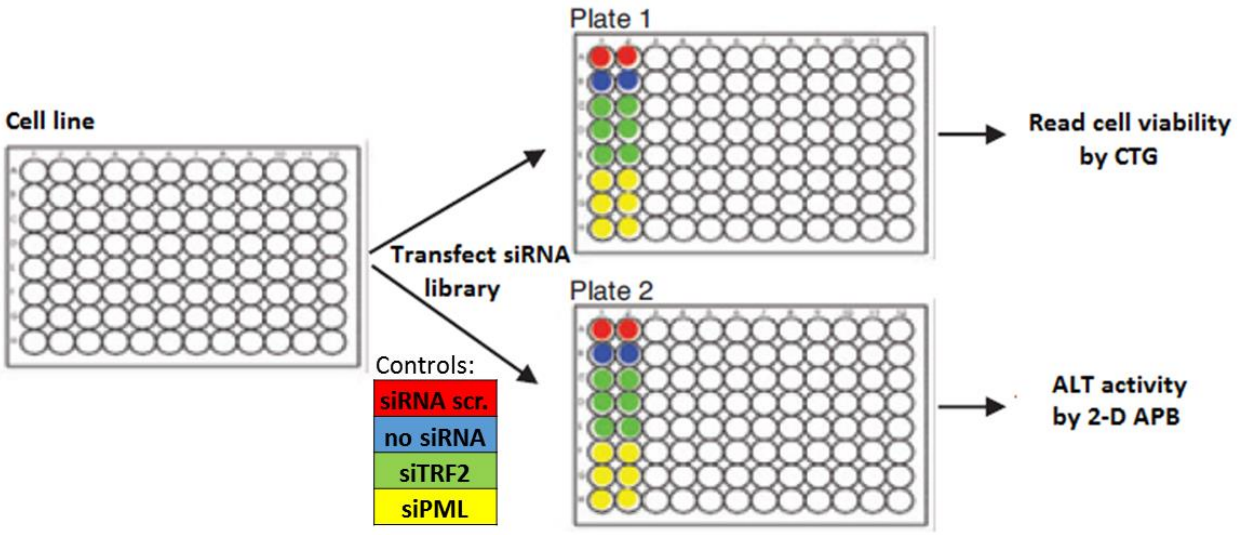

Figure 7. Strategy for siRNA screen using a library created from published ALT-associated gene targets. Cancer cell lines will be subjected to siRNA screen for several genes with at least 2 plates for each screen such that one plate will be assayed for APBs and the other for cell viability. Each plate will have columns 1 and 2 reserved for controls, as outlined above. See also Supplementary Table 1 for the genes of interest for validating the high-throughput assays for ALT activity.

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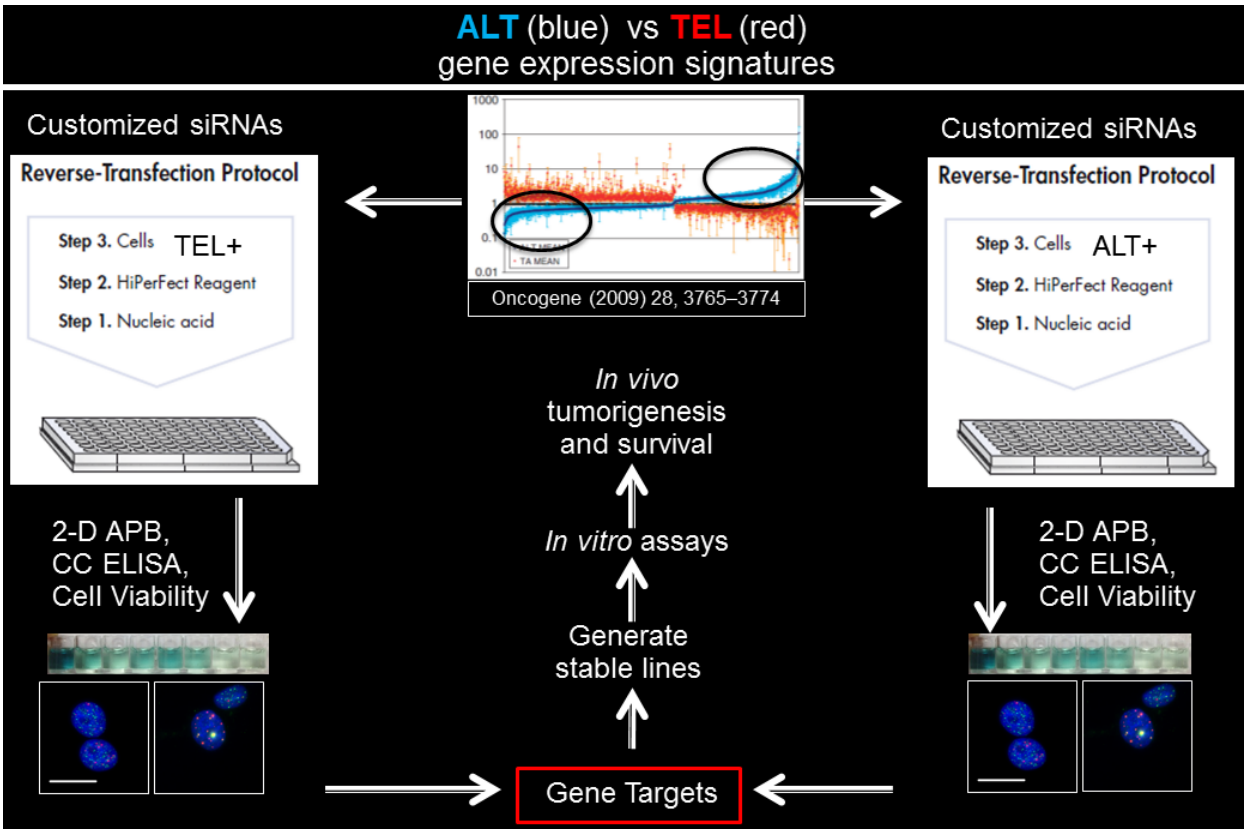

Figure 8. Workflow diagram of the long-term OncoSENS strategy for high-content screening and validation of ALT-associated genes. Microarray gene expression datasets from TEL+ vs ALT+ cells will serve as gene target inputs for our siRNA screen using both types of cancer cells. Genes overexpressed in ALT+ cells but downregulated in TEL+ cells will be knocked down in ALT cells via siRNAs and analyzed using high-content assays. Similarly, genes overexpressed in TEL+ but not in ALT+ cells will be downregulated in TEL+ cells via siRNA. The gene targets identified this way will be used to generate stable cell lines for further *in vitro* and *in vivo* testing to confirm ALT-associated activity and their potential as a therapeutic candidate.

Specific aims

1. Validate high-throughput ALT activity assays via RNA interference screens on ALT+ cells targeting genes that have already been found in the literature to modulate ALT activity.
2. Using our high-content ALT assays, perform siRNA screen on ALT+ cells for the genes overexpressed in ALT+ but not TEL+ cells.
3. Using our high-content ALT assays, perform siRNA screen on TEL+ cells for the genes overexpressed in TEL+ but not ALT+ cells .

Timeline and Milestones

- Purchase cell lines, high-content screening fluorescent microscope, cell counter, luminometer, reagents, primary and secondary antibodies, order siRNA library (1 month).
- Preliminary experiments to determine proper methods for CTG viability assay (ATP control curve), cell line seeding and culture into 96-well plates for 72-96 hours, primary and secondary concentrations for immunolabeling, optimizing APB colocalization script for automated counting (2 months).
- Successfully complete run of 2-D APB and CTG viability assays with HeLa and U2OS cell lines controls to repeat previously established siRNA APB screening results.[25,27,34] (2 months).
- Perform 2-D APB and CTG assays on the panel of siRNA library-treated ALT+ cells for the genes identified in Supplementary Table 1 (2 months).
- Perform 2-D APB and CTG assays on the panel of siRNA library-treated ALT+ cells for the genes identified as overexpressed in ALT+ cells and tumors[48] (3 months).
- Perform 2-D APB and CTG assays on the panel of siRNA library-treated TEL+ cells for the genes identified as overexpressed in TEL+ cells and tumors[48] (3 months).
- Data analysis identification of gene targets, and manuscript/abstract submission to share discoveries with other researcher in the cancer research field (throughout).

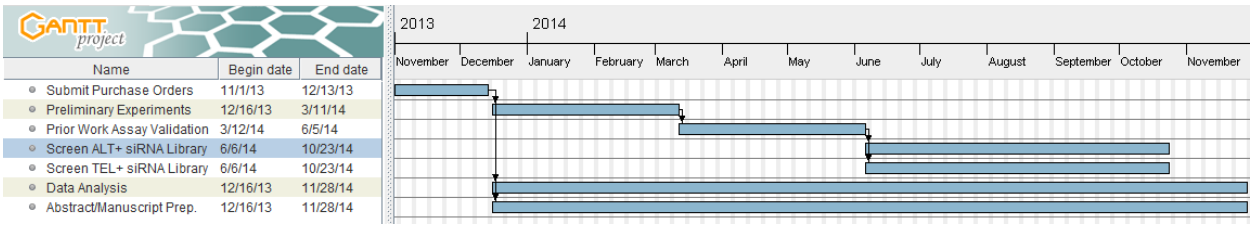

Figure 9. This project is planned to take 1 year.

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Research Methods

Cell lines, Transfection, siRNA library

A panel of three ALT+ cell lines and three TEL+ cell lines will be obtained from ATCC. The **REQUIRES FINAL UPDATED FORMAT** siRNA library detailed in Supplementary Table 1 will be obtained from **TBD ORGANIZATION** in 96-well plate format. The gene silencing and cell viability determination methods from a recent high-throughput screen of telomerase inhibitors[19] will be used. Stable cell lines will be generated from genes of interest identified by high throughput screening will be performed as previously described[19]

ELISA-based CC Assay Development

Cells will be treated as per an established C-Circle expansion protocol and various detection, probing, cleaving and expansion techniques will be tested. Incorporation testing will include ddATP, biotin dATP, DIG dUTP, dUTP. Sample treatment may involve heating, telomeric PCR primer expansion, vortexing, uracil DNA glycosylase treatment, duplex specific nuclease, sonication, denaturation. Detection methods screened will include PCR and ELISA.

2-D APB Assay

Colocalized TRF2 and PML will be labeled as described previously[36] and detected in a high throughput fashion by collapsing 3-D stacks into 2-D images and performing image analysis with a Molecular Devices script.

CTG Viability Assay

Cell viability will be assessed with the CTG viability assay as per manufacturer protocol.

Telomere Length Determination

Telomere length will be determined with an established qPCR-based detection method[49].

CC Assay

The C-Circle assay will be performed as previously described by the Reddel group[26] with alterations of luminescent detection and DNA shearing methods that we have developed at SENS.

Budget

Facility		
	Rent and Utilities	$22,500.00
Reagents		
	siRNA Library	$17,000.00
	Transfection	$3,152.00
	Immunofluorescence	$17,000.00
	CTG Viability	$2,000.00
	Cell Lines	$3,000.00
Equipment		
	Countess Cell Counter	$5,348.00
	Promega Luminometer	$17,000.00
Miscellaneous		
	Travel/Hotel	$8,000.00
	Conferences/Publications	$5,000.00
Total		$100,000.00

Table 1. We are requesting $100,000 for this project.

Scientific Advisors

Scientific Advisor #1?

We plan on asking Matthew O'Connor to be an advisor because of his relevant telomere experience.

Scientific Advisor #2?

We plan on asking Ehud Goldin to be an advisor because of his relevant experience developing a Genome-Wide RNAi Screen for Lysosomal Storage Disorders.

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Personnel

David Halvorsen

- LEF Member, Alcor Member, LEF Teens and Twenties conference attendee
- OncoSENS Research Associate
- TeloDDR conference attendee, France 2012

Haroldo Silva

- Bioengineering Ph.D. from UC Berkeley
- OncoSENS Team Lead
- Developed the OncoSENS high-throughput C-circle ELISA and APB assays

Thomas Hunt

- Volunteer OncoSENS researcher since 2011
- Youngest Thiel 20 under 20 Fellowship finalist (?)

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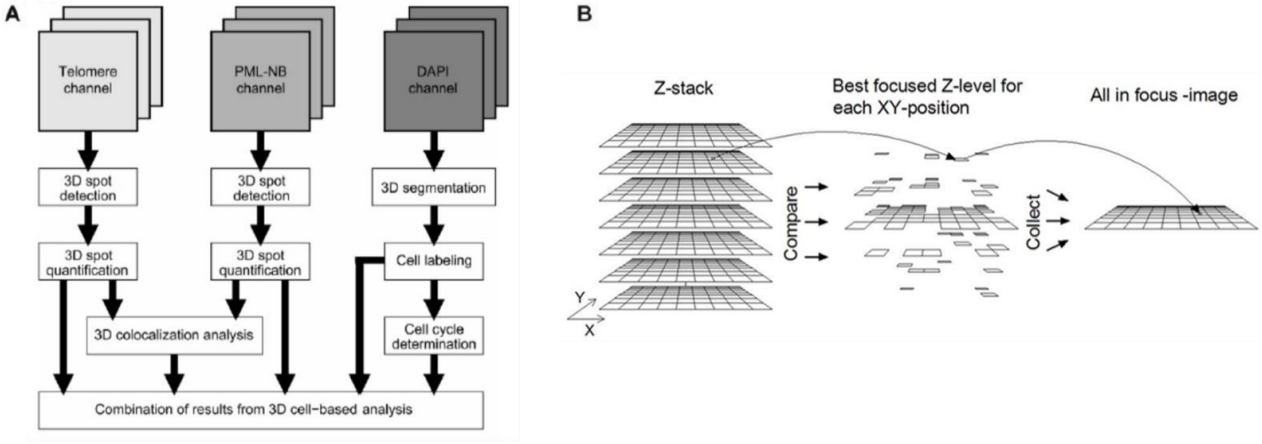
Appendix A: Supplementary Information



Supplementary Figure 1. Comparison of image acquisition techniques used by the current 3-D APB colocalization assay[34] (A) and the two-dimensional 2-D APB assay that we have developed in collaboration with Molecular Devices (B).

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Supplementary Table 1. Genes of interest for initial validation of high-throughput screenings. All the genes below have been found in previous publications to have some impact on ALT cells or ALT activity by different assays such as APB, CC, T-SCE, t-circles or ECTRs, TRFL, C-rich overhangs, and proliferation. These genes will be used in the first screen to validate the high-throughput version of the APB assay before we perform any further screening of gene candidates.

Gene (s)	TMM	Intervention	Result	Assays	Reference
53BP1	ALT	siRNA	decreased	APB	Osterwald 2012
ASF1a	ALT	siRNA	decreased	APB	Jing 2011
ATM	ALT	siRNA	decreased	APB	Osterwald 2012
BLM	ALT	siRNA	decreased	TRFL, cell growth	Bhattacharyya 2009, Temime-Smaali 2008
BRCA2	ALT	Knockdown	decreased	T-SCE	Sapir 2011
HIRA	ALT	siRNA	decreased	APB	Jing 2011
hPOT1 & hTERT homologs	TEL	mutation	increased	CC, TRFL	Lackner 2012, Cheng 2012
hTERT	ALT	Overexpression	decreased	APB, ECTR, TRFL, CC	Plantinga 2013, Perrem 2001, Henson 2009
Ku70	ALT	shRNA	decreased	t-circles, proliferation	Li 2011
Ku80	ALT	shRNA	decreased	t-circles, proliferation	Li 2011
MMS21	ALT	siRNA	decreased	CC, T-SCE, APB, TRFL	Henson 2009, Potts 2007, Osterwald 2012
Mre11	ALT	shRNA	decreased	TRFL	Zhong 2007
NBS1	ALT	siRNA, shRNA, mutation	decreased	APB	Jiang 2005 & 2007, Zhong 2007, Wu 2003
PGC-1b	ALT	shRNA	Cell Death	Viability	Hu 2012
Rad17	ALT	siRNA	not significant	APB	Osterwald 2012
Rad50	ALT	siRNA, shRNA	decreased	TRFL	Potts 2007, Zhong 2007
Rad51	ALT	siRNA	decreased, increased	APB, C-overhangs	Potts 2007, Oganesian 2011
Rad52	ALT	siRNA	increased	C-overhangs, CC	Oganesian 2011
SMC5	ALT	siRNA	decreased	CC, T-SCE, APB, TRFL	Henson 2009, Potts 2007
SMC6	ALT	siRNA	decreased	T-SCE, APB	Potts 2007
SOD2	ALT	shRNA	Cell Death	Viability	Hu 2012
Sp100	ALT	Overexpression	decreased	CC, APB, TRFL	Henson 2009, Jiang 2005
SUMO 1	ALT	siRNA	decreased	APB	Osterwald 2012
SUMO 2	ALT	siRNA	decreased	APB	Osterwald 2012
SUMO 3	ALT	siRNA	decreased	APB	Osterwald 2012
HP1a	ALT	siRNA	decreased	APB	Jing 2011
HP1c	ALT	siRNA	decreased	APB	Jing 2011
p21	ALT	siRNA	decreased	APB	Jing 2011
TopoIII	ALT	siRNA	cell death	Viability	Temime-Smaali 2008
TRF1	ALT	siRNA	decreased	APB	Osterwald 2012
TRF2	ALT	shRNA	decreased	TRFL	D'Alcontres 2007
Ubc9	ALT	siRNA	decreased	APB	Osterwald 2012
XRCC3	ALT	siRNA	increased	C-overhangs	Oganesian 2011
ZScan4	ALT	siRNA	decreased	Proliferation	Zalzman 2004

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Appendix B: Equipment Quotes

E8032	**GloMax®-Multi+ Detection System Base Instrument with Shaking** (Note: you must include at least one module below to be functional)	**$10,000**
E9032	GloMax®-Multi+ Detection System Base Instrument with Heating and Shaking (Note: you must include at least one module below to be functional)	$12,000
E8041	**GloMax®-Multi+ Luminescence Module**	**$7,000**
E8051	GloMax®-Multi+ Fluorescence Module (5 optical kits included)	$4,800
E8061	GloMax®-Multi+ Visible Absorbance Module (450-750nm range with filters at 450nm, 560, 600, 750nm and 2 open positions for customization).	$2,500
E9061	GloMax®-Multi+ UV-Visible Absorbance Module (200-1100nm range with filters at 260nm, 280, 450, 560, 600, 750nm. 2 positions are customizable.)	$6,000
E7071	Single Injector System for GloMax®-Multi Detection System	$2,300
E7081	Dual Injector System for GloMax®-Multi Detection System	$4,326
E6531	Luminescence Light Plate- standard	$1,006
SA3030	GloMax®-Multi+ Base Instrument Service Agreement, 1 year	$3,465
SA3040	GloMax-Multi+ Injector Instrument Service Agreement, 1 year	$497
TS2680	GloMax®-Multi+ Preventative Maintenance	$1,452
	Total	**$17,000**

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sens research foundation
reimagine aging

Quotation

Nexcelom Bioscience LLC
360 Merrimack Street, Building 9
Lawrence, MA 01843

Phone: 978-327-5340
Fax: 978-327-5341
Email: sales@nexcelom.com
www.nexcelom.com

Quotation No.:
Q130827-09

David Halvorsen SENS Research Foundation Mountain View, CA 94041	Email: david.halvorsen@sens.org Phone: (650) 938-6100 x1787 Fax:	Date: 8-27-2013 Rep: Nexcelom Sales Rep Phone: (978) 327-5340 Expires: 10-31-2013

FOB: Lawrence, Massachusetts, USA	Terms: Net 30
Shipping Method: UPS Ground	EIN: 30-022-9049
Delivery Time: 4 weeks after PO	

Product Part Number	Product Description	Quantity	Unit	Unit Price	Subtotal
Cellometer Auto T4 Plus	Cellometer Auto T4 Plus Cell Counter. Includes instrument, software, and pack of 75 slides.	1	Each	$4,900.00	$4,900.00
Discount - T4 Purchase By	Discount - T4 Purchase By Specific Date *	1	Each	($972.00)	($972.00)
CHT4-SD100-014	Disposable, all plastic cell counting chambers for Cellometer cell counters. Packed with protective film. Remove protective film before use. Case of 900 slides for 1,800 counts.	1	Case	$1,350.00	$1,350.00
SH2	Shipping and Handling	1	Each	$70.00	$70.00
				TOTAL	$5,348.00