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TITLE: High-Content FRET-FLIM Screening in Inhibitors of Oncogenic Transcription by C-Myc in Breast Cancer

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14. ABSTRACT

To develop a novel high content screen to identify inhibitors that block Myc:TRRAP interaction, in the first year of this project we constructed several fluorescent fusion protein constructs of Myc and TRRAP, and evaluated their ability to bind and engage in fluorescence energy transfer (FRET) in vivo. All year-one tasks were completed in the first year of the grant. During the second year we discovered that expression of Myc-CFP was toxic to some cells and in other cells the Myc-CFP did not show the expected blue fluorescence. Due to the short half-life of Myc most of the CFP-Myc proteins were turning over before the CFP matured and became fluorescent. To overcome this unexpected issue, we introduced a mutation (T58A) to increase the stability of the CFP-Myc fusion protein and we evaluated additional cell lines, as well as new expression constructs. Conditions were identified in which sufficient CFP-Myc folded correctly and became fluorescent. To define Myc:TRRAP co-regulated target genes we established a Myc-dependent transformation system in the MCF10A cells and conducted Myc-specific ChIP-on-chip in these cells with and without ectopic Myc expression. Expression profiling of these cells was also conducted and data analysis revealed the subset of genes whose promoter was bound and regulated by Myc. During validation of the compound screen we discovered an engineering problem with the FRET-FLIM screening device that prevented the analysis of large numbers of samples. To rectify this we designed and built a pulse stretcher for the FLIM-Opera screening instrument.

15. SUBJECT TERMS
Myc, TRRAP, breast cancer therapeutics, FRET-FLIM, small molecules, high throughput screen

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Abstract:

There is an urgent need for novel anti-breast cancer therapeutics. Our hypothesis is that by identifying small molecules that target the Myc oncogene, we will develop an effective therapeutic that will improve breast cancer patient care and contribute to the eradication of disease. Our OBJECTIVE is to identify compounds that can be used to selectively inhibit the oncogenic activity of Myc by inhibiting its interaction with one of its key binding partners TRRAP. To this end, we aim to 1) develop a novel high content screen to identify inhibitors that block Myc:TRRAP interaction; 2) determine the transcriptional signatures of Myc:TRRAP target genes; 3) screen drug and chemical libraries to identify compounds that disrupt Myc:TRRAP interaction; and, 4) validate lead compounds that disrupt Myc:TRRAP interaction and block the transformation potential of breast cancer cells. To this end, in the first year of this grant we constructed several fluorescent fusion protein constructs of Myc and TRRAP, and evaluated their ability to bind and engage in fluorescence energy transfer (FRET) in vivo. We identified FRET pairs that were functional and established methodology using novel instrumentation that will enable the high throughput screening of chemical libraries. In addition, we established Myc and TRRAP chromatin immunoprecipitation (ChIP) in the MCF10A cell line. We completed on time all tasks scheduled for the first year of the project.

During the second year, our objectives were to conduct the screen and further define Myc:TRRAP co-regulated target genes in breast cancer. These will be described in detail below:

i) To conduct the screen we aimed to use cell lines stably expressing the Myc-CFP and TRRAP-YFP FRET pairs. When establishing these stable systems, we were surprised to find that the stable expression of Myc-CFP was toxic to some cells and in other cells the Myc-CFP did not show the expected blue fluorescence. Moreover, cells died in response to stable ectopic expression of the TRRAP-YFP fragment that corresponded to the Myc-binding domains of TRRAP. To overcome these unexpected issues, we evaluated additional cell systems, as well as new expression constructs that were designed to enable stable cell lines to be developed that constitutively and conditionally express the Myc and TRRAP fusion proteins, respectively. These studies revealed that due to the short half-life of Myc most of the CFP-Myc proteins were turning over before the CFP matured and became fluorescent. To increase the stability of the CFP-Myc fusion protein we introduced a mutation (T58A) that increased the half-life of the fusion protein such that sufficient CFP-Myc folded correctly and became fluorescent. Unfortunately this exacerbated the difficulty of making stable cell lines expressing both Myc and TRRAP as they rapidly shut off expression of the exogenous genes. We therefore, resorted to expressing the TRRAP proteins by transient transfection to evaluate the Myc-T58A-CFP and TRRAP-YFP pairs that would show FRET with high sensitivity and specificity. To accomplish this in a high-throughput format for screening required optimization of automated transfections. Once a suitable protocol was identified and optimized it was automated and validated on the robotic liquid handlers. In validating the screen we discovered an engineering problem with the FRET-FLIM screening device that prevented the analysis of large numbers of samples. To rectify this we designed and built a pulse stretcher for the laser. For this portion of the project, we have overcome several unexpected obstacles and advanced a novel screening system that can be used to identify Myc;TRRAP inhibitors.

ii) To define Myc:TRRAP co-regulated target genes we have established a Myc-dependent transformation system in the MCF10A cells and conducted Myc-specific ChIP-on-chip in these cells with and without ectopic Myc expression. Expression profiling of these cells was also conducted and data analysis revealed the subset of genes whose promoter was bound and regulated by Myc. This list was prioritized by then identifying which of these Myc target genes was associated with patient outcome. These are now undergoing validation at the level of Myc binding by quantitative ChIP. Those that validate will also be assayed for TRRAP-specific ChIP and this cohort will then be tested for their functional role in Myc-dependent transformation of breast epithelial cells. To further profile the target genes to which Myc recruits TRRAP, we will conduct TRRAP-specific ChIP-on-chip in the presence and absence of the regions of TRRAP shown in this study to bind Myc. These dominant negative fragments will add a level of specificity to our TRRAP ChIP-on-chip assays that will enable Myc-dependent TRRAP-recruitment to be profiled genome-wide in breast cancer. The key reagents and technologies required for this research have now been established. Full completion of these goals was not achieved in the second year of funding because of the time and effort required to rectify the many unexpected problems associated with expression of the fusion proteins, as described above. Overall, we have successfully established the tools and technologies that can be used to identify inhibitors of Myc;TRRAP interaction and evaluate

whether these inhibitors disrupt Myc;TRRAP interaction at co-regulated target genes that drive the transformation of breast cancer.

Keywords: Myc, TRRAP, breast cancer therapeutics, FRET-FLIM, small molecules, high throughput screen

Introduction

Background: Recent advances in our understanding of the molecular basis of oncogenesis provide a unique opportunity to design novel effective anti-breast cancer therapies that target pathways essential for cell growth and survival. However, the choice of pathways is crucial. Recent successes such as Gleevec and Herceptin that target kinase pathways and growth factor receptors respectively are useful for treating only a very narrow spectrum of cancers. For example, Herceptin is effective only in a specific subset of breast cancers, those over-expressing the Her2 protein. To treat a broader spectrum of breast cancers it is essential to target key nodes downstream of multiple signaling cascades. The Myc oncoprotein, is a regulator of gene transcription that drives the neoplastic process in 50% of all breast cancers (1). Evidence in mouse models shows that inhibiting Myc can eradicate even existing tumors, making it an especially attractive target for the development of novel therapeutics (2). Myc activity is dependent upon protein-protein interactions.

Hypothesis: We hypothesize that targeting the dependence of Myc oncogenic activity on specific protein:protein interactions will result in anti-Myc therapeutics of high sensitivity and specificity. Recent success in targeting protein-protein interactions supports this idea as interaction inhibitors are now advancing to patient care. Our OBJECTIVE is to identify compounds that can be used to selectively inhibit the oncogenic activity of Myc by inhibiting its interaction with one of its key binding partners TRRAP.

Specific Aims: Develop a novel high content screen to identify inhibitors that block Myc:TRRAP interaction. Determine the transcriptional signatures of Myc:TRRAP target genes. Screen drug and chemical libraries to identify compounds that disrupt Myc:TRRAP interaction. Validate lead compounds that disrupt Myc:TRRAP interaction and block the transformation potential of breast cancer cells.

Body

With support from the DOD, the research outlined in the original proposal progressed in a steady and productive manner as expected in the first year of funding. However, a series of unanticipated problems arose in the second year that required re-designing and assembling new constructs expressing CFP-Myc and TRRAP-YFP. The new constructs were iteratively optimized and procedures were developed to automate the assay. The automated assay was validated for screening. A pulse stretcher was designed and tested to improve the optics in the FLIM-Opera high content screening instrument. To outline the accomplishments to date, the tasks outlined in the original Statement of Work, of the proposal are itemized below (italics) and a progress report for each task provided. New tasks that were required to deal with unanticipated problems caused by the short half-life of the CFP-Myc fusion protein, unstable expression of the CFP-Myc and TRRAP-YFP fusion proteins as well as laser damage to the optical fibers on the Opera have been added and clearly indicated below.

Statement of Work

Task 1: Develop a novel high content screen to identify inhibitors that block Myc:TTRAP interaction (months 1-18)

a) *Penn:Clone chimeric constructs into pCMV10 (or pcDNA3.1) for transient expression of fusion proteins in 293TV cells.* (*Months 1-3 for the first set of constructs.)*

Completed: We generated a panel of constructs in the transient transfection vector pcDNA3.1 in order to optimize FRET between the donor cerulean fluorescence protein (CFP) and acceptor citrine (yellow) fluorescence protein (YFP) when fused to Myc and TRRAP, respectively. A schematic of the constructs generated is provided in Figures 1 and 2. Fusions were generated with the fluorescent protein both 5' and 3' to Myc and TRRAP. To ensure the added fluorescent protein did not interfere with Myc:TRRAP interaction, pairs of these fusions were evaluated for interaction by co-immunoprecipitation in transiently transfected 293TV cells (Figure 3).

b) *Andrews: Evaluate paired interactors to obtain an efficient FRET signal. (Months 2-6 for the first set of constructs.)*
Completed: We evaluated a series of acceptor donor pairs from Task 1a for FRET-FLIM signal and identified a suitable pair (Figures 4 & 5).

c) *Penn/Andrews: To further refine and maximize FRET, linkers will be sequentially added to the spacer region between the fluorescence proteins and the test proteins. FRET-FLIM will be evaluated with modified fusion proteins. (Months 3-9)*
Completed: Using our Opera HCS instrument, our data showed that changing the nature and the length of the spacer region between the protein of interest and the fluorescent protein could dramatically improve FRET-FLIM (Figure 6). Thus, in addition to the fusion proteins described in Task 1a above, we also generated a panel of constructs with different "linker" regions, between the fluorescent protein and the TRRAP fragments in order to refine and maximize FRET (Figures 1 and 2). These were analyzed for FRET-FLIM using transient transfection into 293 TV and MCF-7 cells.

d) *Penn/Andrews: The optimal donor:test pair will be introduced into the Tet-inducible MCF-7 and MDA-MB-231 cell lines. (Months 8-24)*
Nearly Complete: Evaluation of the Tet-inducible MCF-7 and MDA-MB-231 cells showed that the tet-regulated Myc parental cells was leaky and inducibility was quickly lost, thus precluding the usefulness of this system. As an alternative approach we evaluated stable expression of the CFP-Myc fusion using expression vectors with a variety of selectable markers (puro, neo, blast; see Fig 8B for specifics) for expression and selection in 293 TV, MCF-7, SHEP, HO15.19 (Myc null) cells. Much to our surprise the CFP-Myc fusion protein could be stably expressed, but the fusion protein was toxic to some cells. Moreover, in those cells that tolerated expression, the blue fluorescence was not evident in the cells stably expressing the CFP-Myc fusion protein. After much brainstorming over this unexpected result, our hypothesis was that the short half-life of Myc was driving the rapid turnover of the fusion protein and that the CFP component could not fold and fluoresce within this short time frame. To test our hypothesis and further develop the screening system, we evaluated and adopted a Myc mutant (T58A) that has a longer half-life than wild-type Myc (Figure 7) and developed new CFP-Myc-T58 fusion proteins (Figure 8). These were transiently and stably expressed a variety of cells (Figure 9, 10 and data not shown). CFP-Myc fluorescence was indeed increased by using the T58A Myc mutant and this basal fluorescence was further potentiated when protein stability was further enhanced by exposing the cells to either lower temperature conditions or proteasome inhibitor (Figure 11). With our hypothesis validated, we went on to evaluate the feasibility of using the new Myc-T58A fusion proteins for the screen. To this end, the fluorescence lifetime of the new Myc-T58A-CFP donor was measured in the presence of negative and positive controls, as well as the series of TRRAP-YFP acceptors (Figure 12). After conducting several biological replicates, the FRET efficiency was established for these pairs (Figure 13). To further describe the work conducted to overcome the unexpected lack of CFP-Myc fluorescence when expressed stably in cell, we have described the work done as new tasks/goals that were conducted in the second year of this grant.
d-1) New Goal: Create and test a new series of plasmids encoding CFP-Myc fusion proteins with a stabilizing mutation in the Myc sequence resulting in the expression of Myc-T58A-CFP. (months 12-16)
Completed: Expression of the stabilized fusion proteins greatly increased CFP-fluorescence in optimization experiments. See Figures 7-10)
d-2) New Goal: Automation of transient transfections to generate cells suitable for high-content screening (months 16-24).
Completed: A variety of transfection reagents and cell lines were tested for automated delivery of plasmid DNA into cells and expression of CFP-Myc and YFP-TRRAP fragments. Culture conditions (temperature, medium, cell density etc.) were optimized for co-expression of CFP-Myc and YFP-TRRAP fragments,,
d-3) New Goal: Optimizing cell culture conditions to stabilize CFP-Myc sufficiently to permit proper folding and fluorescence from CFP (months 16-24)
Completed: Both the addition of proteasome inhibitors and growth of cells at 25 degrees instead of 37 degrees resulted in increased CFP-fluorescence, although the two together were not additive. See Figure 11.

d-4P New Goal: Screen optimal donor:acceptor pair using cells expressing stable Myc-T58A-CFP donor and transient TRRAP-YFP acceptors.
Completed: Optimal pair was established by measuring the change in fluorescent lifetime of the donor (Figure 12). After repeating the experiment several times, the FRET efficiency of each pair was established (Figure 13)

e) Andrews/Fradin: Accurate decay curves will be generated for CFP-Myc and compared to an existing CFP-C3-YFP control protein (50% FRET) using cell lysates from Task1d above (months 3-7). These curves will be used to optimize detection of FRET by FLIM in live cells (months 6-8). Confirm FRET-FLIM measurements of affinity of the Myc:TRRAP interaction in live cells by fluorescence fluctuation analysis (months 7-12).
FRET-FLIM parameters established analytically will be used to optimize detection windows for ultra-fast detection of FRET-FLIM in live cells in the Opera High Content Screening instrument (months 7-12).
Completed: Accurate decay curves have been generated for CFP-Myc and compared to CFP-C3-YFP controls. Optimization of FRET-FLIM detection using the Opera HCS instrument has been completed (Figure 14 and data not shown).

f) *Andrews/Fradin: Localization and binding affinities will be measured for CFP-Myc and YFP-TRRAP in MCF-7 and MDA-MB-231 cells (from 1.d above) using FRET-FLIM and Fluorescence Fluctuation Analyses (months 12-18).*
Partially Completed. FRET-FLIM localization experiments were completed and demonstrated that the interaction of CFP-Myc and YFP-TRRAP occurred in the nucleus, as expected. Fluorescence Fluctuation analyses were not possible due to the lack of stable cell lines expressing the fusion proteins.

Task 2: Determine the transcriptional signatures of Myc:TRRAP target genes (months 9-14)
We have characterized the MCF10A cell system as a novel model of Myc-dependent transformation. Using this non-transformed breast epithelial cell line, we will determine the transcriptional signatures of Myc:TRRAP target genes. Ectopic expression of human Myc transforms MCF10As, such that they are able to form colonies in soft agar (Figure 15B). Interestingly, when we harvest growing MCF10A cells expressing either empty vector control or ectopic Myc and assay for Myc protein, the levels are remarkably similar (Figure 15A, compare lanes 1 and 2). However, when growth factors are removed from the media for one hour, there is a robust decrease in endogenous Myc protein in the control cells as compared to those expressing deregulated, ectopic Myc (Figure 15A, compare lanes 3 and 4). Thus, the potentiated transformation of the MCF10A cells is due to the deregulated expression of ectopic Myc expression in these cells.

a) *Penn: Optimize sonication conditions for these cell types to achieve genomic DNA fragments <500 bp in length. (month 9)*
Completed: We have optimized the sonication conditions for MCF10A cells, such that we are able to achieve genomic DNA fragments 100-1000 base pairs in length (Figure 16).

b) *Penn: Conduct ChIP. (months 9-11)*
Completed: We have optimized ChIP conditions for Myc and TRRAP in MCF10A cells. This has been confirmed using Q-PRC for positive control target genes (e.g CAD) and negative control genes (e.g. chromosome 21 E-box) (Figure 17). Successful Myc ChIP is conducted using 10 million cells and 1.5 ug of our homemade N262 anti-Myc antibody. TRRAP ChIP can also be conducted when 10 million cells are immunoprecipitated with 2.5 uL of homemade TRRAP antibody.

c) *Penn: Conduct CoC using anti-Myc and IgG control, as well as anti-TRRAP and IgG control prepared from four independent biological replicates, of asynchronously growing MCF-7 and MDA-MB-231 cells +/- induction of TRRAP(F8). PCR amplify Chip'd DNA, label with Cy3 and Cy5 and hybridize to 12K CpG island microarray. Scan arrays.(months 11-12)*

Nearly Complete: We did not use MCF-7 and MDA-MB-231 cells because we further evaluated the tet-inducible system in these cells and found it was very leaky. We moved on to use the MCF10A cells for this work because of our finding that they were transformed in a Myc-dependent manner. We have recently completed ChIP-on-chip experiments for Myc in our MCF10A cell system, both in cells expressing vector control and expressing ectopic Myc. To increase sensitivity and specificity, as well as augment genome coverage of the ChIP-on-chip assay, we have conducted these experiments using the Agilent human promoter tiling arrays, instead of our homemade CpG island array. To achieve substantial significance and deeply probe the arrayed features, ten biological replicates were completed for each cell line (see schematic Figure 18). In addition, expression profiling of four biological replicates of each cell line was also conducted using the Affymetrix platform. The data has been analysed and we are currently validating these hits. We prioritized our analysis by identifying those Myc target genes that regulate expression and are associated with patient outcome. For the latter component of this analysis we exploited publicly available mRNA datasets of primary breast cancer patients that are linked to patient outcome data. Biological replicates for TRRAP ChIp-on-chip have been prepared. Because TRRAP is bound by many transcription factors, we aim to identify those target genes whose regulatory regions are bound by TRRAP in a Myc-dependent manner. We will achieve this by conducting the TRRAP-specific ChIP-on-chip in the presence and absence of inducible dominant negative fragments of TRRAP that encode the Myc-binding domain, validated by co-immunoprecipitation and FRET-FLIM in this study. For further description see Task 2e below.

d) *Penn/Jurisica: Analyze data to identify target genes bound by TRRAP in a Myc-dependent manner. (months 11-12)*
In progress – please see Task 2c and Schematic (Figure 18).

e) *Penn: Validate novel genes shown to be bound by Myc with and without the TRRAP co-activator. Validate Myc:TRRAP co-bound targets with the use of inducible TRRAP dominant negative reagent that disrupts Myc:TRRAP interaction at the level of chromatin (months 12-14).*
In progress. The originally proposed work in MCF-7 and MDA-MB-231 cells was not possible due to the leakiness of the tet-inducible system in those cell lines. To overcome this obstacle, the TRRAP fragments which we have shown to interact with Myc (fragments 1260 and 1690), as well as a fragment that has been shown to not interact with c-Myc (F1) were cloned into the pMEP4 inducible system. Using this system, fragments will be under the control of a metallothionein promoter, which can be induced by treatment with a divalent heavy metal ion, zinc or cadmium. We have introduced these constructs in MCF10A cells, and isolated clones. Unfortunately, we were not able to identify clones that showed robust inducibility of the TRRAP dominant negative fragments. To overcome this issue, a novel inducible system responsive to 4-hydroxytamoxifen was established as an alternative approach, and was shown to successfully induce the fragments when stably expressed in MCF10A and 293TV cells (Figure 19 and data not shown). We chose to conduct this work in the MCF10A cells that show Myc-dependent transformation to provide direct relevance to breast cancer. A schematic outlining the progress to date with the ChIP-on-chip work is shown (See Figure 18).

Task 3: Screen drug and chemical libraries to identify compounds that disrupt Myc:TRRAP interaction (months 8-24)

a) *Andrews: Validate screening assay – demonstrate negative and positive controls can be distinguished with a z-factor of .6-1.0 (months 8-24).*
In progress: Validation of the screening assay is underway. Figures 5 and 13 show the mean and standard deviations for the FLIM measurements expressed as lifetimes (Figure 3) and FRET efficiency (Figure 13). The error bars are sufficiently small that a very good z-factor was obtained. A change in lifetime larger than 300 ps was obtained which is sufficient for certain detection of an interaction between the proteins in live cells and sufficient for screening.
a-1) New Goal: Fully automate transient transfection, imaging and data analysis.
Completed: After optimization of transient transfection procedures these were automated and validated for screening (Months 14-20).

a-2) New Goal: Design and construct a pulse stretcher for the pulsed laser on the FLIM-Opera. Completed: An unanticipated technical limitation of the instrumentation was discovered. The high intensity of the short laser pulses burned out the optical fibers used to guide the light to the sample. After 4 months of experimentation it was determined that a pulse stretching device is required prior to the fiber entrance. In collaboration with Qiyin Fang in the Department of Biomedical Engineering a pulse stretcher has been designed, tested and is being assembled onto the Opera (Months 20-24). Final validation cannot be performed until the pulse stretcher is fully validated (Figure 20).

b) *Andrews/Collins: Screen LoPAC & Prestwick compound libraries of off-patent drugs (Andrews/Collins/Schimmer) and 1000 compounds from the Chembridge library with cell clones expressing interacting fluorescence proteins compared to the same cells expressing only the CFP-fusion protein. Estimate detection efficiency for CFP FRET-FLIM compared to direct effects of compounds on CFP fluorescence lifetime. Determine anticipated range in data due to direct fluorescence from library compounds (this is expected to be small as the compound must fluoresce with excitation and emission overlapping CFP). A validated screen with z-factor 0.5-1.0 will be ready by month 14.* Yet to do.

c) Andrews/Collins: Conduct Screen and analyze data (Months 14-20). Screen Maybridge and Chembridge libraries. Five dilutions for each compound and controls (Andrews/Collins/ Schimmer, months 14-18). Yet to do.

d) Andrews/Collins: Rescreen potential hits by FLIM for disruption of FRET and for short term cell viability indicative of no general cytotoxicity (months 16-18). Yet to do.

e) Andrews/Fradin: Measure compound dependent change in binding constants in live cells including MCF-7and MBA-MB-231 cells. Assay effects of selected compounds on Myc-TRRAP affinity and subcellular localization by FRET-FLIM, confocal microscopy and fluorescence fluctuation analysis (months 18-24). Yet to do.

Task 4: Validate lead compounds that disrupt Myc:TRRAP interaction and block the transformation potential of breast cancer cells. These experiments will begin as soon as hits start to be identified (months 14-24).

a) Penn: Evaluate the inhibitors identified from the screen (Aim 3) for their potential anti-proliferative effects in Myc null cells using the MTS assay. HO15.19 cells will be plated in 96 well plates, the next day cells will be exposed to compound at 3 concentrations relative to the amount of drug required to block Myc:TRRAP FRET. Cell viability will be measured at 3 days post drug. Those compounds that do not trigger an anti-proliferative response in HO15.19 cells will be further evaluated (months 14-20). Yet to do.

b) Penn: Evaluate whether inhibitors block Myc-induced colony growth assays of MCF-10A cells. Compounds that block MCF10Amyc cells from anchorage-independent growth will be further evaluated (months 14-20). Yet to do.

c) Penn: Evaluate the molecular response of MCF-7 and MBA-MB-231 cells to potential Myc:TRRAP inhibitors. ChiP analysis of novel and known positive control Myc- and TRRAP-bound target genes (identified in Aim 2) will be conducted in cells exposed to the concentration of compound shown to disrupt Myc:TRRAP interaction. The molecular kinetics of response will be monitored over time to evaluate whether the compound functions like TRRAP(F8) to block TRRAP precipitation (months 16-24). Yet to do.

d) Penn: Full CoC analysis will be conducted with those compounds that are not cytotoxic to Myc null cells, block Myc-induced transformation of MCF10A cells and block Myc:TRRAP interaction at the level of chromatin in the regulatory regions of established target genes. With the kinetics of response data (in 4c above), the concentration and time of treatment will be established for

the CoC analysis. MCF-7 cells will be treated with inhibitor and CoC analysis conducted as before comparing anti-Myc with IgG and anti-TRRAP with IgG in asynchronously growing cells exposed to vehicle control or inhibitor. Four biologically independent assays will be conducted using the 12K CpG island array and results scanned (months 16-21). Yet to do.

e) Penn/Jurisica: Array analysis will be conducted and results compared to response of TRRAP(F8) conducted in Aim 2. The compounds that closely match the molecular effects of TRRAP(F8) will be further analysed with MDA-MB-231 cells (months 16-24). Yet to do.

Key Research Accomplishments

1. **Constructed and evaluated several Myc and TRRAP fragements (1260, 1690, F1) as fluorescent fusion proteins for their interaction and FRET in vivo.**
2. **Established FLET-FLIM for high throughput screening conditions using the opera high content screening instrument**
3. **ChIP-on-chip and expression profiling experiments are complete for Myc in the MCF10A breast cell system**
4. **Myc target genes identified by ChIP-on-chip and expression profiling were prioritized by identifying those genes associated with patient outcome**
5. **Constructed and evaluated stabilized CFP-Myc-T58A fluorescent fusion proteins for interaction with YFP-TRRAP fragments and for FRET measurements.**
6. **Automated transient transfection for expression of Myc and TRRAP fragements (1260, 1690, F1) as fluorescent fusion proteins.**
7. **Optimized cell culture conditions to maximize fluorescence from CFP-Myc.**
8. **Designed and constructed a pulse stretcher for the Opera to prevent burn out of the optical fibers that deliver light to the sample for FRET FLIM measurements.**

Reportable Outcomes

i) Andrews, D.W**.**, Linking genotype and phenotype using automated image analysis of fluorescence micrographs. International Society for Nuclear Medicine Annual Meeting, Toronto, ON, June 13, 2009.(invited presentation)

ii) Collins T.J., Wasylishen, A., Penn l., and Andrews D.W., High-content screening for inhibitors of oncogenic transcription by c-Myc in breast cancer. Annual Meeting of the Terry Fox Foundation Research Initiative, Vancouver, BC, May 22-24, 2009.

iii) Collins T.J., Wasylishen, A., Penn l., and Andrews D.W., High-content screening for inhibitors of oncogenic transcription by c-Myc in breast cancer. Annual Meeting of the Ontario Institute for Cancer Research, Toronto, ON, Feb 22-24, 2009.

iv) Collins T. J. FRET-FLIM measurements in High Content Screening. PerkinElmer User's group meeting at Cambridge Healthtech Institute's 6th Annual High Content Analysis Conference. San Francisco, CA, January 5-9, 2009. (invited presentation).

v) Collins T.J., Wasylishen, A., Penn l., and Andrews D.W., High-content screening for inhibitors of oncogenic transcription by c-Myc in breast cancer CBCRA Reasons for Hope Conference Vancouver, BC, April, 2008.

vi) Penn, L., Wasylishen, A., Ponzielli, R., Bros, C., Boutros, P., and Andrews, D. Understanding and targeting Myc oncoprotein function in breast cancer. CBCRA Reasons for Hope Conference Vancouver,BC, April, 2008.

vii) Collins T.J., Wasylishen, A., Penn l., and Andrews D.W., High-content FRET-FLIM screening for inhibitors of oncogenic transcription by c-Myc in breast cancer, Era of Hope 2008, Baltimore, MD, June 25-28, 2008.

viii) Penn, L., Wasylishen, A., Ponzielli, R., Bros, C., Boutros, P., and Andrews, D. Understanding and targeting Myc oncoprotein function in breast cancer. Era of Hope 2008, Baltimore, MD, June 25-28, 2008.

ix) Andrews, D.W., Non-traditional approaches to high content analysis using live cells, Assays and Cellular Targets, San Diego, CA, October 17-19, 2007. (invited presentation)

x) Andrews, D.W., Novel approaches to high content analysis using live cells, Gulf Coast Consortium for High Content Screening, Houston, TX, January 28, 2008. (invited presentation)

xi) Collins T.J., High-Content Screening of Intracellular Processes by High-Speed FLIM and FRET **High-Content Analysis 2008,** January 14-17, 2009, San Francisco, CA (invited presentation)

xii) Penn, L. "Extreme Makeover: Using Myc to kill tumor cells – exploiting target genes and apoptosis"; SickKids Program in Cell Biology, Toronto, ON, May 2nd, 2008.

xiii) Penn, L., Wasylishen, A., Ponzielli, R., Bros, C., Boutros, P., and Andrews, D. Understanding and targeting Myc oncoprotein function in breast cancer. Medical Biophysics Student Research Day, Toronto, ON, June 23, 2008

xiv) Penn, L. "Basic research is driving a revolution in cancer diagnosis and treatment", OICR Public Lecture, MaRS Centre, Toronto, ON, June 10, 2008

xv) Penn, L. "Molecular Revolution in Cancer Diagnosis and Treatment", St. Andrew's Club, Canadian Cancer Society, November 2, 2007.

Conclusions

In conclusion, we completed all year 1 goals during the first year of funding and in the second year we made significant progress towards accomplishing the original objectives of this grant. Specifically, in the first year we constructed and evaluated several fluorescent fusion proteins of Myc and TRRAP. We have shown that certain TRRAP fragments (e.g. 1260) interact with Myc and show FRET in vivo. To identify the target genes directly regulated by Myc and TRRAP in breast epithelial cells, we have conducted Myc-specific ChIP-on-chip in the MCF10A breast cell system under non-transformed and transformed conditions, and are presently conducting TRRAP-specific ChIP-on-chip under conditions to distinguish Myc-specific recruitment of TRRAP to specific target genes thought to be critical to breast cancer. Because the cells we were using would not tolerate long-term expression of the fusion proteins, in the second year we screened additional cell lines and evaluated both constitutive and inducible expression systems for the fluorescent fusion proteins. We automated transient transfection conditions and optimized culture conditions by inhibiting the proteasome to increase fluorescence from CFP-Myc. During screen validation we identified an engineering shortcoming in the design of the FLIM Opera high content screening instrument. The Opera high content screening instrumentation was evaluated and a new pulse stretcher designed, constructed and tested. The pulse stretcher has recently been added to the instrument. FRET-FLIM was shown to be a viable approach for chemical library screens for the highly challenging objective of screening for inhibitors of Myc and TRRAP.

References

1. Oster SK, Ho CS, Soucie EL, Penn LZ. The myc oncogene: MarvelouslY Complex. Adv Cancer Res. 2002;84:81-154.

2. Ponzielli R, Katz S, Barsyte-Lovejoy D, Penn LZ. Cancer therapeutics: targeting the dark side of Myc. Eur J Cancer. 2005 Nov;41(16):2485-501.

3. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, Brink R, Leverkus M, Tergaonkar V, Schneider P, Callus BA, Koentgen F, Vaux DL, Silke J. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell. 2007 Nov 16;131(4):682-93.

Appendices

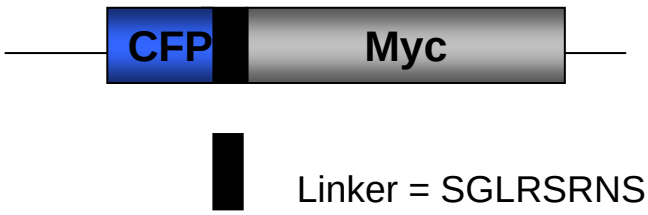
N/A

Supporting Data:

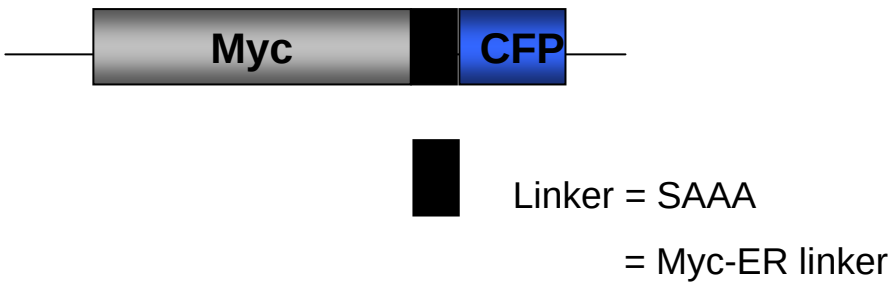
Figures 1-20

Figure 1

CFP-Linker-Myc



Myc-Linker-CFP



Notes:
-Myc has an internal nuclear localization signal (NLS)
-These constructs are not flag-tagged

Figure 1: Cerulean-Myc constructs generated
Schematic representation of Cerulean-Myc (CFP-Myc) fusion proteins currently under evaluation. These constructs have been generated in both transient and stable expression vectors (see Figure 8B for details).

Figure 2

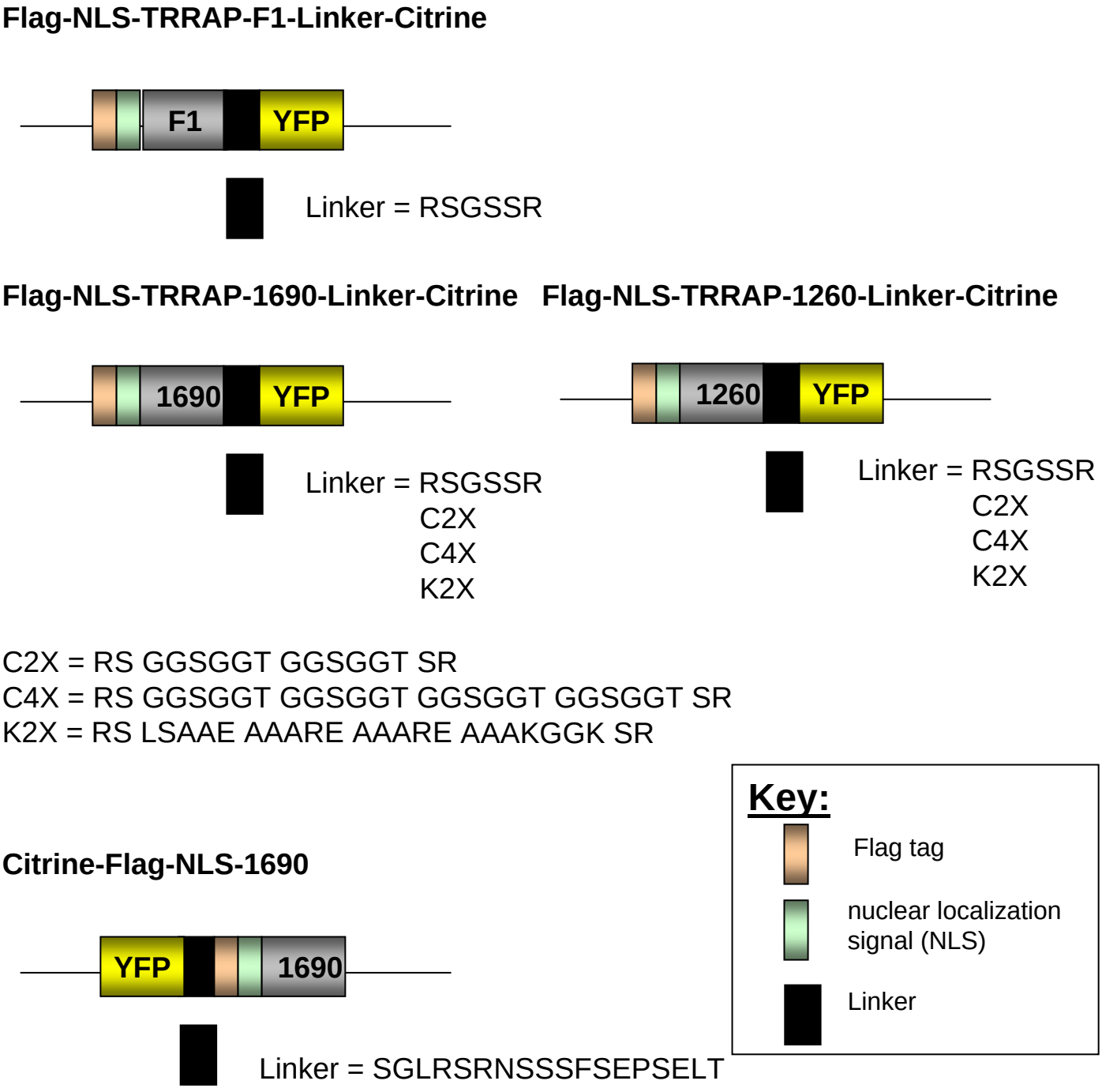


Figure 2: TRRAP-YFP constructs generated
Schematic representation of TRRAP-Citrine (TRRAP-YFP) fusion proteins under evaluation. Three different regions of TRRAP have been used in these constructs (F1, 1690, 1260). These constructs have been generated in transient expression vectors.

Figure 3

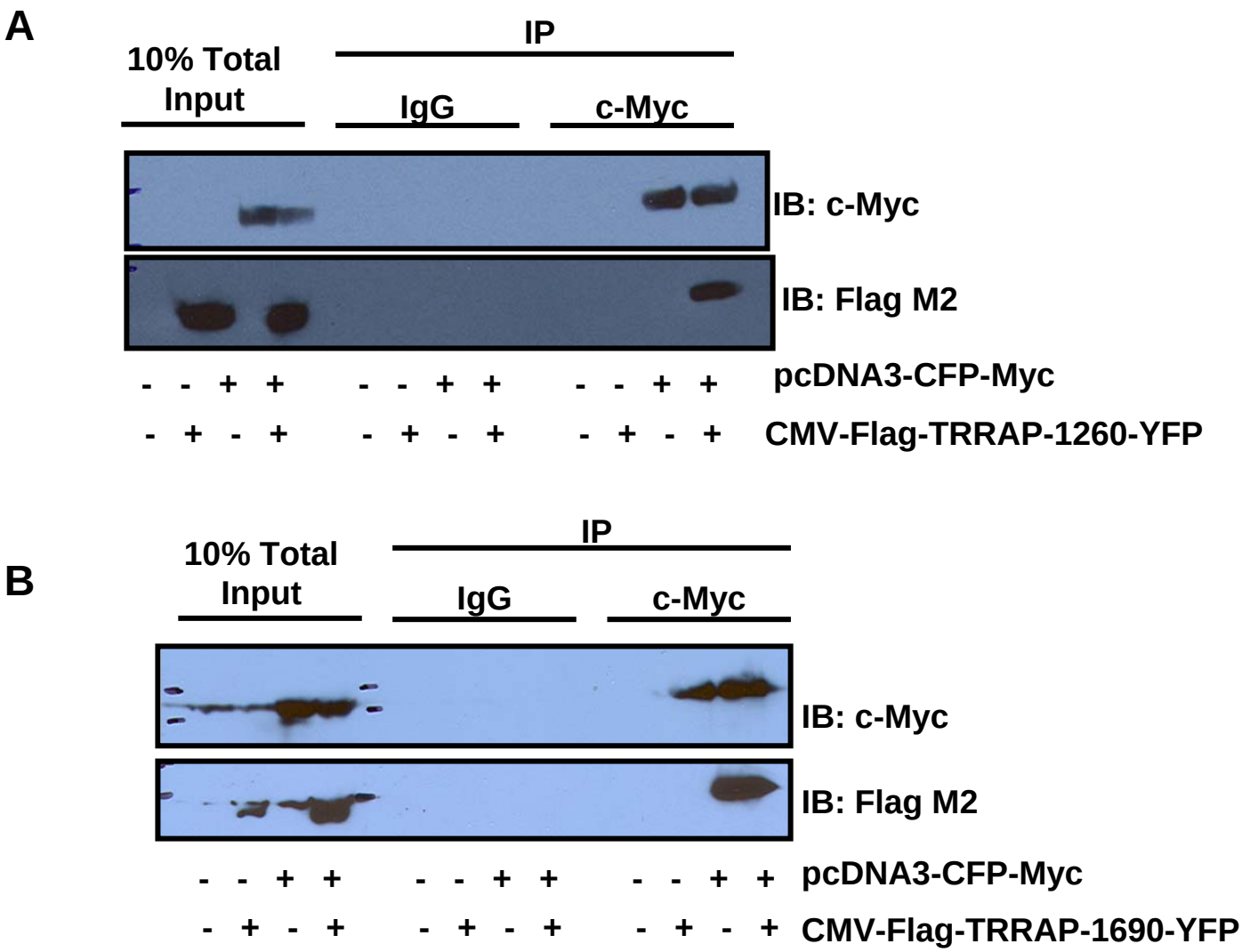
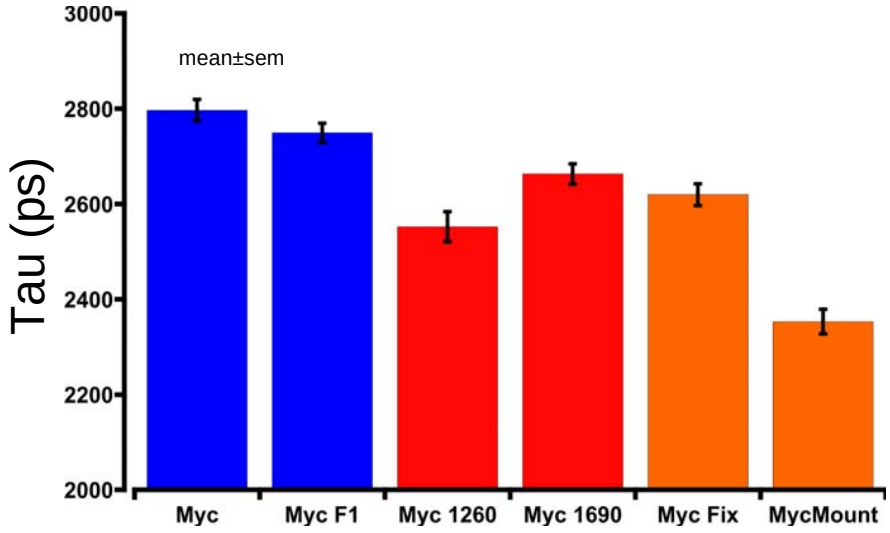


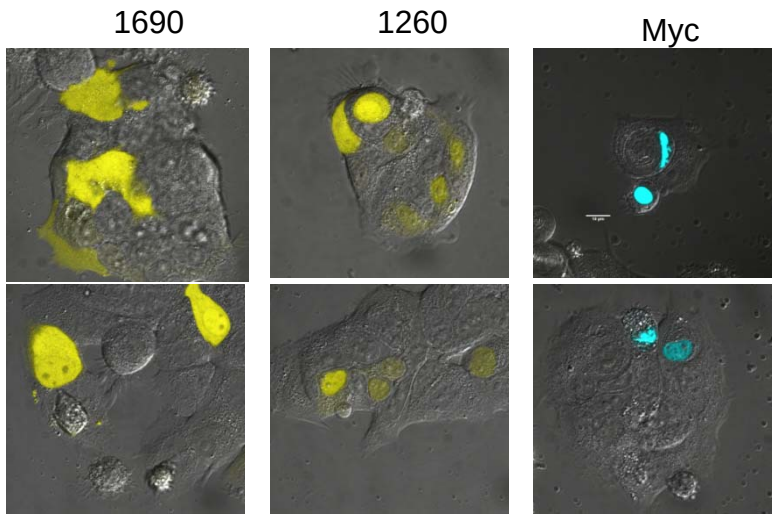
Figure 3: Characterization of fluorescent fusion proteins

A) The CFP-Myc and 1260-YFP fusion proteins interact in vivo. 293TV cells were co-transfected with vectors containing these fusions. Lysates were then immunoprecipitated with control or c-Myc antibodies, resolved using SDS-PAGE and immunoblotted with anti-Flag and anti-Myc antibodies. B) The CFP:Myc and 1690:YFP fusion proteins interact in vivo. 293TV cells were co-transfected with vectors containing these fusions. Lysates were then immunoprecipitated with control or c-Myc antibodies, resolved using SDS-PAGE and immunoblotted with anti-Flag and anti-Myc antibodies.

The TRRAP constructs used in these experiments are those that contain the RSGSSR linker.

Figure 4





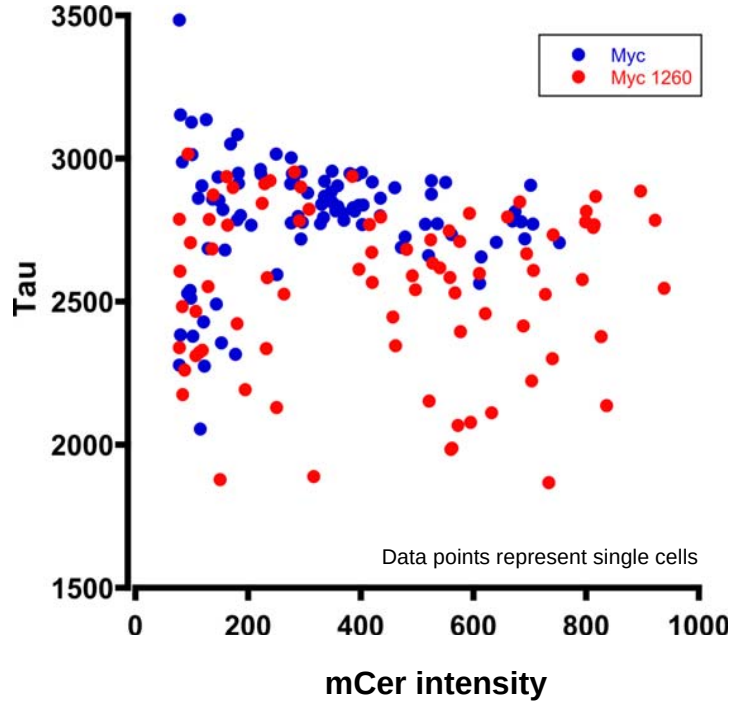
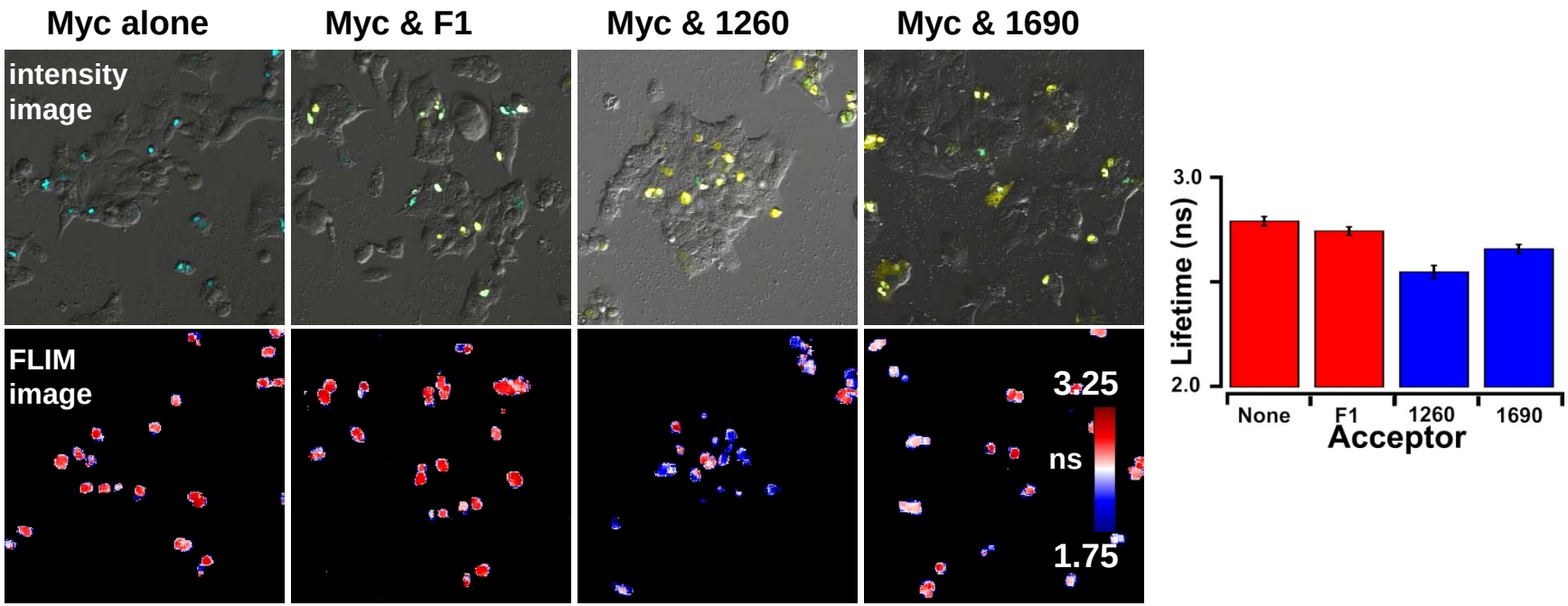


Figure 4: Evaluation of FRET-Pairs

The histograms demonstrate that the average lifetime (Tau) for the Myc-Cerulean construct drops from 2.8 ns to 2.5 ns (for Myc/TRRAP1260). This change in lifetime is sufficient for screening. However, the orange bars show that both fixation and mounting media decrease the lifetime of Myc-CFP unacceptably. The scatter in the data (graph above) shows that there is too much noise in low expressing cells therefore a stable cell line is required. The micrographs show that cells expressing the constructs are sick making it difficult to create a stable cell line. Thus, we are generating stable cell lines with and inducible YFP-TRRAP fluorescent fusion proteins.

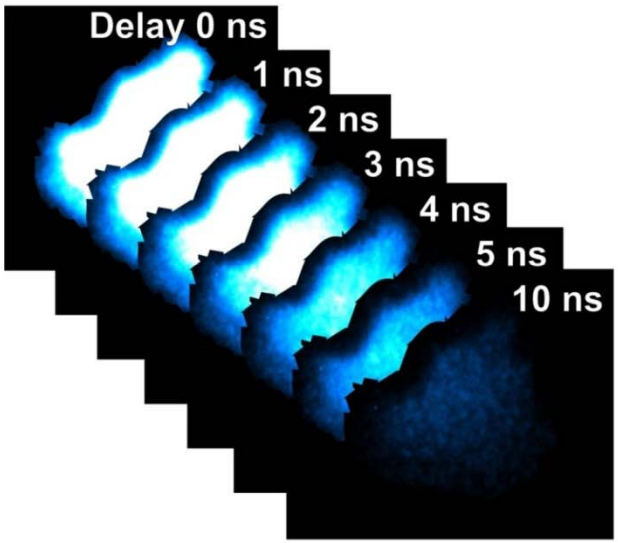
Figure 5



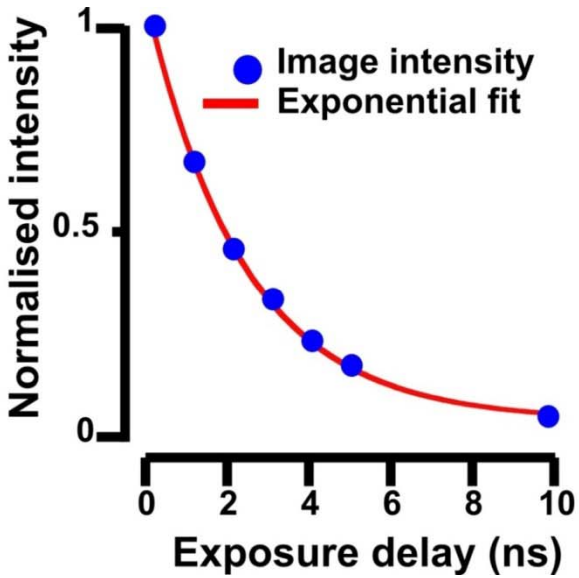
Fragment	Mean lifetime of mCer-Myc (ns ± sem)
Alone (neg ctrl)	2.80 ± 0.02
F1	2.75 ± 0.02
1260	2.55 ± 0.03
1690	2.66 ± 0.02

Figure 5: Evaluation of optimal FRET pairs for FLIM in live cells
Initial tests show that YFP-TRRAP fragments (yellow) will bind to CFP-Myc (cerulean) in live cells as shown by FRET between the CFP-MYC and YFP-1260 but not with the negative controls (Myc alone (None) and F1). YFP-1690 shows intermediate results to date and requires further optimization. The change in lifetime is sufficient for screening (histogram and table) and there is little variability in the data when using live cells. However, these cells also do not tolerate stable expression of the constructs well enough for high throughput screening. Trouble-shooting and optimization of this system was the focus on the second year of funding.

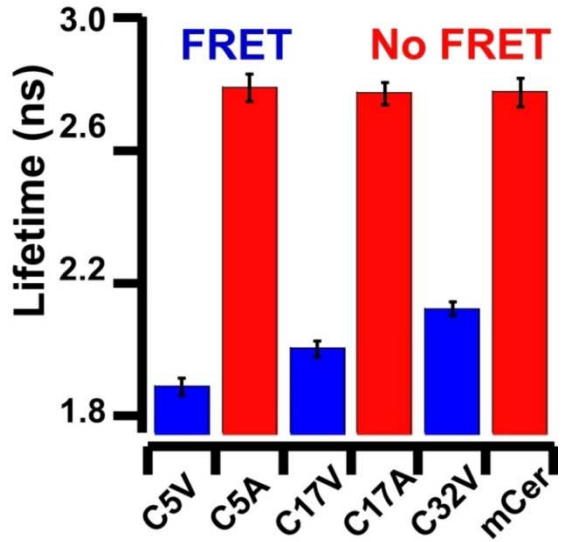
Figure 6



High speed data acquisition from 7 points provides using the Opera HCS instrument.



High speed data acquisition from 7 points provides an accurate estimation of FLIM. We have extended this to two measurements with custom gates and the resulting FLIM estimates are acceptable and can be acquired fast enough for screening (0.5 seconds).



C5V indicates CFP-5 residue spacer-YFP, C5A indicates CFP with the 5 residue spacer alone (negative control). Data for 5, 17 and 32 residue spacers are shown. Thus, nature and length of spacer is an important parameter to optimize to achieve maximal FRET. Importantly, the Opera HSC instrument can detect small changes FRET, showing that this approach can be used for the screen.

Figure 6: Optimization of FRET-FLIM detection using the Opera HCS instrument
Measurements of standards with different length spacers (5, 17, 32 residues) between CFP and YFP fluorescence proteins show that the Opera HCS instrument high speed FLIM acquisition system can be used to measure FLIM accurately at high speed. This is the first demonstration of automated FLIM with sufficient speed for screening.

Figure 7

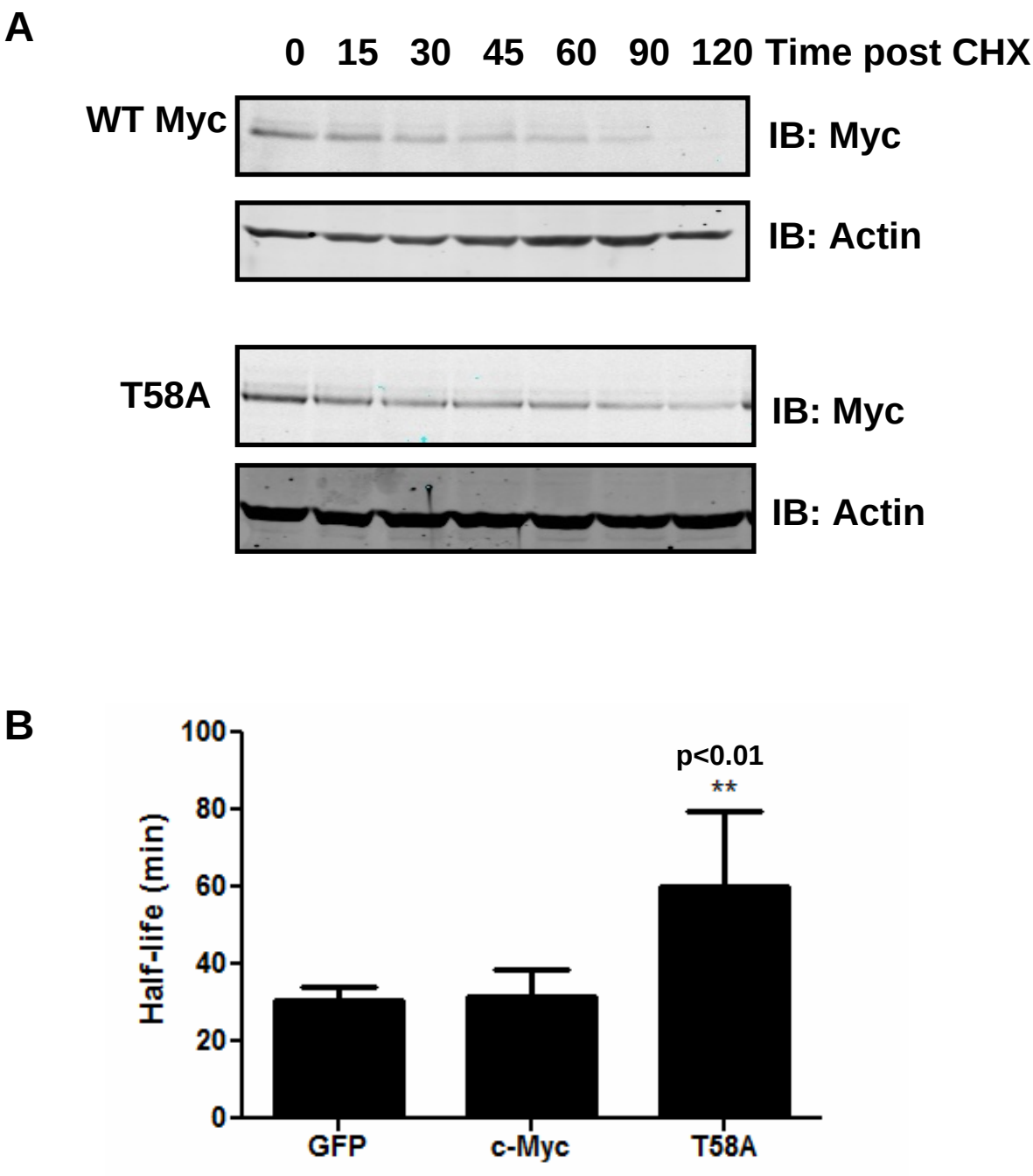


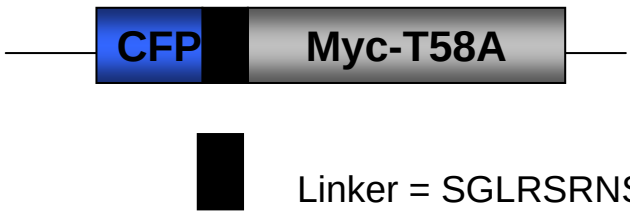
Figure 7: The T58A point mutation increases Myc protein stability

A)Cyclohexmide half-life experiments were performed in MCF10A cells expressing control GFP vector, wild-type Myc, or Myc-T58A. Cells were treated with cycloheximide and whole cell extracts prepared at the indicated time points. Lysates were resolved using SDS-PAGE, and immunoblotted for Myc and actin as a loading control. Western blots were scanned using an Odyssey scanner (LiCOR). B) Western blots were quantified using ImageJ software (NIH), and Myc half-life was determined. This experiment was conducted 3-7 times, *Columns*, mean; *error bars*, SD. **, p,0.01, Student's t-test.

Figure 8

A

CFP-Linker-Myc-T58A



Linker = SGLRSRNS

Myc-T58A-Linker-CFP

Linker = SAAA

= Myc-ER linker

B

		pcDNA3.1	pBabe-puro	pWZL-blast	pSelect-blast	pBabe-neo
Group I fusions wild-type Myc	**CFP-Myc**	√	√	√	√	√
	CFP:Myc (ER linker)	√		√		
	Myc:CFP	√	√	√	√	
Group II fusions Myc-T58A	**CFP:Myc-T58A**	√		√	√	
	CFP:Myc-T58A (ER linker)	√		√		
	Myc-T58A:CFP	√		√	√	

Figure 8: Myc-CFP constructs generated with stabilizing T58A point mutation
A) Schematic representation of Cerulean-Myc (CFP-Myc) fusion proteins containing T58A point mutation currently under evaluation. B) Summary table of all CFP:Myc fusions generated with vectors indicated.

Figure 9

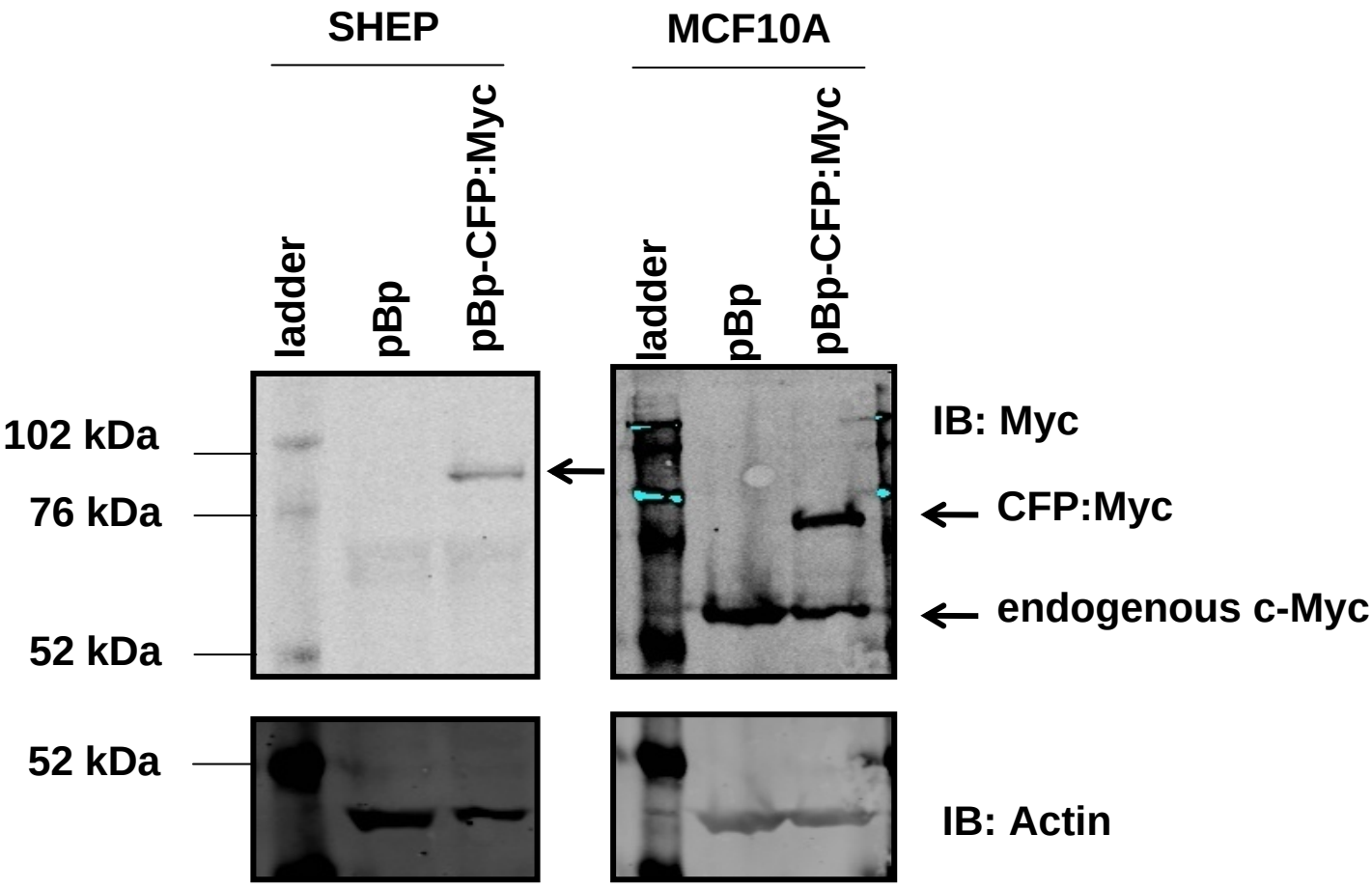


Figure 9: Stable expression of CFP-Myc fusions in SHEP and MCF10A cells
Cells were infected with pBabe-puro-CFP-Myc, and selected in puromycin. Whole cell extracts were prepared, resolved using SDS-PAGE, and immunoblotted to detect the CFP:Myc fusion protein. Both CFP:Myc and endogenous Myc are indicated with arrows.

Figure 10

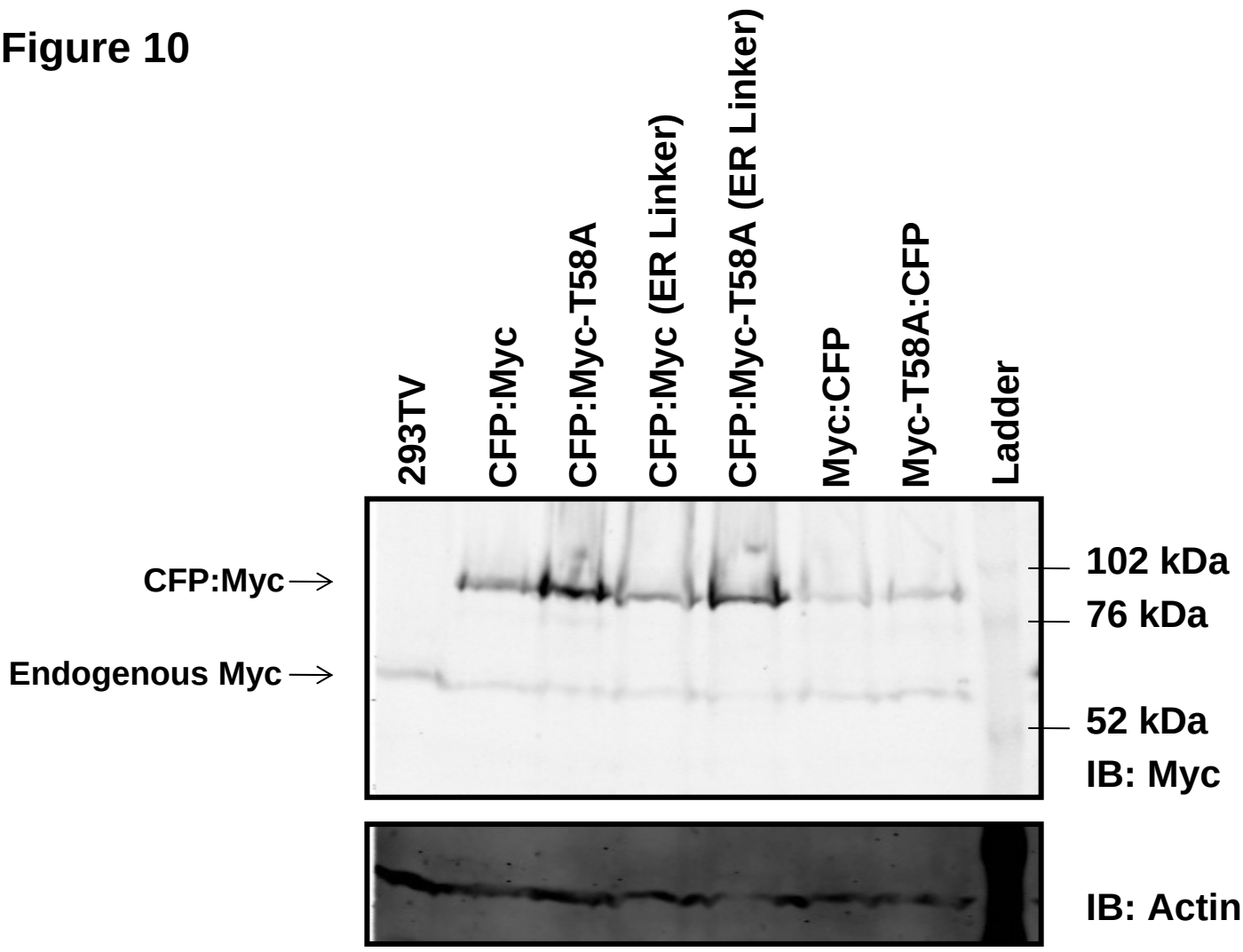
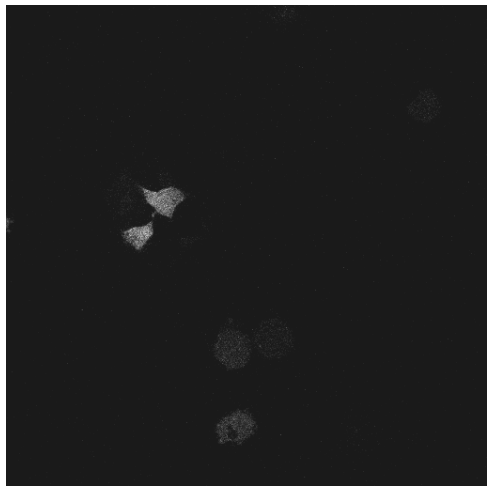


Figure 10: Stable expression of CFP-Myc fusions in 293TV cells
293TV cells were infected with pWZL-blast-Myc-CFP constructs. Cells were selected in blasticidin, and whole cell lysates prepared. Lysates were resolved using SDS-PAGE and immunoblotted for Myc. Both endogenous Myc and Myc-CFP were detected and are indicated with arrows.

Figure 11

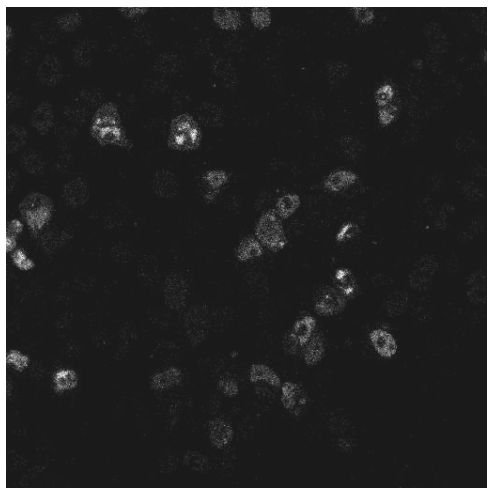
A

37˚C



B

4h at 25˚C



C

4h with MG132
Proteasome
Inhibitor at 37˚C

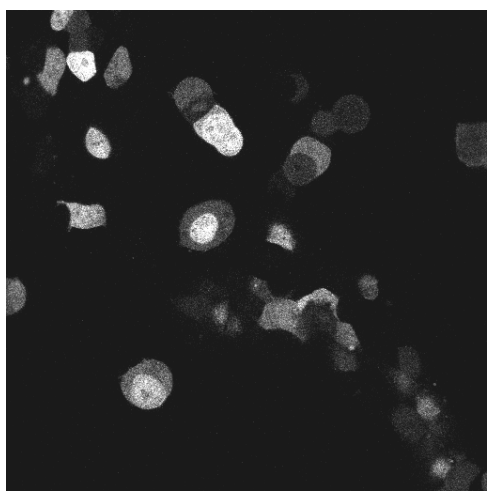


Figure 11:Optimized cell culture conditions to stabilize CFP-Myc in 293TV cells
293TV cells stably expressing pWZL-blast-Myc-T58A-CFP were evaluated for CFP fluorescence under several cell culture conditions. A) Cells were cultured normally, at 37ºC, and exhibit modest fluorescence. B) Cells were cultured for 4 hours at 25ºC and resulted in increased CFP fluorescence. C) Cells were cultured for 4 hours in the presence of proteosome inhibitor, MG132, also increased CFP fluorescence.

Figure 12

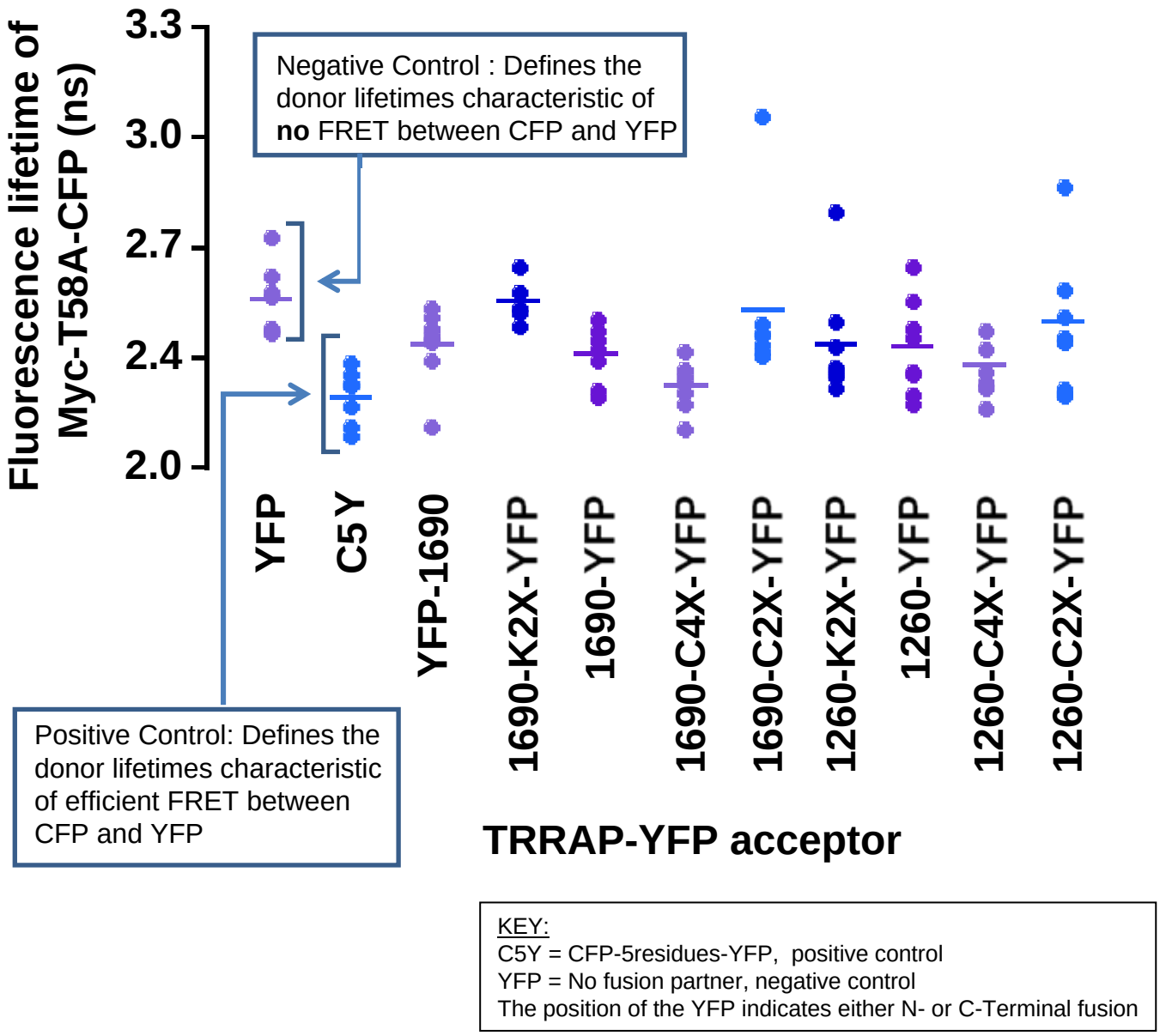


Figure 12: Evaluation of FRET between Myc-T58A-CFP and TRRAP-YFP fusions
293TV cells were transfected with pcDNA3.1-Myc-T58A-CFP and our panel of TRRAP-YFP fusions. The fluorescence lifetime of CFP was measured and used to evaluate interaction between Myc and TRRAP.

Representative data from a single experiment is presented here. Each data point represents the mean fluorescence lifetime from an individual well within the experiment, with at least 6 wells evaluated for each pair of constructs.

Figure 13

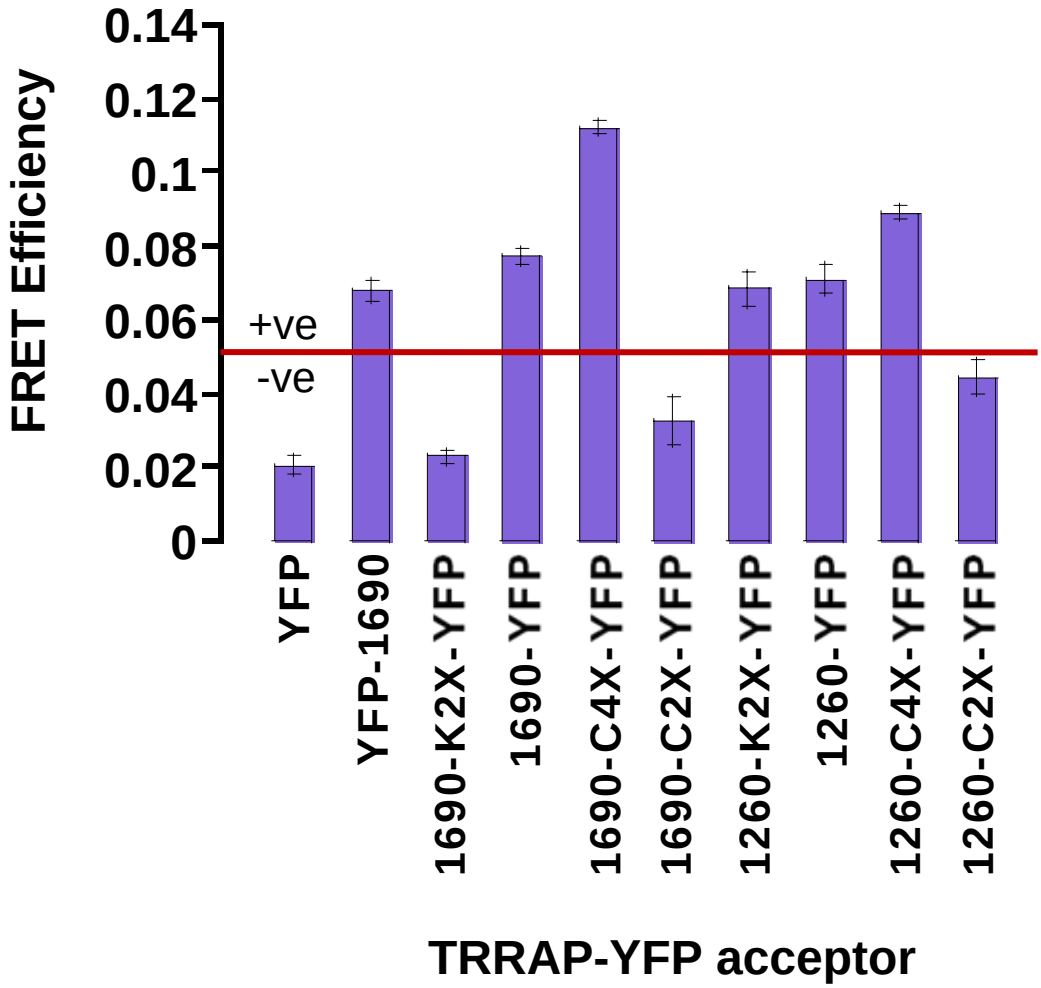


Figure 13: Evaluation of FRET between Myc-T58A-CFP and TRRAP-YFP fusions
293TV cells were transfected with pcDNA3.1-Myc-T58A-CFP and our panel of TRRAP-YFP fusions. The FRET efficiency was measured and used to evaluate interaction between Myc and TRRAP across several independent experiments. *Columns*, mean; *error bars*, SEM for three biological replicates.

From these experiments, it was determined that Myc-T58A-CFP & 1690-C4X-YFP, as well as Myc-T58A-CFP & 1260-C4X-YFP are suitable FRET pairs.

Figure 14

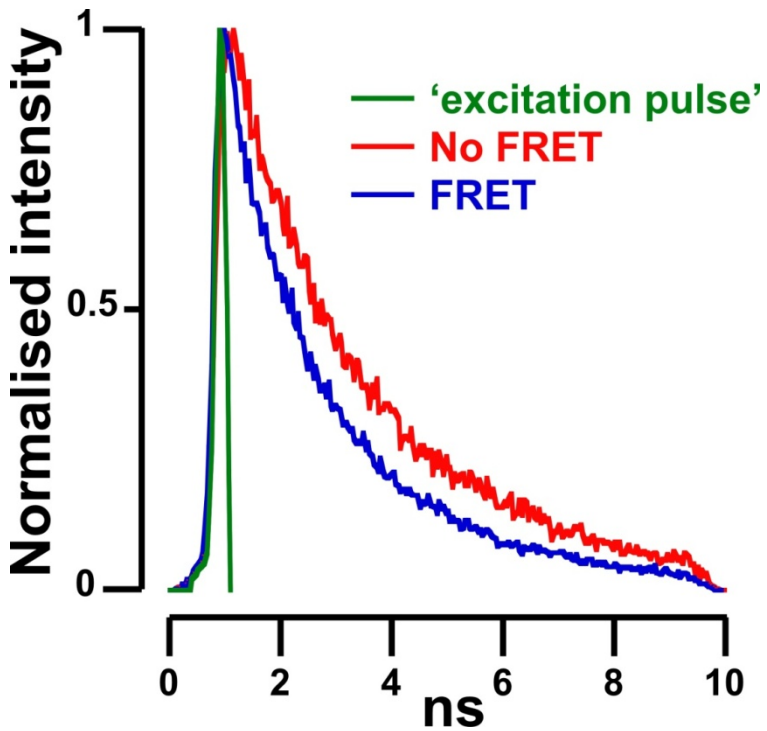


Figure 14: Sample decay curve for measuring FRET between CFP and YFP accurately
FLIM generates images of the cell where the intensity of each pixel corresponds to the average lifetime of the fluorescence protein at that location. Accurate decay curves have been generated for FRET between Cerulean (CFP) and Citrine (YFP) fluorescence proteins.

FRET results in a shortening of the lifetime of the donor fluorophore. The reduction in lifetime is proportional to the amount of FRET as measured by the 'FRET-efficiency'.

Figure 15

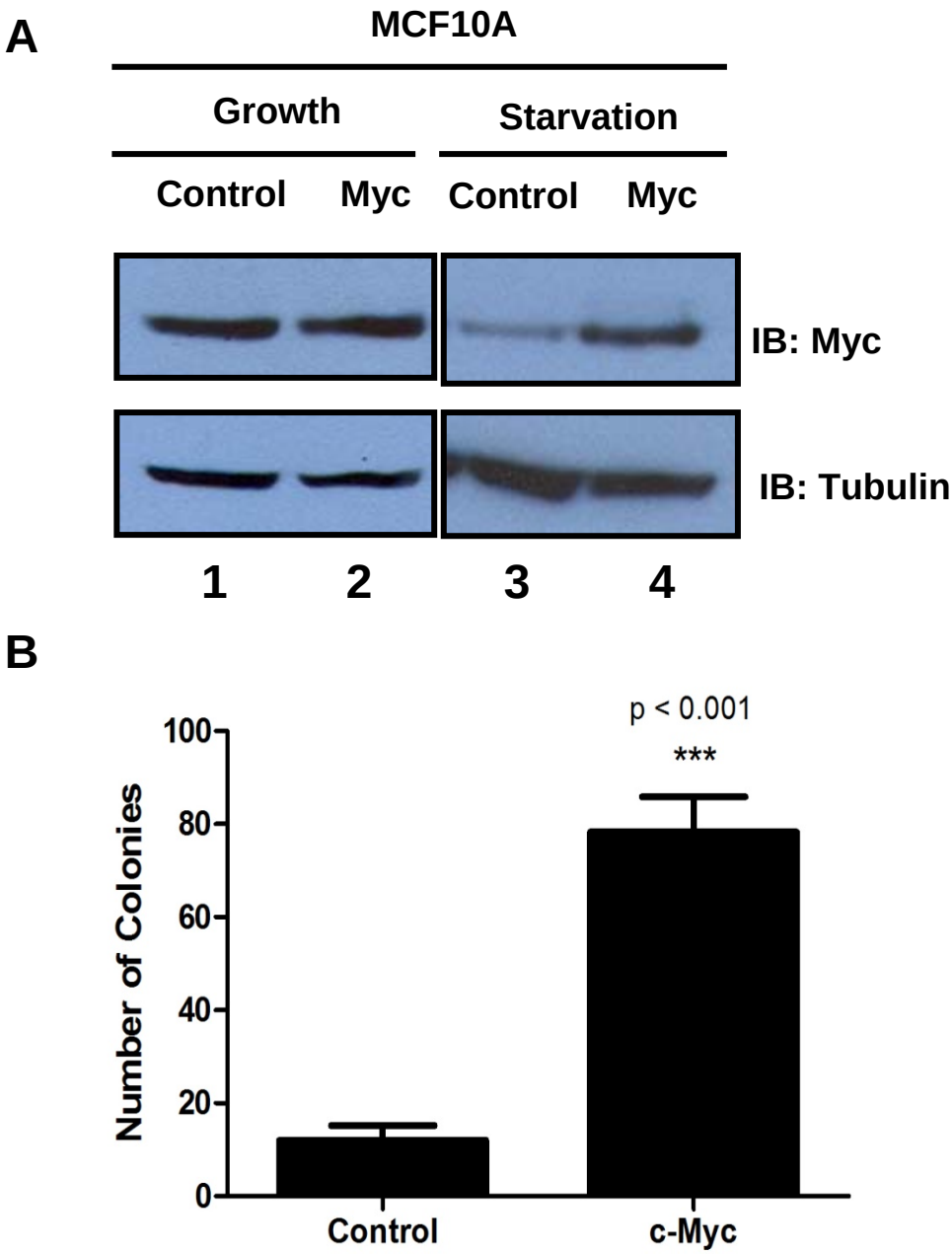


Figure 15: Identification of MCF10As as a model system for Myc-dependent transformation

A) Ectopic expression of Myc in MCF10A cells does not lead to higher levels of protein expression in asynchronously growing MCF10A cells, when measured by western blotting, compare lanes 1 and 2. When growth factors are removed from the media for one hour, cells expressing empty vector exhibit a decrease in endogenous Myc protein, while the level of deregulated Myc in ectopically expressing cells remains high, compare lanes 3 and 4. B) Anchorage-independent growth of MCF10A cells is increased with ectopic expression of wild-type Myc, as measured by colony formation in soft agar. This experiment was conducted three independent times. *Columns*, mean; *error bars*, SD. ***, p,0.001, Student's t-test.

Figure 16

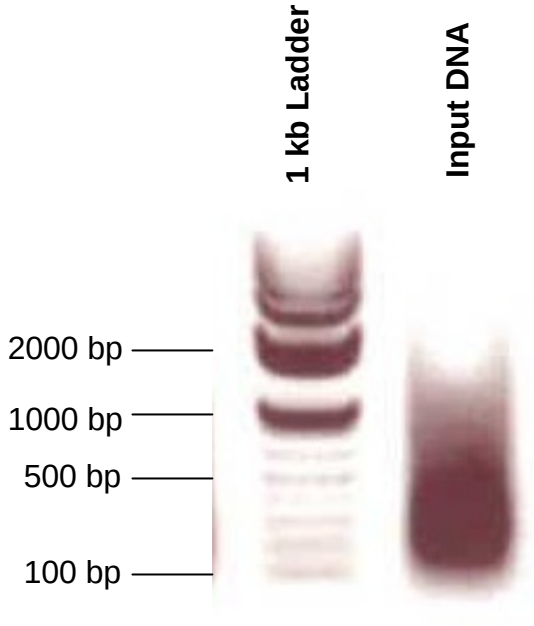


Figure 16: Optimized sonication of MCF10A cells
Nuclear pellets are resuspended in nuclei lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors) at a concentration of 100 million cells per 1 mL of buffer. The samples are sonicated with the Bioruptor (Diagenode) at high setting for eleven 30 second pulses, with 30 second rests between pulses. A sample of input DNA was run on a 1% agarose gel to evaluate the size of the genomic DNA fragments, and indicate DNA fragments of desired length (100-1000 base pairs).

Figure 17

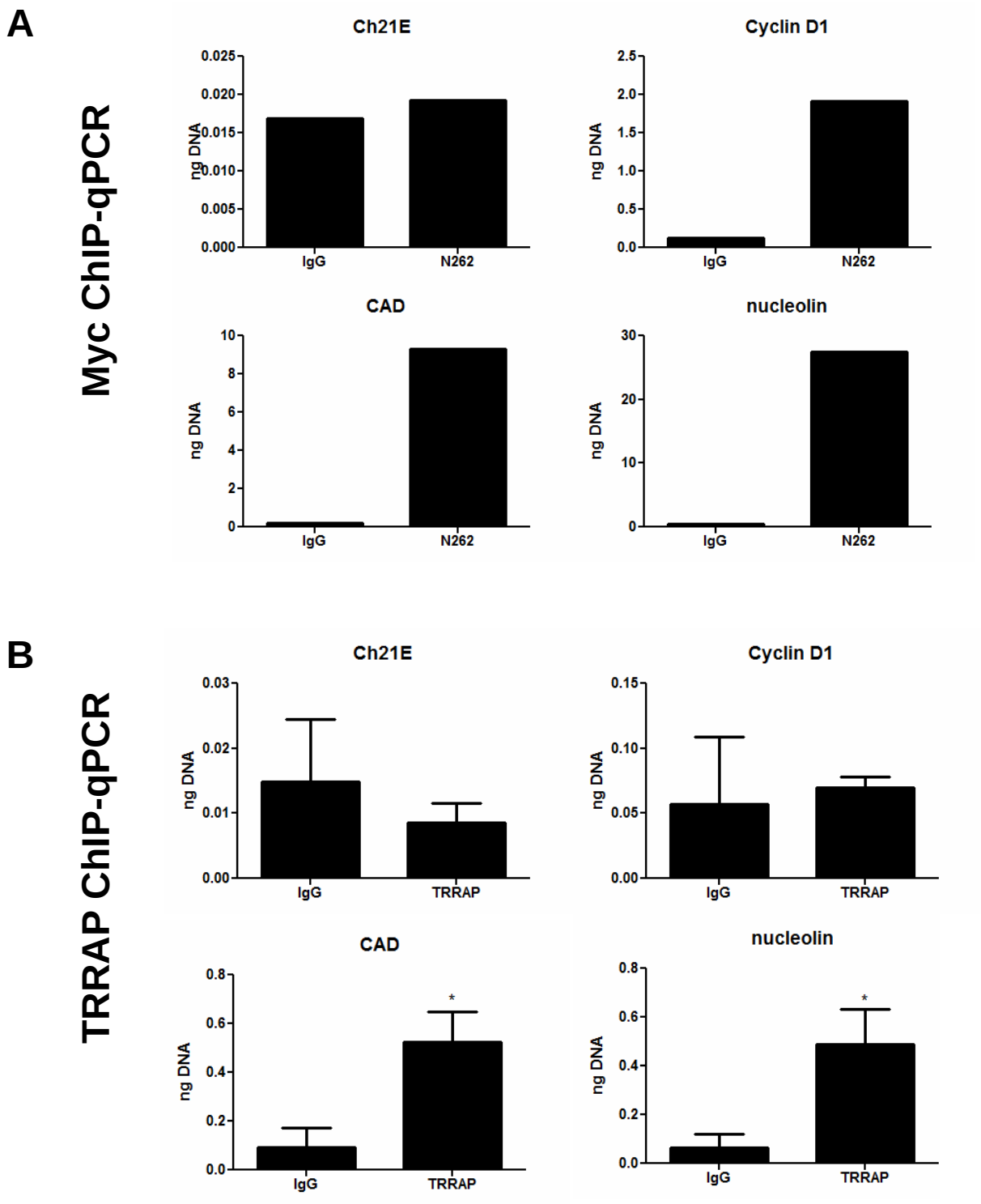


Figure 17 :Myc and TRRAP ChIP in MCF10A cells

We have optimized ChIP conditions for Myc and TRRAP in MCF10A cells. This has been confirmed using qPCR for positive control targets (e.g. CAD) and negative control genes (e.g. Chromosome 21 E-box (Ch21E)). Through this we have also identified targets bound by Myc, but not TRRAP (e.g. Cyclin D1), as well as targets common to both Myc and TRRAP (e.g. nucleolin).

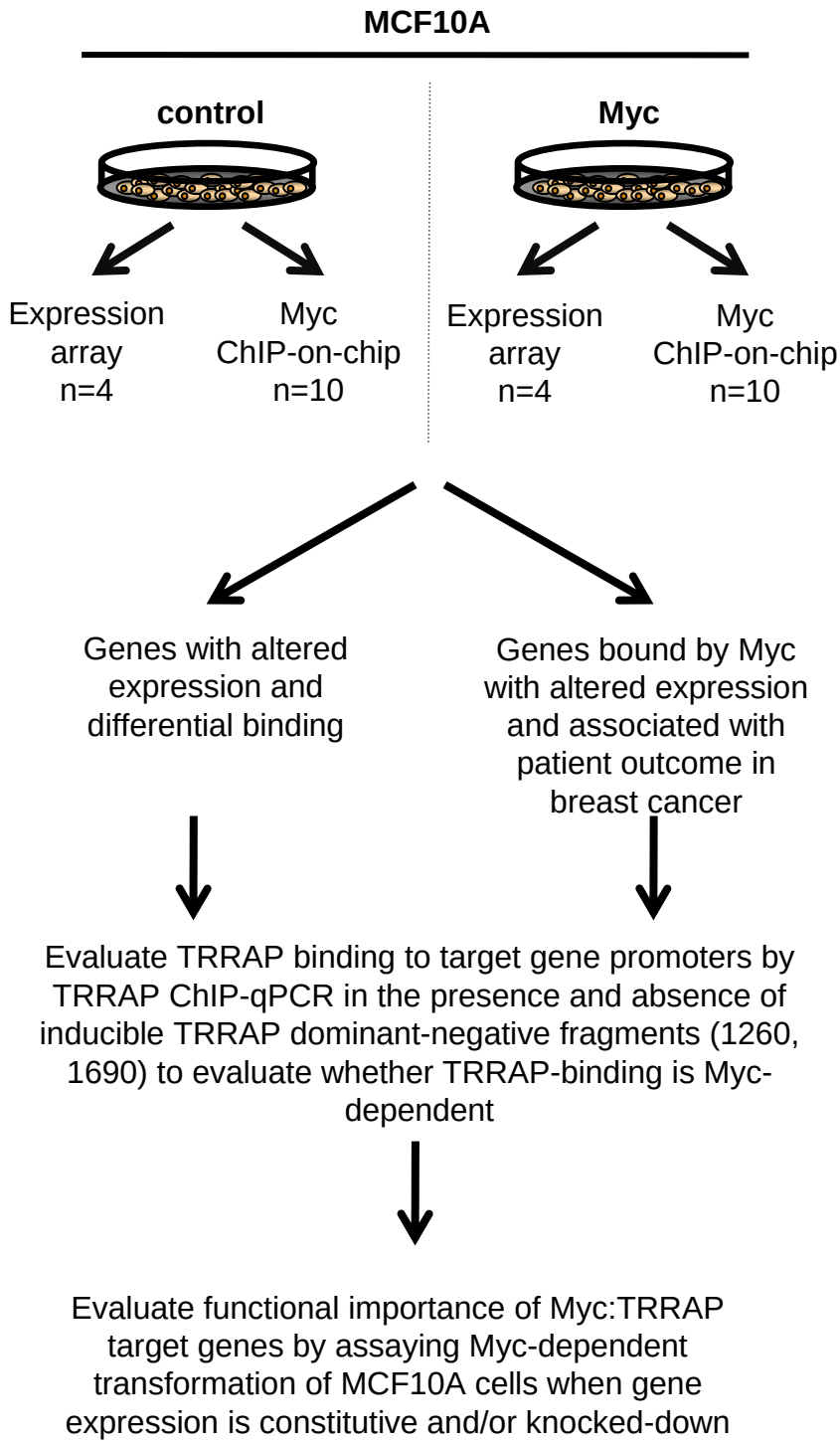
Figure 18: Identifying Myc-TRRAP target genes in the MCF-10A cells that undergo Myc-dependent transformation

1. Myc ChIP-on-chip and expression array analysis of MCF10A cells - complete

2. Data analysis - complete

3. Identify Myc:TRRAP target genes - ongoing

4. Evaluate functional role of Myc:TRRAP target genes to Myc-dependent transformation



To complement the strategy outlined above, we will also conduct TRRAP-specific Chip-on-chip in the presence and absense of Myc:TRRAP inducible dominant negatives TRRAP fragments (1260 and 1690) to profile TRRAP targets that are dependent on Myc for recruitment to chromatin and potential causal to breast cancer

Figure 19

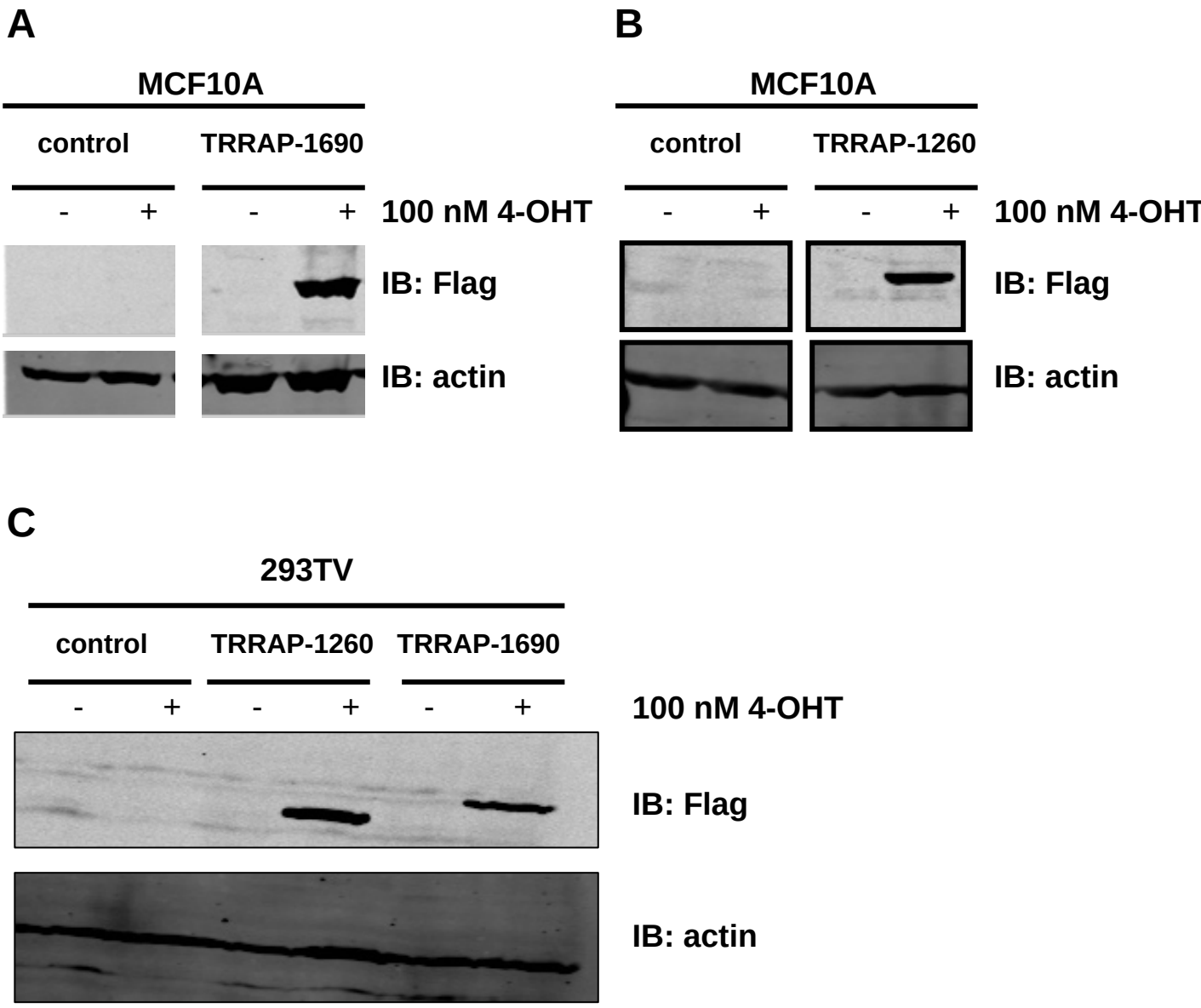
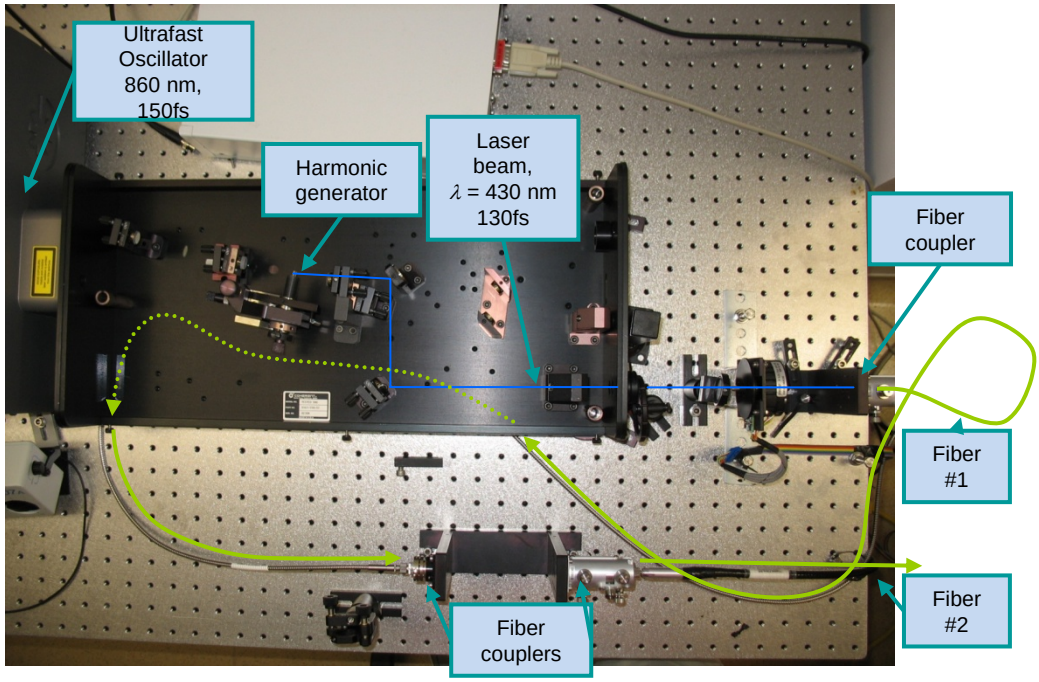


Figure 19: Inducible expression of TRRAP-1690 and TRRAP-1260 in MCF10A and 293TV cells

Cells were infected with the inducible lentivral system containing TRRAP-1260, TRRAP-1690, or empty vector control. Cells were selected in hygromycin and puromycin and cell pools were induced with 100 nM 4-OHT or ethanol control for 24 hours. Cell lysates were prepared and resolved using SDS-Page. TRRAP fragments were detected by immunoblotted with Flag antibody.

Figure 20

Before modification



After modification

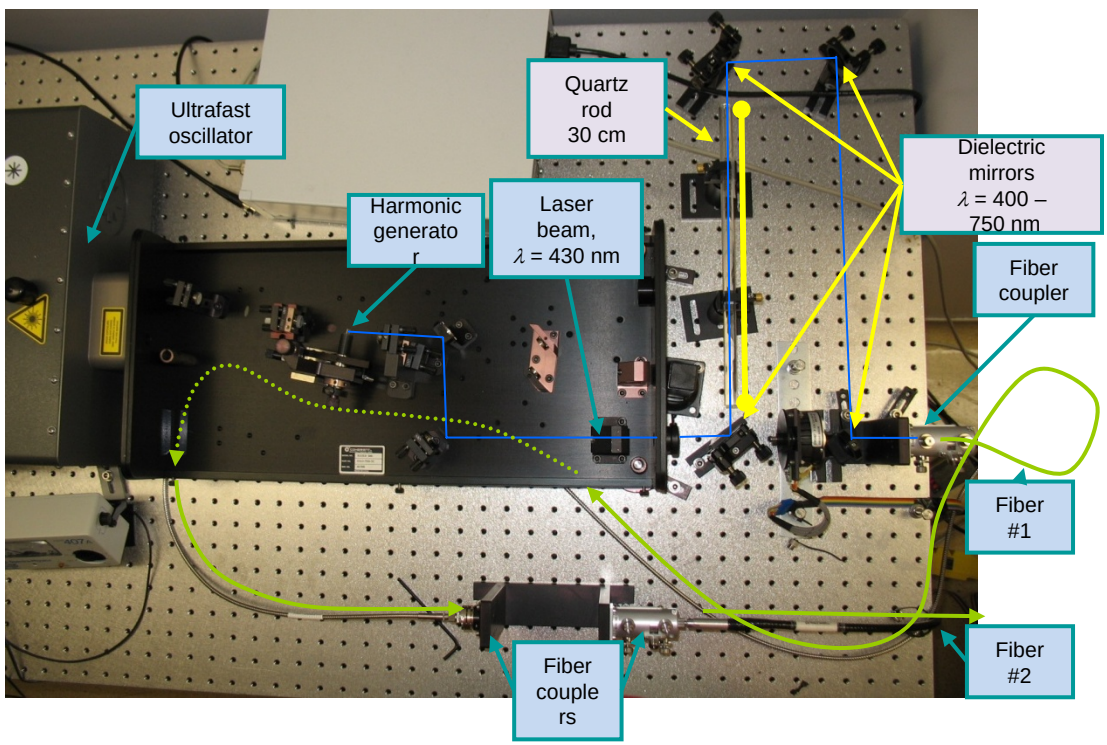


Figure 20: Addition of pulse stretcher to FLIM system