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14. 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. In the first year of this grant we have constructed several fluorescent fusion protein constructs of Myc and TRRAP, and evaluated their ability to bind and engage in fluorescence resonance energy transfer (FRET) in vivo. We have identified FRET pairs that are functional and established methodology using novel instrumentation that will enable the high throughput screening of chemical libraries. In the course of our work, we have shown that the cell systems we were aiming to use are unfortunately sensitive to expression of the fusion proteins. To overcome this unexpected issue, we are evaluating additional cell systems, as well as new expression constructs that will enable stable cell lines to be developed that constitutively and conditionally express the Myc and TRRAP fusion proteins, respectively. We have completed all tasks that we aimed to achieve in the first year of the grant and we are well positioned to fulfill the objectives of our proposal by the end of the second year of this grant.

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

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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 has progressed in a steady and productive manner as expected in the first year of funding. 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.

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 for transient expression of fusion proteins in 293TV cells.* (*Months 1-3 for the first set of constructs.)*
Complete: We have generated a panel of constructs 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 to date is provided in Figures 1 and 2. Fusions have been generated with the fluorescent protein both 5' and 3' to Myc and TRRAP. Additionally, we have 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.

Pairs of these fusions have been 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.)*
Nearly Complete: We have evaluated a series of acceptor donor pairs from a) for FRET-FLIM signal and identified a suitable pair (Figure 7). Further pairs are being examined in different cell lines. As shown in Figure 7 artifacts introduced by fixation required us to perform all of the optimization in live cells. While this has slowed our progress slightly the results obtained so far are excellent.

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)*
Complete: Please see 1a above and Figures 1 to 3.

d) *Penn/Andrews: The optimal donor:test pair will be introduced into the Tet-inducible MCF-7 and MDA-MB-231 cell lines. (Months 8-12)*
Nearly Complete: An optimal donor:test pair was identified by transient transfection in MCF-7 cells (Figure 8). This pair (CFP-Myc and YFP-1260) gives a sufficiently large change in FLIM signal to use for screening. Therefore, we have begun making a cell line expressing these proteins to use for screening. Unfortunately the fragments that gave the best FLIM data were toxic when expressed in MCF-7 and several other cell lines. We used a c-myc null cell line and did not observe toxicity but expression of the constructs was not stable. We are currently changing the promoter and selectable marker on the plasmid to resolve these expression problems.

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 1.d 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).
Complete: Accurate decay curves have been generated for CFP-Myc (Figure 8) and compared to CFP-C3-YFP controls (Figures 9 and 10). Experiments using fluorescence fluctuation analysis have not been started because stable cell lines expressing the fluorescence proteins have not yet been generated. Optimization of FRET-FLIM detection using the Opera HCS instrument has been completed (Figure 10).

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).* Yet to do.

Task 2: Determine the transcriptional signatures of Myc:TRRAP target genes (months 9-14)

Complete: 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 4B). 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 4A, 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 4A, compare lanes 3 and 4).

a) *Penn: Optimize sonication conditions for these cell types to achieve genomic DNA fragments <500 bp in length. (month 9)*
Complete: We have optimized the sonication conditions for MCF10A cells, such that we are able to achieve genomic DNA fragments <500 base pairs in length (Figure 5). 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.

b) *Penn: Conduct ChIP. (months 9-11)*
Complete: We have optimized ChIP conditions for Myc and TRRAP in MCF10A cells. This has been confirmed using Q-PCR for positive control target genes (e.g CAD) and negative control genes (e.g. chromosome 21 E-box) (Figure 6).

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 have recently completed ChIP-on-chip experiments for Myc in our MCF10A cell system, both in cells expressing vector control and expressing ectopic Myc. Five biological replicates were completed for each cell line, and results are currently being analyzed. Biological replicates for TRRAP are currently being prepared. 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.

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

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($_{F8}$) as a dominant negative reagent that disrupts Myc:TRRAP interaction at the level of chromatin (months 12-14).*
In progress. 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) have been 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. We are currently screening these clones for their inducibility. As an alternative approach, we will use an inducible lentiviral system recently described (3). Once we have identified positive clones and optimized induction conditions, we will then assay the ability of the induced fragments to interfere with the c-Myc:TRRAP interaction through Co-IP experiments. Following this, we will conduct ChIP experiments to validate Myc:TRRAP co-bound targets in the presence of these fragments.

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 8-14).*
In progress: Validation of the screening assay is underway. Figure 8 shows the mean and standard deviations for the FLIM measurements. The error bars are sufficiently small that a very good z-factor can be anticipated. We are also evaluating other ways to evaluate the FLIM data at high speed for screening (number of pixels with lifetime above a threshold, etc.). Final validation cannot be performed until a stable cell line is ready (see 1d and 2e).

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 experiments are underway for Myc and TRRAP in the MCF10A breast cell system

Reportable Outcomes

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

ii) 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, April, 2008.

iii) Collins T.J., Wasylishen, A., Penn I., 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, June 25-28, 2008.

iv) 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, June 25-28, 2008.

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

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

vii) 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)

viii) 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.

ix) 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, June 23, 2008

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

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

Conclusion

In conclusion, we have made significant progress during our first year of funding and we are well on our way to accomplishing the original objectives of this grant. Specifically, in the first year we have 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. Because the cells we were using will not tolerate long-term expression of the fusion proteins, we are screening additional cell lines and evaluating both constitutive and inducible expression systems for the fluorescent fusion proteins. To identify the target genes directly regulated by Myc and TRRAP in breast epithelial cells, we have established conditions for ChIP-on-chip in the MCF10A breast cell system under non-transformed and transformed conditions, and are presently conducting these experiments and their analyses. The Opera instrumentation has been evaluated and shown to be a viable approach for the chemical library screens that we will complete in year 2 of this grant.

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

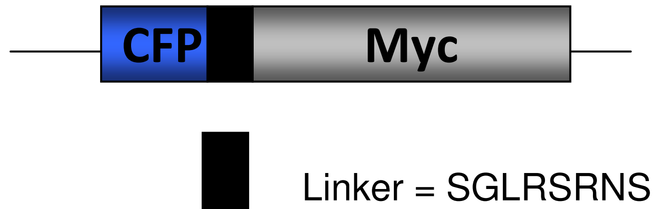
N/A

Supporting Data

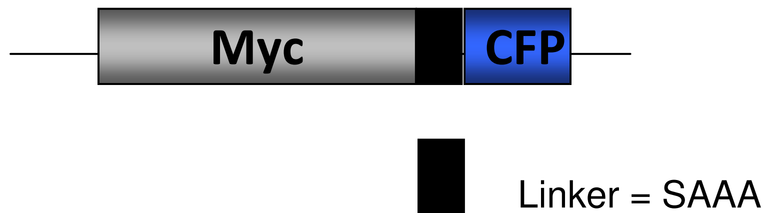
Figures 1-10

Figure 1

CFP-Linker-Myc



Myc-Linker-CFP

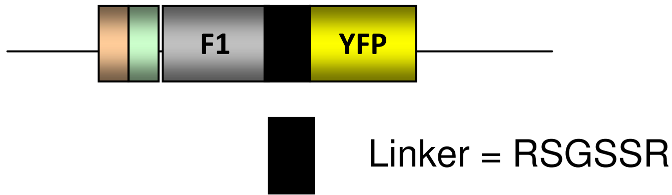


Notes:
-Myc has an internal nuclear localization signal (NLS)
-These constructs are not flag-tagged

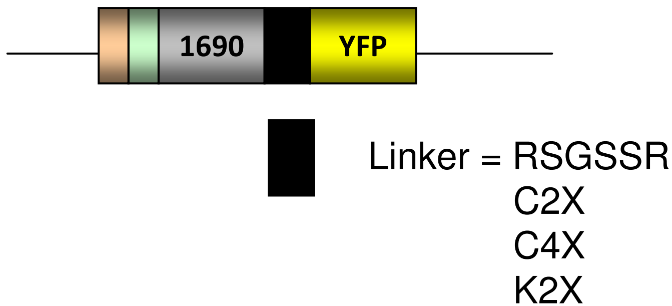
Figure 1: Cerulean-Myc constructs generated
Schematic representation of Cerulean-Myc (CFP-Myc) fusion proteins currently under evaluation. These constructs have been generated in the transient expression vector, pcDNA3.1.

Figure 2

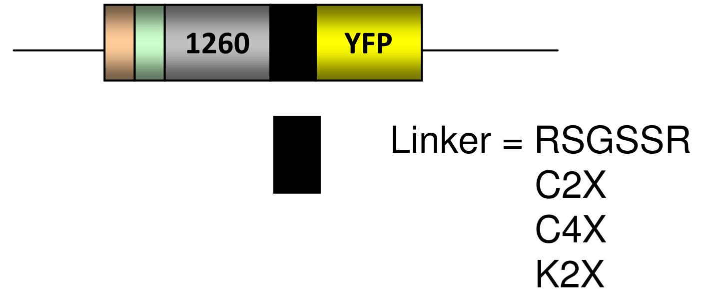
Flag-NLS-TRRAP/F1-Linker-YFP



Flag-NLS-TRRAP/1690-Linker-YFP

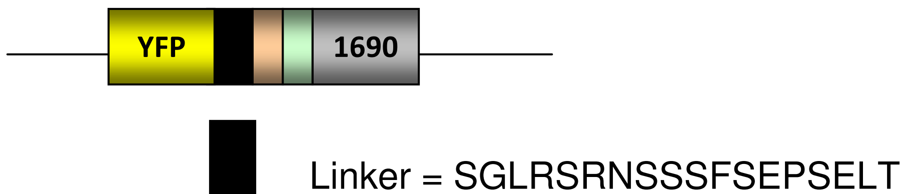


Flag-NLS-TRRAP/1260-Linker-YFP



C2X = RS GGSGGT GGSGGT SR
C4X = RS GGSGGT GGSGGT GGSGGT GGSGGT SR
K2X = RS LSAAE AAARE AAARE AAAKGGK SR

YFP-Flag-NLS-1690



Key:

- Flag tag
- nuclear localization signal (NLS)
- Linker

Figure 2: TRRAP-YFP constructs generated
Schematic representation of TRRAP-Citrine (TRRAP-YFP) fusion proteins currently under evaluation. Three different regions of TRRAP have been used in these constructs (F1, 1690, 1260). These constructs have been generated in the transient expression vector, pCMV10.

Figure 3

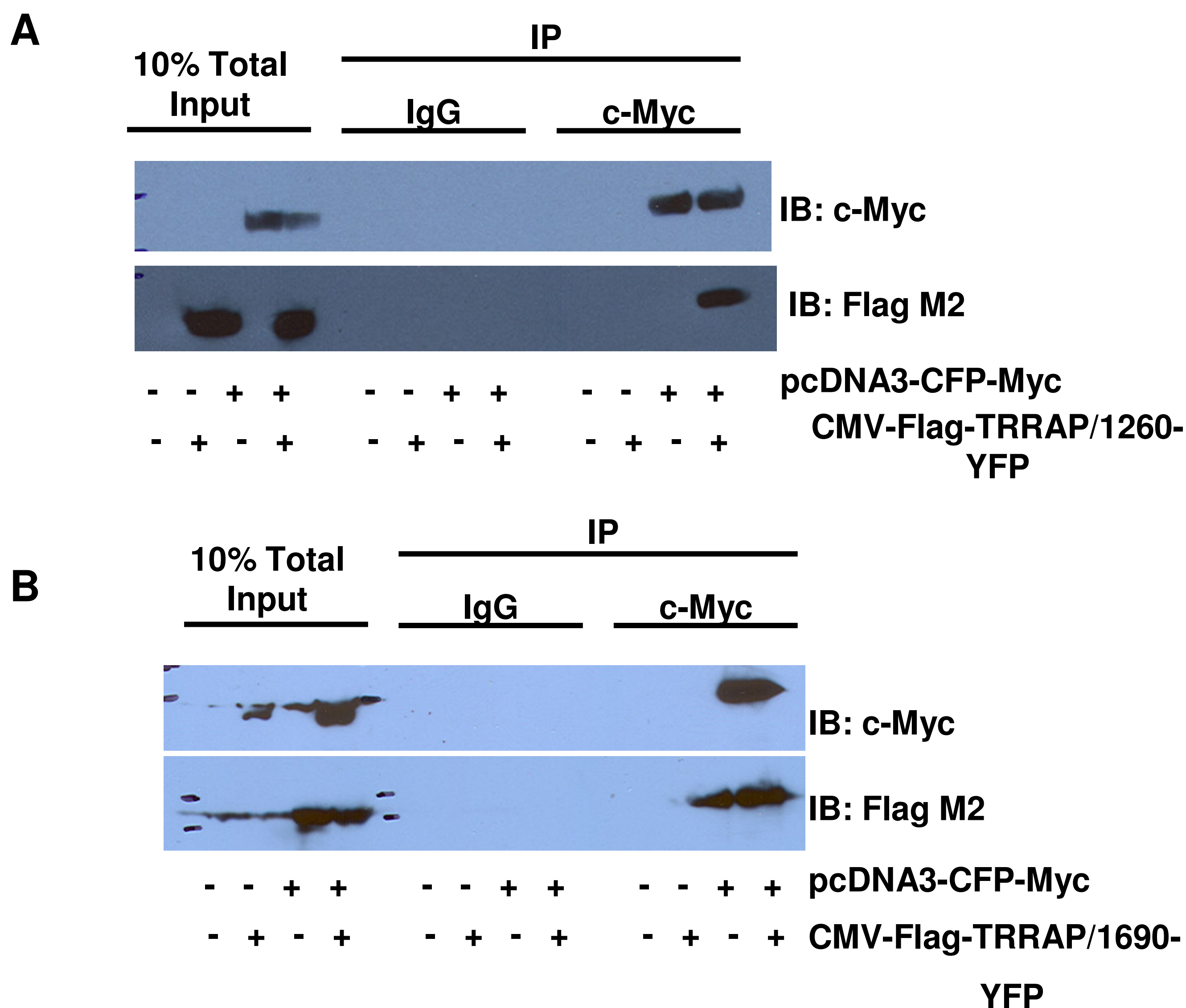


Figure 3: Preliminary characterization of fluorescent fusion proteins
A) The CFP-Myc and 1260-YFP fusion proteins interact *in vivo*. 293TV cells were co-transfected with vectors containing these fusions. Lysates were then immunoprecipitated with control or c-Myc antibodies, resolved using SDS-PAGE and 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 with contain the RSGSSR linker.

Figure 4

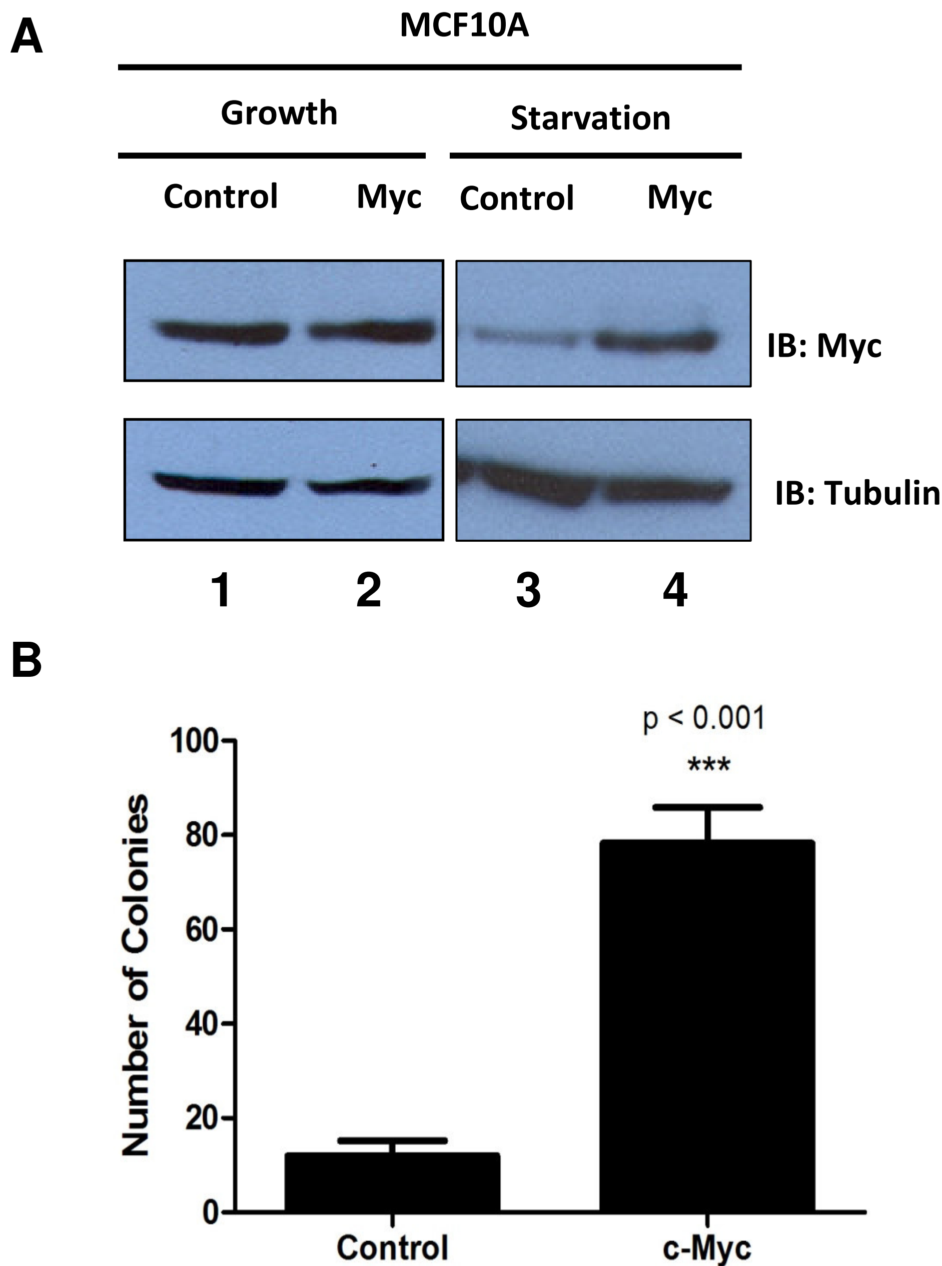


Figure 4: Identification of MCF10As as a model system for Myc-dependent transformation
A) Ectopic expression of Myc in MCF10A cells does not lead to higher levels of protein expression in asynchronously growing MCF10A cells, when measured by western blotting, compare lanes 1 and 2. When growth factors 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.

Figure 5

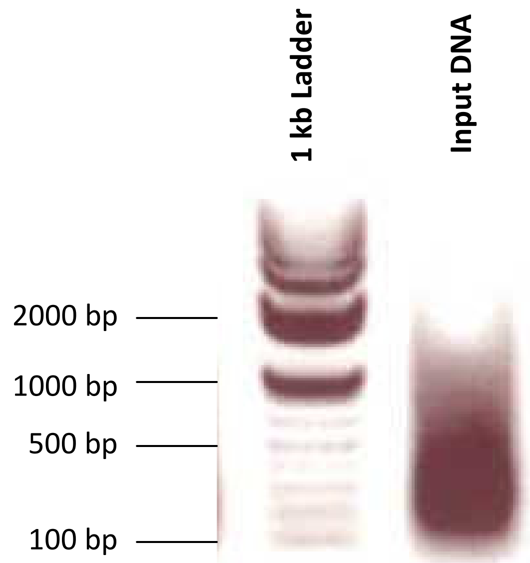


Figure 5: Optimized sonication of MCF10A cells
Nuclear pellets are resuspended in nuclei lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors) at a concentration of 100 million cells per 1 mL of buffer. The samples are sonicated with the Bioruptor (Diagenode) at high setting for 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.

Figure 6

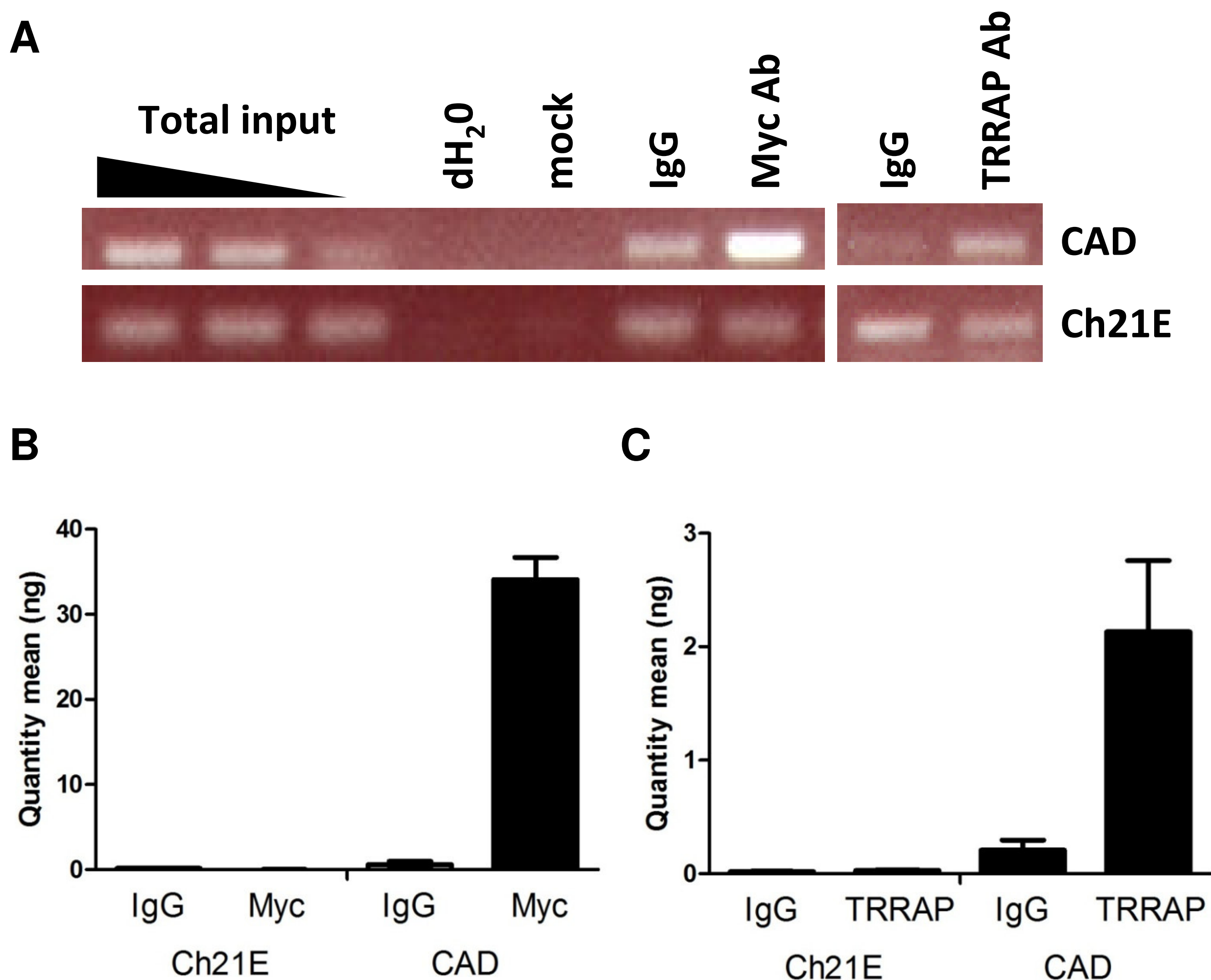
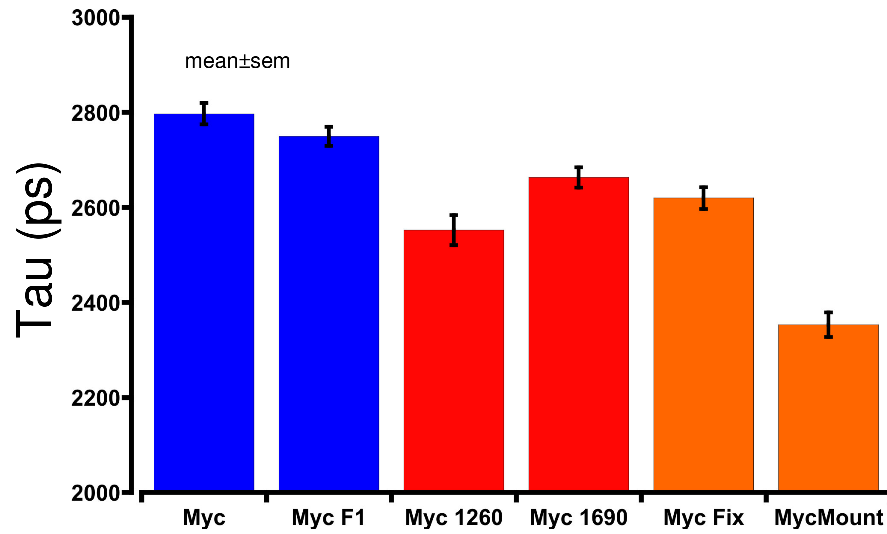
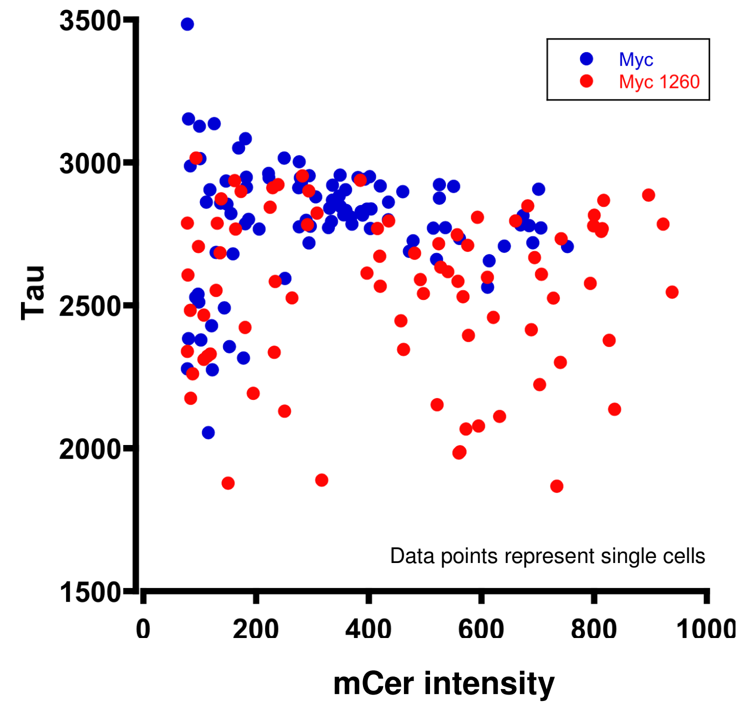


Figure 6: Optimized Chromatin Immunoprecipitation of Myc and TRRAP in MCF10A cells
A) PCR specificity check of representative chromatin immunoprecipitation (ChIP) reactions. CAD is a previously established Myc target, and a gene we have demonstrated to be bound by TRRAP. A chromosome 21 E-box, previously evaluated not to be bound by Myc is used as a negative control. Enrichment for CAD in the Myc and TRRAP lanes indicates optimized experimental conditions. Water serves as a PCR control, and the mock sample as a ChIP control. B) Quantitative PCR analysis of ChIP reactions. *Columns*, mean; *bars*, SD for technical replicates. ChIP-on-chip experiment are currently underway in the MCF10A model system to identify targets bound by Myc and contribute to transformation.

Figure 7. Evaluation of FRET-Pairs





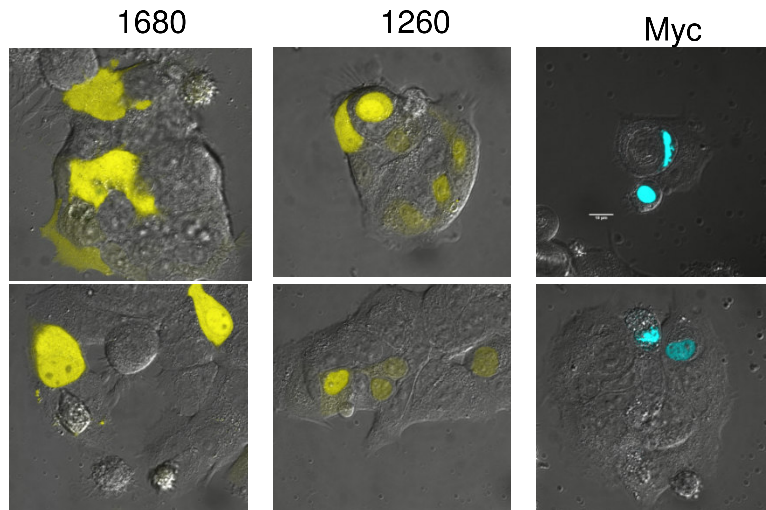
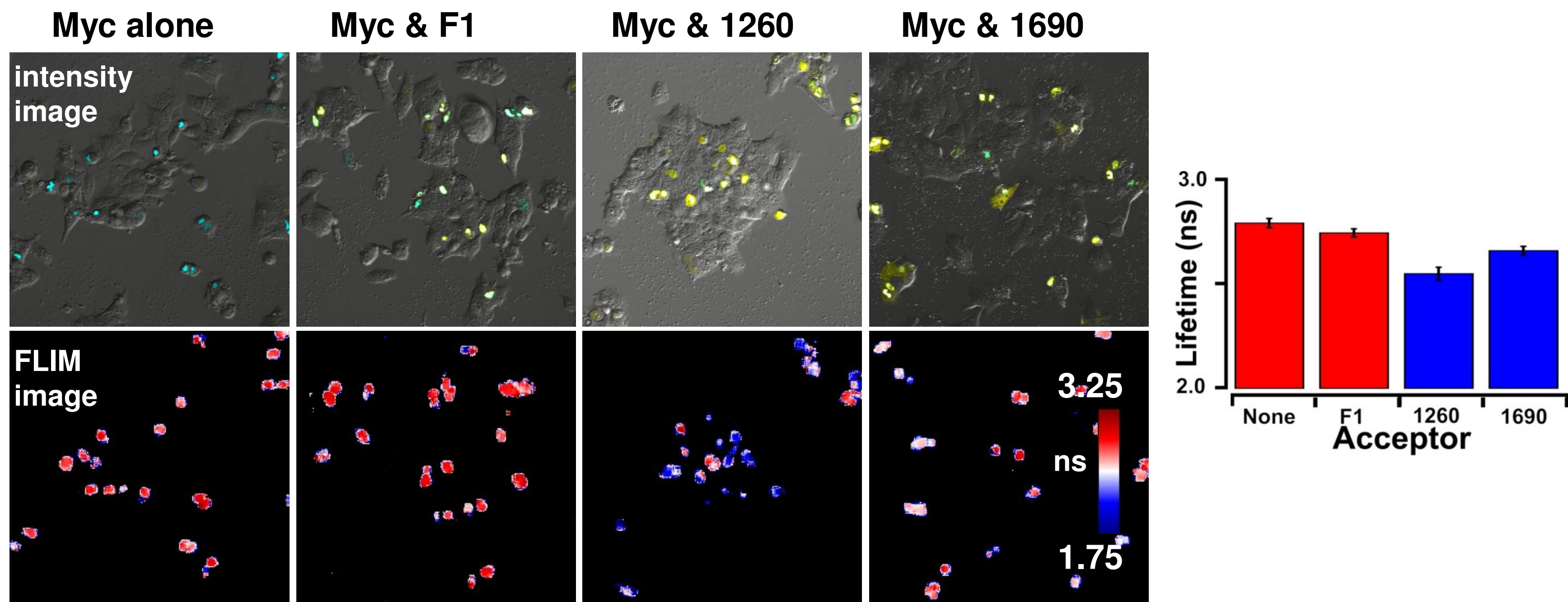


Figure 7: Evaluation of FRET-Pairs. The histograms demonstrate that the average lifetime (Tau) for the Myc-Cerulean construct drops from 2.8 ns to 2.5 ns (for Myc/TRRAP1260). This change in lifetime is sufficient for screening. However, the orange bars show that both fixation and mounting media decrease 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 constitutive CFP-Myc and inducible YFP-TRRAP fluorescent fusion proteins.

Figure 8. Evaluation of optimal FRET pairs for FLIM in live cells



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

Figure 8. 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 negative controls (alone 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 expression of the constructs well enough. We need to continue to optimise the FRET-efficiency while using different linkers, relocating the CFP and YFP and test other cell lines to generate a system that can be used for high throughput screening.

Figure 9. Sample decay curve for measuring FRET between CFP and YFP accurately.

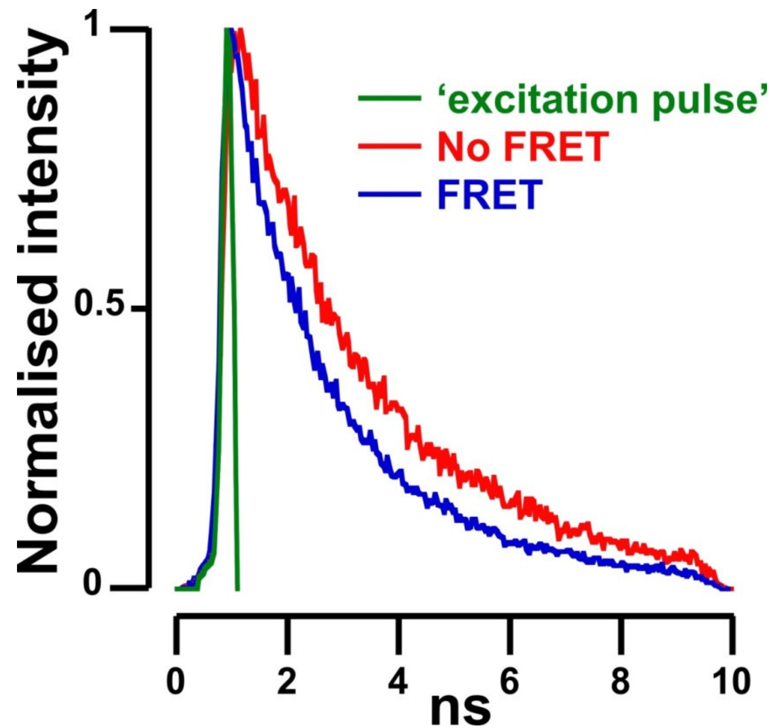
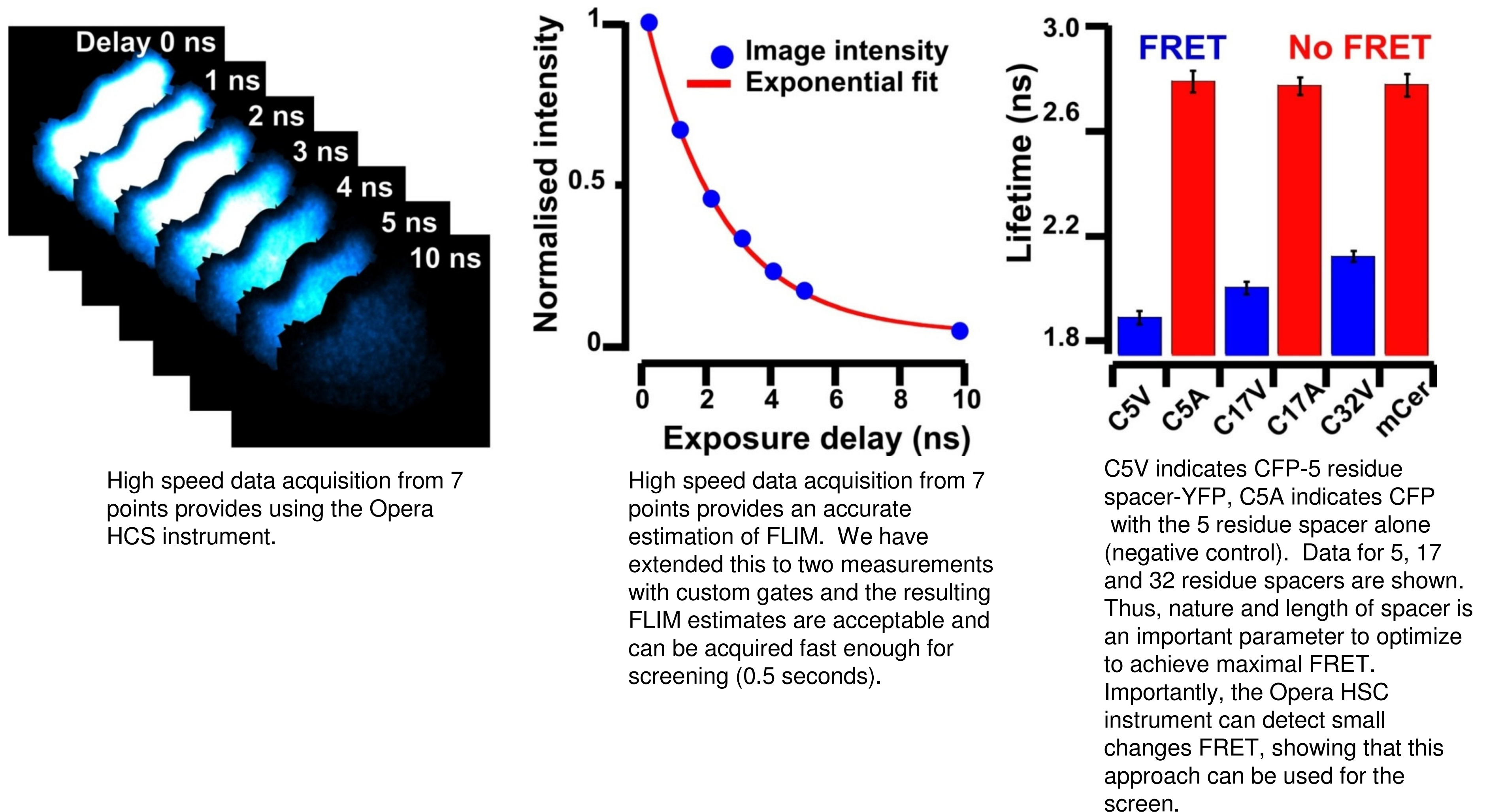


Figure 9. Sample decay curve for measuring FRET between CFP and YFP accurately.
FLIM generates images of the cell where the intensity of each pixel corresponds to the average lifetime of the fluorescence protein at that location. Accurate decay curves have been generated for FRET between Cerulean (CFP) 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 10. Optimization of FRET-FLIM detection using the Opera HCS instrument



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 10. 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.