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This predoctoral fellowship application was initiated by Mr. Dan Clark and recently continued by Ms. Sonalee Athavankar. The objective has been to analyze biological pathways involved in breast cancer proliferation and study small molecules with the potential to modulate these pathways. We have created whole cell assays useful for studies of protein tyrosine kinase enzymes, which comprise a major class of drug targets implicated in a subset of breast cancers. We used these assays to study inhibitors of these enzymes in recombinant yeast cells engineered to be permeable to anticancer agents. We have also used recombinant yeast to identify and characterize protein tyrosine kinases isolated from human cDNA libraries. Current efforts are focused on the covalent modification in living cells of proteins involved in breast cancer proliferation.

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Research Summary.....	4
References.....	10
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusions.....	
Appendices.....	

Research Summary

Specific protein-protein interactions are critical for cellular survival. Small molecules that modulate these interactions can function as drugs to promote or prevent human disease. This predoctoral research has focused on the analysis, identification, and modulation of protein-protein interactions involved in the proliferation of breast cancer.

We initially tested the hypothesis that combinatorial chemical libraries of cyclic peptides expressed in yeast using the split-intein mediated circular ligation of peptides and proteins (SICLOPPS) genetic system¹ would yield potent, stable, and cell-permeable modulators of estrogen receptor (ER)-mediated gene expression. These compounds were designed based on segetalin natural products, which are cyclic peptides that exhibit estrogenic activity in animals.²⁻⁴ Promising compounds were proposed to be evaluated in whole-cell assays as potential non-steroidal hormonal regulators of breast cancer. However, we found that the SICLOPPS system did not function in recombinant yeast as hoped, and this approach was abandoned.

Another more successful project involved the reconstitution of protein-protein interactions initiated by protein tyrosine kinase (PTK) enzymes in recombinant yeast. These enzymes catalyze the transfer of the gamma phosphoryl group of ATP to specific tyrosine residues of target proteins, and initiate protein-protein interactions involved in cellular signaling that are critical for numerous cellular functions.^{5,6} Aberrant PTK activity is involved in numerous human diseases including breast cancer.^{6,7} To study these enzymes, we published the construction of a yeast

tribrid system expressing a potentially universal PTK substrate as a tool for the evaluation of PTKs involved in disease.⁸ We also published a manuscript describing a modification this system to identify chemotherapeutically relevant compounds that inhibit PTK activity.⁹

As shown in Figure 1, we recently combined this yeast tribrid system with a fluorescent reporter gene to discover proteins with PTK activity by screening a human cDNA library. This approach was designed as an alternative to conventional methods for screening cDNA libraries with yeast genetic systems, which can be highly time-consuming pursuits involving the transformation of the cDNA library, growth for several days to generate colonies, combination and redistribution of colonies, and analysis of cDNA protein products for the desired activity. We envisioned that fluorescence activated cell sorting (FACS) of yeast expressing a fluorescent reporter gene might be used to streamline cDNA library screening. Flow cytometry-based techniques such as FACS were proposed to allow quantification of individual yeast cell characteristics based on fluorescence detection. The ability to evaluate single cells could potentially streamline screening of cDNA libraries.

We hypothesized that a fluorescent reporter gene could be used with a "One-Tube" method to circumvent traditional yeast transformation, plating, and harvesting steps to simplify cDNA screening. As shown in Figure 2, this method involved transformation of yeast with the genetic system and cDNA library of interest, selection of desired transformants in liquid culture, induction of protein expression to trigger fluorescent reporter gene expression in the presence of appropriate protein-protein interactions, and FACS to isolate the most fluorescent yeast cells. After

growth of sorted yeast cells, the gene of interest can be identified by sequencing. To accomplish this objective, yeast optimized green fluorescent protein¹⁰ (yEGFP) was incorporated into the yeast reporter plasmid pSH18-34.¹¹ This new reporter plasmid was validated as shown in Figure 3. The yEGFP reporter was found to provide excellent signal-to-noise ratios by flow cytometry was incorporated into a yeast tribrid system to identify PTKs from a cDNA library. Using our one-tube method for screening a human cDNA library isolated from lymphocytes, the lymphocyte-specific PTK Fyn was identified, thus validating the utility of this approach. These results have been accepted for publication in the journal ChemBioChem.¹²

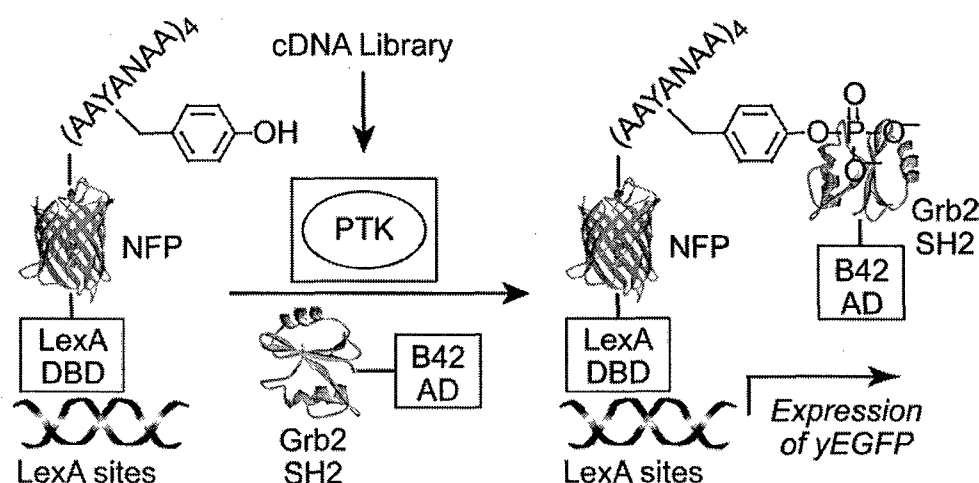


Figure 1. Schematic representation of the yeast tribrid system employed in these studies. This assay reports PTK activity by expression of the fluorescent yEGFP protein. NFP: Nonfluorescent protein. (AAYANAA)₄: Tetrameric PTK substrate.

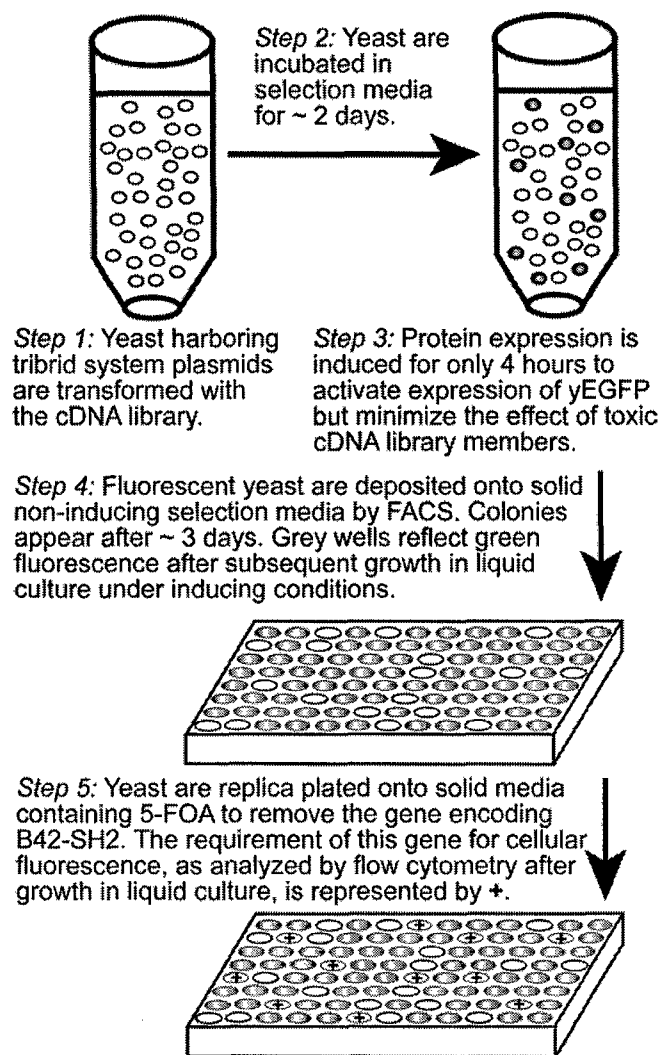


Figure 2. "One-tube" protocol for screening cDNA libraries against a yeast tribrid system to identify protein tyrosine kinases.

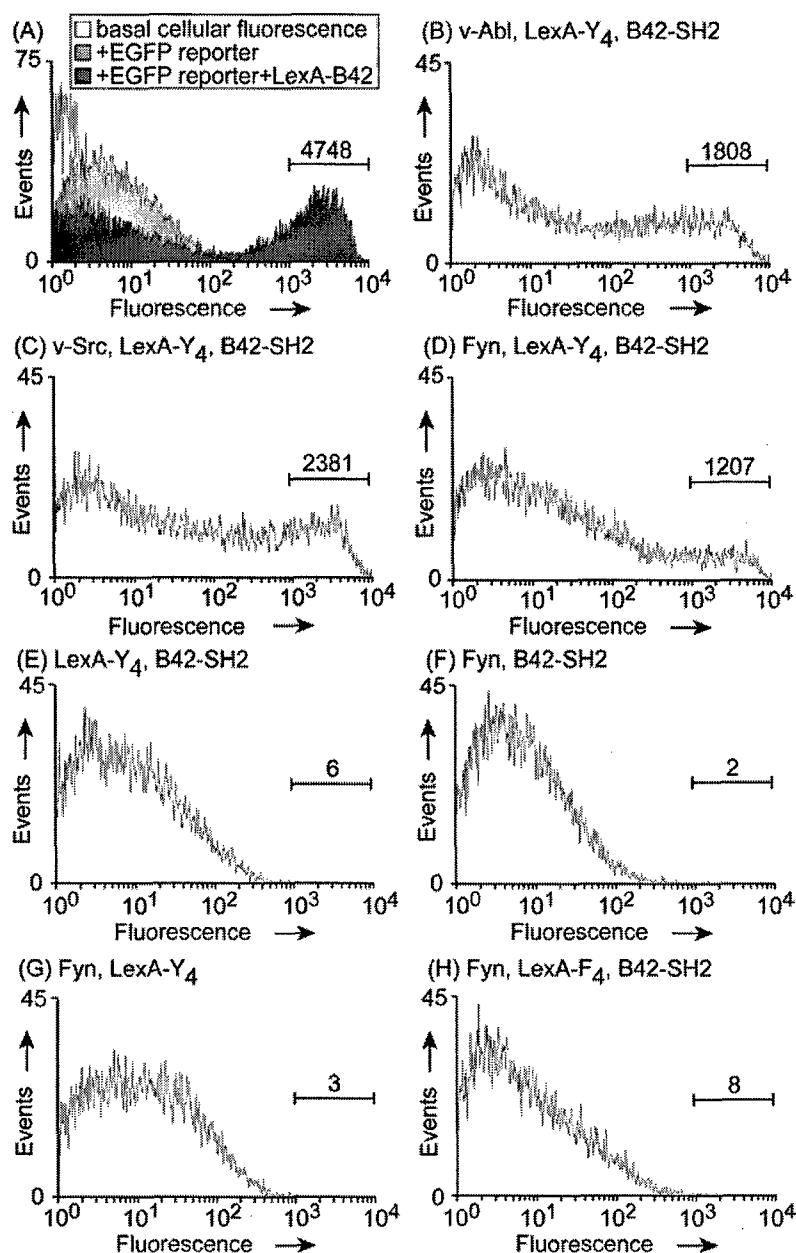


Figure 3. Analysis of yeast transformed with a fluorescent reporter gene by flow cytometry. The gate shown quantified the number of cells out of 15,000 in the fourth (top) decade of fluorescence. Panel A: Validation of the fluorescent reporter gene. The basal fluorescence of yeast lacking the reporter gene is colored white. The background fluorescence contributed by the *yEGFP* reporter alone is light gray, and the maximal fluorescence activated by a B42-LexA fusion protein is dark gray. Panels B-D: Expression of the *yEGFP* reporter gene in yeast tribrid systems. Positive controls: v-Abl (237-630) (Panel B), v-Src (137-526) (Panel C) and Fyn kinase (Panel D). Panels E-H: Fyn kinase omission control experiments. LexA-Y₄ represents the tetrameric tyrosine-containing universal substrate. LexA-F₄ represents a tetrameric phenylalanine-containing control sequence.

The research supported by this fellowship is currently focused on the development of a method to covalently modify and subsequently track proteins involved in breast cancer proliferation. We are studying covalent biotinylation mediated by the biotin protein ligase BirA as an approach to transfer molecules of interest to short 15-amino acid avitag peptide substrates. When this avitag peptide is fused to proteins such as the estrogen receptor, the BirA enzyme can biotinylate a conserved lysine residue within the avitag peptide and provide a handle for tracking this protein. To extend this approach to fluorescent labeling of proteins involved in breast cancer proliferation, we are working to chemically link biotin derivatives to fluorophores that enable modification of these proteins in living cells. This research may provide an important new approach for the fluorescent labeling of proteins involved in breast cancer proliferation.

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Key Research Accomplishments

- Created a yeast tribrid system for the detection and analysis of protein tyrosine kinase activity.
- Created of an ERG6-knockout strain of yeast useful for the identification of small molecules that modulate protein tyrosine kinases expressed in yeast.
- Developed and validated of a fluorescent reporter gene functional in yeast
- Developed a "One-Tube" protocol for the transformation and evaluation of cDNA libraries in PTK yeast tribrid systems
- Validated the utility of the one-tube protocol combined with the fluorescent reporter gene by cloning the PTK Fyn from a human cDNA library.

Reportable Outcomes

Manuscripts Published:

Clark, D. D. and Peterson, B. R. "Fluorescence-Based Cloning of a Protein Tyrosine Kinase with a Yeast Tribid System" *ChemBioChem*, **2005**, Accepted for publication

Clark, D. D. and Peterson, B. R. "Analysis of Protein Tyrosine Kinase Inhibitors in Recombinant Yeast Lacking the *ERG6* Gene" *ChemBioChem* **2003**, *4*, 101-107.

Clark, D. D. and Peterson, B. R. "Rapid Detection of Protein Tyrosine Kinase Activity in Recombinant Yeast Expressing a Universal Substrate" *J. Proteome Res.* **2002**, *1*, 207-209.