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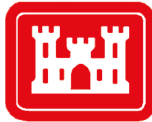

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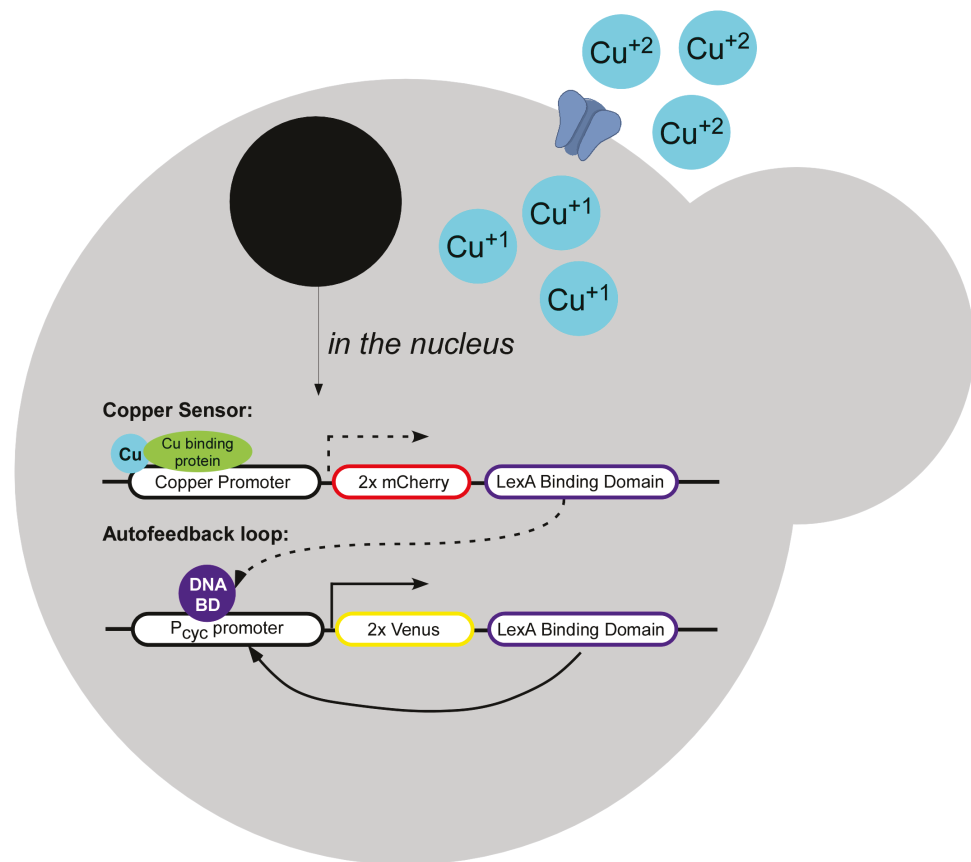
Development of a Genetic Memory Platform for Detection of Metals in Water

Use of mRNA and Protein Destabilization Elements as a Means to Control Autoinduction from the *CUP1* Promoter of *Saccharomyces cerevisiae*

Jamie L. Luther, Holly V. Goodson, and Clint M. Arnett

May 2018



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Development of a Genetic Memory Platform for Detection of Metals in Water

Use of mRNA and protein destabilization elements as a means to control autoinduction from the *CUP1* promoter of *Saccharomyces cerevisiae*

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Under Project 447761, “Genetically Engineered Memory Circuits as Bio-alarms”

Abstract

As of 2017, the United Nations estimated that globally, over 80% of all wastewater is released into the environment without treatment. Often, the water is contaminated with toxic heavy metals, which ultimately enter potable water supplies. Contamination is especially prevalent in low- and middle-income countries, a fact that poses a significant threat to indigenous populations as well as to deployed troops in these areas. Standard methods for detecting trace levels of metals in water requires expensive equipment and highly trained personnel, both of which most developing countries lack. To address this issue, authors designed a fluorescent yeast biosensor capable of detecting a model heavy metal, copper. The biosensor was responsive to copper at concentrations from 1 to 100 ppm. However in the absence of copper, autoinduction was observed. To decrease autoinduction, researchers explored the use of various mRNA and protein destabilization motifs such as adenylate-uridylate-rich elements and peptide sequences rich in proline, glutamic acid, serine, and threonine. Preliminary results demonstrated that the addition of destabilizing mRNA had no effect on levels of autoinduction within the system. However, integration of protein destabilizing amino acids effectively reduced autoinduction, but not without also decreasing the dynamic range of the sensor system.

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Preface

This study was conducted for the U.S. Army Engineer Research and Development Center Environmental Quality/Installations (ERDC-EQ/I) Basic Research Program under P2 Project # 447761, "Genetically Engineered Memory Circuits as Bio-alarms." The technical monitor was Dr. Elizabeth Ferguson, lead Technical Director for ERDC-EQ/I.

The work was performed by the Environmental Processes Branch (CNE) of the Installations Division (CN), U.S. Army Engineer Research and Development Center, Construction Engineering Research Laboratory (ERDC-CERL). The CERL principal investigator was Mr. Clint M. Arnett and at the time of publication, Mr. Harold G. Anderson was Chief, CEERD-CNE; and Mr. Donald K. Hicks was Acting Chief, CEERD-CN. Dr. Elizabeth Ferguson, CEERD-EM-J, was the Technical Director for ERDC-EQ/I. The Interim Deputy Director of ERDC-CERL was Ms. Michelle J. Hanson, and the Interim Director was Dr. Kirankumar V. Topudurti.

This research was conducted through a Cooperative Research and Development Agreement (CRADA) between ERDC-CERL and the University of Notre Dame under CRADA c-16-CERL-01 and was supported in part by appointments at the Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through a cooperative agreement between the U.S. Department of Energy and ERDC-CERL.

COL Bryan S. Green was the Commander of ERDC, and Dr. David W. Pittman was the ERDC Director.

1 Introduction

1.1 Background

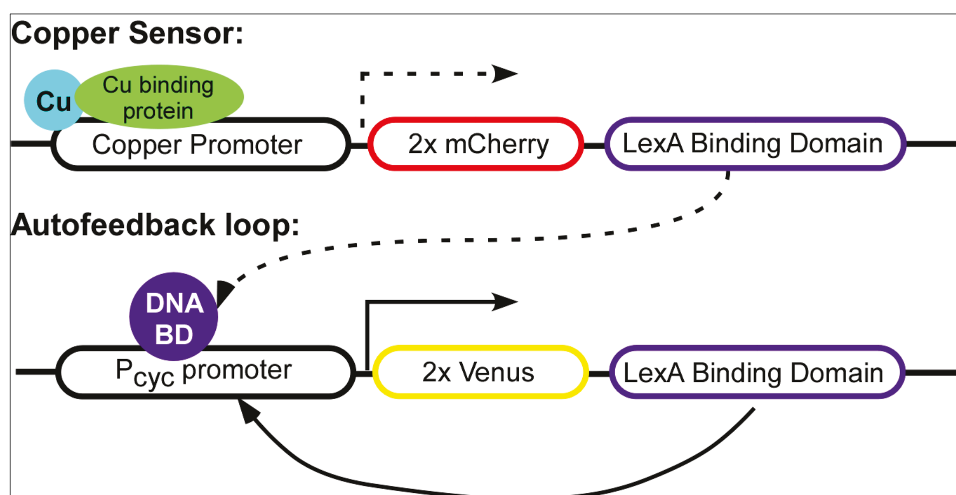
As of 2017, the United Nations (UN) estimated that 80% of all wastewater is released into the environment without treatment. Often, the water is contaminated with heavy metals. This is a global issue, especially in low and middle-income countries (LMICs), where only 8%–28% of wastewater is treated, due lack of infrastructure and funding (United Nations World Water Assessment Programme 2017). Most often, metals enter the water supply via poorly treated domestic, industrial, and agricultural wastewater. Common toxic metal contaminants include arsenic, lead, copper, iron, cadmium, and zinc (Chowdhury et al. 2016). Current standard methods for detecting heavy metals in water include atomic absorption spectroscopy (AAS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES). Comprehensive metal analysis of a water samples using ICP-OES can cost in excess of $100,000, and it requires a trained technician to operate and maintain the instrument (Wilbur 2005). However, many LMICs lack the laboratory infrastructure to conduct comprehensive water screening using standard methods, which increases risk of exposure to toxins. This not only adversely affects the local population but also U.S. troops stationed in these areas. Thus, robust, low-cost technologies to detect trace levels of heavy metals in water supplies are needed.

To address this issue, a biosensor engineered in *Saccharomyces cerevisiae* (yeast) was designed, capable of detecting copper at parts per million (ppm) levels. Copper was chosen for initial sensor development because it is a relevant drinking water contaminant (U.S. EPA 2016), the yeast copper-responsive *CUP1* promoter has been well characterized (Etcheverry 1990; Maya et al. 2008), and yeast cells have been genetically engineered to create biosensors capable of responding to a wide range of analytes (Wang 2006; Adeniran et al. 2015). Additionally, *S. cerevisiae* are an advantageous chassis for such biotechnologies because they are inexpensive, easy to propagate, tolerant to pH and temperature fluctuations, and highly sensitive and specific towards a target analyte. They also have a nonthreatening public perception and are amenable to long-term storage in a dried state (Baronian 2004). Previous research has demonstrated that genetically modified yeast are capable of detecting trace amounts of analyte

even after storage for a year in a hydrogel matrix on paper, making them ideal for use in austere environments (Weaver et al. 2013).

The structure of the biosensor is based on a yeast memory system developed by the Pamela Silver Laboratory at Harvard (Ajo-Frankin et al. 2007). Transient exposure of yeast to an analyte above a threshold concentration initiates a gene expression cascade, which allows the system to yield a sustained response without an additional supply of analyte. Similarly, the yeast copper biosensor was composed of two chromosomally encoded components: copper sensor and autofeedback loop (Figure 1). Ideally, exposure to copper above a threshold concentration will induce production of the red fluorescent protein (RFP) mCherry and the deoxyribonucleic acid (DNA) binding protein LexA, which in turn binds the *CYC1* promoter of the autofeedback loop of the second component. This induces production of the yellow fluorescent protein (YFP) Venus and another copy of DNA binding protein, which stimulates its own sustained production. Thus, transient exposure of the engineered yeast to copper above a threshold concentration yields a sustained response. The bistable design of this genetic circuit allows the yeast to “remember” exposure to copper, even after its removal from the system.

Figure 1. Copper memory platform overview. In response to copper, the first component of the copper sensor produces the RFP mCherry and a DNA binding protein. The DNA binding protein induces the second component of the system, producing the YFP Venus and self-sustaining DNA binding protein production (autofeedback loop).



Although similar memory systems have been designed to detect galactose and DNA damaging agents (Burrill and Silver 2011), the yeast biosensor described here is the first of its kind to have memory of exposure to a

metal analyte. The sensor construct will serve as a scaffold to design yeast sensitivity to other toxic metals such as lead and arsenic, which both have more complex genetic machinery. Previous work by the Holly Goodson Laboratory at the University of Notre has identified multiple genetic arsenic-responsive elements that could be engineered into the system (unpublished data). The technology could be utilized by Department of Defense (DoD) personnel, industry, regulatory agencies, field workers, and wastewater treatment facilities across the globe to monitor water quality at a fraction of the traditional cost.

1.2 Objective

The objective of this work was to create a *S. cerevisiae* genetic memory system capable of detecting copper at levels less than 10 ppm in water sources.

1.3 Approach

The genetic components which contribute to the copper response in *S. cerevisiae* are relatively simple and well characterized, making it ideal for proof of principle. However, it is known that the copper response in *S. cerevisiae* suffers from autoinduction, which could lead to a false positive response. To decrease autoinduction and increase system dynamic range, researchers explored the use of messenger ribonucleic acid (mRNA) and protein destabilization motifs.

To achieve this work's objective, multiple copper-sensitive, fluorescent *S. cerevisiae* strains were constructed using standard molecular biology techniques. Strains differed by number and type of mRNA or protein destabilization element. Additionally, certain strains contained the memory genetic loop in combination with mRNA or protein destabilizers. The first task was to use fluorescence microscopy to screen each strain for the desired phenotype. Cells were grown to mid-log phase, induced with copper for a fixed time period, and analyzed for fluorescence. While microscopy can screen small populations of cells, it cannot provide details about entire populations in a high-throughput manner. The second task was to characterize the fluorescence of larger cellular populations (10^6 cells) using flow cytometry. This technique was used to determine the percentage of cells that were fluorescent relative to the total population and to what extent (intensity). The third task was to characterize strains using a fluorescence

plate reader, which has the advantage of being able to analyze a large cellular population over the course of time.

A complete discussion of the work's materials and methods is contained in Chapter 2.

2 Materials and Methods

2.1 Materials and reagents

Materials and reagents were purchased as follows: Corning 96-well clear-bottom black polystyrene microplate, 37% (volume/volume [v/v]) formaldehyde and D-galactose (Fisher Scientific, Hanover Park, IL); yeast nitrogen base, without amino acids (Bioworld, Dublin, OH); dextrose (BD Diagnostics, Franklin Lakes, NJ); drop-out mix without yeast nitrogen base (US Biological, Salem, MA); low-fluorescence yeast nitrogen base without amino acids, folic acid, and riboflavin (Formedium, Norfolk, United Kingdom); and 4% (weight/volume [w/v]) copper (II) sulfate solution (Sigma Aldrich, Milwaukee, WI). All other chemicals and supplies used were of the highest quality available and were purchased from major commercial vendors.

2.2 *S. cerevisiae* growth conditions

All yeast strains were cultured overnight in a shaking water bath at 30°C and 250 revolutions per minute (rpm) in 125 mL baffle flasks with the appropriate drop-out medium, as previously described (Amberg et al. 2005). Then, 0.5 mL of overnight culture was used to inoculate 25 mL of fresh media. Cells were grown to log phase, optical density at 600 nm (OD_{600}) of 0.3 to 0.5, before induction with copper sulfate. Where applicable, supplies and media where sterilized by either autoclaving at 120°C for 15 min or passing through a 0.2 μm filter prior to use.

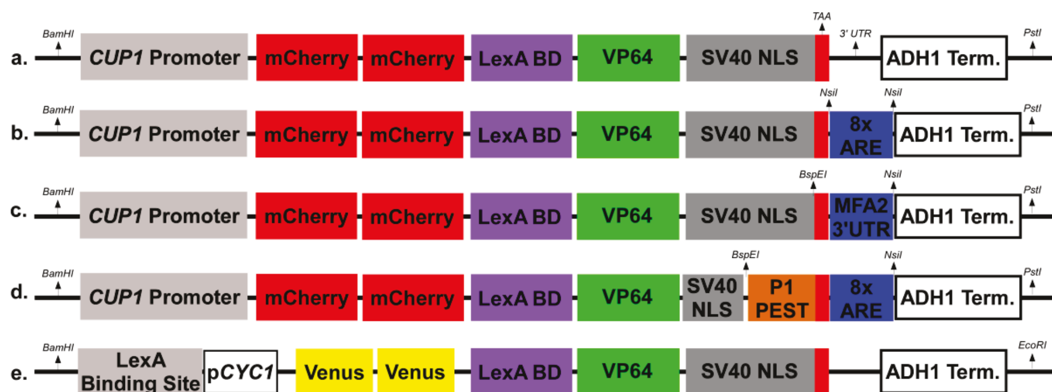
2.3 Yeast plasmid and strain construction

Memory system sequences, integrative plasmids, and *S. cerevisiae* strain PSY580APSY580A (MATa, ura3-52, trp1Δ63, leu2Δ1) were obtained from the Pamela Silver Laboratory at Harvard Medical School (Boston, MA). The *CUP1* yeast copper promoter was used to replace the galactose promoter (*GAL1*) in the sensor gene of the original memory system (Ajo-Frankin et al. 2007). The copper sensor gene and autofeedback loop were synthesized and cloned into the integrative plasmids pRS305 and pRS306, respectively, by GenScript (Piscataway, NJ). The copper sensor gene was cloned into pRS305 using restriction enzymes BamHI and PstI. The autofeedback loop was cloned into pRS306 using BamHI and EcoRI. The native cup1 sensor platform consisted of the copper promoter, two copies of

the mCherry fluorescent protein, LexA DNA binding domain, VP64 activator, SV40 Nuclear Localization Signal (NLS), stop codon, and the ADH1 terminator (Figure 2a). This sequence was integrated at the LEU locus of PSY580A to create strain "cup1." The N1 destabilized cup1 sensor was integrated at the LEU locus to create strain "cup1-8xARE" (Figure 2b). The resultant plasmids were referred to as pRS305-cup1 and pRS306-autofeedback. Plasmid pRS305-cup1-8xARE was created by cloning the N1 mRNA destabilization motif (adenylate-uridylate-rich [ARE]) 5'-UUAUUUAUU-3' from *c-fos* into the 3' untranslated region (UTR) of the copper sensor gene (Zubiaga et al. 1995), directly after the stop codon using the NsiI restriction enzyme (Figure 2c). Short oligonucleotide sequences containing the ARE motif flanked with NsiI sites were used for cloning. The clone with the greatest number of ARE inserts was chosen. Plasmid pRS305-cup1-MFA2 was created by cloning the 3' UTR from the MFA2 gene directly after the stop codon of the cup1 sensor as shown in (Figure 2d). The 3' UTR sequence from MFA2 was obtained from the Saccharomyces Genome Database (SGD).* This sequence was synthesized and cloned into pRS305 by Genscript (Genscript Biotech, Piscataway, NJ) using the restriction enzymes BspEI and NsiI. Plasmid pRS305-cup1-8xARE-PEST (proline, glutamic acid, serine, threonine sequence) was created by cloning 8xARE in tandem with P1 PEST at the C-terminus of the copper sensor (Figure 2e). The P1 PEST sequence was obtained online from the commercially available plasmid, pd1EGFP-N1. The 8 copies of the ARE and P1 PEST were synthesized by GenScript and cloned into pRS305 using BspEI and NsiI restriction enzymes. Copper sensor and autofeedback genes were integrated into PSY580APSY580A at the LEU2 and URA2 loci, respectively, using a standard protocol to create the strains "cup1-autofeedback" and "cup1-8xARE-PEST-autofeedback" (Sikorski and Hieter 1989). All chromosomal integrations were confirmed using colony polymerase chain reaction (PCR), as described by Bruno (2008). (Insert sequences and plasmid maps are shown in this report's appendix).

* https://www.yeastgenome.org

Figure 2. Summary of sequences cloned into yeast integrative plasmids, pRS305 and pRS306; (a) native cup1 sensor gene with the copper promoter, 2 copies of the mCherry fluorescent protein, LexA DNA binding domain, VP64 activator, SV40 Nuclear Localization Signal (NLS), stop codon, and the ADH1 terminator, (b) cup1-8xARE, N1 destabilized stabilized cup1 sensor, (c) cup1-MFA2, MFA2 3' UTR destabilized cup1, (d) cup1-8xARE-PEST, N1 and P1 PEST destabilized cup1, and (e) autofeedback loop which differs from the cup1 sensor by a LexA DNA Binding Site, P_{cyc} minimal promoter, and 2 copies of Venus fluorescent protein.



2.4 Fluorescence microscopy and ImageJ quantification

Yeast cells were screened for mCherry fluorescence using a Nikon TE-2000U epifluorescence microscope equipped with a 100x oil immersion objective (Nikon Instruments Inc., Melville, NY). Live cells were applied to a glass slide with cover slip, spotted with immersion oil, and imaged. An X-Cite 120 lamp (Excelitas Technologies, Fremont, CA) was used as a light source. Excitation and emission was achieved by using a Nikon Cy3HQ filter cube equipped with 535/585 nm excitation filter and 590/630 nm emission filters. A Hamamatsu Digital Camera C1140 (Hamamatsu Photonics K.K., Hamamatsu City, Japan) was used to take images. All images were taken with the same exposure and 1x1 binning. Images were saved as 16-bit, uncompressed tagged image files (TIF) with 2048 x 2048 pixels. The brightness and contrast of each image (Figures 3, 6, 8, 11) were normalized for comparison within each experiment and cell mean intensity was quantified using ImageJ software* (Rasband 1997–2016). The elliptical tool was used to draw a circle around 10 cells in each slide. The mean intensity was measured via the analyze menu contained within ImageJ.

* A public-domain, Java-based image processing program developed at the National Institutes of Health.

2.5 Flow cytometry

Yeast cells were screened for Venus and mCherry fluorescence using a BD LSRFortessa X-20 cell analyzer (BD Biosciences, San Jose, CA). Cells were grown and treated as previously described. After induction with copper for 2 hr, cells were fixed with formaldehyde at a final concentration of 4% (v/v) for 20 min at room temperature. Following fixation, cells were pelleted, resuspended in 1x phosphate-buffered saline (PBS) buffer, and stored at 4°C for less than 24 hr. Venus fluorescence was detected using a 488 nm excitation in conjunction with fluorescein isothiocyanate (FITC) emission at 530 nm. Fluorescence from mCherry was detected using a 561 nm excitation in conjunction with the phycoerythrin (PE)-Texas Red (610 nm emission) bandpass filter. Cell populations (10^6 cells) were analyzed using FCS Express 6 Plus software (De Novo Software, Glendale, CA). Cells were gated relative to a negative control strain (PSY580APSY580A) lacking either fluorescent reporter.

2.6 Plate reader

A BioTek Synergy H1 Microplate reader (Winooski, VT) was used to monitor the development of Venus and mCherry fluorescence over a 24 hr time period. Cells were grown as previously described to OD_{600} of 0.3 to 0.4 before dilution with appropriate media to $OD_{600} \approx 0.05$. To each well of a Corning 96-well plate, 180 µL of dilute yeast culture plus 20 µL of appropriate copper stock was added. Cells were grown in the 96-well plate at 30°C using continuous orbit at 800 cycles per minute. Venus and mCherry fluorescence was monitored using 500 and 580 nm excitation and 530 and 610 nm emission, respectively. Absorbance at 600 nm was measured to monitor cell growth.

3 Results and Discussion

3.1 Expression from the copper sensor gene is leaky in the absence of inducer

The *CUP1* promoter is a strong, inducible promoter that is endogenous to *S. cerevisiae*, and it is often used to drive heterologous expression of proteins in cells by the addition of copper to the growth media (Labbé and Thiele 1999). However, the promoter is subject to autoinduction in the absence of copper (Tauseef et al. 1984; Peng et al. 2015; Rugbjerg et al. 2015). To confirm the leaky nature of the *CUP1* promoter, log phase *S. cerevisiae* cells expressing the cup1 sensor gene (Figure 2a) were treated with 0 to 100 ppm copper for 2 hr prior to fluorescence microscopy (Figure 3). An increase in mCherry fluorescence was clearly observed as the concentration of copper was increased in the system in a dose-dependent manner. ImageJ was used to quantify the mean fluorescence intensity from cells in each slide (Figure 4). The microscopy images and ImageJ quantification demonstrate that the system responds robustly to copper over 3 orders of magnitude. However, the untreated sensor (0 ppm copper) demonstrates autoinduction with roughly double the fluorescence of a negative control.

Figure 3. Fluorescence microscopy of cells expressing the cup1 sensor treated with 0 to 100 ppm copper; (a) PSY580A negative control, (b) cup1 sensor treated with 0 ppm copper, (c) 1 ppm copper (c), (d) 5 ppm copper, (e) 10 ppm copper, and (f) 100 ppm copper.

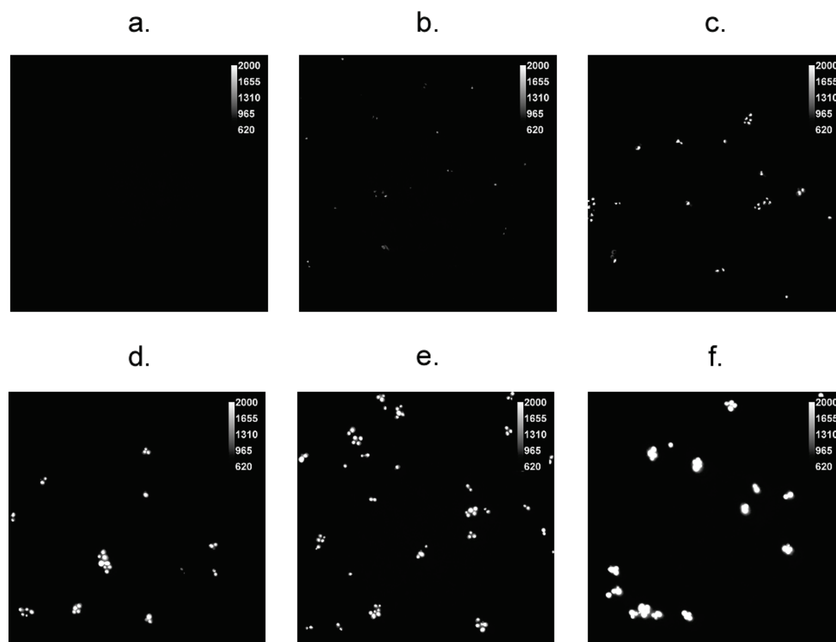
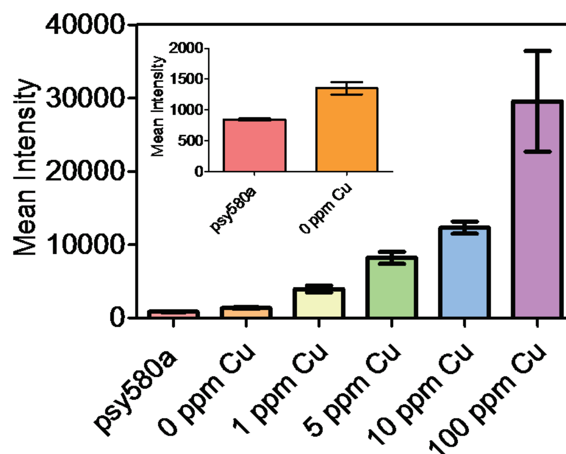
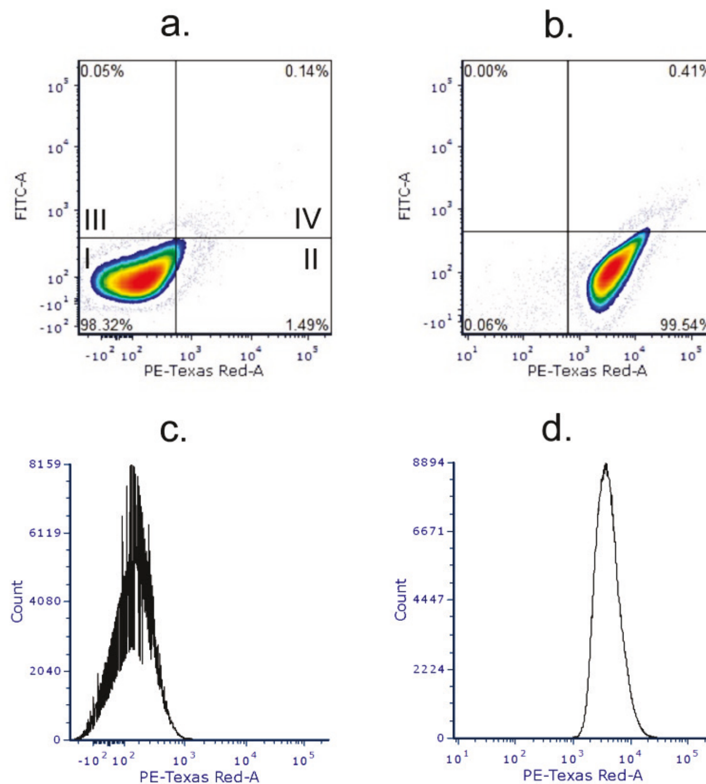


Figure 4. ImageJ quantification of cells expressing the cup1 sensor treated with 0 to 100 ppm copper. Each bar represents the average mean intensity (n = 10 cells). Error bars represent standard deviation. Inset highlights difference in fluorescence between cup1 sensor (0 ppm copper) and the negative control (PSY580A).



One disadvantage to using microscopy to screen a cell population is that it can only analyze a small fraction of the cells per any given slide. To better understand how a larger population of cells were reacting, flow cytometry was performed on 10^6 cells of the untreated (0 ppm copper) cup1 sensor (Figure 5). Quadrant nomenclature in Figure 5a, and Figure 5b was defined as: quadrant I (no fluorescence), quadrant II (mCherry only), quadrant III (Venus only), or quadrant IV (both mCherry and Venus). Flow cytometry demonstrated that a significant portion of the population autoinduced in the absence of copper. Roughly 1.5% of the PSY580a population exhibited fluorescence, whereas nearly 99.5% of the cup1 population showed some degree of fluorescence. The mean mCherry intensities (Texas Red) for the sample populations of negative control strain and cup1 sensor were 163 and 3998, respectively (Figure 5c and Figure 5d). Although a very small percentage of the PSY580A population did weakly fluoresce, it was minimal fluorescence compared to cup1 sensor, which showed strong fluorescence. This analysis clearly demonstrated that nearly the entire cup1 population was leaky and sensitive to autoinduction in the absence of copper.

Figure 5. Flow cytometry analysis of untreated S. cerevisiae cells expressing the cup1 sensor; (a) density plots of PSY580A cells, (b) cells expressing untreated (0 ppm copper) cup1 sensor, (c) histogram representation of mCherry intensity for PSY580A, (d) and histogram representation of mCherry intensity in cup1 sensor.



Autoinduction within the system is problematic in that it can significantly decrease the dynamic range of a bio-based sensor system. Additionally, it can be inhibitory for a memory platform because both gene circuits in the sensor will continuously be in the induced or "on" state in the absence of analyte. In order to create system memory, sensor gene products need to reach a threshold level to successfully activate the autofeedback response in the presence of analyte and remain "off" in the absence of copper. However if the system is always on, analyte-dependent bistable behavior will not be achieved, leading to a false positive response. In order to eliminate autoinduction, mRNA and protein destabilization elements were explored in an effort to reduce initial transcripts and translational products of the cup1 system.

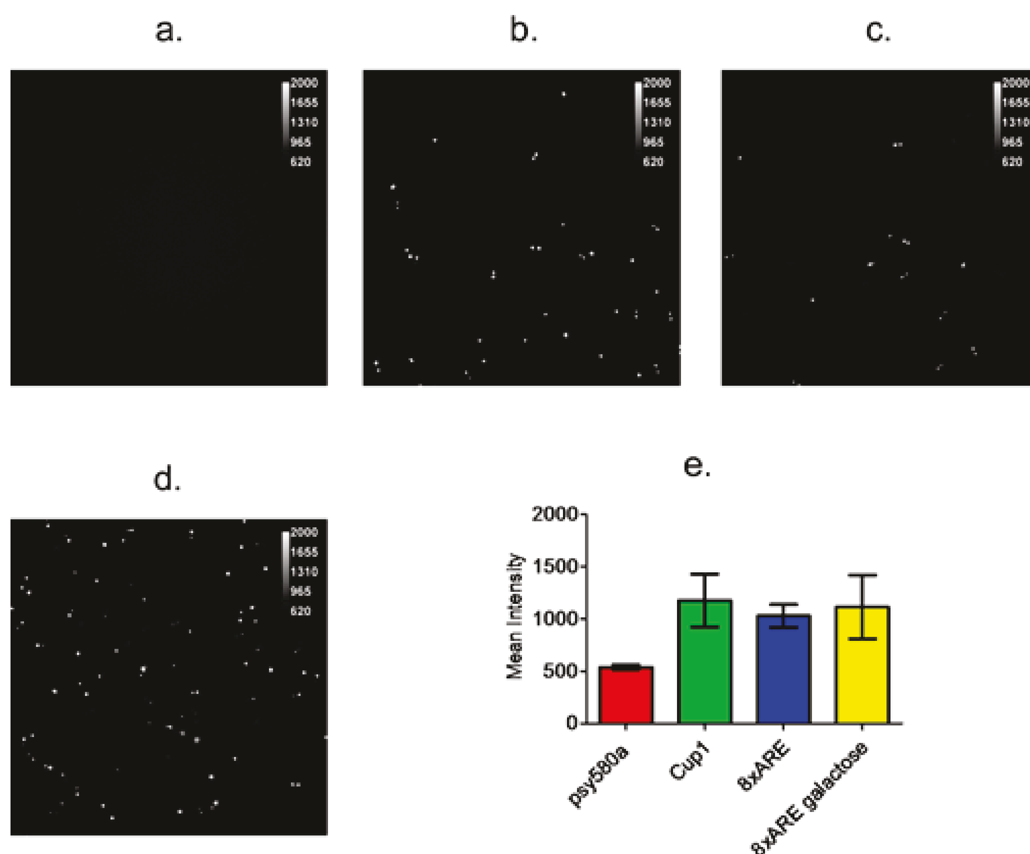
3.2 mRNA destabilization elements as a means to control *CUP1* autoinduction

Short-lived genes involved in cellular proliferation and differentiation, such as cytokines, transcription factors, or proto-oncogenes, are often regulated at the mRNA transcript level. AREs present in the 3' UTR often regulate the decay rate of these unstable transcripts (Chen and Shyu 1995). AREs function by recruiting ARE-sequence-specific proteins to the 3' UTR, which helps to modulate deadenylation of the poly (A) tail (LaGrandeur and Parker 1999; Vasudevan and Peltz 2001; Duttagupa et al. 2003). It has been demonstrated by others that AREs can be used to decrease the half-life of a variety of proteins by modulating their corresponding mRNA levels. For example, the N1 ARE (5'-UUAUUUAUU-3') motif, which is present in the 3' UTR of the *c-fos* proto-oncogene, was inserted into the 3' UTR of a luciferase reporter. Incorporation of this ARE reduced the reporter half-life from 14–17 hr to 6–12 hr (Voon et al. 2005). To control leaky expression from the cup1 sensor gene, 8 copies of the N1 ARE were cloned into the 3' UTR to create the *S. cerevisiae* strain cup1-8xARE (Figure 2b).

Initially, fluorescence microscopy was used to monitor mCherry production of untreated *S. cerevisiae* cells expressing either cup1 or cup1-8xARE (Figure 6). Strains PSY580A and cup1 were grown with 2% glucose and served as the controls. Strain cup1-8xARE was grown with 2% glucose or 2% galactose. Minimal florescence was observed in PSY580A (Figure 6a); however, florescence could be easily seen for cup1 and cup1-8xARE when grown with glucose as a source of carbon and energy (Figure 6b, Figure 6c). The ARE present in the 3' UTR of cup1-8xARE are derived from the unstable *c-fos* proto-oncogene transcript present in mammalian cells. In another study, which sought to determine whether mammalian AREs function in yeast, it was determined that 3' UTR of *c-fos* has a stabilizing effect on transcripts when cells were grown in 2% glucose (Vasudevan and Peltz 2001). However, when cells were grown in media with 2% glycerol/3% lactic acid, 2% galactose, or 2% raffinose as a carbon source, the researchers observed transcript destabilization. To test this hypothesis, cells expressing cup1-8xARE were grown in media with 2% galactose as a carbon source (Figure 6d). ImageJ was used to quantify florescence of each strain. In Figure 6e, error bars represent the average mean intensity (n = 10 cells) and error bars represent standard deviation. Fluorescence microscopy analysis of cells expressing the cup1-8xARE destabilizing motif did not exhibit a significant reduction in fluorescence relative to cup1

when grown on glucose or galactose, indicating the N1 ARE had no apparent overall effect on minimizing autoinduction from *CUP1* promoter. However, it should be noted that changes in mRNA levels do not always correlate with reporter protein abundance (Maier et al. 2009). Thus, it is possible that mRNA levels may be lowered without changing reporter output. Testing this hypothesis will be the focus of future quantitative polymerase chain reaction (qPCR) experiments with cup1-8xARE.

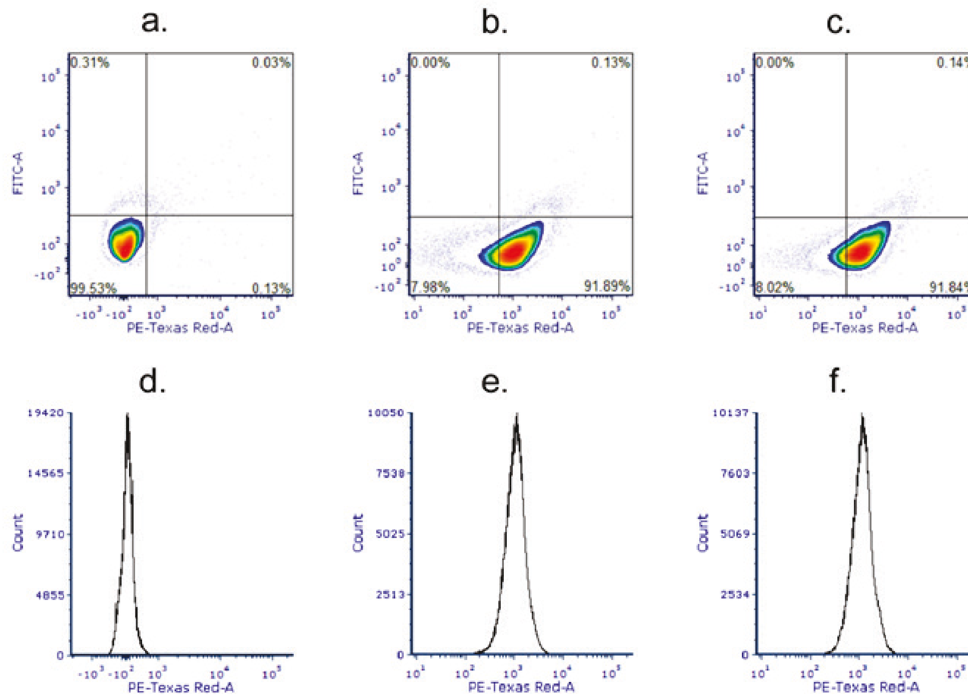
Figure 6. Fluorescence microscopy of untreated (0 ppm copper) cells expressing N1 mRNA destabilized cup1 sensor in glucose or galactose media; (a) PSY580A, (b) cup1 sensor, (c) cup1-8xARE sensor in 2% glucose media, (d) and cup1-8xARE sensor in 2% galactose, (e) ImageJ quantification of cells.



As previously described, flow cytometry analysis was used to confirm microscopy data on untreated strain populations of 10^6 cells (Figure 7). As observed previously, the vast majority (>99%) of the PSY580A population showed no florescence (Figure 7a). Roughly 92% of the cup1 and cup1-8xARE exhibited florescence (Figure 7b and Figure 7c). The average mean mCherry intensities of PSY580A, cup1, and cup1-8xARE were 80, 1847, and 2124 respectively (Figure 7d, Figure 7e, and Figure 7f). Flow cytometry

analysis confirms that N1 mRNA destabilization had no apparent effect on decrease reporter levels in cup1.

Figure 7. Flow cytometry analysis of untreated (0 ppm copper) cells expressing the N1 mRNA destabilized cup1 sensor in glucose media: (a) PSY580A, (b) cup1, and (c) cup1-8xARE. Histogram representations of mCherry intensity; (d) PSY580A, (e) cup1, and (f) cup1-8xARE.



The transcript encoding the *S. cerevisiae* mating pheromone a-factor MFA2 is known to be unstable ($t_{1/2}$ = 3.5 min), and studies have determined that the 3' UTR of the protein is entirely responsible for rapid transcript decay (LaGrandeur and Parker 1999; Vasudevan and Peltz 2001; Muhlrad and Parker 1992; Muhlrad et al. 1994). To further explore mRNA destabilization as a means to control leaky expression from the *CUP1* promoter, the effects of this endogenous ARE were tested. The entire 3' UTR of MFA2 was inserted directly after the stop codon of the cup1 sensor to create cup1-MFA2 (Figure 2c). Again, fluorescent microscopy revealed minimal florescence in PSY580A (Figure 8a), but florescence was easily detected in cup1 and cup1-MFA2 (Figure 8b and Figure 8c). Fluorescence quantification using ImageJ showed no significant decrease in intensity between cup1 and cup1-MFA2, and both strains were shown to autoinduce relative to the control strain PSY580A (Figure 8d). These findings were confirmed using flow cytometry (Figure 9). Of the 10^6 cells interrogated, more than 99% of PSY580A showed no fluorescent signature, and the

mean intensity was only 75 (Figure 9a and Figure 9d). Approximately 97% of cup1 and 99% of cup1-MFA2 populations exhibited red fluorescence (Figure 9b and Figure 9c), and the average mCherry mean intensities were 1840, and 1961, respectively (Figure 9e and Figure 9f). These data demonstrated that the addition of the 3' UTR of the mating pheromone a-factor MFA2 had no quantifiable effect on limiting autoinduction of *CUP1* promoter. However as stated previously, changes in mRNA abundance do not always correlate strongly with protein levels, and qPCR experiments of the cup1-MFA2 transcripts will be required to determine the effect on overall concentrations.

Figure 8. Fluorescence microscopy of untreated (0 ppm copper) cells expressing MFA2-3'UTR mRNA destabilized cup1 sensor; (a) PSY580A, (b) cup1, (c) cup1-MFA2, and (d) ImageJ quantification of cells; each colored bar represents the average mean intensity (n = 10 cells), and error bars represent standard deviation.

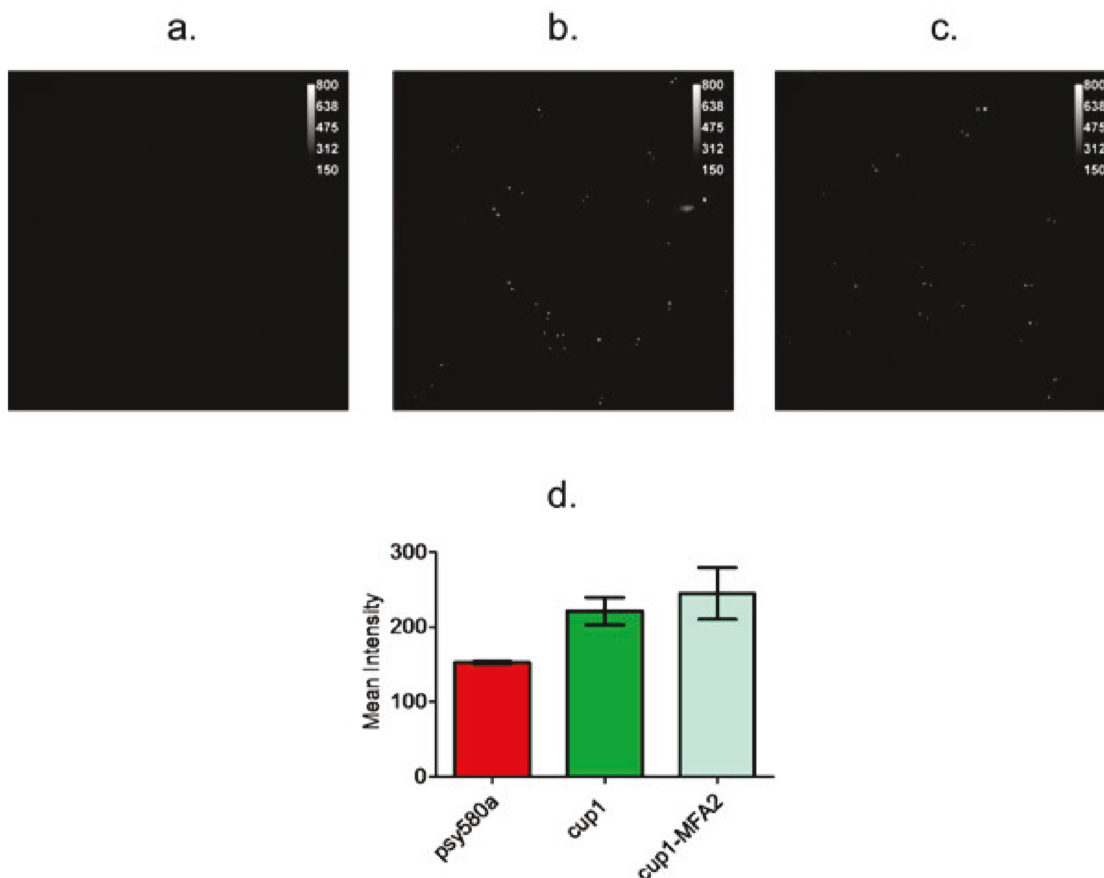
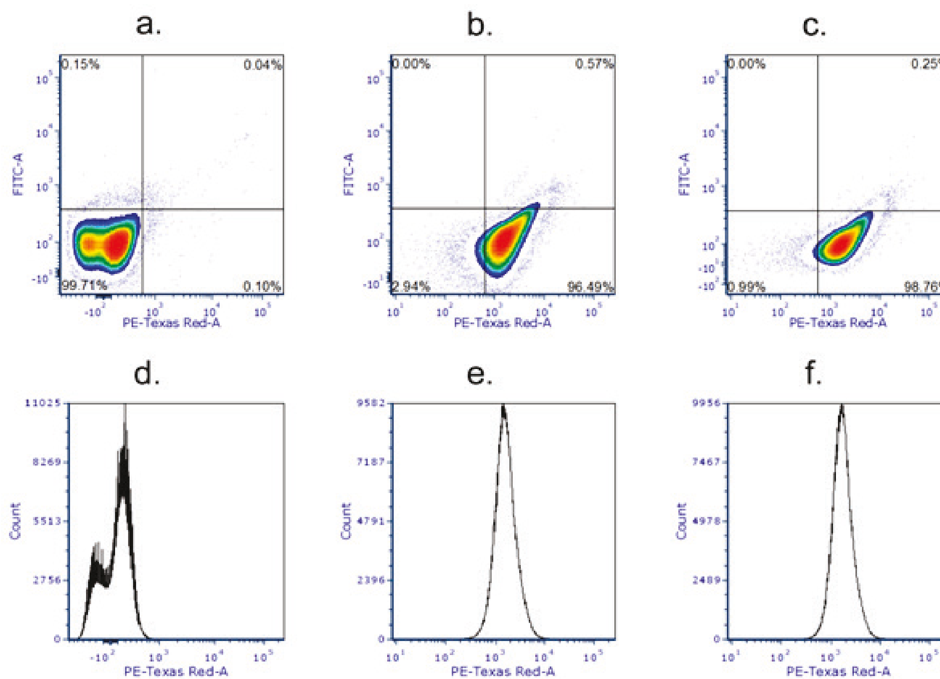
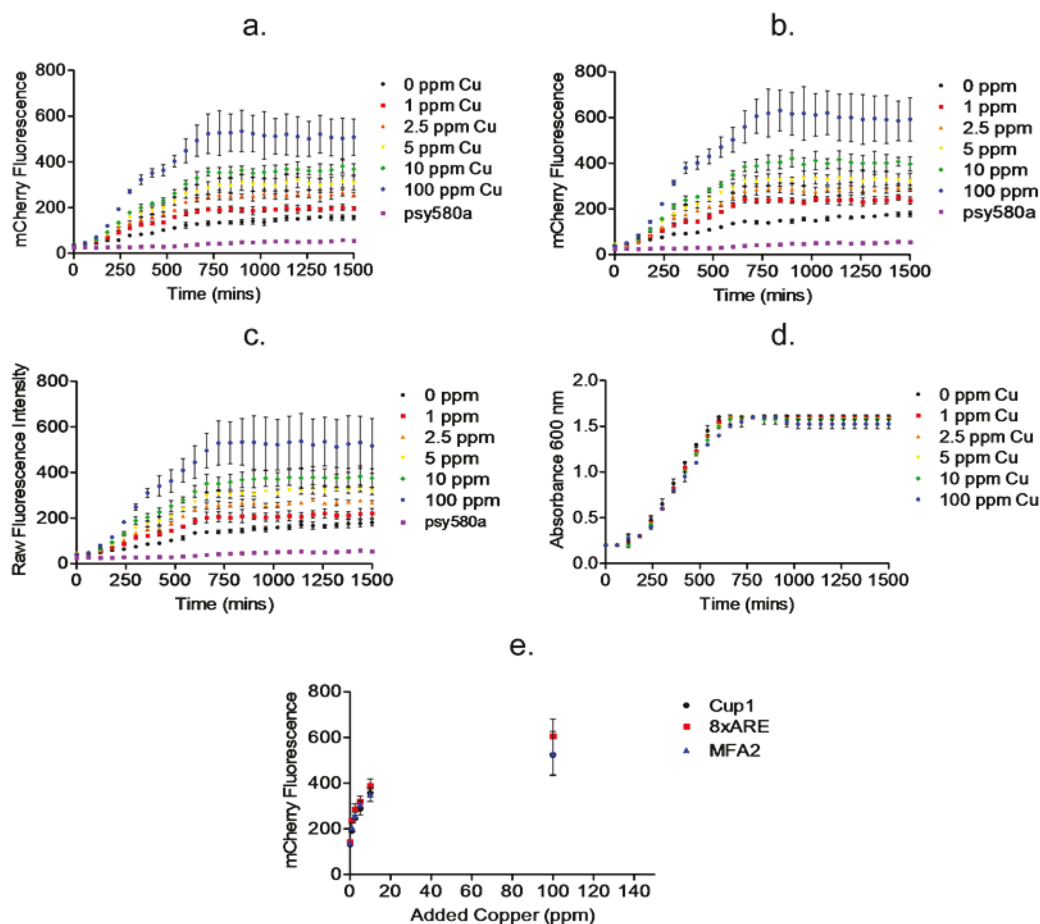


Figure 9. Flow cytometry analysis of untreated (0 ppm copper) cells expressing the MFA2-3'UTR mRNA destabilized cup1 sensor: (a) PSY580A, (b) cup1, and (c) cup1-MFA2. Histogram representations of mCherry intensity: (d) PSY580A, (e) cup1, and (f) cup1-MFA2.



Although both fluorescent microscopy and flow cytometry demonstrated no significant reduction in mCherry fluorescence with either mRNA destabilized strain tested after 2 hr, greater incubation may be required for destabilizing effects to be observed. To determine if the mRNA destabilization motifs had an effect on cup1 autoinduction over time, a fluorescent plate reader was used to monitor mCherry fluorescence over a 24 hr time period, with varying concentrations of copper (Figure 10).

Figure 10. Time course of fluorescence intensity for mRNA destabilized constructs treated with 0 to 100 ppm copper: (a) cup1, (b) cup1-8xARE, (c) cup1-MFA2, (d) absorbance of cup1 at 600 nm, and (e) comparison of mCherry fluorescence from each strain after 12 hr incubation. Each point represents the average fluorescence intensity of 4 technical replicates from one experiment. Error bars represent standard deviation.



Yeast cells expressing cup1, cup1-8xARE, or cup1-MFA2 were treated with 0–100 ppm copper, and then mCherry fluorescence was monitored over 24 hr and fluorescent readings were taken every 2 hr. In addition, growth of cup1 was also monitored by tracking the OD_{600}, which was also used as a measure of toxicity to copper exposure over time. Each engineered strain exhibited a similar dose response to copper from 0–100 ppm, with each showing the greatest mCherry fluorescence when incubated with 100 ppm copper (Figure 10). Maximum growth of cup1 was observed after roughly 12 hr, and copper concentrations up to 100 ppm had little to no apparent effect on growth over the 24 hr incubation period (Figure 10d). Thus, generalized toxicity was believed not to have affected reporter protein output. Intensities of mCherry for all strains correlated with growth, exhibiting

maximum fluorescence after approximately 12 hr, and intensities remained relatively stable for the remainder of the study for each copper concentration tested (Figure 10a, Figure 10b, Figure 10c). The fluorescence of each strain was compared after 12 hr, and in all cases, no significant variations in mCherry intensity were observed between any of the destabilized strains at each copper concentration, indicating no reduction in the sensor transcripts due to mRNA destabilization (Figure 10e).

However, it is important to note that in the Voon et al. (2005) study that used the N1 nonamer to decrease the half-life of a luciferase reporter, the tetracycline-controlled transcriptional activation system was used to control expression. In their system, the addition of the antibiotic tetracycline to the growth medium shuts down transcription by rapidly degrading any remaining mRNA, thus preventing translation of a new reporter. The copper sensor gene design in this study works in the opposite manner, because addition of copper to the growth medium turns on transcription. While the mRNA may have been degraded faster, it is likely accumulating to levels that yield significant levels of reporter, which may explain the lack of repression in cup1. As stated previously, qPCR experiments are necessary to determine if this is the case; nevertheless, alternative destabilization methodologies similar to the Tet-Off system will likely need to be explored to lower or eliminate autoinduction.

3.3 Protein destabilization elements as a means to control *CUP1* autoinduction

Cells are constantly synthesizing and degrading proteins as part of the central dogma of genetics. Many short-lived proteins, such as the transcription factor c-Fos or the metabolic enzyme mouse ornithine decarboxylase (mODC), contain specific amino acid sequences which target them for rapid proteolytic destruction (Loetscher et al. 1991; Salvat et al. 1999). One such motif is a PEST sequence, which is enriched in the amino acids proline (P), glutamate (E), serine (S), and threonine (T) (Rechsteiner and Rogers 1996). The presence of a PEST motif targets a protein for degradation by the proteasome or makes it more susceptible to enzymatic destruction. The PEST motif from mODC has been used in several studies to reduce the half-life of the stable reporter green fluorescent protein (GFP) ($t_{1/2}$ = 26 hr) (Li et al. 1998; Kitsera et al 2007). To decrease autoinduction from the cup1 sensor, we took a similar approach and fused the P1 PEST sequence from mODC (amino acid residues 422–461) to the C-terminus of

cup1-8xARE to create cup1-8xARE-PEST (Figure 2d). Copper dosing experiments with cup1-8xARE were performed as previously described in this section, and fluorescent microscopy and flow cytometry were performed to determine the effect of PEST on the reporter protein mCherry.

Fluorescence microscopy of yeast expressing cup1-8xARE-PEST demonstrated that addition of a PEST sequence significantly decreased autoinduction from the *CUP1* promoter (Figure 11). A decrease in reporter protein intensity was easily visualized in cup1-8xARE-PEST when compared to untreated cup1 (Figure 11b and Figure 11c). Fluorescence was also found to be visually similar to the control strain PSY580A (Figure 11a). In addition, mCherry output remained suppressed when dosed with copper up to 100 ppm (Figures 11d–11h). These visual observations were confirmed by ImageJ analysis, and fluorescence intensities were found to be significantly lower than for untreated cup1 (Figure 12). These findings suggested there was a decrease in autoinduction in cup1-8xARE-PEST; however, that decrease was at the expense of sensitivity as there was no apparent dose response to copper.

Figure 11. Fluorescence microscopy of cells expressing P1 PEST destabilized cup1 sensor; (a) PSY580A, (b) cup1, and (c) cup1-8xARE-PEST with 0 ppm copper, (d) 0.5 ppm copper, (e) 1 ppm copper, (f) 2 ppm copper, (g) 10 ppm copper, and (h) 100 ppm copper.

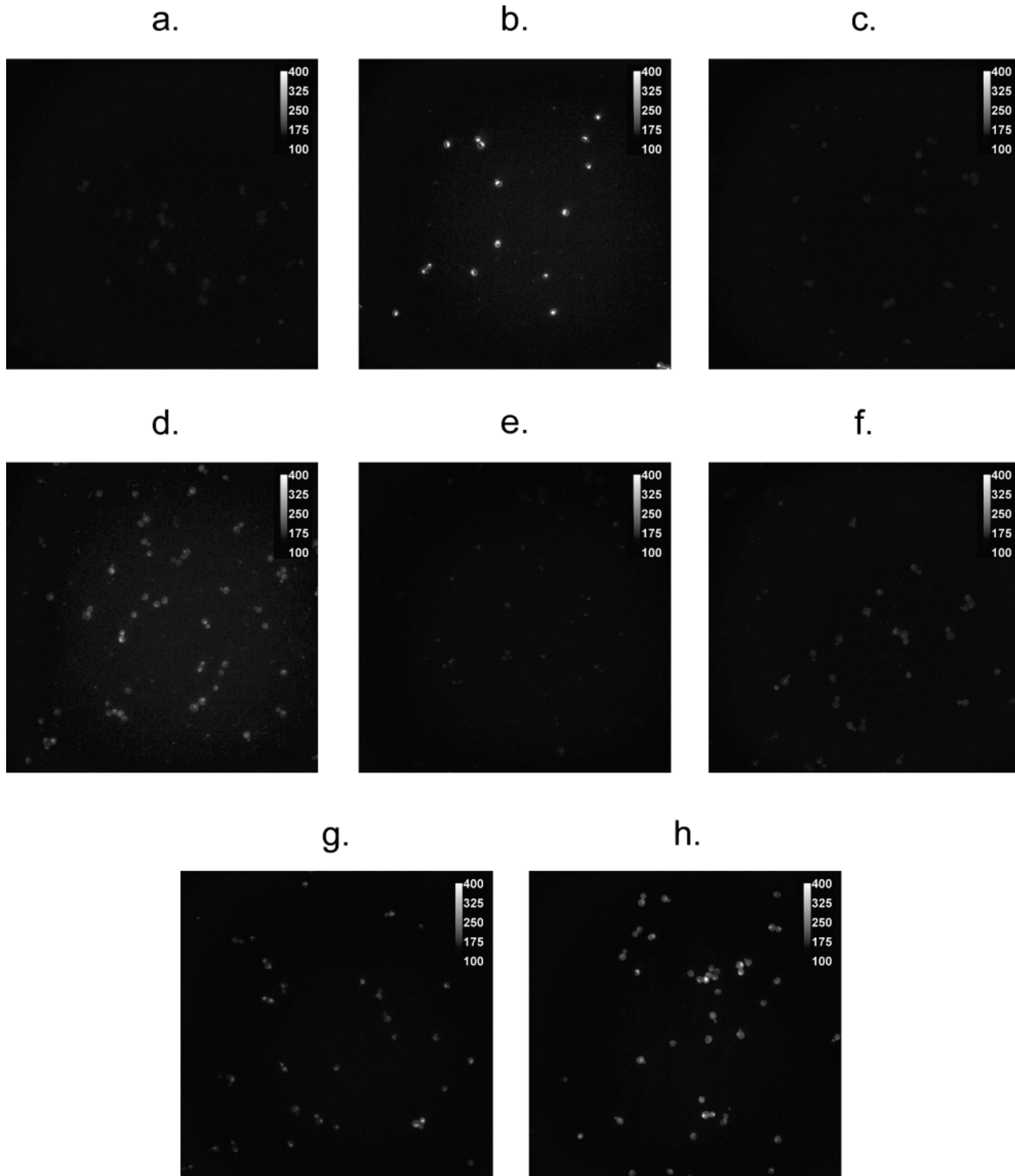
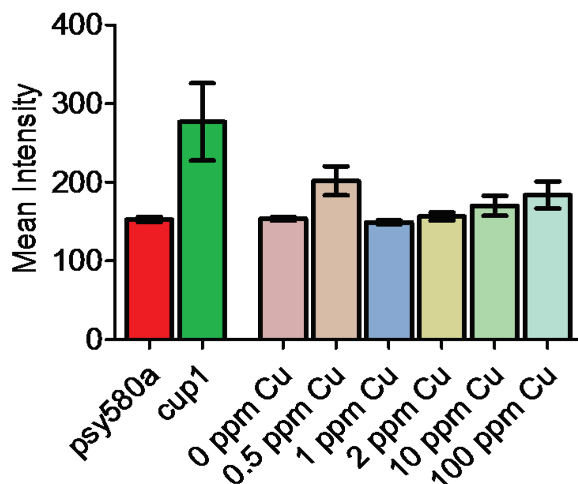
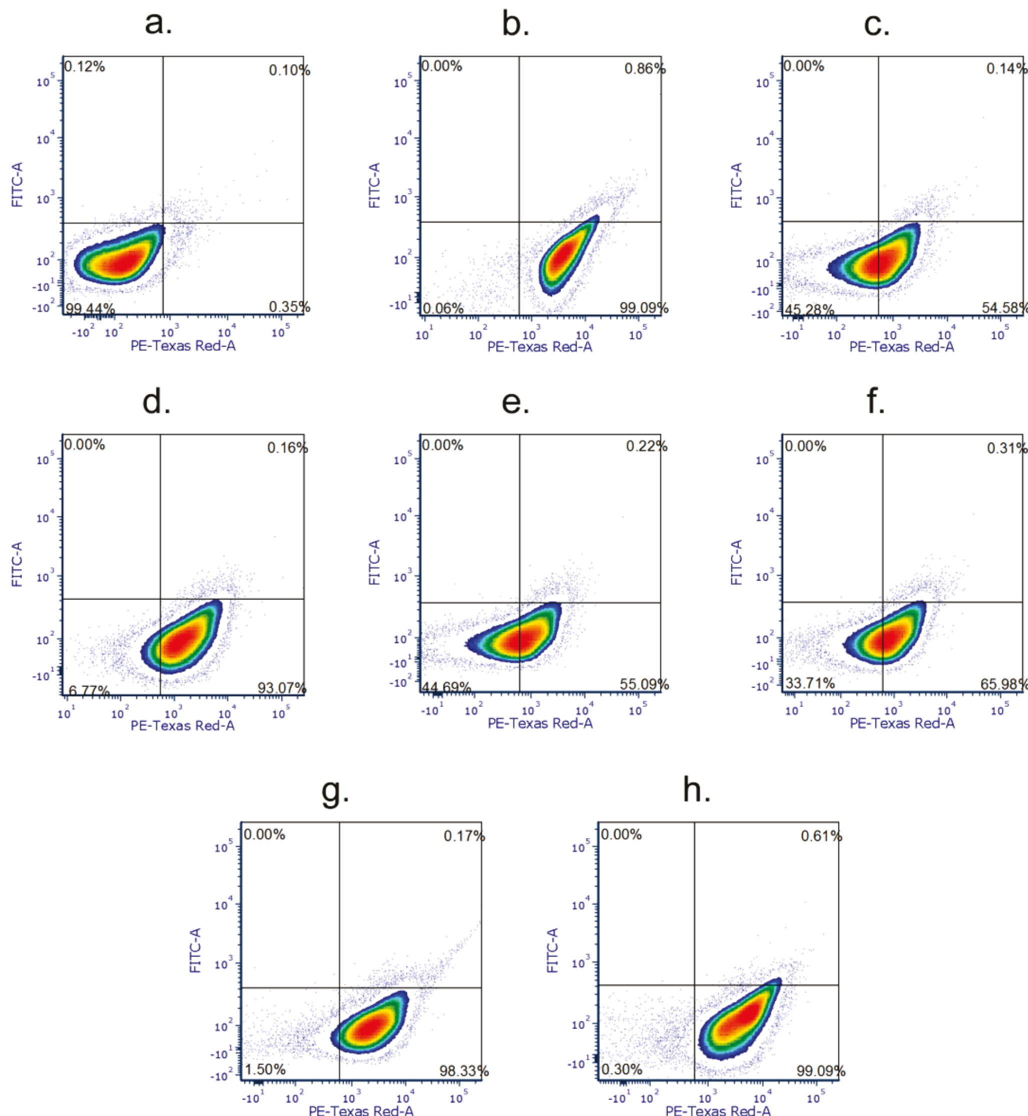


Figure 12. ImageJ quantification of cells expressing the P1 PEST destabilized sensor treated with 0 to 100 ppm copper. Each bar represents the average mean intensity (n = 10 cells). Error bars represent standard deviation. Quantification of cell fluorescence from cup1-8xARE-PEST demonstrates a dose response to copper.



Flow cytometry analysis was performed on 10^6 cells expressing cup1-8xARE-PEST. Similar to previous findings, the pys508a population (negative control) exhibited minimal fluorescence (<1%), whereas nearly the entire cup1 population (>99%) showed fluorescence associated with mCherry (Figure 13a, and Figure 13b). Untreated (0 ppm copper) cup1-8xARE-PEST population demonstrated roughly a 45% reduction in reporter protein (Figure 13c). The median mCherry intensities of PEST-destabilized cells in quadrants I and II were 383 and 847, respectively. The median mCherry intensities of PSY580A and cup1 were 164 and 3874, respectively. Because the reporter protein that is tagged with a PEST sequence will eventually become degraded, it was believed that the less-fluorescent population of cup1-8xARE-PEST expressing cells contains less reporter, due to degradation. Strain cup1-8xARE-PEST appeared to respond to copper to a certain extent in a dose-dependent fashion (Figures 13c–13h), where nearly the entire population exhibited fluorescence at a copper concentration of more than 10 ppm (Figure 13g and Figure 13h). The quadrant II - cup1-8xARE-PEST cells were treated with 1, 2, 10, and 100 ppm copper and had median mCherry values of 976, 1041, 2251, and 3964, respectively. The average median mCherry intensity of all copper-treated cells in quadrant I was 448±37, which is consistent with a weakly fluorescent population of cells. The decreased autoinduction from cup1-8xARE-PEST makes it a good candidate for evaluation as the first component in the memory sensor.

Figure 13. Flow cytometry analysis of cells expressing the P1 PEST destabilized sensor treated with 0 to 100 ppm copper; (a) PSY580A, (b) cup1, (c) cup1-8xARE-PEST treated with 0 ppm copper, (d) 0.5 ppm copper, (e) 1 ppm copper, (f) 2.5 ppm copper, (g) 10 ppm copper, and (h) 100 ppm copper.



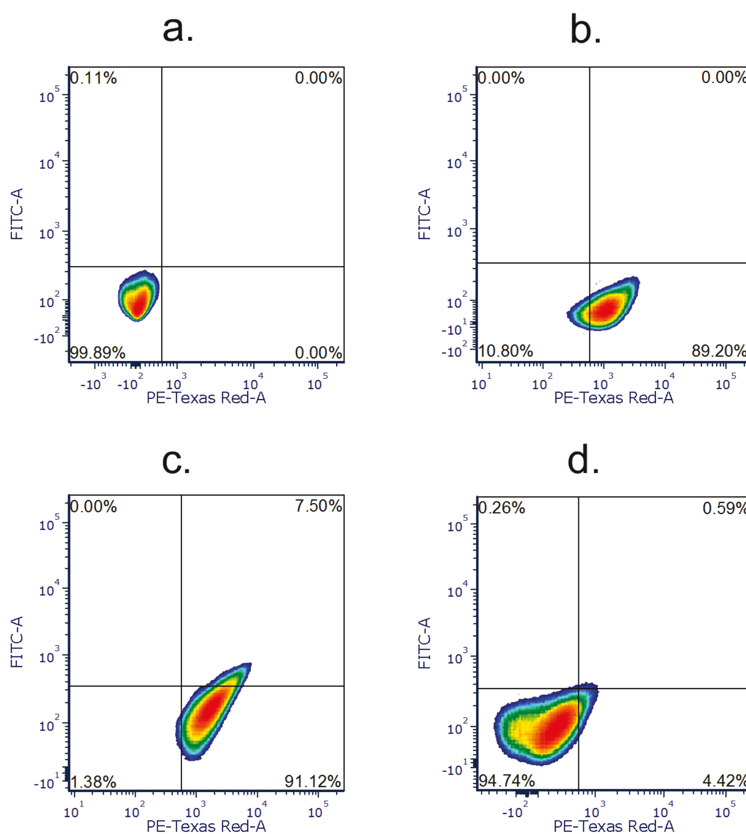
3.4 PEST-destabilized *CUP1* sensor gene used in a memory platform

To create a bistable memory system, expression of activator from the cup1 sensor gene should be off in the absence of copper. Ideally, induction of the cup1 sensor would turn on the autofeedback loop only above a certain threshold concentration of copper. Once the threshold is met, the autofeedback loop will sustain itself even in the absence of copper, providing memory of exposure. However, because of the leaky nature of the *CUP1* promoter, engineering a bistable system is challenging due to autoinduction and subsequent expression of the autofeedback loop in the absence of

copper. Nevertheless, it is not always possible to predict how a system will behave *a priori*. To determine if a copper-sensitive memory system could be constructed using the *CUP1* promoter, we engineered two yeast strains. One strain contained the leaky cup1-sensor with the autofeedback loop (Figure 2a and Figure 2e), and the other strain contained the destabilized cup1-8xARE-PEST sensor with the same autofeedback loop (Figure 2d and Figure 2e). Theoretically, exposure to copper will activate the first component of the system and induce production of RFP mCherry and LexA, and in turn, the proteins will bind with the *CYC1* promoter of the autofeedback loop contained in the second component. This protein binding induces production of Venus and another copy of LexA, which stimulate its own sustained production to provide memory to the system, as was shown in Figure 1.

Flow cytometry analysis of cup1-autofeedback or cup1-8xARE-PEST-autofeedback memory strains is shown in Figure 14. The entire population of the control strain PSY580A exhibited no fluorescence (Figure 14a) and approximately 90% of cup1 population expressed mCherry with a median intensity of 1148, but none of the cells were positive for *both* mCherry and Venus (Figure 14b). This finding was expected because the control strain did not have the autofeedback loop containing YFP. Over 91% of cup1-autofeedback sensor cells expressed mCherry with a median intensity of 1611, and 7.5% were positive for *both* mCherry and Venus with median intensities of 4519 and 475, respectively (Figure 14c). In contrast, only 4% of cup1-8xARE-PEST-autofeedback sensor cells expressed mCherry (with a median intensity of 691), and less than 1% were positive for both mCherry and Venus, having median intensities of 848 and 391, respectively (Figure 14d). Production of mCherry and Venus in the cup1-autofeedback demonstrated the functionality of the two-component memory system, and the significant reduction in cells expressing mCherry and Venus in cup1-8xARE-PEST-autofeedback demonstrated PEST control of autoinduction in the absence of copper.

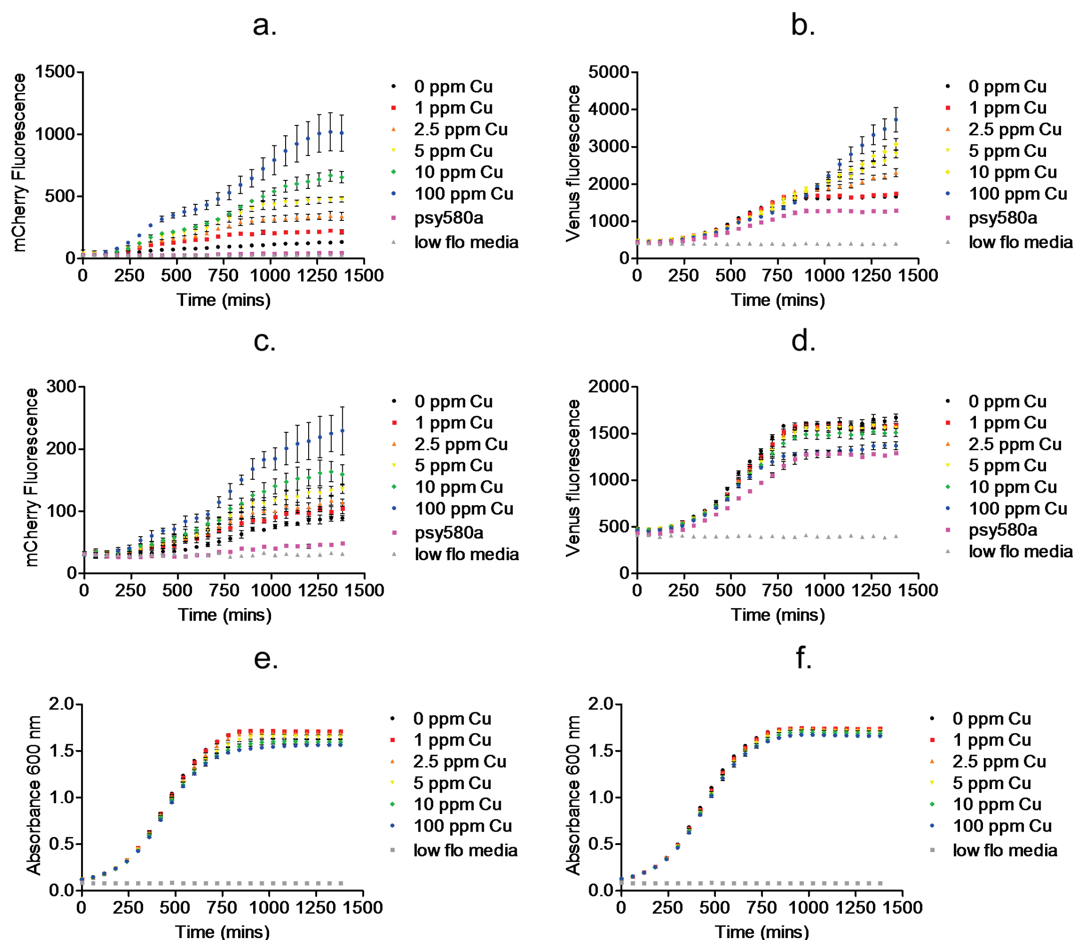
Figure 14. Flow cytometry analysis of untreated (0 ppm copper) cells expressing autofeedback constructs; (a) PSY580A, (b) cup1, (c) cup1-autofeedback, and (d) cup1-8xARE-PEST-autofeedback.



To determine if the autofeedback loop is activated with copper at certain points during growth, a plate reader was used to monitor the development of mCherry and Venus in cells expressing either the cup1-autofeedback sensor or the cup1-8xARE-PEST-autofeedback sensor (Figure 15). Both autofeedback sensors were found to produce a dose response from 0 to 100 ppm in the presence of increasing copper. This became apparent after roughly 6 hr of growth. Dose like differences in Venus fluorescence began after 10 hr of growth. After 16 hr of growth, the Venus fluorescence of cup1-autofeedback increased significantly for cells treated with 2.5, 5, 10, or 100 ppm copper (Figure 15b). However, by this time, the Venus fluorescence of cup1-8xARE-PEST-autofeedback had already begun to stabilize (Figure 15d). Most of the prominent changes in mCherry fluorescence took place in the log phase of cell growth, while most changes in Venus fluorescence took place near when the cells were in stationary phase (Figure 15e and Figure 15f). This delay in Venus fluorescence likely results from delayed accumulation of autofeedback loop components and reporter maturation. In *S. cerevisiae*, YFP has been documented to take up to 40 min to

fold and mature (Gordon et al. 2007). Both strains containing the autofeedback loop responded to copper by producing mCherry in a dose-dependent manner. The fluorescence intensity of the PEST-destabilized strain was much less overall (i.e., lower dynamic range). Cup1-autofeedback produced Venus in a dose-dependent manner, particularly at concentrations greater than 2.5 ppm. Venus levels from cup1-8xARE-PEST-autofeedback were not considerably greater than a negative control. As with the previous evaluation, no significant differences in growth could be attributed to the inserted constructs, and no apparent toxic response was exhibited by increasing concentrations of copper (Figure 15e and Figure 15f). Ultimately, these results demonstrate that the PEST destabilization autofeedback loop was turned on in the absence of copper.

Figure 15. Time course of fluorescence for autofeedback strains treated with 0 to 100 ppm copper; (a) mCherry fluorescence cup1-autofeedback, (b) Venus fluorescence cup1-autofeedback, (c) mCherry fluorescence cup1-8xARE-PEST-autofeedback, (d) Venus fluorescence cup1-8xARE-PEST-autofeedback, (e) cup1-autofeedback growth curves, and (f) cup1-8xARE-PEST-autofeedback growth curves.



However, it is important to note that although copper was excluded from the medium, trace amounts about the experiments done in this study was that copper levels in the components used to create the yeast media were not controlled. This was done in order to determine if a copper-sensitive memory platform could be created without stringent growth requirements. At minimum, the yeast media contains 0.04 ppm copper as a contribution from the nitrogen base. This minimum does not include the sugar, double deionized water (ddH_2O), or drop-out mix. It may be worth using ICP-OES to verify copper levels before each experiment. To rule out effects from trace levels of copper, future experiments will be performed in media prepared from low-fluorescence, copper-free nitrogen base, and 18 MΩ water. Copper levels will be verified using ICP-OES.

4 Conclusions

We have developed an *S. cerevisiae* sensor for the detection of copper. The sensor responds robustly to treatment with 0–100 ppm copper. However, in the absence of copper the system is still highly active. For detection of copper in drinking water sources, a leaky system is not ideal. We explored the use of various mRNA and protein destabilization elements to in an effort to eliminate background signal from the system. We determined that the addition of the P1 PEST protein destabilization element to the copper sensor dramatically reduced background expression from the system. The destabilized sensor responds robustly to copper in excess of 1 ppm. This is beneficial because the World Health Organization (WHO) states that safe drinking water should not contain more than 2 ppm copper. However, despite destabilization, the system still remains on in the presence of low levels of copper. Our results demonstrated that the second component in the memory platform, the autofeedback loop, is still turned on in the absence of copper, despite destabilization efforts. Thus, due to the pervasive leaky nature of the copper promoter, it is not certain whether that will be a possibility. An important point to note is that the copper levels were not controlled in the media used to culture the yeast in the experiments outlined in this report. At minimum, the media could have contained about 0.04 ppm copper. To rule out effects of sources of exogenous copper, future work will focus on studying the behavior of the copper memory platform in the total absence of exogenous copper. This work will be accomplished in conjunction with the ICP-OES analysis.

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Appendix: DNA Sequences and Vector Maps

Native cup1

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Cup1-8xARE

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Cup1-MFA2

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Cup1-8xARE-PEST

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Autofeedback loop

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Figure A1. Plasmid pRS305 (www.snapgene.com/resources).

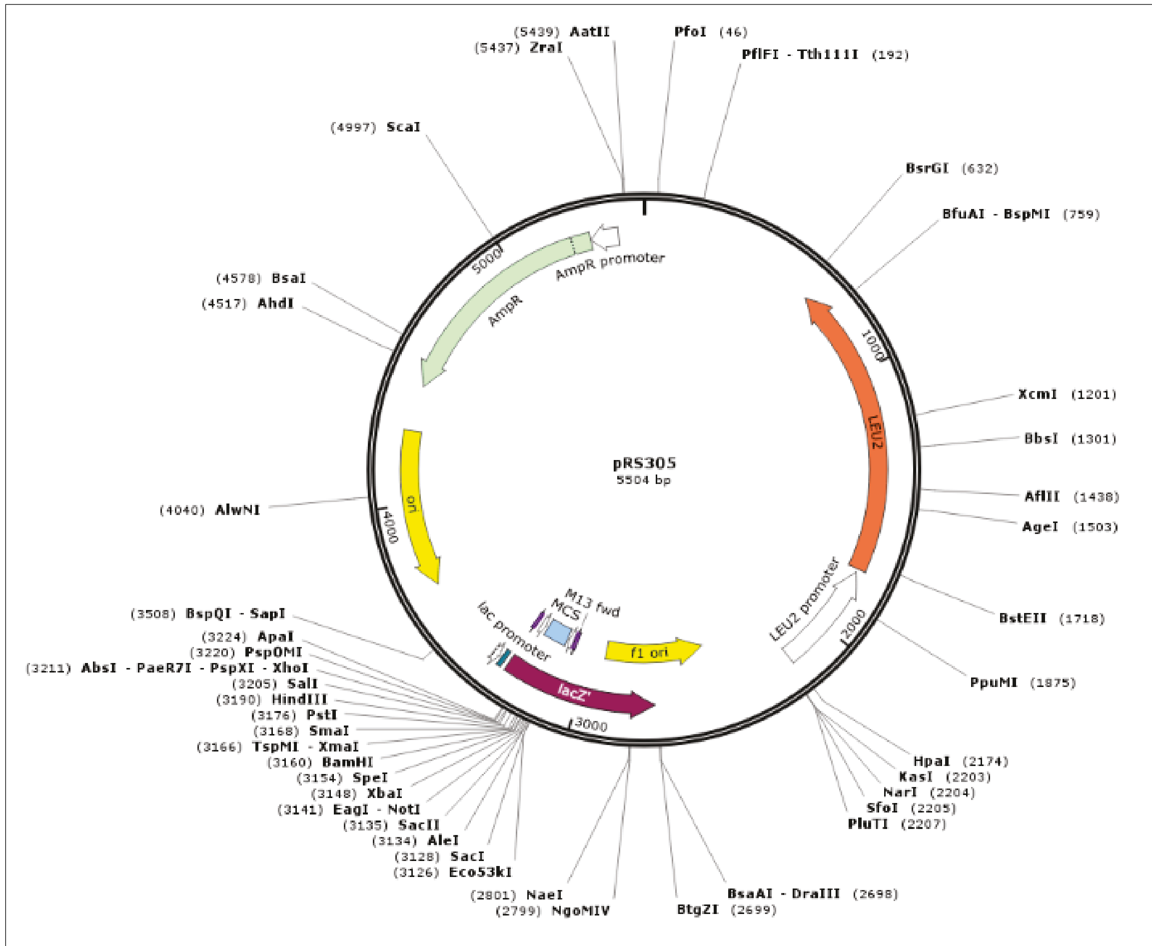


Figure A2. Plasmid pRS306 (www.snapgene.com/resources).

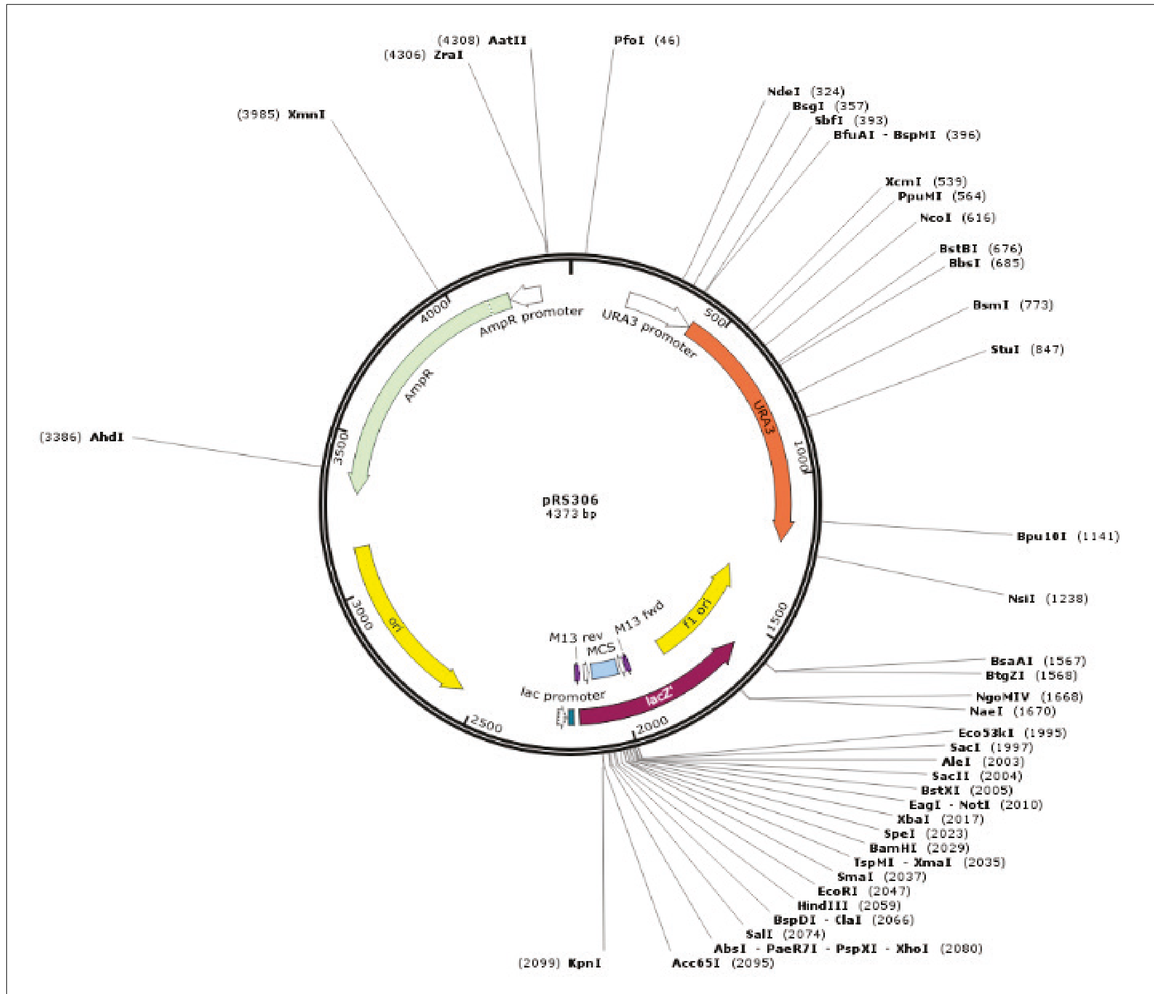
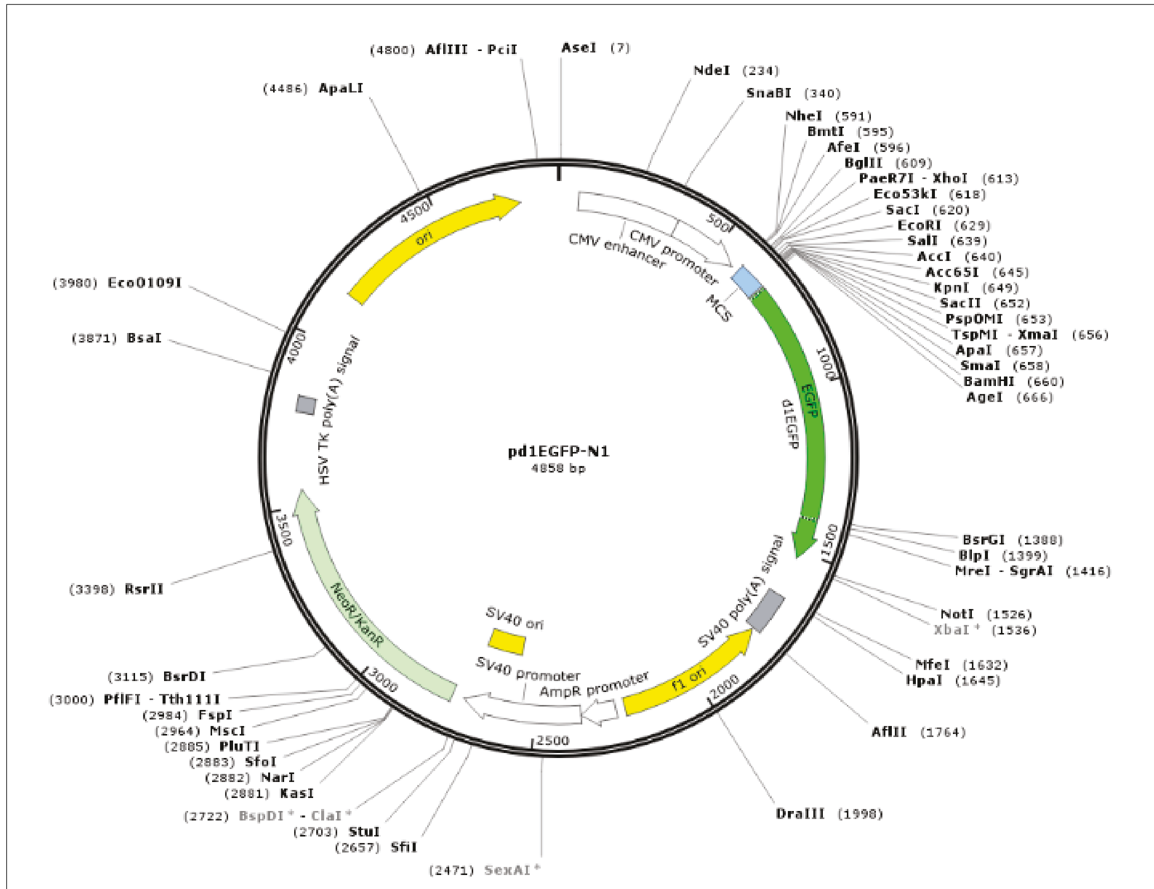


Figure A3. Plasmid pd1EGFP-N1 (www.snapgene.com/resources).



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14. ABSTRACT

As of 2017, the United Nations estimated that globally, over 80% of all wastewater is released into the environment without treatment. Often, the water is contaminated with toxic heavy metals, which ultimately enter potable water supplies. Contamination is especially prevalent in low- and middle-income countries, a fact that poses a significant threat to indigenous populations as well as to deployed troops in these areas. Standard methods for detecting trace levels of metals in water requires expensive equipment and highly trained personnel, both of which most developing countries lack. To address this issue, authors designed a fluorescent yeast biosensor capable of detecting a model heavy metal, copper. The biosensor was responsive to copper at concentrations from 1 to 100 ppm. However in the absence of copper, autoinduction was observed. To decrease autoinduction, researchers explored the use of various mRNA and protein destabilization motifs such as adenylate-uridylate-rich elements and peptide sequences rich in proline, glutamic acid, serine, and threonine. Preliminary results demonstrated that the addition of destabilizing mRNA had no effect on levels of autoinduction within the system. However, integration of protein destabilizing amino acids effectively reduced autoinduction, but not without also decreasing the dynamic range of the sensor system.

15. SUBJECT TERMS

Water—Pollution, Drinking water—Contamination, Heavy metals, Water quality, Water--Sensory evaluation, Biosensors

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