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University of California, Riverside

Undergraduate Research Journal

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UC RIVERSIDE UNIVERSITY OF CALIFORNIA

University of California, Riverside
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From the Administration

Across the sciences, social sciences and humanities, the opportunity to participate in undergraduate research and creative activities can open magical new worlds for students at the University of California, Riverside.

Many UCR students have the chance to explore new concepts, investigate complex questions and advance and test their own hunches as they learn the rigor of the scientific method, the creativity of experimental design, the joy of scholarly research and personal expression, and the discipline and hard work of writing. We are proud that by the time they graduate, more than 50 percent of UC Riverside undergraduates will have participated in faculty-mentored research or creative projects.

It is a pleasure to present this year's *Undergraduate Research Journal*, which showcases the academic discoveries and creative endeavors of some of our talented undergraduates. I invite you to share the journeys they detail here. I know you will be inspired, as I am, by the quality of the work they have achieved.

Kim A. Wilcox
Chancellor

You hold in your hands the Tenth Annual *UC Riverside Undergraduate Research Journal*. It provides a selective, peer-reviewed venue featuring the very best faculty-mentored undergraduate research and scholarship on our campus.

I want to congratulate the young scholars whose work appears herein. The process of discovery can be filled with excitement but also with frustration, as we search for the golden threads that tie together the ideas we have been pursuing and the findings that have emerged from our work. During this process, we travel a path that no one has been on before. The journal article is the culmination of that process—a formal presentation to our community of peers and mentors of what we found on that journey. Authors, place this volume on your bookshelf. Pull it down occasionally from the shelf to re-read and to remind yourself of the journey you traveled. I wish you many more such journeys in the future.

I know this edition of the *Journal* will be read by many more people than authors and editors. When you read it, I hope you will feel pride in the accomplishments of these UCR students. These students have decided to make the most of their college years as a time for professional development in research. They have looked for the big picture and they have sweated the details. In the future I hope that many more UCR students will see the opportunities for growth that faculty-mentored research can provide and that many more of our faculty will find ways to help their students experience the growth that a successfully sustained research project so often brings.

The Student Editorial Board, under the general supervision of the Faculty Advisory Board, has led the peer review process with the strong results you see before you. I would like to thank Gladis Herrera-Berkowitz, Director of Student Success Programs, for conscientious, caring, and timely organizational work that helped to bring this edition of the *Journal* to fruition. Thanks also to UE student coordinators, Julianne Rolf and Sierra LaPoint, who assisted in the production of the *Journal*.

This is the last edition of the *Journal* that will appear during my tenure as Vice Provost. I feel a special gratitude to have worked over the last five years with so many creative and hard-working students, staff, and faculty on undergraduate research projects. These projects have been central to our growing reputation as the UC campus most engaged with and most successful in fostering undergraduate research, scholarship, and creative activity.

Best regards,

Steven G. Brint
Vice Provost for Undergraduate Education
Professor of Sociology

UCR Undergraduate Research Journal

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"It is our great honor to present UCR's Tenth Annual Undergraduate Research Journal, which represents the passion and commitment to undergraduate research and creative activity of the student authors, their faculty mentors, the Student Editorial Board and the Faculty Advisory Board. Congratulations to the students who put forth the effort to get published and to the Editorial Team for their diligent work in ensuring that the published articles met high standards and represented the best work from our university. We are proud to have worked with such an amazing team of devoted and enthusiastic individuals and to contribute to the legacy of excellent undergraduate research that UCR has cultivated for the past decade."

- Student Editorial Board

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From the Faculty Advisory Board

Since UCR's *Undergraduate Research Journal* started, it has published almost 120 scholarly articles across many fields. These papers represent the commitment of many of our undergraduates to performing independent research as part of their experience at UCR. The *Journal* thus fills a critical need for our students. More often than not, undergraduate research forms part of a larger work with many contributors, which can mean a dilution of the student's contributions as well as a longer wait time between completion of the work and its publication. With the *Undergraduate Research Journal*, our students can write about their specific research findings and get first-author credit. They can publish before the end of the academic year, and gain the experience of seeing their manuscript go through a peer-review and publication process just like articles in a standard research journal. When the paper becomes a part of a student's professional experience, it contributes to their record of scholarly achievement in a special way.

The *Journal* is managed primarily by undergraduates who form the Student Editorial Board, working with members of the Faculty Advisory Board. We owe a debt of gratitude to the students for their professionalism and dedication to the timely review and preparation of the articles you see here. We are also grateful for the participation of the members of the Faculty Advisory Board in guiding the student reviewers.

If you are interested in publishing your undergraduate research at UCR, consider submitting to our next issue!

Dr. Morris F. Maduro
Chair of the Undergraduate Research Journal Faculty Advisory Board
Professor of Biology

About the Cover...

"Follow You Into the Wild" by Aishwarya Veerabahu

I believe that nature's beauty is not always obvious or easy. It often demands a colossal effort to learn its ways and appreciate its finesse. For example, I had to get on wet dirt ground and wedge myself underneath a bush with my back against the jagged edge of a rain gutter in order to capture this photo. It is of a plant that is known to be thigmotropic—it grows in response to stimuli the plant touches. To me this photo represents the adventure of pursuing knowledge—the wilderness of all that we have yet to discover. For when a question is asked that has no answer, the unknown stimulates our sense of curiosity. Scientists, just as this wild plant has done and will always do, instinctively respond to that stimulus. To grow toward it. To learn. To enlighten others in turn. To pursue knowledge. To follow science into the wild.

Instagram: @aishwarya_photography

Aishwarya Veerabahu

Department of Biology

Aishwarya is a second-year biology major and is working towards a career as a cardiothoracic surgeon. She enjoys photography and music as creative outlets. She is currently pursuing research in the field of organic chemistry and is learning to play the drums. She gratefully attributes the multifold growth in her passion for science to the support from her peers, teaching assistants, professors, and to the caliber of excellence and enthusiasm they bring to UCR every day.

Adult *C. elegans* Exhibit Physiological Abnormalities When Early Gut Development is Partially Compromised

Kollan Doan and Morris F. Maduro
Department of Biology

ABSTRACT

The nematode *Caenorhabditis elegans* displays developmental robustness, such that nearly all embryos show normal development across a range of conditions. We are investigating properties of adults derived from embryos that are partially compromised for a very early step in the specification of the intestine (gut) primordium. As the gut provides essential nutritional functions to the animal, we hypothesize that is there are any abnormalities remaining in these adults, they might be detectable through indirect measurements of characteristics that depend on normal metabolism and physiology. Here, we examine adults derived from strains in which early embryonic gut development has been partially compromised and quantify three physiological properties: resistance to oxidative stress by exposure to hydrogen peroxide, mean rate of pharyngeal pumping, and average life span. Our results show that when specification is mildly compromised, oxidative stress resistance increases by 35%, pharyngeal pumping decreases by 11%, and life span decreases by 20%. In a more severely compromised strain, oxidative stress resistance decreases by 11%, pharyngeal pumping decreases by 27%, and life span decreases by 23%. The results show that function of the intestine, and general metabolic health, are indeed affected when gut specification is compromised. We propose that in *C. elegans*, proper function of the adult gut requires robust early progenitor specification, and that there are limits to the ability of later gut development to compensate for early perturbations in specification.

Keywords: *C. elegans*, gut specification, differentiation, adult defects, longevity, oxidative stress, pharyngeal pumping, metabolism

FACULTY MENTOR

Morris F. Maduro
Department of Biology

Morris F. Maduro is a Professor in the Department of Biology and a past recipient of a Distinguished Teaching Award at the University of California, Riverside. His work focuses on the ways in which genes direct the development of animals, using the nematode *C. elegans* as a model system. Undergraduates are regularly involved in research in his laboratory.

AUTHOR

Kollan Doan
Department of Biology

Kollan Doan is a second-year Cell, Molecular, and Developmental Biology major. His research investigates the relationship between gene regulatory networks and gut specification in the model system, the nematode *Caenorhabditis elegans* under the guidance of Dr. Morris Maduro. He is a member of CNAS Science Ambassadors and Delta SIFY in which he works with outreach to inspire underprivileged students in pursuing STEM careers. He plans to apply to medical school within the next few years.

Kollan Doan

INTRODUCTION

The nematode *Caenorhabditis elegans* is an ideal model system for genetic studies due to its short life span and simple anatomy. Embryonic development takes approximately 12 hours at 25°C, whereupon the embryos hatch as first-stage larvae (L1). Approximately 26 hours after hatching and three larval molts (L1, L2, and L3), the animal enters the fourth-stage larvae (L4) whose prominent feature is the developing vulva. After 10 hours and a final molt, the animal develops to a young adult. The young adult matures to a full adult capable of egg-laying after eight hours. The adult consists of approximately 1000 cells, ~300 of which are neurons (Sulston et al. 1983). The *C. elegans* intestine has been a useful model for organ development and function. The fully developed intestine is a simple tube consisting of 20 cells at the end of development with a total of 34 nuclei at adulthood. The gene network that controls specification of the gut progenitor, a single embryonic cell called E, is well understood (Maduro 2006). The E cell is specified through the activation of several genes in simple gene network. The maternal factor SKN-1 activates the zygotic *med-1,2* GATA factor genes whose products contribute, along with input from maternal factors POP-1 and PAL-1, to the activation of the *end-1,3* genes to specify the E fate (Maduro et al. 2001, 2007). Downstream of specification, the GATA factors ELT-2 with contribution from ELT-7 maintain the intestinal fate, through initial activation of their genes by END-1,3 and autoregulation of *elt-2,7* to maintain their expression (McGhee et al. 2009; Fukushige et al. 1998). The simplest interpretation of this model is that gut development follows two phases: an early specification phase that gives the gut progenitor cells their identity, followed by a differentiation phase that maintains the intestinal fate.

In this work, we are interested in the extent to which the differentiation phase is self-correcting for mild defects in specification. In many systems, when early embryonic development is partially compromised, organ development undergoes canalization to result in an otherwise normal tissue (Waddington 1942). It is thought that canalization normally buffers stochastic differences among embryos that result from changes in the environment and variations

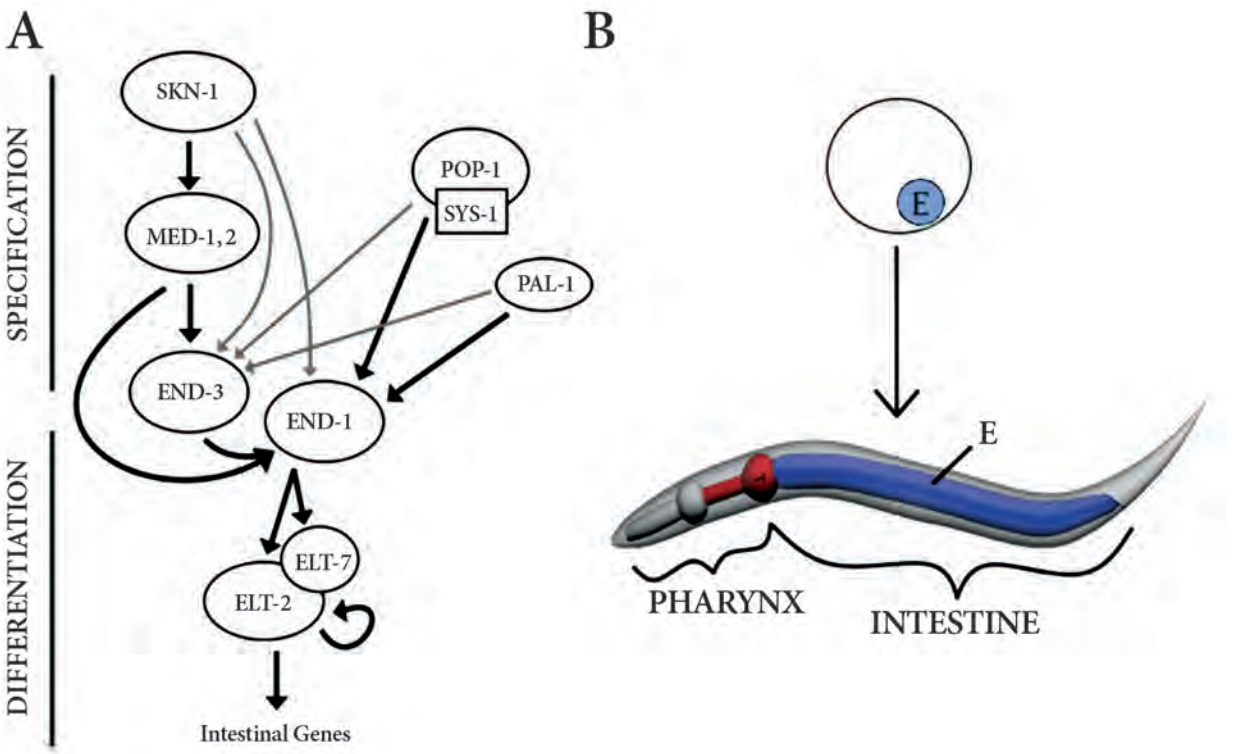

Figure 1: *A)* C. elegans *gene regulatory network for the specification of E showing required convergent upstream inputs of SKN-1, POP-1/SYS-1, and PAL-1 (Maduro 2008). B) Progenitor cell E produces the entire intestine (colored blue).*

Table 1: *Summary of strains used in experiments. Data from Maduro et al. 2007, 2015.*

Strain	Description	% of Embryos Making Any Amount of Gut Multicultural
MS1810	*end-1*(-), *end-3*(-) double mutant carrying wild-type *end-1*(+) and *end-3*(+) transgene, used as a control	100% (n>200)
MS1809	*end-1*(-), *end-3*(-) double mutant carrying *end-1*(+) and *end-3*(+) transgene, lacking binding sites for the MED proteins	75% (n=459)
MS404	*med-1*(-), *end-3*(-) double mutant	42% (n=251)

in gene expression. Unlike most other systems, the *C. elegans* embryo is "mosaic": if an embryonic cell is unable to adopt a specified lineage or is missing entirely, the normal descendants will not be replaced (Sulston et al. 1983). When the gene network that specifies gut in *C. elegans* is partially compromised, not all E lineage cells adopt a gut fate in all embryos (Maduro et al. 2015). The worms that do create a functional gut and survive into adulthood exhibit a variety of visible defects that suggest metabolic function is impaired, but this has not yet been tested directly (Maduro et al. 2015).

In this study, we determined the degree to which survival of partial gut differentiation manifested a change in the physiological state of animals compared to controls, focusing on three quantifiable behaviors with implications in metabolism. The first of these was resistance to oxidative stress. Endogenous or exogenous reactive oxygen species (ROS) can damage various components of the cell. These radicals, resulting from oxygen consumption during metabolism, can impair function by oxidizing macromolecules (Golden et al. 2002). *C. elegans* can be subjected to oxidative stress by exposure of compounds to assess for the ability to withstand free radicals. Hence, we evaluated survival time of animals in liquid buffer containing 3 mM hydrogen peroxide. The second behavior measured was pharyngeal pumping. To consume nutrients, usually bacteria or single-celled fungi, *C. elegans* larvae and adults pump food into the intestine using the pharynx, an organ that is functionally equivalent to the human esophagus. The rate of pharyngeal pumping is regulated depending on the presence of food, and function of the pharynx muscles themselves depends on the overall health of the animal (Croll 1978; Horvitz et al. 1982; Chiang et al. 2006). We measured the rate of pharyngeal pumping on agar plates in the presence and absence of food (bacteria) by direct microscopic observation. The third behavior measured is life span. The ability of *C. elegans* to survive under laboratory conditions is directly related to their overall health (Golden et al. 2002). To measure life span, we maintained animals on agar plates in the presence of food, eliminating animals that ceased movement and became unresponsive to touch.

METHODOLOGY

Nematode Strains and Animal Handling. Three strains were used, consisting of a control (MS1810) and two strains that exhibit compromised gut specification: MS1809 and MS404. Shown in Table 1, MS1809 has mild defects in gut specification such that its embryos make some amount of gut approximately 75% of the time. Strain MS404 has more severe defects in gut specification and whose embryos make gut approximately 42% of the time. Strains were age-synchronized by rinsing and bleaching of gravid adult worms. Synchronized embryos were collected via centrifugation then pipetted onto petri dishes consisting of Nematode Growth Medium (NGM) and seeded with OP50, an *E. coli* bacterial strain that is standard for growing *C. elegans* (Brenner 1974). Embryos were left to hatch and raised at 23°C for approximately three days until late L4/early adulthood was reached. Age-synchronized adult worms were randomly picked and allocated for use in all experiments. Assays for oxidative stress resistance, pharyngeal pumping rate, and life span were performed in triplicate according to previously established procedures, summarized below (Kenyon et al. 1993; Larsen 1993; Chiang et al. 2006).

Oxidative Stress Resistance. To measure resistance to oxidative stress, commercially available 3% Hydrogen Peroxide (H_2O_2) was diluted down from approximately 882 mM to 3 mM in M9 buffer. Fifty microliters of 3 mM H_2O_2 were pipetted into six wells each per strain of a 96-well microtiter plate. Four worms at late L4/early adulthood

Kollan Doan

were allocated per well across six wells for a total of 24 worms per strain. Observations lasted over a period of eight hours beginning with t = 0 hours and recording the number of worms alive after each hour. The assay was repeated three times and the number of worms alive each hour was summed to determine the average number of worms alive each hour per strain. Student's t-test was used to determine significance in differences between control and compromised strains.

Pharyngeal Pumping. To determine pharyngeal pumping rates, 70 worms per strain, synchronized at late L4/early adulthood stage, were transferred to new plates. Half were transferred to plates seeded with OP50 as food with the other half transferred to unseeded plates with no food. Observations began 30 minutes after transferring worms to plates to allow for acclimation. The pharyngeal pumping rate was defined as the number of contractions in the pharyngeal terminal bulb in a time of one minute. An ANOVA test was use to analyze the differences in pharyngeal pumping rate among and between the control strain and compromised strains.

Life Span. Animals grown under the conditions described above were selected for longevity assay as follows. Fifty age-synchronized worms at late L4/early adulthood from each strain were transferred to new, seeded plates every day for three to four days until egg-laying ceased. The worms were then transferred every two to three days to new plates until all worms were deceased, ascertained by cessation of movement and lack of response to gentle touch. Observations were taken daily, beginning with day one of adulthood, and the number of worms that were still alive on each subsequent day was recorded. Worms that died prematurely due to hatching of embryos inside the mothers, or who escaped the agar surface, were censored from the results. Student's t-test was used to determine significance between control and compromised strains.

RESULTS

Resistance to Oxidative Stress. We evaluated survival of adults in the presence of 3 mM hydrogen peroxide (H_2O_2). Mean survival time was calculated from the survival of individual animals over an eight-hour time period.

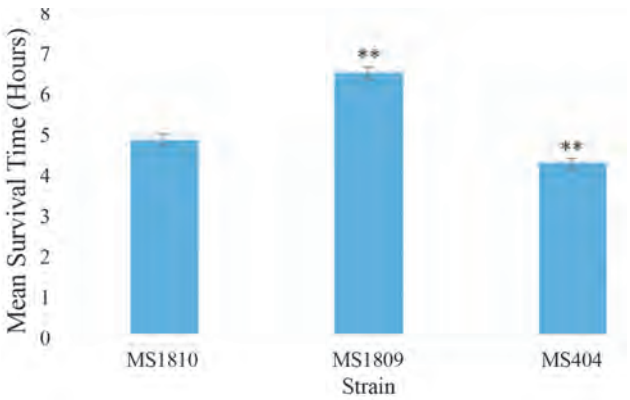

Figure 2: *The mean surviva:l time of adults in 3 mM hydrogen peroxide. **Values were significant between wild-type and compromised strains.*

The mean survival time of control MS1810 animals was 4.8 ± 0.13 hours. We found that the mean survival time of MS1809 was higher, 6.5 ± 0.15 hours. Student's t-test determined that this increase was significant ($P < 0.0001$). While MS1809 experienced increased survival in H_2O_2, MS404, a highly gut compromised strain, showed a decrease in mean survival time of 4.25 ± 0.14 hours compared to the control and mildly compromised strain. Statistical analysis showed that the susceptibility of MS404 to oxidative stress was significant ($P = 0.0033$).

Pharyngeal Pumping Rate. We measured the rate of pharyngeal pumping of our strains in the presence and absence of bacteria as described in Figure 3. In the

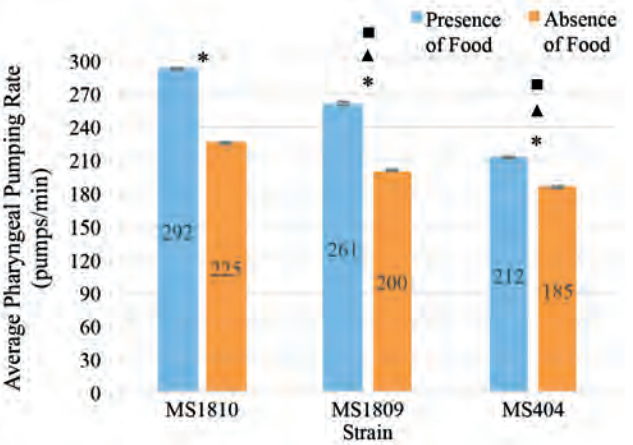

Figure 3: *The average pharyngeal pumping rate of MS1810, MS1809 and MS404 in the presence and absence of food (mean ± SEM). *Values were significant between groups in the presence and in the absence of food. ▲Values were significant when compared to wild-type in the presence of food. ■Values were significant when compared to wild-type in the absence of food.*

presence of food, the pharyngeal pumping rates across the three strains MS1810 (control), MS1809, and MS404 were measured to be 292 ± 1.5, 262 ± 1.7, and 212 ± 1.2 pumps/minute, respectively. In the absence of food, the pharyngeal pumping rates were 225 ± 1.3, 200 ± 1.1, and 185 ± 1.2 pumps/minute respectively. The decreases seen across all three strains were consistent, confirming that as the gut specification became more compromised, surviving adults showed significantly slower pharyngeal pumping rates ($P < 0.0001$).

Life Span. Using the control strain MS1810 as a baseline, we determined that both strains exhibiting embryonic gut defects experienced decreases in adult life span. The average life spans of MS1810, MS109, and MS404 were 10.7 ± 0.88, 8.5 ± 0.63, and 8.2 ± 0.52 days, respectively, as shown in Figure 4. We determined that the average life span of MS1809 adults was 20% lower ($P = 0.0515$) and that of MS404 was 23% lower ($P = 0.0178$) than the control.

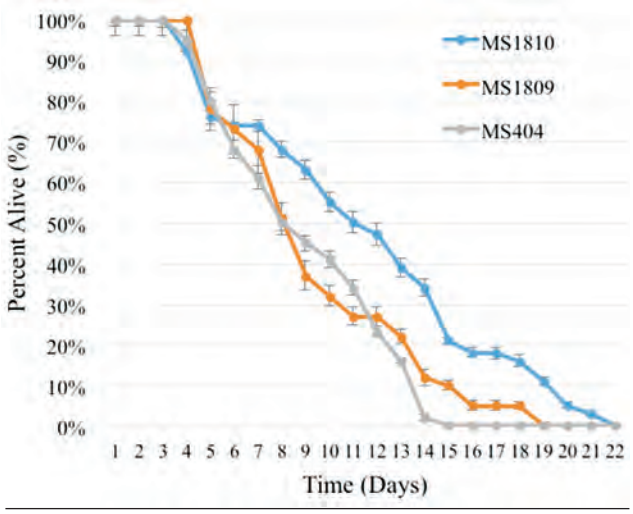

Figure 4: *Adult life span of the control strain to gut compromised strains. Differences between groups were significant ($P = 0.0237$).*

DISCUSSION

We found that partial gut differentiation generally led to physiological defects with more severe abnormalities exhibited from a more compromised strain, with one exception described below. Our data shows that *C. elegans* animals are unable to compensate for early gut specification defects, resulting in mild phenotypic abnormalities in the adult. This suggests that the developing intestine does not compensate for the consequences of partial specification, even though a relatively normal morphology gut is produced (Maduro et al. 2015). It was reported that the hypomorphic specification adults exhibit a slight increase in the number of gut nuclei, an increase in approximately five extra nuclei on average. These extra nuclei do not appear to be the direct cause of the physiological defects, as gain-of-function mutants in *cdc-25.1* that result in an even greater number of nuclei have not been observed to have other defects in gut function (Kostić et al. 2002).

In the mildly compromised strain MS1809, we observed an increase in resistance to oxidative stress, while in the more severely compromised strain MS404, we observed a decrease. This result was unexpected for two reasons. First, the other two behaviors measured, pharyngeal pumping and life span, both decreased in the compromised strains. Second, resistance to oxidative stress has generally been observed to correlate with an increased life span, not a decreased one (Kenyon 2010). We measured oxidative stress in MS1814, a strain with similar genotype to MS1809 but constructed in parallel, and found the same results as with MS1809 (data not shown), suggesting that the increased oxidative stress resistance in MS1809 is not the result of a spurious background mutation. Rather, the results suggest that a mild perturbation of gut specification results in the activation of a pathway that increases resistance to oxidative stress, but a stronger compromise blocks this pathway. A further experiment to determine whether pathways involved in oxidative stress resistance are indeed activated in MS1809 but not MS404 could be used to test this hypothesis of degree in gut specification.

The decreases in pharyngeal pumping and life span, and the changes in resistance to oxidative stress, collectively suggest that metabolic function is altered in the specification-compromised strains. Consistent with this, unpublished results from our laboratory show that the MS1809 and MS404 strains exhibit increases in lipid storage in the intestine. Excess lipid storage is analogous to an increase in fat cells in mammals, suggesting that our *C. elegans* strains have altered metabolism in favor of energy storage. We interpret our collective results to mean that partially-compromised gut specification results in a change to an alternate metabolic state that affects metabolism, causing changes in a variety of physiological properties such as resistance to oxidative stress and overall health. This metabolic state is similar, but not identical, to effects observed

under dietary restriction—when normal animals are subjected to limited caloric intake, they normally exhibit increases in life span and resistance to oxidative stress (Yu 1996). The results also indicate that the effects of partial gut specification on differentiation are complex, likely representing changes in a variety of pathways that cause diverse physiological abnormalities. Further experiments will be directed at identifying the major regulatory pathways that have changed as a result of compromised specification.

CONCLUSION

Through characterization of abnormalities in animals that survive partial gut specification, we were able to detect multiple phenotypic defects that suggest that proper metabolism in adults requires proper early progenitor specification. Gut function plays a fundamental role in pathogenesis of various metabolic diseases, including obesity and diabetes, which suggests that at least some of the time, these diseases may result from mild defects in early embryonic development. Our work suggests that *C. elegans* provides a platform for understanding the connection between early-acting gene networks for gut specification and the consequences on development and adult intestine function.

ACKNOWLEDGMENTS

I would like to thank Dr. Morris Maduro for his guidance, mentorship, and continued support throughout my project. I would also like to extend my thanks to Gina Broitman-Maduro and Hailey Choi for their invaluable input and insight.

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Fabrication and Optical Microscopy Observation of the Electrochemical Lithiation of Polysilicon Thin Films

Cody A. Gonzalez and Sandeep Kumar

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ABSTRACT

One of the primary issues that restricts the development of new materials for next generation lithium ion batteries (LIB) is performance degradation over the lifecycle of the battery. Our goal is to elicit clear understanding of the mechanisms responsible for the degradation of performance that will help in designing better materials for electrodes and improve their performance. The goal of this study is to observe, through optical microscopy, the electrochemical lithiation of silicon thin films on devices. These devices are fabricated for use in a transmission electron microscope (TEM) to further understand the behavior of the material. We are developing an *ex situ* TEM LIB setup that would allow us to understand the material degradation during lithiation (charge) and delithiation (discharge). Silicon is the primary material being studied due to its possessing the largest specific capacity of any anode for LIBs. Through the use of photolithography, deposition, and etching techniques, these devices are created and then lithiated to be observed in an optical microscope. These *ex situ* experiments are performed wherein a constant voltage is applied across the device setup to observe the deformation mechanics of nanoscale silicon. Attempted lithiation has yielded successful results, with discoloration of the polysilicon viewed, given prolonged exposure to Lithium and Ionic Liquid Electrolyte. The discoloration is thought to be the result of the lithiation of the polysilicon thin films and future work will include confirmation of successful lithiation through TEM observation of the lithiated polysilicon.

Keywords: Lithium-ion battery, battery electrodes, silicon, thin films, energy storage

AUTHOR

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Cody Gonzalez is a senior in Mechanical Engineering. He is interested in material characterization of micro electro-mechanical systems (MEMS) and nanotechnology. He joined Sandeep Kumar's Multi-Physics Lab in the summer of 2014. He has developed an interest in novel materials for lithium ion batteries and the application of nanotechnology in novel sensors.

FACULTY MENTOR

Sandeep Kumar

Department of Mechanical Engineering

Professor Kumar received his Bachelor of Technology degree in Mechanical Engineering from the Regional Engineering College, Kurukshetra, India, and his Master of Science degree from the Indian Institute of Technology, Delhi, India. He completed his PhD degree at Pennsylvania State University under the guidance of Professor Aman Haque. His doctoral research explored *in situ* TEM studies on mechanical properties and deformation mechanisms in nanoscale thin films, and multilayer hard coatings. His publishing credits include thirteen peer-reviewed journal papers, three peer-reviewed conference papers and nine technical conference presentations.

Cody A. Gonzalez

1.0 INTRODUCTION

One of the foremost issues facing Lithium Ion Battery (LIB) development is performance degradation due to repeated electrochemical cycling. In particular, battery performance degrades as a result of a loss of electrical contact as well as due to a permanent lithiation of a small portion of silicon. This is particularly true when bulk silicon is lithiated. However, little is known about the deformation mechanics that occur when nanoscale silicon is lithiated. The primary goal of this study is to develop an LIB setup to allow for observation via optical microscopy of the lithiation of silicon thin films. In the long term, the objective of this research is to create an *in situ* LIB setup to allow for observation via transmission electron microscopy (TEM) of the lithiation of silicon thin films.

Currently, graphite is the primary material used in the construction of LIB anodes, although silicon has a much higher specific capacity to hold charge. However, silicon is much more brittle than graphite and cracks under the stress incurred during cycling when used at a bulk scale. This occurs because silicon expands roughly 300 percent volumetrically when lithiated (Wang et al., 2012). The experiments undertaken will lithiate nanoscale silicon instead of bulk scale silicon to counter the stress resulting from rapid volumetric expansion. The reduction in scale will lower the internal stresses that result from the large volumetric expansion. This research will enable the study of the fundamental deformation mechanics of nanoscale silicon during lithiation through the characterization of such fundamental material properties as the resistance and thermal conductivity of the lithiated polysilicon. In the very long term, this fundamental characterization has the possibility to lay the groundwork for future studies of polysilicon as a superior electrode for utilization in LIBs.

2.0 BACKGROUND

As technology advances exponentially in the amount of power it uses, energy storage technology has lagged significantly behind. One reason for this is the aforementioned inherent low specific capacity of graphite with a maximum theoretical capacity of 372 mA h g^{-1} (Tarascon & Armand, 2001). Silicon, however, has a great potential to act as an electrode with a theoretical capacity of upwards of 4200 to 4500 mAh/g (Baranchugov et al., 2007; Boukamp, Lesh, & Huggins, 1981). Studies by Baranchugov have shown good performance using Li/Si cells containing an Ionic Liquid Electrolyte (ILE) with a stable Si electrode capacity of about 3000 mA h g^{-1} and a relatively low irreversible capacity, which should indicate a possibility for multiple cycling (Baranchugov, Markevich, Pollak, Salitra, & Aurbach, 2007). However, we want to understand the deformation mechanics of nanoscale polysilicon because there has been no answer to the proposed research question: "How does nanoscale thin film silicon deform and react to lithiation?"

Mechanical disintegration is suspected to be one of the chief culprits of rapidly decreasing capacity if cycled over twenty times when amorphous silicon thin films are utilized (Bourderau, Brousse, & Schleich, 1999). Other theories propose additional reasons for the capacity fading over multiple cycles, such as the formation of a Solid Electrolyte Interface (SEI), irreversible intercalation, and loss of electrical contact in addition to the pulverization from the large volumetric expansion.

The primary issue encountered in characterizing the lithiation of silicon is that it is much more brittle, volumetrically expands much more than graphite, and cracks under the stress incurred during cycling when used at a bulk scale. Carbon is able to form four covalent bonds due to its four electrons in the second shell. Similar to carbon, silicon typically forms four bonds; however, unlike carbon it can accept additional electrons and form up to five or six bonds. These additional bonds are at least partially responsible for the large volumetric expansion.

In order to counter the stress resulting from rapid volumetric expansion, two different methods of accommodation can be undertaken. These involve either increasing the overall strength of silicon through the use of silicon composites, or reducing the size of the silicon through utilization of silicon nanoparticles, nanowires, or nanofilms (Dimov, Kugino, & Yoshio, 2003). The use of carbon in silicon composites to increase the strength of the silicon would drastically lower the effective specific capacity of the silicon, therein limiting the maximum specific capacity. Nanoparticles

lose electrical contact easily due to the volumetric expansion of silicon, and embedding the nanoparticles in a carbon matrix is not effective due to cracking of the carbon fiber from silicon nanoparticle expansion (Gu et al., 2012). Nanowires allow for volumetric expansion and are convenient for *in situ* TEM studies; however, they are not immediately applicable to development of LIBs. Chan, et. al. performed a study that circumvented the issues of volumetric expansion with the use of silicon nanowires, but failed to lithiate the Si nanowires in a way that accurately simulated operation of a LIB (Chan et al., 2008). Thus, it is necessary to study nanofilms as they have the most immediate potential to act as an electrode in a LIB. This research aims to characterize how nanoscale polysilicon lithiates in order to pave the way for its use in LIBs. However, there are many different methods for material characterization.

There are several popular methods that are used to characterize materials: SEM, TEM, X-ray Diffraction (XRD), and energy-dispersive spectroscopy (EDS). SEM equipped with backscattered detectors is used to characterize silicon in addition to use of energy-dispersive spectroscopy to investigate particle morphology (Xiao et al., 2010). XRD is also used to determine the phases present in films in addition to the use of SEM to examine the microstructure and chemical composition of the silicon thin films (Dimov et al., 2003; Maranchi, Hepp, & Kumta, 2003; Shi, Barker, Saïdi, & Koksbang, 1996).

Optical microscopy is the method this experimental setup will use to observe the lithiation of silicon thin films, as has been done before through TEM with nanowires (Gu et al., 2012; Liu & Huang, 2011; Woo, Park, Hwang, & Whang, 2012). The TEM allows for the most accurate morphologic, compositional and crystallographic information on samples; however, for the purpose of this experiment, lithiation confirmed through optical changes must suffice. As there is not sufficient time for an in depth study through TEM for this experiment, optical microscopy is a quicker way to obtain measurable success. In particular, *in situ* TEM studies will allow for the observation of these effects in real time and so will follow this research pending successful optical change or discoloration of the thin films. TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen, in this case polysilicon, interacting with it as the beam passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a Charge Coupled Device (CCD) camera. TEM allow us to see and observe various material deformation and electrochemical process at very high resolution. *In situ* TEM experiments introduce a challenge since a TEM chamber can accommodate only a 3 mm diameter size experimental setup. Due to this difficulty, optical microscopy will be used as the primary mechanism while a micro-electromechanical system (MEMS) based *in situ* TEM micro LIB setup is developed for this purpose.

3.0 METHODS

The MEMS devices will be fabricated through a process wherein a thin silicon wafer is treated with three lithography steps, in between each the wafer is etched, deposited with gold as an electrode, and etched on the back side respectively.

3.1 Front-Side Silicon Oxide and Polysilicon Deposition

To begin, a 300 micron thick silicon wafer is coated with approximately 3000 Angstroms of silicon oxide using Plasma Enhanced Chemical Vapor Deposition (PECVD). Polysilicon is then deposited uniformly using Low Pressure Chemical Vapor Deposition (LPCVD) at 620°C for 30 minutes. This yielded a deposition of approximately 150 nm of P-Si, measured through the use of a Dektak profilometer.

3.2 Electrode Lithography

AZ 5214 reversible photoresist is spun on the wafer to allow for the transfer of the electrode pattern. This pattern is then transferred to the wafer using a photolithography recipe involving exposure, reverse exposure, and development.

During the second step of the wafer processing, 10 nm of chromium and 100 nm of gold are deposited through electron-beam evaporation on top of the wafer to provide a surface for electrical contact. The liftoff is then performed sequentially using acetone, isopropanol, and wafer to

remove the gold sitting on the photoresist. The acetone dissolves all photoresist, and because the photoresist at 1.4 microns is much thicker than the gold at 100 nm or 0.1 microns, the photoresist holding the gold is easily removed while the gold deposited on the surface (on the chromium used to improve adherence) remains unaffected.

3.3 Back-Side Lithography

After the gold liftoff, a much thicker photoresist, SPR220, is used to coat the bottom side of the wafer to prepare for back side etching. Reactive Ion Etching (RIE) is used to etch away the P-Si and silicon oxide not covered by the spun SPR220 photoresist. This allows for the oxide covered by the photoresist to remain intact to enable Deep Reactive Ion Etching (DRIE) of silicon at the locations where the silicon oxide was etched away. This back side etching of silicon makes the devices nearly free-standing, except for the oxide remaining on the top of the wafer. This silicon oxide is etched away using Hydrofluoric acid Vapor Etching (HFVE) which makes the devices completely free-standing and able to be tested *in situ* via TEM.

3.4 Optical Microscopy and TEM Setup

Once the wafers are processed completely and the devices are left free-standing, the next phase of the project begins, which involves the lithiation of silicon thin films and their observation via optical microscopy. Further research would involve the creation of the TEM setup for study of the electrochemical lithiation of nanoscale silicon. The implementation of the TEM Holder setup in Fig. 1 will allow for the satisfying of our final objective of *in situ* TEM observation of the pulverization of silicon upon electrochemical cycling of lithiation and delithiation.

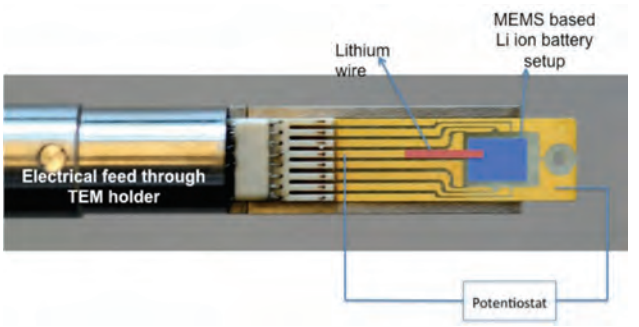

Figure 1: In situ *TEM Li ion battery characterization setup*

However, the objective of this study will focus on an *ex situ* observation through optical microscopy. Both the fabricated devices and additional chips of gold on polysilicon are being tested for lithiation by both applying a voltage difference and letting the lithium, ILE, and P-Si remain in contact for extended periods of time.

An *ex situ* experiment is developed, where the devices which hold the silicon can be lithiated and then placed in the TEM for observation of the *ex situ* TEM studies of the silicon post-lithiation. In order to create the *ex situ* study and prepare for the *in situ* study, thin strips of Lithium wire have been prepared in a glovebox and are then placed on the 150 nm thick portion of polysilicon of the devices. To perform the *ex situ* study, the device is placed on a metal contact surface with wires soldered to provide an electric connection. After the device is placed, a lithium wire is placed in contact with the silicon and then a silver paste is spread over it to ensure an electrical connection.

4.0 RESULTS

Initial successes yielded both successful wafer processing and the discoloration of the silicon. The wafer processing proved successful and upon lengthy exposure to ILE and Lithium, the P-Si changed color in what is expected to be the lithiation of the P-Si. In Fig. 2a, a device of half polysilicon (green) and half gold is seen. The P-Si sits on top of the full layer of gold. After repeated experiments and controls, the discoloration persists when lithium or lithium and ILE has prolonged contact with the P-Si, as shown in Fig. 2b. As is seen in Fig. 2a, the silicon is initially a bright emerald color, and after lithiation a dark grey color forms uniformly around the piece of silver lithium. A solder bead for electrical contact is seen at the

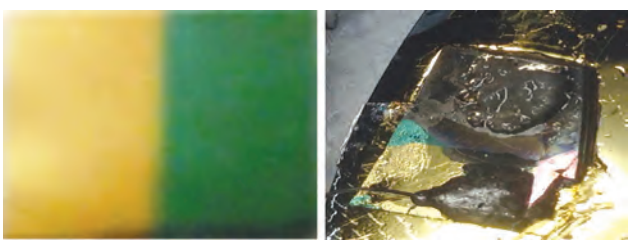

Figure 2: *a) Gold/Polysilicon Device without lithiation; b)* Ex situ *TEM Li ion battery characterization setup post lithiation*

bottom of Fig. 2b. In the case of Fig. 2b, the bright green polysilicon color can still be seen in part of the device that the suspected lithiation has not spread to. Additional observation of this lithiation of P-Si will be done in a TEM.

A successful method of device fabrication has been created wherein a thin silicon wafer is treated with the approach covered in the methodology section. Once the wafers are processed completely and the devices are left free-standing, the next phase of *ex situ* studies has begun. This is done as an introductory study and proof of concept for the goal *in situ* TEM setup for study of the electrochemical lithiation of nanoscale silicon.

The implementation of the TEM Holder setup in Fig. 1 will allow for the satisfying of the initial objective of *in situ* TEM observation of the pulverization of silicon upon electrochemical cycling of lithiation and delithiation.

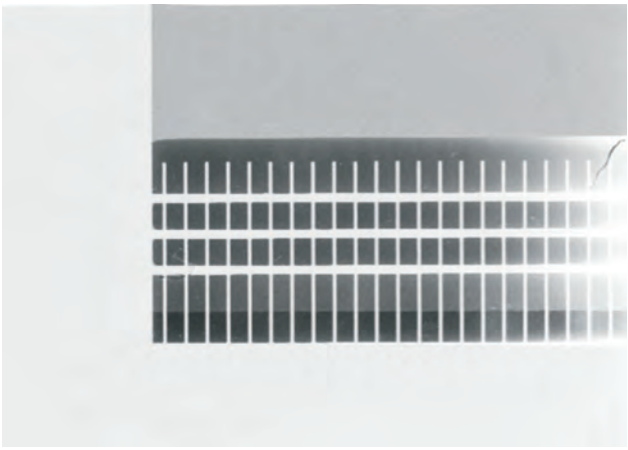

Figure 3: *Entire device of 3 mm X 3.8 mm dimensions at 74X magnification in SEM*

Additionally, several SEM pictures were taken of a device. Fig. 3 is a micrograph taken at 74X magnification of the device in its entirety. The mesh bridge visible in the top right corner of the device allows for more direct electrical contact between the polysilicon and the gold surface on the bottom of the device, where a wire will be soldered on to provide the necessary potential to lithiate the P-Si.

5.0 DISCUSSION

Two major obstacles have been encountered thus far. First, the initial use of sputtered Si at room temperature proved an issue because the silicon became oxidized due to its small grain size. This proved problematic when the wafer was exposed to HFVE where the hydrofluoric acid vapor etches away oxides, including the oxidized silicon removing the material intended for study. To solve this issue, polysilicon was deposited using LPCVD instead of sputtering at a high temperature to increase the grain size of P-Si and reduce the chance of silicon oxidation. After successfully withstanding a direct dip in Buffer Oxide Etcher (BOE), it was concluded that the LPCVD deposited P-Si was not oxidized and would be acceptable.

The second obstacle concerns the DRIE process, which is the last step prior to HFVE. The majority of the devices are being over etched when exposed to the DRIE process and after being released through HFVE, the vast majority of the devices break across the bridge, as seen in Fig. 4b. As this is nearly 3 mm of unsupported silicon, this is suspected to break due to residual stress and a lack of additional support structure. The proposed solution to this issue is to further separate the bridges, shown in Fig. 4b, and add cross-bracing to support the bridge, which would connect the lower conducting portion of the device to the P-Si for the application of an electric potential. It was found that for several of the devices that had a different etch design for the back-side were being under etched, so the bridges were separated further apart to allow for enough space to etch at approximately the same rate as the remainder of the devices.

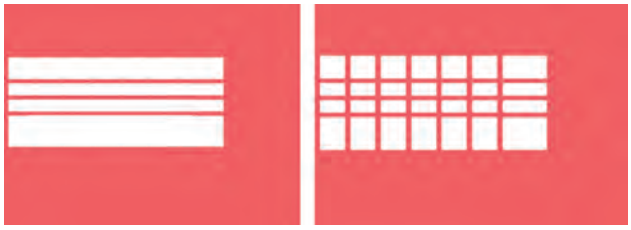

Figure 4: *a) Device design without support; b) Modified Device design with support*

This study has yielded a successful device fabrication procedure with the exception of the issue with the DRIE process. This issue was solved by adding cross-bracing to the back-side lithography mask to provide additional support for the devices once they are made free-standing. The grid is created to allow enough open space to allow for optimum resolution in the TEM. Further study of the second LIB wafer to be processed has yielded high resolution SEM images.

Many of the devices produced from this second wafer are being used to continue with the *ex situ* study in preparation for the *in situ* TEM study. As previously stated, the most significant result thus far is the potential successful lithiation of P-Si. After repeated experiments, the devices and chips have both been successfully discolored after prolonged exposure to lithium and ILE. The mechanism responsible for this discoloration of the P-Si after exposure to lithium is expected to be the diffusion of lithium throughout the P-Si. Further study through observation in a TEM before and after the suspected lithiation will prove whether the lithium is diffusing into the silicon structure.

Future studies will focus on study of the suspected successful lithiation and the *in situ* TEM observation of the thin film P-Si. Once successful observation of the lithiation has been fulfilled, additional studies are planned to be conducted on the thermal and electrical properties of the lithiated polysilicon. In the long term, the wafer processing established in the methodology has the ideal far-reaching application of being utilized as a template for many more *in situ* and *ex situ* studies of nanoscale materials.

7.0 ACKNOWLEDGEMENTS

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Structural Analysis of BipA, a Key Player in Eubacterial Pathogenicity

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Department of Biochemistry

ABSTRACT

Bacterial infections are a major source of global morbidity. What distinguishes pathogenic bacteria from harmless strains is the presence of virulence factors. These factors are critical during cellular stress response, such as during starvation or infection of a host. A key player in the expression of virulence factors is the eubacterial protein BipA, a translational GTPase. To modulate the production of pathogenic molecules, BipA associates with the small ribosomal subunit, one of two major components of the ribosome. During starvation, the cellular concentration of alarmone guanosine-3', 5'-bis pyrophosphate (ppGpp) increases dramatically. This influx allows ppGpp to bind to BipA and change its binding specificity from ribosomes to the small ribosomal subunit. To understand how ppGpp influences BipA's binding specifity, we hypothesize that ppGpp induces a structural change when bound to BipA. We present structures of full-length BipA from *Escherichia coli* in apo, GDP-, and ppGpp-bound forms solved through X-ray crystallography. Analysis indicates the ppGpp-bound form of BipA is identical to the structure of the GDP-bound form. These results suggest an additional binding partner, along with ppGpp, is necessary to change the binding preference of BipA. Understanding the mechanics of BipA may provide insight on other translational GTPases in eubacteria.

Keywords: GTPase, ppGpp, BipA, *Escherichia coli*, X-ray crystallography, ribosome, virulence factors, stress

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Dr. Gregor Blaha is an Assistant Professor in the Department of Biochemistry. His research focuses on bacterial physiology and its response to environmental changes. Currently, his lab investigates the coupling of transcription and translation by combining biochemical and biophysical methods with genetic approaches.

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Joseph Hahm is a fourth-year Biochemistry student. He is a Supplemental Instruction (SI) leader, working to both assist students in understanding the complexities of physics and guide other SI leaders into becoming strong role models. Working in the Blaha lab since July 2013, Joseph investigates the structures of various eubacterial proteins involved in transcription and/or translation. He plans to take his lab-honed problem-solving skills to medical school with sights on becoming a forensic pathologist.

Joseph Hahm

INTRODUCTION

In enteropathogenic *Escherichia coli*, bactericidal/permeability-increasing inducible protein A (BipA) has been linked to the expression of virulence genes and the avoidance of the host defense mechanism (1-4). BipA is a highly conserved translational guanosine triphosphate hydrolase (GTPase) present in eubacteria with a genome size larger than 2.8 x 10^6 base pairs. For comparison, model organism *E. coli* K-12 has a genome size of 4.6 x 10^6 base pairs (5). GTPases have the same core structure but switch between an inactive conformation, favored when guanosine diphosphate (GDP) is bound, and an active conformation, favored when guanosine triphosphate (GTP) is bound.

BipA has been found to also bind to guanosine-3′, 5′-bis pyrophosphate (ppGpp), a key regulator of virulence and pathogenicity in eubacteria. ppGpp stalls replication, reshapes the transcriptome, and modulates translation (6). When the cell is starved for nutrients (e.g., amino acids, phosphates, nitrogen, etc.), intracellular concentrations of ppGpp increase exponentially (7). During starvation, BipA has two distinct forms, switching between associating with the small ribosomal subunit or the ribosome (8, 9). We hypothesize that ppGpp binds to BipA when the level of ppGpp is high during stress. This binding induces a structural change in BipA and switches its preference from the ribosome to the small ribosomal subunit.

In order to demonstrate the effect of ppGpp on BipA, we solved the molecular structure of BipA bound to ppGpp and compared the structure with that of BipA bound to GDP. Both nucleotide bound structures should differ from one another and from the apo protein. Our results reveal that ppGpp binding to BipA does not induce a specific structural changes in BipA. This insight suggests that ppGpp and another binding partner must both be present in order to switch the binding specificity of BipA.

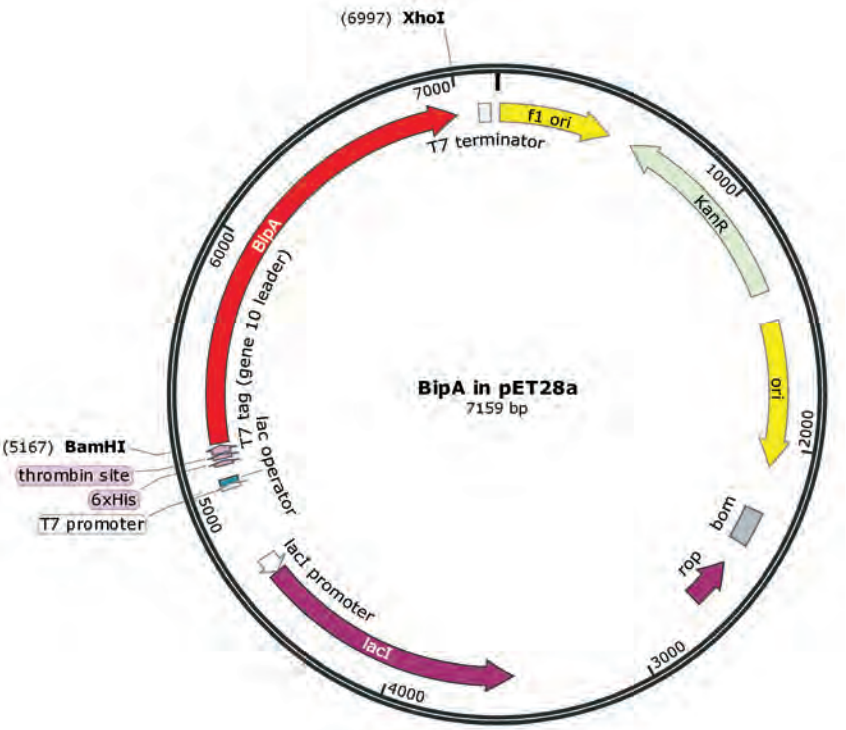

Figure 1. *Vector map of full-length bipA gene cloned into pET28a vector. The same vector is used for BipA-CTF, with the full-length gene replaced by the sequence of the fragment.*

METHODOLOGY

Full-length BipA is naturally found in *E. coli*. To ensure BipA expression outcompeted all other protein expression, we introduced the gene through a vector and chemically induced its expression. The following describes this procedure.

Cloning, protein overexpression, and protein purification of full-length BipA. The DNA sequence of full-length BipA (amino acid residues 1–607) from *E. coli* K12 MG1655 was inserted into the pET28a vector (Novagen) between BamHI and XhoI restriction sites by in-fusion cloning (see Figure 1 for vector map of final construct). The plasmid DNA was transformed into *E. coli* T7 Express cells (New England Biolabs). Cells were grown in the presence of 30 µg/mL Kanamycin in Lennox broth. Protein overexpression was induced at mid-log growth phase with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for an additional 20 hours at 16°C before being harvested and flash frozen in liquid nitrogen. Cells were stored at -80 °C.

Cells containing overexpressed full-length BipA were resuspended in lysis buffer (25 mM HEPES-NaOH, 50 mM Glycine, pH 8.0). Cell suspension was lysed with three passages through an Emulsi-flex C3 homogenizer at 15,000 psi. Target protein was separated from cell debris using centrifugation at 30,000 relative centrifugal force (rcf) for one hour at 4°C. Clarified cell lysate was loaded onto a 5 mL HisTrap column (GE Healthcare). The column was washed with 1.5 M NaCl and eluted with 200 mM imidazole. The eluate was buffer exchanged multiple times into lysis buffer before loading onto a 20 mL DEAE column (GE Healthcare). Full-length BipA was eluted with a linear gradient of 0 to 600 mM NaCl. Protein content of each elution fraction was analyzed by SDS-PAGE. Fractions containing >90% full-length BipA were pooled, concentrated, buffer exchanged into storage buffer (10 mM HEPES-NaOH, 20 mM Glycine, pH 8.0), and stored at -80°C until further use.

Cloning, protein expression, and protein purification of BipA-CTF. The DNA sequence of the C-terminal fragment of BipA (amino acid residues 306–607) from *E. coli* K12 MG1655 was inserted into pET28a vector between BamHI and XhoI restriction sites by in-fusion cloning. The plasmid DNA was transformed into E. cloni® BL21 (DE3) cells (Lucigen). Cells were grown in the presence of 30 µg/mL Kanamycin in MDAG liquid media (10). Protein overexpression was induced at mid-log growth phase with 0.5 mM IPTG. Cells were grown for an additional 20 hours at 22°C before being harvested and flash frozen in liquid nitrogen. Cells were stored at -80°C.

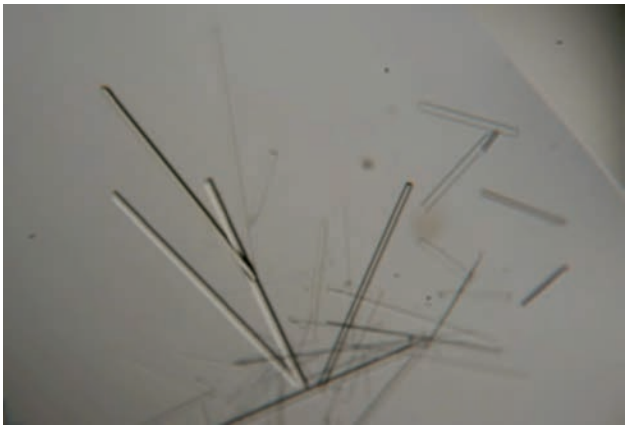

Figure 2. *Crystal of full-length BipA. Crystallization only occurred in the presence of* $[Co(NH_3)_6]C_{l3}$.

Cells containing overexpressed BipA-CTF were resuspended in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 0.28 mM PMSF, pH 8.0). Cell suspension was lyzed with five passages through an Emulsi-flex C3 homogenizer at 7,500 psi. Target protein was separated from cell debris using centrifugation at 200,000 rcf for two hours at 4°C. Clarified cell lysate was loaded onto a 5 mL HisTrap column (GE Healthcare). The column was washed with lysis buffer, 500 mM NaCl, and 20 mM imidazole. BipA-CTF was eluted with a linear gradient of 20 to 300 mM imidazole. Protein content of each elution fraction was analyzed by SDS-PAGE. Fractions containing >95% BipA-CTF were pooled, concentrated, buffer exchanged multiple times into storage buffer (5 mM Tris-HCl, pH 8.0), and stored at 4°C until further use.

Crystallization of full-length BipA. Full-length BipA was crystallized by vapor diffusion out of a sitting drop consisting of a 1:1 ratio of 6 mg/mL protein to well solution (100 mM Tris-HCl, 2% [w/v] PEG 6000, 5 mM $[Co(NH_3)_6]Cl_3$, pH 8.0) at 20°C. Crystal quality was enhanced by micro-seeding to yield crystals with dimensions up to 800 x 100 x 100 µm in three days (see Figure 2). Complexes of BipA with either GDP or ppGpp were formed by soaking crystals of full-length BipA overnight in a solution of 100 µM GDP or 50 µM ppGpp in the presence of 1 mM $MgAc_2$, respectively. Crystals were stabilized by addition of ethylene glycol in the well solution, then periodically increasing its concentration over time. Once the final glycol concentration reached 45% [v/v], crystals were flash frozen in liquid nitrogen.

Crystallization of BipA-CTF. BipA-CTF was crystallized by vapor diffusion out of a sitting drop consisting of a 10:1 ratio of 7 mg/mL protein to well solution (3.04 M sodium formate, 200 mM Tris-HCl, 1 mM $MgBr_2$, pH 7.6) at 20°C. This method yielded crystals with dimensions up to 1,000 x 550 x 300 µm in three to seven days. Crystals were soaked in a well solution containing an additional 30% [v/v] propylene glycol for 10 seconds before flash freezing in liquid nitrogen.

Data collection. Full-length BipA protein crystals were shipped to beamlines 5.0.1 and 5.0.2 at the Advanced Light Source at Lawrence Berkeley National Laboratory while BipA-CTF protein crystals were shipped to beamline 24-ID-C at the Advanced Photon Source at Argonne National

Joseph Hahm

Laboratory for remote X-ray crystal diffraction data collection. (For an image of the data collected, see Figure 3).

Structure determination. Data was processed with HKL-2000 (11) and MOSFLM (12). The structures of apo full-length BipA and BipA-CTF were solved by molecular replacement using BipA from *Vibrio parahaemolyticus* (PDB ID: 3E3X) as the search model. The structures of the nucleotide-bound states were solved by molecular replacement using the apo full-length BipA structure as the initial search model. The phase quality of the molecular replacement solutions was improved by density modification. Model bias was reduced by calculating composite omit maps with simulated annealing of torsion angles as implemented in PHENIX program suit (14). Each model was rebuilt with the molecular graphics program Coot (15) and refinement was achieved with iterative cycles in PHENIX and REFMAC5 (14, 16).

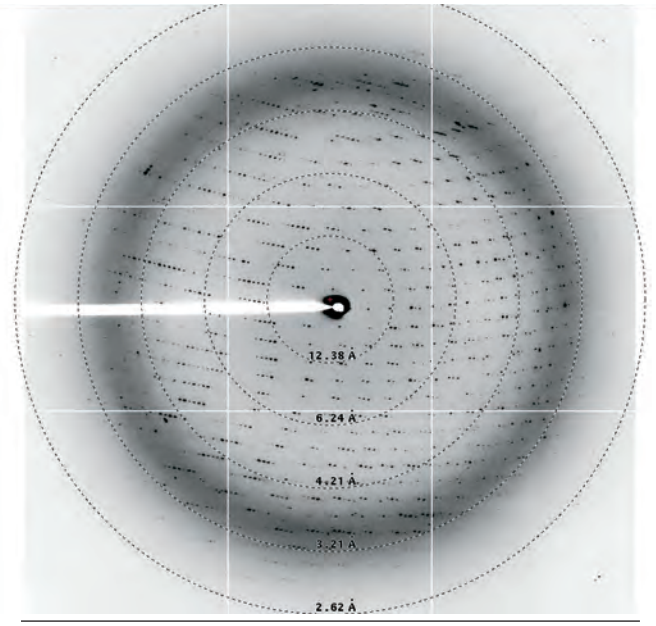

Figure 3. *Diffraction Pattern Of Full-Length BipA. This crystal diffracted to a resolution of 2.6 Ångstroms.*

RESULTS

Crystal structure of full-length BipA. Full-length BipA crystallizes in the $P2_1$ space group with two copies in the asymmetric unit. The structure was solved and refined to a resolution of 2.6 Å. Full-length BipA can be subdivided into five domains (see Figure 4, top). Domain I (amino acid residues 1–202) resembles the common motif of a G domain found in translational GTPases, consisting of a central six-stranded β-sheet surrounded by five α-helices (17). Domain II (amino acid residues 203–302) has the recognizable OB-fold, a five-stranded β-sheet coiled to form a closed β-barrel (18). Domain III (amino acid residues 303–385) and V (amino acid residues 386–482) share the same double-split β-α-β motif observed in other ribosomal proteins (19). The C-terminal domain (CTD) of BipA (amino acid residues 483–607) incorporates a mixture of eight β-sheets and two α-helices. Unfortunately, a polar region (amino acid residues 540–555) extending from the distal end of the CTD is disordered. To resolve this disordered region, we crystallized BipA-CTF, consisting of domains III, V, and the CTD (see Figure 4, bottom).

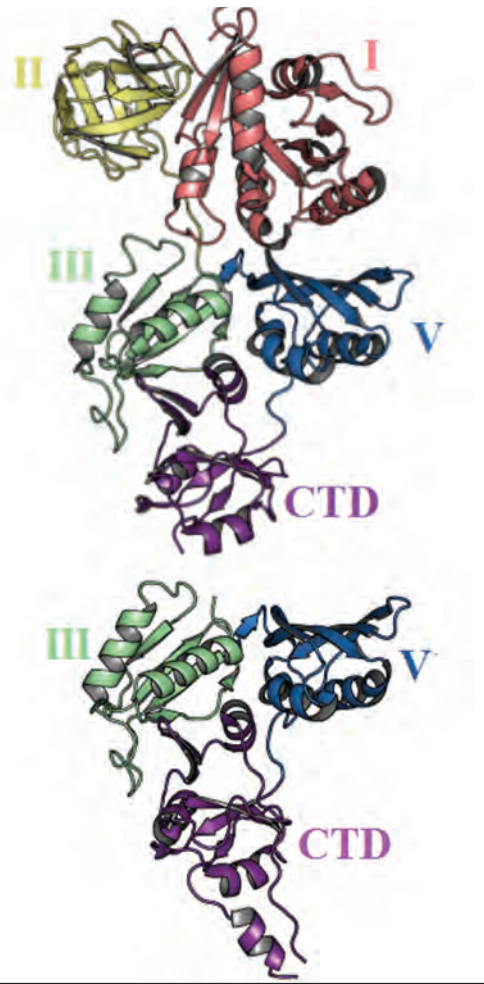

Figure 4. *Structural models of full-length BipA and BipA-CTF. Top) Full-length BipA with five domains I, II, III, V, and CTD displayed in salmon, yellow, green, blue, and purple respectively. Bottom) BipA-CTF with domains III, V, and CTD displayed in the same color scheme.*

Crystal structure of BipA-CTF. BipA-CTF crystallizes in the $P4_12_12$ space group. The structure was solved and refined to a resolution of 2.5 Å. The disordered region of the full-length protein structure (amino acid residues 540–555) was successfully solved (see Figure 5, top). The overall structure of BipA-CTF resembles that of the solved full-length BipA structure. However, domain V is rotated towards the CTD and coordinated by a magnesium ion located close to the pivot point. Another magnesium ion lies in the loop connecting domain V and the CTD. The location of the Mg^{2+} ions increases the positive surface charge that interfaces with ribosome binding (see Figure 5, bottom) (20).

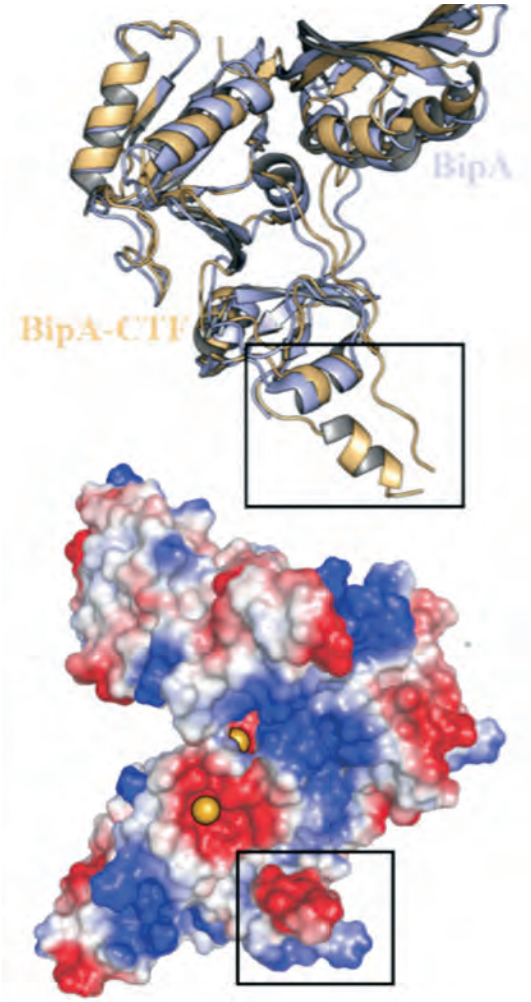

Figure 5. *Significance of solving the structure of BipA-CTF. Top) The superimposition of BipA (lavender) onto BipA-CTF (gold) reveals the resolved disorder region (boxed) of the full-length BipA structure. Bottom) An electrostatic surface model of BipA-CTF with its two critical Mg^{2+} ions (yellow). The boxed region is the surface that interfaces with ribosome binding.*

Crystal structures of GDP- and ppGpp-bound BipA. The structure of GDP-bound BipA was solved to a resolution of 3.1 Å. The GDP nucleotide is bound in domain I similarly to other translational GTPases. The G-1 box motif (amino acid residues ^{12}AHVDHGKT^{19}) (single-letter code) wraps around the phosphate groups of the guanosine nucleotides. BipA also contains the highly conserved DX2G motif (^{74}DTPG^{77}), responsible for coordinating the critical catalytic Mg^{2+} ion. Moreover, hydrophobic sequence motifs ^{128}NKVD^{131} and ^{166}SAL^{168} provide hydrophobic interactions and hydrogen bonds to stabilize the guanosine base (see Figure 6, left) (21).

The structure of ppGpp-bound BipA was solved to a resolution of 3.3 Å. Surprisingly, this structure is identical to that of BipA bound to GDP (Figure 6, right). The amino acid residues form the same interactions with ppGpp as with GDP. The additional 3′-pyrophosphate of ppGpp extends towards the 5′-pyrophosphate and does not interact with any of the amino acids of BipA.

DISCUSSION

During stress, when the level of ppGpp is high, the binding preference of BipA changes from ribosomes to small ribosomal subunits, suggesting ppGpp could regulate BipA's binding specificity (9). ppGpp acts as an effector molecule, selectively binding to a protein and influencing its enzymatic activity, usually observed as a structural change (8). Therefore, to determine the effect of ppGpp binding on BipA's affinity to the small ribosomal subunit, we solved the structures of apo, GDP-, and ppGpp-bound BipA.

Both the structures of GDP- and ppGpp-bound BipA are identical (see Figure 6, right). Furthermore, they are analogous to the structure of the nucleotide-free protein (not shown). NMR studies of a related translational GTPase, IF2, confirm that the GTPase domain is identical between the GDP- or ppGpp-bound states (22). However, in the case of BipA, not only is the GTPase domain identical, but the entire protein adopts the same structure when bound to either GDP or ppGpp.

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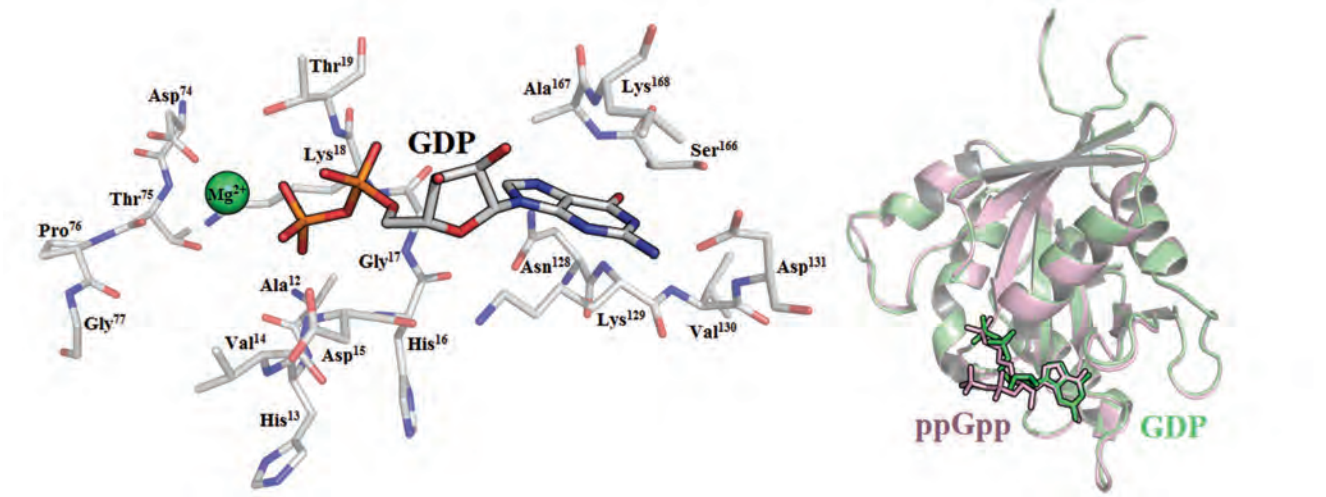

Figure 6. *Guanosine nucleotide bound states of BipA. Left) Stick representation of the critical amino acids responsible in stabilizing GDP in the active site with its coordinating Mg^{2+} ion (green). The GI motif wraps around the phosphate groups (red and orange) while the SAL and NKVD sequences surround the guanosine base. To the left, the DTPG groups position the Mg2+ ion. Right) The superimposition of GDP-bound BipA structure (green) onto the ppGpp-bound BipA structure (pink) highlights no structural difference in the GTPase domain.*

Our results suggest that in order to alter BipA's binding specificity, an interaction with ppGpp and another effector molecule is necessary. Similar mechanisms have been proposed for other translational GTPases when bound to GTP. Studies suggest the binding preference of GTPases only change in the presence of both the nucleotide and a corresponding binding partner, i.e. a ribosome for EF-G, an aminoacyl-tRNA for EF-Tu (23). In the case of BipA, the small ribosomal subunit may be its binding partner. Discerning BipA's influence on the small ribosomal subunit, and in turn, the ribosome, may determine the structural mechanics of eubacterial translation. Future work includes crystallizing BipA with the small ribosomal subunit to confirm a conformational change, and binding preferences.

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A Story A Story: Caribbean Myth and History in the Poetry of Anne Sexton

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ABSTRACT

This research paper examines a poem written by Anne Sexton nine months before her suicide in October 1974. The poem, "Rowing," quotes from the Afro-Caribbean myth of Anansi the Spider, and it alludes to the journey of the Ashanti people from West Ghana. The Ashanti retained their cultural traditions and stories even as they were transported in chains to the Caribbean during the terrible Middle Passage. The discovery of such a crucial influence on one of her late poems will change the conventional wisdom that Sexton is known for her "confessional" poetry and for her containment within white culture. Until now, Sexton's involvement with myth, fairy tale, and folk tale has usually been viewed as an engagement with European culture, but the evidence of her use of African materials in "Rowing" indicates that the poet was moving from European culture to African and Caribbean cultures at the end of her career and life. I include a close reading of "Rowing" to reveal the many associations Sexton made with the journey of the Ashanti people, and I research the poetry and music of several important Caribbean artists popular in the 1960s and 70s, such as Kamau Brathwaite and Bob Marley, to demonstrate how the emerging global and postcolonial consciousness influenced Sexton. Her interest in African and Caribbean history and myth has been overlooked. It is significant because her turn toward the story of Anansi represents a change in her consciousness and a bridge to a different future.

Keywords: Anne Sexton; African and Afro-Caribbean folklore; Eurocentric perspective; changing global consciousness; Ashanti of West Ghana; dub poetry; Anansi the Spider

FACULTY MENTOR

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Steven Gould Axelrod is a Distinguished Professor of English at the University of California, Riverside. He has received the university's Distinguished Teaching Award. He is the author of *Robert Lowell: Life and Art* (Princeton University Press, 1978; Legacy Edition, 2015), *Robert Lowell: A Reference Guide* (G. K. Hall, 1982), and *Sylvia Plath: The Wound and the Cure of Words* (Johns Hopkins University Press, 1990). He is co-editor of *The New Anthology of American Poetry*, Volumes 1-3 (Rutgers University Press, 2003, 2005, 2012). Axelrod is currently editing (with Grzegorz Kosc) a groundbreaking volume, *The Memoirs of Robert Lowell*, for Farrar, Straus and Giroux.

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Jill Goldstein Hoo is a fourth-year student in the Department of English. In the 1970s she penned songs for jazz and blues artists Les McCann and Esther Phillips. She discovered Anne Sexton's use of Afro-Caribbean materials while a student with Professor David Lloyd; he encouraged her to work with Professor Steven Axelrod in order to expand her knowledge of Sexton and the emerging global and postcolonial consciousness of the 1960s and 70s that would have influenced Sexton.

Jill Goldstein Hoo

INTRODUCTION

In Greek mythology, Kharon (Χάρων) rows the souls of the newly deceased across the River Styx. "Rowing," the first poem in Anne Sexton's collection of poetry titled *The Awful Rowing Toward God*, written nine months before her suicide in October 1974, would seem at first reading to be a metaphor for the mythological journey of the soul across the water to the underworld of the dead. Toward the end of "Rowing," Sexton writes, "and God was there like an island I had not rowed to" (A. Sexton "Rowing" 24), echoing the theme illumined in much of her later poetry, that of a quest for a god whose grace she could not obtain. Nevertheless, despite Sexton's common association with Greek and European mythologies, she actually drew inspiration in "Rowing" from precolonial Afro-Caribbean folklore. She used Afro-Caribbean narrative and motifs to communicate themes of cultural oppression and personal despair.

Global Myths, Folk Legends and Fairy Tales

According to poetry expert Steven Gould Axelrod, Distinguished Professor of English at the University of California, Riverside, "Until she wrote 'Rowing,' Sexton's involvement with myth, fairy tale, and folk tale has always been viewed as an engagement with European culture, because Sexton's earlier volume, *Transformations*, was based on European myths, mainly as recounted by the Grimm Brothers" (Axelrod). Sexton is further associated with European tradition in her similarities to Sylvia Plath: both used techniques of Freudian analysis to turn their poetry into a locus for self-exploration, and both wrote poems, although years apart, titled "Cinderella," retelling the story and remaking the image of a "charwoman," as Sexton called her, to challenge the superficiality of the happy-ever-after fantasy. Their work was seminal in expressing dissatisfaction with the social order, but the emerging global postcolonial consciousness – which blends with the more Eurocentric consciousness in the 1960s and 1970s – was beginning to change everything.

The Caribbean Artists Movement

Sexton's turning to Caribbean myth represents a departure from the work done by Sylvia Plath, and other celebrated "confessional" poets of the 1950s and 1960s, such as Robert Lowell, John Berryman and W.D. Snodgrass. In 1967, when Sexton was invited to the London Poetry International to read from *Live or Die*, her Pulitzer Prize winning book of poetry, the United States was embroiled in the disastrous Vietnam War. Demonstrations against the war were staged in Washington, D.C., as well as in small towns across America. At the same time feminists were demanding equality, not just in the workplace, but at home and in marriage, themes Sexton had been exploring in her poetry with a radical vision. She was invited to read her poetry at the Festival at a time of great political and social change.

While the Festival of Poetry was going on, another major literary and cultural movement called the Caribbean Artists Movement was happening in London (CAM). CAM was founded to create a forum for writers, artists and critics from the English-speaking Caribbean, resident at that time in the United Kingdom. Talks, discussions, conferences, recitals and art exhibitions provided an opportunity to explore new directions in Caribbean arts and culture at a time of political and social change. CAM officially launched with a public reading by Edward Kamau Brathwaite, one of its founders, from his work, *Rights of Passage*. This book was published in 1967, followed by *Masks*, and *Islands*, and then in 1973, the books were published as a trilogy titled *The Arrivants*. The publication in London and New York of a trilogy containing the work of a Caribbean poet is an important example of the ways in which African and Afro-Caribbean poets, such as Brathwaite, were creating a global postcolonial consciousness alongside the Eurocentric perspective of mid-twentieth century poets, such as W.H. Auden, Jon Stallworthy, Stephen Spender, and many more who were in attendance at the festival.

Popularity of Dub Poetry

Sexton, as an artist working in Boston and New York, would have been influenced by the poetry and music from the Caribbean, and she would have heard of Brathwaite while she was in London. Brathwaite, who was born in Barbados, read his work over a musical track of various West African rhythms, blues, jazz, ska, and calypso. He was not the only Caribbean artist making an impact in the Western world by featuring dub poetry, the use of poetry over music written specifically for performance. Linton Kwesi Johnson, the

originator of dub poetry (whose songs were on the charts in Great Britain), Mighty Sparrow (called the Calypso King of the World), and the legendary Jamaican poet, folklorist and educator, Miss Lou Bennett (who appeared at some of the same poetry readings as Sexton), were artists whose intention was to disrupt the cultural imperatives of the white, hegemonic class. Across the ocean, Bob Marley was in New York singing, "Get up, stand up, stand up for your rights / Get up, stand up, don't give up the fight" (Marley 1-2). At one of his concerts in Boston, where Sexton lived, the police were so afraid of riots that they moved Marley's Friday concert night to a Sunday afternoon in hopes of keeping college students under crowd control.

Sexton was writing poetry that demonstrated some of the same resistance to patriarchal and economic oppression as the Caribbean poets. After the London poetry festival, she began performing her own poetry dubbed over rhythm and sound the way Brathwaite had done at CAM. According to Sexton's biographer, Diane Middlebrook, after Sexton returned from London, she formed a rock group, "Anne Sexton and Her Kind," named after one of her most famous poems, and within the year began reciting her own work to music (Middlebrook 286). "People flock to Bob Dylan, Janis Joplin, and the Beatles, they are the poets of the English-speaking world," Sexton told her daughter (L. Sexton 147). It is entirely in keeping with her awareness of cultural imperatives that she would consider forming a rock band and begin performing dub poetry.

Music was not the only attraction of the Caribbean. Sexton vacationed often in Bermuda at the Cambridge Shores, located in the Sargasso Sea, near the Caribbean, where she could sit for hours in the sun by the pool and be pampered by hotel staff. The staff were descendants of slaves brought to Bermuda by the Spaniards in the seventeenth century, although the Africans, Irish and Native American Indians never willingly accepted their status of slaves. It appears that Bermuda became a place for Sexton to encounter African and Caribbean culture at a comfortable distance. Interestingly, there are no books by Afro-Caribbean or African-American writers and poets among Sexton's possessions that are now archived at the library of the Harry Ransom Center, at the University of Texas in Austin. We do know, however, that she read *A Story A Story*, the precolonial African and Afro-Caribbean myth that Sexton cited to inform her poem, "Rowing."

"Let It Go. Let It Come."

A Story A Story is a children's book, winner of the 1970 Caldecott Medal, written and illustrated by Gail E. Haley (Haley). It is about Anansi the Spider, a myth that originated with the Ashanti people of West Ghana. In the biography of her mother, *Searching For Mercy Street*, Linda Gray Sexton relates that browsing in bookstores with her mother was one of her fondest memories, and that she and her mother had loved fairy tales all their lives. Their shared love of these stories led to creation of *Transformations*, which Sexton then dedicated to Linda (L. Sexton 114, 148). In the Caribbean myth, Anansi is a lovable trickster, half spider, half human being, who wants to buy all the stories of the world from the Sky God, Nyami. Nyami keeps the stories in a golden box by his throne, but once Anansi proves his worth, and after he pays the asking price, Nyami gives him the stories. The little spider then takes the golden box of stories back to the people of his village, and when he opens the box all the stories scatter to the corners of the world.

"Rowing" begins with the same lines that appear in Haley's book: "A story, a story; let it come let it go" (Haley). In "Rowing," Sexton adds parenthesis and changes the order of the verbs in the first two lines of her poem: "A Story, A Story! (Let it go. Let it come.)" (A. Sexton "Rowing" 1-2). Sexton also cites the ending of Haley's book: "This is my tale which I have told, if it be sweet, if it be not sweet, take somewhere else and let some return to me" (Haley). This last line references the stories God gave to Anansi and the way Anansi shared the stories with his people; it is also a strategy of resistance employed by Anansi to control the powerful forces arrayed against him. While Sexton repeats the words from Haley's version, she adds the line, "As the African says" (A. Sexton "Rowing" 45), which pays homage to the origin of the story at a time when white-identified poets usually did not honor black tradition, and she ends her poem "with me still rowing" (A. Sexton "Rowing" 49).

Sexton uses the retelling of a slave narrative as a framework for expressing her suicidal despair, a theme she expressed in much of her poetry. This poem represents a new stage

in her representation of psychological anguish, in that she communicates it through metaphors and language derived from African and Caribbean myths rather than from myths basic to the Anglo-European culture that had previously sustained and shaped her. Sexton finds a powerful metaphor for her own alienation, depression, and struggle for creative expression in the texts African-American and African-Caribbean poets composed to reflect the suffering of slavery and Jim Crow conditions, and their efforts to build a postcolonial identity. In giving expression to her own feelings, she also draws attention to, and finds inspiration in, the historical struggle of the African diaspora.

It must be stated that Sexton does not claim that her family's experience was similar to the families from West Africa that were kidnaped and transported to the Caribbean, where the original Amerindian people – Taino, Siboney, Carib, and Arawak – had been decimated. Within 30 years of Columbus' discovery, one million people died and it became necessary for the conquistadores to import new labor. The people of Ashanti, Congo, and Nigeria were then imported into the Caribbean where they were generally worked to death.

Sexton's use of the Anansi myth follows the tradition of other postmodernists who, according to Michael North, interpret the culture of the third world through the lens of their own culture. In *The Dialect of Modernism*, North explains in a study of racial masquerade that "Anansi's aim is to protect himself by cringing before the white master, lying, avoiding the issue and misleading him" (North 107). The story personifies the Ashanti's inability to speak their own language in the Caribbean and the compelled act of speaking the language of their oppressors. It is important to point out the differences between the quest of poets like Sexton to revisit their childhoods through the medium of myth and fairy tale, and the quest of the Ashanti people to challenge the colonial past and reinterpret it. The story of Anansi in the Caribbean is not a quest for selfhood, but for a nation coming into being.

Anansi the Trickster

Anansi mimics the language of his white oppressor in order to control him, and eventually the white man mimics the crafty Anansi in return. Sexton may have turned to a story about a creature who receives gifts from a god in order to explore her own search for a god she could believe in, and in so doing she seeks to find herself through black myth rather than white. On the file folder of first drafts for *The Awful Rowing Toward God*, in which "Rowing" is the first poem, she notes, "these poems were started 1/10th/73 and finished 1/30th/73 (with two days out for despair, and three days out in a mental hospital)." In her own words she explained that the poems, all first drafts, "were written in a frenzy of despair and hope." Afterwards, she sent a copy of the poems to her agent with a note of explanation that the book was "a bit 'odd' but after all, I didn't do it, the typewriter did it" (A. Sexton *Self Portrait* 390, 403).

The typewriter held totemic meaning for Sexton, and it provides a clue as to why she turned to a story about a creature who receives his stories from a god who keeps them in a box near his throne. Sexton shared a story with several friends about how she went to a priest seeking solace, and he told her that God was in her typewriter. Maxine Kumin, the Pulitzer Prize winning poet, and Sexton's friend, included the story in her forward to the publication of *The Complete Poems of Anne Sexton* (xxiii). Kumin maintains that his simple words gave the poet the desire to continue living and writing, and that he made the poems in *The Awful Rowing Toward God* possible.

Still Rowing

A close reading of "Rowing" reveals many associations Sexton may have made to the journey of the Ashanti people from West Ghana. Her story begins at the assembly line of an automobile factory. "I was stamped out like a Plymouth fender / into this world" (A. Sexton "Rowing" 3-4). The simile compares the speaker to a part in the production line, which is then thrust with an enjambment "into this world." Slaves also were "stamped" into the hold of vessels that took them across the ocean to the new world. The idea of enslavement then appears in the next two lines of her poem, with "the crib" as a metaphor for a prison with bars: "First came the crib / with its glacial bars" (A. Sexton "Rowing" 5-6). Another etymological meaning for crib is an aid to translation. Once again, this may signify Sexton's concern with the ways in which her individuality was stolen and suppressed throughout childhood.

She felt silenced as a child, molded into conformist behavior: "Then there was school, / The little straight rows of chairs, / Blotting my name over and over" (A. Sexton "Rowing" 9-10). Coincidentally, these lines are very similar to Kamau Brathwaite's lines in his poem, "Legba," which appears in his trilogy *The Arrivants*, and relates the experience of a black child in the white man's school in Barbados: "they go to school to the head- / master's cries, / read a black- / board of words, angles, lies" (Brathwaite 17-21). While Sexton did not experience racism, she did feel constrained as an independent woman in a patriarchal culture and as a person with psychological disabilities attempting to survive in a society intolerant of deviations from the norm.

Her associative gifts came directly from a troubled and chaotic life that she used to uncloak the cult of domesticity and other cultural narratives of a privileged American lifestyle, so intimidating to her both emotionally and physically. Physically, she felt bullied and disempowered, as if she were "undersea all the time, / a stranger whose elbows wouldn't work." (A. Sexton "Rowing" 12-13). Sexton was struggling with both depression and her place in society. The depression was connected, certainly in her own understanding and undoubtedly in reality, to the limits placed on her because of her gender.

But she grew: six times in the following lines she repeats the words "I grew," and she employs the word "but" many times, to say perhaps that her growth was at the cost of her humanity.

> but I grew
> like a pig in a trenchcoat I grew,
> and then there were many strange apparitions,
> the nagging rain, the sun turning into poison
> that, saws working through my heart,
> but I grew, I grew,
> and God was there like an island I had not rowed to,
> still ignorant of Him, my arms and my legs worked,
> and I grew, I grew, (18-26)

It is a fearful image, with Sexton castigating the person she has become by comparing herself to a pig, perhaps a metaphor for greedy consumption and inner filth. It may also be an allusion to the way the invaders introduced the raising of pigs on the islands, with the pigs responsible for destroying native flora and crops.

Rats, too, figure in Sexton's representation of her journey. In several of her earlier poems she used the rat as a metaphor for her sick self, but this poem requires another interpretation: the horrific ordeal of the people from the African continent who were forced into the holds of ships infested with rats. It is estimated that 20 to 30 million people who crossed the Atlantic during the Middle Passage died of trauma, drowning, or disease. Others place this number much higher. The narrator wisely does not compare herself to a slave; rather, she compares herself to a pig and then to a rat. But even though she is filled with self-disgust, she avers that God will embrace her. A clue to the latent meaning in this verse is its allusion to her favorite and oft used palindrome, "rats live on no evil star." By the reversal of "rats" into "star" she opens the door from "the hold" to the transcendent.

> But there will be a door
> and I will open it
> and I will get rid of the rat inside of me,
> the gnawing pestilential rat
> God will take it with his two hands
> and embrace it. (39-44)

Sexton, by her own admission, did not proof read the poems contained in *The Awful Rowing Toward God*, the way she did with all her previous work, perhaps because of her failing mental health, and she was dealing with the reluctance of a Eurocentric publishing industry to address the issue of racial injustice and genocide. Whether the omission was intentional or not, her poem mirrors the Caribbean experience. Sexton was not indifferent to the violence caused by the colonizers and the patriarchal hegemony that was established on the Caribbean islands.

"Ananse" by Brathwaite

Following is a verse from Brathwaite's poem "Ananse," published in *The Arrivants* a few years before Sexton wrote "Rowing." Although one cannot say for sure that Sexton read this poem, it is likely that she did. Brathwaite's speaker endowed the mythic black creature with a transformative potential that would have resonated with Sexton:

> Now the poor hang him up in the ceiling,
> their brooms cannot reach his hushed corner
> and he sits with the dust, desert's rainfall of soot,
> plotting a new fall from heaven

threading
threading
the moon
moonlight stories (165-166).

Brathwaite elaborates on the cultural connections between the West and the African and Caribbean peoples in an interview conducted by Nathaniel Mackey in *The Art of Kamau Brathwaite* (23-24):

> You see, as a Caribbean person, we start with the ruins and our responsibility is to rebuild those fragments into a whole society…So that when Africans come into the Caribbean one is very much aware that they have not only brought their own culture with them but that culture begins to relate to the culture of the European, the conquistador, the Amerindian, and right away we are at the threshold of a new kind of social structure, a new kind of cultural imagination.

In Sexton's poem alluding to the Anansi myth, she resurrects Ashanti cultural materials, not so much as a consumed object but as an Afro-Caribbean way of organizing experience.

It is not surprising that Sexton would be drawn to global myths, folk legends, and fairy tales. What is surprising, however, is that such a crucial influence on one of her key poems has remained largely unknown. It is a symptom of how the literature and poetry of Africans and African-Americans was marginalized or abstracted during the 60s and 70s.

CONCLUSION

The use of the Anansi myth and the inclusion of African and Afro-Caribbean material demonstrates how the emerging global and postcolonial consciousness of the 1960s and 70s influenced Sexton. It also subtly changes the conventional wisdom that Sexton is known for her "confessional" poetry and for her containment within white culture. In a very real sense, Sexton's poem is a collaboration between the poet and the multitude of creators, most of them anonymous, who generated the story of Anansi over the centuries. The fact that the Anansi story was popularized in the 1980s by children's entertainer Raffi—an artist of Egyptian-Armenian origins, naturalized Canadian citizenship, and international perspective—only adds to the sense of shifting geographical and cultural centers in the work of Sexton, and in North American verbal expression more generally (Axelrod). Sexton apparently borrowed from the Anansi story for her own purposes, but the cultural product she generated assures the continuation of a story originally told by village priests around the campfires on the banks of the Senegal River. Her work connects her to a precolonial, colonial, postcolonial and global tradition through Anansi.

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Utilizing FRET in the Development of an *in vitro* SUMOylation Assay for High-Throughput Screening

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ABSTRACT

Förster Resonance Energy Transfer (FRET) is an extensively used assay in biomedical research for understanding biological phenomena and conducting high-throughput drug candidate screenings. Here, we investigate the efficacy of FRET in the SUMOylation signal transduction pathway that occurs in eukaryotes, which has been linked to Influenza A, breast cancer, and other diseases. By studying the implications for determining FRET's efficacy, researchers can further develop this study and utilize this method to accurately screen a multitude of proteins for discovering potential drug inhibitors in the SUMOylation pathway as well as other ubiquitin-like pathways. Through molecular cloning, we created constructs for each protein involved in the SUMOylation cascade reaction: Aos1, Uba2, Ubc9, SUMO1, with and without fluorescence-tags. Later, we were able to express, purify and characterize each protein. Subsequent assessments of *in vitro* SUMOylation assay involving a truncated protein of known SUMOylation substrate-RanGap were then conducted in a high-throughput manner. Aos1 from the E1 complex had the lowest yield compared to the other constructs, but still provided sufficient biochemical activity to conduct the assay. According to the SDS-PAGE results, Uba2 contained a fair amount of impurities, but still provided sufficient activity to form the E1 complex with Aos1. From FRET, we were able to note an increase of $\geq$ 200% over the initial value. Additionally, Em_{FRET_Max} was determined with 95% confidence to be $(2.38 \pm 0.09) \times 10^6$ RFU with a coefficient of variation of 0.067. Ultimately, these results demonstrate an effective and working method to provide accurate molecular screenings and characterizations.

Keywords: Förster Resonance Energy Transfer (FRET), high-throughput screening (HTS), SUMOylation, SUMO, CyPet, YPet, Aos1, Uba2, Ubc9, SUMO1, RanGap1c

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INTRODUCTION

The SUMOylation pathway is a post-translational modification in eukaryotes that utilizes small ubiquitin-like modifiers (SUMOs) and regulates many aspects of cell physiology, including: cell-cycle, transcription, nuclear-cytosolic transport, chromosome dynamics, apoptosis, ribosome biogenesis, and DNA replication/repair.[1] A single mutation in one of these processes can lead to unwanted cell proliferation, such as the growth of malignant cells. These SUMO proteins covalently conjugate to other proteins in the pathway where mature forms are then created from cutting, folding, or adding certain functional groups through a cytosolic enzymatic cascade in a similar way to ubiquitination. Figure 1 displays the overall SUMOylation pathway and how SUMO1 undergoes the forward cycle and interacts with three unique complexes.

Pre-SUMO1 is initially cleaved into its mature form by SENPs (sentrin specific proteases) displaying a double-glycine-residue motif. The SUMO activating enzyme complex E1, a heterodimer composed of Aos1 and Uba2, then undergoes an ATP-dependent step to adenylate the C-terminal carboxyl group of SUMO1, releasing a pyrophosphate. SUMO1 is transferred to the thiol group of the catalytic cysteine of Uba2, releasing AMP and Aos1. Next, SUMO1 reacts by transferring a cysteine residue from Ubc9 in the E2 complex to the active site, forming another high-energy thioester bond. Lastly, SUMO1 is transferred from the E2 complex and is conjugated to a substrate (in our case, it is YPet-RanGap1c) at a lysine residue with the help of E3 ligases.

We will be utilizing the Förster Resonance Energy Transfer (FRET) assay, a distance-dependent interaction method by which energy is transferred non-radiatively between a donor and an acceptor fluorescent molecule by means of intermolecular long-range dipole-dipole coupling with overlapping emission and excitation spectra.[2] To measure FRET, a fluorometer first generates a specific wavelength of light to excite the donor molecule. The donor then produces an emission wavelength that is used as the excitation source for the acceptor molecule. FRET is then recorded as the measured emission from the acceptor molecule. The fluorometer software graphically displays these FRET emission values in terms of relative fluorescent units (RFUs) with respect to time. FRET technology can provide accurate measurements of molecular proximity at distances of 10-100 Å and is highly efficient if the two fluorophores are positioned within the Förster radius.[3] FRET is a unique, highly-sensitive, and homogenous method in high-throughput screening (HTS), and unlike other methods, it is easy to operate, has quicker assay times, and is cost-effective. Because FRET efficiency is dependent on the inverse sixth power of the intermolecular distance, FRET assays are a widely used technique for investigating specific transient molecular interactions and reaction intermediates involving multiplex enzymatic cascades in a variety of biological phenomena.[3]

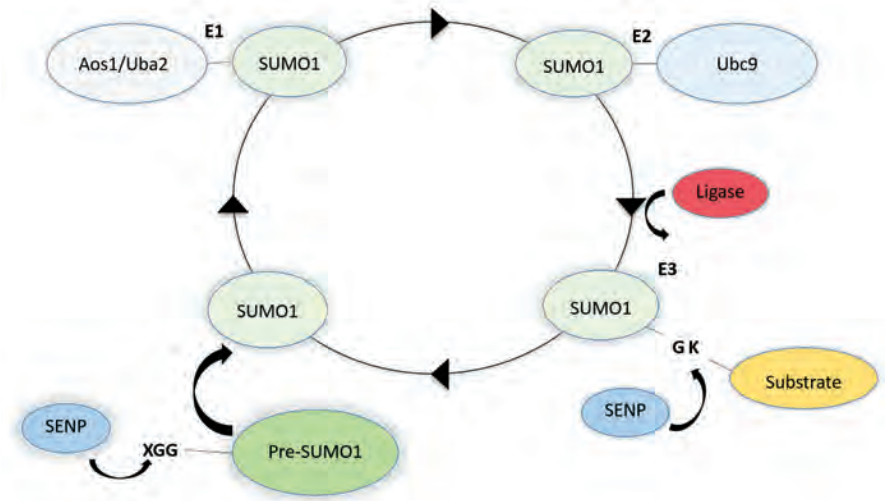

Fig. 1: *General diagram of the SUMOylation pathway showing all 3 major enzyme complexes.*

Other methods for HTS, specifically reporter gene assays and yeast two-hybrid (Y2H) assays, have limitations of their own that make them less efficient. Although reporter gene assays are also a cost-effective HTS platform, they require long incubation periods, have difficulty achieving antagonist detection due to reporter accumulation, and can have high false positive rates since the signal event is distal from receptor activation.[4] Though Y2H assays provide direct identification of interacting protein pairs without

further downstream experiments, it is rather less cost-effective and is a more labor-intensive approach.[5]

FRET assays also have a few limiting factors, such as low signal-to-noise ratio resulting from a cell's intrinsic fluorescence background and requirement of overlapping emission spectra. However, when successfully implemented, FRET can be readily applied to detect molecular interactive events, making it an invaluable technique for biological research. Our objective is to utilize a FRET assay on the recombinant fluorescent proteins for characterization and identification, which we can then use to analyze the performance of this method. By producing working constructs of the major proteins in the pathway and testing their activity, we can develop this assay to measure and determine the fluorescence emissions. By achieving highly sensitive expected results via FRET, we can conclude that this assay is an advantageous method over other assays for HTS within the field of study.

MATERIALS AND METHODS

Transformation into BL21 via Electroporation

Electrical transformation via electroporation was performed, in which pET 28(b) vectors encoding the Aos1 (~40 kDa), Uba2 (~71 kDa), Ubc9 (~18 kDa), and SUMO1 (~11.5 kDa) genes, some fluorescently tagged with CyPet or YPet (both ~27 kDa), were transfected into *Escherichia coli* strain BL21 (DE3) competent cells. 20 µL of BL21 cells were mixed with 1 µL of a pre-made DNA ligation mixture. This mixture was then transferred to a cuvette and a 1.8 kV shock was applied. Immediately after, 250 µL of SOC medium was added. The bacterial cells were then incubated in a shaker at 37°C for one hour and 10 cm LB agar plates with ampicillin antibiotic at 100 µg/mL were preheated in a 37°C incubator. The transformation mixtures were uniformly spread on the LB agar plates and incubated at 37°C overnight.

Protein Expression and Purification

From the culture plates, bacterial colonies were then inoculated into 10 µL culture tubes containing LB with 1 mL of the appropriately added antibiotic per 1 mL of LB. After shaking the tubes at 250-300 rpm at 37°C overnight, the media was then inoculated into 1 L flasks containing 2XYT media with antibiotics. The flasks shook at 37°C for three hours. The cells were then induced by addition of IPTG and the flasks were shaken at 180-250 rpm at 25°C for 14-16 hours. The bacterial culture media were transferred into 250 mL bottles that underwent centrifugation at 7,000 rpm for three minutes at 4°C to create cell pellets; the supernatants were then poured into a waste container. The bacterial cell pellets were re-suspended in 15 mL of Binding Buffer by vortexing and were then transferred into 50 mL centrifuge tubes and placed on ice. Next, the mixtures were each sonicated for 10 minutes at five-second intervals. The tubes were then centrifuged at 35,000 rpm for 30 minutes to pellet down cellular debris and inclusion bodies. Lastly, the protein supernatant solutions were collected in 50 mL conical tubes.

To perform protein purification, we used 10 mL columns washed with autoclaved milli-Q water and transferred 200 µL of QIAGEN Ni^{2+}-NTA agarose beads and let the solutions flow through naturally. Next, the protein supernatants were transferred into the column and were allowed to drain through while the proteins bonded to the Ni^{2+} beads. Washing Buffer I, II, and III were poured through the columns once, twice, and once respectively to purify the proteins. The purified proteins were then eluted with 400 µL of an Elution Buffer.

Table 1: *Composition of the buffers used during the protein expression and purification process.*

Binding Buffer	Wash Buffer 1	Wash Buffer 2	Wash Buffer 3	Elution Buffer	Dialysis Buffer
5mM Imidazole	300mM NaCl	1.5M NaCl	0.5M NaCl	500mM Imidazole	1M DTT
500mM NaCl	20mM Tris-HCl, pH = 7.9	20mM Tris-HCl, pH = 7.9	20mM Tris-HCl, pH = 7.9	500mM NaCl	1M Tris-HCl pH = 7.4
20mM Tris-HCl, pH = 7.9	--	0.5% Triton	10mM Imidazole	20mM Tris-HCl, pH = 7.9	2M NaCl

After collecting all the proteins, the solutions were injected into separate dialysis cassettes and dialyzed in a buffer overnight and aliquoted into 1.5 mL Eppendorf tubes (buffer contents shown in Table 1).

Protein Gel Electrophoresis and Concentration Determination

To determine protein concentrations, a Bradford assay was performed utilizing the Thermo Scientific BioMate 3 UV/VIS 6-cell holder spectrophotometer. Protein purity was also checked via SDS-PAGE. 15 mL of loading buffer (0.25 M Tris-HCl (pH = 6.8), 8% SDS, 30% glycerol, 0.02% Bromophenol Blue, and 0.2 M DTT) was mixed with 5 mg of protein solution in 1.5 mL Eppendorf tubes. Autoclaved milli-Q water was then added to fill the solution to a total volume of 30 mL. The protein samples were then heated at 100ºC for five minutes. The samples were pipetted into the SDS-PAGE gel and ran for 1.5 hours at 100 V. After electrophoresis, the gel was then placed in staining buffer (0.25% Coomassie Brilliant Blue R-250, 50% Methanol, 10% Acetic Acid, 40% water) for one hour. Destaining buffer (50% Methanol, 10% Acetic acid, 40% water) was then used until the bands were visible. Lastly, a picture of the gel was taken using the UVP Bioimaging system to analyze the band purities and confirm the proteins' molecular weights.

Förster Resonance Energy Transfer Assay

The fluorescence and FRET emissions of the proteins were determined using the Molecular Devices Flexstation II^{384} benchtop scanning fluorometer and the SoftMax Pro Acquisition and Analysis software. As a proof of concept, we monitored if the SUMOylation cascade would take place with our purified proteins. Using a black Greiner 384-well microplate, two samples were prepared, one being a negative control. Each well contained 49.5 µL of 1X SUMO buffer solution (150 mM NaCl, 50 mM Tris-HCl (pH = 7.4), 1 mM DTT, 4 mM MgCl2), 1 µL each for CyPet-SUMO1, Aos1, Uba2, and Ubc9, and 0.5 µL of YPet-RanGap1c (RanGap1c ≈ 18 kDa). Lastly, 6 µL of 10 mM ATP, or autoclaved milli-Q water for the negative control sample, was added and mixed for a total volume of 60 µL. The microplate was then placed into the fluorometer and incubated at 23.6°C. We then ran a kinetic reading in which the emission intensities at three wavelengths were collected: 475 and 530 nm at excitation wavelength of 414 nm, and 530 nm at excitation wavelength of 475 nm.[6] The fluorescence emission was monitored for one hour and the emission intensities were collected every 10 seconds. After we observed that SUMOylation occurred from our FRET results, we prepared diluted protein samples of 5 µM and set up a 60 µL reaction, in which our working concentrations in each well were 1 µM of CyPet-SUMO1 and YPet-RanGap1c and 0.5 µM of the Aos1, Ubc9, and Uba2 enzymes (refer to Table 2). To calibrate our empty Greiner 384-well plate, we ran an endpoint reading. We then prepared 13 samples in our microplate, each with 8 µL of 1X SUMO buffer solution, 12 µL of CyPet-SUMO1 and YPet-RanGap1c, and 6 µL of Aos1, Ubc9, and Uba2. Lastly, 10 µL of 10 mM ATP, or autoclaved milli-Q water for one negative control sample, was added and mixed in

Table 2: *Absorbance and concentration values of the proteins in the SUMOylation cascade including our working concentrations used for the SUMOylation and FRET assays.*

Protein	Absorbance	Concentration [µM]	Concentration [mg/ml]	Working Plate Concentrations [µM]
CyPet-SUMO1	0.817	51.5974	0.3238	1.000
Aos1	0.534	26.7898	0.2754	0.500
Aos1	0.337	17.4325	0.2217	--
Ubc9	0.598	68.4506	0.3622	0.500
YPet-Ubc9	0.693	33.6842	0.2077	--
Uba2	0.571	16.3613	1.1617	0.500
YPet-RanGap1c	1.065	75.8222	1.4501	1.000

each well. The microplate was then placed into the fluorometer and incubated at 37°C. We then ran a kinetic reading and collected emission intensities at the same aforementioned wavelengths. The fluorescence emissions of all 13 samples were monitored for 90 minutes with emission intensities collected every 30 seconds. After the emission intensities were corrected by subtraction of background fluorescence from calibration of the 384-well microplate, the Em_{FRET_Max} was determined and compared.

RESULTS

Protein Purity and Concentration

Once we managed to express, purify, and collect our protein samples, we ran a concentration check on each of them. According to Table 2, YPet-RanGap1c provided the greatest absorbance value (1.065) while our Aos1 samples gave low absorbance values (0.534 and 0.337).

The concentration of each protein was calculated from the respective absorbance values using an equation generated from a standard curve. To check the purity of our samples, we calculated each protein to 5 mg from the concentrations of the Bradford assay and ran them on a 10% SDS PAGE gel. After the gel was destained, allowing us to see the bands, we compared the protein band molecular weights to the GoldBio BLUEstain protein ladder shown in Figure 2.

According to this, we were able to note that each protein was well within the range of their expected molecular weight values with minor exceptions.

Protein Activity and FRET Assay

As a proof of concept and to ensure the proteins will work together during our FRET assay, we performed a SUMOylation assay kinetic measurement along with a negative control. After running the kinetic reading for one hour, we were able to see the FRET emission significantly increase by approximately 163.48% compared to the negative control having a negligible increase of 14.08% shown in Figure 3.

Before we began our FRET assay, we created separate stocks of our proteins diluted to working concentrations shown in Table 2. In Figure 4, we were able to see the FRET emissions after the high-throughput assay kinetic reading from a total of 13 wells with one being a negative control lacking ATP.

The percentage increase of the 12 samples for the FRET data were calculated by subtracting each final emission value by its respective initial emission value and dividing by the same respective initial value. By taking the average of each aforementioned percentage increase, the mean percentage increase was then calculated to be 199.59% within a 95% confidence interval of 184.55% to 214.64%. Em_{FRET_Max} was also determined to be (2.38 ± 0.09) x 10^6 RFU within a 95% confidence interval based on the RFU data of Figure 4. The high-throughput coefficient of variation for Em_{FRET_Max} was calculated to be 0.067 by dividing the standard deviation over the mean. Using the Z-factor equation from Zhang, et al. 1999, our

Fig. 2: *10% Polyacrylamide gel results from SDS-PAGE running at 100 V for about 90 minutes.*

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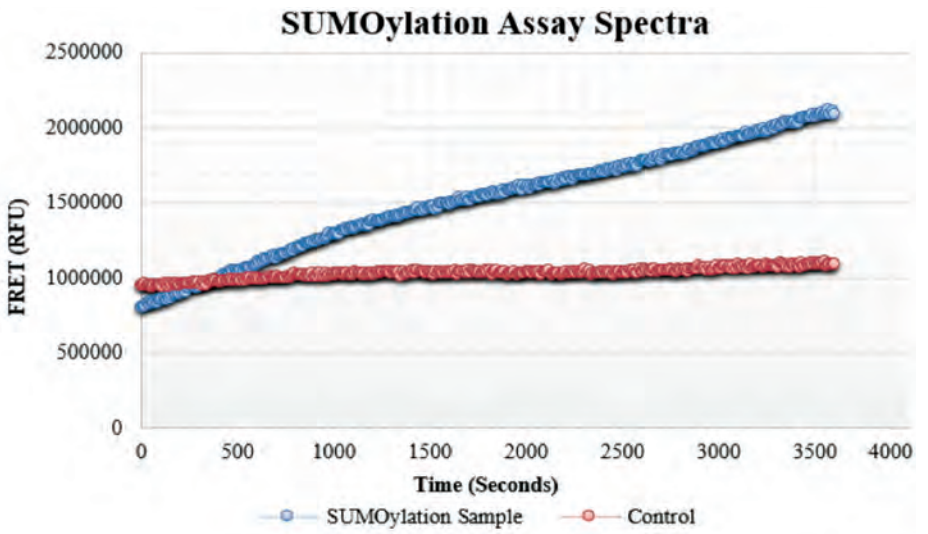

Fig. 3: *SUMOylation assay kinetic measurement displaying a working construct of the proteins.*

Z-factor value was calculated to be 0.643.[7] This diversity of statistical results provides different forms of the same concept allowing for an accurate procedural performance.

DISCUSSION

The SUMOylation pathway is a post-translational modification in eukaryotes that employs SUMO proteins and can control aspects of cell physiology.[1] In our research, we developed a high-throughput assay for the SUMOylation cascade using FRET technology. In our previous experiments, Aos1 has yielded lower concentrations than some of the other proteins in the pathway. Thus, we expressed and purified two separate batches and experimented with the higher concentrated sample. In addition, though YPet-Ubc9 was not used in the final FRET assay, we expressed and purified a sample to ensure acceptable activity of our CyPet-SUMO1 protein. Furthermore, Ubc9's molecular weight is slightly shifted when compared to the ladder, but is due to the fact that Ubc9 has extra histidine residues that add 1 kDa to its molecular weight. Uba2 is another protein that can be difficult to purify, and although the activity of Uba2 was sufficient enough for the SUMOylation cascade to occur, its purity was not the best according to our protein gel. A proposed method we believe that will improve the purity of Uba2 is performing the purification process twice. After performing the first purification and dialysis, we propose that the protein supernatant left in the dialysis cassette be taken

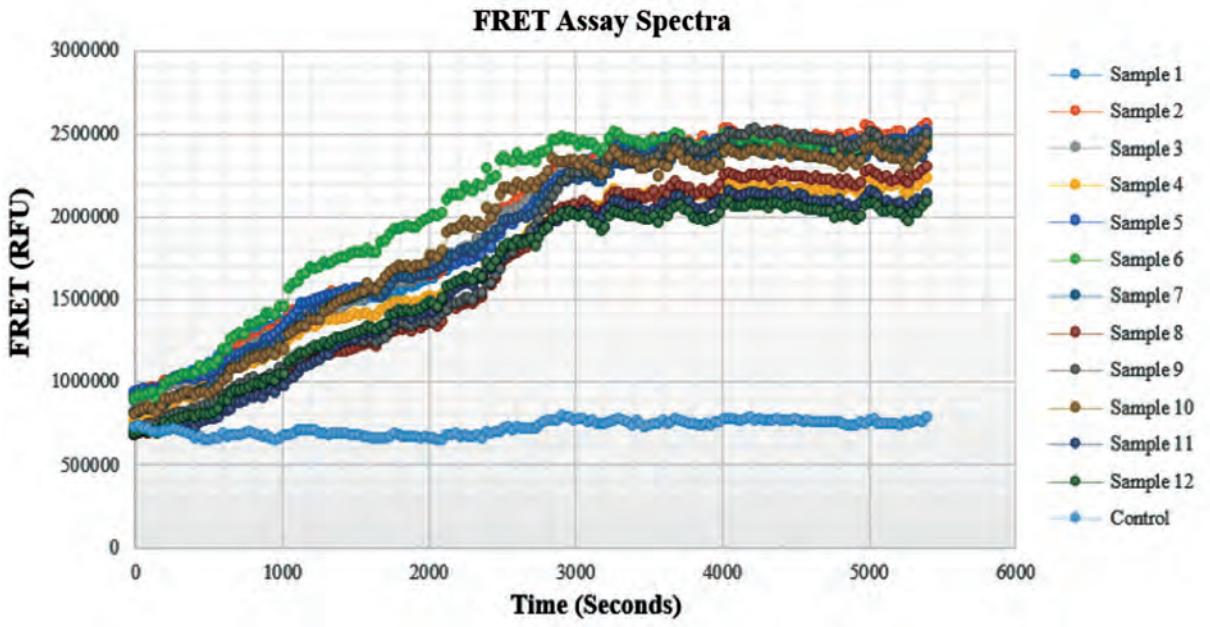

Fig. 4: *Kinetic measurement of the high-throughput FRET assay data from 13 wells revealing the emissions in comparison with an ATP-lacking negative control. The spectra take into consideration the data that was corrected via subtraction of the endpoint measurement of the background fluorescence from the 384-well plate.*

out and poured back into the 10 mL column with 200 µL of Ni^{2+} beads. Wash Buffers I, II, and III can then be added into the columns only once each. After all wash buffers drain through the column, the purified proteins can be eluted and pipetted into cassettes for overnight dialysis and aliquoted. However, because of this double purification process, there may be less volume of protein supernatant to collect. To collect a sufficient amount of protein, we suggest using volumes greater than 1 L of 2XYT media with antibiotics during protein expression. Despite minor protein impurities and concentrations, the results indicate these issues did not significantly affect the assay. The increase of ~200% in our FRET data demonstrates that our methods work since the increase is directly correlated with the formation of product and that they can be performed reliably. The concept of the Z-factor, a screening window coefficient, is used to characterize the quality or performance of a HTS assay where $-1 < Z < 1$. According to Zhang, et al. 1999, our calculated Z-factor value is well within the $0.5 \leq Z < 1$ range in which the quality of our HTS assay is considered to be an "excellent" assay.[7]

Recent studies have shown that the SUMOylation pathway plays a critical role in regulating the transcriptional mechanisms that maintain the basal breast cancer phenotype and also has the potential to be a novel therapeutic target for treating Multiple Myeloma.[8,9] SUMOylation has also been linked to the Influenza A virus nucleoprotein (NP), which allows for intracellular trafficking of the NP and viral growth.[10] Ultimately, having an in-depth understanding of the SUMOylation pathway and applications of FRET can lead to further research in identifying novel SUMOylation inhibitors, which may be an effective treatment strategy for cancers and other diseases.

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The Dynamic Social Agency of the Kālacakra Sand Mandala

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ABSTRACT

The Tibetan Buddhist initiation rite known as the Kālacakra, or "Wheel of Time," famously requires the construction and dissolution of an elaborate sand mandala—a multi-tiered cosmological map made from millions of grains of colored sand. While the intricate design and ephemeral nature of the mandala have long piqued the interest of Buddhists and non-Buddhists alike, the history of Kālacakra scholarship has either focused on historical and philosophical accounts of the ritual, or has offered a semiotic didacticism for deciphering the mandala's encoded symbols. These approaches fail to consider the lived experiences of those individuals who interact with the mandala spiritually and secularly. Based on qualitative research conducted among the mandala's new and diverse publics, including tantric disciples, refugees, converts, and casual observers, this paper examines the integration of the Kālacakra into the transnational cultural landscape by weaving the experiences of non-practicing publics through an exploration of the practitioner's ritual engagement. I argue that the mandala functions with a secondary social agency that affords specific modes of subject formation, and that by engaging with the mandala in particular ways, individuals construct and negotiate the many overlapping worlds they inhabit. Such a perspective makes evident that the conventional historical and semiotic approaches to Kālacakra scholarship neglect how the mandala functions ethnographically. I therefore offer a method for understanding the myriad social functions of the mandala that maps more fully onto the complex realities expressed by the mandala's many engaged publics.

Keywords: Tibet, Buddhism, ritual, mandala, agency, affordances

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INTRODUCTION

The Kālacakra, or "Wheel of Time," (Tib. *duskhor*) initiation rite revolves around the creation and ritual destruction of a complex artistic representation of enlightened experience known as the sand mandala.[1] It is little noted that this material focus of the Kālacakra ritual is more to tantric practitioners than a sacred symbol: it is thought to possess its own agency, constructing a religious reality into which the initiated enter. Unique among the otherwise restrictive *Anuttarayogatantra*, or "Highest Yoga Tantra" traditions, (Tib. *rnal 'byor bla na med pa'i rgyud*), the Kālacakra has a history of large-scale public performances governed by the sociocultural and geopolitical needs of the Tibetan people as they struggle against persecution and indifference.[2] In the hands of prominent Tibetan religious figures such as the Dalai Lama, the public performance of this tantric rite has expanded since the Chinese annexation of Tibetan cultural regions in the 1950s and the global diaspora of Tibetan refugees down to the present.[3] Based on qualitative research conducted among Kālacakra practitioners in several Tibetan-American Buddhist communities, this paper examines the integration of this practice into the American cultural landscape through the mandala's invention of new and diverse publics. To do this, I weave considerations of the non-practicing publics through my explanation of the practitioner's ritual engagement. I argue that the mandala functions with a secondary social agency that affords specific modes of subject formation, and that by engaging with the mandala in particular ways, individuals construct and negotiate the many overlapping worlds they inhabit. Such a perspective makes evident that the conventional historical and semiotic approaches to Kālacakra scholarship neglect how the mandala functions ethnographically. I therefore offer a method for understanding the function of the mandala that maps more fully onto the expressed realities of the mandala's engaged publics.

In the summer of 2015, I attended the annual Kālacakra retreat at Dü Khor Choe Ling ("Land of Kālacakra Study and Practice"), the new dedicated retreat space for the Namgyal Monastery Institute of Buddhist Studies in Ithaca, New York. There I observed the construction of the sand mandala (Fig. 1) and spoke with members of its many varied publics. Traditionally, this mandala would have only been constructed during a full initiation rite—that is, for the purpose of conferring initiation into the Kālacakra lineage onto Tantric adepts.[4] In recent decades, however, the mandala is also being constructed and dissolved for general audiences in otherwise secular spaces.[5] Although the knowledge contained within the mandala and its ritual is highly specialized and sacred, the preservation of Tibetan culture is of the utmost importance. The Dalai Lama himself notes that

> Although some mandalas … can be openly explained, most are normally supposed to be kept secret. Consequently, many speculative and mistaken interpretations have been published by people who viewed them simply as works of art. Because the severe misunderstandings that can then arise are more harmful than a partial lifting of secrecy, I have given a number of initiation rites in recent years, and many Westerners have become acquainted with the Kālacakra tradition.[6]

The retreat at Namgyal did not offer an initiation and was not a secular performance, but rather was an opportunity for previously initiated students to receive in-depth teachings from the Geshes on the philosophical and technical aspects of Kālacakra practice.[7] The students, a group of international converts, listened eagerly, scribbling notes during lessons and carefully modeling the speech

Fig. 1: *Monks constructing Kālacakra sand mandala, Dü Khor Choe Ling, Ithaca, NY. All photos taken by author.*

and gestures of the monks while in the Shine Room, the monastery space dedicated to imagery and ritual. They are a community, connected to each other through their shared participation in global Kālacakra-related events and their mutual desire to achieve authenticity in their practice, despite their language barriers. All of the students at the retreat had taken at least one initiation from the Dalai Lama and had been engaged in the study and practice of Kālacakra for several years.[8] Coming from such diverse linguistic and cultural backgrounds, these students each had unique stories about when, where, and how they first encountered the Kālacakra mandala, but without exception they all attested to being moved first and foremost by its aesthetic beauty, symbolic complexity, and technically masterful construction.

MANDALA AS OBJECT

For most *Anutarrayoga* traditions, a painted three-dimensional, or visualized, mandala of the meditational deity may be used; however, for the Kālacakra initiation, only a mandala made of colored powder or sand is permitted.[9,10] The creation of the mandala is an incredibly complex and ritualized process, with each stage requiring special recitations and gestures. This can take weeks to complete—at Namgyal, the monks took shifts, funneling sand and chanting from 7am to 10pm every day for ten days (Fig. 2). The sand is painstakingly placed in a precise arrangement, the meaning of which is complex, deeply layered, and self-referential. Within its multi-tiered symbolism is a depiction of what is generally called the Inner, Outer, and Other, or the Internal, External, and Alternative Kālacakra, which respectively depict the energy flows within the human body; the components of reality, such as time, space, elements, and beings; and another place not bound by space and time, in which humans may engage with, and eventually become, the divine.[11]

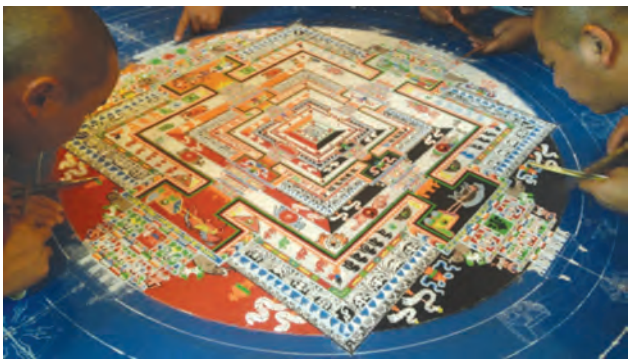

***Fig.** 2: Monks funnel sand into a precise design.*

But how can we best understand the mandala as it functions in actual practice? The monks are not the only ones the mandala encounters in its short lifetime, so how does the mandala act in the lives of its other publics, and what approach might allow us to consider its multifaceted existence more fully? Viewers of the mandala do not enter the monastery to read the cryptic messages encoded in the mandala's iconography. On the contrary, the mandala is an entity with which they interact—some are transfixed by its exotic beauty and impermanent nature, some imbue it with cultural pride and nostalgia, and some know it to be a participatory member of their ritual practice. This being the case, a straight-forward semiotic analysis of the mandala's meaning seems inadequate at best. These publics act *through* the mandala, which provides them with the means of spiritual, epistemological, and ontological transformation. Thus, if we are to understand the mandala, and its purpose and function in the lives of those individuals who interact with it, we ought to favor an approach that centers on action and engagement, rather than symbolism alone. Only in this way will the dynamic social being of the mandala be adequately addressed.

Especially helpful for such an approach is the work of Alfred Gell, who asserts in *Art and Agency* that "the action-centered approach to art is inherently more anthropological than the alternative semiotic approach because it is preoccupied with the practical mediatory role of objects in the social process, rather than with the interpretation of objects 'as if' they were texts."[12] This insight is crucial for understanding the Kālacakra mandala as it functions in both spiritual and secular contexts. Such an analysis necessarily focuses on "the network of relationships surrounding [the mandala] in specific interactive settings" and therefore provides the basis of a framework that more truly reflects the lived experience of Kālacakra practitioners.[13]

THE MANDALA AS AGENT

Much of the scholarship on the Kālacakra up to now has considered the ritual historically or philosophically, and those works that do consider the materiality of the mandala

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***Fig.** 3: Kālacakra and Vishvamata.*

typically take a semiotic approach to deciphering its esoteric symbolism. It is easy to see why: reading the mandala textually does provide details that help make the map legible to those outside the mandala's culture of origin. Attempting to understand the mandala through a rote explanation of its iconographic detail, however, reduces the mandala to a mere signifier of some other, truer thing out in the world. This affords the mandala purpose other than to signal a referent, a claim that implicitly contradicts the interactions described by its different publics. In practice, the mandala does much more than simply signify something outside of itself. In fact, this approach negates the fact that the mandala is precisely *not* one thing. It is not, for example, merely a coded index of symbolic meanings that can be read as a highly specialized encyclopedic map of space and time. Nor is it only a representation of the cosmic palace of a deity, suggesting through reference the existence and ultimate importance of the deity itself over and above its signifier. On the contrary, its resident deity, Kālacakra, who is an embodiment of the enlightened body, speech, and mind of a Buddha, along with his consort, Vishvamata, and their cohort of supporting deities, are actually made manifest *through* the mandala: without the mandala, the invocations of the tantric adepts would be fruitless, as it is the mandala that creates the alternative space-time within which the deities can reside.

The manifestation of the deities made possible by the mandala is what allows for the ontological transformation of the initiate, who, by mentally constructing the mandala-palace and navigating through its many levels, comes to unite with Kālacakra and embody his simple form. By conscientiously deconstructing the aggregates of the self, the ritual's initiate, or *yogin*, is able to re-construe his or her identity in the form of divine wisdom and compassion—through the death of the human 'self,' one may be reborn a god.[14] Underlying this practice is the belief that Buddhahood is already latent within the individual, and therefore not essentially different from the state that must be overcome: "The inner kinship of all beings forms the basis for the complicated Tantric system of analogies and correlations, and through 'thought and action by analogy' death and rebirth ultimately lead to an awareness of blissful emptiness and the attainment of an (immaterial) divine body."[15] This likeness is enacted through rigorous meditative practices that "mediate the dynamic tension between the twin notions of conventional reality (Tib. *kundzob-denpa*) of the lifeworld and the ultimate reality (Tib. *dondam-denpa*) of its emptiness."[16] The mandala, then, creates and exists within a liminal space between these two realms, a place in which the temporary humanity of the *yogin* literally realizes the inherent sameness he shares with Kālacakra, his *yidam*, or tutelary Buddha.[17] This experience is only possible because of the mandala and the alternative space-time it creates. In order to understand the mandala's agency more thoroughly, and the role it plays in the social realities of its constituents, we need to break down the interactions taking place.

Gell's notion of object-agency is essentially relational—there is an agent acting and there is a recipient of those actions.[18] Yet, according to Gell, the recipient cannot fulfill its role without the intervention of some material form: "Objectification in artefact-form is how social agency manifests and realizes itself, via the proliferation of fragments of 'primary' intentional agents in their 'secondary' artefactual form."[19] This means that the materiality through which we exercise our agency becomes an extension of our 'primary' agency in such a way that the materiality itself can be seen as a 'secondary' agent. Without some physical mediation, our intentions will not become actions, and the recipient will not successfully receive the desired effects of our agency. Thus, while Gell asserts "objects are not 'self-sufficient' agents, but only 'secondary' agents in conjunction with certain specific (human) associates," through such an assertion, he also attests to the crucial role of materiality in any agentive expression.[20] Since it is through our agency that we

construe ourselves as social beings, the materiality with which we do so fundamentally shapes the actions we take, and in turn the people we are. In other words, our identities are informed by our material interactions.

Gell's notion suggests that while the mandala does not have the type of intentional agency its human counterparts have, the humans themselves are knowable as agents in this context only through their interaction with the mandala. Consider the Kālacakra students. They are not merely people: they distinguish themselves from the majority through their knowledge and practice of this particular ritual. Their Tibetan holy books (Tib. *pecha*), their bells and vajras, and all their other precious ritual implements are part of this distinction. We cannot refer to them as Kālacakra students without simultaneously referencing these materials and the social context and religious practices they imply.[21] Kālacakra students are capable of being the kind of religious agents they are "only because of the artefacts they have at their disposal, which, so to speak, turn them from mere people into [religious beings] with extraordinary powers. Their kind of agency would be unthinkable except in conjunction with the spatio-temporally expanded capacity for [religious transformation] which the possession and use of [ritual implements like the mandala] makes possible."[22]

This approach is helpful, as it lets us go beyond the rote translation of symbols to emphasize the nature of the social interaction between mandala and public(s). Social interaction is the place where culture is made and maintained, and it is through our interactions with people and things that we construct the many overlapping worlds we inhabit. The mandala is actively engaged in this process, mediating our conceptions of reality in order to convert intentions into effective action. But what kind of mediation is possible here, and how do these specific modes of engaging this particular material activate culturally specific means of subject formation? Or, in other words, how are the identities of these publics afforded by and cultivated through interaction with the mandala? To answer this question, we must look more closely at the interactions themselves.

THE MANDALA AND ITS AFFORDANCES

In thinking about how particular constellations of sociocultural factors create specific human results, Webb Keane emphasizes "the affordances of interaction and the objectifications they can induce."[23] Specifically he suggests that the materials with which individuals interact, and their unique mechanisms for understanding those materials, produce the field of possibility within which those individuals may act.[24] Importantly, they do not determine how the individual will act, but they do inform and limit the potential range of actions one may take in response to them. In terms of the Kālacakra mandala, this means that the possible agentive expressions of each individual are framed by the mandala's own materiality in conjunction with the matrix of religious, cultural, social, intellectual, and philosophical meanings *afforded* to the individual. For example, within its ritual context, the mandala affords the means for an individual to be reborn as a transcendent being. The ritual context alone offers this possibility. To a non-initiate, the mandala simply cannot function in this way, as the requisite factors for such perception and agency are not available.

This is important, as the materials in play and their associations "serve as affordances only *in particular combinations* and *relative to particular observers*."[25] The mandala by itself can be seen as just a carefully arranged pile of sand. However, given the Kālacakra tradition, replete with the texts, teachings, and practices that govern the mandala's ritual uses, the initiate is afforded the opportunity to see the mandala as a conduit for divinity and to act accordingly. Members of other publics—the monastery's volunteers, the casual museum-goers, myself as a scholar—cannot engage with the mandala in this particular way; we simply do not have the necessary combination of ingredients. Nevertheless, we each inadvertently assemble a unique arrangement of factors that shape our own interactions with the mandala. The museum-goer, for example, may find herself enriched from her experience of viewing the mandala and observing the monks in their practice of creation. For her, interacting with the mandala may afford a view of herself and her world as contrasted against the Tibetan monks and their foreign way of life. Her sense of identity, her place in the world, her

knowledge of the world itself, are all augmented in this interaction. The mandala may not be a conduit for divinity in this case, as its soteriological function and eschatological claims may never exist for her, and thus cannot serve as the impetus for any potential action. Still, simply seeing the mandala is a form of engagement that alters her awareness of herself and her world.

Given the range of possible responses to the mandala, its particular materiality is at once similar and very different things—it is the thing that provides a path to Buddhahood, plants the seed of the dharma in passersby, or is simply a beautiful and moving work of exotic cultural art. Still, these possibilities do not predetermine the responses themselves: "The affordances are properties of the [mandala] vis-à-vis human action. As such they are real, and exist in a world of natural causality, but they do not cause people to respond to them in any particular way."[26] The way in which an individual understands oneself in relation to the mandala determines how that individual will choose to express their agency, within the potentialities afforded. This relationship, between mandala and individual, therefore governs not only the range of possible actions, but also the sense of self that is produced as a result of those actions. The Kālacakra students, in calling themselves Kālacakra students, exemplify how the mandala's affordances can structure how one sees and describes oneself. In the same way, each public, and each member of each public, positions itself uniquely in terms of the mandala, given the factors afforded to it, and in so doing they construct their own identities in relation to the mandala, as well as their notion(s) of the world(s) they inhabit and navigate.

It is this variety of relationships and responses to the mandala's affordances that determines the publics themselves. I have used the word 'publics' here to denote these different types of responses, though it should be mentioned that these publics are not discrete entities and many people and publics engage with the mandala in multiple ways simultaneously. This is especially true of those for whom the mandala affords more—that is, those who act within a larger range of potentialities. These individuals inhabit a series of overlapping worlds, which inform their identities recursively. So, while the casual observer may never learn enough about the mandala for it to afford certain actions (at least in this lifetime), for the monk who has spent years studying and practicing, the Kālacakra mandala is at once myriad things: it is map of all time and space, a depiction of the human body, the divine residence of a host of enlightened beings, and a portal for one's own nirvana; its construction and destruction are acts of great compassion and its radiance can plant the seed of the dharma in all who behold it; and significantly, it serves as a means to generate more global awareness of the Tibetan plight in the name of cultural preservation. The mandala affords all of these realities simultaneously for some, whose identities are fundamentally formed and altered through their informed engagement with its materiality. Any engagement with the mandala, then, fundamentally contributes to the construction and negotiation of worlds, whether by dedicating oneself to the ritual practice, or by adding dimensions to one's global awareness through recognizing the worlds of others.

CONCLUSION

The means and methods of interaction the mandala exhibits with its diverse publics demand that we reassess our conventional modes of analysis. While a semiotic approach to the mandala does afford certain insights into the cosmological reality in which its ritual practitioners function, it does not attend to the many ways the mandala is functioning interactively in the lives of its wider audience. Speaking with members of the mandala's many publics makes evident that the mandala is an active participant in the constitution of those individual's identities and worlds, and increasingly so, as the mandala is more and more thoroughly integrated into the American (and transnational) cultural landscape. The dominance of public-specific didacticism, whether in cultural histories, religious philosophies, or public displays, reduces the scholarship on the Kālacakra mandala to a falsely segmented and separated set of data. By reading the mandala in such limited ways, we negate the reality expressed through the mandala itself via its interactions with its publics. This then renders our approach to understanding the mandala inherently incomplete, and in disregarding the lived experiences the mandala affords, we unjustly impose a hierarchy onto the potential meanings it encompasses, and by proxy onto lives of our ethnographic informants. Here, I

have offered an approach that evokes both Gell and Keane in an attempt to observe the actual functioning of the mandala in the lives of its spiritual and secular audiences. The Kālacakra mandala exists within many spheres, each of which affords many potential means for engagement, and in order to understand how it is affecting and affected by its many publics, we must not insist on the one most comfortable reading.

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*All photos taken with permission by author at the Kālacakra retreat hosted at Dü Khor Choe Ling in Ithaca, NY, summer 2015.

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The Effects of Mindfulness Meditation on Adolescents' Stress Management

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ABSTRACT

Adolescence is a period of significant stress during which youths' confidence in their coping capabilities can greatly influence the way they approach stressful situations. While previous research examined the importance of such coping self-efficacy in effective stress management, few studies have explored ways to improve this skill in youth. One possible method is mindfulness meditation (MM). MM guides the participant into focusing on their natural breath and thought, cultivating an attitude of non-judgment. The present study examined how key components of MM improve coping self-efficacy by impacting cognitive control systems (e.g., flexibility of thought) and emotion-related regulatory processes. We hypothesized that MM would improve youths' impulse control and focus, cognitive reappraisal, and self-acceptance to foster greater perceived self-efficacy. We examined these effects in 29 adolescents (ages 11 to 16, M_{age} = 13.97, 17 girls) before, during, and after participation in a week-long summer camp involving an immersive mindfulness training curriculum. During the week, the youths participated in mindfulness-based workshops in addition to normal camp activities. On the first and last days of camp, they completed measures of inhibitory control and self-reports assessing aspects of their emotion regulation. Findings showed that MM may foster meaningful improvements in youths' belief in their coping ability, ability to reframe negative situations, ability to control dominant responses, and acceptance of self. Overall, this study shows that MM has the strong potential to improve coping self-efficacy and foster effective coping strategies for positive well-being.

Keywords: Mindfulness; coping self-efficacy; adolescence; stress management; emotion regulation; developmental psychology

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Dr. Davis's research focuses on how children and adolescents learn to cope with stress and regulate their negative emotions. She studies the development of emotion regulation skills using a biopsychosocial framework, with the goals of explaining what children can do to alleviate negative emotions, when and under what circumstances they can do these things, and why emotion regulation relates to healthy and unhealthy functioning.

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Akhila Nekkanti is a fourth-year Neuroscience student whose interests lie in exploring the efficacy and accessibility of mindfulness-based intervention models for youth at risk for psychopathological problems. Through the Chancellor's Research Fellowship and immeasurable support from her graduate and faculty mentors, she was able to research the impact of mindfulness meditation on youths' regulatory functioning. She plans to use this invaluable experience to pursue a PhD in Clinical Psychology and eventually develop a widely-accessible intervention model for underserved communities.

Akhila Nekkanti

INTRODUCTION

Biological changes and newly burgeoning social challenges are characteristic of adolescent life. These changes both create a significant source of stress and provide the opportunity for youth to develop critical coping strategies for successful adaptation (Buchanan, 1992; Dumont et al., 1997; Semple et al., 2009). As adolescents begin to expand their horizons and become more aware of the social, economic, and political world (Inhelder & Piaget, 1958), they also face the challenges of adjusting to these potentially stressful changes in adaptive ways (Dumont, 1999).

A significant contributor to youths' successful management of stress is *coping self-efficacy*, the belief in one's capability to exercise control over their own emotional functioning (Bandura, 1992). When stressors become overwhelming, adolescents can be at risk for such emotional and behavioral problems as anxiety, depression, substance abuse, and poor academic achievement (Beato-Fernandez et al., 2007; Crosnoe et al., 2004). Positive self-efficacy relies heavily on cognitive and emotional processes and enables us to perceive adverse stressors as challenges that can be overcome. In the face of physical, cognitive, or social challenges, a strong sense of self-efficacy promotes both physiological and psychological well-being by impacting multiple cognitive and affective domains (Bandura, 1994). For example, when dealing with a social or environmental stressor (e.g., family problems, academic pressure), the perception of having the control and ability to manage one's life can serve as a significant buffer of the stressor's negative impact (Aschbacher et al., 2009). High levels of relative coping self-efficacy have been shown to foster a solution-focused attitude that guides effective performance (Bandura, 1994), whereas low levels foster an attitude of self-criticism and hopelessness (Jerusalem et al., 1992). Related, emotional states impact people's judgment of their coping deficiencies and capabilities, with positive moods enhancing their perceived self-efficacy and allowing the situation to be interpreted in a more positive way (DeLongis et al., 1988).

The present study aimed to assess the impact of a one-week mindfulness meditation (MM) training intervention on socioemotional and cognitive functioning in adolescents. Mindfulness meditation training is an increasingly popular component of many interventions that cultivates the habit of perceiving both positive and negative life events purposefully and non-judgmentally (Kabat-Zinn, 2011). Because MM aims to alter one's perception of the stressor as well as one's perception of their own "present moment" thoughts and sensations (Cullen, 2011; Grossman, 2004), it may be an effective means of improving adolescents' ability to manage stressors. While many previous studies have demonstrated the importance of coping self-efficacy for physical well-being and cognitive functioning, few have examined potential methods for improving coping self-efficacy in youth.

Mindfulness Meditation as a Method of Improving Coping Self-Efficacy. Mindfulness therapy is based on the idea that people are generally unaware of their moment-to-moment thoughts (Grossman, 2004); it teaches sustained focus on ongoing mental phenomena without comparison or evaluation to enable a less critical and more accurate perception of daily events (Brown, 2003). MM practice often combines open-monitoring meditation techniques with focused-attention techniques, fostering the ability to maintain deliberate attention to momentary experiences while developing an attitude of kindness and acceptance (Davidson et al., 2015, Grossman et al., 2004). The practice cultivates two skills: a) the ability to focus on a single phenomenon (e.g., one's breath), reducing the impact on attention that distracting thoughts may have, and b) the ability to monitor thoughts nonjudgmentally, improving regulation of moment-to-moment emotions (Lutz et al., 2008). This emphasis on nonjudgmental awareness of present-moment thought and determined focus addresses the symptoms of poor self-efficacy mentioned previously: an overly self-critical attitude and a lack of motivation to direct solution-focused behavior (Bandura, 1993). Many interventions have successfully employed MM to reduce performance anxiety, pain, and a range of behavioral problems (Bishop et al., 2004). Given the different skills developed by MM training, it is worth examining how MM may impact multiple domains of adolescent development that are essential for supporting the development of coping self-efficacy and stress management.

By fostering both cognitive and emotional control (Prakash et al., 2015), MM has the potential to improve youths' successful regulatory functioning broadly. Cognitive control is the ability to control one's thoughts and mental functions, switch flexibly between thoughts, and maintain attentive focus (Carlson & Wang, 2007). Closely related to this general skill is a more specific technique known as cognitive reappraisal, a sophisticated strategy for managing emotions that employs the use of significant cognitive control to hold a current interpretation of a situation in mind and then reframe it in a different way (Ochsner et al., 2005). In stressful situations where people have little control over the outcome (e.g., a loved one is seriously ill), cognitive reappraisal becomes especially beneficial as it allows the person to change the emotional impact of the situation and regain a sense of control (Troy, 2010). Although MM has been shown to impact both cognitive control and emotion regulation in adults (Zylowska et al., 2008), little research has examined how MM impacts adolescents' cognitive control and emotion management abilities, and whether changes in these skills relate to youths' perceived or actual coping abilities. Of the existing studies on the impact of mindfulness in adolescents, the majority focus on clinical samples conducted primarily in the school setting (Black et al., 2009). The current study addresses this gap.

The Present Study. Although MM has been shown to reduce symptoms of anxiety, depression, and chronic stress in adults, little research has examined its impact on key components of adolescents' stress management. The overarching goal of this study was to examine how MM training impacts different aspects of regulatory functioning to improve adolescents' coping abilities. Specifically, we focused on examining the following questions:

1) Are there changes in adolescents' coping self-efficacy over the week?
2) How does MM relate to adolescents' cognitive control and emotion regulation?

We assessed the influence of a one-week MM training camp on youths' regulatory skill as evidenced by short-term longitudinal change in their cognitive and emotional regulation abilities. We looked specifically for effects on youths' emotion-related coping and self-perception. The purpose of the study was to understand how MM may improve adolescents' coping abilities and their confidence in their coping abilities by examining how it impacts specific components of stress management: impulse control and focus, emotional reappraisal, and self-kindness.

METHODS

Participants. 29 adolescents (ages 11 to 16, M_{age} = 13.97, 17 girls) participated in a research study at a summer camp conducted in collaboration with the *Tools for Peace* organization and their *Stop, Breathe, & Think (SBT)* curriculum. This Los Angeles-based non-profit organization's *SBT* curriculum emphasizes mindfulness, meditation, and compassion-in-action to guide youth into becoming aware of and managing their actions and reactions positively.

Procedure. Two months before the camp, parents received a package including a letter describing the study, a copy of the consent form, and a copy of the assent form to review. Interested parents signed and returned the consent form ahead of their child's arrival to the summer camp. Prior to participation in any research activities, campers assented to participate in the research study. On the first and last days of camp, adolescents completed computer tasks designed to measure their inhibitory control, and a series of self-reports assessing different aspects of their emotion regulation and self-compassion. During the week, they engaged in camp activities (e.g., morning discussion circle, yoga, music, mandala work) and *SBT* workshops, where they learned to settle their minds and work with difficult emotions through active discussion, collaborative activities, and practicing MM.

Materials. Adolescents completed the *Emotion Regulation Questionnaire* (*ERQ;* Gross et al., 2003), a 10-item survey measure designed to assess differences in the habitual use of two emotion regulation strategies: cognitive reappraisal and expressive suppression. They also completed the *Self Compassion Scal*e (*SCS*; Neff, 2003), which is a 26-item questionnaire designed to assess participants' attitudes toward themselves during instances of pain or failure. Of the six subscales on this measure, only the self-kindness subscale was used in this study.

Cognitive Control Measures. Youth completed a Day/Night (D/N) computer task at the beginning and end of

the camp week as a component of the cognitive control assessment (Gerstadt et al., 1994; Best et al., 2010). D/N is a neutral task (i.e., does not elicit an emotional response) in which participants see images of a sun or a moon (20 trials) and must press a button associated with the opposite image (e.g., if a sun is shown, they press the button for moon). Accuracy scores from this task provided a measure of "cool" (emotionally neutral) effortful control, the capacity to purposefully suppress a dominant response to perform a subdominant response. Participants also responded to single-item measures of their ability to focus *before camp* and *since camp started* on a 5-point Likert scale (1 = Not at all well, 5 = Very well). We created a cognitive control composite by standardizing and summing the change scores of youths' D/N accuracy and their responses on the 5-point Likert scale over the week. Change scores for the D/N inhibitory control task were measured by subtracting the number of correct responses made at the start of camp from the number of correct responses made at the end of camp.

Emotional Control Measures. The composite of emotional control was created by standardizing and combining changes in perceived ability to handle feelings before camp and since camp started (1 = Not at all well, 5 = Very well), with use of cognitive reappraisal using the ERQ from the first day of camp to the last day of camp. Change scores measuring their ability to handle feelings were created by subtracting scores for *before camp* from *since camp started*.

Self-Acceptance Measures. As an indirect measure of self-acceptance, we coded and summed the total number of positive descriptors youths used to describe themselves in the qualitative survey they completed towards the end of camp. We created a self-acceptance composite by combining these scores with change scores for self-kindness using the SCS from the first day of camp to the last day of camp.

RESULTS

Were there changes in adolescents' coping self-efficacy over the week? We conducted paired samples t-tests to examine youths self-reported improvements in our direct measures of coping self-efficacy over the course of the camp week. Overall, campers self-reported an increase in how well they handled feelings from *before camp* (M = 2.80, SD = 1.00) to *since camp started* (M = 4.08, SD = 0.812; $t(24) = -5.297$, $p < 0.001$, $d = -1.404$), and in their confidence in their ability to handle feelings from before (M = 3.00, SD = 1.00) to during camp (M = 3.88, SD = 1.16; $t(24) = -2.916$, $p = 0.008$, $d = -0.810$). Bivariate correlations revealed that more positive qualitative self-descriptors were related to greater increases in the ability to handle feelings ($r = 0.534$, $p = 0.009$) and greater increases in cognitive reappraisal ability ($r = 0.438$, $p = 0.029$).

How does MM relate to adolescents' cognitive control and emotion regulation? Youth who demonstrated greater increases in cognitive control also demonstrated greater increases in use of cognitive reappraisal strategies (r = 0.400, p = 0.047) as well as increases in their subjective ability to handle feelings ($r = 0.397$, $p = 0.049$), suggesting that changes in cognitive control were related not only to emotion regulation strategy use, but coping self-efficacy as well. Although cognitive control was not directly related to self-kindness, we found that youths who demonstrated an increase in cognitive reappraisal ability over the course of the camp week showed significantly greater increases in self-kindness ($M = 0.382$, $SD = 0.533$) compared to those who did not increase in their cognitive reappraisal ($M = -0.217$, $SD = 0.486$; $t(21) = 2.82$, $p = 0.010$, $d = 1.174$) and used significantly more positive descriptors about themselves in their qualitative responses ($M = 3.64$, $SD = 2.67$) than those who didn't increase in cognitive reappraisal ($M = 0.909$, $SD = 0.701$; $t(23) = 3.285$, $p = 0.003$, $d = 1.397$). This suggests a positive association between gains in emotion regulation (in particular, use of cognitive reappraisal, which requires greater cognitive control) and gains in self-acceptance (self-kindness and positive self-descriptors).

DISCUSSION

The present study examined the impact of MM on adolescents' stress management (cognitive and emotional regulation) and whether these effects were associated with improved perceived coping self-efficacy in youth. First, we examined whether the MM training improved coping self-efficacy in youth. Results showed that youths not only improved in their perceived ability to handle their feelings, but also in their confidence in their ability to

handle feelings. These improvements were also associated with their use of positive self-descriptors and reappraisal strategies, an indication that the youths were able to see themselves and their abilities in a more positive light after participating in the MM training. Although the relatively short time frame of assessment and lack of a control group were important limitations of the current study (and inherent to the summer camp setting), results suggest that in conjunction with improvement in relevant aspects of cognitive control and emotion-related coping, MM is associated with improvement in youths' coping self-efficacy.

We found that gains in cognitive control during the week of MM training influenced youths' ability to use reappraisal strategies to control their emotions. These results are in line with previous work suggesting that reappraisal requires cognitive sophistication (Troy, 2010; Buhle, 2014; Goldin, 2009) and is associated with a more adaptive cognitive-emotional profile. (Mauss et al., 2007). Overall, our findings suggest that MM can influence both cognitive and emotional regulation via enhancements in cognitive control (e.g., flexibility of thought) and reframing.

In recognizing that one's ability to control disturbing thoughts and perceptions (cognitive control) may improve the way youth deal with emotionally stressful situations, we then strove to examine the impact of this improvement on self-acceptance. Overall, results indicated that although improvements in cognitive control may correspond with cognitive reappraisal ability, they did not directly relate to self-acceptance. Adolescents who demonstrated an increase in cognitive reappraisal over the week, however, showed significantly greater increases in self-acceptance compared to those who did not increase in reappraisal. Improvements in cognitive control may indicate improvements in the ability to control automatic processes (i.e., automatic responses to negative stressors), a key component in employing reappraisal strategies (Uleman, 1989) but not directly involved in fostering self-compassion. Reappraisal, on the other hand, encourages the perception of a negative situation in a more positive light, a strategy that directly impacts one's perception of self (Troy, 2010). MM may indirectly foster greater self-acceptance by improving cognitive control and subsequently enhancing one's ability to perceive stress in a positive light.

Limitations and Future Directions. There are two central limitations to this study that arise because of our focus on a summer camp for adolescents. First, the length of the MM intervention may be too short to reveal long-term effects of the training. Previous research on long-time meditators (Easterlin, 1988) shows that consistent long-term mindfulness practice has a marked impact on overall well-being. Still, our results show that even a brief MM intervention can confer short-term benefits, such as improved regulatory skill. Second, this study did not include a control group of adolescent campers who were not involved in MM training. Because the campers completed mindfulness training practices (meditation, yoga, mandala) along with typical camp activities like swimming, horseback riding, and arts and crafts, the improvements in stress management cannot be solely attributed to MM. However, previous studies on the impacts of mindfulness in adolescents in the school setting with a control group show similar improvements in coping with distress (Black et al., 2009). Future MM studies could strengthen the existing findings by incorporating a control group and follow-up time points to examine longer-term effects of the training. The present study provides new ideas about the impacts of MM in healthy adolescents and has implications for a more scientific understanding of MM as a method to improve coping self-efficacy and stress management in youth.

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The Where of Happiness: Cross-Cultural Comparison of Happiness and Situational Experience

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ABSTRACT

Previous research has observed increases in happiness after participation in activities such as expressing gratitude and performing kind acts (Lyubomirsky & Layous, 2013). Although this research has helped psychologists to better understand the concept of happiness, the majority of the studies that have been conducted were limited to W.E.I.R.D. populations (Westernized, Educated, Industrial, Rich, Democratic; Henrich, Heine & Norenzayan, 2010). To further assess happiness across cultures, the current study investigates the relationship between situational experiences and national levels of happiness. Participants were recruited from 21 countries (total N = 5,552; males = 2029; females = 3523; mean age = 21.65 years) were asked to evaluate their experiences of situations at 7 pm the previous night using the 89 items of the Riverside Situational Q-sort (see Appendix I).We then correlated their situational experiences with country-level scores of national happiness (World Happiness Report, 2015). Results indicated that individuals from countries with the highest levels of happiness tended to describe their experience of situations as raising issues of power, causing small annoyances, and being criticized. Results also indicated that individuals from countries with the lowest levels of happiness tended to describe their situations as including opportunities to express unusual ideas, being emotionally arousing, and having important, minor details. These results underscore the complexity of both situational experience and happiness when studied globally. These results suggest cultural variables that affect variation in happiness within a single country may not apply when studying happiness across cultures.

Keywords: Situation, culture, countries, happiness, international, identification, experience

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Zizhong (David) Xiao is a third-year Psychology student. His research focuses on social, personality, and positive psychology. He currently works under faculty including Dr. David Funder, Dr. Howard Friedman, Dr. Rachel Wu, Dr. Sonja Lyubomirsky, and Dr. Kate Sweeny. David will be presenting his research at the Stanford Undergraduate Research Conference and the Association for Psychological Sciences Convention. He hopes to become a leader in positive psychology research and win a National Science Foundation grant someday.

Zizhong (David) Xiao

INTRODUCTION

The emergence of positive psychology

The desire to achieve everlasting happiness is one of the few universal human behaviors (Lyubomirsky, 2008). Positive Psychology, the study of happiness and optimal human functioning, was formally established as a discipline when Martin Seligman introduced the concept during the 1998 American Psychological Association Presidential Address (Linley, Joseph, Harrington & Wood, 2006). Since Seligman's (1998) introduction, Positive Psychology has become a rapidly growing subarea of psychological research. On-going positive psychology research has observed increases in happiness following participation in positive activities such as expressing gratitude, performing acts of kindness, cultivating optimism, and savoring the present moment (Lyubomirsky & Layous, 2013). Additionally, numerous studies have shown that happiness is associated with and precedes many successful life outcomes, such as marriage, work performance, and health (Lyubomirsky, 2008).

Although these studies are highly informative, most positive psychology research is conducted within so-called "WEIRD" countries (Westernized, Educated, Industrial, Rich, Democratic; Henrich, Heine & Norenzayan, 2010), and thus may lack generalizability to the whole global population. Therefore, more recently, psychologists have attempted to find evidence across cultures regarding the aspects of various cultures that contribute to happiness (Easterlin & Sawangfa, 2010). For example, Hagerty and Veenhoven (2003) demonstrated that higher national Gross Domestic Product (GDP) is related to higher levels of average national happiness. The purpose of this study is to explore other aspects of cultures, specifically, characteristics of situational experience, that might be relevant to happiness.

Situational experiences assessment

Human behavior is affected by two factors: the person and the situation. Recent empirical research in positive psychology has focused more on the former rather than the latter, with numerous studies showing that certain personality traits are associated with individual levels of happiness (Furnham & Cheng, 1997). For example, Diener, Sandvik, Pavot, and Fujita (1992) found a positive correlation between extraversion and happiness. However, research has paid less attention to the relationship between people's experience of daily situations and happiness. This research project attempts to address this gap.

The relative lack of research on situational experiences associated with happiness is possibly due to the lack of a comprehensive assessment tool. To address this issue, the Riverside Situational Q-sort (RSQ) aims to assess psychologically meaningful characteristics of the situation (Guillaume et al, 2015). The RSQ includes 89 attributes of situations (e.g., "social interaction is possible," (RSQ #56) "affords an opportunity to ruminate, daydream or fantasize," (RSQ #49) and "situation includes intellectual or cognitive stimuli" (RSQ #2) (Guillaume et al, 2015). Participants sort each of these 89 items in to one of 9 categories ranging from extremely characteristic (category 9) to extremely uncharacteristic (category 1) of their situation at 7 pm. To see a complete list of the Riverside Situational Q-sort, refer to *Appendix I*. This data will supplement current relationship on associating various human behaviors and happiness levels.

Measuring national level of happiness

For this study, the national average of happiness for 21 countries was obtained from the 2015 World Happiness Report (WHR). The WHR research scientists utilized the Cantril ladder survey method by asking a representative sample population of a specific country (typically 2,000 – 3,000 participants/country, variation of participant sample sizes depends on the total population size of certain countries) questions regarding to their subjective happiness and self-reported life satisfaction level (The World Happiness Report, 2015). Examples of the questions within the survey include:

- "All things considered, how satisfied are you with your life as a whole these days? Use a 0 to 10 scale, where 0 is dissatisfied and 10 is satisfied."
- "Did you experience happiness during a lot of the day yesterday?"
- "Did you experience enjoyment during a lot of the day yesterday?"

They repeated the same steps for the 156 countries within the United Nations. Following the data-collection, the WHR researchers compiled all the responses into a number ranging from 1- 10 that indicates the national average of happiness for each country. The purpose is to specify which country is happier than another on a ranking system. For example, Switzerland scored 7.59, which is happier than Singapore, which scored 6.8. Singapore, in turn, is happier than France, which scored 6.56 and so on (The World Happiness Report, 2015). By examining this data set from the WHR, we can use the national averages of happiness levels and then associate it with the situational experience data in order to complement our understanding of the relationship between holistic human behaviors and happiness levels.

Purpose of this study

The goal of this research is to compare average ratings of situational experience and national levels of happiness across 21 different countries. We hypothesize that situations experienced have a direct effect on national average of happiness level. This will give us a more nuanced understanding of happiness. Based on our current understandings from Positive Psychology research, we hypothesize the following:

Countries with higher levels of happiness will tend to experience characteristics of situations where:

- Close personal relationships are present or have the potential to develop within the situation (e.g., participant is on a date) (RSQ #51). This hypothesis is based on previous positive psychology research, which indicates social network development is linked to higher levels of wellbeing (Lyubomirsky, 2008).
- Situation includes intellectual or cognitive stimuli (e.g., books, lectures, intellectual conversation) (RSQ #53). This hypothesis is based on previous positive psychology research, which indicates those specific stimuli are mechanisms that can transform people's minds to be in the state of flow. Flow is defined as a "state in which people are so involved in an activity that nothing else seems to matter; the experience is so enjoyable that people will continue to do it even at great cost, for the sheer sake of doing" (Csikszentmihalyi, 1990). Research shows being in the state of flow increases overall life satisfaction (Csikszentmihalyi, 1990).
- Someone may need help (RSQ #10); since this situational characteristic allows the opportunity for others to perform acts of kindness. This hypothesis is based on previous positive psychology research, which indicates acts of kindness increase people's happiness (Lyubomirsky & Layous, 2013).

Countries with lower levels of happiness will tend to experience characteristics of situations where:

- Situation involves social comparison (e.g., participant is at a party) (RSQ #78). This hypothesis is based on previous positive psychology research, which indicates social comparison is negatively associated with happiness (Lyubomirsky, 2008).
- Situation includes potential for immediate gratification of desires (e.g., food, shopping, and sexual activities) (RSQ #55). This hypothesis is based on previous positive psychology research, which indicates aspects of hedonic pleasure such as material goods, money, and sex are not related to long-term well-being and happiness (Lyubomirsky, 2008).
- Things are happening quickly (RSQ #20). This hypothesis is based on previous positive psychology research, which indicates fast-paced economic growing countries usually score lower on overall happiness levels due to stressful work demands (Weiting, Diener, Aurora & Harter, 2008).

METHODS

Participants

Participants were drawn from a larger study, The World at 7:00: Comparing the experience of situations across 20 countries (Guillaume et al., 2016). Each collaborator of the larger project recruited participants from their respective research institution. In total, there were N = 5,552 (Female = 3523; male = 2029; mean age = 21.65 years). Table 1 provides the details of the countries studied and the participants (Guillaume et al., 2016).

Table 1: *Participant Samples from 21 Countries*

Country	N (Total)	Female	Male	Mean Age
Australia	141	109	32	20
Austria	87	71	16	25
Canada	191	126	65	21
China	1565	854	711	22
Czech Republic	220	159	61	28
Denmark	126	102	24	23
Estonia	314	251	63	26
Germany	70	55	15	27
Italy	144	75	69	23
Japan	227	107	120	20
Netherlands	258	220	38	20
Poland	97	73	24	24
Russia	101	80	21	21
Singapore	158	109	49	21
Slovakia	98	86	12	22
South Africa	114	62	52	23
South Korea	103	69	34	22
Spain	108	78	30	22
United Arab Emirates	83	42	41	21
United Kingdom	107	75	32	21
United States	1218	727	491	20

Assessment of Participants' Cross-Cultural Situational Experiences

Participants from the 21 different countries went to a website built by the University of California, Riverside International Situations Project Lab. The participants were first asked to provide an open-ended response describing what they were doing the previous night at 7 pm. Specifically, they were asked to describe (1) where they were, (2) who they were with, and (3) what they were doing (Guillaume et al., 2016). Examples of participants' responses include:

Country	Time	Experience of Situations at 7PM
Estonia	7.00 PM	At about 7.00 I was sitting in the sauna with my grandmother, adding some steam and whisking.
United States	7.00 PM	We were smoking cigarettes and drinking wine.
Japan	7.00 PM	I sang using the karaoke box with my friend.

Following the description of their situational experiences, participants quantified this situation using the RSQ (described above; see *Appendix I)*.

Assessment of National Average of Happiness

The national average of happiness of the 21 countries was derived from the World Happiness Report (World Happiness Report, 2015). The exact numbers of the 21 countries studies are listed in *Table 2*.

Table 2: *National Average of Happiness of the 21 countries*

Country	National Average of Happiness
Australia	7.28
Austria	7.2
Canada	7.43
China	5.14
Czech Republic	6.51
Denmark	7.53
Estonia	5.43
Germany	6.75
Italy	5.95
Japan	5.99
Netherlands	7.38
Poland	5.79
Russia	5.72
Singapore	6.8
Slovakia	5.99
South Africa	4.64
South Korea	5.98
Spain	6.33
United Arab Emirates	6.9
United Kingdom	6.87
United States	7.12

Data Analysis

The 89 RSQ items rating from each participant's responses were averaged within each of the 21 countries, and then correlated with national levels of happiness. The data was quantified and analyzed through the R statistical analysis program. Using R, we repeatedly ran the correlations with random rearrangements of countries to prevent the possibility of a spurious correlation—a significant

correlation by mere chance. Correlations between average national placements of RSQ items and national happiness are reported only when the number of significant correlations exceeds chance (Sherman & Funder, 2009).

RESULTS

Our analyses uncovered unexpected relationships between characteristics of situations experienced by countries with higher country-level happiness scores and countries with lower country-level happiness scores. Specifically, contrary to our hypotheses, results indicate that individuals from countries with the highest levels of happiness (e.g., Denmark and Canada; 7.53 and 7.43, respectively) on average, tend to report situational experiences that involve power (see RSQ #79), include one or more small annoyances (see RSQ #34), and entail being criticized (see RSQ #16) ($r = 0.59$, $r = 0.70$ and $r = 0.58$, respectively). Also contrary to our hypotheses, results indicate that individuals from countries with the lowest levels of happiness levels on average (e.g., China and South Africa; 5.14 and 4.64, respectively) tend to report situational experiences that include opportunities to express unusual ideas or points of view (see RSQ #41), are potentially emotionally arousing (see RSQ #83), and in which minor details are important (see RSQ #11) ($r = -0.54$, $r = -0.53$, and $r = -0.45$, respectively).

DISCUSSION

The results of the current study failed to support our hypotheses based on previous research. The associations between national levels of happiness and aspects of situational experience were counterintuitive and unexpected. For the countries with high levels of happiness, we originally hypothesized that individuals within happier countries would tend to experience situations involving the emergence of social networks (see RSQ #51), include intellectual stimuli (see RSQ #53), and have opportunities to perform acts of kindness (see RSQ #10). However, our data found that individuals in happier countries tended to experience situations characterized by power issues (see RSQ #79), including minor annoyances (see RSQ #34), and being criticized (see RSQ #16). For the countries with lower levels of happiness, we originally hypothesized that individuals in countries with lower levels of happiness will tend to experience situations that involve social comparison (see RSQ #78), fulfill hedonistic desires (food, sex, and money) (see RSQ #55), and are characterized by a fast pace of activity (see RSQ #20). However, we found that individuals in countries with lower levels of happiness reported experiencing situations where people are allowed to express unusual points of view (see RSQ #41), which are emotionally arousing (see RSQ #83), and in which minor details are important (see RSQ #11).

IMPLICATIONS

To better understand what caused our counter-intuitive results, we re-examined the situational experiences participants described in the countries with higher levels of happiness and countries with lower levels of happiness. On average, 7 pm situations within countries with higher levels of happiness tend focus on family (e.g., "I was at home having dinner with my family", and "I was watching TV at home with my family"). Therefore, the power structure of families may cause people to rate issues of power highly on the RSQ. Also, on average, 7 pm situations within countries with higher levels of happiness tend to relate to studying (e.g., "I was at home studying…" and "I was taking notes…"). Thus, the constant pressures of studying may cause frustrations; which may cause people to rate high degrees of small annoyances highly within the RSQ. Lastly, on average, 7 pm situations within the countries with higher levels of happiness tend to be associated with work (e.g., "I was at work at McDonalds…", and "Overtime working in the office"). Whenever one is in a professional environment, one is more likely to be criticized by supervisors or co-workers, which in turn may cause participants of happier countries to rate their situational experiences with high degrees of being criticized on the RSQ.

Furthermore, on average, 7 pm situations within countries with lower levels of happiness tend to be associated with peers (e.g., "I was with my classmates in the bedroom chatting…" and "I was drinking beers with four friends …".) Thus, high levels of peer interaction create opportunities for people to express unique perspectives, which may cause people to rate chances to show unusual

points of view highly on the RSQ. Also on average, 7 pm situations within countries with lower levels of happiness tend to relate to stressful occupational activities (e.g., "I was studying for exams" and "I work at night in hospital"). Therefore, these demanding tasks may cause aggravation in the countries with lower levels of happiness, which leads participants to rate their 7 pm situations with high emotional arousal on the RSQ. Lastly, on average, 7 pm situations within countries with lower levels of happiness tend to focus on intricate tasks (e.g., "Giving high school students extra lessons" and "I was doing an experiment for my master's project…"). Therefore, these detail-oriented tasks require high attention to details, which lead participants to rate highly on situations with "minor details are important". These explanations, drawing from examples of situational experiences in both countries with higher levels of happiness and countries with lower levels of happiness, may provide a background to explain the counter-intuitive results of this study; however, we are not able to draw any substantial conclusions from them. These results may be due to a variety of factors, such as: countries with higher levels of happiness are pressured to work and excel, since most of the countries with higher levels of happiness are industrialized. In contrast, since most of the countries with lower levels of happiness are developing, individuals are pressured to work in order to strengthen economic development. However, further data-analysis needs to be performed on people's situational experiences before we can accept those explanations as valid and firm conclusions.

Overall, the results of this study are counter to what we expected; however, they emphasize the complexity of both situational experience characteristic and happiness when studied across the globe. Additionally, this study shows that variables such as situational experience, which are relevant to individual variation in happiness within a country, may not apply when comparing happiness across countries. Clearly, more cross-culture studies need to be done to understand the relationship between human behavior and happiness across the world.

LIMITATIONS

First, we only assessed situational variables while ignoring the behavioral variables (which can be further tested through the Riverside Behavioral Q-sort). Furthermore, we only measured situational experiences at one time (7 pm). The results might have been different if we had instructed the participants to record multiple situational experiences across the time of the day. Lastly, our study only utilized university age participants (20-28 years old). Thus, a more diverse age group would be required to reach better generalizability. Therefore, further studies need to be implemented to look at cross-cultural variables that construct the national average of happiness.

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APPENDIX I

Riverside Situational Q-sort Version 3.15

1. Situation is potentially enjoyable.
2. Situation is complex.
3. A job needs to be done.
4. Someone is trying to impress P.
5. Someone is trying to convince P of something.
6. P is counted on to do something.
7. Talking is permitted.
8. Talking is expected or demanded.
9. P is being asked for something.
10. Someone needs help.
11. Minor details are important.
12. Situation evokes values concerning lifestyles or politics.
13. Affords an opportunity to demonstrate intellectual capacity. (e.g., an intellectual discussion, a complex problem needs to be solved)
14. Situation is uncertain.
15. Another person (present or discussed) is under threat.
16. P is being criticized, directly or indirectly.
17. Someone is attempting to dominate or boss P.
18. Situation is playful.
19. Introspection is possible. (e.g., the atmosphere allows or encourages reflection upon deeply personal issues)
20. Things are happening quickly. (Low placement implies things are happening slowly.)
21. Someone (present or discussed) is unhappy or suffering.
22. A reassuring other person is present.
23. P is being blamed for something.
24. A decision needs to be made.
25. Rational thinking is called for.
26. Situation calls for self-restraint.
27. Situation involves competition.
28. Affords an opportunity for P to do things that might make P liked or accepted.
29. Others are present who need or desire reassurance.
30. Situation entails frustration. (e.g., a goal is blocked)
31. Physical attractiveness of P is relevant.
32. It is important for P to make a good impression.
33. Situation would make some people tense and upset.
34. Situation includes one or more small annoyances.
35. Situation might evoke warmth or compassion.
36. A person or activity could be undermined or sabotaged.
37. It is possible for P to deceive someone.
38. Someone else in this situation (other than P) might be deceitful.
39. Situation may cause feelings of hostility.
40. People are disagreeing about something.
41. Affords an opportunity to express unusual ideas or points of view.
42. Situation contains physical threats.
43. Situation contains emotional threats.
44. Situation raises moral or ethical issues. (e.g., a moral dilemma is present; a discussion of morality)
45. A quick decision or quick action is called for.
46. Situation allows a free range of emotional expression.
47. Others present might have conflicting or hidden motives.
48. Situation entails or could entail stress or trauma.
49. Affords an opportunity to ruminate, daydream or fantasize.
50. Situation has potential to arouse guilt in P.
51. Close personal relationships are present or have the potential to develop.
52. Someone other than P is counted on to do something.
53. Situation includes intellectual or cognitive stimuli. (e.g., books, lectures, intellectual conversation)
54. Assertiveness is required to accomplish a goal.
55. Situation includes potential for immediate gratification of desires. (e.g., food,shopping, sexual opportunities)
56. Social interaction is possible.
57. Situation is humorous or potentially humorous. (if one finds that sort of thing funny)
58. P is the focus of attention.
59. Situation includes sensuous stimuli. (e.g., touch, taste, smell, physical contact)
60. Situation is relevant to bodily health of P. (e.g., possibility of illness; a medical visit)
61. Success in this situation requires self-insight.
62. P controls resources needed by others.
63. Others present a wide range of interpersonal cues. (e.g., body language, tone of voice, social signals)
64. Situation includes behavioral limits. (e.g., rules or social norms that might or might not be challenged)
65. Situation includes aesthetic stimuli. (e.g., art, music, drama, beauty)
66. Situation is potentially anxiety-inducing.
67. Situation makes demands on P. (either explicitly or implicitly)
68. Affords an opportunity to express or demonstrate ambition.
69. Situation might make P feel inadequate.
70. Situation includes stimuli that could be construed sexually.
71. Situational demands are rapidly shifting.
72. P is being abused or victimized.
73. Members of the opposite sex are present.
74. Potential romantic partners for P are present.
75. Situation has potential to arouse competing motivations.
76. Situation is basically simple and clear-cut.
77. Affords an opportunity to express charm.
78. Situation involves social comparison.
79. Situation raises issues of power. (for P or others present)
80. Affords an opportunity to express masculinity.
81. Others may need or are requesting advice from P.
82. Independence or autonomy of P is questioned or threatened.
83. Situation is potentially emotionally arousing.
84. Affords an opportunity for demonstrating verbal fluency. (e.g., a debate, a monologue, an active conversation)
85. People who are present occupy different social roles or levels of status.
86. P is being pressured to conform to the actions of others.
87. Success requires cooperation.
88. P is being complimented or praised.
89. Affords an opportunity to express femininity.

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