LUMINESCENCE ACTIVITY AS A BIOSENSOR OF

ISOBUTYRALDEHYDE PRODUCTION

IN CYANOBACTERIA

By

Jill Mecklenborg

Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements for

the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

August, 2011

Nashville, Tennessee

Approved:

Carl H. Johnson

Robert L. Galloway, Jr.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The Growing Importance of Biofuels

The worldwide energy consumption rate is projected to increase 49% by 2035.

To date, the world's increasing energy demands have been met primarily by fossil fuels

(i.e., coal, oil, natural gas). However, concerns about climate change, fossil fuel price

increases, security of oil supply, and depleting reserves of easily-extractable oil direct

efforts toward implementation of sustainable energy practices. (Energy Information

Administration, 2010)

Novel technologies utilize the earth's natural resources such as wind, ocean tides,

sunlight, and geothermal heat as renewable sources of energy.   Still, liquid fuel remains

the primary fuel source of the industry and transportation sectors and is expected to

remain the world's largest source of energy (Energy Information Administration, 2010).

There exists, therefore, a tremendous need for the development and industrialization of

energy-dense biofuels to replace the petroleum-based fuels currently in consumption.

Biofuels are defined as any fuel derived from biomass (biological material from living or

recently-living organisms), and they possess much potential as a substitute for gasoline

and diesel fuels. Meeting energy demands in a sustainable fashion will require the

development of a large-scale, cost-effective, and carbon-neutral energy system (Lewis &

Nocera, 2006).

Bioethanol and biodiesel.

Liquid biofuels include ethanol and biodiesel. Ethanol is drinking alcohol and is made from the fermentation of sugars, while biodiesel is produced by the transesterification of lipids (Demirbas, 2009).

Ethanol, a two-carbon alcohol, is currently the primary biofuel in use, with production exceeding fifteen billion gallons per year (Fischer et al., 2008). It serves largely as an additive in petroleum-derived gasoline, with its concentration varying greatly. While ethanol has a higher octane rating and produces lower emissions, its energy content is only two-thirds that of gasoline per unit. Efficient ethanol consumption requires a high compression engine that is not widely available. Further, existing methods of production are energy and cost intensive, involving several steps. The first and most expensive step is procurement of simple sugars from the organic carbon source, which is typically carried out by the addition of enzymes. Various yeast strains then convert the sugars into ethanol through a process of fermentation, distillation, and dehydration (Service, 2010).

Biodiesel refers to fuel consisting of long-chain esters derived from organic oils or fats. Its energy content exceeds that of ethanol and increases as hydrocarbon chain length increases. Biodiesel is most often made by chemically reacting lipids with alcohols. Common feedstocks include the oil of soy beans, rapeseed, sunflowers, peanuts, and palm trees, while innovative research is examining the possibility of production by algae and other microorganisms. Biodiesel is Europe's dominant renewable fuel.

Food crops.

Ethanol is manufactured predominantly from corn-derived starch (in the U.S.) and sugarcane-derived sucrose (in Brazil), but can also be produced from the polysaccharides of potato and rice plants. Biodiesel is also manufactured chiefly from food crops, with soybean oils being the most commonly used. Despite high production numbers achieved, there are several foreseeable problems with widespread employment of food crop-based biofuels.

First, there exist some basic concerns regarding the availability of food and water to the world's growing population. As land is chosen for farming either food crops or ethanol-producing crops, food supply could become limited and prices inflated. The obvious solution is to extend farming to meet the world's food needs, but extensive farming of these crops would worsen water pollution and cause localized water shortages. The current corn farming process requires a great deal of water, fertilizer, and pesticides. Corn ethanol and soybean biodiesel irrigation require two to three orders of magnitude more water than the oil extraction and refinement process. Water usage would be reduced if seed companies are successful in engineering drought-tolerant corn strains that would maintain their yields through extended dry spells. (Service, 2009)

Second, there are concerns that the process of clearing sizeable portions of land for farming would result in significant carbon emissions. Although biofuel feedstocks utilize CO_2, the clearing of carbon-rich habitats for farmland can actually increase greenhouse gases (Searchinger et al., 2008). As soil and plant biomass store large amounts of terrestrial carbon, the burning and decomposition of natural habitats will

3

result in significant CO_2 release into the atmosphere (Fargione et al., 2008; Sheehan, 2009).

Existing methods of production are cost and energy intensive. There is the obvious cost of farming the food crops, but fermentation and transesterification are costly procedures also. In ethanol production, the starch (comprised of many glucose units joined by glycosidic bonds) and sucrose (a disaccharide derived from glucose) and fructose must first be broken down into simple sugars (e.g., glucose and fructose). This is usually carried out by the addition of enzymes, which represent a major cost component of the overall process. Ethanol derived from corn actually requires more energy input than it produces, with one liter of 99.5% corn ethanol requiring 46% more fossil energy than the energy produced as ethanol. Biodiesel from soybean plants requires 27% more energy (Service, 2010; Pimentel & Patzek, 2005).

Lignocellulosic biomass.

In addition to edible polysaccharides, sugarcane and corn also contain lignocellulosic biomass that can be made into biofuel. Lignocellulose is the non-edible fibrous or woody portion of plants. It is the feedstock for the paper and textile industries, but it can also be broken down into simple sugars and fermented to produce cellulosic ethanol. Plants providing high yields of biomass per acre, such as switchgrass and miscanthus, can be grown at low cost with minimal agricultural effort. In contrast to food crops, these plants can be harvested multiple times each year and require significantly less water and fertilizer. While production of ethanol from biomass would potentially alleviate some of the aforementioned problems, new difficulties arise

4

pertaining to the conversion of lignocellulosic material into sugar monomers. (Somerville et al., 2010)

Lignocellulose is composed of cellulose and hemicellulose polysaccharides tightly bound to lignin. It thereby forms the structural framework of plant cell walls, providing strength and rigidity to the stems, stalks, and trunks of plants. Cellulose, the most abundant of the three, entails thousands of β-D-glucose units arranged in a linear chain, making it tough and resistant to hydrolysis. Hemicellulose consists primarily of pentoses (the most abundant being xylose), but also contains some hexoses and uronic acids. It has a highly branched arrangement and contains fewer sugar units, rendering it unstructured and weak in comparison to cellulose. Lignin, an amorphous polymer comprised of phenylpropanoid compounds, crosslinks with cellulose and hemicellulose fibers via ether and ester bonds. (Klemm et al., 2005)

To extract the fermentable sugars, the celluloses and hemicelluloses must first be disconnected from the lignin and then hydrolyzed into simple monosaccharides. This process involves a costly combination of acid and cellulolytic enzyme treatments. Due to its robust structure, obtaining lignocellulosic sugar monomers is more difficult and less effective than obtaining starch and sucrose monomers. Yet another challenge involves the high percentage of pentose sugars in the hemicellulose fibers. While the hexose sugars of cellulose, starch, and sucrose are fermented by typical yeast strains, pentose sugars remain difficult to ferment. Engineering yeast strains to metabolize pentose sugars is a leading area of cellulosic ethanol research efforts. (Service, 2010; Jeffries & Jin, 2004)

Cellulosic ethanol has the advantage of abundant and diverse raw material (including the woody portions of food crops), potentially relieving some of the land use concerns. Nevertheless, current sugar procurement and fermentation techniques are cost-intensive, with only 40% of the biomass energy content converted to ethanol (Service, 2010). For cellulosic biofuel production to be economically viable, heterotrophic microbes must be engineered to increase the rate and yield of both cellulose conversion and sugar fermentation (Fischer et al., 2008).

Heterotrophic microbes.

Heterotrophic microbes are being studied as a means of alternative fuel production, due to the ease of expanding their use to industrial-scale bioprocessing. New metabolic pathways that control cellulose degradation and biofuel production are being discovered and manipulated in an effort to yield the most efficient production of biofuel from cellulose. Using recombinant technology, heterotrophic microbes could potentially be engineered to perform direct conversion of lignocellulosic material to biofuel. Thus, it may be feasible for a single microorganism to do the job of what would previously have required multiple cultures. Producing biofuels directly from cellulose, known as consolidated bioprocessing, is believed to reduce costs substantially compared a multiple step approach. (Alper & Stephanopoulos, 2009)

Microorganisms involved in fungal decomposition of plants and termite digestion possess the innate ability to degrade lignocellulosic materials and convert the hydrolyzed products to ethanol. Various enzymes are secreted and the genetic pathways encoding for these enzymes are being explored through genetic recombination (Alper &

6

Stephanopoulos, 2009). Yeast wild-type *S. cerevisiae* strains readily ferment hexose sugars via glycolysis, but contain no innate pathway for pentose fermentation. Recent metabolic engineering studies on *S. cerevisiae* strains expressing pathways for pentose fermentation have enabled rapid and efficient fermentation of xylose (via a fungal xylose isomerase pathway) but require further optimization of the arabinose fermentation pathway (van Maris, et al., 2006; Fernandes & Murray, 2010).

Cellulolytic anaerobic *Clostridia* strains are naturally able to produce cellulase, hydrolyze cellulose, and ferment sugars (including xylose). Yet these bacteria also naturally produce acetate and lactate, reducing the efficiency of ethanol production (Demain et al., 2005). Metabolic engineering of *Clostridia* has proven difficult in comparison to that of non-cellulolytic yeast and *E. coli* strains (Fischer et al., 2008). *C. cellulolyticum*, however, has been successfully engineered to increase ethanol production by 53% (Guedon et al., 2002).

Microbes have also been metabolically engineered to make higher alcohols, which possess greater energy content than ethanol. Atsumi et al. have engineered *E.coli* to produce isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol from glucose. The general approach involved modification of the amino acid biosynthesis pathway to increase production of the 2-keto acid specific to the desired alcohol. With regard to isobutanol production, the acetolactate synthase enzyme (*alsS*) of *Bacillus subtilis,* having a higher affinity for pyruvate than the corresponding *E.coli* enzyme, was overexpressed resulting in a 1.7-fold increase in isobutanol production. Genes responsible for generation of by-products that compete for pyruvate were deleted to further increase production. The result was a strain of *E.coli* capable of overproducing

isobutanol, with a metabolic engineering approach applicable to the production of other alcohols and possibly other organisms. (Atsumi et al., 2008)

Using that same metabolic strategy, *C. cellulolyticum* was recently engineered to synthesize isobutanol directly from cellulosic material. Strains were grown on agar plates containing either crystalline cellulose or cellobiose, an intermediate disaccharide product of cellulose. Despite a spontaneous frameshift mutation in the *alsS* sequence resulting in a truncated protein, the strain produced isobutanol at levels of 140 mg/L on cellobiose and 400 mg/L on cellulose, a significant increase compared to the empty vector strain which reached just 17 mg/L and 30 mg/L respectively. The WT engineered strain produced 364 mg/L on cellobiose and 660 mg/L on cellulose. Difficulties relating to the gene expression system and enzymatic activity measurements were encountered with *C. cellulolyticum*, but such difficulties were not present with *E. coli*. (Higashide et al., 2011)

Regardless, heterotrophic microbes require an organic carbon compound source. Whether the sugars come from starch, cellulose, simple glucose, or some other source, the carbon remains an expensive medium. Feedstocks are the bulk of the cost in these biofuel applications, comprising approximately 80% of the cost. This cost includes producing, harvesting, storing, and transporting the organic carbon source. (Babcock, 2007; Dismukes et al., 2008)

Algae.

Photosynthetic capability is a promising target for biofuel generation, as photoautotrophs do not require an organic medium. The growth rate of algae is much

faster than that of plants, potentially allowing for more efficient fuel production.

However, algae require a lot of water and atmospheric levels of CO_2 aren't high enough

to spur exponential growth. Moreover, the algal photosynthetic approach requires several

intermediate steps for recycling CO_2 into usable fuels and an extensive extraction

process, thereby resulting in high production costs (McKinlay & Harwood, 2010;

Sheehan, 2009).

Ethanol from algae is possible by converting starch (the storage component) and

cellulose (the cell wall component). Lipid production by algae seems a more efficient

means given that algae lipid energy content greatly exceeds that of ethanol. Thus, algae

hold the potential for producing both ethanol and biodiesel, with lipids in algae oil made

into biodiesel and carbohydrates converted to ethanol. (Gordon & Polle, 2007)

Stephens et al. conducted industrial feasibility studies and sensitivity analyses to

find that cost-effective technologies have not yet been successfully integrated and

optimized. It was concluded that algal biomass may become an attractive option for

biofuel production if metabolic engineering can boost productivity to offset the expensive

extraction process. (Stephens et al., 2010)

Cyanobacterial Biofuel Production

Phototrophic microbes hold much promise in the development of biofuels, as the

utility of photosynthesis bypasses the need for harvesting, transporting and

deconstructing plant-derived biomass to provide simple sugars. Unlike heterotrophic

microbes, their growth is inexpensive, requiring only a salt medium.

Cyanobacteria, also known as blue-green algae, are photoautotrophic prokaryotes, capable of converting solar energy into chemical energy and fixing CO_2 via photosynthesis. Cyanobacteria are more efficient solar energy converters than plants, thriving across a greater range of photon intensities, while exhibiting greater light capture efficiency due to the ability to capture light all year. They exhibit diversity in metabolism and habitat, with some species thriving in salt water. They would need to be grown in a land-based photobioreactor system, which is still expected to have a large area footprint. However, this could be placed on non-farmable land, thereby resolving the conflict between using land for biofuel or food crops (Angermayr et al., 2009). The major foreseeable problem with photoautotrophic growth is the rate limiting step of CO_2 assimilation (Zhou & Li, 2010). However, this issue may be overcome, as genome alteration of bacteria requires less extensive measures compared to that of plants. Also, with phototrophic microbes, modifications to their nutrition and environmental surroundings can be easily made in an effort to maximize production (Dismukeset al., 2008).

Ideally, one cyanobacteria strain could be genetically engineered to produce high-energy biofuels, thereby simplifying the production process and eliminating the need for several steps to reach the end product. Whatever the end product, conversion to liquid fuel will require technologies simpler than those associated with breaking down the cellulose of biomass. To improve biofuel production, metabolic engineering strategies would need to re-direct photosynthetic energy away from sugar production and toward the production of a biofuel. (Stephanopoulos, 2007)

Hydrogen.

Hydrogen gas itself can be used as fuel. Hydrogen is an intrinsic end-product of cyanbacterial fermentation, as electrons produced by photosynthesis are directed toward H_2 production. Cyanobacteria are able to produce H_2 through native hydrogenase, native nitrogenase, and an introduced hydrogenase. However, these enzymes are inactivated by O_2, thereby limiting the efficient production of H_2. Thus, research has resorted to separating H_2 production from photosynthesis, through use of specialized cells with low O_2 concentrations and O_2-depleting sulfur deprivation. Metabolic engineering has increased H_2 production through disruption of Calvin cycle flux. Photobiological H_2 production has managed to surpass rates of current crop-based biofuels. The opportunity remains for cyanobacterial production of higher-energy fuels, but it is possible that hydrogen gas production could also be optimized, for use as an additional fuel source. (Angermayr et al., 2009; McKinlay & Harwood, 2010)

Isobutyraldehyde.

One such higher-energy fuel of increasing interest is isobutanol. Isobutyraldehyde (IBA) can be converted to isobutanol and other hydrocarbons with chemical catalysts. Advantages of isobutyraldehyde/isobutanol biofuel over ethanol include: higher energy density, higher octane number, less corrosion to pipelines due to lower hygroscopicity (ability of a substance to extract water molecules from the surrounding environment), and an easier purification process due to its high vapor pressure. (Atsumi et al., 2008)

Stemming from their earlier work with *E.coli,* Atsumi et al. engineered

Synechococcus elongates PCC7942 to produce isobutanol directly from CO_2. First, the

gene encoding for keto acid decarboxylase, an enzyme that catalyzes the conversion of a

keto acid precursor (2-ketoisovalerate) to isobutanol, was inserted and expressed under

the control of the isopropyl-β-D-thiogalactoside (IPTG) inducible promoter *P*trc. Flux to

2-ketoisovalerate was increased when the *alsS* gene from *Bacillus subtilis* and the *ilvC*

and *ilvD* genes from *E. coli* were inserted. These genes express enzymes involved in the

conversion of pyruvate to ketoisovalerate. (Atsumi et al., 2009)

Rubisco catalyzes the CO_2 fixation reaction in the Calvin cycle. Overexpression

of the *rbcLS* gene enhanced Rubisco enzyme activity *in vitro* and improved

isobutyraldehyde/isobutanol production. While the engineered strain showed growth

retardation with high levels of isobutanol, the isobutyraldehyde production strain

produced constantly for nine days in the system. The rate of production of IBA was

higher than those reported for ethanol, hydrogen, or algal lipid production. The

coordinating increase in Rubisco activity and isobutyraldehyde production suggest that

CO_2 fixation is the rate-limiting step. Further optimization of CO_2 fixation would likely

improve IBA production. (Atsumi et al., 2009)

Additional high-energy fuels.

The cyanobacteria *Synechocystis* was modified to produce isoprene, an energy-

rich 5-carbon hydrocarbon used industrially in synthetic rubber polymers. The

heterologous expression of plant isoprene synthase (*IspS*) enabled photosynthetic

isoprene generation. Codon optimization of the DNA sequence conferred an advantage

in *IspS* gene expression, allowing for overexpression of the gene and increasing the yield of isoprene. (Lindberg et al., 2010)

Upon discovery of a new alkane synthesis pathway in cyanobacteria, C13-C17 alkane/alkene mixtures were produced in *E.coli*. Orf1593 and orf1594 genes were found to produce an acyl-acyl reductase and an aldehyde decarbonylase, implicating these as the genes responsible for the conversion of fatty acid intermediates to alkanes and alkenes. To test this hypothesis, the alkane operon from cyanobacteria was expressed in *E. coli,* which is known not to produce alkanes. The result was a strain of E. coli that could produce alkanes, proving that these genes and enzymes can be altered to control direct conversion to alkane biofuels. (Schirmeret al., 2010)

Additionally, C10-C18 fatty acids were overproduced and secreted into medium by metabolic engineering of *Synechocystis* PCC 6803. First an acyl-acyl carrier protein was introduced and found to increase fatty acid production. This was followed by a series of genetic alterations that further enhanced increased fatty acid production. More modifications were made by deleting the S layer from cell envelopes and weakening peptidoglycan layers. This was successful in increasing secretion of the fatty acids. The increased fatty acid production and secretion seen in mutants suggests that fatty acid secreting cyanobacteria may be a capable technology for renewable biofuel production. (Liu et al., 2010)

Optimization of Biofuel-Producing Cyanobacteria

It is important to note that cyanobacteria can complete more than one growing cycle in a single day, with some species doubling in just a few hours of culture.

Identification and manipulation of these metabolic pathways could increase their biofuel-producing capacity. Increasing knowledge of their physiology, development of advanced technologies in photobioreactor design, and manufacture of high-energy products would improve the economic feasibility of the cyanobacterial production system.

Manipulation of circadian pathways.

In cyanobacteria, as well as in the majority of organisms there exist genetically programmed daily cycles, known as circadian rhythms. These clocks consist of intricate mechanisms of chemical reactions responsible for regulating the timing of cellular events. Circadian pathways control metabolic processes, including cell division. (Mori & Johnson, 2001)

By growing strains in competition with each other, cellular fitness was determined to be maximal when phasing of circadian processes closely matched that of environmental (light-dark) cycles (Ouyang et al., 1998). It was found that the *S. elongatus* PCC7942 strain with an inactivated circadian clock outgrows its wild-type counterpart under constant light (Xu et al., 2000). However, in 12 hour light-dark cycles, the wild-type strain outgrows the inactivated strain.

Circadian gene expression is regulated by KaiA, KaiB, and KaiC proteins. Some metabolic proteins, such as the D1 protein of the photosystem II reaction center, encoded by *psbA1*, have been found to be circadian-controlled (Johnson & Golden, 1999; Liu et al., 1995). It is unknown whether any of the genetic pathways controlling biofuel production are circadian-controlled.

Bioluminescence.

Bioluminescence refers to the production and emission of visible light in a living organism. It is controlled by a chemical reaction that oxidizes luciferins, a class of light-producing organic substrates. This conversion of chemical energy to radiant energy is direct and efficient, meaning that very little heat is given off in the process. Luminescence is also termed cold light.

Bacterial luciferin is a reduced flavin mononucleotide ($FMNH_2$). The luminescence reaction is catalyzed by luciferase and requires the oxidation of $FMNH_2$ and a long-chain aldehyde (Hastings, 1983). The result is emission of excess free energy in the form of a blue-green light, as represented by the following reaction: $FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + light$ (490nm). Long-chain aldehydes (C8-C14) result in the most stable reactions.

Bioluminescence has been used as an *in vivo* biosensor in cyanobacteria to identify genes involved in circadian pathways. The luciferase gene (*luxAB*) is linked to the gene of interest and the luminescence pattern monitored (Kondo, et al., 1993; Liu et al., 1995).

The *alkJ-luxAB* with *blaP* promoter was recently found to express luminescence in the presence of aldehydes and alcohols. To do so, *alkJ* produces an alcohol dehydrogenase that converts C5-C12 linear primary alcohols to aldehydes. Luminescence increased linearly with increased concentrations of IBA. The long-chain aldehydes produced can be converted to alcohols, which possess a high energy density. (Minak-Bernero et al., 2004)

A novel idea.

Genetically engineered cyanobacteria provide a promising system for both biofuel production and CO_2 utilization. It remains unknown as to which genetic pathways contribute to aldehyde production and to what extent. The idea proposed here is to manipulate circadian pathways in an effort to optimize IBA production, as this has not yet been studied. The goal is to find the strain able to maximally produce isobutyraldehyde, with a procedure applicable to the production of other biofuels. The amount of product could be measured by luminescence levels.

CHAPTER II

A HIGH-THROUGHPUT LUMINESCENCE SCREENING SYSTEM

Explication

The immediate objective is to find the cyanobacterial mutant or mutants that

result in optimal biofuel production. There are several factors to consider: amount of

intended product, purity of intended product, and development of conditions that would

halt or cease production. Therefore, it is essential to screen a large library of mutants

possessing the potential to produce the desired product. Since the eventual goal is to

produce large quantities of a biofuel for widespread use, it is likely that the preeminent

mutants will be those producing the greatest amounts of the intended biofuel throughout

an extended period of time.

Bioluminescence as a reporter.

It is first necessary to find a means of quantifying the amount of biofuel produced.

Bacterial luciferase fused to a promoter sequence produces luminescent colonies that can

be imaged and quantified. Bioluminescence has been used to detect circadian rhythms in

mutant colonies through analysis of images taken over a period of time. Thereby,

luminescence serves as a real-time reporter of circadian gene activity. Bioluminescence

could be used to detect the amount of biofuel production in a similar manner.

Long-chain aldehydes are the substrate of a luciferase reaction, producing a

complimentary alcohol along with light emission. These aldehydes and alcohols can be

used as high-energy biofuels.   Of primary interest is detection of isobutyraldehyde production.

Using a cooled-CCD camera capable of sensitive light detection, luminescent colonies can be detected and their luminescence quantified. Bacterial luminescence on agar has been found to increase with known increasing concentrations of isobutyraldehyde vapor. Thus, in comparing mutant luminescence levels of biofuel-producing cyanobacteria, it would be possible to determine the amount of isobutyraldehyde produced, e.g. the amount of IBA in each dish. Those mutants with the highest luminescence values over time will be those optimal for isobutyraldehyde fuel production.

High-throughput screening.

High-throughput screening (HTS) allows a researcher to rapidly identify active genetic mutations modulating a particular bimolecular pathway. HTS provides continuous real-time data collection. It is especially useful for screening a large library of genetic mutations.

Automation is a critical operative element of an effective and well-designed high-throughput apparatus. Such a device would make use of motorized mechanical components, a means of signal detection, and a data processing system, with functionality controlled by automated software. Thereby, an HTS system can simultaneously incubate and analyze numerous plates, allowing for the parallel screening of thousands of mutant colonies.

System Development

Automated luminescence measurements in liquid culture.

Bioluminescence measurements were first automated using the Apple II microcomputer in the early 1980s, with the advent of the "Taylortron." Fluorescent lamps provided the illumination necessary for cyanobacteria to undergo photosynthesis in a liquid culture environment. This original apparatus consisted of a linear arrangement of thirty 20-mL vials, each in a temperature-controlled holder. A phototube measured the luminescence of each sample, moving from one position to the next under the control of custom software. The automated system allowed for luminescence measurements to be taken over long periods of time, thereby allowing for detection of certain luminescent rhythms. (Taylor et al., 1982)

An updated "Taylortron" apparatus showed that luciferase activity can accurately report circadian rhythms in cyanobacteria. Gene expression activity was reported with bacterial luciferase (*luxAB)* fused to a promoter gene. Period and phase changes were investigated by resetting the circadian clock with dark pulses, thus resetting the luminescence pattern. This provided evidence for the existence of circadian cycles in prokaryotes in liquid culture. (Kondo et al., 1993)

Circadian rhythm detection on agar plates.

The following two studies did not employ mechanical components, but are significant in providing evidence of circadian rhythm detection via bioluminescent cyanobacteria imaged on agar plates.

Subsequently, luciferase was shown to be useful as a bioluminescence reporter for individual bacteria colonies. Colonies grown on agar plates were found to display a rhythm similar to that of bacteria in liquid culture. A cooled-CCD camera (8-bit resolution) took repeated images of a single agar plate. Images were analyzed for luminescence intensity changes over time. Individual colonies were recognized as ovals by the custom software, and luminescence data was taken from the selected regions of interest (ROIs). Mutants with varying period lengths were identified. (Kondo & Ishiura, 1994)

Additional studies conducted around the same time showed transgenic Arabidopsis plants utilizing a firefly luciferase construct to be capable of reporting circadian patterns. A video imaging system was used to visualize images of plant seedlings grown on an agar plate in low-light conditions. Luciferase bioluminescence was measured over time and mutants with varying period lengths were detected. This system provided evidence that circadian rhythm luminescence could also be imaged from plant seedlings grown on agar plates. (Millar et al., 1995)

Automated luminescence measurements on agar plates.

This research led to the development of a high-throughput apparatus that could screen for circadian rhythm mutations on multiple agar plates. This "Kondotron" consisted of a stepper-motor-controlled turntable, containing twelve channels for agar plates. The apparatus employed a Macintosh-based system with a custom driver-interface. Using this high-throughput system, cyanobacteria colonies were screened after

mutagenesis; short (as low as 16 hour) and long (up to 60 hour) mutants were identified. (Kondo et al., 1994)

<div align="center">Current System</div>

Hardware.

Given significant advances in technology over the last thirty years, the current system employs a commercially-available stepper motor and driver, a micro-machined turntable, and a modern PC computer. The system features a turntable with channels for twelve individual petri dishes or plates. Mechanical aspects are similar to the original system, but the addition of a commercially available motor-driver interface (EMP400, Oriental Motors) allows for all table functions to be handled by the computer, as opposed to the manually switching of a physical control box. The EMP400 controller is connected to the computer through a serial port.
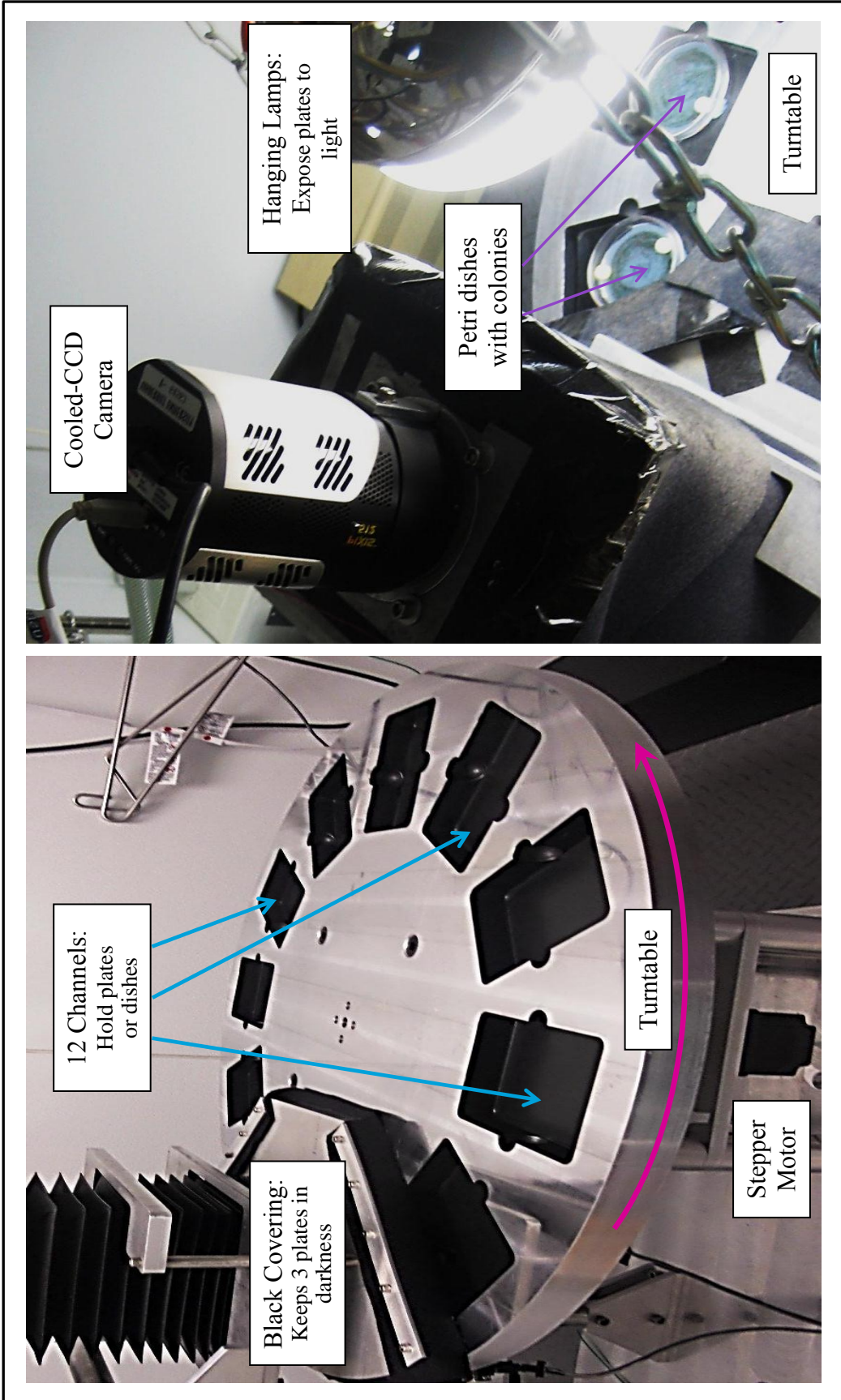
The current system employs the PIXIS 512 camera (Princeton Instruments), which is a CCD camera water-cooled to -70$^{\text{o}}$C. This camera has 16-bit resolution, as opposed to the previously used 8-bit resolution. The larger dynamic range provides an improved ability to distinguish quantitative differences in colony luminescence.

Software capabilities.

The software is PC-based and run via LabView (National Instruments), providing the user with an efficient operative interface. All turntable movement is now controlled with the software program, with options for controlling the table independently of the
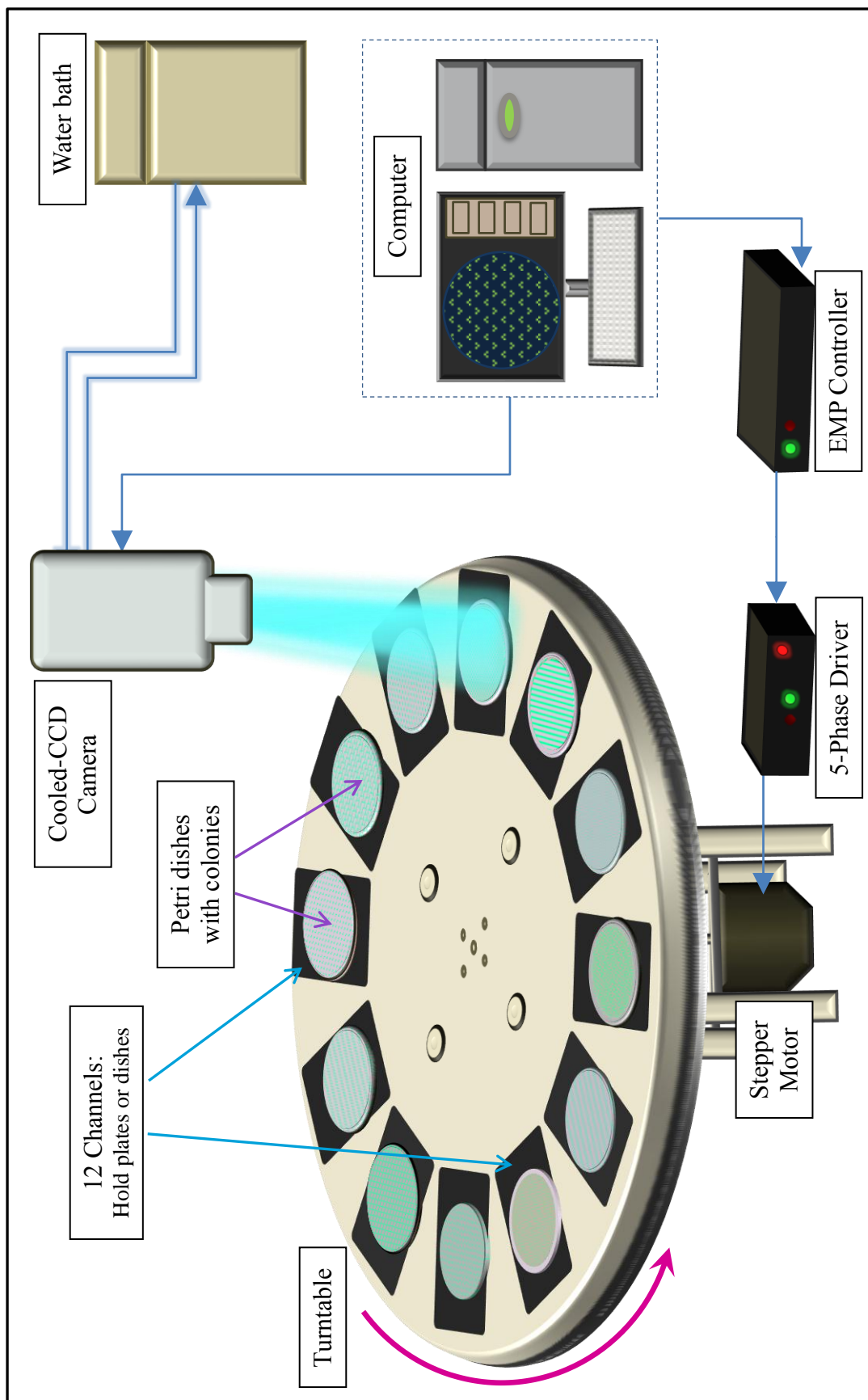
Figure 2.1. Schematic depicting hardware configuration of Kondotron. The turntable is controlled by a stepper motor and driver, with the EMP Controller allowing for computer communication. The computer also controls camera function.

Labels on figure: Water bath, Computer, EMP Controller, 5-Phase Driver, Cooled-CCD Camera, Petri dishes with colonies, 12 Channels: Hold plates or dishes, Turntable, Stepper Motor

Figure 2.2. Photos depicting hardware configuration of Kondotron. The turntable is shown with stepper motor beneath. Petri dishes are positioned in the channel holders. Lights hang from the ceiling to illuminate petri dishes, while a black accordion coveringkeeps the dishes in darkness during camera exposure.

automated acquisition series program via a unique user interface ("Motor Control"). Serial communication between the computer and the EMP400 makes use of National Instrument's VISA (Virtual Instrument Software Architecture).

The software consists of two additional options independent of the automated series. The "Focus" tab allows the user to continuously capture images, outputting them in a fraction of a second. This feature allows for the user to properly focus the camera when making adjustments to the camera lens or distance between the camera and the turntable. The "Single Image" tab is capable of capturing an image, saving an image at a specific location, and opening a previously saved image. The feature is useful in acquiring images of plates at various lengths to determine optimal exposure time.

The most complex of the software features is the automation of a series of images ("Ktron Program"), which combines image acquisition, turntable movement, and image processing. While an image is being acquired, the previous image is displayed for processing. The program can be paused at any time to allow for additional processing time. Once running, this program will continue to acquire and save images until it is stopped.

There now exists the ability to move individual colonies in the middle of an experiment, so as to account for any drifting that might occur if the plate were to dry out or crack during the experiment. Multiple colonies can also be moved as a single unit, compensatory for accidental or purposeful movement of a plate.
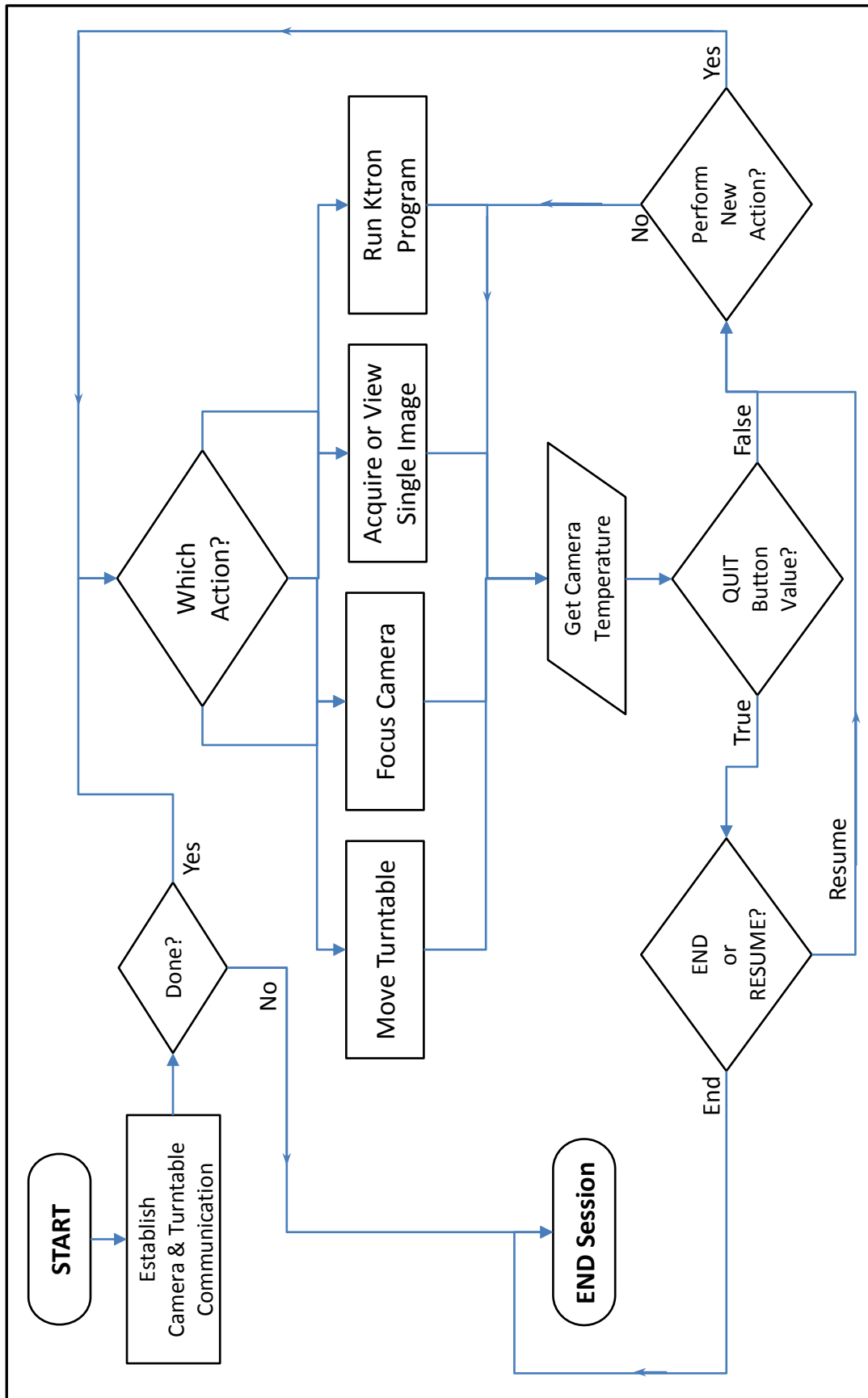
Figure 2.3. Flow chart depicting general software connections of new program. The software allows for turntable control and image acquisition independent of the automated program. The program can be exited at any time.
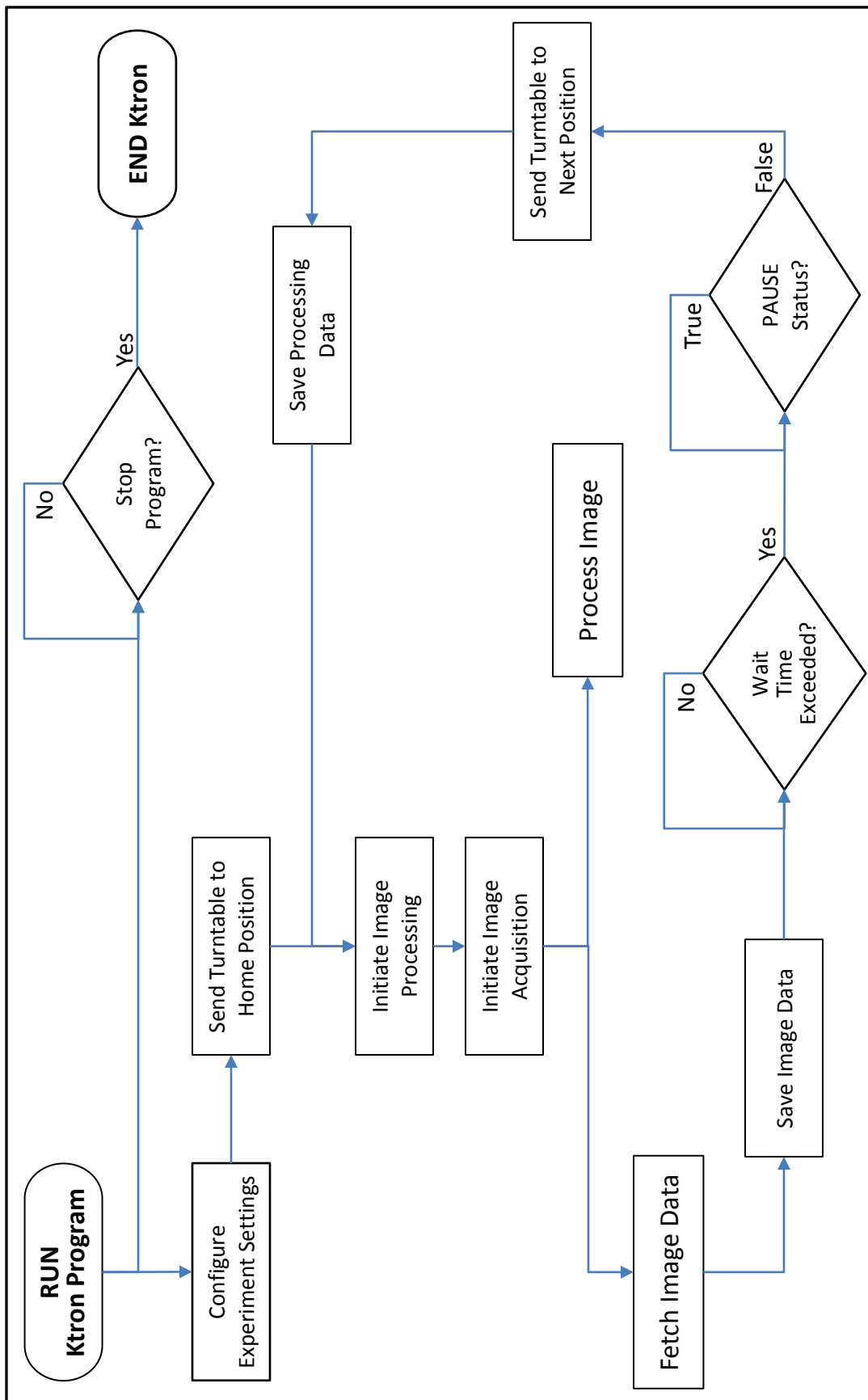
Figure 2.4. Flow chart depicting software connections of automated Kondotron program. The turntable is sent to "Home" position at the start. When not paused and the wait time is exceeded, the table moves to the next channel. Image acquisition and processing occur simultaneously.

Image Acquisition.

Images are acquired using the Scientific Imaging ToolKit (SITK) software

package, which is commercially available (RCubed Software) and run through LabView

VIs. The SITK package employs pvcam software (designed for Princeton Instruments

cameras) to send commands to the PIXIS 512 camera. The SITK software is configured

to initialize the camera, check the camera temperature, set the exposure time, control

shutter functioning, perform online background subtraction, and save the resulting image.

Under the "Single Image" tab, the user can select whether or not acquire and

subtract a background image. When the "Ktron Program" is running, a background

image is taken at the beginning of each cycle, with the SITK software ensuring the

camera shutter remains closed for the duration of the exposure. The software then

subtracts this image from all subsequent images in that cycle, saving each image with the

background subtracted.

Image Processing.

The image is opened and processed using LabView's Vision Assistant package.

Basic functions consist of brightness/contrast adjustments, colony display options, and

image zooming. The brightness and contrast of an image can be adjusted to allow for

improved colony selection, while the colonies can be selected as either a white outline or

a filled black oval with the option to display colony numbers.

Selection of colonies is improved, due to use of new image analysis techniques,

including various thresholding and morphological operations. Thresholding can be

automatic (considers local pixel values) or manual (the user sets the threshold to a

particular value and all pixels above that value are selected). A series of up to three

morphological transformations can be applied to the thresholded image,   including

"Dilate," "Open," and "Close." The colony particles can be separated further by applying

a Watershed Transform or simple erosion at the particle edges. Finally, the colonies are

filtered by size, eliminating any colony selections above or below the user-set value.

When the user clicks on a colony, a plot is displayed depicting that colony's

intensity over the time course of the experiment. Up to five colonies can be selected for

comparison, each displayed on the same image using different colors. The plot scale

automatically adjusts to colony intensity values and can be displayed with or without an

additional plot (gray) of the plate's average colony intensity over time.

Analysis.

Analysis software affords for additional calculations and manipulations. The

program provides calculations of the minimum, maximum, mean and integral

luminescence for each colony over the course of the experiment. Period, phase, and

luminescence could be adjusted to account for all data or a desired portion of the acquired

data. Baseline calculations could also be adjusted in this manner.

A slideshow containing a plot and calculations of each colony is displayed, with

the abilities to pause and control slideshow speed. Specific images can be saved with the

displayed contrast and brightness settings and opened in other commonly-used programs,

such as *Microsoft Office*, *Windows Live Photo Gallery*, and *Paint*. Similarly, a series of
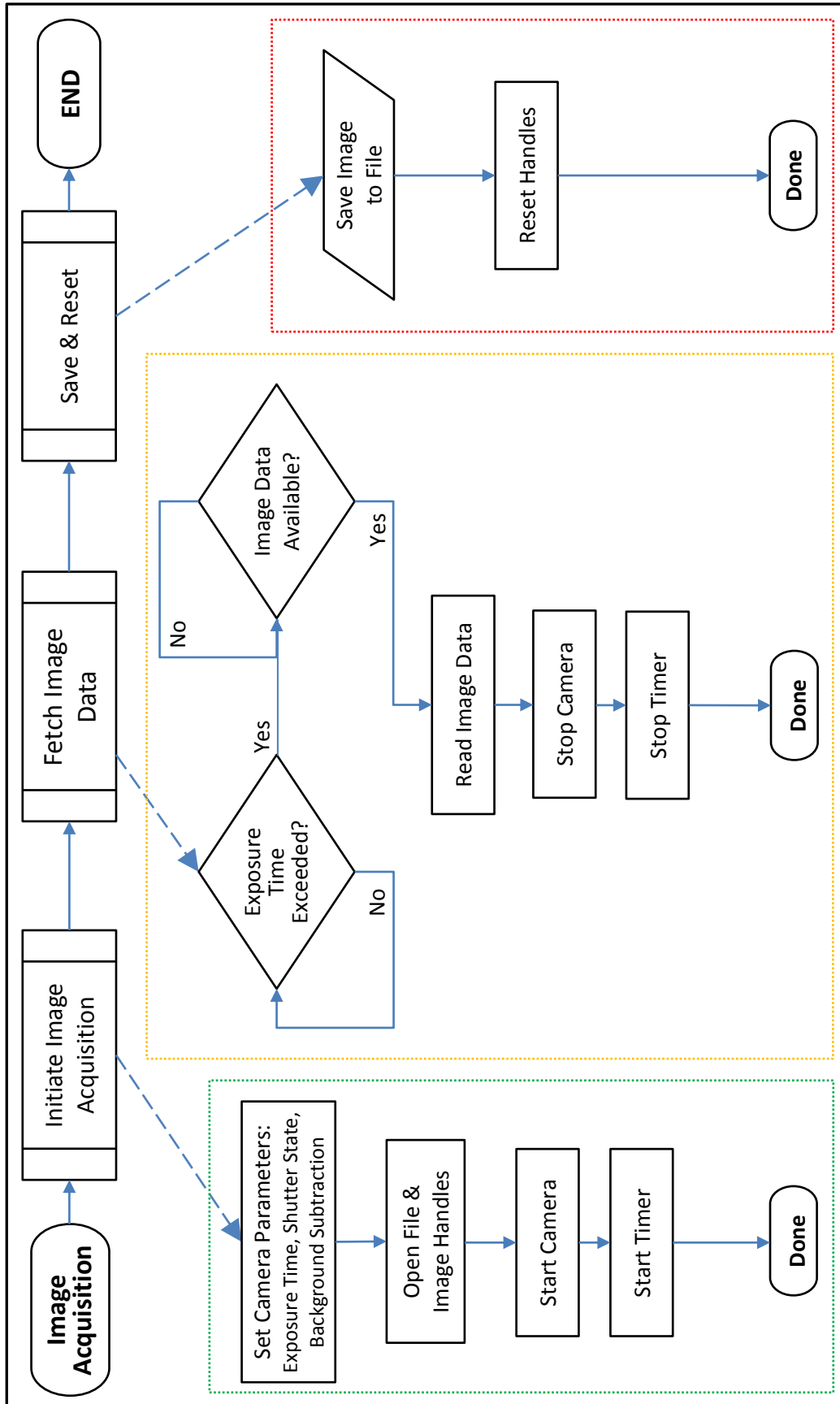
Figure 3.5. Flow chart depicting image acquisition procedure. This series of steps occurs when acquiring an image both within and outside of the automated program. "Initiate Image Acquisition" configures the camera and starts the exposure. "Fetch Image Data" consists of a loop that continually checks for image data once the exposure time has been exceeded. Finally, the images is saved and camera configurations are reset.
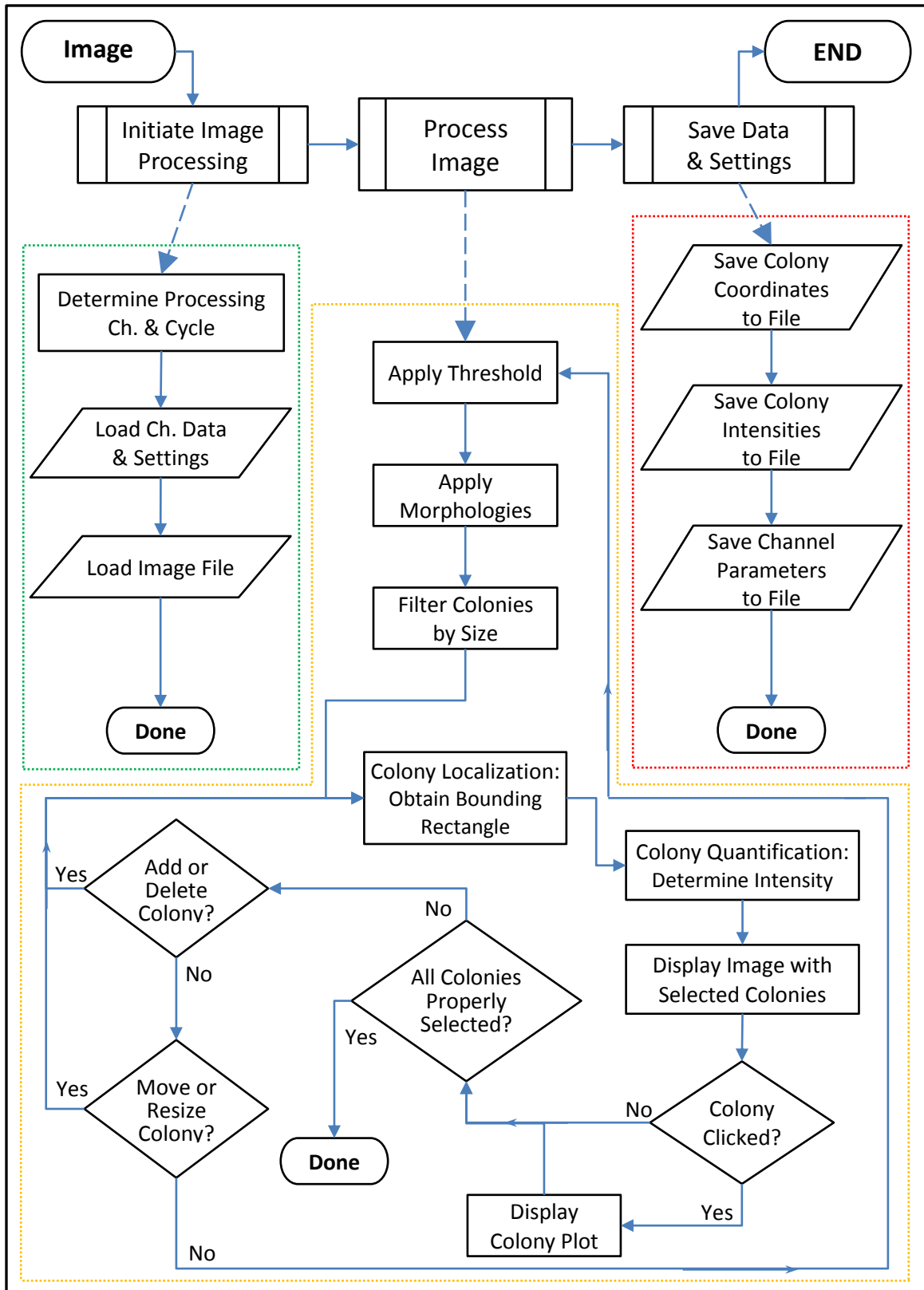
Figure 2.6. Flow chart depicting image processing procedure. The newly acquired image is loaded and processing occurs according to parameters. If colony selection displayed is not optimal, parameters can be adjusted or colonies can be added, deleted, moved, or resized.

images can be saved as an Audio/Video Interleaved (AVI) file. The user is able to save

the entire image or a selected portion.

CHAPTER III

FEASIBILITY OF LUCIFERASE ACTIVITY AS A BIOSENSOR OF
ISOBUTYRALDEHYDE PRODUCTION IN CYANOBACTERIA

Gas chromatography and high-performance liquid chromatography are the current
methods most commonly used to quantify alcohols and aldehydes. Such techniques are
expensive, time-intensive, and low-throughput.

Therefore, we have designed a method to use luminescence as a non-invasive
gauge of isobutyraldehyde ($(CH_3)_2CHCHO$) production. This is possible because the
substrate necessary for the bacterial luciferase reaction is an aldehyde. While optimal
aldehyde chain length is between eight and fourteen carbons, isobutyraldehyde, with its
two methyl groups on the carbonyl carbon, is shown to be an acceptable substrate. This
bioluminescence reporter system will be used to select for mutants exhibiting enhanced
aldehyde production. The aldehydes can then be enzymatically converted to alcohols that
can be used as fuel.

Preparatory Methods

Two different cyanobacterial promoters were attached to luciferase: *psbAI*p::*luxAB*
and *trc*p::*luxAB*. Individual cyanobacteria colonies were seeded onto agar in petri dishes.
The plates were exposed to different concentrations of isobutyraldehyde (IBA) vapor, by
means of a cap in the dish containing 0.2mL IBA dissolved in mineral oil.

Results

Initial experiments involved eight plates, each containing cyanobacteria strains with the two aforementioned promoters. Colonies were exposed to increasing concentrations of IBA for various exposure lengths.

It was found that as the concentration of IBA vapor increases, luminescence activity also increases. Cyanobacteria with the *trc* promoter initially exhibit greater luminescence activity than those with *psbAI* promoter. *trc* cyanobacteria exhibit some detectable luminescence at 0% IBA, while *psbAI* do not. *psbAI* cyanobacteria increase in intensity over several days to reach intensity levels comparable to *trc* cyanobacteria, but *trc* cyanobacteria do not appear to increase in intensity significantly throughout the same time course.
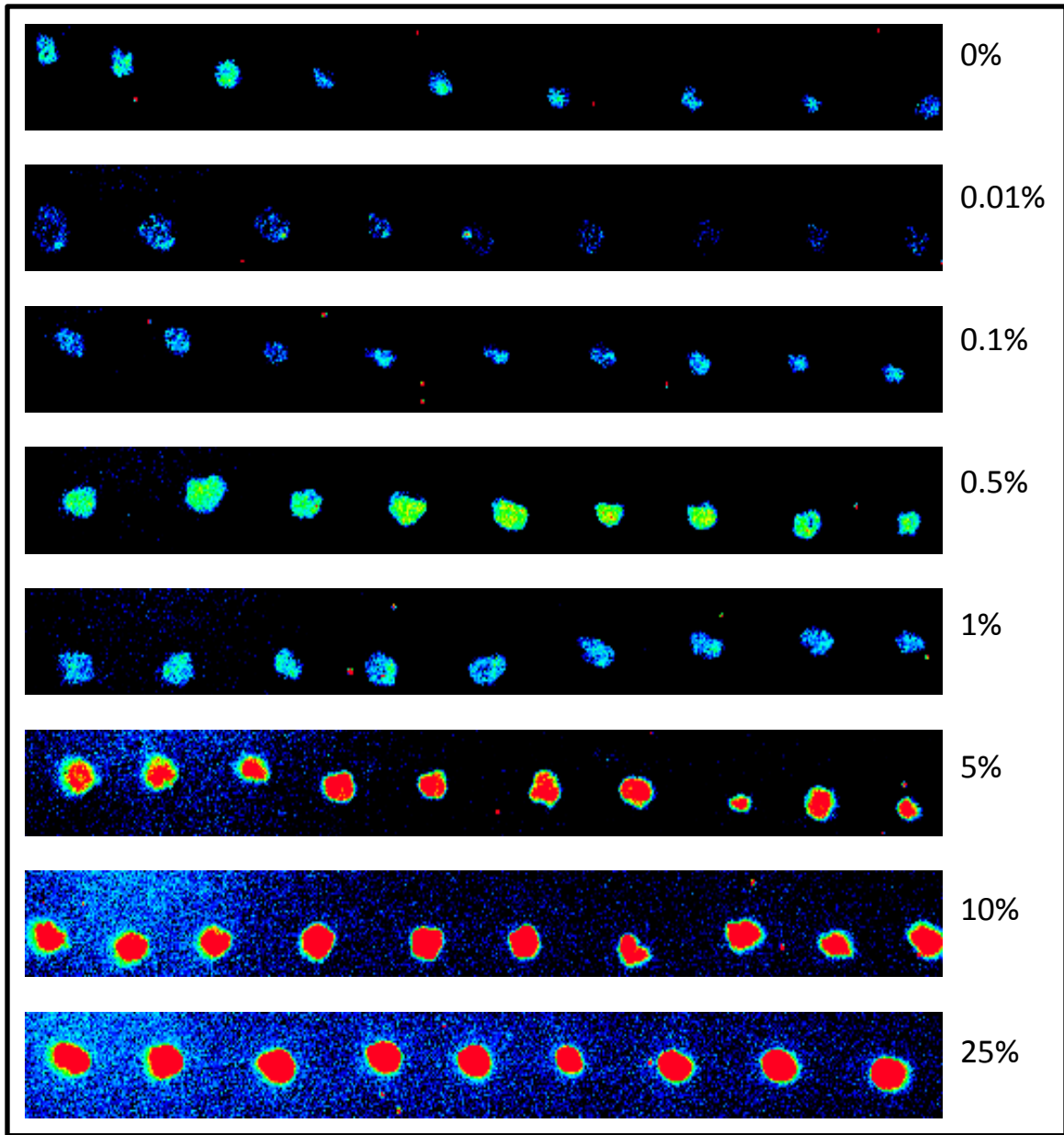
Figure 3.1. 15-minute exposures of *trc*p::*luxAB* at eight different IBA concentrations. Plates contain 0%, 0.01%, 0.1%, 0.5%, 1%, 5%, 10%, and 25% IBA. Mention that colony sizes were equivalent.
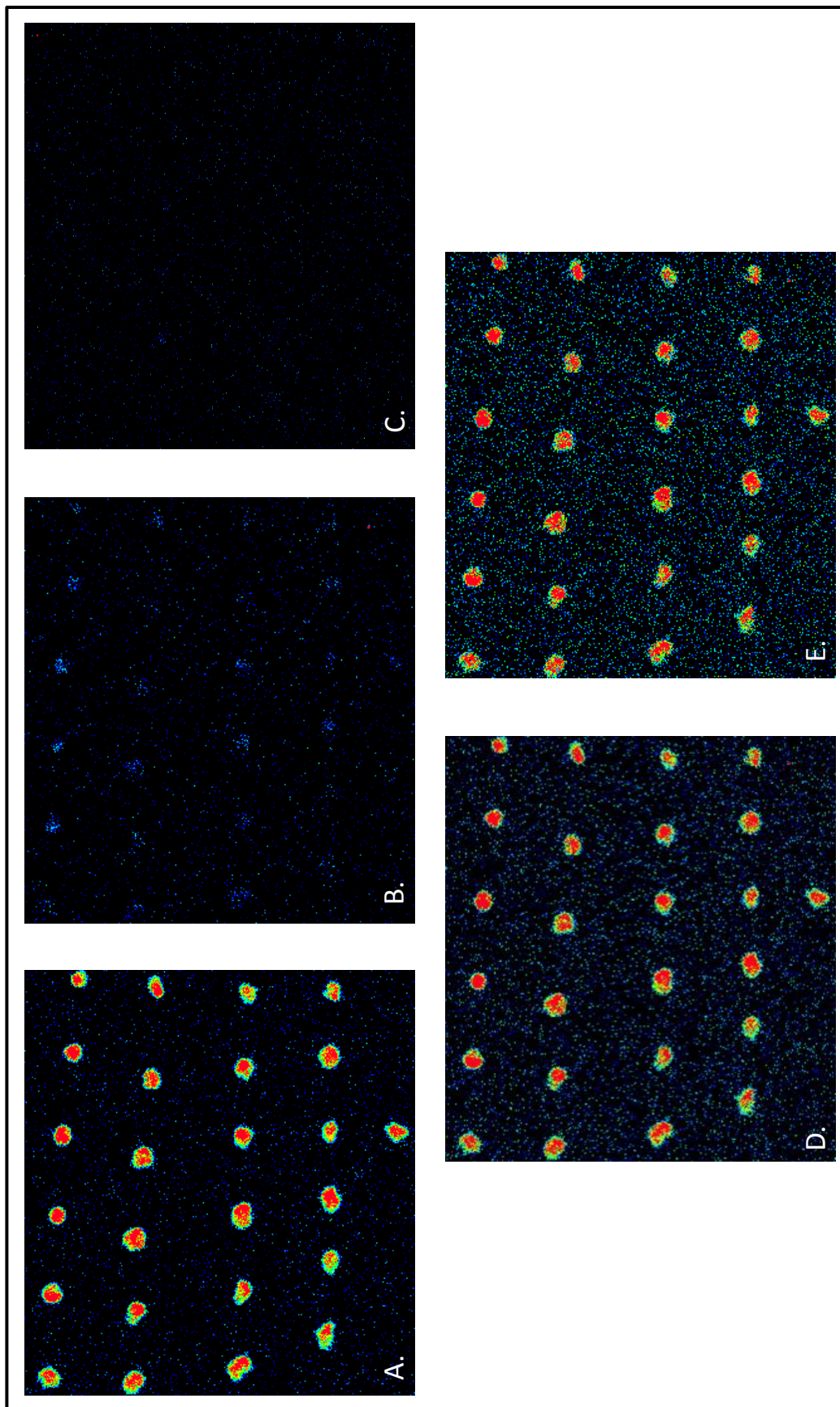
Figure 3.2. *trcp::luxAB* cyanobacteria colonies with 5% IBA. A series of three successive 1-minute exposures (A, B, C). Compiled images, equivalent to a 2-minute exposure (D) and a 3-minute exposure (E).
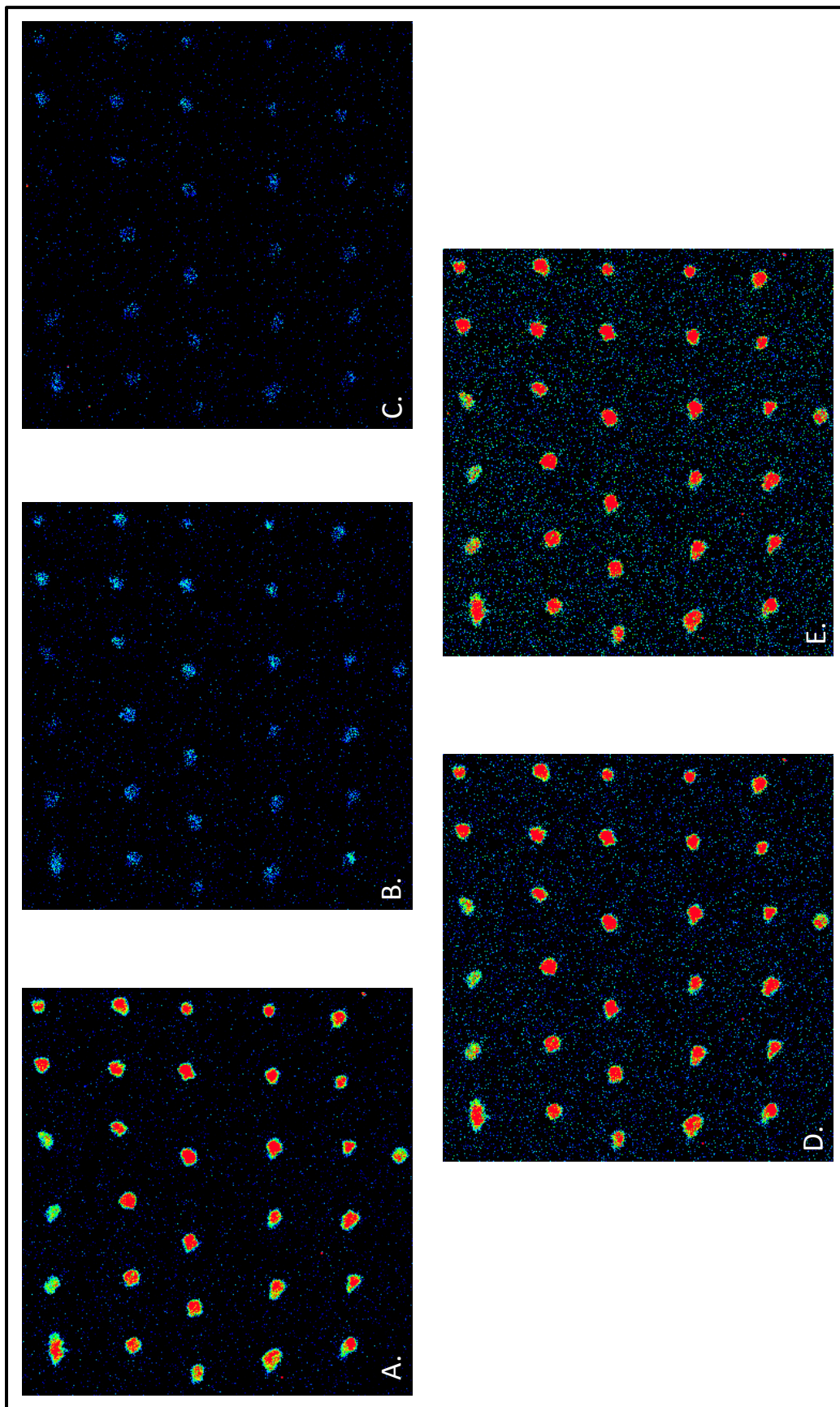
Figure 3.3. *psbAI::luxAB* cyanobacteria colonies with 5% IBA. A series of three successive 1-minute exposures (A, B, C). Compiled images, equivalent to a 2-minute exposure (D) and a 3-minute exposure (E).

CHAPTER IV

CONCLUSION

Summary of Experimental Results

This high-throughput luminescence reporter system allows for efficient screening and monitoring of isobutyraldehyde-producing mutants.

Future Prospects for Biofuel Production

Isobutyraldehyde ($(CH_3)_2CHCHO$) is a branched aldehyde consisting of just two methyl groups on the carbonyl carbon, but affinity for $FMNH_2$ increases with increasing chain length. Ideal binding affinity is with a linear aldehyde of chain length between eight and fourteen carbons. Expectations are that performance of this system will improve if long-chain aldehydes, alcohols, or alkanes are the desired end-product. With increased affinity, a greater percentage of the aldehyde will to bind to the $FMNH_2$, resulting in improved sensitivity of luminescence reporting.

Further, alcohols, alkanes, and aldehydes with longer chains provide more energy than their short-chain counterparts. The energy contained in the hydrocarbons of a long chain, a highly branched chain, or an aromatic ring is greater than that of short-chain functional groups (such as methyl and ethyl groups). High-energy content of such products is appealing for biofuel applications. We plan to test additional alkanes, alcohols, and aldehydes using the established system.

A Future Application: Kondotron to Screen for Fluorescence in Bacteria

Limitations of current fluorescence screening techniques.

Microplate readers are the primary screening technique for fluorescence in bacteria. Plate readers employ a liquid media, which is convenient in that most bacterial samples can be easily introduced into and grown in this type of medium. High density microplates (384- or 1536-well microplates) are typically used for screening applications.

Bacterial colonies, however, do not form in a liquid suspension, and a plate reader is unable to measure the fluorescence of bacterial colonies on circular agar plates. Agar is the most convenient way to grow bacteria because it does not require the laborious manual transfer of bacterial cultures into each well of a microplate. Plating bacteria onto agar plates is advantageous for screening purposes, because bacteria grow into single colonies, each a clone of a single bacterium.

Fluorescent probes are commonly used in applications today. Fluorescence-activated cell sorting has been used to isolate bacteria grown in liquid media that have undergone mutations, thereby permitting analysis of an entire bacterial population in real time (Abuaita & Withey, 2010). Fluorescence-based cytometric beads allow for the desired gene expression profile to be rapidly detected in liquid media, but this methodology is not high-throughput (Leach, Stroot, & Lim, 2010).

Measuring fluorescence in bacterial colonies.

Use of the "Kondotron" to measure fluorescence would allow bacteria to be grown on plates. By using short wavelength LEDs (emitting blue excitation light) and putting an interference filter (for green light emission) on the current camera setup, it

may be possible to establish a high-throughput screening method for bacterial fluorescence.

The application of fluorescence detection to the system would involve a few additional components. Fluorescent measurements would require an excitation system (the first optical system) to illuminate the colonies using a specific wavelength selected by an optical filter. An emission system (a second optical system) would be needed to collect the emitted fluorescent light. This light is then separated from the excitation light using a filter, and the fluorescent signal is measured with a light detector, such as a photomultiplier tube (PMT). This application would provide greater sensitivity, as well as greater application range, given the wide selection of fluorescent labels available today. Luminescence reporter systems, with their light emissions being the result of a chemical reaction instead of light excitation, are simpler optically than their fluorescence counterparts, as they only require a light detector and do not require a light source.

APPENDIX A


KONDOTRON SOFTWARE SPECIFICS

This appendix consists of five figures depicting the LabView block diagrams used
to code the newly developed program. It is meant to be an extension of the "Current
System" section of Chapter 2, and, as such, clear and direct parallels exist.
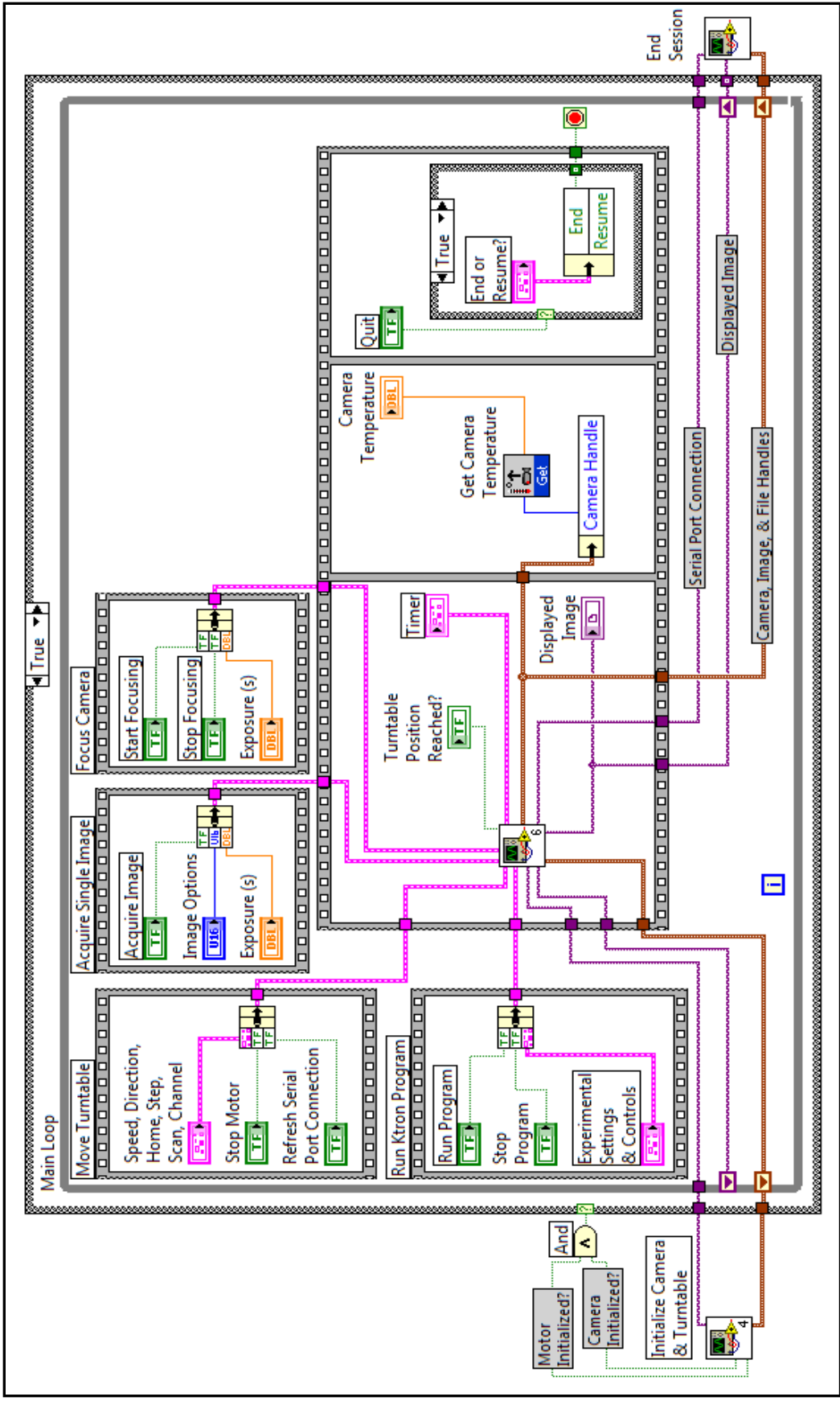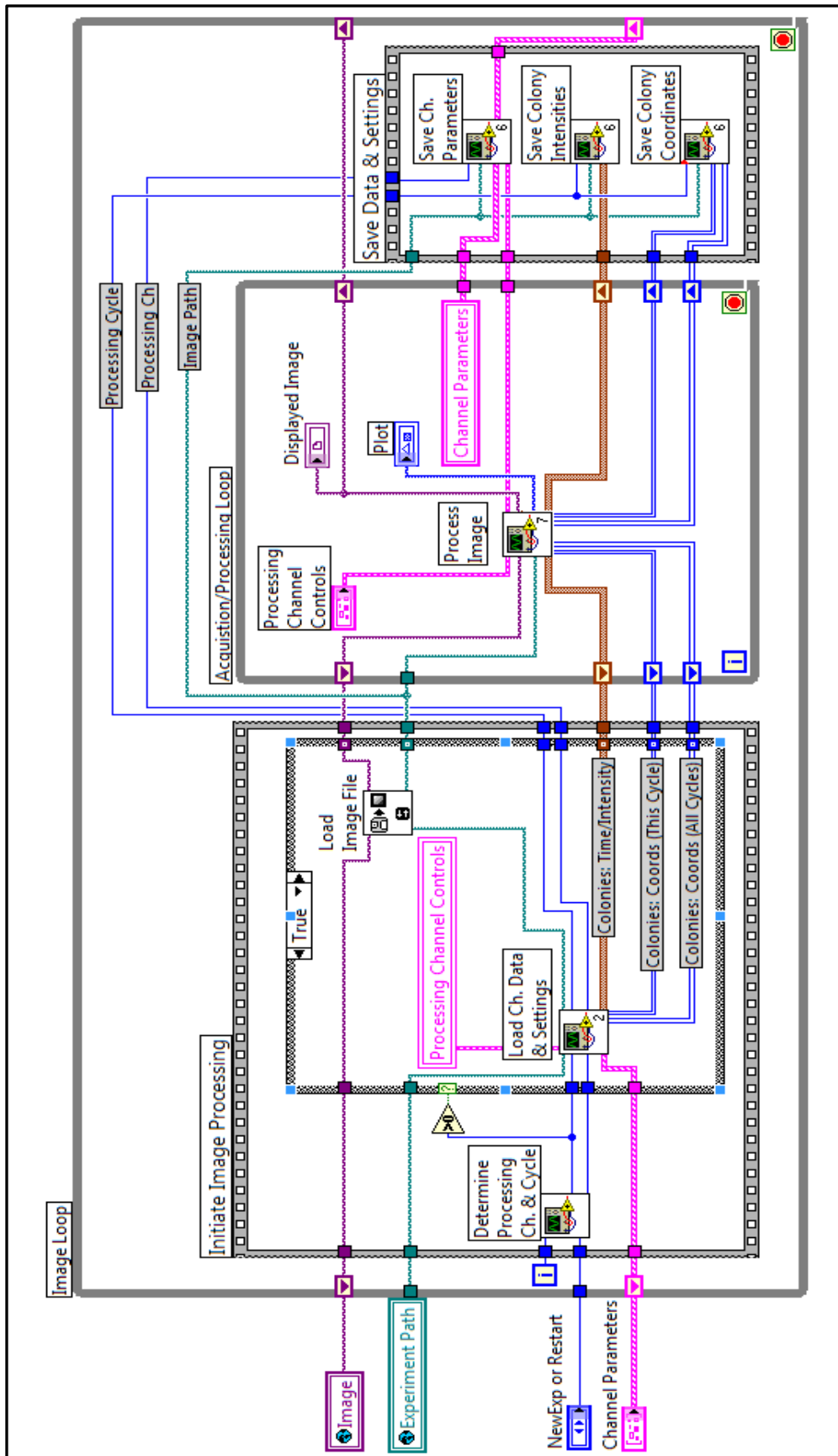
Figure A.1. Labview block diagram depicting software connections of new program. Once the camera and motor are successfully initialized, a while loop is entered. From there, any of four options can be selected, ended, and another selected. The camera temperature is checked constantly. The program can be exited at any time.
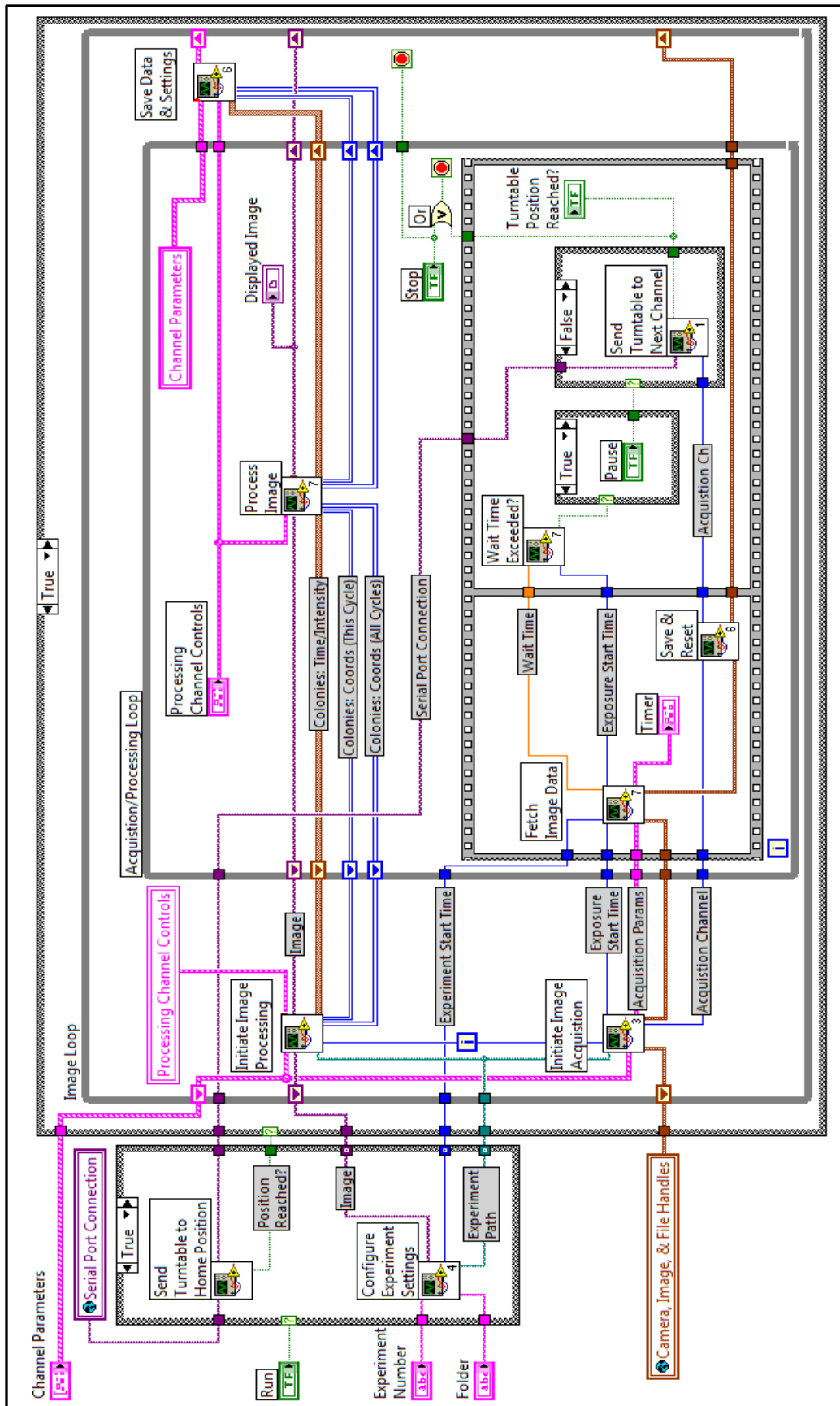
Figure A.2. Labview block diagram depicting automated Kondotron program. The turntable is sent to "Home" position and settings configured at the start. Initiation of acquisition and processing are conducted in the "Image Loop." Image acquisition and processing occur simultaneously within the "Acquisition/Processing Loop." When not paused and the wait time is exceeded, the table moves to the next channel.
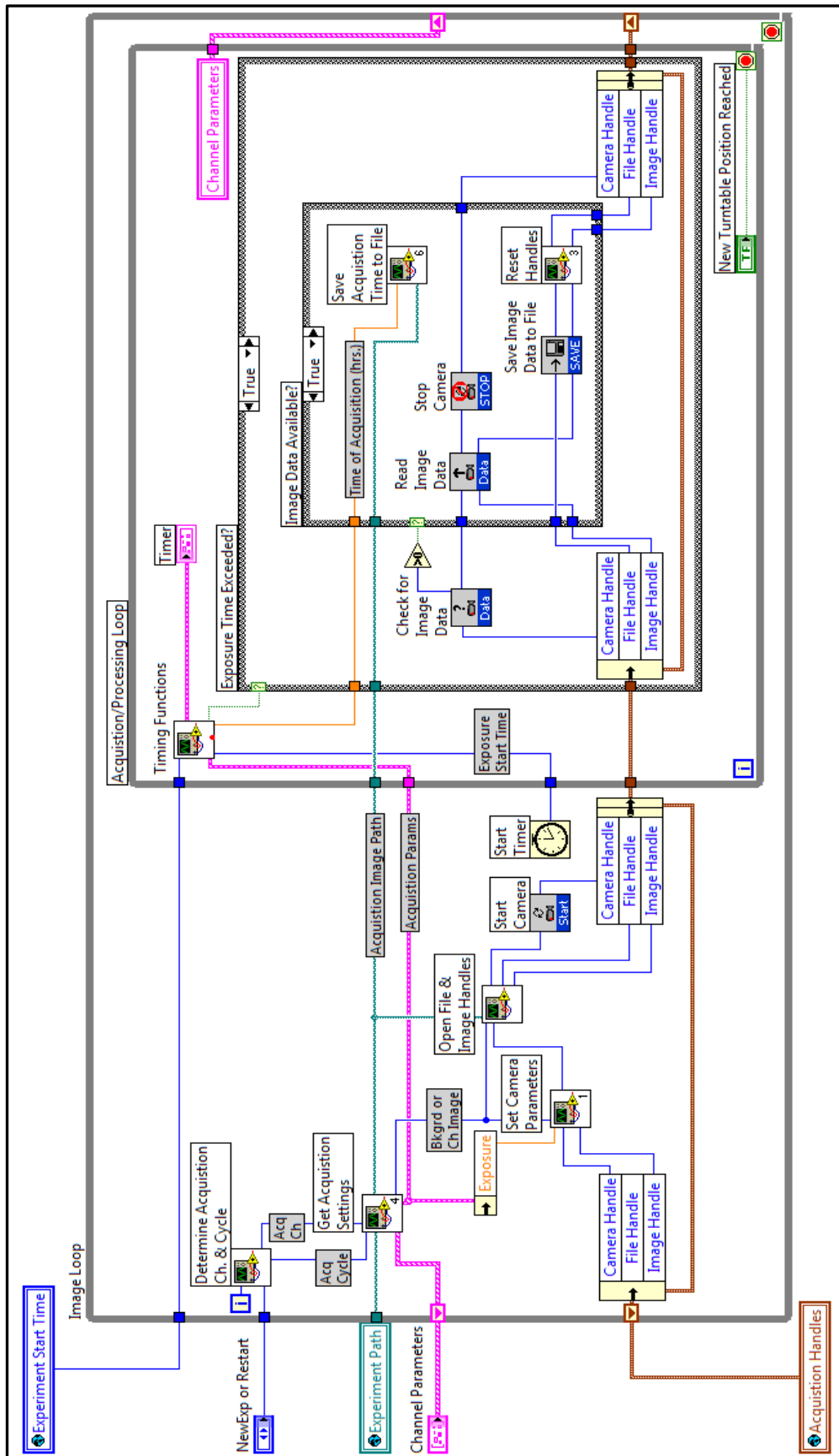
Figure A.3. Labview block diagram depicting image acquisition procedure. The initiation steps occur in the main "Image Loop," configuring the camera and starting the exposure. Within the "Acquisition/Processing Loop," the time is calculated and displayed. Once the exposure time has been exceeded and image data is available, the camera is stopped and the image is read and saved. When the new turntable position is reached, the "Acquisition/Processing Loop" ends, returning to the initialization steps to initialize for a new image.

Figure A.4. Labview block diagram depicting overview of image processing procedure. Processing is initiated within the "Image Loop," just as with acquisition initialization, whereby the most recently acquired image and any previous colony selections are loaded. If necessary, additional processing occurs according to parameters in the "Acquisition/Processing Loop." Finally, the processing parameters, colony locations, and colony intensities are saved.
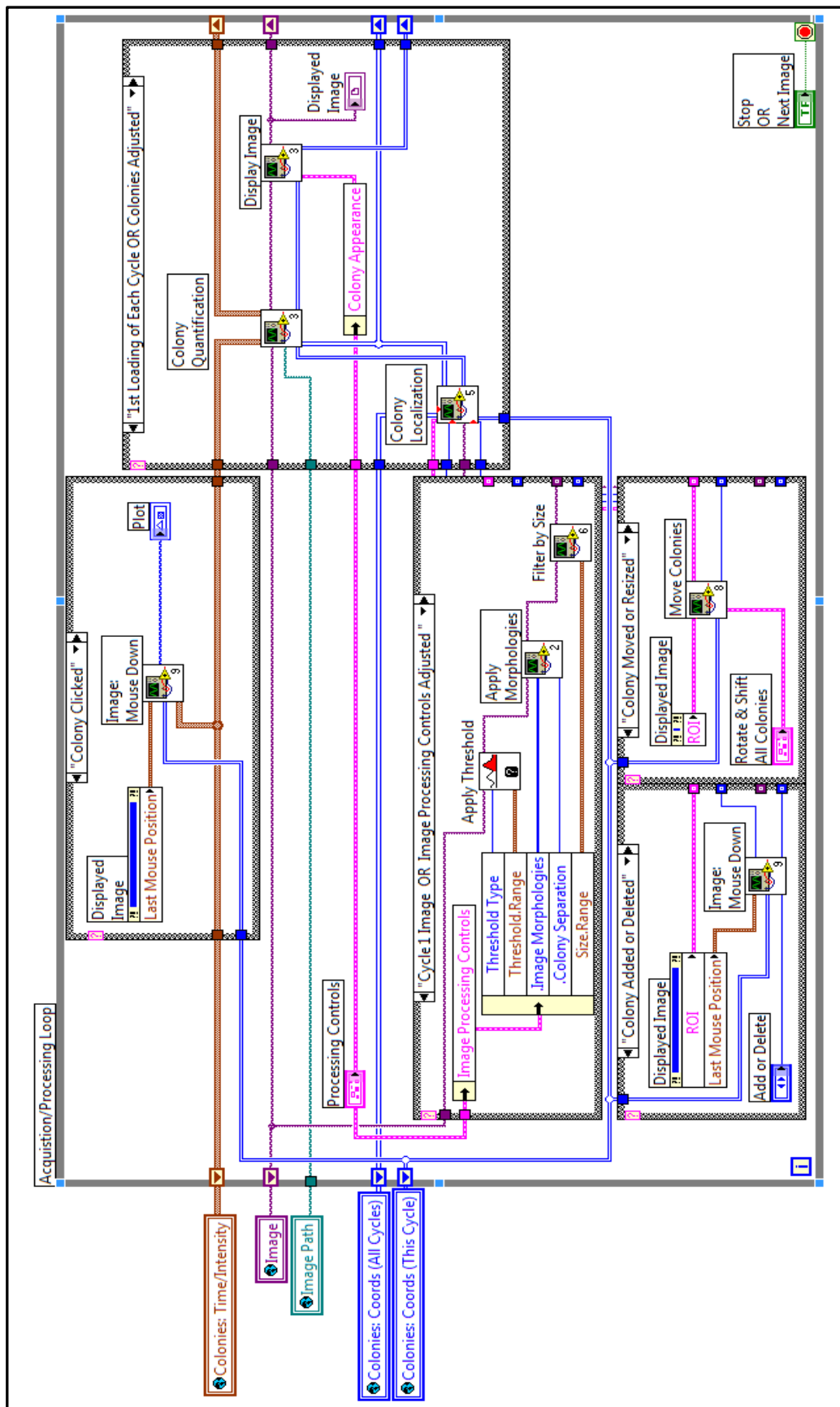
Figure A.5. Labview block diagram depicting details of image processing. Once processing has been initialized, a colony can be clicked and its intensity versus time plot displayed. If colony selection is not optimal, processing parameters can be adjusted via changes in thresholding, morphology transformation, or size filtration. Colonies can also be added, deleted, moved, or resized.

USER MANUAL FOR OPERATION OF PROGRAM

Introduction

Following is an instruction manual for both new and veteran users of the

Kondotron program.   This manual was written by Yao Xu with modifications by Carl

Johnson and Jill Mecklenborg. The Johnson group has developed a custom high-

throughput screening system with a turntable and CCD camera. This apparatus can

distinguish as many as 1,000 colonies per plate and has the capacity to automatically

monitor 12 plates. Therefore, ~12,000 colonies can be screened or monitored each time,

The Johnson lab has successfully applied this automated apparatus to isolate and

characterize a variety of clock mutants in cyanobacteria.

Operational Manual

Before Using JKtron

- Check waterbath's water level (If the level is low, please refill waterbath Miliipore water before AND during experiment running whenever the level is low)
- Check UPS if the battery is low or dead
- Clean turning table
- Keep the JKtron room clean
- Complete JKtron experiment worksheet (see the last page of manual)

Fig. 1. Main Menu

Main Menu of the JKtron Program

As shown in Fig. 1, there are four categories of function:

(1) Motor Control --- for table turning control
(2) JKtron Program --- for JKtron experiment setup
(3) Focus Camera --- for camera's focus and zooming
(4) Single Image --- for taking individual images

Note: On top of the tab menu shows total running time, exposure counter (seconds), current temperature of the camera, indicator for positioning (turns green when a position is reached), and QUIT (click it to stop running the program).

Basic Operations of the JKtron Program

1. Click "**Jktron program.vi**" on desktop to open the program.

2. Click "**Motor Control**" on the tab menu to move the table (Fig. 2):
   - (1) Choose desired direction (Counterclockwise *vs.* clockwise) and speed (Fast vs. Slow) of motor
   - (2) Select "Scan," Step," "Home," or "Channel (0 ~ 11)" to start the motor moving with the desired function.
   - (3) Click "Stop Motor" to stop the table from turning immediately.

     Note: Table must pass "Home" position before properly selecting a particular channel. The position indicator will light up once the table has reached the desired location and the motor has stopped. Click "Refresh" to refresh the motor's connection to the computer. *This should only be used when the movement function buttons do not result in motor movement.*

3. Upload plate samples into table's wells

4. Select "**JKtron Program**" tab on the tab menu

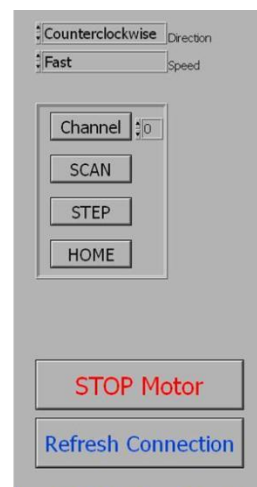5. Choose "**Experiment Info**" from the pull-down menu (Fig. 3).
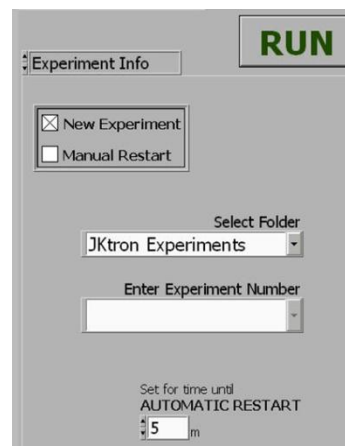
Fig. 2. Motor Control

Fig. 3. Experiment Info

Note: This pull-down menu has 3 options and is located on the left of the control panel below the "RUN" button (Fig. 4)
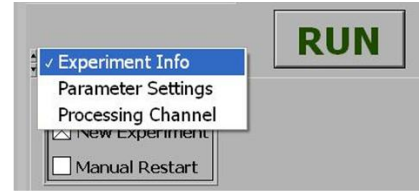

6. Mark "New Experiment" If a new JKtron experiment is being started

Fig. 4. JKtron Program Tool Bar

7. Choose the folder you would like to save your experiment in.

    Note: Usually, JKtron experiment data are saved to a folder named "JKtron Experiments."

8. Enter an experiment # (*e.g.* JK0001, JK0002, JK0003, …)

9. Input an "Automatic Restart" time (in minutes) in case **the program** (instead of power outage problem) is hung up due to an unexpected reason, *e.g.* 5 min, 10 min, 20 min, etc.

10. Choose "**Parameter Settings**" from the pull-down menu (Fig. 4)

11. Set parameters for each of the channels that are being tested (Fig. 5), including (see Advanced Operations for more details):
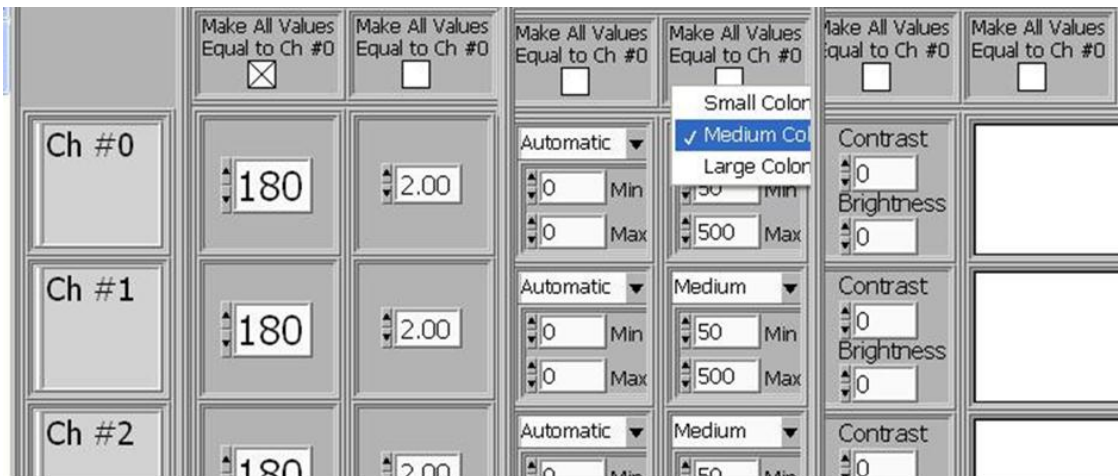
Fig. 5. Parameter Settings

1) "*Exposure*" time (in seconds, *e.g.* 180 for 3 min),
2) "*Start Time*" (in hours) for the starting time of the 1st data recording point in constant light (*e.g.* 3.5 hrs),
3) "*Threshold*" value --- There are three options from the pull-down menu:
    i)   "*Automatic*" for automatic selection
    ii) "*Manual –Dim"* for dimer colonies (min = 20; max = 20,000)
    iii) "*Manual –Bright"* for bighter colonies (min = 200; max = 20,000)
4) "*Size*" for colony size selections from the pull-down menu:

48

i) Small colonies
ii) Medium colonies
iii) Large colonies

5) "*Contrast/Brightness*" for colonies' image appearances (*Note*: this setting will not affect data's collection and values)

6) "Sample notes for each channel" for your own record.

Note: Changing the threshold type or colony size will automatically change the associated min and max values.

To make settings the same for all channels, tick the box located above each parameter to have all values the same as Ch#1. Un-tick this box to make adjustments to channel parameter values.

12. Push "**RUN**" button to start the JKtron running program.

Note: Motor will go the "Home" position. If motor was previously moving, it will stop and then go to the "Home" position. The position indicator will light up once the "Home" position has been reached.

Control panel will be disabled until background image and Ch#0 image are completed.

Once the motor has moved to Ch#1, the image for Ch#0 will appear, the control panel will become enabled, and "Processing Channel" will be selected from the pull-down menu throughout the Experiment.

Fig. 6. Start or stop JKtron

13. To end an experiment, push the "QUIT" button, located at the right side of the control panel above the tab menu (Fig. 6).

You will then be given the choice to "End Now" or "Resume." The program will continue to run until "End Now" is selected.

Advanced Operations of the JKtron

1. Switching control panel during experiment:
As shown in Fig. 4, "Parameter Settings" and "Experiment Info" can be viewed by changing the value of the pull-down menu. These values serve only as indicators and cannot be changed while the experiment is running. The exception is "Channel Notes" under "Parameter Settings."

The contents of the "**Processing Channel**" page will be selected and displayed each time a new image is shown (**Fig. 7**). Adjustments to the image and colonies are made here and can be made at any time throughout the experiment.

Fig. 7. Processing Channel

2. "PAUSE" function
The "PAUSE" button replaces the "RUN" button after the experiment has started. Push the "**PAUSE**" button to remain on a particular Image or channel for an extended period of time. For example, pause running until completion of a manual colony selection.
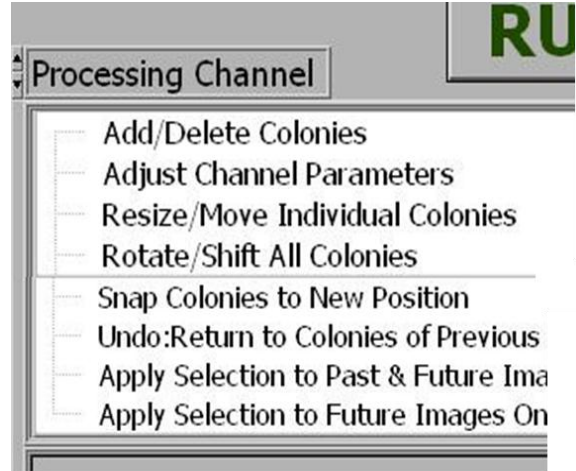
Should the "PAUSE" button accidentally be clicked or the user have clicked the button and left the room, the experiment will resume according to the pre-set parameters after 30 minutes.

3. Data Checking
- Click on a colony to display its intensity plot.
- Click on a new colony and its plot will replace the previous plot.
- Click anywhere else on the screen to get the plot to disappear.
- Right-click the plot to indicate whether you would like the average intensity of all selected colonies on that plate to be show.
- Hold down the control key and click colonies to view up to five different colonies on one plot.

4. Adjust appearance of image
Appearance of image can be manually adjusted using "**Brightness & Contrast**." (Fig. 8). Positive values will make the image brighter and show more contrast. Negative values will make the image darker and show less contrast. A more negative brightness value will make the background of the image appear more black.

These settings do not affect intensity values of the image. Settings will be saved and applied to future images of that channel.

5. Adjust appearance of colonies
Select display the colonies as **Filled** (black) or **Outlined** (white) option to display **colony number** on image.
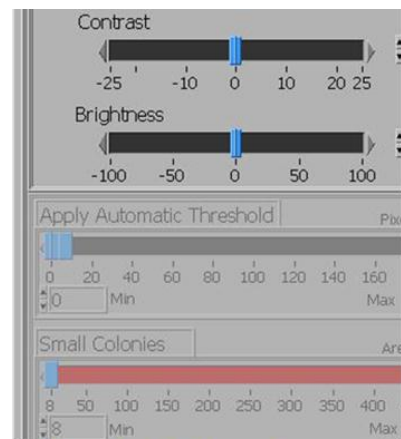
Fig. 8. Adjust image

6. Zooming and panning

Click the **magnifying glass** icon on the image tools menu to zoom in on the image (Fig. 9). Click on the image on the location at which you would like to zoom. Repeat to zoom in further.

Note: The image tools menu is at the bottom right of the image and consists of "Zoom to Fit" and icons of a magnifying glass, an arrow, a hand, and an oval.

To move to different locations on the image, use the **image scrollbars** or click the **hand icon** and drag.

Fig. 9. Zooming

Click "**Zoom to Fit**" to fit the entire image on the screen.

Adjust colonies selected by choosing different options on the colony selection menu. Colony selection can be changed at any time throughout the experiment.

Note: The colony selection menu is located on the "Processing Channel" page, directly under the pull-down menu. Adjust the scrollbar at the right of the menu to show more.

7. "Add/Delete Colonies"

This is the default setting and will be selected at the beginning of each image,with "**Delete Colony**" and "**Add Colony**" unticked and the oval tool selected (Fig. 10). This option allows for the manual addition and deletion of selected colonies.

1) **Deleting colonies**
When a colony is deleted, it is deleted from all previous images also. Intensity values of the average plot will change, although the change may be slight.

Fig. 10. Add/Delete

- Hover the mouse over or click the unwanted colony. Push **Delete** on the keyboard.

- Tick "**Delete Colony.**" Click the colony to be deleted. This method is best for quick deletion of colonies.

2) **Adding colonies**
When a colony is added, the new colony's coordinates are applied to all previous images to obtain intensity values for that colony. The colony plot and average plot will indicate as such. There are several ways to add colonies:

- First check that the oval icon on the image tools menu is selected. It is impossible to add colonies if this is not selected.

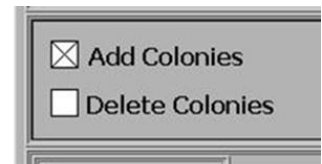- Draw an oval around the colony to be added, starting from the center of the colony and expanding outwards.

51

- Push **Insert** on the keyboard. Use the mouse to double click the newly drawn oval.

- Before drawing the oval, tick "**Add Colony**" on the control panel (Fig. 9). Then draw the oval as above. When the mouse is lifted, the colony will be added immediately.

   No adjustments can be made after the mouse is lifted, but the colony can be deleted according to the above directions. This method is best for quick adding of colonies and is recommended for use only after the user has developed proficiency in adding colonies.

8. "Adjust Channel Parameters"

Choose this option to adjust the parameters for automatic selection of colonies. The program automatically saves parameters for selection and applies new colony selections to all previous images of that channel before moving to the next image (Figs. 5 & 11).

1) "**Threshold**"

Adjust "**Threshold**" via pull-down menu and sliders. It is important to know that "Threshold" is the first parameter to be applied when processing the image. Here are some tips:

Fig. 11. Adjust Channel Parameters

- Check intensity levels of the image to get an idea of what values to use when applying a manual threshold.

- The resultant image after thresholding is binary, but that binary image will never be shown on the screen. Pixel values between the "Min" and "Max" values are 1 (selected pixels) while pixel values outside "Min" and "Max" are 0 (unselected pixels).

- The "Min" value indicates that every pixel in the image above that value will be selected and considered as part of a colony. Values below "Min" will not be considered part of a colony.

- The "Max" value indicates that every pixel below that value (and above "Min") will be selected and considered part of a colony, while every value above will not. This value should only be lowered in the event that the user notes some very high intensity levels that are not part of the colonies.
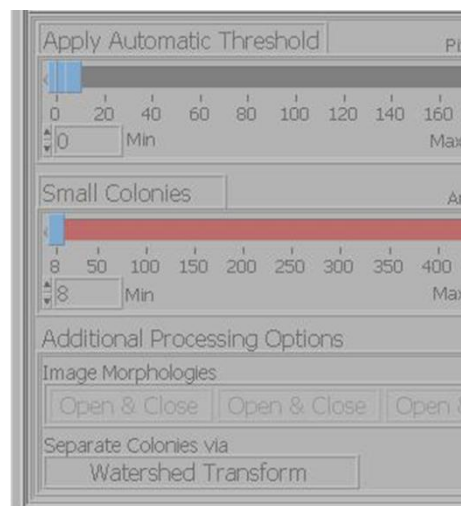
- "**Automatic Threshold**" accomodates for varying background intenisty within an image. No adjustments to "Min" and "Max" can be made.

- "**Manual Threshold - Dim**" provides the user with a truncated slide, allowing for greater accuracy in threshold value selection at lower levels

- "**Manual Threshold - Bright**" provides a scale with a greater range of values, allowing the user to choose higher threshold values with the slider.

2) "Colony Size"

Adjust the "**Colony Size**" filter via pull-down menu and sliders. "Colony Size" is the last parameter applied to the image.

- "Min" is the smallest value (in pixels) that a colony can be. Decreasing allows for smaller colonies, sometimes selecting noisy pixels as colonies. Increasing eliminates smaller colonies, but a value too high can eliminate small colonies.

- "Max" is the largest value that a colony can be. Decreasing eliminates any colonies larger than the value.

- "**Small Colonies**" applies a unique filter to the image suited for selection of small colonies. The scale is truncated to allow for accurate selection of smaller "Min" values.

- "**Medium Colonies**" provides a more expanded slide scale, ideal for selection of medium colonies.

- "**Large Colonies**" provides a very expanded scale, necessary only when selecting larger colonies.

3) More options for colony morphologies

Other operations are often useful in the selection of **small** colonies, but may be minimally useful in selection of medium or large colonies. These operations are applied to the binary image after thresholding but before filtering the colonies by size. The default is no additional operations.

Morphologies are applied first, with a maximum of three, applied in the order chosen. These options are described below:

- "**Dilate**" increases the size of the selected area by increasing the area around the boundary. This is in opposition to an **Erosion**, which decreases the size of a selected area by decreasing the area around the boundary.

  If two colonies have boundaries close in proximity, "Dilate" may result in indentification of these two colonies as one colony.

  Similarly, if one larger colony is identified as two smaller colonies, applying the dilate function may result in identification of just one colony.

  Applying an "Open," "Close," or "Open & Close" function to an image before applying a "Dilate" function can work to smooth boundaries and separate colonies further before dilating them.

  - ➢ It seems especially useful when application of an automatic threshold results in the selection of colonies that are slightly smaller than the actual colonies. Applying a dilation (or multiple dilations) in this case will likely increase the size of the colonies selected to produce the desired result.

  - ➢ If "Dilate" is applied to an image that has been thresholded manually, it is often necessary to increase the "Min" value of that threshold.

- "**Open**" performs an erosion followed by a dilation. Because the erosion is first, it functions to remove small groups of selected pixels that are rather isolated from other selected pixels. The dilation is only performed on the selected pixels that remain after the erosion.

- "**Close**" performs a dilation followed by an erosion. Because the dilation is first, it functions to fill small holes within a single colony or merge two separate colonies into one. It then applies an erosion to make the resultant cluster of pixels smaller by decreasing the area along its boundary.

- "**Open & Close**" uses dual combinations of "Open" and "Close." This function calls several combinations of erosions and dilations, thereby smoothing colony boundaries. Sometimes this function can result in colony boundaries that are too smoothed out and no longer properly fit the shape of the actual colony.

Colony Separation is applied after all morphologies.

- "**Separation via Watershed**" applies a filter based on the original image to the binary image.

  The original image is partitioned based on its topographical surface to create a filter image separated into non-overlapping segments. When the filter is applied to the binary image, selected pixels are divided into separate colonies according to the boundaries of the segments.

Although segments never overlap, the resultant selected colonies may
overlap slightly, because all colonies are converted to ovals.

- "**Separation via Erosion**" separates selected pixels of the binary image that touch
each other by narrow isthmuses.

9. "**Resize/Move Individual Colonies**"

This option allows the user to resize and/or move a particular colony. When the mouse
is moved over a colony's boundaries, a double-headed arrow will appear. Clicking
allows for resizing of the colony by dragging the mouse.

When the mouse is on the center of a colony, two double-headed arrows
in the shape of a cross. Clicking allows the user to drag the colony to a
new location. New colony selections are automatically saved to be applied to future
images.

10. "**Rotate/Shift All Colonies**"

When selected, a new control will appear in place of "Add Colony" and "Delete
Colony." Rotate all colonies a specified number of degrees. Increase values to rotate in
the CW direction and decrease values to move in the CCW direction.

Shift all colonies a specified number of pixels. Increase values to move colonies up and
right. Decrease values to move left and down. New colony selections are automatically
saved to be applied to future images. Once the colonies have been set to a specific
location, all values are reset to indicate the newly selected position as zero.

11. "**Snap Colonies to new Position**"

This option is to be used when a plate has been moved for any reason. Selecting this
option will result in the immediate movement of colonies to a new location.

This function moves all colonies together, keeping them at approximately the same
relative positions. Use "Resize/Move Individual Colonies" to further adjust the location
of selected colonies.

If this option is selected on an image with very low contrast, it is unlikely that the
program be able to find the new location of colonies.

Scroll the mouse over the image to compare the intensity level of the background to that
of the colonies. If these values are only a few pixels different, then this function may
not produce the desired result.

It is recommended to use the "Rotate/Shift All Colonies" and "Resize/Move Individual Colonies". functions to obtain the desired colony selection.

If selection of this function should produce an undesired result, simply choose "Undo: Return to Colonies of Previous Cycle."

New colony selections are automatically saved to be applied to future images.

Special Attention: Please be very careful when use "**Rotate/Shift All Colonies**" and **"Snap Colonies to new Position**".

12. "**Apply Colony Selection to Future Images**"

Selection will apply current colony coordinates to all future images, thereby keeping all past intensity values the same but using new colony coordinates to determine future intensity values.

This is the default for "Resize/Move," Rotate/Shift," and "Snap." It is not necessary to select this option after such colony adjustment. Selecting "Add/Delete Colonies" following such colony adjustment will result in the automatic application of this function.

Should the user want the new colony coordinates to be applied to previous images after one of these functions, it is necessary to select "Apply Colony Selection to Past & Future Images."

13. "**Apply Colony Selection to Past & Future Images**"

Selection will apply current colony coordinates to all past and future images, thereby updating the intensity values associated with previous cycles.
When performed at a later cycle, this function may take several seconds to complete.

This is the default option when "Adjust Parameters" is selected. It is not necessary to select this option after adjusting parameters. Selecting "Add/Delete Colonies" following parameter adjustment will result in the automatic application of this function.

14. "**Undo: Return to Colonies of Previous Cycle**"

Selection of this option will delete all adjustments made during the current cycle and return to the selected colonies of the previous cycle. Each colony's intensity values will be returned to the values previously determined, and the colony plots will indicate as such.

Focus Camera

Click "**Focus Camera**" tab

Set "**Exposure**" time (in seconds)

Adjust height of camera to focus

Click "**Focus**" to start succession of images. The images will be displayed continuously in grayscale in a separate window.
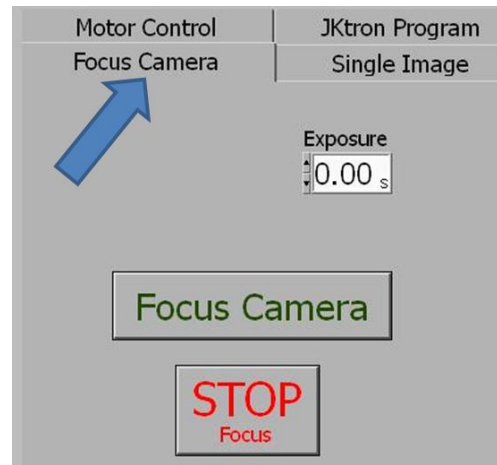
Click "**STOP**" to stop the camera.

Fig. 12. Focus Camera

Single Image

Click "**Single Image**" tab

Set "**Exposure**" time (in seconds)

Choose a shutter control option
Normal *vs.* Closed (for **background** images)

- Indicate what to save the image as by (1) entering a location and name in the space provided or (2) clicking on the file folder icon to the right of the space and selecting a name and location via the dialog box.
- If you would like to subtract a background image, (1) enter a location and name or (2) click on the associated file folder icon select via dialog box.
- Click "**Acquire**" to start the exposure.
- The time in the upper right corner will indicate the image exposure time.

Fig. 13. Single Image

- When the exposure is complete, the most recent image will appear in color on the large area to the right of the control panel.
- The appearance of this image can be adjusted using the "Brightness & Contrast" box.
- The image will also appear in a separate window (in grayscale). When you take multiple images, these also appear in separate windows, allowing for quick comparison of different exposure times.
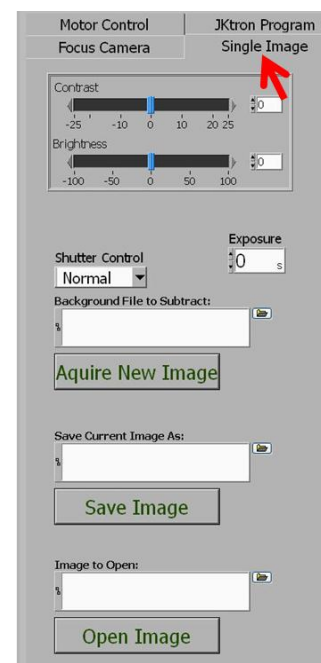
57

- The images in the separate windows will only be closed upon clicking the "**Close All Images**" button.

Troubleshooting

1. Hang-up problem

If an ongoing experiment is frozen or hung up due unexpected reasons, the JKtron needs to be restarted manually

- Select "Experiment Info" tab

- Mark "Manual Restart" box

- Choose the folder your current data has been saved

- Open the JKtron experiment # that needs to be resumed

- Enter "Automatic Restart" time in minutes (e.g. 10 min)

- Push "RUN" to start.
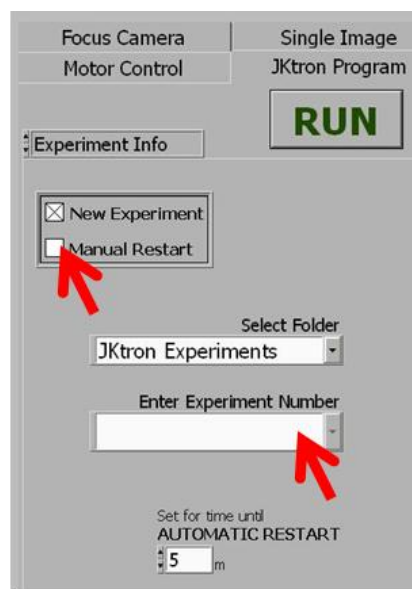
Fig. 14. Manual Restart

2. Table-turning problem

If the turning table cannot be controlled by the motor-controlling program, the communication should be reset.

- Click "**Motor Control**" on the tab menu

- Click "Refresh" to refresh the the motor's connection to the computer (Fig. 2).

  Note: This should only be used when the movement function buttons do not result in motor movement.

JKtron **Exp**. # _____   User: _____   Date: _____

Project:

_____

Protocol:

Temperature: _____; Light: _____ ;   Startime: _____

Plate #	Sample Description	Start in LL	Thresh old	Min	Max	Note
0						
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						

Keys for special Channels:

REFERENCES

(2010). *Energy Information Administration: International Energy Outlook 2010.* US
    Department of Energy.

Abuaita, B., & Withey, J. (2010, November 19). Genetic screening for bacterial mutants
    in liquid growth media by fluorescence-activated cell sorting. *J Microbiol
    Methods*.

Alper, H., & Stephanopoulos, G. (2009, October). Engineering for biofuels: exploiting
    innante microbial capacity or importing biosynthetic potential? *Nature Reviews,
    7*, 715-723.

Andrew J. Millar, I. A.-H. (1995, February 19). Circadian Clock Mutants in Arabidopsis
    Idenitfied by Luciferase. *Science, 267*, 1161-1163.

Angermayr, A. S., Hellingwerf, K. J., Lindblad, P., & Teixeira de Mattos, M. J. (2009).
    Energy biotechnology with cyanobacteria. *Current Opinion in Biotechnology, 20*,
    257-263.

Atsumi, S., Hanai, T., & Liao, J. C. (2008, January). Non-fermentative pathways for
    synthesis of branched-chain higher alcohols as biofuels. *Nature, 451*(3), 86-90.

Atsumi, S., Higashide, W., & Liao, J. C. (2009, December). Direct photosythetic
    recycling of carbon dioxide to isobutyraldehyde. *Nature Biotechnology, 27*(12),
    1177-1180.

Babcock, B. A. (2007, Spring). High Crop Prices, Ethanol Mandates, and the Public
    Good: Do They Coexist? *Iowa Ag Review, 13*(2).

Demain, A., Newcomb, M., & JH, W. (2005, Mar). Cellulase, clostridia, and ethanol.
    Microbiol Mol Biol Rev, 69(1), 124-154.

Demirbas, A. (2009). Political, economic and environmental impacts of biofuels: A
    review. *Applied Energy, 86*, S108-S117.

Dismukes, C. G., Carrieri, D., Bennette, N., Ananyev, G. M., & Posewitz, M. C. (2008).
    Acquatic phototrophs: efficient alternatives to land-based crops for biofuels.
    Current Opinion in Biotechnology, 19, 235-240.

Energy Information Administration. (2010). *International Energy Outlook 2010.* US
    Department of Energy.

Fargione, J., Hill, J., Tilman, D., Polasky, S., & Hawthorne, P. (2008, February). Land
    Clearing and the Biofuel Carbon Debt. *Science, 319*, 1235-1238.

Fernandes, S., & Murray, P. (2010, Nov). Metabolic engineering for improved microbial pentose fermentation. *Bioeng Bugs., 1*(6), 424-428.

Fischer, C. R., Klein-Marcushamer, D., & Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metabolic Engineering, 10*, 295-304.

Gordon, J., & Polle, J. (2007). Ultrahigh bioproductivity from algae. *Applied Microbiology Technology, 71*, 969-975.

Guedon, E., Desvaux, M., & Petitdemange, H. (2002). Improvement of cellulolytic properties of Clostridium cellulolyticum by metabolic engineering. *Appl. Environ. Microbiol., 68*, :53–58.

Hastings, J. (1983). Biological diversity, chemical mechanisms, and the evolutionary origins of bioluminescent systems. *Journal of Molecular Evolution , 19*(5), 309-321.

Higashide, W., Li, Y., Yang, Y., & Liao, J. C. (2011, April). Metabolic Engineering of Clostridium cellulolyticum for Production of Isobutanol from Cellulose. *Applied and Environmental Microbiology, 77*(8), 2727-2733.

Jeffries, T., & Jin, Y. (2004, Feb). Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biotechnol., 5*, 495-509.

Johnson, C., & Golden, S. (1999). Circadian programs in cyanobacteria: adaptiveness and mechanism. *Annu Rev of Microbiol , 53*, 389–409.

Klemm, D., Heublein, B., Fink, H.-P., & Bohn, A. (2005). Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *ChemInform, 36*(36).

Kondo, T., & Ishiura, M. (1994, Spril). Circadian Rhythms of Cyanobacteria: Monitoring the Biological. *Journal of Bacteriology, 176*(12), 1881-1885.

Kondo, T., Strayer, C. A., Kulkarnit, R. D., Taylor, W., Golden, S. S., Ishiura, M., et al. (1993, June). Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. *Plant Biology, 90*, 5672-5676.

Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Shinsuke, K., & Ishiura, M. (1994, November 18). Circadian Clock Mutants of Cyanobacteria. *Science, 266*, 1233-1236.

Leach, K., Stroot, J., & Lim, D. (2010, October 29). Same-Day Detection of E. coli O157:H7 Recovered from Spinach Using Electrochemiluminescent and Cytometric Bead Array Biosensors. *Appl Environ Microbiol.*

Lewis, N. S., & Nocera, D. G. (2006, October). Powering the planet: Chemical challenges in solar energy utilization. *PNAS, 103*(43), 15729-15735.

Lindberg, P., Park, S., & Melis, A. (2010). Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. *Metabolic Engineering, 12*, 70-79.

Liu, X., Brune, D., Vermaas, W., & Curtiss, R. I. (2010). Production and secretion of fatty acids in genetically engineered cyanobacteria. *PNAS*, 1-6.

Liu, Y., Golden, S., Kondo, T., Ishiura, M., & Johnson, C. (1995). Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. *J Bacteriol, 177*(8), 2080–2086.

McKinlay, J. B., & Harwood, C. S. (2010). Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology, 21*, 244-251.

Millar, A. J., Carre, I. A., Strayer, C. A., Chua, N.-H., & Kay, S. A. (1995, February 19). Circadian Clock Mutants in Arabidopsis Idenitfied by Luciferase. *Science, 267*, 1161-1163.

Minak-Bernero, V., Bare, R. E., Haith, C. E., & Grossman, M. J. (2004, July). Detection of Alkanes, Alcohols, and Aldehydes Using Bioluminescence. *Biotechnology and Bioengineering, 87*(2), 170-177.

Mori, T., & Johnson, C. H. (2001). Circadian programming in cyanobacteria. *Cell & Developmental Biology, 12*, 271-278.

Ouyang, Y., Andersson, C., Kondo, T., Golden, S., & Johnson, C. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proc Natl Acad Sci USA , 95*, 8660–8664.

Pimentel, D., & Patzek, T. W. (2005). Ethanol Production Using Corn, Switchgrass, and Wood; Biodiesel Production Using Soybean and Sunflower. In *Biofuels, Solar and Wind as Renewable Energy Systems* (pp. 373-394).

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., et al. (2006, January). The Path Forward for Biofuels and Biomaterials. *Science, 311*, 484-489.

Schirmer, A., Rude, M. A., Popova, E., & del Cardayre, S. B. (2010, July). Microbial Biosynthesis of Alkanes. *Science, 329*, 559-562.

Searchinger, T., Heimlick, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., et al. (2008, February). Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emission from Land-Use Change. *Science, 319*, 1238-1240.

Service, R. F. (2009, October). Another Biofuels Drawback: The Demand for Irrigation. *Science, 326*, 516-517.

Service, R. F. (2010, August). Is There a Road Ahead for Cellulosic Ethanol? *Science, 329*, 784-785.

Sheehan, J. (2009, December). Engineering direct conversion of CO2 to biofuel. *Nature Biotechnology, 27*(12), 1128-1129.

Somerville, C., Youngs, H., Taylor, C., Davis, S. C., & Long, S. P. (2010, August). Feedstocks for Lignocellulosic Biofuels. *Science, 329*, 790-792.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science, 315*, 801-804.

Stephens, E., Ross, I. L., King, Z., Mussgnug, J. H., Kruse, O., Posten, C., et al. (2010, February). An economic and technical evaluation of microalgal biofuels. *Nature Biotechnology, 28*(2), 126-128.

Taylor, W., Wilson, S., Presswood, R., & Hastings, J. (1982). Circadian Rhythm Data Collection with the Apple II Microcomputer. *Journal of Interdisciplinary Cycle Research, 13*, 71-79.

van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., et al. (2006, Nov). Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. *Antonie van Leeuwenhoek , 90*(4), 391-418.

Wijffels, R. H., & Barbosa, M. J. (2010, August). An Outlook on Microalgal Biofuels. *Science, 329*, 796-799.

Xu, Y., Mori, T., & Johnson, C. (2000). Circadian clock-protein expression in cyanobacteria: rhythms and phase-setting. *EMBOJ , 19*, 3349–3357.

Zhou, J., & Li, Y. (2010, March). Engineering cyanobacteria for fuels and chemicals production. *Protein Cell, 1*(3), 207-210.