A FAR-FIELD RADIO FREQUENCY EXPERIMENTAL EXPOSURE SYSTEM FOR YEAST: CREATING A NEW STANDARD

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

This paper presents a framework for investigating the potential effects of radiofrequency (RF) exposure on gene expression and cellular processes. Utilizing Schizosaccharomyces Pombe (*S. pombe*) as a model organism and specialized equipment to create a standard test environment for continued research in this field. Key methods include environmental monitoring, RF antenna configuration, and power density mapping. Results demonstrate the feasibility of the proposed experimental setup. Future directions involve variations in RF frequency and exposure time, as well as exploring underlying molecular mechanisms. This framework aims to lay the groundwork for future experimentation in this field. It emphasizes the need for rigorous scientific inquiry and highlights the potential implications for understanding biological responses to RF exposure. This work provides a roadmap for further investigation into RF effects on gene expression, facilitating advancements in scientific understanding without conducting an actual experiment.

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DEDICATION

This work is dedicated to the unwavering support and encouragement of those who have been instrumental in shaping my journey.

To my parents, John and Laurie Nevin, your confidence in me has been a guiding light. Your endless encouragement and sacrifices have laid the foundation for my success, and I am forever grateful for your love and support.

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This work stands as a testament to the love and support of my family and partner, whose faith in me has made all things possible. Thank you for believing in me even when I doubted myself.

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LIST OF ABBREVIATIONS

CO2 .....Carbon Dioxide.

DNA .....Deoxyribonucleic Acid.

IEEE .....Institute of Electrical and Electronics Engineers.

mRNA .....Messenger RNA.

O2.....Oxygen.

ppm .....Parts per million.

RF.....Radiofrequency.

RNA .....Ribonucleic Acid.

RX.....Receive.

SAR.....Specific Absorption Rate.

S. pombe.....*Schizosaccharomyces pombe*.

TF.....Transcription Factor.

tRNA .....Transfer RNA.

TX .....Transmit.

LIST OF SYMBOLS

ρ.....Density.

λ.....Wavelength.

Ae.....Effective aperture.

dm .....Incremental mass.

dV.....Volume element.

dW.....Incremental energy.

f .....Frequency in Hertz.

Gr .....Gain of the receiving antenna.

Gt.....Gain of the transmitting antenna.

Pr .....Power at the receiving antenna.

Pt .....Power at the transmitting antenna.

R.....The distance between the transmitting antenna and the yeast.

R1.....The distance between the transmitting antenna and the incubator shelf in the bottom position.

R2.....The distance between the transmitting antenna and the incubator shelf in the middle position.

R3.....The distance between the transmitting antenna and the incubator shelf in the top position.

S .....Power density.

t .....Time.

v.....Velocity.

1. INTRODUCTION

"Radiation is energy that moves from one place to another in a form that can be described as waves or particles" [10]. Radiation exists at a range of energies (frequencies) which can be referred to as the electromagnetic spectrum. There are two types of radiation: nonionizing and ionizing radiation. Ionizing radiation is at the "high" energy end of the spectrum and can detach electrons from atoms or molecules therefore causing changes at the atomic level. Exposure to ionizing radiation is proven to cause cell damage and in worst cases death. Nonionizing radiation is the "low" energy end of the spectrum and cannot cause electrons to detach from atoms or molecules. At certain energy levels, nonionizing radiation can induce molecular motion, such as rotation or vibration, which increases their temperature. With an eye toward this heating effect, the Institute of Electrical and Electronics Engineers (IEEE) has established an exposure limit below which nonionizing radiation is considered safe [15, 24]. The specific thresholds are discussed later. Research on whether nonionizing radiation might have effects upon the human body other than heating, such as altering gene expression, thus far has not reached a consensus. With the explosion of consumer technology devices containing radios, continuing the investigation is crucial. To determine if there truly is an effect of radiofrequency (RF) exposure on gene expression, a standardized test environment is one critical tool.

1.1. Background of Radiofrequency

RF is a range of frequencies: 3 kHz to 300 GHz. This range falls in the middle of the nonionizing radiation portion of the electromagnetic spectrum. People are exposed to RF from natural and nonnatural sources. Outer space, the sun, the sky, and the Earth are all natural sources of RF. These natural sources make up a very small portion of daily exposure. As technology has progressed, there has been a dramatic increase in RF sources over the past 20

years [4]. Human made sources of RF radiation include broadcasting radio and TV signals, transmitting signals from cordless telephones, cell phones and cell phone towers, satellite phones, and 2-way radios, radar, Wi-Fi, Bluetooth® devices, and several other sources [24]. These sources output at different frequencies across the range of radiofrequency. This rise in sources, and therefore the increase in exposure, increases the need for continued exploration into the long-term effects.

1.1.1. Field Characteristics

Four characteristics important to RF radiation are frequency, wavelength, specific absorption rate (SAR), and power density. Wavelength (λ) is related to the frequency (f) and velocity (v) by the expression:

$$\lambda = fv \tag{1}$$

SAR is a measure of the rate of RF energy absorbed by the body. Mathematically, SAR is described as the "time derivative of the incremental energy (dW) absorbed by (dissipated in) an incremental mass (dm) contained in a volume element (dV) of a given density (ρ).

$$SAR = \frac{d}{dt}\left(\frac{dW}{dm}\right) = \frac{d}{dt}\left(\frac{dW}{\rho dV}\right) \tag{2}$$

SAR is expressed in units of watts per kilogram (W/kg)" [15]. Power density is the "amount of power per unit area normal to the direction of propagation, usually expressed in watts per square meter (W/m^2) or milliwatts per square centimeter (mW/cm^2)" [15].

To calculate power density, use the following relationship between power received and effective aperture:

$$S = \frac{Pr}{Ae} \tag{3}$$

where S represents the power density, Ae is the effective aperture, and Pr is the power at the receiving antenna. The Friis equation can be used to calculate the expected power to at the receiving antenna (Pr):

$$\text{Pr} = Pt\frac{Gt*Gr*\lambda^2}{(4\pi R)^2} \quad (4)$$

where

Pt = power at transmitting antenna

R= distance between antennas

Gr = gain of receiving antenna

Gt = gain of transmitting antenna

To determine effective aperture, Ae:

$$Ae = \frac{\lambda^2}{4\pi}Gr \quad (5)$$

Equations 3, 4, and 5 are derived from [23].

SAR and power density are crucial factors to ensure safe levels. RF energy absorption into the body depends on the conductivity of the tissue which, in turn, depends on the composition of the tissue. In other words, different parts of the human body will have different SAR levels. According to the IEEE standard, the maximum power density is a function of frequency. For example, the maximum power density for 0.003 – 0.1 MHz range is between 100 and 1000000 mW/cm^2 and the maximum power density for 300 – 3000 MHz range is f/1500 mW/cm^2. Given an electromagnetic field of 2.45 GHz the latter relationship would be used to calculate a maximum power density of 1.6 mW/cm^2. However, like most rules, there are exceptions. Electromagnetic field strengths may be exceeded in cases of low SAR levels [15].

1.1.2. Field Creation

An RF field can be created using an antenna, “defined as the structure associated with the region of transition between a guided wave and a free-space wave, or vice versa” [13]. More simply, an antenna converts electrical power into radio waves or radio waves into electrical power. There are many types of antennas with a variety of properties.

1.2. Overview of Gene Expression

A gene is the fundamental unit of heredity. Gene expression is the process by which information encoded in a gene is turned into a function. Figure 1, adapted from [7], illustrates the intricate process in which genes go from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) to a protein. This process is the central dogma of molecular biology and can be broken down into two parts: transcription and translation. Transcription is the process from DNA to RNA. Translation is the process from RNA to protein. Changes to seemingly minor parts of these processes could have significant impact on the resulting genes that are expressed.

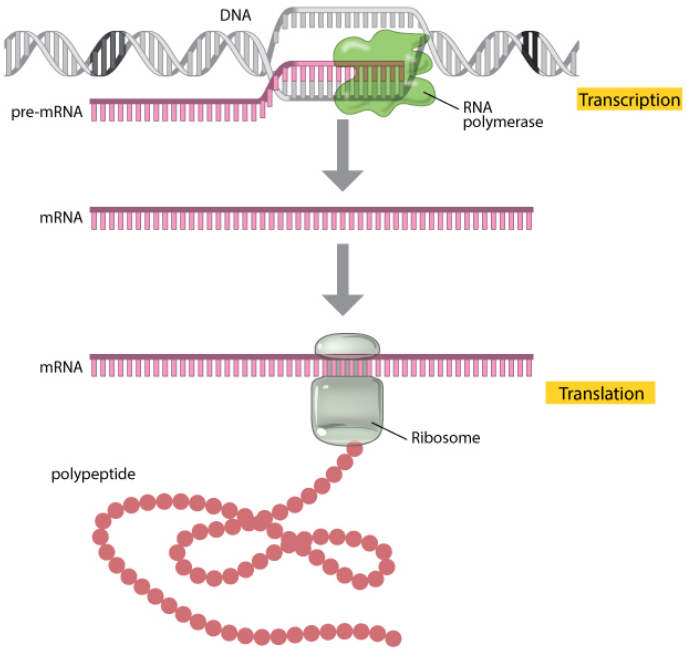


Figure 1. Illustration of transcription and translation.

1.2.1. DNA and RNA Explained

DNA has a double helix structure composed of nitrogen bases, phosphate, and sugar, (also known as a nucleotide) held together by covalent (phosphodiester) bonds and hydrogen bonds. In addition, DNA is antiparallel, meaning the two strands that make up the helix are oriented in opposite directions. As seen in Figure 2 [3], if one strand is in the 5' to 3' orientation in the helix the other strand would have to be in the 3' to 5' orientation.

Figure 2. Illustration of Antiparallel DNA.

The hydrogen bonds occur between the nitrogen bases and are known as base pairing. DNA coils around histones which are proteins to form chromosomes. When DNA is tightly coiled around the histones, DNA is not easily accessible for transcription to occur which also described as heterochromatin. When DNA is loosely coiled around the histones, DNA is accessible, and transcription can occur easily which is also described as euchromatin (see Figure 3 [9]). RNA has many similarities with DNA. The main differences are RNA is single stranded and has one different nitrogen base.

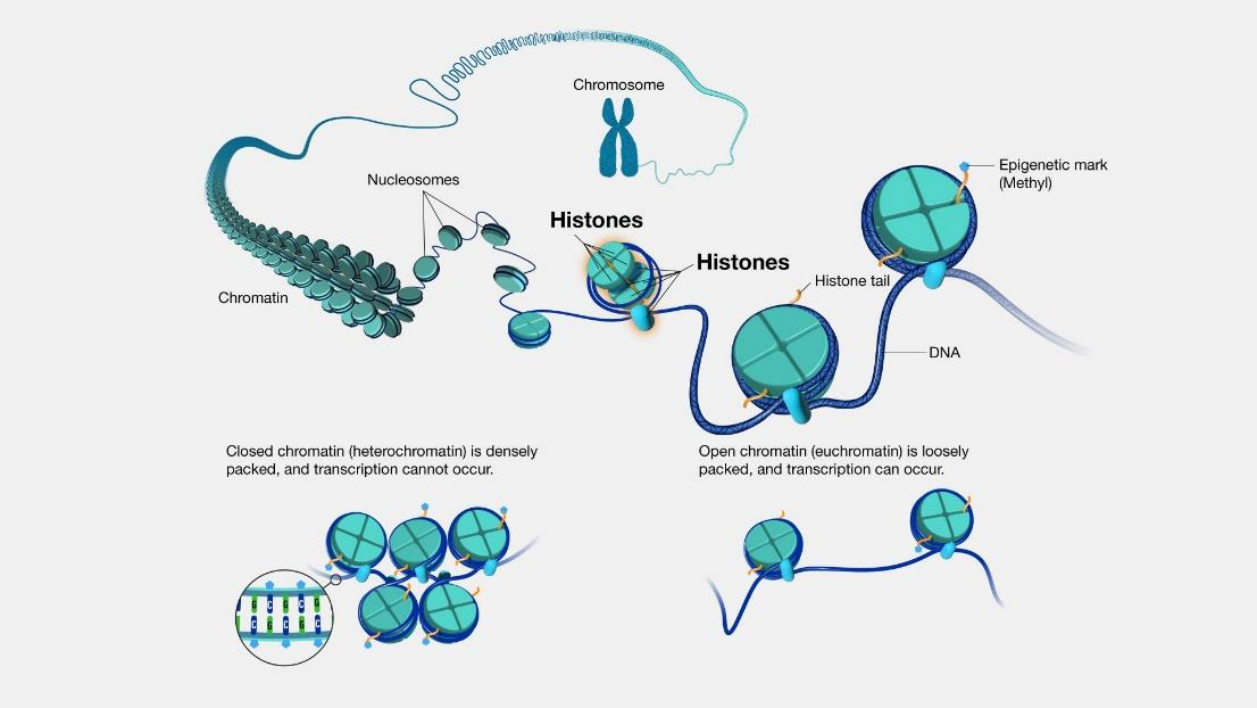


Figure 3. Illustration of Chromosomes and Chromatin Structure.

1.2.2. Transcription

Transcription has three steps: initiation, elongation, and termination. Initiation begins when RNA polymerase binds to the promoter region of DNA near the beginning of a gene. Then, the RNA polymerase separates the strand of DNA into the template strand and the coding strand (see Figure 4a [17]). Next, elongation is when the RNA polymerase builds the complementary RNA transcript 5’ to 3’ as it moves along the template strand 3’ to 5’ (see Figure 4b [17]). Lastly, once RNA polymerase reaches the terminating sequence, the RNA transcript is released (see Figure 4c [17]). RNA transcript is as known as pre-messenger RNA that must be processed into messenger RNA (mRNA).

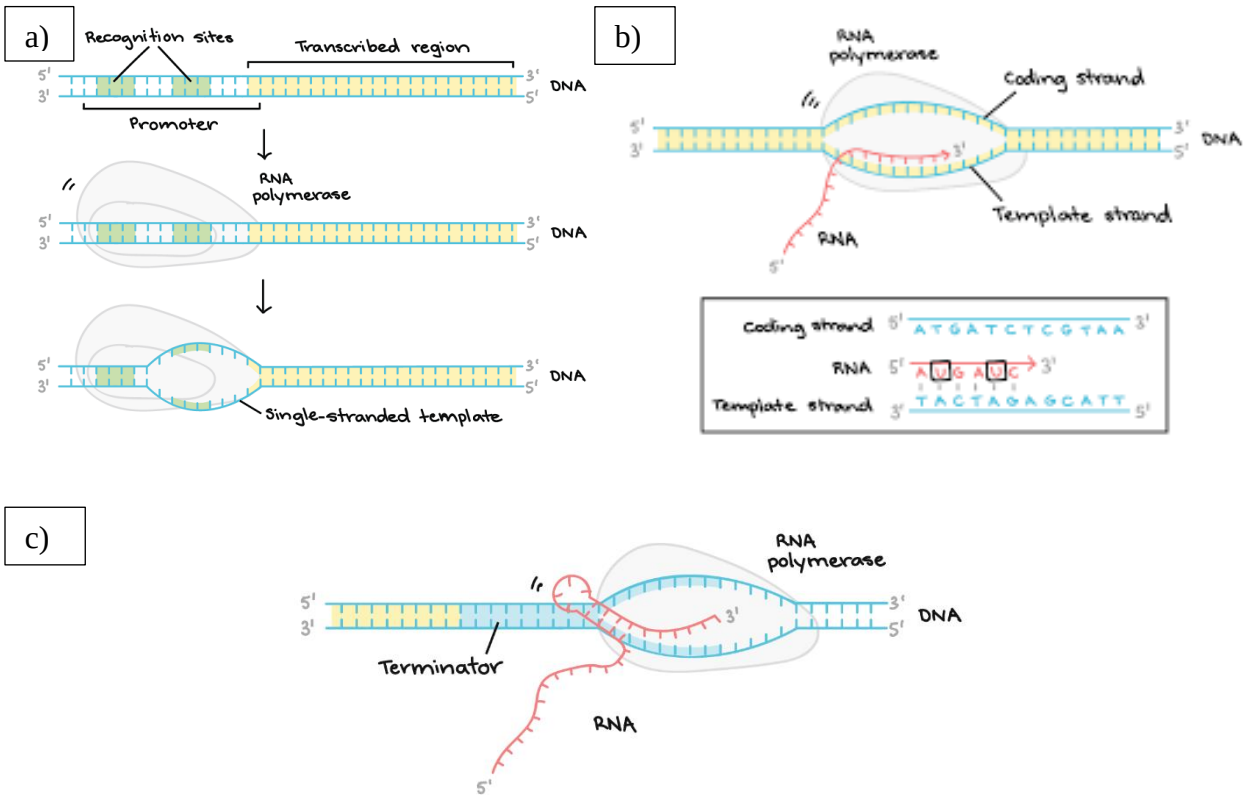


Figure 4. (a) Transcription Initiation. (b) Transcription Elongation. (c) Transcription Termination.

Transcription factors (TFs) are important to this process and include two types: general and regulatory. General TFs bind to the core promotor and regulatory TFs bind to the proximal control elements which are segments of DNA before the promoter region (see Figure 5 [19]). Every cell has the same general TFs, but different cell types have different regulatory TFs. Also known as activators or repressors, regulatory TFs can inhibit or stimulate what genes are expressed. RNA polymerase can only bind to the promoter when general TFs are present. Activators help general TFs and/or RNA polymerase bind to the promoter whereas the repressors inhibit binding. TFs are activated by signal transduction pathways or cell surface receptors.

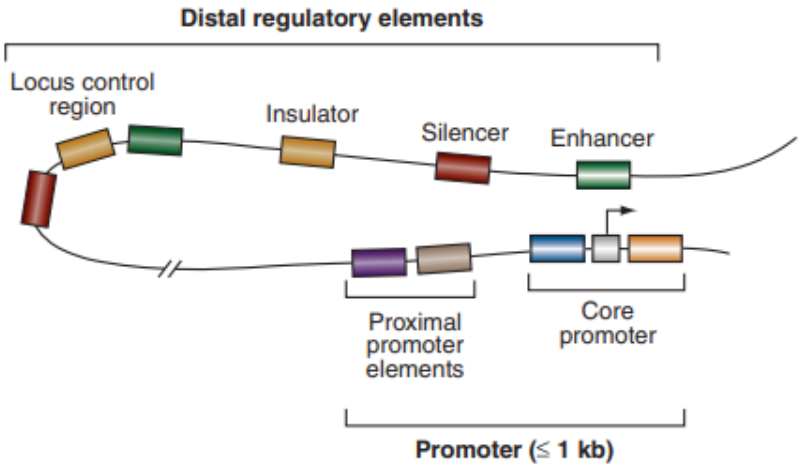


Figure 5. Binding Sites for Transcription Factors.

1.2.3. RNA Processing

Before translation, pre-messenger RNA is processed into mRNA by adding a cap to the 5' end of the strand and then a poly-A tail is added to the 3' end. Next up is splicing. The RNA strand is made up of two types of smaller strands: introns and exons. Introns are sections that do not carry information to build a protein and exons are sections that do. This step is where the introns are removed or "spliced" out, and the exons are "glued" back together. Having this additional step in gene expression allows for different mRNA strands to be made from the same gene, also known as alternative splicing.

1.2.4. Translation

Translation is the process of translating that processed sequence of mRNA into a sequence of amino acids, creating a polypeptide which becomes a protein, depicted in Figure 6 [8]. Three nucleotides in the mRNA make up a codon and there are 64 possible combinations. However, only 61 codons "code" for a particular amino acid. The other three codons are used as stop signals to let the cell know the protein is complete. Even though there are 61 "coding" codons, there are only twenty amino acids. This means there may be several codons that code for the same amino acid except for the start codon. The start codon only codes the amino acid

methionine. In translation these codons are read from 5’ to 3’ by transfer RNAs (tRNAs) which have the anticodon. Anticodons are complementary to the mRNA codon and bind to the mRNA via base pairing. tRNA also carries the specified amino acid. Translation also has three steps: initiation, elongation, and termination. First, initiation is the formation of the initiation complex. To form this complex, the tRNA with the start codon and therefore methionine must attach to the small ribosomal unit. Then, together they bind to the 5’ cap and begin moving in the 3’ direction until they reach the start codon. Finally, the large ribosomal unit binds to complete the initiation complex. Elongation is the process in which the polypeptide chain grows into a protein. The mRNA shifts through the ribosome one codon at a time allowing a new tRNA to come bind to the codon and its attached amino acid to form a bond to the previous one creating a polypeptide. Termination begins when the stop codon is read. Proteins known as release factors disrupt the enzyme that creates the bonds between each amino acid and adds a water molecule. This causes the polypeptide chain to be released from the tRNA and become a protein.

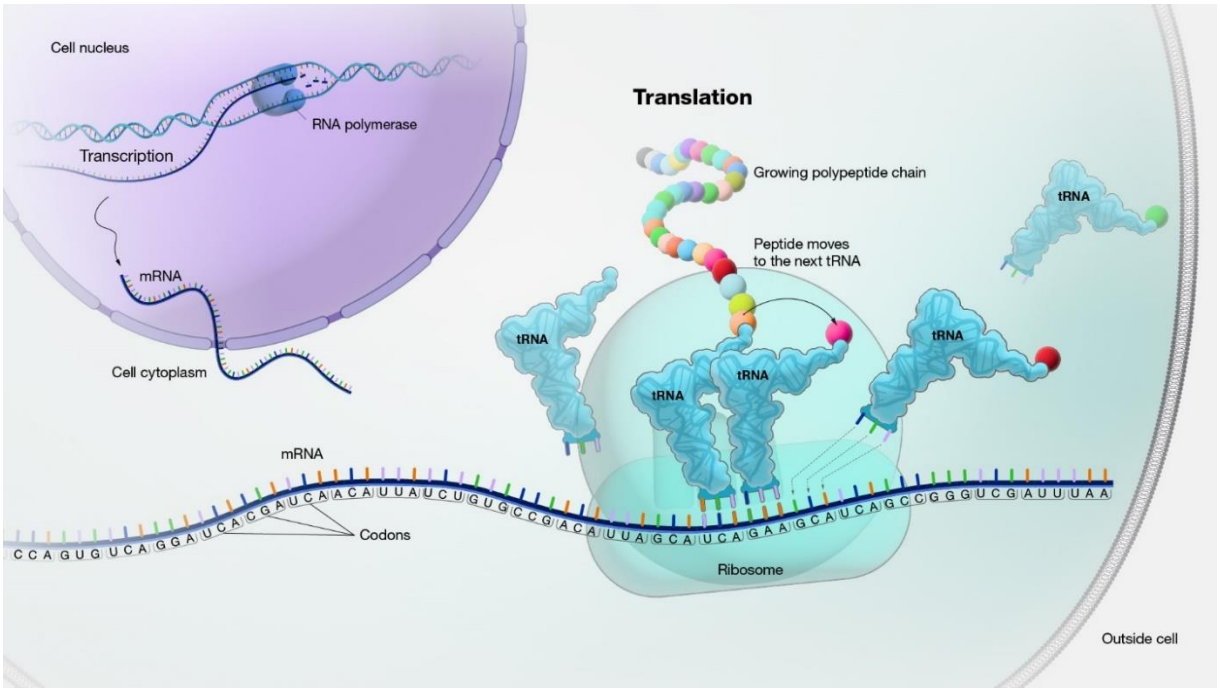


Figure 6. Translation.

1.2.5. Epigenetics

Epigenetics is also important to consider when looking at how genes are expressed. Epigenetics is the study of how cells control gene activity without changing the sequence of DNA. In other words, it is the investigation into any mechanism that can control what genes are activated or repressed without changing the DNA sequence. Epigenetic mechanisms include methylation of DNA and histone modification. These mechanisms cause changes in the chromatin structure. Factors that affect these mechanisms include "physiological and pathological conditions and by the environment" [1] like diet and exercise.

DNA methylation is when a methyl group is added to DNA by bonding to the nitrogen base known as cytosine (see Figure 7 [22]) in humans. When DNA methylation occurs in the promoter region the gene is suppressed through two different mechanisms. First, there is the assembly of methyl-binding domain-based protein complexes which prevent the binding of transcription factors. Second, methyl-binding domain proteins recruit histone deacetylase which leads to another mechanism: histone modification [18]. Most DNA methylation is essential for normal development, but when dysregulated can contribute to diseases like cancer [16].

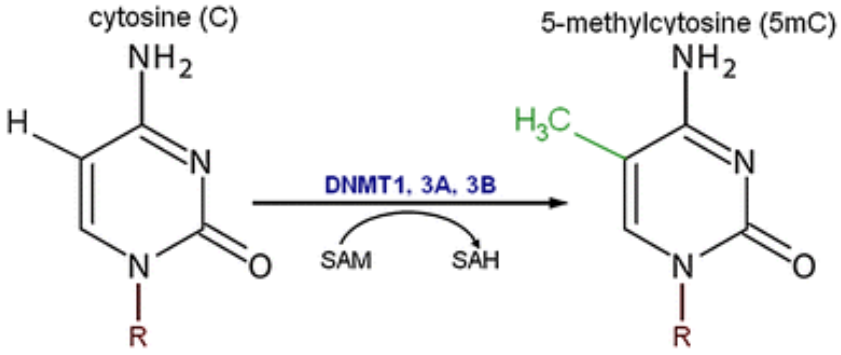


Figure 7. DNA Methylation.

"The most significant histone modifications include acetylation and methylation" [18]. As previously mentioned, histones are the proteins DNA wrap around to create chromosomes.

Histones can go through acetylation or deacetylation which is when an acetyl functional group is added or removed, respectively, to a lysine (amino acid) residue on a histone. High acetylation contributes to less dense chromatin region, euchromatin, therefore increased the accessibility to DNA for transcription to occur while low acetylation has the opposite effect. Histone methylation is when a methyl group is added to the lysine or arginine residue. The effects of histone methylation are dependent on the location of the amino acid residue and number of methyl groups.

1.2.6. Complexity of Gene Expression and Signal Pathways

As noted previously, TFs are activated by signaling pathways. There are other molecules involved in the process of gene expression that are also related to these pathways which means this mechanism must be considered. A signaling pathway is described as "a series of chemical reactions in which a group of molecules in a cell work together to control a cell function, such as cell division or cell death" [21]. An imbalance in these molecules, such as oxidative stress, can cause DNA damage. Oxidative stress occurs when there are more free radicals than antioxidants in a cell. A free radical is an unstable molecule, meaning it exists with an unpaired electron. An antioxidant is "a substance that protects cells from the damage caused by free radicals" [20] by giving them an electron (see Figure 8 [26]). Oxidative stress can cause lipid peroxidation which "leads to the formation of oxylipins which is shown to modulate signaling associated genes" [6]. Lipid peroxidation is when free radicals "steal" electrons from lipids. In other words, DNA can be damaged due to disruptions to the signaling pathways that play a role in how genes are expressed.

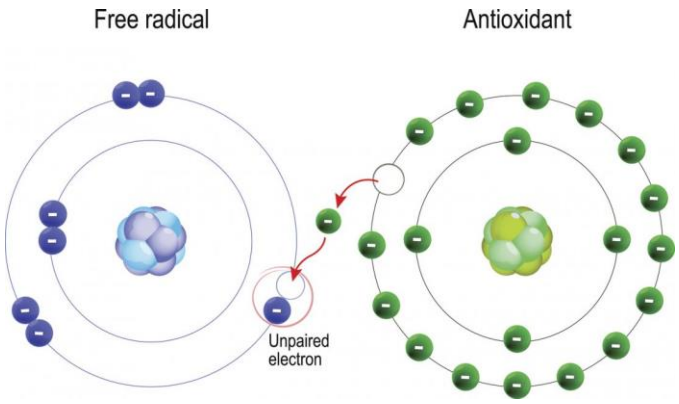


Figure 8. Free Radical and Antioxidant.

2. REVIEW

The lack of consensus in the scientific community on how or if the exposure to RF can cause biological changes is due to the multitude of methods used and therefore variation in procedure leading to a lack of reproducibility [2, 11, 12, 28]. In addition to the various testing methods used, the complexity of how a cell expresses its genes leads to many hypotheses of what RF does to the process. The methods and results of several studies will be discussed here.

As seen in Table 1, in three of the four studies, the researchers completed *in vivo* studies and the fourth was *in vitro*. *In vivo* means the experiment is carried out using the whole living organism and *in vitro* refers to experiments that occur outside a living organism. Two of the *in vivo* studies were with mice and the third was with rabbits. In one study the mice were unrestrained meaning they were free to move around [12] and in the other the mice's movement was restricted by a small plexiglass cage during the exposure [2]. The rabbit study was also unrestrained, but they were exposed individually [11]. The *in vitro* study used the cell culture of mice [28].

Table 1. Summary of Study Characteristics.

Study	Reference	Animal	*In vivo/In vitro*	Environment
1	12	Mice	*In vivo*	Unrestrained group
2	28	Mice	*In vitro*	N/A
3	11	Rabbit	*In vivo*	Unrestrained individual
4	2	Mice	*In vivo*	Restricted

The exposure and field characteristics of each study were also very different and can be seen in Table 2. The first study shows an average whole-body SAR level of 0.3422±.00034 W/kg which exceeds the IEEE standard of 0.08 W/kg, however, this study shows the power density is below 1.6 mW/cm^2. The second study also exceeds the IEEE standard with an average whole-body SAR of 2 W/kg; however, it is the safety limit for mobile emission radiation by the

Internation Commission on Non-Ionizing Radiation Protection [28]. The third study states that "the evaluated data was 14 ± 0.5 V/m" which is field strength, and this measurement is simple to convert to its corresponding power density [11]. The relationship is as follows:

$$power\ density = \frac{(field\ strength)^2}{impedance} \quad (8)$$

The conversion is based on free space conditions where the impedance is 377 Ohms.

$$power\ density = \frac{(\frac{V}{m})^2}{\text{impedance}} = \frac{(14)^2}{377 Ohms} = 0.05\ \text{m}W/cm^2$$

The corresponding power density would be 0.05 mW/cm^2 which does fall below the maximum power density deemed safe by the IEEE standard. The fourth study provides spatial peak SAR values averaged over 10 grams of tissue instead of average whole-body SAR. These values are below the IEEE standard of 4 W/kg [2, 15].

The test setup in each study has similarities, but they are vastly different. As mentioned in [12] a standardized test environment would be beneficial and hopefully result in easily reproducible and conclusive results. A standardized setup would also allow researchers to choose which variable they would like to investigate and see how the results change.

Table 2. Summary of Exposure/Field Characteristics.

Study	Exposure			Field	
	Continuous/ Intermittent	Daily Duration (minutes)	Time Period	Frequency (MHz)	Average whole-body SAR (W/kg)
1	Continuous	N/A	31 days	2450	0.3422±.00034
2	Intermittent	N/A	24 hours	1800	2
3	Intermittent	15	7 days	1800	None given
4	Continuous	120	6 months	900, 1800, 2100	0.0543, 0.0246, 0.0235*

*Spatial peak SAR was provided.

In addition to the setup of each experiment, the analyses also have their differences. Both studies [12] and [28] use methods to analyze genes and quantify the expression levels before and

after exposure. In [12], the researchers used RNA-seq and in [28] they used real time quantitative polymerase chain reaction. Both studies [11] and [2] measure MDA and 8-OHdG before and after exposure to determine if DNA damage has occurred. Study [2] also does a single cell gel electrophoresis, measures nitric oxide, total antioxidant status, total oxidant status, and calculates the oxidative stress index. They used a single cell gel electrophoresis because it is "a fast, sensitive, and relatively simple method used to detect DNA damage at the single cell level" [2]. Nitric oxide levels were also investigated because nitric oxide "acts as a mediator in central nervous system neurons and plays a role in the realization of many brain functions, is one of the most important reactive nitrogen derivatives formed in biological systems" [2]. In addition, the total antioxidant status and total oxidant status are measured to calculate the oxidative stress index which is a "sign of degree of oxidative stress" [2]. Each analysis method is different; however, they all relate to gene expression.

2.1. Potential Mechanisms Affected by Radiofrequency

Currently, many researchers believe that nonionizing radiation such as RF does not have the ability to affect biological mechanisms. However, in all four studies the researchers present results that show otherwise [2, 11, 12, 28]. Each study showed significant differences in their control versus exposed groups.

Seen in Table 3, both [12] and [28] were able to show a change in the quantity of some genes. In [12], *Hansen et al* found that most genes were not affected by the RF exposure, but the genes that were affected showed two types of responses: digital and analog. Digital in the sense that a gene was "switched on" or "switched off" and analog meaning that genes were significantly up or down regulated [12]. In [28], *Zhao et al* noted comparable results to that in [12]. Researchers found 34 genes of the 1200 investigated were affected by the exposure to RF,

24 up-regulated and 10 down-regulated. The methods in these studies show a change, but give no indication to the exact mechanism by which gene expression is affected by RF.

In both [11] and [2] the researchers were able to quantify DNA damage. In [11], *Guler et al* found that MDA and 8-OHdG levels had increased significantly in the pregnant and non-pregnant rabbits, but not the newborns. They conclude that the exposure "resulted in releasing secondary messengers, such as free radicals, leading to oxidative destruction in lipids and DNA molecules" [11]. However, they postulate the newborns were not affected due to the depth of penetration. In [2], *Alkis et al* found higher DNA damage and oxidative stress indicators in the exposure groups and concluded that "900-, 1800-, and 2100-MHz RFR emitted from mobile phones may cause oxidative damage, induce increase in lipid peroxidation, and increase oxidative DNA damage formation in the frontal lobe of the rat brain tissues" and "2100-MHz RFR may cause formation of DNA single-strand breaks" [2]. These two studies do not directly show that RF affects gene expression because they did not measure gene quantities, but DNA is integral to the process of gene expression. In addition, they provide insight into the exact mechanism affected by RF that could lead to a changed level of gene expression. Studies [19] and [2] suggest that RF could affect the number of free radicals.

Table 3. Summary of Previous Study Results.

Study	Results
1	Digital or analog in certain genes
2	24 up-regulated and 10 down-regulated, 1,166 unaffected
3	DNA damage detected
4	DNA damage detected

In a separate paper [5], the authors propose four mechanisms to be further investigated on these topics:

1. Transcription factor binding kinetics are altered as the protein energy is more or less favored.
2. Enzyme kinetics are altered as the protein energy is more or less favored.
3. DNA regulatory sites become more or less available.
4. Downstream protein affects result in feedback (positive or negative) which trigger gene expression.

Expanding upon the third potential mechanism is a discussion of epigenetics and connects to idea four. As previously mentioned, epigenetics includes mechanisms that change chromatin structure. A change in the structure will change the accessibility of DNA regulatory sites like the promoter region where RNA polymerase and TFs bind to allow for transcription to occur. Chromatin structure is also controlled by proteins, so if those genes are damaged downstream, the structure and therefore gene expression could be changed. In addition, if genes of TFs are unable to be expressed or their expression level changes due to a change in chromatin structure, the overall level of transcription could be greatly affected. This also applies to other proteins crucial to the transformation of DNA to RNA to protein.

Proteins are the functional unit of organisms. The function of a protein is directly related to its shape. A protein goes through conformational changes as part of various biological processes. These conformational changes are triggered by inputs such as ligand binding, chemical modification, and a change in environment. It should be investigated to determine if RF could affect this process by causing unintended conformational changes in protein resulting in a change in their function. A potential way the environment could change due to RF is an increase in free radicals which is found could be true from studies [11] and [2].

A standardized environment is needed to further the effort in this field to determine the specific biological mechanism of action influenced by radiofrequency exposure that alters gene expression.

2.2. Model Organism Selection

Before designing a standardized environment for this purpose, a model organism must be selected since different organisms have different requirements for life. Fission Yeast *Schizosaccharomyces Pombe (S. pombe)* is a great candidate for several reasons. First, animal studies like rabbit or mice are more expensive because they require more care and setup and longer duration. Second, the animal must be euthanized to complete most studies. Using yeast allows researchers to perform extensive investigations over shorter spans of time with minimal cost and without killing animals. This would increase the repeatability and reproducibility of a study which would further the topic significantly and, hopefully, giving more traction to their individual conclusions.

S. pombe is a model organism because it is a rod-shaped unicellular eukaryote that shares many features with humans such as gene structures, chromatin dynamics, prevalence of introns, and control of gene expression through pre-messenger RNA splicing, epigenetic silencing, and RNAi pathways. The genome of *S. pombe* is made of 12.6 billion base pairs which are split across three chromosomes. There are 5118 genes identified as protein encoding of which 3539 are homologous to humans.

In addition, *S. pombe* is easy to grow. The yeast can grow in a temperature range of 18 to 36 degrees Celsius in a minimal or rich medium. As previously mentioned, using yeast would result in faster testing due to its doubling time of two to four hours. This could allow for

significantly shorter durations of radiofrequency exposure. It is also important to note that the doubling time of a yeast cell is related to temperature.

3. MATERIALS AND METHODS

The following section outlines the materials and methods employed in conducting the experimental investigation. A range of specialized equipment was used to create controlled experimental conditions and gather accurate data. The equipment includes precision instruments for generating radiofrequency signals, monitoring environmental parameters, and ensuring the stability of the experimental setup. Detailed descriptions of the equipment specifications and system design verification protocols are provided to ensure transparency and reproducibility of the research findings. These methodologies lay the foundation for rigorous experimentation and scientific inquiry into the impact of radiofrequency on gene expression and cellular processes.

3.1. Materials

Table 4 presents a comprehensive list of the equipment employed in the experimental investigation, along with their respective make, model, and specifications. Each piece of equipment plays a unique role in facilitating different aspects of the experiment, ranging from generating radiofrequency signals to monitoring environmental parameters and ensuring the stability and reproducibility of the experimental setup.

Table 4. Equipment Used

Name	Make/Model/Specifications
Analog Signal Generator	Agilent/N5181A 100 kHz – 3 GHz
Spectrum Analyzer	Agilent E4407B 9 kHz – 26.5 GHz
Flat Patch Antenna	Taoglass Limited FXUB66 Ultra-Wide Band Antenna 700-6000 MHz 12.04 by 5.04 cm
Coax Cable	UTIFLEX Micro-Coax 26.5 GHz
Power Amplifier	Mini-Circuits (ZHL-30W-252-S+) 700 to 2500 MHz
Incubator	VEVOR Reptile Incubator 25 L Capacity 5-60 degrees Celsius constant temperature
MicroSD Module	Adafruit MicroSD Card Breakout Board
MicroSD Card	Adafruit 512 MB MicroSD Card
MicroSD to USB Adapter	Wansurs USB3.0 Micro SD Card Reader and SD Card Reader (2 in 1) to USB Adapter
CO2 Gas Sensor	DFRobot SEN0159
O2 Gas Sensor	DFRobot SEN0465
Humidity Sensor	TDK CHS-MSS 20-85±5% Relative Humidity
Everyday Humidity Sensor	ThermoPro TP50 10-99±2~3% Relative Humidity
Temperate Sensor	NTC Thermistor
Arduino Mega	Arduino Mega 2560 Rev3
Power Supply Adapter Cord	9V 1A Power Adapter AC DC Cable Cord for Arduino UNO MEGA 2560 1280
Solderable Breadboard	LampVPath PCB Prototype Board
Power Supply	MASTECH HY5020EX 50V DC 0-20 A
Epoxy	Loctite Epoxy Putty All Purpose Repair, 2 oz, 1, Stick, White
Wire	22 Gauge Single Core
Resistors	10k +/- 5% Axial Through-Hole Resistor
5V Module	Power Supply Module 3.3/5V for Arduino

3.1.1. Incubator

To provide a temperature-controlled environment for yeast it is common to use a plate incubator, however, plate incubators have their pitfalls. First, they are most often made of metal which would distort the radiofrequency field central to this experiment. Second, laboratory grade incubators are expensive. In addition, a port is needed to feed a cable into the incubator to create

the RF field meaning the incubator needs to come with a port or will need one cut. These limitations lead to the use of an inexpensive, plastic incubator.

3.1.2. Radiofrequency Antenna

Since the incubator is small, the size of the antenna was a key characteristic. The easiest route is a flat patch antenna also known as a microstrip antenna. They are small and easy to manufacture or buy. A patch antenna has three layers: ground plane, substrate, and patch. The antenna can be easily placed onto the bottom of the incubator, keeping it in place with its adhesive backing. A power amplifier, signal generator, power supply, and the necessary cables are used to create the RF field. The three coax cables used to create and verify the test environment were measured for insertion loss using a network analyzer, and results are depicted in Table 5. A spectrum analyzer and another antenna are used to verify the output of the transmitting antenna.

Table 5. Insertions Loss of Coax Cables Measured at 2.45 GHz.

Number	Length	Insertion Loss (dB)
1	Long	0.71
2	Long	0.72
3	Short	0.40

3.1.3. Monitoring System

To ensure the stability of the experimental environment and gather data throughout the experiment, an environmental monitoring and logging system was developed. The monitoring system consists of an Arduino Mega microcontroller, 5V module, and various analog sensors strategically placed in the incubator. Ports were created to feed wires into the enclosure and epoxied in place. Only analog sensors were used to simplify the code requirements.

3.1.3.1. Choice of Arduino Mega

The Arduino Mega microcontroller was selected due to its ample quantity of analog pins, which allow for the simultaneous connection of multiple sensors without the need for additional multiplexing hardware. This simplifies the setup and reduces complexity while ensuring efficient data collection. The Arduino Mega was powered by a power supply adapter cord shown in Table 4. The 5V module is powered by another power supply adapter cord into a wall outlet.

3.1.3.2. Data Logging Capability

To log data without the need for continuous connection to a laptop or external device, an SD card module was integrated with the Arduino Mega. This module enables the storage of sensor data as a text file, providing a convenient and portable means of data storage. Each data entry is estimated to be 50 bytes, and with data logged every minute, there will be 60 entries per hour. Over the course of a 72-hour experiment, there will be a total of 4,320 entries, resulting in an estimated total size of 216,000 bytes. This falls well below the storage capacity of the microSD card, which is 512 MB, ensuring ample space for data storage without exceeding the card's capacity.

3.1.3.3. Environmental Parameters Monitored

The monitoring system is designed to oversee crucial environmental factors essential for yeast growth and to ensure consistency between the control and experimental incubators. These parameters encompass temperature, carbon dioxide (CO2), oxygen (O2), and humidity levels within the incubator. Eight thermistors were strategically placed throughout the incubator to monitor temperature variations across different regions. This enables the detection of any temperature gradients or fluctuations that may impact yeast growth. Specific sensors are employed to continuously track CO2, O2, and humidity levels within the incubator. Although the

incubator does not actively control these parameters, monitoring them allows researchers to ensure similar environments exist between the control and experimental incubators, thereby minimizing potential confounding variables. Figure 9 depicts the location of these sensors in the incubator.

a) b)

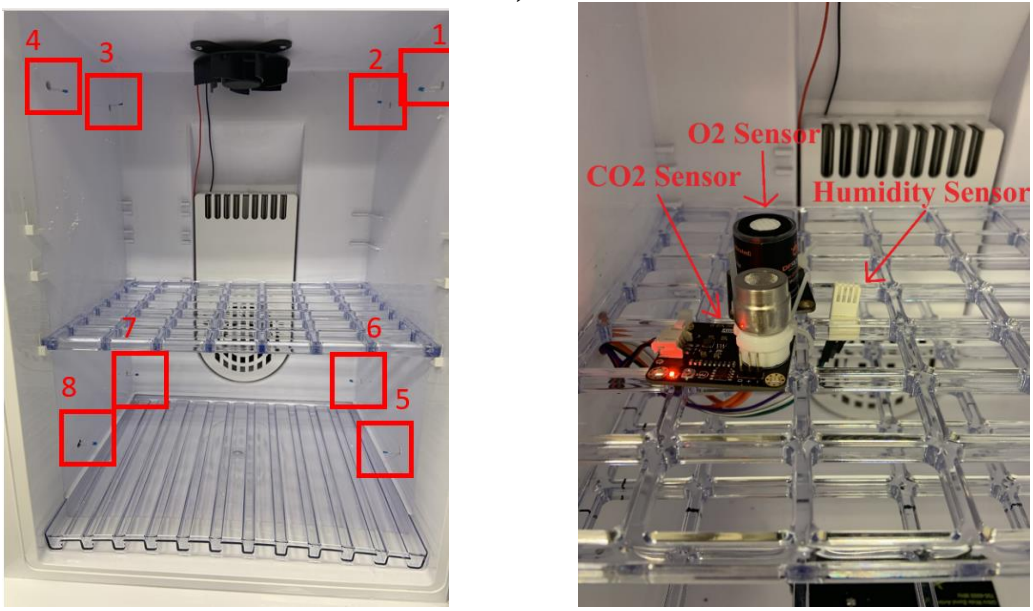


Figure 9. Placement of Sensors in the Incubator. (a) Thermistor Arrangement Inside the Incubator. (b) Placement of CO2, O2, and Humidity Sensors.

By implementing a robust monitoring system with the Arduino Mega and a variety of analog sensors, researchers can accurately track and record key environmental parameters throughout the experiment. This ensures the results of any experiment completed to determine the effect of radiofrequency on gene expression are not due to other environmental factors. For detailed implementation, including the Arduino code utilized in this monitoring system, please refer to the provided appendix (see Appendix A). Table 6 and Table 7 represent the logged data for this test environment over the course of roughly 10 minutes from each of the sensors individually and an average of the eight thermistors to get an approximate overall temperature within the off incubator.

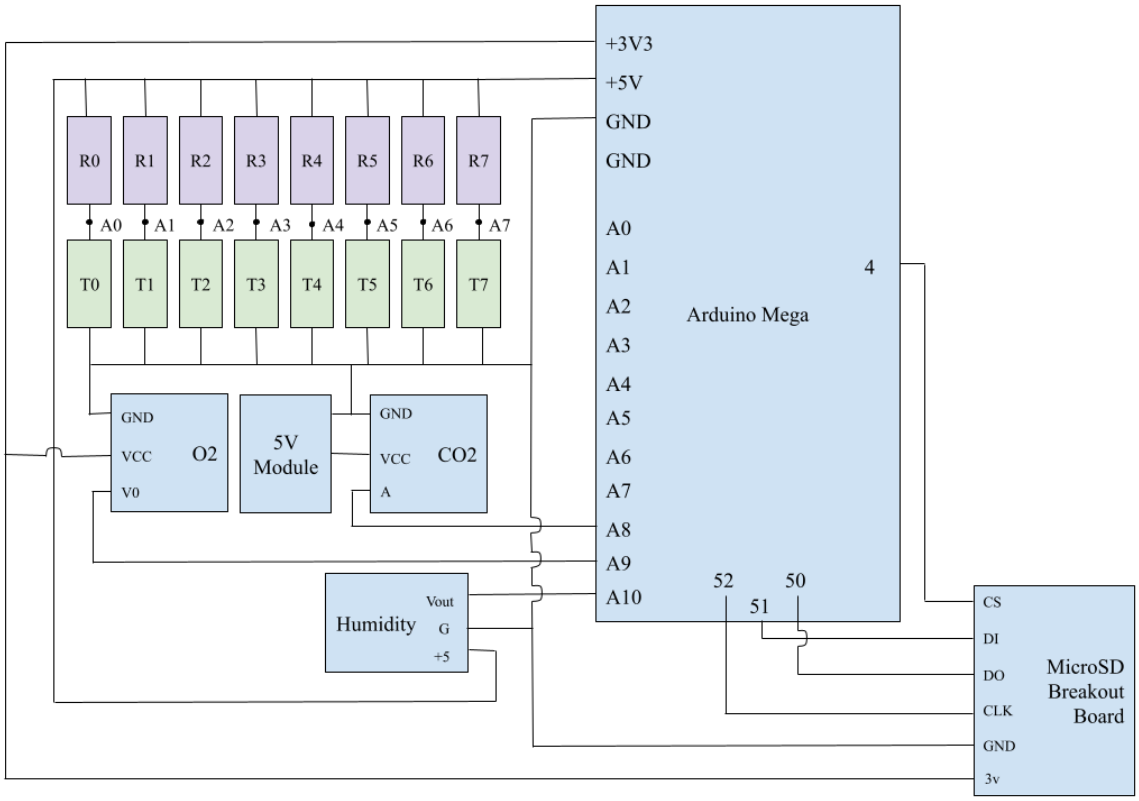
Table 6. Temperature Monitoring in Degrees Celsius in Incubator.

Time (Hours)	Temp 1	Temp 2	Temp 3	Temp 4	Temp 5	Temp 6	Temp 7	Temp 8	Temp Average
0.013	24.64	24.33	25.36	25.26	23.72	23.92	24.03	24.03	24.41
0.033	24.64	24.33	25.46	25.26	23.82	24.03	24.03	24.13	24.46
0.050	24.64	24.44	25.46	25.36	23.82	24.03	24.13	24.13	24.5
0.067	24.74	24.44	25.46	25.36	23.82	24.03	24.13	24.13	24.51
0.084	24.74	24.44	25.57	25.36	23.92	24.03	24.03	24.13	24.51
0.100	24.85	24.44	25.57	25.46	23.92	24.03	24.23	24.23	24.59
0.117	24.85	24.44	25.57	25.46	23.92	24.13	24.23	24.23	24.6
0.134	24.85	24.54	25.57	25.46	23.92	24.13	24.23	24.23	24.62
0.151	24.85	24.54	25.67	25.57	23.92	24.13	24.23	24.23	24.64
0.167	24.85	24.54	25.67	25.57	24.03	24.13	24.23	24.23	24.65

Table 7. CO2, O2, and Humidity Levels in Incubator.

Time (Hours)	CO2 (ppm)	O2 (%)	Humidity (% Relative Humidity)
0.013	650	21.50	41.95
0.033	662	21.50	41.44
0.050	667	21.50	46.49
0.067	676	21.50	43.46
0.084	678	21.40	41.45
0.100	693	21.50	44.98
0.117	700	21.50	47.00
0.134	690	21.50	42.45
0.151	703	21.40	41.95
0.167	703	21.50	47.51

Schematic 1 provides a visual representation of the connection and integration of the environmental monitoring system within the experimental setup. This schematic illustrates the arrangement and interconnection of various components, including sensors, the Arduino Mega, and storage device, to facilitate comprehensive environmental monitoring throughout the experiment.



Schematic 1. Schematic of Environmental Monitoring System.

3.2. Environment Verification

3.2.1. Incubator Characterization/Thermistor Monitoring

The performance of the incubator was systematically evaluated across a range of temperatures to assess its suitability for maintaining a stable environment for yeast growth. It is essential to understand how well the incubator can regulate temperature, especially considering the sensitivity of yeast to environmental conditions. The temperature range chosen for characterization aligns with the requirements for yeast growth, spanning from 18 to 36 degrees Celsius. This range encompasses the optimal growth temperature for the specific yeast strain under investigation, which in this case is *S. pombe*. The Arduino code used for the characterization described in this section is provided in the appendix for reference (see Appendix B).

To characterize the incubator, temperature measurements were taken at various set points within the specified range, and results are shown in Table 8. The incubator was allowed to stabilize at each temperature setting before recording temperature data and logging for five minutes. The stabilization period was kept minimal, corresponding to the time required for the incubator to reach the desired temperature setting and stabilize enough for temperature readings. Temperature averages were calculated after five minutes at each set temperature to assess the stability and uniformity of temperature distribution within the incubator. Additionally, temperature differentials between different regions within the incubator were analyzed to identify any temperature gradients or inconsistencies. It was observed that the incubator maintained relatively stable temperatures within the desired range, with slight variations noted at higher temperatures. These variations indicate limitations in the incubator's temperature control mechanisms, particularly at elevated temperatures.

Table 8. Temperature Averages After Five Minutes in Degrees Celsius.

Set Temperature	Min(ave) all	Max(ave) all	Min(ave) top 4	Max(ave) top 4	Min(ave) bottom 4	Max(ave) bottom 4	Min(diff)	Max(diff)
18	18.45	20.29	17.58	19.98	19.25	20.61	1.3	2.8
20	19.98	21.40	19.45	21.31	20.48	21.49	0.4	1.71
22	21.67	22.34	21.44	22.30	21.89	22.40	0.3	0.71
24	22.99	23.17	23.29	23.47	22.70	22.91	1.02	1.12
28	26.45	26.95	26.19	26.79	26.63	27.15	0.73	1.35
32	28.78	32.30	30.17	34.05	27.28	30.55	4.05	6.45
36	31.61	35.71	33.26	37.82	29.79	33.70	4.73	7.62

Following the initial measurements, a three-hour extended stabilization period was implemented at 36 degrees Celsius (see Table 9). This provides valuable insight into the incubator's performance over time, especially at higher temperatures where stability might be more challenging to maintain. This longer stabilization period allowed for thorough equilibration

of the internal temperature within the incubator, minimizing any transient fluctuations and ensuring a more accurate representation of the incubator's performance over time.

Table 9. Characterization with Different Stabilization Periods in Degrees Celsius.

	Min(ave) all	Max(ave) all	Min(ave) top 4	Max(ave) top 4	Min(ave) bottom 4	Max(ave) bottom 4	Min(diff)	Max(diff)
Minimal	31.61	35.71	33.26	37.82	29.79	33.70	4.73	7.62
3 Hours	31.66	35.45	32.76	36.93	30.57	33.98	3.4	5.42

Since the RF antenna emits energy, it is important to consider the heat it can produce. In Table 10, the results are shown before and after 3 hours with the antenna on, but with the incubator off. This is crucial for understanding and mitigating experimental variables that may affect yeast growth conditions.

Table 10. Effect of Antenna Heat on Incubator without Power.

Time (hours)	Min(ave) all	Max(ave) all	Min(ave) top 4	Max(ave) top 4	Min(ave) bottom 4	Max(ave) bottom 4	Min(diff)	Max(diff)
0	23.95	24.53	23.90	24.41	24.00	24.72	0.72	1.44
3	25.25	26.22	24.95	25.96	25.36	26.48	1.66	3.42

Table 8 demonstrates that the incubator exhibits slightly greater difficulty in maintaining higher temperatures, with variations becoming more pronounced as the set temperature increases. In addition, Table 10 shows that the presence of the RF antenna introduces additional heat into the system, contributing to temperature fluctuations within the incubator. To further describe the influence of the antenna on the incubator's temperature regulation, Table 11 shows results with the antenna on and off at 36 degrees Celsius. By contrasting these conditions, we can assess the extent to which the antenna affects the heating dynamics of the incubator, providing valuable insights for optimizing experimental protocols and mitigating potential confounding variables.

Given the additional heat emitted by the antenna seen in Table 10 but the lack of temperature difference seen in Table 11 it is apparent that the incubator's control mechanism can compensate. By implementing such a procedure, potential sources of experimental variability can be effectively mitigated, ensuring the reliability and accuracy of the results. This proactive approach enhances the integrity of the experimental setup and underscores the commitment to maintaining stringent standards of scientific rigor. Thus, by carefully addressing these considerations, the optimization of experimental conditions can be achieved, facilitating robust and reproducible scientific inquiry.

Table 11. Effect of Antenna Heat on Incubator with Power.

Antenna	Min(ave) all	Max(ave) all	Min(ave) top 4	Max(ave) top 4	Min(ave) bottom 4	Max(ave) bottom 4	Min(diff)	Max(diff)
Off	31.66	35.45	32.76	36.93	30.57	33.98	3.4	5.42
On	31.82	35.76	32.40	36.67	31.16	34.98	2.89	5.67

3.2.2. CO2, O2, and Humidity Monitoring

The incubator cannot control CO2, O2, and humidity. However, monitoring these parameters allows researchers to ensure similar environments exist between the control and experimental incubators.

3.2.2.1. CO2 and O2

According to the American Society of Heating, Refrigerating and Air-Conditioning Engineers CO2 levels should be maintained under 1,000 parts per million (ppm). In addition, since people exhale CO2, occupied buildings will have higher concentrations of CO2 [14]. A normal atmosphere contains 20.9% oxygen by volume, and spaces below 19.5% should not be entered without appropriate accommodations. The level of oxygen can be reduced by the replacement of oxygen by other gases [27]. For example, exhaling results in CO2, which will replace some oxygen within the space near the sensor, decreasing the oxygen level. In other

words, when exhaling near a CO2 sensor, the CO2 level should increase and exhaling near an oxygen sensor should decrease levels. Table 12 shows the CO2 and O2 levels in an empty room for 5 minutes and then 5 minutes of an individual breathing near the sensor. The results demonstrate the anticipated relationship between CO2 and O2 levels: exposure to exhaled CO2 leads to an increase in CO2 levels and a decrease in O2 levels.

Table 12. CO2 and O2 Levels in an Empty Room vs. Occupied Room: Sensor Test Results.

Time (minutes)	CO2 level (ppm)	O2 level (% volume)
1	553	21.50
2	535	21.50
3	534	21.50
4	534	21.50
5	524	21.50
6	10579	20.81
7	8129	20.81
8	5822	21.01
9	3753	21.20
10	6246	21.10

3.2.2.2. Humidity

According to the Environmental Protection Agency, humidity levels should range from 30 to 50 percent relative humidity indoors [25]. To verify the reading of the humidity sensor used in the experimental setup an additional sensor meant for everyday use in the home was used. Table 13 shows the results from both sensors over the course of ten minutes. The results show similar readings that fall within the excepted indoor range.

Table 13. Humidity Sensor Comparison in % Relative Humidity.

Time (minutes)	Test Environment Sensor	Home Sensor
1	34.37	37
2	34.37	37
3	34.37	37
4	34.37	37
5	34.37	37
6	33.86	37
7	33.86	37
8	34.37	37
9	33.86	37
10	33.86	36

3.2.3. Power Density of RF Field

Field characteristics change depending on distance, making it crucial to determine the ideal location to place the yeast. Figure 10 depicts the three possible shelf positions for the yeast to grow in the incubator.

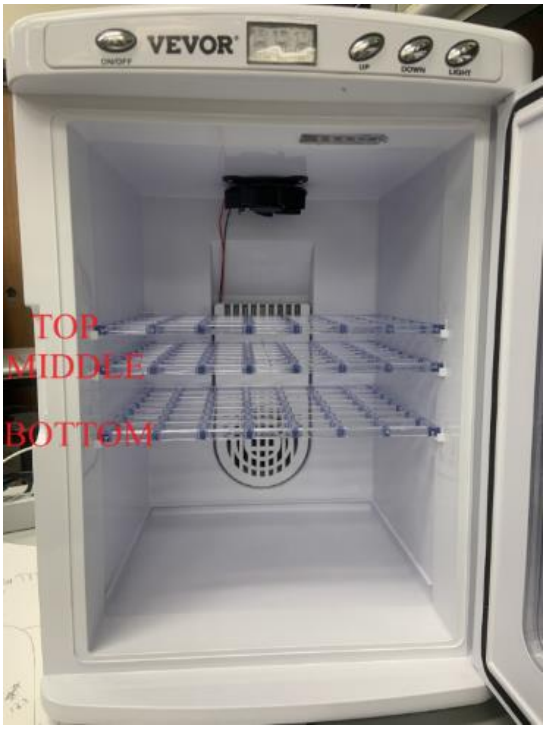


Figure 10. Incubator Shelf Positions.

As previously mentioned, the IEEE standard states that below 1.6 mW/cm^2 is safe for 2.45 GHz in an uncontrolled environment. For this test environment, it is crucial to ensure the field created is below this threshold. Figure 11 illustrates the system used to calculate the power density created by the transmitting (TX) antenna using a spectrum analyzer to measure the power at the receiving (RX) antenna. The bottom shelf is 14.3 cm away from the TX antenna (R1). The middle shelf is 19.6 cm away from the TX antenna (R1). The top shelf is 22.6 cm away from the TX antenna (R1).

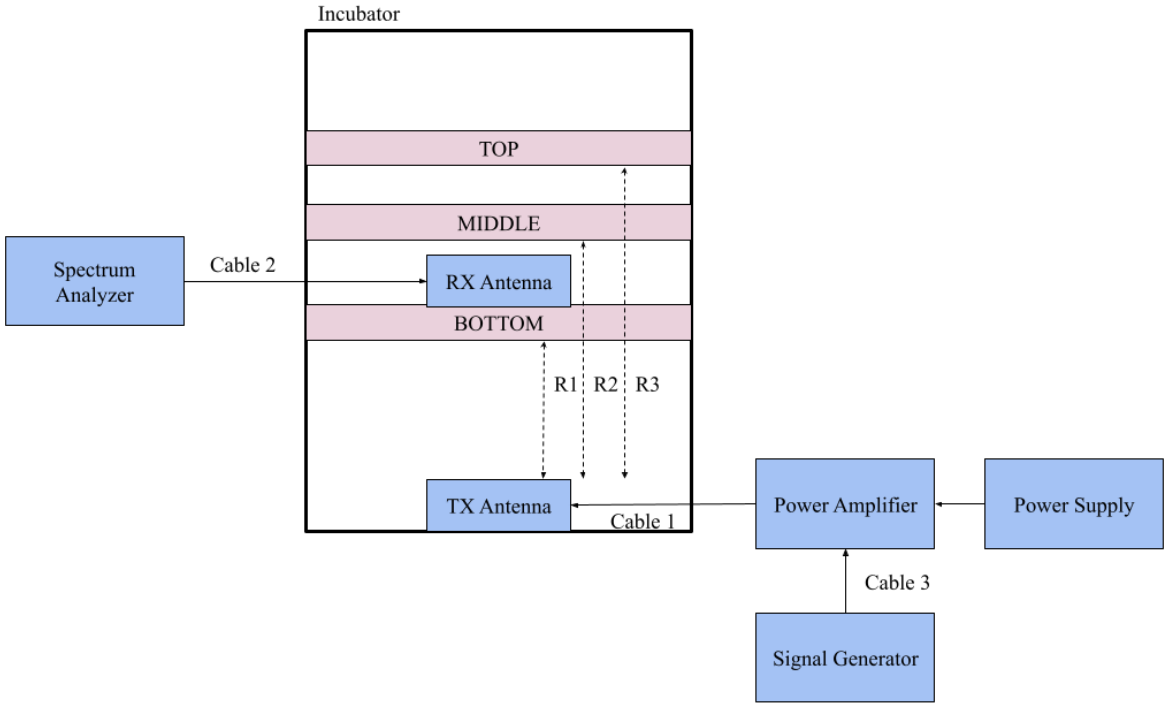


Figure 11. Power Density of Field Verification Layout.

Figure 12 illustrates the power transmitted by the TX antenna. The RF power amplifier is itself powered by a bench-top power supply set to 28 V. The RF output on the signal generator is set to 2.45 GHz and -14.4 dBm which feeds into the power amplifier via cable 3 which has an insertion loss of 0.40 dB, meaning the power into the power amplifier is -14.8 dBm. The power

amplifier adds a gain of +50 dB resulting in +35.2 dBm and goes through cable 1 which has an insertion loss of 0.71 dB meaning the TX antenna receives +34.49 dBm (2811.90 mW).

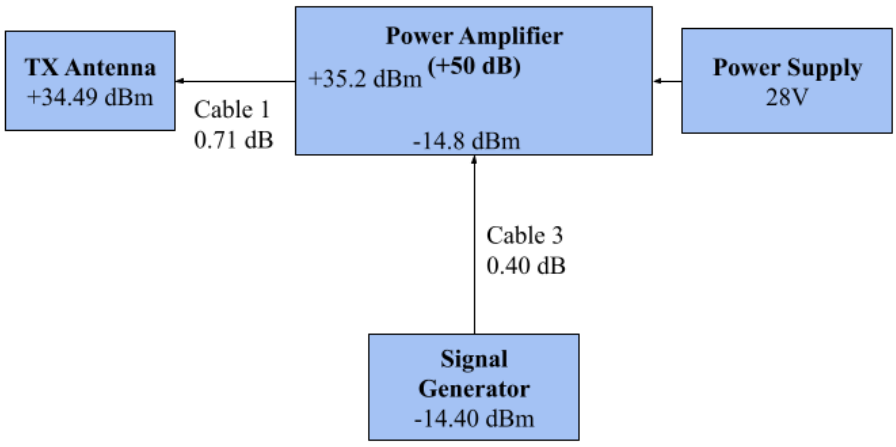


Figure 12. Power Transmitted Diagram.

Equation 4, the Friis equation, can be used to calculate the expected power to be received by the RX antenna. As shown above, the power transmitted is 2811.90 mW. For this setup both transmitting and receiving antenna are the same, with a peak gain at 2.4 GHz of 2.24 (+3.5 dBi) according to the datasheet. Using equation 1, to find wavelength given frequency is 2.45 GHz and velocity is the speed of light.

$$\lambda = fv = 2.45\ x\ 10^9\ Hz\ (2.998\ x\ 10^{10}\ cm/s) = 12.24\ cm$$

For example, if R= R1 from Figure 11 the power received can be calculated using equation 4.

$$\text{Pr} = Pt\frac{GtGr\lambda^2}{(4\pi R)^2} = 2811.90\ \ mW * \frac{2.24 * 2.24 * (12.24\ cm)^2}{(4\pi * 14.3\ cm)^2} = \ 65.46\ mW$$

Pr is 65.46 mW (+18.16 dBm), 34.84 mW (+15.42 dBm) and 26.21 mW (+14.18 dBm), respectively for R1, R2 and R3. To determine power density, first calculate effective aperture using equation 5, given wavelength is 12.24 cm and Gr is 2.24 (+3.5 dBi).

$$Ae = \frac{\lambda^2}{4\pi}Gr = \frac{(12.24\ cm)^2}{4\pi} * 2.24 = 26.71\ cm^2$$

Then, using equation 6 calculates the power density on the bottom shelf (R1).

$$S = \frac{Pr}{Ae} = \frac{65.46\ mW}{26.71\ cm^2} = 2.45\ mW/cm^2$$

Making the expected power densities 2.45, 1.03, and 0.98 mW/cm^2, for R1, R2, and 3 respectively. These are the estimated power densities given the antenna at 2.45 GHz is close to peak gain given by the datasheet at 2.4 GHz.

Figure 13 shows the breakdown of how each shelf is measured. The antenna is 12.04 by 5.04 cm, and the incubator shelves are roughly 66 by 52 cm. This allows the antenna to be positioned in eight locations on a shelf with no overlap and minimal additional space.

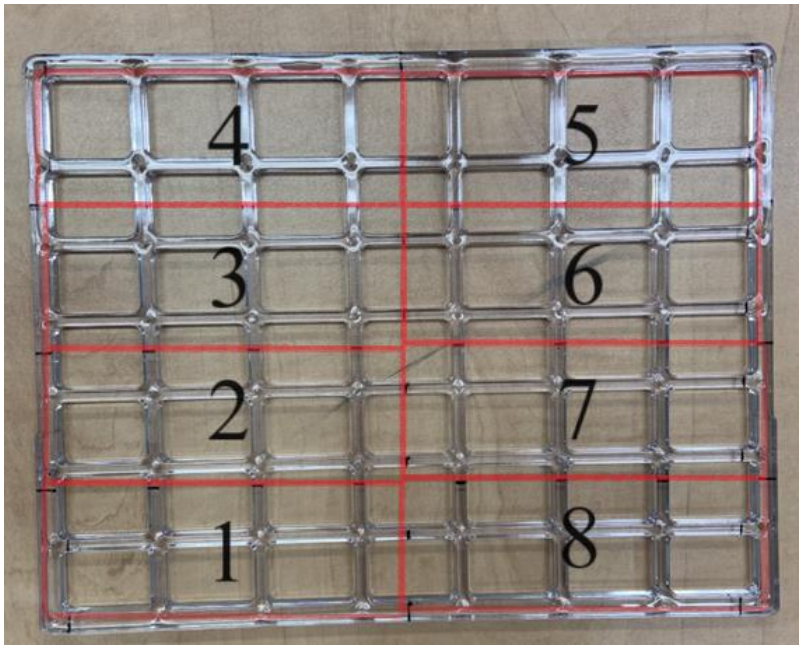


Figure 13. 4 by 2 Grid of Incubator Shelves Measured by Receiving Antenna.

Figure 14 depicts the placement of the RX antenna at position one on the bottom shelf of the incubator, with the coaxial cable carefully positioned to maintain straight alignment for optimal signal reception and the position of the transmitting antenna.

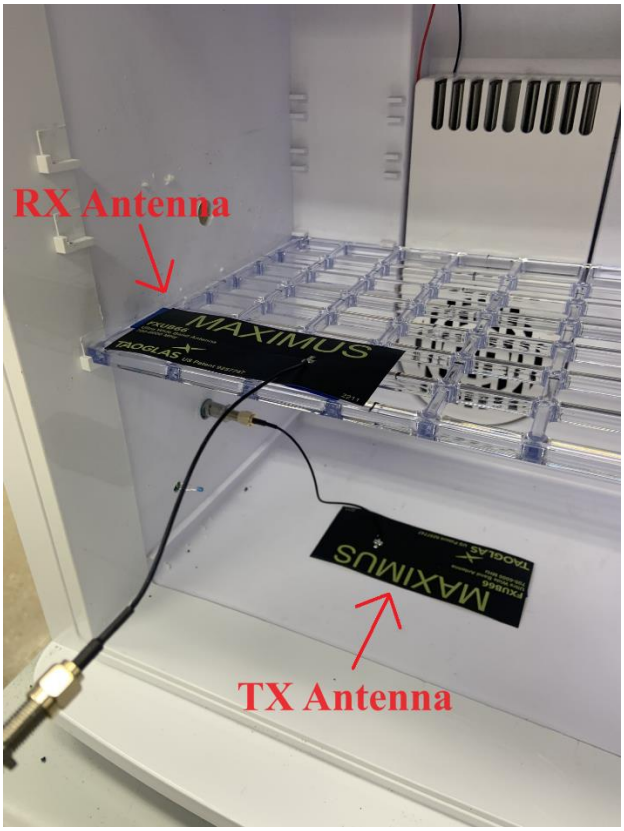


Figure 14. Transmitting and Receiving Antenna Placement.

Table 14 shows the power level read by the spectrum analyzer, corrected power level after accounting for insertion loss of cable two, and the calculated power density in each possible position in the incubator. Positions six through eight on the bottom shelf are within 0.1 dBm of each other, making this region the best for petri dish placement. This is because of the uniformity across the region and its proximity to the IEEE power density limit. Only one shelf should be used for petri dishes, because inter-shelf power differences are much larger than intra-shelf power differences.

Table 14. Power Read by Spectrum Analyzer in Decibel-Milliwatts (dBm).

Shelf	Position	Power (dBm)	Power Compensated (dBm)	Power Density (mW/cm^2)
Bottom	1	+9.0	+9.72	0.35
	2	+10.3	+11.02	0.47
	3	+10.3	+11.02	0.47
	4	X	X	X
	5	+10.1	+10.82	0.45
	6	+14.3	+15.02	1.19
	7	+14.3	+15.02	1.19
	8	+14.2	+14.92	1.16
Middle	1	X	X	X
	2	+8.7	+9.42	0.33
	3	X	X	X
	4	X	X	X
	5	X	X	X
	6	+13.1	+13.82	0.90
	7	+13.9	+14.62	1.08
	8	+13.1	+13.82	0.90
Top	1	X	X	X
	2	+8.1	+8.82	0.29
	3	X	X	X
	4	X	X	X
	5	X	X	X
	6	+9.9	+10.62	0.43
	7	+12.8	+13.52	0.84
	8	+12.6	+13.32	0.80

Note: X denotes unstable regions.

The power levels calculated for each shelf height are different than measured because the gain of the antenna in different positions varies. The X denotes the regions within the incubator in which the power level was in constant fluctuation, making it difficult to measure and not ideal for yeast growth. Since the power density does not exceed the IEEE standard SAR level does not need to be calculated. In addition, based on the power density findings the CO2, O2, and

humidity sensor placement were chosen to be on the middle shelf, in positions two and three, to be close to the yeast but in locations not ideal for testing.

4. FUTURE DIRECTIONS/EXPERIMENTATION

To ensure any results obtained from the use of this test method are solely from the RF source provided, the surrounding environment should be measured. A potential method is to use spectrum analyzer to track peaks for the approximate duration the experiment will run. If the peak is below the maximum power density reading from the field density mapping step, then anechoic material or other methods are not needed to shield the environment from other RF sources. This shows that the highest power density the yeast would be exposed to is from the TX antenna not the surrounding environment.

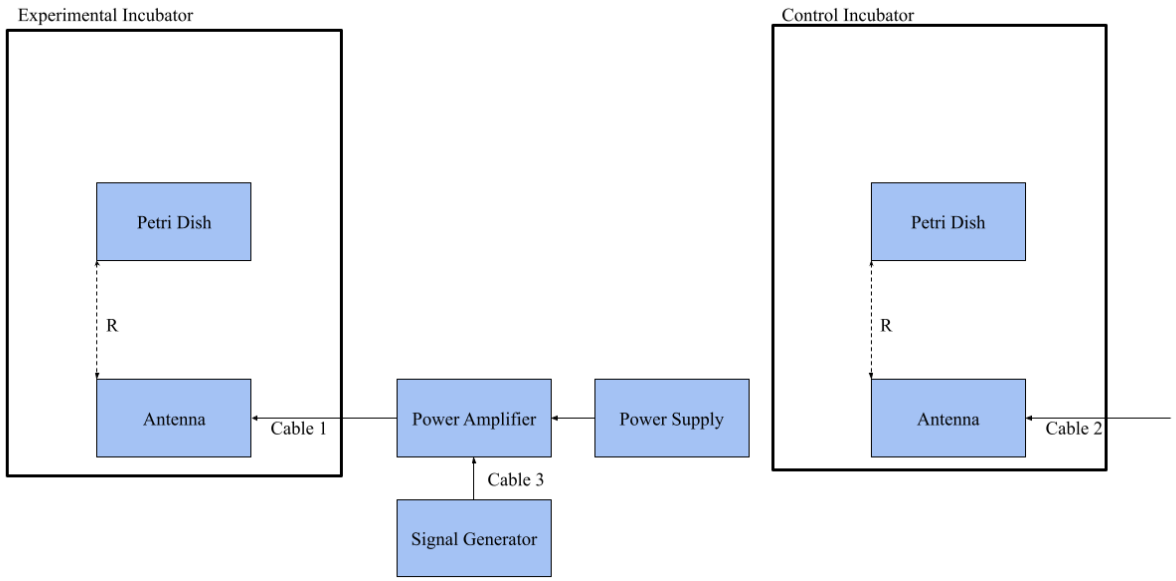


Figure 15. Layout of Experimental Setup.

Figure 15 illustrates the setup for an environment using the setup described in this paper. To ensure the yeast can survive in the environment created is a simple colony count before and after growing in the incubator for a specific time. This step is important to show that the colonies can grow in their environment before we add more variables. The next steps will involve conducting experiments similar to those described in references [12] and [28] to facilitate

comparison of the results. Specifically, the focus will be on examining the quantity of genes before and after exposure. If the outcomes align with those reported in the referenced studies, it will provide additional evidence supporting the hypothesis that radiofrequency affects gene expression. However, these experiments alone do not elucidate the mechanisms underlying the observed effects on gene expression, necessitating further investigation.

Following the comparison experiments, two potential avenues for further exploration emerge. First, similar experiments will be conducted, with variations in the frequency or exposure time of the radiofrequency. This approach aims to determine the specific roles played by these factors in gene expression. Such findings are significant, as variations in the affected genes under different frequencies or exposure durations could have profound implications for future medical treatments of genetic disorders. By identifying the genes susceptible to RF-induced changes and understanding the parameters influencing these alterations, novel therapeutic strategies may emerge.

Secondly, additional experiments will be designed to investigate the underlying mechanisms of how gene expression is affected. This includes examining the quantity of free radicals, analyzing the structure of proteins crucial to the regulation of gene expression, and exploring changes in chromatin structure. These investigations aim to provide insights into the molecular pathways through which radiofrequency influences gene expression, thus enhancing our understanding of the broader biological effects of RF exposure.

These proposed future directions outline a comprehensive research strategy aimed at further describing the impact of radiofrequency on gene expression and its underlying mechanisms, with implications for both basic science and potential therapeutic interventions in genetic disorders.

5. CONCLUSION

Using the proposed test method and associated procedures would allow easier access for researchers in this field due to the lower cost associated with yeast and a plastic incubator and the simplicity. In addition, being able to have shorter experiments would allow for the research on radiofrequency exposure to develop at a faster rate. Since the effects of radiofrequency on gene expression are unknown, the more experiments conducted at a high level will allow conclusive evidence to either cement current beliefs or change them.

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APPENDIX A. ENVIRONMENTAL MONITORING SYSTEM ARDUINO CODE

```
#include <SPI.h>
#include <SD.h>

#define MG_pin A8
#define DC_gain 8.5

#define read_sample_interval 50
#define read_sample_times 5

#define zero_point_voltage 0.318
#define reaction_voltage 0.030

float CO2Curve[3] = {2.602, zero_point_voltage, (reaction_voltage / (2.602 - 3))};

const int chipSelect = 4; // Pin connected to the CS (Chip Select) pin of the SD card module

// Analog input pins for an O2 sensor
const int o2SensorPin = A9;

// Array of analog input pins for thermistors
const int thermistorPins[] = {A0, A1, A2, A3, A4, A5, A6, A7};  // Analog pins for thermistors
const float thermNominal = 10000.0;  // Nominal resistance at 25 degrees C
const float temperatureNominal = 25.0;  // Nominal temperature for thermistor
const int seriesResistor = 10000;  // Value of the series resistor

File dataFile;

// Function prototype
float readHumidity();

void setup() {
  // Initialize serial communication
  Serial.begin(9600);

  // Wait for 45 seconds before initializing SD card and creating the file
  delay(45000);

  // Initialize SD card
  if (!SD.begin(chipSelect)) {
    Serial.println("SD card initialization failed.");
    return;
  }

  Serial.println("SD card initialized successfully.");
```

```
  // Create a new file for logging
  dataFile = SD.open("data.txt", FILE_WRITE);
  if (dataFile) {
    Serial.println("Logging data...");
    dataFile.println("Time (Hours), CO2(ppm), O2(%), Temp 1 (°C), Temp 2(°C), Temp 3 (°C),
Temp 4 (°C), Temp 5 (°C), Temp 6 (°C), Temp 7 (°C), Temp 8 (°C), Temp(Ave) (°C), Humidity
(%)"); // Header
    dataFile.close();
  } else {
    Serial.println("Error opening data.txt for writing.");
  }
}

void loop() {
  // Record timestamp
  unsigned long currentTime = millis();

  // Wait for 1 minute before taking the first reading
  if (currentTime < 60000) {
    delay(60000 - currentTime);
  }

  // Read and print CO2 concentration
  float co2Concentration = readCO2Concentration();
  Serial.print("CO2 Concentration: ");
  Serial.print(co2Concentration);
  Serial.println(" ppm");

  // Read and print O2 concentration
  float o2Concentration = readO2Concentration();

  // Read and print thermistor temperatures
  float thermistorTemperatures[8];
  readThermistorTemperatures(thermistorTemperatures);

  // Read and print humidity
  float humidity = readHumidity();

  // Log data to SD card
  logData(currentTime, co2Concentration, o2Concentration, thermistorTemperatures, humidity);

  // Delay before next reading
  delay(60000);
}
```

```
void logData(unsigned long timestamp, float co2Concentration, float o2Concentration, float
thermistorTemperatures[], float humidity) {
  dataFile = SD.open("data.txt", FILE_WRITE);
  if (dataFile) {
    // Write data to the file
    // Convert timestamp to hours with decimals
    float timeHours = timestamp / 3600000.0;
    dataFile.print(timeHours, 3);
    dataFile.print(", ");
    dataFile.print(co2Concentration);
    dataFile.print(", ");
    dataFile.print(o2Concentration);
    dataFile.print(", ");
    for (int i = 0; i < 8; i++) {
      dataFile.print(thermistorTemperatures[i]);
      dataFile.print(", ");
    }
    // Calculate and print average temperature
    float totalTemperature = 0.0;
    for (int i = 0; i < 8; i++) {
      totalTemperature += thermistorTemperatures[i];
    }
    float averageTemperature = totalTemperature / 8.0;
    dataFile.print(averageTemperature);
    dataFile.print(", ");
    dataFile.println(humidity);
    dataFile.close();
  } else {
    Serial.println("Error opening data.txt for writing.");
  }
}

float readCO2Concentration() {
  float volts = MGRead(MG_pin);
  int percentage = MGGetPercentage(volts, CO2Curve);
  return percentage;
}

float MGRead(int MG_pin) {
  int i;
  float v = 0;

  for (i = 0; i < read_sample_times; i++) {
    v += analogRead(MG_pin);
    delay(read_sample_interval);
  }
```

```
  v = (v / read_sample_times) * 5.0 / 1023;
  return v;
}

int MGGetPercentage(float volts, float pcurve[]) {
  if ((volts / DC_gain) >= zero_point_voltage) {
    return -1;
  } else {
    return pow(10, ((volts / DC_gain) - pcurve[1]) / pcurve[2] + pcurve[0]);
  }
}

float readO2Concentration() {
  int o2Value = analogRead(o2SensorPin);
  float Vout_O2 = o2Value * (5.0 / 1023.0); // Convert analog value to voltage
  float Vout0_O2 = 1.5;
  float Vout1_O2 = 1.0;

  // Calculate O2 concentration using the provided formula
  float o2Concentration = (10.0 / (Vout1_O2 - Vout0_O2)) * (Vout_O2 - Vout0_O2);
  return o2Concentration;
}

void readThermistorTemperatures(float temperatures[]) {
  for (int i = 0; i < 8; i++) {
    int rawADC = analogRead(thermistorPins[i]);
    float resistance = seriesResistor / (1023.0 / rawADC - 1);
    float steinhart;
    steinhart = resistance / thermNominal;     // (R/Ro)
    steinhart = log(steinhart);                // ln(R/Ro)
    steinhart /= 3380.0;                       // 1/B * ln(R/Ro)
    steinhart += 1.0 / (temperatureNominal + 273.15); // + (1/To)
    steinhart = 1.0 / steinhart;               // Invert
    steinhart -= 273.15;                       // Convert to Celsius
    temperatures[i] = steinhart;
  }
}

float readHumidity() {
  // Code to read humidity from your humidity sensor and return the value
  int humiditySensorPin = A10;
  int sensorValue = analogRead(humiditySensorPin);
  float voltage = sensorValue * (5.0 / 1023.0);

  // Replace these values with the characteristics of your sensor
  float sensorMin = 0.0;  // Minimum voltage
```

```
  float sensorMax = 1.0;  // Maximum voltage

  // Calculate humidity percentage directly
  float humidityPercentage = ((voltage - sensorMin) / (sensorMax - sensorMin)) * 100.0;

  // Ensure the humidity percentage is within valid bounds
  humidityPercentage = constrain(humidityPercentage, 0, 100);

  return humidityPercentage;
}
```

APPENDIX B. INCUBATOR CHARACTERIZATION CODE

```
// Array of analog input pins for thermistors
const int thermistorPins[] = {A0, A1, A2, A3, A4, A5, A6, A7};  // Analog pins for thermistors
const float thermNominal = 10000.0;  // Nominal resistance at 25 degrees C
const float temperatureNominal = 25.0;  // Nominal temperature for thermistor
const int seriesResistor = 10000;  // Value of the series resistor

unsigned long lastReadingTime = 0;  // Timestamp of the last reading
unsigned long lastAnalysisTime = 0;  // Timestamp of the last analysis
unsigned int readingsCount = 0;  // Counter for the number of readings
const unsigned int maxReadings = 30;  // Maximum number of readings before analysis
const unsigned long readingInterval = 10000;  // Reading interval in milliseconds (10 seconds)

float minAverageTemperature = 1000.0;  // Initialize with a high value
float maxAverageTemperature = -1000.0;  // Initialize with a low value

float minFirstFourAverage = 1000.0;  // Initialize with a high value
float maxFirstFourAverage = -1000.0;  // Initialize with a low value

float minLastFourAverage = 1000.0;  // Initialize with a high value
float maxLastFourAverage = -1000.0;  // Initialize with a low value

float minTemperatureDifference = 1000.0;  // Initialize with a high value
float maxTemperatureDifference = -1000.0;  // Initialize with a low value

void readAndPrintThermistorTemperatures();

void setup() {
  // Initialize serial communication
  Serial.begin(9600);
  lastReadingTime = millis();
}

void loop() {
    unsigned long currentTime = millis();
   // Perform measurements every 10 seconds
  if (currentTime - lastReadingTime >= readingInterval) {
    readAndPrintThermistorTemperatures();
    lastReadingTime = currentTime;
    readingsCount++;
  }

  // Check if it's time to perform analysis
  if (readingsCount >= maxReadings && currentTime - lastAnalysisTime >= readingInterval) {
    // Reset the readings count
```

```
    readingsCount = 0;

    // Print the analysis results
    printAnalysisResults();
    lastAnalysisTime = currentTime;
  }
}

void printAnalysisResults() {
    // Print the minimum and maximum average temperatures
    Serial.print("Minimum Average Temperature: ");
    Serial.print(minAverageTemperature);
    Serial.println("°C");

    Serial.print("Maximum Average Temperature: ");
    Serial.print(maxAverageTemperature);
    Serial.println("°C");

    Serial.print("Minimum First Four Average Temperature: ");
    Serial.print(minFirstFourAverage);
    Serial.println("°C");

    Serial.print("Maximum First Four Average Temperature: ");
    Serial.print(maxFirstFourAverage);
    Serial.println("°C");

    Serial.print("Minimum Last Four Average Temperature: ");
    Serial.print(minLastFourAverage);
    Serial.println("°C");

    Serial.print("Maximum Last Four Average Temperature: ");
    Serial.print(maxLastFourAverage);
    Serial.println("°C");

    Serial.print("Minimum Temperature Difference: ");
    Serial.print(minTemperatureDifference);
    Serial.println("°C");

    Serial.print("Maximum Temperature Difference: ");
    Serial.print(maxTemperatureDifference);
    Serial.println("°C");

  //resetting the minimum and maximum values for the next 5-minute interval
     minAverageTemperature = 1000.0;
     maxAverageTemperature = -1000.0;
     minFirstFourAverage = 1000.0;
```

```
    maxFirstFourAverage = -1000.0;
    minLastFourAverage = 1000.0;
    maxLastFourAverage = -1000.0;
    minTemperatureDifference = 1000.0;
    maxTemperatureDifference = -1000.0;
}

void readAndPrintThermistorTemperatures() {
  float totalTemperature = 0.0;
  float totalFirstFourTemperature = 0.0;
  float totalLastFourTemperature = 0.0;
  float minTemp = 1000.0;  // Initialize with a high value
  float maxTemp = -1000.0;  // Initialize with a low value

  for (int i = 0; i < 8; i++) {
    int rawADC = analogRead(thermistorPins[i]);
    float resistance = seriesResistor / (1023.0 / rawADC - 1);
    float steinhart;
    steinhart = resistance / thermNominal;     // (R/Ro)
    steinhart = log(steinhart);                  // ln(R/Ro)
    steinhart /= 3380.0;                         // 1/B * ln(R/Ro)
    steinhart += 1.0 / (temperatureNominal + 273.15); // + (1/To)
    steinhart = 1.0 / steinhart;                 // Invert
    steinhart -= 273.15;                         // Convert to Celsius
    Serial.print("Thermistor ");
    Serial.print(i + 1);
    Serial.print(" Temperature: ");
    Serial.print(steinhart);
    Serial.println("°C");

    // Track the minimum and maximum temperatures
    minTemp = min(minTemp, steinhart);
    maxTemp = max(maxTemp, steinhart);

    // Accumulate temperature for average calculation
    totalTemperature += steinhart;

    // Accumulate temperature for the first four thermistors
    if (i < 4) {
      totalFirstFourTemperature += steinhart;
    }
    // Accumulate temperature for the last four thermistors
    else {
      totalLastFourTemperature += steinhart;
    }
  }
```

```
  // Calculate and print average temperature
  float averageTemperature = totalTemperature / 8.0;
  Serial.print("Average Temperature: ");
  Serial.print(averageTemperature);
  Serial.println("°C");

  // Update minimum and maximum average temperatures
  minAverageTemperature = min(minAverageTemperature, averageTemperature);
  maxAverageTemperature = max(maxAverageTemperature, averageTemperature);

  // Calculate and print average temperature for the first four thermistors
  float firstFourAverageTemperature = totalFirstFourTemperature / 4.0;
  Serial.print("Average Temperature (First Four): ");
  Serial.print(firstFourAverageTemperature);
  Serial.println("°C");

  // Update minimum and maximum average temperatures for the first four thermistors
  minFirstFourAverage = min(minFirstFourAverage, firstFourAverageTemperature);
  maxFirstFourAverage = max(maxFirstFourAverage, firstFourAverageTemperature);

  // Calculate and print average temperature for the last four thermistors
  float lastFourAverageTemperature = totalLastFourTemperature / 4.0;
  Serial.print("Average Temperature (Last Four): ");
  Serial.print(lastFourAverageTemperature);
  Serial.println("°C");

  // Update minimum and maximum average temperatures for the last four thermistors
  minLastFourAverage = min(minLastFourAverage, lastFourAverageTemperature);
  maxLastFourAverage = max(maxLastFourAverage, lastFourAverageTemperature);

  // Calculate and print temperature difference
  float temperatureDifference = maxTemp - minTemp;
  Serial.print("Temperature Difference: ");
  Serial.print(temperatureDifference);
  Serial.println("°C");

  // Update minimum and maximum temperature differences
  minTemperatureDifference = min(minTemperatureDifference, temperatureDifference);
  maxTemperatureDifference = max(maxTemperatureDifference, temperatureDifference);
}
```