2024 IRES Scientific Report: Development and Practice of Gene Editing and Cell Culture Techniques – IR-LEGO, B-gTemp, and "Gel on Dish"

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Abstract

As part of the National Science Foundation Award #1952513, experiments focused on optics and genetics were conducted over two months at the National Institute of Basic Biology in Okazaki, Japan. Microscopy and imaging were heavily emphasized during this program, as several different instruments were used, including spinning disk confocal microscopy, scanning laser confocal microscopy, atomic force microscopy, and light sheet fluorescent microscopy. Several genetic techniques, such as plasmid restriction, ligation, transformation, and transfection, were performed to create transgenic cells and medaka expressing various fluorescent proteins, such as mCherry, mNeonGreen, RFP, and GFP. These editing plasmids also contained gene sequences for the expression of heat shock response factors. The expression of heat shock response factors is fundamental to the IR-LEGO gene editing system, as the system works by triggering heat shock response factors with an infrared laser to induce the transcription of genes by the heat shock promoter. Along with the editing of plasmids, experiments were also conducted involving the forming of acrylamide gel plates cross-linked with laminin to mimic the extracellular matrix during cell culture.

Introduction

The Fundamentals of Microscopy

Microscopy is the field of study that allows humans to see objects that are too small for the naked eye and is heavily dependent on the behavior of light. Light is a type of electromagnetic radiation that can act as both a particle and wave, which carries visual information that can be sensed by the human eye to produce images in the brain, allowing humans to see. The fact that light can act as a wave is important, as many aspects of a microscope rely on this property of light. The wavelength of light describes the distance between two peaks in a wave of light and is indirectly related to the energy of that light. Different wavelengths of light can interact with different kinds of matter to perform a wide variety of behaviors, which can be leveraged to perform a function such as magnifying an image. Light can also interact with itself, as two wavelengths in contact with each other will combine. Light waves that are in phase have aligned peaks and troughs and will add together to create a larger wave, and the opposite is true for light waves that are out of phase, resulting in phenomena known as constructive and destructive interference, respectively. The ability of light to interact with matter and other light waves allows researchers to manipulate the information being carried by that light to produce virtual images that allow us to see into the world of the microscopic.

To accomplish this, several properties of light need to be manipulated so that the image of a microscopic specimen can be magnified to a level that is both visible and clear. Many of these properties allow for light coming from a source to be diverted, spread out, and refocused, with each change in the direction of light contributing to the quality of the image. Light will reflect, refract, and diffract within a microscope, changing its direction and concentration at every stage. When light strikes a smooth surface, it can bounce off that surface at the same angle

at which it hits the surface, which is known as reflection. Mirrors allow for light to be directed through a non-linear path to an ultimate destination, with some mirrors, such as dichroic mirrors, selectively reflecting specific wavelengths of light to act as a filter. Refraction is when light bends as it travels from one medium into another, such as from air to glass, and is the fundamental principle of how lenses work. Lenses within a microscope are designed so that the path of light is directed from the source, through the specimen, and into the eye of the observer. Magnification of an image is possible by the fact that light bent through a lens will produce a virtual image that appears larger to the observer than the real image. Diffraction is the property of light where a beam will spread out after passing through a narrow space or around an edge. When light passes through multiple apertures within the same plane, the light spreading out from those openings will interfere with itself, both constructively and destructively, creating a pattern known as an Airy Disk that depicts where light is being amplified or diminished. When light interacts with a sample, the light will diffract to create an Airy Disk, with each ring of the disk around the center carrying visual information from light that diffracted due to interacting with the sample. The numerical aperture of a lens describes the angle at which the lens can gather incoming light, with a higher numerical aperture leading to more diffracted light being gathered, and a clearer image being produced due to the increased amount of visual information present in the diffracted light. The clarity of an image (or the distance at which two different objects can be identified) is known as the resolution.

Many properties of the visual information received by our eyes exist due to the various ways in which certain molecules interact with light. These characteristics of the interactions between light and the sample can depend on the behavior of electrons within matter. Chemical bonds form when two atoms share electrons, and some bonds allow more freedom of movement

for electrons than others. In a covalent bond between two atoms, electron orbitals will overlap to initially form a rigid sigma bond, but additional bonds between the same two atoms will create pi bonds. Molecules that contain multiple pi bonds within their structure contain many electrons that are relatively more free to move around, such as from a ground state to an excited state. Since these electrons require less energy to be raised to an excited state, they allow for weaker types of light with longer wavelengths, such as visible light, to interact with these molecules. These types of electron-light interactions can lead to visible light being absorbed or scattered, which are all factors in how light reaches the eyepiece. Absorption leads to the sensation of color, as the light of a wavelength that contains the exact amount of energy to be absorbed by an electron will not be visible to the human eye, as it does not reflect off the absorbing substance. Light scattering occurs when light collides with matter which causes the light waves to spread out in three-dimensional space.

Fluorescence and Fluorescent Microscopes

A specific way in which pi-bond electrons are used in microscopy is through the concept of fluorescence. Fluorescence occurs when light absorbed by an electron causes that electron to rise to an excited state, and then emits some of the energy when dropping back to the ground state. The energy released in this transition from excited to ground comes in the form of a photon of light with a longer wavelength than the incident light used to excite the electron. Fluorescence is important to microscopy, as many different kinds of microscopes exist to take advantage of fluorescence. When compared to microscopes that use light directly from the source to send the image to the observer, fluorescent imaging techniques instead rely on the light being emitted from the source of the fluorescence. The advantage this provides is that the emitted light is

traveling in all directions, rather than the direction of the incident light, and also has a longer wavelength than the incident light as well. This allows for the eyepiece to be positioned at an angle perpendicular to the direction of the light source, as well as facilitates the use of filters to select specific wavelengths of light. These features of fluorescent microscopes prevent light that is bright and unfocused from reaching the observer, creating images that are clear and easily visible. The fluorescence of a sample is also dependent on the quantity of fluorescent molecules within that sample, which means that fluorescence can also be used for quantitative measurements.

In biology, another advantage of fluorescence is that compounds capable of fluorescence can be introduced into an organism, for a wide variety of applications. An important factor in why fluorescence is used for biological experiments is that many types of fluorescent molecules exist that are not cytotoxic, allowing for active monitoring of living organisms. Fluorescent probes can also be designed to recognize specific sites within a cell, such as if an antibody is used with a fluorescent molecule attached to it. Highly specific probes allow for the determination of the location of specific targets and can be used to monitor the movement of those targets during a time-lapse. Many different kinds of fluorescent proteins exist, so DNA can be edited for the expression of these kinds of proteins. This means that organisms that naturally do not fluoresce much or at all can be genetically modified to fluoresce, making them easier to see under a microscope. During this program, several different fluorescent microscopes were used, which include spinning disk confocal microscopes, scanning laser fluorescent microscopes, and light sheet microscopes.

Transfection of NIH3T3 Cells with Plasmids Containing Fluorescent Protein Genes

One strategy to genetically modify a eukaryotic organism is to introduce foreign DNA that contains the gene of interest into the nucleus of a cell so that it is used by the organism's own DNA transcription system. One type of technique to accomplish this goal is known as transfection, which is defined as the addition of nucleic acids to the nucleus of a cell artificially, and without the help of a viral infection. One type of DNA vector commonly used for genetic engineering via transfection is the plasmid, which is a circular sequence of DNA commonly found in bacteria. The advantage of using plasmids is that they can be easily edited with restriction enzymes and ligation proteins, and are quickly cloned with the help of bacterial culture. After culturing bacteria such as *E. coli* that contain the edited plasmid, a high number of plasmids can be extracted for future transfection.

To demonstrate the introduction of an edited plasmid carrying genes for the expression of fluorescent proteins into a cell, a plasmid will be allowed through the cell's outer membrane and inner nuclear membrane to be placed in a position to be transcribed. In this experiment, Lipofectamine LTX reagent from a Thermo Fisher kit will be used to perform the transfection using the procedure provided by Thermo Fisher. Lipofectamine LTX reagent is a transfection agent that utilizes lipid vesicles that surround plasmids and are taken into the cell through endocytosis to transport the plasmids into the nucleus for transcription and is known for low toxicity and high efficiency. NIH3T3 cells will be transfected with plasmids containing genes for the fluorescent proteins mNeonGreen, mCherry, and TaqBFP. Plasmids were extracted from *E. coli* with a Nippon Genetics FastGene Plasmid Mini Kit. Gel electrophoresis will be used to confirm that the plasmids are the correct size. After transfection, the cells were left in culture for about 72 hours, and the cells with new DNA were monitored under a Leica SP8 microscope to confirm that the genes for fluorescent proteins were successfully transfected.

Plasmids containing mNeonGreen, mCherry, heat shock response factor, and hygromycin resistance genes

Before transfection, the plasmids being transported to the cell's nucleus need to be constructed from other existing plasmids to create the desired effect in the transgenic organism. Before lab work could begin, the ApE plasmid editing software was used to determine that the YK6 backbone plasmid would be used since it would provide the backbone of the final plasmid, the HygR hygromycin resistance gene, as well as contain the mouse HSP70 promoter and SV40 poly-A promoter needed for transcription. In addition to the YK6 plasmid, plasmids containing genes for the mNeonGreen and mCherry fluorescent proteins will also be used. The restriction enzymes BamHI, HindIII, and XbaI will be used for their compatibility with the plasmids being restricted and to prevent the restriction of important genetic sequences. A Nippon Genetics FastGene Plasmid Mini kit was used to extract and purify plasmids for restriction. Gel electrophoresis was used to confirm the success of restriction and ligation. After gel electrophoresis, the correct plasmid fragments need to be identified based on the estimated fragment size from the ApE computer diagram and will be ligated after extraction. Once the plasmids are ligated, *E. coli* will be used as the subject of transformation so that the plasmids can be cloned for later transfection into medaka.

Cross-Linking of Laminin to Glass Slides

During cell culture, cells are normally seeded on plastic plates for proliferation. However, cells grown on culture plates may not behave similarly to cells within the human body due to the

lack of an extracellular matrix. In this experiment, a glass slide is modified to resemble the extracellular matrix by crosslinking laminin to its surface. This is achieved by utilizing RCA cleaning and plasma treatment to make the surface of a glass slide highly hydrophilic. The hydrophilic glass is then treated with vinyltrimethoxysilane to coat the surface of the glass with vinyl groups, making the glass slide highly hydrophobic. After the addition of the vinyl groups to the surface of the glass slides, the slides are coated with an acrylamide-based gel that covalently bonds to the vinyl groups, creating an acrylamide-coated slide. Through treatment with sulfo-SANPAH and laminin, the laminin used during the experiment crosslinked with the acrylamide surface of the glass, creating an environment designed to better replicate the extracellular matrix than a standard cell culture plate. To investigate the difference in cell behavior on a laminin-coated plate compared to a standard cell culture control plate, images were taken of 40 individual cells per experimental group, and the area and aspect ratio of each cell was measured using ImageJ. The stiffness of each plate was also measured with an atomic force microscope, to compare the change in gel stiffness to cell behavior.

IR-LEGO treatment of mCherry medaka

Within cells, there exists a mechanism known as the heat shock response that is triggered when the cell requires defense against dangerous environmental conditions such as heat. The heat shock response is activated when heat shock response factor proteins are activated by heat, and then act as a transcription factor for proteins that are meant to protect the cell from the factors threatening it. In IR-LEGO, a cell can be genetically engineered to contain sequences within its DNA that are transcribed when the heat shock response factors are activated. The purpose of this modification is to grant control of the timing and location of the expression of

specific genes to the researcher. By directing an infrared laser at the region of interest, heat can be generated within a localized area to activate heat shock-mediated gene expression without killing the cells (Kamei 2009). Medaka carrying the genes for the expression of mCherry via heat shock transcription factors were subject to IR-LEGO to activate the fluorescent protein genes within the cells of the fish. Both skin and muscle tissue were subjected to lasers that ranged in power from 20.6 mW to 24.9 mW. To confirm that the activation of gene expression was successful, light sheet microscopy was used to image the regions of the medaka's body that were subject to the laser.

Experimental Procedures

Transfection of NIH3T3 with plasmids for expression of fluorescent proteins

To transfect plasmids into NIH3T3 mouse embryonic fibroblast cells, the cells first needed to be seeded at the correct confluence for the experiment. Before the transfection, a culture of NIH3T3 cells in DMEM culture media had to be passaged to achieve the correct number of cells for transfection. For cell passaging for a 6 cm cell culture dish, the DMEM media was first aspirated from the culture. The plate was then washed with 4 mL of PBS, which was equal to the original volume of cell media in the plate. After the PBS wash, the PBS was aspirated and replaced with 1 mL of 0.05% trypsin solution. After 5 minutes of treatment with trypsin, the cells had become suspended and were transferred with a pipette to a tube for centrifugation. The cells were centrifuged for 3 minutes at 1200 rpm. After centrifugation, the trypsin was removed from the cell pellet at the bottom of the tube, and the cells were

resuspended in 5 mL of DMEM. The cells were counted using a hemocytometer so that enough cells were seeded for 70-90% confluency after a weekend in an incubator at 37 degrees Celsius.

To obtain plasmids for transfection, the protocol from the Nippon Genetics FastGene kit was followed. *E. coli*. which had been modified to contain plasmids of the expression of the fluorescent proteins mCherry, mNeonGreen, and TaqBFP was cultured before the experiment and was allowed to proliferate on agar to form colonies. After colonies formed, 4 colonies for each fluorescent protein were picked up and transferred to separate tubes of LB broth and centrifuged at 20000 rpm for 2 minutes. After centrifugation, the LB broth was replaced with 200 μL of mP1, and vortexed until dissolved. 200 μL of mP2 was added to each tube, and the tubes were inverted gently. 300 μL of mP3 was added to each tube and the tubes were inverted again. The tubes were then spun down at 13000 rpm for 2 minutes, and the supernatants were transferred to spin columns. The spin columns were spun down at 13000 rpm for 2 minutes, and the flowthrough was discarded. 400 μL of mP4 was added to each spin column, and the columns were spun down at 13000 rpm for 30 seconds twice, with the flowthrough being discarded both times. 600 μL of mP5 was then added to each spin column, and the columns were down at 13000 rpm for 30 seconds. The column membranes were then dried by spinning them down at 13000 rpm for 2 minutes. To elute the plasmids, 50 μL of mP6 was added to each column, and the membranes were incubated for 2 minutes at room temperature. The columns were then spun down at 13000 rpm for 2 minutes, and the flowthrough was kept for each column. The concentration of each type of plasmid was then measured using a NanoDrop instrument.

After measurement of plasmid concentration, gel electrophoresis was used to determine if the plasmids had the correct genetic sequence before transfection. Before electrophoresis, 10 μL of restriction enzymes were added to each sample, and incubated overnight at 37 degrees

Celsius. 1% agarose gel with tris-acetate-EDTA was prepared in a mold to create the wells where the plasmids would be loaded. Before loading, 20 μL of each sample was mixed with 4 μL of 6X Loading Dye to make them visible in the gel. The gel was run at 100 V for 10 minutes.

After gel electrophoresis confirmed that the plasmids were the correct size, 2500 ng of plasmid from each sample was mixed with 125 μL of OptiMEM, 7.5 μL of LTX, and 2.5 μL of Plus Reagent. The DNA and LTX mixtures were incubated for 5 minutes at room temperature and were added to 2 mL of DMEM culture media. The NIH3T3 cells cultured to 70-90% confluency were then given the LTX culture media for 1 day of incubation. Normal DMEM media replaced the LTX cell culture media, and the cells were incubated for another 3 days. After the incubation period, a Leica SP8 microscope was used to image the cells to determine if the fluorescent proteins were being expressed.

Plasmids containing mNeonGreen, mCherry, heat shock response factor, and hygromycin resistance genes

Before the editing process began in the lab, the ApE software was used to model the individual plasmids that would be cut to create the final product. The YK43 backbone plasmid, the YK6 plasmid encoding for HygR hygromycin resistance, and the plasmids containing the mCherry and mNeonGreen fluorescent proteins were modeled to determine the best restriction enzymes for cutting without damaging the target genes. For the first attempt at restriction, the plasmids Kpn1, HindIII, and XbaI were chosen. A negative control run in parallel with *E. coli* that were given plasmids without DNA inserts showed that the plasmids did not re-ligate on their own.

For the first round of editing, the plasmids for the backbone and HygR gene were chosen and would be cut with KpnI, XbaI, and HindIII. After the plasmids and restriction enzymes were incubated for about 3 hours with CutSmart buffer, the restricted DNA fragments were run through gel electrophoresis. After determining the locations of the target fragments in the gel by their size, the protocol of a FastGene gel extraction kit was followed to purify the plasmids after gel electrophoresis. The extracted plasmids were then allowed to ligate at 16 degrees Celsius for 30 minutes. After ligation, the DNA was transfected to *E. coli* through temperature shocking the bacteria in the presence of the plasmids. After the *E. coli* had taken up the new DNA, it was spread across agar gel culture plates to incubate overnight. After the *E. coli* had proliferated and formed colonies, the plasmids were extracted from the bacteria using the protocol from the Nippon Genetics FastGene extraction kit. After extraction, the concentration of DNA for the mCherry and mNeonGreen plasmids was measured using a NanoDrop instrument.

For the second round of restriction, the newly formed plasmids and the plasmids for hygromycin resistance were restricted with the same restriction enzymes as the first round. The procedure was followed for the second round of restriction as with the first round. After the new fragments were ligated to the backbone, PCR was run on the plasmids for about 1.5 hours, with 1-5 minutes for the initial stage of denaturation, and about 20-40 cycles of denaturation, annealing, and extension of the DNA. After this round of plasmid editing, the negative control came back alive, which showed that no DNA was inserted and that the procedure needed to be changed.

After returning to the ApE software to find a different approach to the design of the plasmid editing procedure. It was determined that a procedure would be followed where the YK6 plasmid would be used as the source of the HSP70 promoter and the HygR gene and would have

the fluorescent protein genes inserted into it. The BamHI, HindIII, and XbaI restriction enzymes were chosen to perform the restrictions. The same procedure as the first attempt was followed for restriction, extraction, and transformation to produce *E. coli* with the edited plasmids.

Laminin-coated glass slides

To begin preparing the laminin-coated slides, glass slides first need to go through RCA cleaning to make them highly hydrophilic. First, glass slides are immersed in acetone, ethanol, methanol, and MilliQ water one at a time, and each time for 3 minutes in an ultra-sonicator. After these steps, the slides are immersed in a 1:1:5 mixture of hydrogen peroxide, ammonium, and MilliQ water for 3 minutes with ultra-sonication. The slides are then put in a water bath for 30-60 minutes at 60 degrees Celsius. After the water bath, the slides are rinsed with MilliQ water 10 times and are then dried in a vacuum oven at 70 degrees Celsius overnight.

For silanization, the hydrophilic glass slides are placed in a vinyltrimethoxysilane solution (190 mL toluene, 10 mL vinyltrimethoxysilane) and a shaker overnight. The solution is then removed and replaced with pure toluene and is ultra-sonicated for 3 minutes. The slides are then ultra-sonicated in pure ethanol and then in MilliQ water for 3 minutes each. The slides are then rinsed with MilliQ water 10 times and are then dried in a vacuum oven at 70 degrees Celsius overnight. The glass slides subject to this reaction should be coated in vinyl groups and highly hydrophobic.

To bind acrylamide gel to the hydrophobic slide, a 10 mL stock solution (Stock A) of 16% w/v acrylamide (AA) was prepared by mixing 1.6 g of AA with 10 mL water. Another 10 mL stock (Stock B) of 16% w/v acrylamide and 1.6% w/v bis-acrylamide (bis-AA) was prepared

by mixing 1.6 grams of AA and 0.16 grams of bis-AA with water. Mixtures of (16%, 0.8%), (16%, 0.4%), (16%, 0.2%), and (16%, 0.1%) AA to bis-AA were prepared by mixing (500 mL A, 500 mL B), (750 mL A, 250 mL B), (875 mL A, 125 mL B), and (937.5 mL A, 62.5 mL B) together. Stages were prepared on wet paper towels, and the hydrophobic glass slide was placed on the stage. 4 μL of APS and 0.9 μL of TEMED were added to 200 μL of an (AA, bis-AA) solution, and the solution was vortexed for 10 seconds. 50 μL of the solution was pipetted onto the silanized slide, and the AA mixture was pressed flat with a hydrophilic glass slide. The gel mixture was allowed to sit for 15 minutes until the hydrophilic slide was taken off. This procedure was performed for each concentration of bis-AA, and each acrylamide gel slide was rinsed with MilliQ water for 1 hour 3 times. After the slides were rinsed, an atomic force microscope (AFM) was calibrated with a uniform glass slide. The gel was inserted into the AFM and the stiffness was measured by testing three locations per slide and finding Young's modulus for each location.

To begin the process of cross-linking, the acrylamide gels were first stuck to culture plates with silicon-based glue. The gel slides were then rinsed with HEPES buffer under UV light for 15 minutes. Sulfo-SANPAH was prepared by first preparing 56 μL aliquots of 50 mg/mL solutions of sulfo-SANPAH in DMSO. 504 μL of HEPES buffer was then added to an aliquot of sulfo-SANPAH, and 140 μL of the mixture was pipetted on each gel, and placed under UV for 8 minutes, with light shaking after 4 minutes. After treatment with sulfo-SANPAH, the gel slides were rinsed with 2 mL of HEPES buffer 4 times, 2 mL of PBS 4 times, and again with 2 mL of HEPES buffer 4 times. Afterward, the slides were rinsed again with 4 mL of PBS at 4 degrees Celsius with shaking for 15 minutes 4 times. 430 μL of laminin solution prepared before the experiment was then added to each gel slide, and the slides were placed in a 4 degrees

Celsius shaker overnight. The laminin was then aspirated, and 4 mL of 10 mM tris buffer was added to the plates, and they were put in the 4 degrees Celsius shaker overnight.

Once rinsed, the laminin-coated plates were seeded with 20000 cells each from a stock culture. After the cells were given time to adhere to the plate, they were imaged with an Olympus SZX16 microscope. Enough pictures were taken of the cell culture so that 40 individual cells could be counted. After the images were taken, ImageJ was used to measure the area and aspect ratio of the cells.

IR-LEGO in mCherry medaka

To begin IR-LEGO, the instrument was first turned on, and the focus plane was adjusted to focus the infrared laser. The power of the laser was also adjusted by changing the settings of the power source and emission filters. Before the medaka were inserted into the instrument, the laser was turned on to measure the laser strength in room conditions. The laser's intensity was measured so that it was possible to use powers of 15X (23.9 dB), 17.5X (24.3 dB), and 20X (24.8 dB). 4 sample medaka larvae and 2 control medaka larvae (positive and negative) containing heat shock response factor-activated RFP and GFP fluorescent protein expression were collected from their tanks and anesthetized in MS222. Once the fish was not responding to physical stimuli, it was placed under the laser. The laser was directed so that 3 locations in both muscle and skin tissue were hit with the three different laser strengths in each fish.

The activation of fluorescent proteins in the medaka was first checked after the IR-LEGO procedure with an Olympus SZX16 microscope. The positive and negative controls were checked first, followed by the fish treated with IR-lEGO. To prepare fish for viewing under the

microscope, the medaka were anesthetized in MS222, and physically immobilized in viscous methyl cellulose solution. Due to difficulty in identifying fluorescence attributed to IR-LEGO, the experiment moved on to the Leica SP8 microscope for stronger magnification and resolution. After using the Leica SP8 and not seeing a clear pattern of fluorescence from IR-LEGO, a Zeiss Z.1 light-sheet microscope was used to make a 3D model of the fluorescence emitted by the medaka. Preparing the medaka for the Zeiss Z.1 first required anesthetizing the medaka in MS222. The anesthetized medaka was then drawn into a syringe, which was then inserted into the sample holder for the microscope. Once the medaka was in the instrument, the correct region of the body was found by moving the sample holder in 3-dimensions, which was followed by imaging.

Results

Transfection of NIH3T3 with plasmids for expression of fluorescent proteins

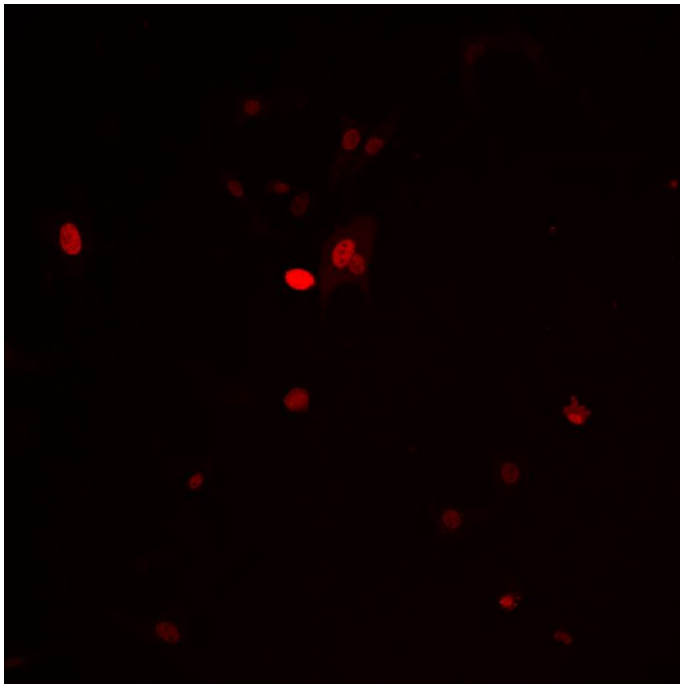


Figure 1. NIH3T3 cells transfected with plasmids carrying the mCherry fluorescent protein gene.

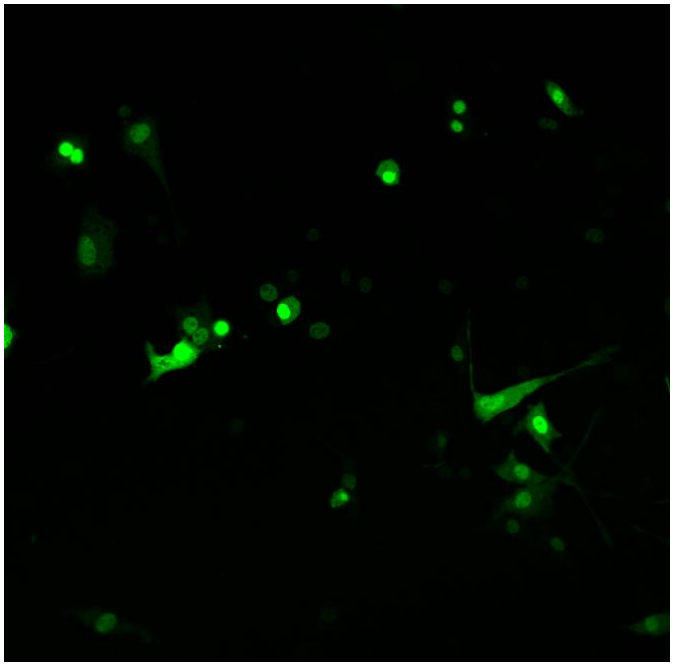
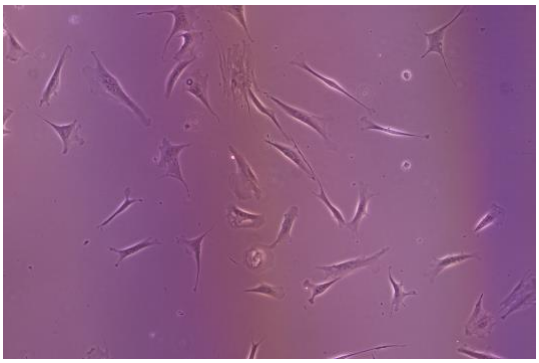


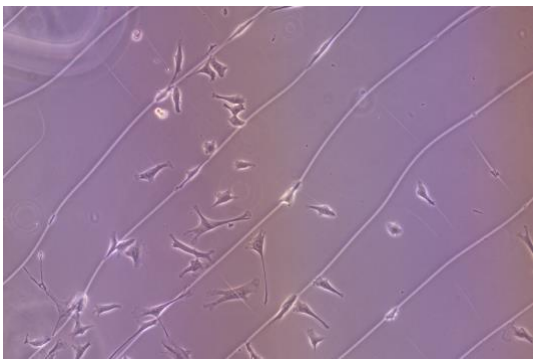
Figure 2. NIH3T3 cells transfected with plasmids carrying the mNeonGreen fluorescent protein.

Cross-Linking of Laminin to Glass Slides

A Glass



B 0.05% bis-AA



C 0.1% bis-AA

D 0.2% bis-AA

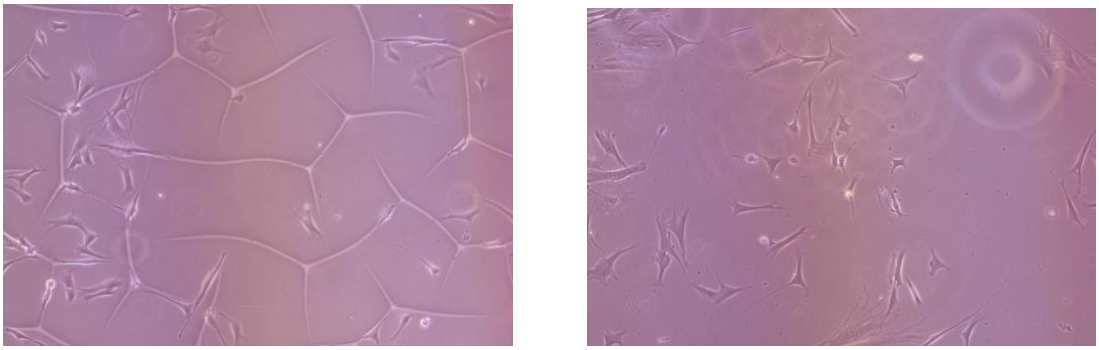


Figure 3. Cells cultured on gels of differing thickness.

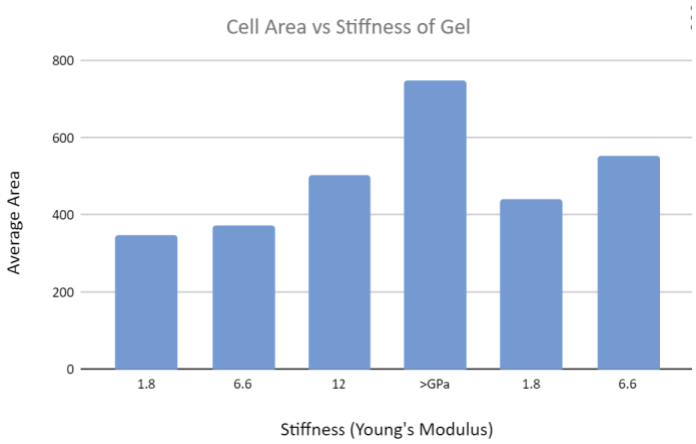


Figure 4. Results of AFM measurement of stiffness versus cell area measurements.

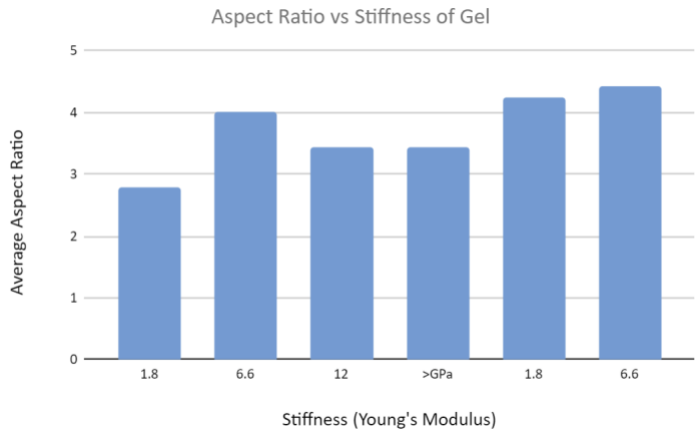


Figure 5. Results of AFM measurement of stiffness versus cell aspect ratio measurements.

IR-LEGO treatment of mCherry medaka

A - Positive Control B – Negative Control C – 20 mW IR-LEGO

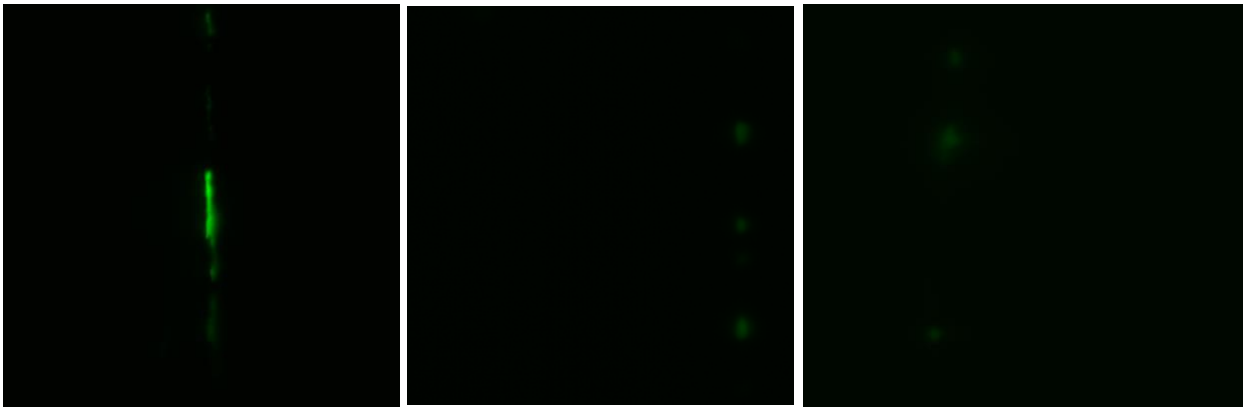


Figure 6. Results of Light Sheet Fluorescence Microscopy Imaging of Medaka that had been Subject to a 20 mW IR Laser

Discussion

Transfection of NIH3T3 with plasmids for expression of fluorescent proteins

After the imaging of the NIH3T3 cells with the Leica SP8 scanning laser confocal microscope, it was observed in Figures 1 and 2 that the cells had expressed the fluorescent proteins that corresponded to the gene that each plasmid carried. In Figure 1, the cells that were transfected with the plasmid for mCherry expressed fluorescence within the range of 600 nm after being excited with light at around 588 nm. In Figure 2, the cells that were transfected with the plasmid for mNeonGreen expressed fluorescence within the range of 520 nm after being excited with light near 500 nm. This experiment demonstrates the successful transfection of a

bacterial plasmid into a eukaryotic cell and also shows the practicality of fluorescence in biological research.

Plasmids containing mNeonGreen, mCherry, heat shock response factor, and hygromycin resistance genes

The final product of this experiment was a plasmid that contained the genes for the heat shock promoter, mCherry and mNeonGreen fluorescent proteins, and the hygromycin gene. After completing the procedure, the plasmids were ready to use for transfection or microinjection in a future experiment. This experiment demonstrated the procedure for DNA restriction, ligation, and transformation, as well as provided hands-on experience in gel electrophoresis, and using the technique qualitatively to confirm the results of gene editing.

After initially not being able to follow through on the original plan for restricting the plasmids with XbaI, KpnI, and HindIII, another procedure was created to adjust for possible sources of error in the previous experimental design. A possible source of error in this experiment was the proximity between restriction sites, as not enough space may have been allowed for more than one restriction enzyme on the plasmid at certain points during the restriction procedure. This experiment was important, as it demonstrated the flexibility of plasmid restriction, where if one strategy does not provide an intended result, another type of plasmid or restriction enzyme can be considered to achieve the same purpose.

Cross-Linking of Laminin to Glass Slides

From the stiffness data presented in Figures 3 and 4, it can be observed that gels that are less stiff lead to cells that are smaller and have a more rounded shape. This change in cell shape at different stiffnesses shows that cells respond to different environments. This means that to better model cell behaviors, cells in cultures could be placed in environments more similar to the human extracellular matrix than a flat plastic surface. In the future, the different combinations of acrylamide and bis-acrylamide concentrations could be explored to determine which one is the most effective at improving accuracy to the human body. Different types of extracellular matrix factors other than laminin could also be explored.

IR-LEGO in mCherry medaka

At the end of this experiment, no change in fluorescence in the medaka could be identified. Possible reasons for the unintended results could be that the laser intensity was too strong and killed the cells rather than harmlessly triggering the heat shock response. Another factor could be that not enough time was given to the cells to divide and proliferate to create a signal visible to the naked eye. In the end, several techniques were demonstrated throughout this experiment, including the general operation procedure of IR-LEGO, and how to use a light sheet microscope to create a 3-dimensional image of the fluorescence of a medaka.

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References

Deguchi, Tomonori, Mariko Itoh, Hiroko Urawa, Tomohiro Matsumoto, Sohei Nakayama, Takashi Kawasaki, Takeshi Kitano, et al. “Infrared Laser-Mediated Local Gene Induction in Medaka, Zebrafish, and Arabidopsis Thaliana.” *Development, Growth & Differentiation* 51, no. 9 (2009): 769–75. https://doi.org/10.1111/j.1440-169X.2009.01135.x.

Kamei, Yasuhiro, Motoshi Suzuki, Kenjiro Watanabe, Kazuhiro Fujimori, Takashi Kawasaki, Tomonori Deguchi, Yoshihiro Yoneda, et al. “Infrared Laser–Mediated Gene Induction in Targeted Single Cells in Vivo.” *Nature Methods* 6, no. 1 (January 2009): 79–81. https://doi.org/10.1038/nmeth.1278.