Detecting Gene Expression *In-Vivo* Using Differential Laser Absorption

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Abstract:

The goal of this thesis is to find the best and most cost-effective laser based technique available for imaging gene expression for genetically manipulated (transgenic) mouse brains *in vivo*. While there are quite a few techniques for imaging *in-vivo* gene therapies, such as, Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET), these and similar protocols have problems imaging microscopic processes. Therefore the decision was made to implement a laser based optical imaging system with Differential Laser Absorption being the version. Other techniques, specializing in either *in-vitro* or *in-vivo* imaging are addressed with their advantages and disadvantages.

Introduction:

The goal of this thesis project is to be able to detect develop a system that would be able to detect the gene expression of new genes introduced into the brain of a mouse through gene therapy. Before addressing how this goal is to be accomplished, a background on the uses, goals and problems with current gene therapy technology is needed.

The goal of gene therapy is to supplement or replace the function of mutated genes with the correct genetic information.[1] Gene Therapy was first envisioned as a treatment for just inherited diseases and disorder; however the current view is that any genetic disorder, from cancer to arthritis, could be treated in the future using some implementation of this type of therapy. Currently there are two protocols for that have been developed for gene therapy: *ex vivo* and *in vivo*.[1] In *ex-vivo* version of gene therapy, the subjects cells are extracted with the new genes being inserted in vitro, then reinserted into the subject. In the *in vivo* method, the new genetic material is inserted directly into the necessary place.

There are problems, however, with current gene delivery technology. Two things can summarize these issues: lack of cell specific targeting and low transmission and transfection efficiency. These two issues are very closely connected. The lack of cell specific targeting is an is self explanatory while the low transfer efficiency basically means that a great deal of excess genetic material needs to be introduced into the system of the subject in order to have a hope of effectiveness. This problem will be greatly lessened as ability to specify genes improves.

Next comes the issue of detection of the success or failure of gene therapy. Typically, a marker or reporter genes is used to "report" the quality of the effectiveness of the transfer of the

genetic material. These marker genes are usually foreign; do not naturally occur in the host, where the gene therapy is applied to allow for easier detection. There are issues with using foreign marker genes. The biggest problem comes from a possible immunogenic or the immune system response. If the immune system acts to eliminate the foreign marker gene, then detection of the quality of the gene transfer could be limited.[1] There is a method available that allows one to detect whether or not the gene therapy was successful and gene expression is occurring without having marker genes. When the fully functioning genetic material is introduced into the subject, it also includes the ability to code for a specific protein that has fewer immunogenic properties. Now that we have addressed what gene therapy is, now is the time to address the current technologies used to detect and image the marker genes or protein expressions.

Molecular imaging is a relatively new and emerging field that deals with the imaging and detection of diseased tissue on the cellular and genetic level rather than on the macroscopic level. The future goal is to easily examine molecular processes and gene expression of cells, in their natural and living state, without causing physical harm to the organism. However, the primary method currently used to test for gene expression is tissue analysis.[1] Tissue analysis examines macroscopic physical, physiologic, and metabolic changes for tissue differentiation between normal and diseased tissue instead of identifying the DNA mutations or specific DNA sequences responsible. Furthermore, many *in vitro* analytic techniques that do focus on such genetic factors are either not applicable or not as effective in vivo. Current *in vitro* analytic techniques of genetic factors include Northern Blot for RNA, Southern Blot for DNA, Western Blot and immunostaining for protein expression, and polymerase chain reaction (PCR) and restriction fragment length polymorphism mapping (RFLP) for DNA fingerprinting.[1]

Blotting is the transfer of nucleic acids and/or proteins from a gel strip to a specialized, chemically reactive matrix on which the nucleic acids, etc. may become covalently bound in a pattern similar to that present in the original gel. Since this can only happen externally, this method cannot be adapted for our purposes. Immunostaining is the testing of sections of tissue for specific proteins after removal by applying a dye as an indicator. This also is strictly inapplicable. With PCR, it is possible to amplify small amounts of DNA for DNA fingerprinting, to map genomes, and to isolate specific genes. PCR, however, requires slicing of DNA sequences and centrifuges out DNA, which will destroy the cell. This is also is not feasible for use in living cells. RFLP is done when DNA is cut or digested into fragments by a restriction enzyme, then the fragments are separated and then blotted. This also cannot be done when imaging gene expression *in vivo.*

Examples of techniques designed specifically with *in vivo* imaging are single photon emission tomography (SPECT), positron emission tomography (PET), and Magnetic Resonance Imaging (MRI). These imaging methods, however have their own problems. Both SPECT and PET techniques are radionuclide techniques, which means that they using ionizing radiation in order to detect and image. While both techniques have a high enough resolution in order to detect and image the protein produced by the expressing gene, the radiation could be too much could be too much for extended exposure to the subjects.[5] While the MRI imaging technique does not have the same radiation issues, the problem here is that MRI imaging does not currently have the resolution necessary to image the gene expression.[1]

There is a method of detection could work for our purposes. Optical imaging and detection by light absorption can and has been used for gene expression. The protocol for this type of optical imaging is placing the region where gene therapy occurred between the laser and a

detector. The protein produced when the specific gene is expressed absorbs the laser beam. (See Figure 1) This allows for the relatively easy detection of gene expression. If gene therapy was successful, then less light intensity would be detected upon leaving the subject then was originally beamed at the subject.

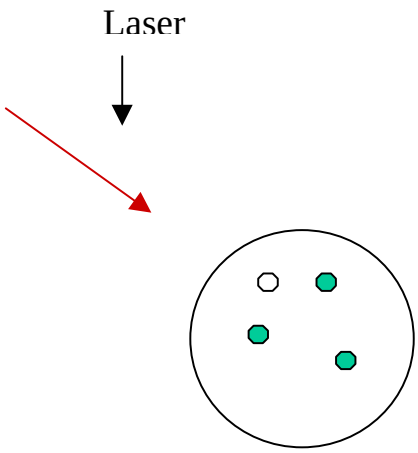

Figure 1: Partial diagram used to show principle of laser absorption. White circle is non expressing gene while green is protein from expressing gene.

However, this model is over simplified and does not take into account real world problems. There are two serious problems with the present model: 1) Neither humans nor mice are transparent and 2) There is always severe scattering.

There are two basic reasons why some objects are not transparent. One reason is that the object absorbs all the visible light that impacts it. The second reason is that light is strongly scattered. This is the case with such items as milk, foam, and white paint. The vast majority of objects fit in the second category.[6]

The issue of absorption by the skin is a serious problem when trying to image using optical methods. Imaging using visible light is severely limited due to the high coefficient of absorption of hemoglobin, a large component of blood. Hemoglobin has its highest coefficient of absorption

at wavelengths below 650nm. Visible light imaging is limited to only a few hundred microns. This is vastly smaller than the diameter of a mouse head, which is approximately 2 – 4 cm.[2] Moving to the use of infrared light, above 900nm, is equally problematic. At these wavelengths, water has a very high coefficient of absorption. The solution here is to use Near Infrared Light (NIR) or the 800 nm window (See Figure 2). Both hemoglobin and water have their lowest coefficients of absorption in this region of light. In this region living tissue becomes basically transparent. It has been experimentally shown using microwatt lasers that NIR light can travel at least 4-cm. of skull and brain tissue or deep muscle. This is a long enough length to image the processes in a mouse brain.

Within this wavelength window, the dominant problem to the detection of gene expression is the scattering of light. The problem of light scatter is not as simply solved as the absorption issue. There is no wavelength of light were the scattering of light is significantly reduced. The scattering coefficient is the multiplicative inverse of the random-walk step length, which is approximately 1 micron in human tissue.[6]

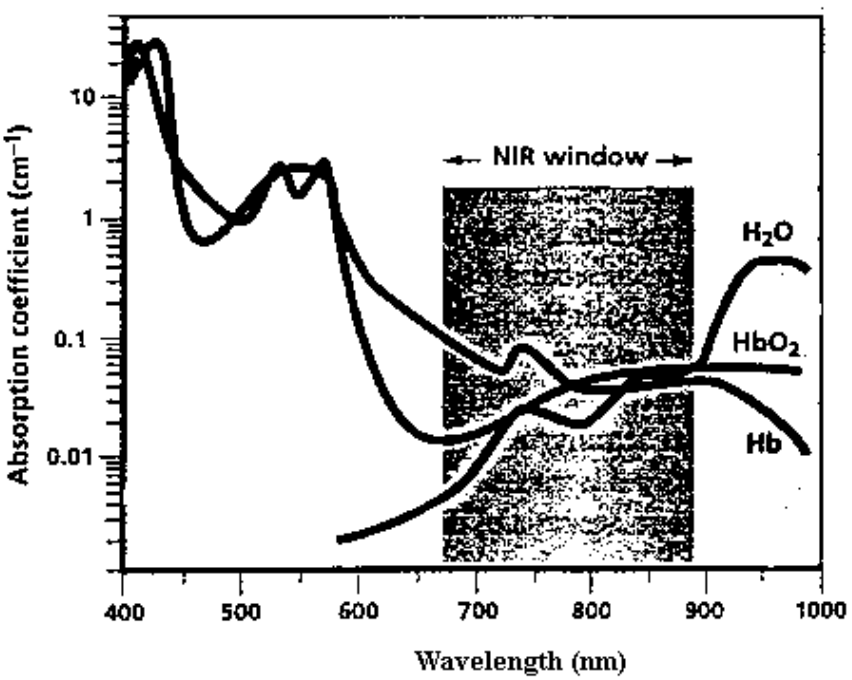

Figure 2: NIR Window, this shows how hemoglobin Hb and water H_20 have their lowest coefficients of absorptions within the window between 650 to 900nm.

The random walk step length is the distance that light can travel upon encountering an object before the light beam is changes directions or is scattered. With a random walk step length so short, there is very little that can be done minimize scatter with the single laser absorption design. Since this is the case, a bigger question must be asked next: How does one differentiate between light that was scattered versus light that was absorbed by the protein produced when specific genes were expressed. One cannot really tell the difference using a single laser absorption system.

The solution is to use a two-laser system. While there are various two-laser protocols available, the plan is to use a differential laser absorption technique to detect the dye produced by a specific protein, when it is expressed. (See Figure 4)

My differential laser absorption system would work in the following way. The two lasers would be oriented at 90-degree angles from each other. Both would be aimed at a dicroic mirror, which would allow both beams to travel approximately the same distance. The two lasers with

only a slight wavelength difference, one with a wavelength equal to the max absorption wavelength of the dye produced by protein when the gene is expressed and the other with a wavelength slightly off the max absorption wavelength. Both lasers would be within the 800 nm window. Both lasers would have the same intensity. Instead of the one detector that would be necessary for a single laser setup, now two detectors are necessary. Due to the wavelength of the light being similar and the intensity of the beams being the same, the "difference" in the beams of light reaching the two detectors could be associated with the beams being absorbed by the special dye. Scattering is no longer a big issue because both laser beams should undergo the same amount of scattering thereby canceling its affects.

Next the scientific literature was checked to verify the feasibility of goals of this project. According to the current scientific literature on a similar laser system, Frequency Modulated (FM) Spectroscopy, [3] a continuous wave laser can be used to detect absorptions as small as 10^{-3} - 10^{-4}. The 10^{-3} number is used in order to be conservative. (In FM Spectroscopy, a laser is modulated; then differences in the differential absorption between the two-sideband waves are detected as absorption).

Experimental Setup

To begin the experiment, I started out by first designing a single laser system to test the precision of my light detector circuit detection that could be obtained by a simple setup. The goal is to see how closely the beam intensities could be but still be able to detect that they are different. (See Figure 3)

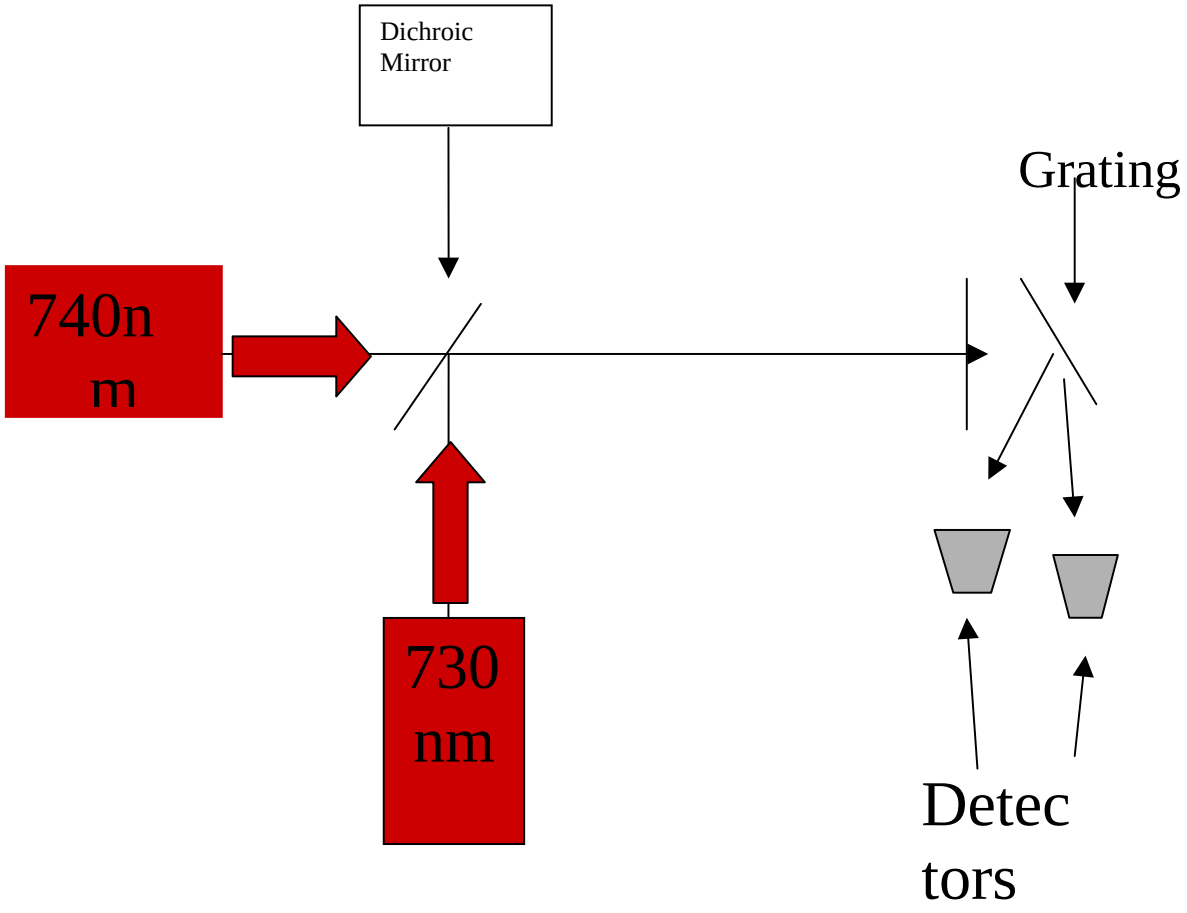

Figure 3: Possible Differential Laser Absorption setup

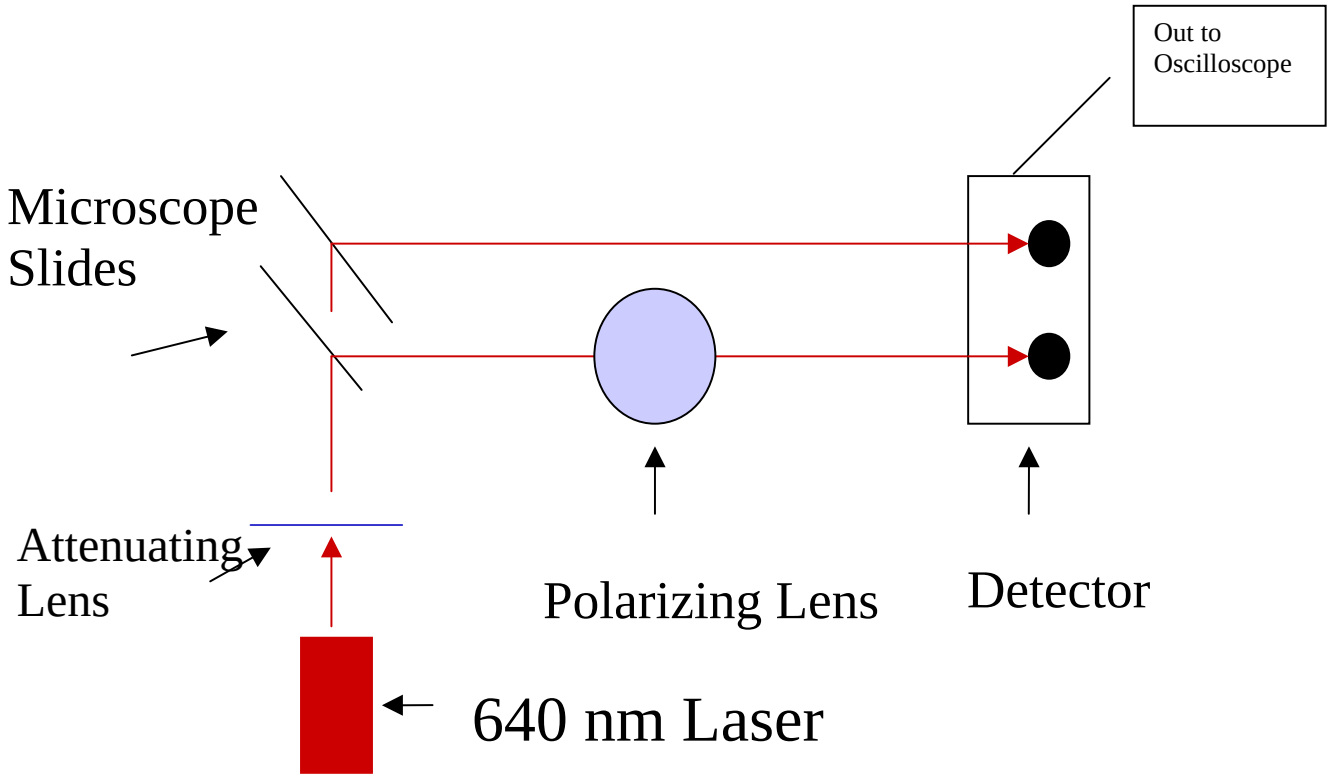

Figure 4: Single Color Laser Setup

The setup consists of a single laser whose beam was first sent through an attenuating lens, for the beam was saturating the detector at the end of the beam's path. Next the beam is reflected off of a microscope slide in order to further attenuate the beam and to split the beam into two separate beams. In this setup, one beam is traveling farther than the other therefore the beam traveling the shorter distance needed to be decreased by a relatively small amount. This was accomplished by mounting a polarizing lens on a rotating stage where degrees were clearly marked. I was able to rotate the circular platform to which the polarizing lens was attached until the oscilloscope read that the intensity of the two beams were the same and slightly alter change until they were no longer equal and see the difference in the oscilloscope.

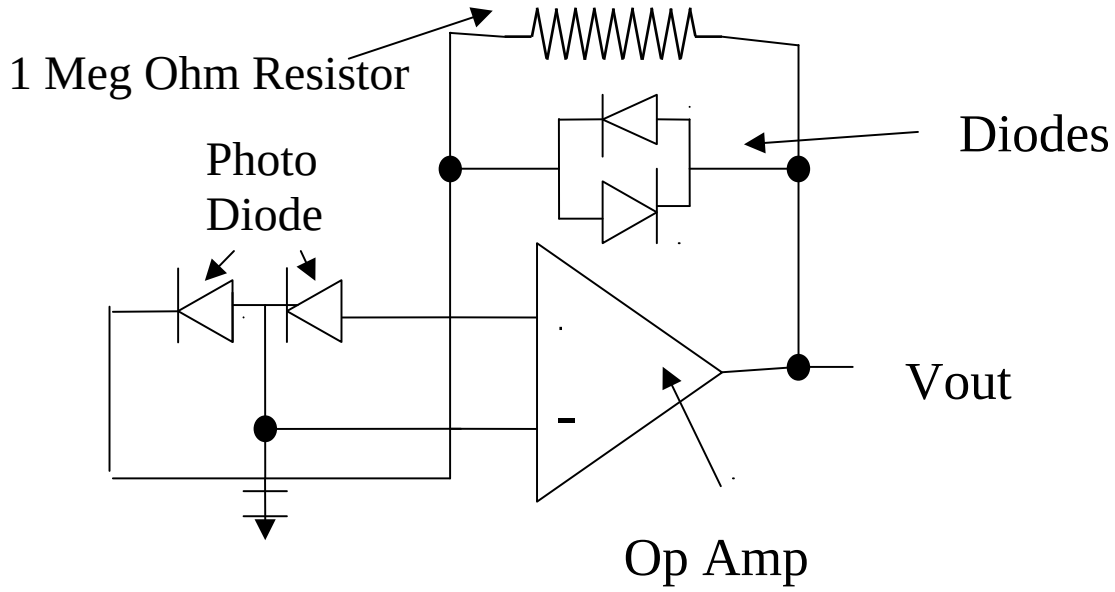

Figure 5: Electronics Setup for Light Balance Detector

Figure 5 is a blown up look at the detector at the end of my setup. This light balance detection circuit works in the following manner. The output voltage is zero if the amount of light entering the two photodiodes is equal. Feedback resistance determines the photoelectric sensitivity. The two regular diodes are placed in opposite and in parallel to each other. This is done so that the output voltage that is seen by the oscilloscope is limited to +/- .5V. This allows the sensitivity to be very high near the balanced state.

Experimental Steps

There were three steps done in order to calculate how fine a difference in intensity between the two laser beams that I was able to detect with the oscilloscope. First, I detected the angle where the front beam, which was shown through the polarizing lens, was completely blocked by the polarizing lens. This became my $\ominus_0$, $\ominus_0$ = 206 degrees. Next I measured how many degrees it was before the two photo diodes read the same intensity from both beams. This measurement became my $\ominus_1$, $\ominus_1$= 201 degrees. These two numbers can be plugged into the equation for intensity.

$$\text{Laser Intensity} = I_0 * \sin^2(\ominus_1 - \ominus_0) \qquad [1]$$

The third step was to measure the minimum angle change detectable. The measurement is know as $\nabla\ominus$, $\nabla\ominus$ = 3 minutes or a degree. (Each degree is split up into 60 minutes and 60 seconds).

The difference in intensity is found by the following equation:

$$\text{Difference in Intensity} = \nabla I = 2 * I_0 * \sin(\ominus_1 - \ominus_0) * \cos(\ominus_1 - \ominus_0) \qquad [2]$$

The fractional change in measurable intensity is the value that I was looking to compare with the number from the scientific literature for fractional absorption using fm spectroscopy, 10^{-3}. This value was calculated using the following equation:

$$\text{Fractional Change in Intensity Measurable} = \triangle I / I = 2 * \sin(\ominus_1 - \ominus_0) * \cos(\ominus_1 - \ominus_0) * \triangle\ominus / I \qquad [3]$$

This reduces to:

$$\triangle I / I = 2 * \cot(\ominus_1 - \ominus_0) * \triangle \ominus \qquad [4]$$

$$\triangle I / I = 2 * \cot(45) * 3'' = 2 * 10^{-3}.$$

This number is in line with the experimental results obtained using FM Spectroscopy. This finding basically allows me to believe that the there is a good possibility that detecting fractional change in intensity measurable using Differential Laser Absorption is feasible.

Future Work

There are two-steps to this process. First, we must figure if we can measure small differential absorption under non-scattering conditions. Secondly, how much is our resolution of the differential absorption affected by scattering. For part one, we first need to know how small of an absorption can be detected. Now we need to know how many dye molecules will be produced by gene when it is actively producing protein and if this will be enough to be detected by our methods.

According to the commonly occurring results, we expect ˜ 4- 8 fluorescing molecules for each protein molecule produced by the specific gene. Fluorescing microscopy routinely observes concentrations of 5 – 20 um / ml of protein. Proteins typically have molecular weights of 10^5g / mole. This leads to a dye concentration of:

4 molecules per protein * [10^{-6}g /ml] / [10^5 g / mole] ˜ 10^{-7}moles per liter or M (molarity).

The dye extinction coefficient for dyes used is $10^5 M^{-1} L^{-1}$, so we expect an absorption length of 1 meter. Therefore, we can detect 10^{-3} absorption over a path length of 1mm. By this calculation, our differential absorption techniques should work. However, the amount of scattering will limit us. To what extent, will be determined.

We are developing a model system of light propagation in human tissue using a glass cuvette filled with methanol and inserting a capillary tube filled with a certain concentration of dye solution. Next, human tissue is simulated with the gradual adding of intralipid, a well-known tissue-simulating medium.[4] The measurement of the differential absorption with a system similar to Figure 3 with a cuvette placed in between the laser and the detector circuit.

There are four questions that I wish to answer when the experiment is constructed. 1) Can I actually measure instead of calculate a 10^{-3} differential absorption? 2) Can this still be done in the presence of severe scattering? 3) What is the minimum dye concentration detectable? 4) What is the maximum intralipid concentration allowable and still be able to detect differential absorption?

Based on the answer to these questions given by the simulation of the mouse brain, I will be able determine the feasibility of carrying this experiment over to the living mouse or if there are addition adjustments that need to be made.

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[6] Arjun Yodh and Britton Chance, "Spectroscopy and Imaging with Diffusing Light", Physics Today **48,** 34-40 (1995).