Differentiating Acute Otitis Media (AOM) from Otitis Media with Effusion (OME) Using Autofluorescence of NADPH in Neutrophils

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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Abstract

Ideally, clinicians will be able to accurately distinguish between Otitis Media with Effusion (OME) and Acute Otitis Media (AOM), which requires antibiotic treatment. However, it is currently difficult for clinicians to differentiate between them. Specifically, diagnoses by general practitioners are unreliable about 27% of the time due to unoptimized diagnosis techniques and subjective diagnosis criteria[1]. Additionally, ~2.2 million cases of AOM occur annually among children, amounting to a direct cost of $4 billion each year due to unnecessary removal of adenoids or tonsils, antibiotic over-prescription, and hearing loss[2]. AOM is an infection of the middle ear fluid behind the tympanic membrane (TM). OME is a condition commonly confused with AOM in which there is fluid present behind the TM that is not infected with bacterias that cause AOM[3]. The high rate of misdiagnosis between AOM and OME can be attributed to current clinical diagnostic methods of visual observation and tympanometry. The goal of this project is to detect infection indirectly by measuring NADPH concentration as an indicator of neutrophil accumulation in response to infection, and therefore develop a proof of concept detection method to be further adapted for implementation in an otoscopic medical device. It resulted in detection of NADPH down to a concentration of ~0.01mM, and a discovery that the relationship between the concentration of NADPH and its fluorescence voltage output is logarithmic. Future development of a device that employs this diagnostic method will help reduce the 27% rate of misdiagnosis, and therefore decrease the annual $4 billion cost associated with AOM. Improved diagnostic accuracy will also increase patient and parent comfort and confidence in their physician.

Introduction

Otitis Media and NADPH

Acute Otitis Media (AOM) is an infection of the middle ear fluid behind the tympanic membrane (TM). It accumulates ~2.2 million cases annually in pediatric patients, and is diagnosed in 80%-90% of children before school age (~5yo)[4]. It is a painful condition that, if left untreated, can lead to development of meningitis, permanent hearing loss, and other more serious conditions[5]. Otitis Media with Effusion (OME) is a condition commonly confused with AOM in which there is fluid present behind the TM, but it is not infected with *Haemophilus influenzae, Moraxella catarrhalis,* or *Streptococcus pneumoniae* - bacterias that cause AOM. On average, AOM is misdiagnosed about 27% of the time by general practitioners and primary care providers, however, among otolaryngologists, misdiagnosis only occurs about 10% of the time[1]. Misdiagnosis of inflammatory ear conditions leads to over- and/or underprescription of antibiotics; another problem that has consumed countless resources for the medical community. In the western world, AOM accounts for the most antibiotics prescriptions[6]. Unstable doctor-patient relationships, pressure from patients, and physician's lack of confidence contribute to increased use of antibiotics. Overprescription of antibiotics can lead to a patient developing resistant bacteria and later make it difficult to treat if AOM reoccurs.

The high rate of misdiagnosis between AOM and OME can be attributed to the method of clinical assessment used to determine presence or absence of infected effusion (fluid) in the middle ear space. In contemporary practice, clinicians use one or a combination of multiple methods for analysis of the TM and contents of the middle ear canal. The provider will use an otoscope either with or without a pneumatic attachment to observe the visual properties of the TM, specifically looking for signs of inflammation such as redness, swelling, presence of a "pus drum" (obvious infected purulence behind the TM), and vasculature (hyperemia). If the provider is using a pneumatic otoscope, they will completely occlude the canal creating a vacuum, and will then use the pneumatic attachment to pump a puff of air

into the space while looking through the otoscope to assess rigidity of the TM. This technique assumes that an infected ear will have little to no tympanic movement due to the pressure exerted by fluid present behind the TM. This technique does not assess the actual contents of the middle ear fluid in any way. A similar technique, tympanometry, is also commonly used when conducting a diagnostic ear exam for Otitis Media. To diagnose with tympanometry, clinicians evaluate TM response to changes in outer ear pressure using a tympanometer which generates a compliance response curve for the patient's TM called a tympanogram. A mostly flat curve indicates little to no movement of the TM, and suggests that there is fluid exerting positive pressure behind the TM in the middle ear, thereby hindering its compliance[7]. This technique also does not assess the actual contents of middle ear effusion. While these diagnostic methods are legitimate for analysis of the elasticity and appearance of the TM, they do not assess the presence or absence of infected fluid in the middle ear space, instead relying on inferences that are only likely to be correct when made by seasoned otolaryngologists. In order to analyze the characteristics of middle ear effusion, a diagnostic method is proposed to assess patient infection status by detecting nicotinamide adenine dinucleotide phosphate (NADPH) as a surrogate measure of neutrophils in the middle ear, thereby indicating if infected fluid is in the space and enabling a physician to make a correct diagnosis.

NADPH, the reduced form of NADP, is a cofactor that donates electrons and hydrogens to reactions catalyzed by some enzymes. It is produced from the pentose phosphate pathway and oxidizes to form[8] NADP+. A primary function of NADPH is the donation of electrons to reduce oxidized compounds in redox reactions such as reductive biosynthesis, detoxification, oxidative defense, and reactive oxygen species (ROS) generation[9,10]. It is also commonly involved in anabolic pathways to create large molecules[9].

When infection is detected by the body in the middle ear space, an innate immune response takes place; neutrophils are considered the first line of this host immune response against invading pathogens[11]. They are primarily attracted to sites of inflammation by chemotactic factors such as C5a, a complement-activated chemoattractant[12,13]. In AOM, neutrophils are also recruited by IL-17A proteins specifically responding to *Streptococcus pneumoniae*[14]. Neutrophils have a high concentration of NADPH, the substrate of the oxidase responsible for the "respiratory burst" of innate immune response release of ROS in activated neutrophils[15–18,41] . When neutrophils are recruited and then activated by pro-inflammatory stimuli, they use NADPH oxidation to donate electrons to make superoxide. Neutrophil activation causes an emergency response to foreign pathogens in the system, and therefore NADPH concentrations can be used as an indicator for infection. To elaborate, NADPH oxidase (NOX) is a membrane-bound enzyme that faces the extracellular space, and is dormant until activated by stimuli (such as bacteria). Once activated, NOX is used as a reaction catalyst to transfer electrons from NADPH to generate ROS. NADPH's role in neutrophil immune response begins with the stimulation the neutrophil by bacterial detection. This leads to NOX2 activation which then catalyzes oxygen reduction with 1 electron sent from NADPH resulting in production of superoxide. Neutrophils then engulf bacteria in phagosomes and release superoxide into the phagosomes through activated NOX2. The superoxide dismutates to hydrogen peroxide which is then used by myeloperoxidase (MPO) to make additional ROS and hypochlorous acid (HOCl) to kill bacteria. NADPH is also integral to the formation of neutrophil extracellular traps (NETs), as ROS generated by NOX2 is used to stimulated MPO which then mediates activation of neutrophil elastase (NE), a product that drives chromatin decondensation by processing histones. This sparks the release of NETs that then capture pathogens, degrade bacterial toxic factors, and kill bacteria[15–19].

Fluorescence

This project relies on autofluorescence of NADPH as a means to detect neutrophil content in middle ear effusion. When light of a certain wavelength is absorbed by a solution or tissue, endogenous fluorophores are excited and emit light of a longer wavelength[20]. Molecules have unique fluorescence spectra based on their electron distribution in the ground state[21]. When a susceptible molecule is excited with a specific wavelength of light their energy state increases to a higher level spurred by the energy absorbed from the light. After excitation, the molecule falls back down to lower energy states in a process known as vibrational relaxation. This process occurs at a slower rate than excitation, and allows the molecule to approach the energy level at which it will fluoresce. As the molecule continues to fall in energy level, it emits a photon at a wavelength unique to its composition. Once it has emitted, the molecule returns to ground state, again at a slower rate than previous steps of the fluorescence process[22]. NADPH specifically is excited with ~360nm wavelength light, resulting in fluorescent emission at ~450nm.

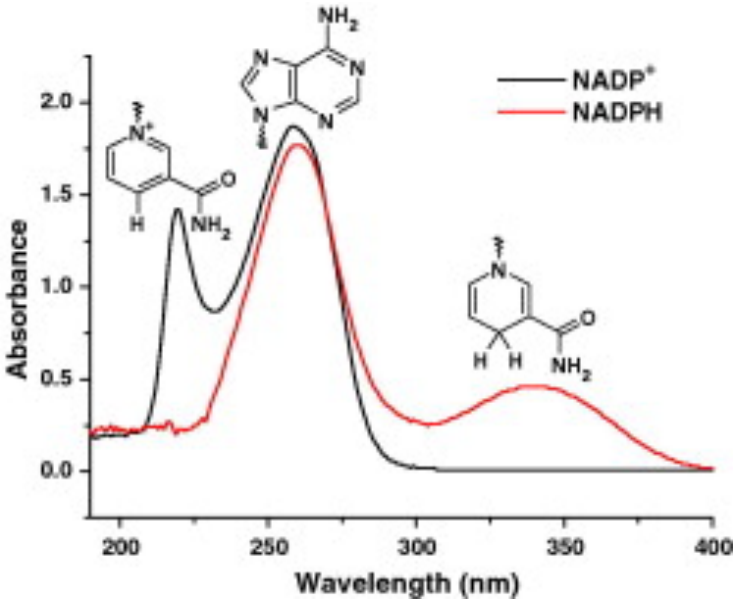


Figure 1: NADPH Fluorescence Spectrum[23]

Prior Art

With regards to previous innovation for diagnosis of OM, there are many existing technologies that attempt to evaluate patients' middle ear status. The most commonly used diagnostic tools are visible light otomicroscopy and pneumatic otoscopy (tympanometry), however these have contributed to the 27% rate of misdiagnosis for OM, as they do not assess the specific molecular contents of effusion, require physician training and practice for accurate usage, and possess limitations in their diagnostic certainty[1]. A number of techniques have been developed to improve diagnosis; they can be subdivided based on their method/mechanism of action into *imaging technologies* and *bioengineering tools*[24].

Recent advances in middle ear imaging modalities use acoustics, radiology, visible light techniques, and near infrared techniques to improve visualization on the middle ear. Acoustic techniques include high frequency ultrasound (HFUS), spectral gradient acoustic reflectometry (SGAR), and transmastoid ultrasound[24]. While HFUS allows for visualization of the middle ear anatomy, it has not been tested *in vivo*, and may not translate through thicker soft tissue. HFUS also only analyzes anatomy, and not the molecule contents of effusion[25]. Similarly, SGAR uses sonar waves to assess presence of effusion, however it can not detect progression or clearance of effusion nor can it differentiate if effusion is infected[26]. Finally, transmastoid ultrasound detects effusion via the mastoid air system, but can not determine if effusion is infected[27].

To continue, the primary radiology-based technique preceding the project is synchrotron radiation phase-contrast imaging (SR-PCI). This method involves phase-shifted beam interference to visualize

middle ear anatomy with improved contrast for soft tissue compared to its competitors (absorption contrast micro-CT), however, it does not image for effusion specifically, and presents standard health risks associated with radiative imaging modalities[28]. The project addresses the limitations of these technologies by evaluating the molecular NADPH content of effusion as opposed to simply assessing middle ear anatomy or the mere presence/absence of effusion. The device eventually developed from the project will also have low health risks associated with its usage as the patient will be exposed to 360nm light for a few seconds at most for fluorescence data to be collected and converted to a readable output.

Visible light and near-infrared techniques include multicolor reflectance imaging, narrow band imaging (NBI), anti-confocal middle ear assessment, and optical coherence tomography (OCT)[24]. Multicolor reflectance imaging uses RGB narrow-band reflectance imaging to visualize middle ear tissue structure, however it is very susceptible to patient movement and image distortion, and does not evaluate effusion contents[29]. Similarly, NBI uses visible light to penetrate tissue at varying depths to indicate hypervascular areas and can identify diseased tissue, however it is a poor diagnostic for AOM because diseased tissue does not necessarily mean there is infected effusion present[30]. Anti-confocal middle ear assessment uses near infrared spectroscopy to analyze inflammatory blood content of middle ear, however it does not precisely evaluate effusion, and has not been tested *in vivo*[31]. In similar fashion, OCT uses near infrared spectroscopy to evaluate TM thickness and displacement as an indication of infected effusion. This technique has had notable success in visualization of middle ear biofilm structure and combined assessment of TM thickness, however it can not image through the ossicles, is uncomfortable for patients as it requires an ear canal seal for data collection, and is inefficient with a long image processing time[32–34]. The project builds on these light-based techniques and addresses their limitations by specifically evaluating the molecular NADPH content of effusion, and the eventual device being minimally invasive and comfortable for the patient to endure.

Bioengineering tools for diagnosis of OM use computerized software to develop an AI training algorithm for diagnosis of OM based on otoscopic images of the TM. The software uses an image database of over 200 TM images that have been diagnosed as AOM or OME, and virtually segments the TM into regions for evaluation of color and shape. The technique claims to have a diagnostic accuracy of >90%, however, it is difficult to be sure that the training images have been given the correct label diagnoses, as they were not confirmed with physical extraction and assessment of effusion contents[35]. This technique is also used for detection of ventilation tubes in the TM, however this function is excessive, as tubes are visible using normal otoscopic evaluation[36]. Additionally, because it involves the use of remote software, bioengineering techniques are susceptible to error due to failed network connection and algorithm errors, and are only as good as the image database used to train them. Overall, bioengineering tools depend on images that may already be misdiagnosed and therefore labeled incorrectly, and pose additional challenges for troubleshooting based on their remote nature. The project addresses these limitations by making a primary diagnosis based on the molecular NADPH content of effusion, and is not dependent on a separate collection of data or images for analysis. It also does not require the use of complex software and AI algorithms to function correctly, and can therefore be easily assessed for sources of failure if it is operating incorrectly.

Materials & Methods

Instrument Design

In order to detect infection indirectly by measuring NADPH concentration as an indicator of neutrophil accumulation, the project team created a proof of concept instrument to be used in

experimental trials. Light from a ~360nm LED illuminator (M365L3, ThorLabs) is collimated and passes through the first lens (125mm focal length) of a beam reducer. The light continues to the dichroic mirror (#34-725, Edmund Optics) where it is reflected through another lens (25mm focal length) into the NADPH-concentrated solution sample, contained in a cuvette in a custom-designed holder. The light emitted from the excited sample (~460nm) is directed back through the previous lens and dichroic mirror and then through another lens (25mm focal length). An emission filter (452nm, #86-351, Edmund Optics) is secured flush to the detecting face of a photomultiplier tube detector (PMT) (Hamamatsu H10721-110, Edmund Optics) to reduce background light.

The PMT is used to detect the emitted ~450nm NADPH fluorescence, but has current as an output. Therefore, the project team designed and built a transimpedance amplifier circuit to convert current to voltage allowing us to measure the fluorescent output in millivolts (mV) using a multimeter (Figure 2). The optical component of the instrument was contained within a box with a blackened interior during experimental trials to reduce background light, and its completed form is shown in Figure 3. In experiments, the project team loaded NADPH solutions of varying concentrations into an empty cuvette, and then enclosed the experiment in the dark box before beginning each trial. The emitted signal was detected by the PMT and then converted to a voltage output by the transimpedance amplifier that was recorded for later data analysis.

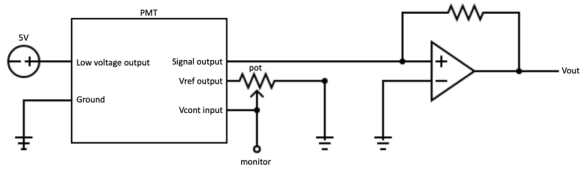


Figure 2A: Electrical diagram.

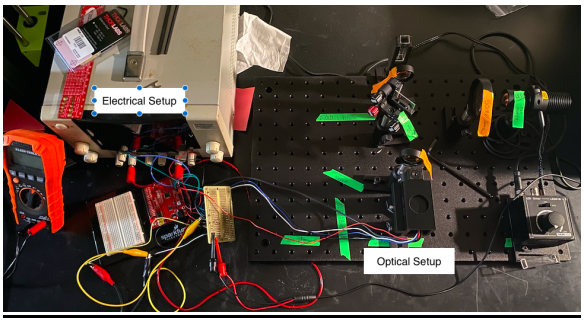


Figure 2B: Instrument overview image.

Figure 2: Instrument Electrical Component

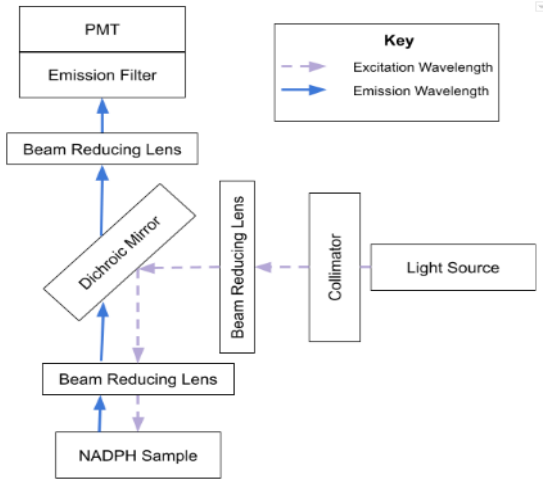


Figure 3: Instrument Optical Component Diagram

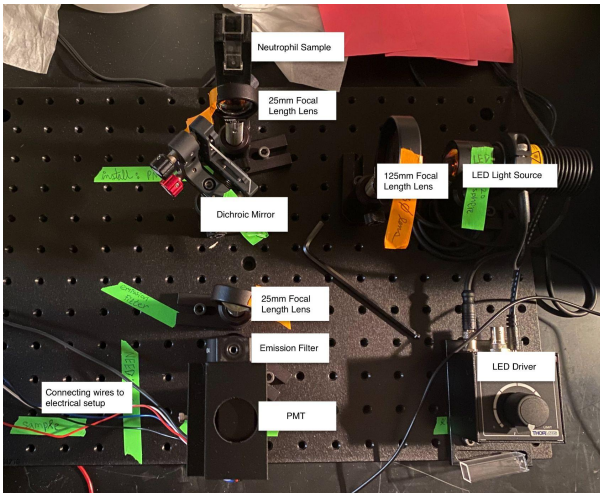


Figure 4: Instrument Optical Component Image

Pre-NADPH Integration Experiment

For the first round of experimentation, the project team conducted trials with an empty cuvette to determine the background noise and level of gain saturation based on the LED driver setting and the transimpedance amplifier adjustable gain level. The project team recorded the transimpedance amplifier output voltage as a function of LED light output level over a wide range of gain values. These data were recorded and analyzed to determine the ideal parameters for gain and LED setting. In Figure 5, the data is plotted as output voltage (mV) versus light output level over four different gain values. The project team analyzed this figure to find the settings where the PMT is sensitive to light, indicated by a visible range in y-values, while ensuring the PMT is not saturated, indicated by a high output voltage plateau in the y-values. Based on these requirements, the project team knew that the 0.936V gain value was not appropriate since it is saturating the PMT while the 0.320V and 0.006V gain values were not appropriate since the PMT is not sensitive enough at this level. The project team determined that a gain of 0.611V and a light output level of 3 were the ideal settings for the PMT to be both sensitive and not saturated.

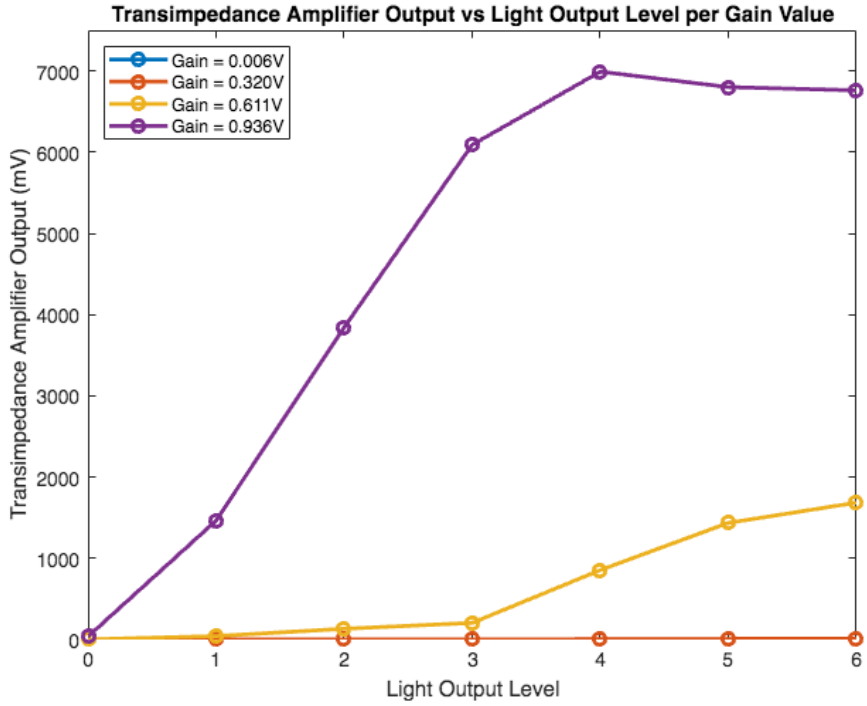


Figure 5: Output Voltage (mV) vs Light Output Level per Gain Value (V)

This plot shows the voltage output recorded across various LED Driver Light Output Levels for various gain values. Using this plot, the project team was able to determine which Gain value and Light Output Level to use for future experimental trials to ensure the setup is both sensitive (wide range of voltage output values) while not saturating the PMT with light (maximum voltage output of ~7000mV).

NADPH Integration Experiments

In order to determine the target concentration for NADPH in solution, the project team reviewed the literature to estimate how much NADPH is present in infected effusion. The total concentration of NADP in hyman normal lymphocytes was found to be 14.5±3.9 pmol/10 million cells[37]. This concentration was divided by the volume of one neutrophil to yield NADPH concentration of a neutrophil[38]: $\frac{14.5*10^{-9}mmol}{10{,}000{,}000\ cells} * \frac{1\ neutrophil}{500*10^{-15}\ L} = 0.0029mM\ NADPH$. Given that there are $2.11*10^6 \pm 7.91*10^5$ neutrophils in 1 ml of purulent (infected) effusion[39], the estimated concentration for NADPH in a suspension of neutrophils adjusting for the volume fraction of cells is approximately:

$\frac{500*10^{-15}L*2110000\,neutrophils}{0.001L} = 3.06 * 10^{-6}mM.$ Once the target concentration was found, 2 stock solutions of NADPH were created with concentrations of ~30mM and ~15mM respectively. These concentrations were tested to determine if the instrument can detect NADPH at high levels of concentration. The transimpedance amplifier output voltage was measured (mV) for two experimental controls (empty cuvette and a cuvette filled with deionized water) and the two NADPH solutions (30mM and 15mM). When comparing the voltage output of the NADPH trials to the control trials in Figure 6, a large increase in output voltage was found after introducing the NADPH indicating that the instrument is detecting NADPH at high concentrations. It was also determined that the output voltage measured for both the 30mM and 15mM solutions were approximately the same, leading to the conclusion that the PMT is saturated at this high level of concentration and the stock solutions must be diluted repeatedly before seeing a relevant change in light detection.

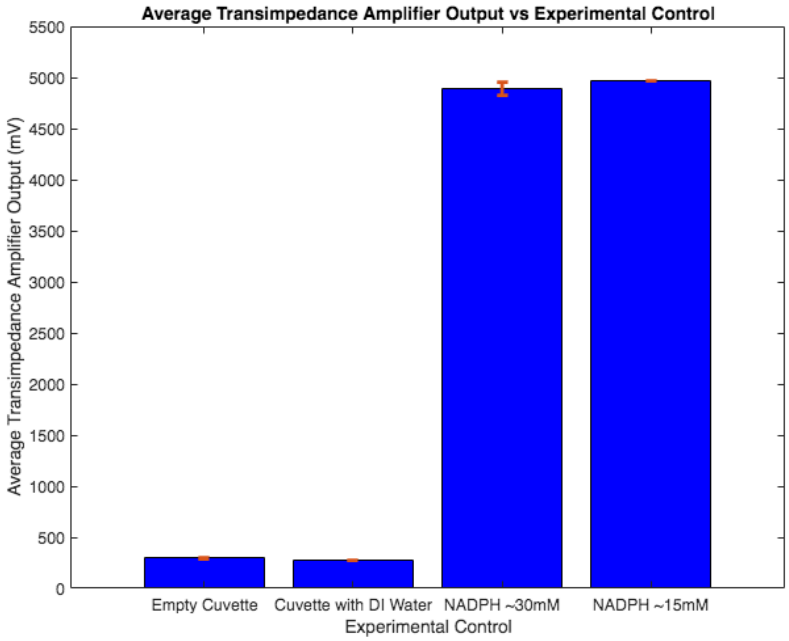


Figure 6: Average Output Voltage (mV) per Experimental Control
To determine if the optical setup is functioning properly and can detect NADPH, the transimpedance amplifier output voltage was measured (mV) detected for an empty cuvette, a cuvette filled with DI water, a NADPH solution of concentration ~30mM, and a diluted NADPH solution of concentration ~15mM. The spike in voltage output when comparing the control values to the NADPH values shows that the optical setup is functioning correctly by fluorescing and detecting the NADPH solution.

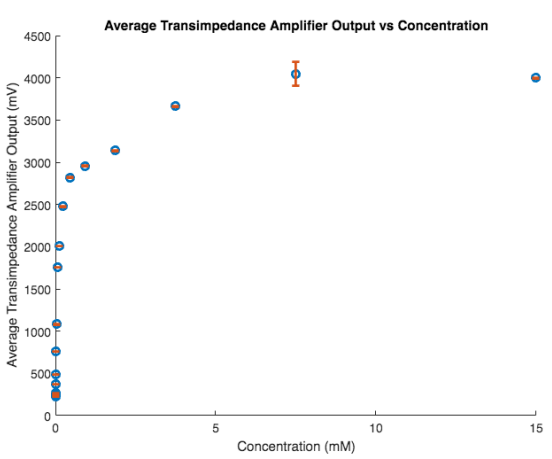
After determining that the instrument design can detect NADPH at high concentrations, the team wanted to find the relationship between NADPH concentration and the transimpedance amplifier output voltage. Based on the last experiment of NADPH integration, it is known that the PMT is saturated at values above 15mM so experimentation began with the 15mM stock solution and apply 2-fold dilutions to the stock solution until the instrument can no longer detect the NADPH in solution. The first experimental trial started with analysis of the 15mM stock solution and then progressed to 2-fold serial dilutions of the NADPH solution in a cuvette. At each concentration, the gain was recorded to ensure that it was ~0.611, the output voltage was recorded before turning on the LED, for a measured noise level to normalize the data, and when the LED was turned on, producing five trials at each concentration level. The team ensured that the LED was only on for a short period of time since the NADPH can photobleach which could lead to a lower concentration over time[40]. Sample dilutions and recordings of the voltage output were recorded until the output plateaued. The point was assumed to be the point at which the instrument saturated with background noise with no NADPH being detected. Lastly, these values were measured for an empty cuvette and a cuvette filled with deionized water, to act as a control. For the second

experimental trial, the experimental method was adjusted to only include a decreased range of concentration values, and to make the NADPH solutions by direct dilution from the stock rather than serial dilution. Then the same measurements were recorded over a reduced range of concentrations and for the two controls.

Results

For the analysis of data, there were two main goals: first, to determine the relationship between the transimpedance amplifier output voltage and NADPH concentration, and second, to gain information on the baseline noise level of the instrument. First, the team plotted the raw data with the output voltage on the y-axis and the NADPH concentration on the x-axis (Figure 7A). The team noticed that the shape of the curve appeared to be a logarithmic relationship between the variables and decided to apply a log-transform on the x-axis data (Figure 7B). By applying the log-transform, the plot turned into a more linear line that was determined would be easier to analyze. The team started by performing a linear regression on the linear portion of the plot and found that the relationship is in fact logarithmic. To confirm this is a proper fit, the root mean square error (RMSE) was calculated for the linear regression to be ~136.91mV. By normalizing this RMSE value to the dataset by using the equation *Normalized RMSE = RMSE/(maximum data point - minimum data point)*, it can be concluded the linear regression is a good fit because the adjusted RMSE value was ~0.038 on a scale from 0 to 1. Next, it was determined which data points show a statistically significant increase in output voltage when compared to the control of a cuvette filled with deionized water by performing an independent two sample t-test for each data point. It was found that the data point with the first significant increase in output voltage was at a concentration of ~0.00183mM. Based on this information, it was determined that the baseline noise level was the average of the output voltages of concentrations lower than ~0.00183mM, which was ~240.39mV. Using the baseline noise level and the linear regression equation, the team was able to determine the noise equivalent concentration to be ~0.004mM by plugging the baseline noise level (*~240.39mV*) into the linear regression equation (*y=1172.0*log(x)+3031.2*) and solving for the concentration, *x*. The noise equivalent concentration tells us the limit of NADPH detection.

For the second experimental trial analysis, the same approach was followed with a few modifications to adjust for the change in concentration range. First, the raw data (Figure 8A) and the log-transformed data (Figure 8B) were plotted, then the linear region was analyzed with a linear regression. The team noticed that the two highest concentration points had a lot of noise, indicated by the large error bars, and that the PMT was near saturation. Excluding these two points, it was found that the relationship between the output voltage and NADPH concentration is logarithmic (*y=798.3*log(x)+1725.5*) and calculated a RMSE value of 90.12mV. The normalized RMSE value was calculated to be 0.043 on a scale from 0 to 1, indicating that both datasets and linear regressions are approximately equally fitted. . Lastly, the baseline noise level was calculated to be ~178.5mV and the noise equivalent concentration to be ~0.0115mM.



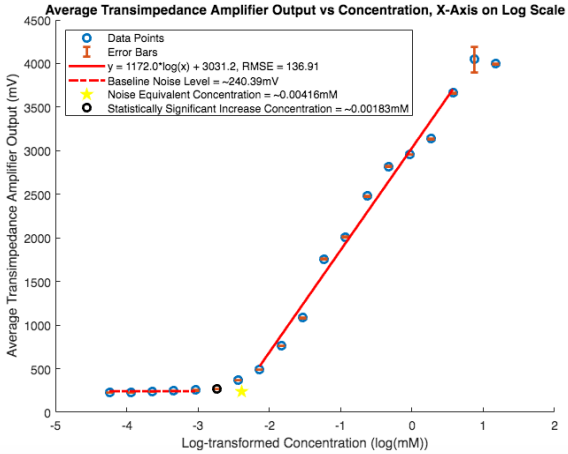
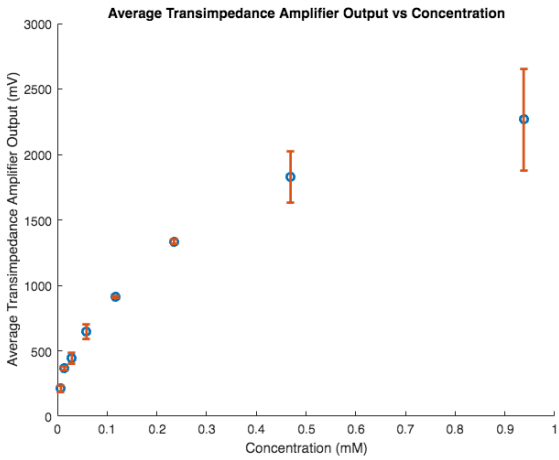


Figure 7A: Output Voltage (mV) vs Concentration (mM) **Figure 7B:** Output Voltage (mV) vs Log-Transformed Concentration (log(mM))

Figure 7: Trial #1, Average Transimpedance Amplifier Output Voltage (mV) vs Concentration (mM)

To determine the relationship between the Transimpedance Amplifier Output Voltage and the NADPH concentration as well as the baseline noise level of the instrument design, the output voltage (mV) was measured across a range of concentration values. The raw data of output voltage versus concentration is plotted in Figure 7A and the log-transformed data of output voltage versus the log of concentration is plotted in Figure 7B.



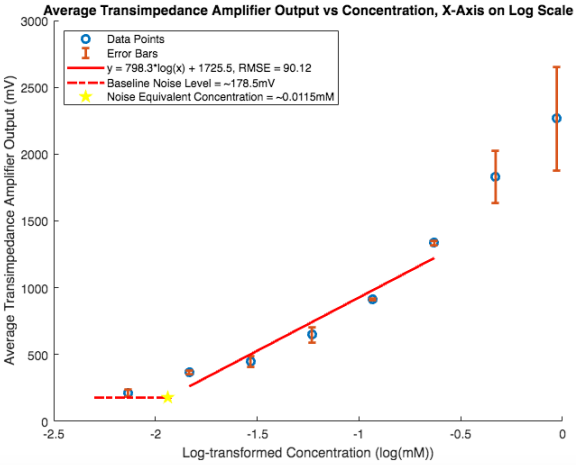


Figure 8A: Output Voltage (mV) vs Concentration (mM) **Figure 8B:** Output Voltage (mV) vs Log-Transformed Concentration (log(mM))

Figure 8: Trial #2, Average Transimpedance Amplifier Output Voltage (mV) vs Concentration (mM)

To determine the relationship between the Transimpedance Amplifier Output Voltage and the NADPH concentration as well as the baseline noise level of the instrument design, the output voltage (mV) was measured across a range of concentration values. The raw data of output voltage versus concentration is plotted in Figure 8A and the log-transformed data of output voltage versus the log of concentration is plotted in Figure 8B.

Discussion

Overview

The goal of this project was to develop a proof of concept method for detection of AOM infection by measuring NADPH concentration as an indicator of neutrophil accumulation. This new diagnostic method will help decrease the ~27% rate of AOM misdiagnosis for general practitioners and primary care physicians, and will therefore reduce the annual $4 billion cost associated with unnecessary antibiotic prescription, surgical procedures, and patient hearing loss. Above all else, this method of detection will

help to increase patient and parent comfort in the clinic, and confidence in their physician, therefore creating a more beneficial clinical experience with reliable diagnosis. The major findings are successful proof of concept in the fluorescence-based detection of NADPH in solution down to a concentration of ~0.01mM, and the determination of a logarithmic relationship between NADPH concentration and fluorescence voltage output.

Impact

The otoscopic device eventually developed from the team's method of detection will have a significant impact on the differential diagnosis of AOM and OME, and will help ensure that patients receive appropriate treatment once their condition has been accurately diagnosed. Improvement from the 27% misdiagnosis rate will help reduce the $4 billion annual cost associated with AOM treatment methods such as antibiotic prescription, surgical removal of adenoids or tonsils, and eventual loss of hearing with recurrence. Knowing that they will receive an accurate diagnosis, pediatric patients and their parents will feel more comfortable in clinical settings, and more confident in their physician to provide them with accurate diagnosis. Patients will also experience more physical comfort by benefitting from appropriate treatment methods, as opposed to not receiving necessary treatment based on incorrect diagnosis that creates more potential for painful recurrence of AOM.

Marketability

The potential market for the eventual device derived from this project is very promising. The Otoscopes Global Market is projected to reach $198.1 million by 2026. Additionally, the device would be marketable in a variety of clinical settings as it could be adapted not only for use by primary care physicians and general practitioners, but also for specialty use in otolaryngology clinics. The method of detection itself also holds potential for adaptation to diagnose infections in other areas of the body such as through the nose and mouth, and an adjustment of the specific excitation wavelength and specific emission wavelength for detection could allow this method to be applied to detection of other molecular compounds responding to infections outside of AOM, or other types of ear infections such as Otitis Externa. Given the fact that >80% of children experience an ear infection before the age of five[4], and that it is a condition that is very painful and can lead to later complications, most pediatric patients and their parents would likely be very interested in a more reliable method for detection and accurate diagnosis of infection.

Conclusion

Completion of this project has resulted in successful proof of concept for detection of AOM infection indirectly by measuring NADPH concentration as an indicator of neutrophil accumulation down to concentration of ~0.01mM. While this concentration is still much greater than the target concentration of ~0.003uM, the simple detection of NADPH through fluorescent emission is a major accomplishment to the goals of this project. This project has also resulted in the determination of a logarithmic relationship between the concentration of NADPH in solution and fluorescence voltage output picked up by the PMT, an important finding as it was previously unknown. These findings will be helpful to future iterations of the project, as they will be used to make further improvements to the detection capabilities of the instrument.

The primary limit on the sensitivity of this instrument is the background signal from the excitation light. In the future, the team would like the instrument to be improved for better reduction of background signal. This could be accomplished by creating a structure to enclose the optical pathway since much of the noise is from scattered excitation light. The project should also continue on to the

design and implementation of a phantom model to mimic the TM and middle ear space that would be the environment for the diagnostic instrument. This will also raise the new challenge of determining the level of penetration of light through the TM itself, and if the detector will be able to pick up on the NADPH emission light, which will likely be at a very low intensity level. Finally, the project should be improved by repeating the same methods and trials as have been done this year, but with a neutrophil-concentrated sample instead of NADPH as a surrogate measure. Using real neutrophils will better mimic the conditions of infected middle ear effusion that has garnered an immune response. By making the necessary improvements, and continuing to optimize the experimental parameters and technical components of the detection instrument, this project can develop into a diagnostic device with potential to save billions of dollars and change Otitis Media diagnosis, and patients' experiences with it, for the better.

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Supplementary Information

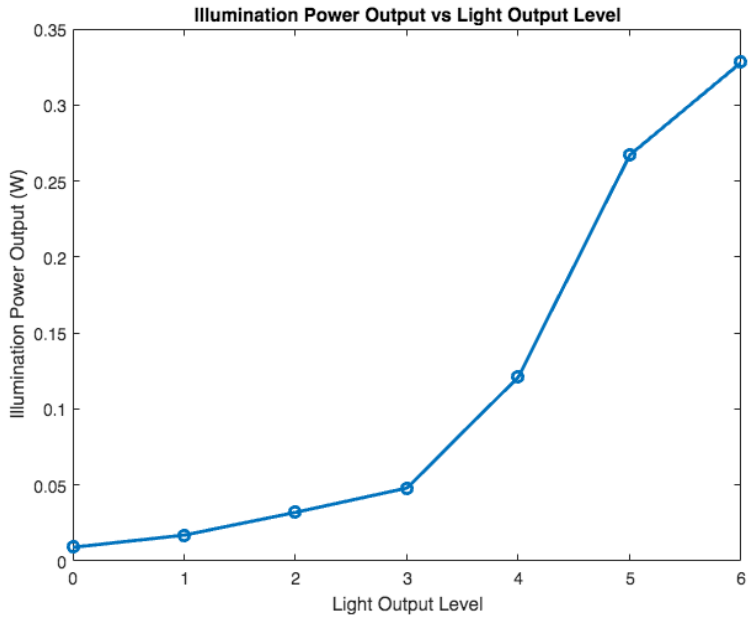

Figure S1: Illumination Power Output (W) vs Light Output Level

This plot shows the Illumination Power Output recorded across various LED Driver Light Output Levels. Using this plot, the project team was able to determine the relationship between the level set on the LED driver and the amount of light actually emitted to ensure the LED driver was working properly.

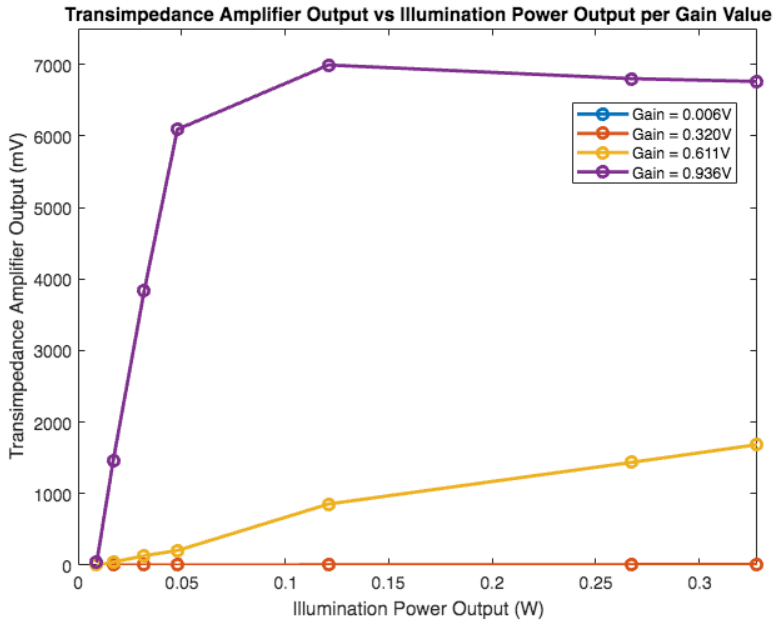

Figure S2: Output Voltage (mV) vs Illumination Power Output (W) per Gain Value (V)

By combining the information in Figure 5 and Figure S1, a plot of Output Voltage (mV) versus Illumination Power Output (W) was created to ensure the proper gain value and light output level was selected for experimentation.