Photoluminescence Detection of 2,4,6-Trinitrotoluene (TNT) Binding on Single Cell Diatom Biosilica.

by
Nathan D. Harms

A THESIS

submitted to
Oregon State University
University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering
(Honors Scholar)

Presented June 2, 2016
Commencement June 2016
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Nathan D. Harms for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on June 2, 2016. Title: Photoluminescence Detection of 2,4,6-Trinitrotoluene (TNT) Binding on Single Cell Diatom Biosilica.

Abstract approved: ____________________________________________________________

Gregory Rorrer

T. pseudonana biosilica and live cells was explored as a biosensor for detection of the explosive compound 2,4,6-trinitrotoluene (TNT) in aqueous solution. The diatom frustule is an intricately nanostructured, highly porous biogenic silica material derived from the shells of microscopic algae called diatoms. This material emits strong visible blue photoluminescence (PL) upon UV excitation. PL-active frustule biosilica was isolated from cultured cells of the marine diatom T. pseudonana, exposed to varying concentrations of TNT, and the PL response was observed using PL spectroscopy. This process was repeated for live T. pseudonana cells. Both experiments showed distinct trends and an attempt at explanation was made.

Key Words: Diatom, Trinitrotoluene, Photoluminescence

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APPROVED:

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Gregory Rorrer, Mentor, representing Chemical, Biological and Environmental Engineering

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Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

______________________________
Nathan D. Harms, Author
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1 – Introduction

Trinitrotoluene (TNT) was first synthesized in 1863 by German chemist Julius Wilbrand and was originally used as a yellow dye. Over time, its explosive properties became known and it became widely used in that capacity. Although TNT is still widely used in today’s society, it is highly toxic and poses a great environmental threat to both humans and wildlife. For example, small doses of TNT can cause the skin to turn bright yellow, anemia, abnormal liver function, and other carcinogenic effects. Because of this, there has been a push in sensing and detection methods of TNT.

Current detection methods include High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS). However, both of these methods are very costly and take up large amounts of time. Due to these inefficiencies, development in novel detection methods have been on the rise; specifically, the use of mesoporous silica. Mesoporous silica has been shown to elicit a photoluminescent response in the presence of ultraviolet light; moreover, this response can be quenched by varying concentrations of TNT. This makes mesoporous silica a viable option when sensing TNT in aqueous environments. (Tao, Yan, Guangtao, Yu, 2008) The only issue is that these synthetic materials take time and effort to manufacture; however, there is a natural option mesoporous silica.

Diatoms are a single-celled algae that possess naturally porous silica shells (frustules) that have micro and nanoscale features that possess unique photonic and optoelectronics properties. (Sumper, Brunner, 2006) Specifically, the frustule of a diatom possesses a similar photoluminescent response to synthetic mesoporous silica. (Qin, Gutu, Jiao, Chang, Rorrer, 2008) The main premise of this research was to test if diatom
biosilica can be used as an alternate to mesoporous silica in TNT detection. Also, live cells were tested as an option as well.
2 – Background Information

2.1 – Diatoms

Diatoms are a prolific class of single-celled algae that possess silica shells or frustules with intricate submicron and nanoscale features. These features have unique photonic and optoelectronic properties. During frustule development, membrane-bound transporters actively take up the soluble silicon in the form of silicic acid. Once inside the cell, silicic acid is converted into silica and molded into nanostructures by protein-mediated condensation reactions within the silicon deposition vesicle.

Nanostructured biosilica from cultured photosynthetic diatom cells have shown to possess photoluminescent properties, exhibiting strong blue light emission upon UV excitation. (LeDuff, Roesijadi, Rorrer, 2016). Furthermore, the cultivation of diatom cells under controlled delivery of soluble silicon and other trace metals offers a route to make nanostructured diatom with controlled photoluminescent (PL) and electroluminescent properties. (Jeffryes, Solanki, Rangineni, Wang, Chang, Rorrer, 2008). These photoluminescent properties have been harnessed for biosensing applications.

*Thalassiosira pseudonana* (*T. pseudonana*) (pictured in figures 1 and 2) is a species of marine centric diatom that was used during the procedure of this lab. *T. pseudonana* was chosen because the Rorrer lab has previously shown that frustule biosilica isolated from cultured diatoms had exhibited PL emission centered around 440-500 nm under UV excitation at 337 nm. Also, it has been shown that antibody
bioconjugated *T. pseudonana* effectively serves as an adequate sensing tool for TNT detection. (Gale, Gutu, Jiao, Chang, Rorrer, 2009).

Figure 1. SEM image of *T. pseudonana* (side view).
Photoluminescence quenching is any process that decreases the fluorescence response of a fluorophore. This occurs either from dynamic quenching or static quenching. Dynamic quenching occurs due to collisions between quenching agents and fluorophores while static quenching occurs when a quenching agent forms a non-fluorescent complex with fluorophores causing a reduction in the photoluminescence response. (Brandt, 2010) Both of these types of quenching are depicted in figures 3 and 4.
Figure 3. A cartoon representation of dynamic quenching. Quenching agents remain in solution and absorb light, thus inhibits the fluorescent response.

Figure 4. A cartoon representation of static quenching. Quenching agents bind to the fluorophore and inhibit the fluorescent response.

Using Raman spectrometry, the quenching effect can be quantified and described using a Stern-Volmer plot (depicted in figure 5). This type of chart plots the concentration of the quenching agent against the measured intensity ratio of the fluorophore through equations 1 and 2.

\[
\frac{F_0}{F} = 1 + K_D [Q] \quad \text{Equation 1}
\]

\[
\frac{F_0}{F} = 1 + K_S [Q] \quad \text{Equation 2}
\]
In equation 1, $F_0$ represents the intensity of the fluorescent response without the presence of a quenching agent, $F$ represents the intensity of the fluorescent response with the presence of a quencher at the concentration $Q$, $K_D$ represents the dynamic quenching constant ($K_D$ is replaced by $K_S$ if the quenching is known to be static quenching – shown in equation 2) and $Q$ is the concentration of the quenching agent. A single quenching process can be deemed as static or dynamic be changing temperature. If slope increases with increasing temperature, then the process dynamic, while if the slope decreases with decreasing temperature, then the process is static. (Brant, 2010). A visual representation of this equation is given below.

![Graph](image)

Figure 5. Stern-Volmer Plot Example. The slope of this plot is equal to $K_D$ or $K_S$ depending on the method of quenching.

In some cases, the effect of a quenching agent is due to a combination of static and dynamic quenching. This results in equation 3 and subsequently in equation 4, a combination of equations 1 and 2. The Stern-Volmer plot then appears to be polynomial as opposed to linear due to the combined quenching. (Brandt, 2010)
\[
\frac{F_0}{F} = (1 + K_D [Q])(1 + K_S [Q]) \\
\frac{F_0}{F} = 1 + (K_D + K_S)[Q] + K_D K_S [Q]^2
\]

Equation 3

Equation 4

Figure 6. Stern-Volmer plot example for combined quenching. This type of plot fits a second order polynomial function.

2.3 – Trinitrotoluene Quenching

Previously, it has been shown that synthetic mesoporous silica elicits fluorescence response and that mesoporous silica exhibits static quenching when in the presence of TNT. (Tao, Yan, Guangtao, Yu, 2008) Based off of this previous research, the idea of using diatom biosilica instead of mesoporous silica arose. (De Stefano, Rotiroti, De Stefano, Lamberti, Lettieri, Setaro, Maddalena, 2009) (Lettieri, Setaro, De Stefano, De Stefano, Maddalena, 2008). The main differences between mesoporous silica and diatom biosilica are the imperfections in biosilica (i.e. biosilica is not entirely SiO$_2$ – occasionally hydroxide or nitrogen groups find their way into the biosilica), and the geometry of silica source (mesoporous silica are typically flat while frustules are not).
3 – Methods

3.1 – Diatom cell culture

Axenic cultures of the photosynthetic marine diatom *Thalassiosira pseudonana* were obtained from UTEX The Culture Collection of Algae (UTEX # LB FD2) and cultivated in foam-stoppered 500 mL flasks with 100 mL of Harrison’s Artificial Seawater Medium (Harrison, Waters, Taylor, 1980) (ASM) enriched 0.050 mM dissolved silicon as Na$_2$SiO$_3$ (Martin-Jezequel, Hildebrand, Brezinski, 2000) (Fanning, Pilson, 1973). Flask cultures were maintained at 22 °C and 21 μE/m$^2$-sec incident light intensity on a 14 hour light 10 hour dark photoperiod, on an orbital shaker at 100 rmp. The cell suspension was subcultured at 10% v/v every 14 days. (Jeffries, Rosenburger, Rorrer, 2013)

The cell suspension was then inoculated to an initial cell number density of 1.8 · 10$^6$ cells/mL. For cell number density, a 0.1 mL aliquot of the cell suspension was diluted in 10.0 mL of saline electrolyte solution and counted on a Beckman Z2 Coulter Counter at a minimum threshold of 4 μm/ Triplicate cell counts were performed on each sample.

3.2 – Frustule Isolation

Intact frustules of *T. pseudonana* cells were isolated by detergent extraction. Live cells were transferred into 50 mL conical tubes and centrifuged at 2000 G, 4 °C for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 5 mL 100 mM EDTA and 1% Igepal CA-630 solution (detergent solution). Organic compounds are extracted at 37 °C for 60 min on an orbital shaker. Suspensions were transferred into 2
mL conical tubes and were collected by centrifugation at 13200 G and 4 °C for 5 minutes.

The supernatant was decanted and the cells were resuspended in 1 mL of the detergent solution and organic matter was extracted for an additional 5 min at 37 °C on an orbital shaker. The cells were collected by centrifugation at 13200 G, 4 °C for 5 minutes. This 5 minute extraction procedure was repeated twice (total of 3 extractions). The remaining detergent solution was decanted and the frustules were washed.

Frustules were washed via resuspension using 1 mL of deionized water followed by immediate centrifugation at 13200 G, 4 °C for 5 minutes. The frustules were washed in water two additional times followed by a wash using 20 mM sodium phosphate and 100 mM EDTA solution at a pH of 7.0. Finally, the frustules were resuspended in 1 mL of phosphate-buffered saline and frozen until needed. Upon use, the frozen frustule suspension was defrosted, centrifuged at 13200 G and 4 °C for 5 minutes, and resuspended in 1 mL of artificial sea medium. (LeDuff, Roesijade, Rorrer, 2016).

3.3 – Sample preparation

0, 10, 20, 30 and 40 mg/L of trinitrotoluene (TNT) in anhydrous acetonitrile were prepared. 10 μL of a TNT solution of interest was combined with 100 μL of cells or frustules, and 890 μL of ASM in a blacked-out 2 mL conical tube. The reaction tubes was placed on a rotisserie rotator for 24 hours.

Cell and frustule samples were deposited on a clean silicon surface so that the biological signal from the biosilica could be resolved. Square silicon wafers (1.0 cm/side)
were cleaned with 70% v/v ethanol, dried under sterile air and placed within a petri dish containing a damp paper towel. A 5 μL of sample (either cell suspension or frustule suspension) was pipetted onto the cleaned silicon wafer where the droplet dispersed to a nominal diameter of 5 mm. The samples were allowed to set for 10 minutes so that the cells could settle on the wafer surface. The deposited samples were kept hydrated in the humidified petri dish and immediately analyzed by micro-photoluminescence analysis.

3.4 – Photoluminescence Measurements

Micro-photoluminescence (μ-PL) measurements on individual living cells or frustules were performed on a Horiba Jobin Yvon Lab Ram HR Confocal Raman Microscope equipped with a Kimmon 325 nm He-Cd laser (IK315R-E, 15 mW average power) (Arteaga-arias, Nahmad, Navarro-Contreras, Encinas, Garcia-Mezaa, 2014) A single cell or frustule on the silicon wafer in the valve up orientation was imaged under a 40X-UV objective of the Olympus BS41 microscope with a 200 μm field of view. The laser spot diameter was determined to be $1.22 \lambda / NA$, where $\lambda$ is the laser wavelength (nm) and NA is the numerical aperture. At 40X magnification and NA set to 0.5, the laser spot diameter at $\lambda = 325$ nm was 0.8 μm (Orellana, Petersen, van den Engh, 2004). The laser spot was manually targeted to the center of the cell. The cell was individually lased at times of 20, 40, and 60 seconds before emission spectra collection. At the end of each lasing time, the emitted light was passed through the 40X-UV objective with a D1 optical density filter. The filtered PL spectra were captured by a Synapse CCD detector (model 354308, 300 grating) with an integration time of 3 seconds. (Gale, Jeffryes, Gutu, Jaio, Chang rorrer, 2011). The process was repeated for 10 individual cells or frustules per TNT
concentration. All spectra were corrected for the silicon wafer background. A photo of the Raman spectrometer is depicted below in figure 7.

Figure 7. Raman spectrometer (LamRAM HR800) used for PL measurements.
4 – Results and Discussion

300 Raman spectra were collected from both the frustule and the live cell samples (150 spectra per experiment). These data were processed using a combination of Visual Basic for Applications (VBA) macros and Matlab script (see Appendix C for VBA and Matlab scripts). The combination of these two scripts resulted in noise-reduced spectra (see Appendix A for noise fitted spectra) that were peak fit (Bradley, 2015) for both the biosilica peak (approximately 500 nm) and the chlorophyll peak (approximately 700 nm) using peakfit.m – a peak fitting software provided by Dr. Tom O’Haver of the University of Maryland at College Park (unpublished). The parameters used for each peak fit are given in Appendix C in the Matlab script. A representative peak fit spectra is given below. Additional peak fits can be provided upon request.

![peakfit.png](attachment:peakfit.png)

Figure 8. Representative peak fitting plot. The top plot represents the raw spectra with the peak fitted curved overlaid. The blue peak represents the raw data, the green curves represent the individual peak fitted curves and the red curve represents the total peak fit curve. The bottom plot represents the residual plot corresponding to the spectra.
From the peak fitted data, the following pieces of information were collected: the wavelength at which the biosilica peak occurred according to the peakfit.m; the peak height of the fitted biosilica peak; and the height of the noise-reduced biosilica peak. The peak fitting software allowed for quick and efficient analysis of the noise-reduced spectra; however, it was erroneous at times. Tabulated peak positions for biosilica were occasionally positioned at wavelengths of approximately $10^{14}$ nm as opposed to traditional 500 nm. Because of this, the determined wavelengths for each peak were averaged and statistical outliers were thrown out. This resulted in between 7 to 10 cells or frustules for each concentration where were used to determine the average intensity for each concentration at each lasing time. Intensity ratios were calculated on the basis of each lasing time and these intensity ratios were averaged within their respective concentration. These average intensity ratios were utilized for Stern-Volmer plots for both frustules and cells. These plots for both frustules and cells are shown below. (Additional Stern-Volmer plots for each lasing time are shown in Appendix D).

![Figure 9. Stern-Volmer plot for the frustule experiment. Error bars present on this plot represent one standard deviation.](image-url)
Figure 10 offers no suggestion that TNT participates in either static or dynamic quenching alone. It may be possible that TNT participates in a combined quenching mechanism in the presence of diatom frustules. However, there has been no previous research to suggest that TNT participates in dynamic quenching, so this theory is most likely invalid. However, it may be possible that frustules contain residual organic material that may be causing interference. Figure 11 shows similar results to figure 11; however, figure 11 is for live cells. Figure 11 strongly suggests dynamic quenching due polynomial trend of the data; however, this is most likely not the case. *T. pseudonana* is known to consume TNT and use it as a nitrogen source when the cell is nitrogen starved. (Crus-Uribe, Rorrer, 2006) Because live cells incubated in a nitrogen depleted and TNT rich solution for 24 hours, this may have caused the live diatoms to become starved for nitrogen, and start breaking down TNT down at lower concentration. Then, at higher TNT concentrations, the live cells began dying due to the high toxicity level of the increased TNT concentration and the photoluminescent response began to be quenched.
The results of this research created more questions. For example, one of the main questions regarding what exactly happens between each of the concentrations chosen. Because the trend observed in both the frustules and live cells is non-linear, it leaves some guess work as to what exactly happens between data points collected. This could be further studied by simply repeating this experimental procedure at additional TNT concentrations. At this moment, the frustules or live cells cannot be used as an adequate TNT detection method.
5 – References

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## Appendix A: Data Tables

Table 1. Processed data for frustule experiment.

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<th>TNT Concentration (mg/L)</th>
<th>Lasing Time</th>
<th>Average Peakfit Intensity (a.u.)</th>
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Table 2. Processed data for live cell experiment.

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<td>1.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>20 sec</td>
<td>1469</td>
<td>622</td>
<td>2.22</td>
<td>1.75</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>40 sec</td>
<td>764</td>
<td>356</td>
<td>1.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 sec</td>
<td>479</td>
<td>239</td>
<td>1.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>20 sec</td>
<td>591</td>
<td>78</td>
<td>5.52</td>
<td>5.91</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>40 sec</td>
<td>244</td>
<td>44</td>
<td>4.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 sec</td>
<td>95</td>
<td>18</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix B: Photoluminescence Spectra

The following spectra were generated from the VBA script given in appendix C. Each plot contains 30 PL spectra for either frustules or live diatoms at varying concentrations of TNT. The 30 spectra come from 10 cells that were lasered 3 times after 20, 40 and 60 seconds. Raw spectral data can be provided upon request.

Figure 11. 0 mg per L TNT, Frustule Experiment.
Figure 12. 10 mg per L TNT, Frustule Experiment.

Figure 13. 20 mg per L TNT, Frustule Experiment.
Figure 14. 30 mg per L TNT, Frustule Experiment.

Figure 15. 40 mg per L TNT, Frustule Experiment.
Figure 16. 0 mg per L TNT, Cell Experiment.

Figure 17. 10 mg per L TNT, Cell Experiment.
Figure 18. 20 mg per L TNT, Cell Experiment.

Figure 19. 30 mg per L TNT, Cell Experiment.
Figure 20. 40 mg per L TNT, Cell Experiment.
Appendix C: VBA and Matlab Script

B.1: VBA Script

Sub RorrerExpt_TableFormat()
    ' RorrerExpt_TableFormat Macro
    ' Keyboard Shortcut: Ctrl+L
    Range("B1").Select
    Selection.End(xlToRight).Select
    Selection.End(xlToLeft).Select
    Range("B1:J1").Select
    With Selection
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlBottom
        .WrapText = False
        .Orientation = 0
        .AddIndent = False
        .ShrinkToFit = False
        .MergeCells = False
    End With
    Range("B2:G2").Select
    With Selection
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlBottom
        .WrapText = False
        .Orientation = 0
        .AddIndent = False
        .ShrinkToFit = False
        .MergeCells = False
    End With
    Range("H2:J2").Select
    With Selection
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlBottom
        .WrapText = False
        .Orientation = 0
        .AddIndent = False
        .ShrinkToFit = False
        .MergeCells = False
    End With
    Range("B3:C3").Select
    With Selection
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlBottom
        .WrapText = False
        .Orientation = 0
        .AddIndent = False
        .ShrinkToFit = False
        .MergeCells = False
    End With
End Sub
End With

Range("D3:E3").Select
With Selection
  .HorizontalAlignment = xlCenter
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = False
End With

Range("F3:G3").Select
With Selection
  .HorizontalAlignment = xlCenter
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = False
End With

Range("B2:G2").Select
ActiveCell.FormulaR1C1 = "Raw Data"
Range("H2:J2").Select
ActiveCell.FormulaR1C1 = "Noise-Reduced"
Range("B3:C3").Select
ActiveCell.FormulaR1C1 = "20 Seconds"
Range("D3:E3").Select
ActiveCell.FormulaR1C1 = "40 Seconds"
Range("F3:G3").Select
ActiveCell.FormulaR1C1 = "60 Seconds"
Range("B1:J3").Select
Selection.Copy
Range("K1").Select
ActiveSheet.Paste
Range("T1").Select
ActiveSheet.Paste
Range("AC1").Select
ActiveSheet.Paste
Range("AL1").Select
ActiveSheet.Paste
Range("AU1").Select
ActiveSheet.Paste
Range("BD1").Select
ActiveSheet.Paste
Range("BM1").Select
ActiveSheet.Paste
Range("BV1").Select
ActiveSheet.Paste
Range("CE1").Select
ActiveSheet.Paste
Range("CE1:CM1").Select
Selection.End(xlToLeft).Select
Range("B1:J1").Select
Application.CutCopyMode = False
ActiveCell.FormulaR1C1 = "Cell 1"
Range("K1:S1").Select
ActiveCell.FormulaR1C1 = "Cell 2"
Range("B1:J1").Select
ActiveCell.FormulaR1C1 = "1"
Range("K1:S1").Select
ActiveCell.FormulaR1C1 = "2"
Range("T1:AB1").Select
ActiveCell.FormulaR1C1 = "3"
Range("AC1:AK1").Select
ActiveCell.FormulaR1C1 = "4"
Range("AL1:AT1").Select
ActiveCell.FormulaR1C1 = "5"
Range("AU1:BC1").Select
ActiveCell.FormulaR1C1 = "6"
Range("BD1:BL1").Select
ActiveCell.FormulaR1C1 = "7"
Range("BM1:BU1").Select
ActiveCell.FormulaR1C1 = "8"
Range("BV1:CD1").Select
ActiveCell.FormulaR1C1 = "9"
Range("CE1:CM1").Select
ActiveCell.FormulaR1C1 = "10"
Range("CE1:CM1").Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Range("A1").Select

End Sub

Sub RorrerExpt_AllInOne()
'
' RorrerExpt_AllInOne Macro
'
' Keyboard Shortcut: Ctrl+Shift+L
'
' A macro designed to do DataFormatting, NoiseReducing, DataSelection and FinalFormatting all in one

Range("B4:B2816").Select
Selection.Cut
Range("A4").Select
ActiveSheet.Paste
Range(_

29
 ).Select
 Selection.Delete Shift:=xlToLeft
 Range("B2:D2").Select
 ActiveCell.FormulaR1C1 = "Raw Data"
 Range("E3:G3").Select
 Selection.Copy
 Range("B3").Select
 ActiveSheet.Paste
 Range("B2:D3").Select
 Application.CutCopyMode = False
 Selection.Copy
 Range("H2:J2").Select
 ActiveSheet.Paste
 Range("N2:P2").Select
 ActiveSheet.Paste
 Range("T2:V2").Select
 ActiveSheet.Paste
 Range("Z2:AB2").Select
 ActiveSheet.Paste
 Range("AF2:AH2").Select
 ActiveSheet.Paste
 Range("AL2:AN2").Select
 ActiveSheet.Paste
 Range("AR2:AT2").Select
 ActiveSheet.Paste
 Range("AX2:AZ2").Select
 ActiveSheet.Paste
 Range("BD2:BF2").Select
 ActiveSheet.Paste
 Range("B1:G1") Select
 Application.CutCopyMode = False
 ActiveCell.FormulaR1C1 = "1"
 Range("H1:M1").Select
 ActiveCell.FormulaR1C1 = "20 Seconds"
 Range("H1:M1").Select
 ActiveCell.FormulaR1C1 = "2"
 Range("N1:S1").Select
 ActiveCell.FormulaR1C1 = "3"
 Range("T1:Y1").Select
 ActiveCell.FormulaR1C1 = "4"
 Range("Z1:AE1").Select
 ActiveCell.FormulaR1C1 = "5"
 Range("AF1:AK1").Select
 ActiveCell.FormulaR1C1 = "6"
 Range("AL1:AQ1").Select
 ActiveCell.FormulaR1C1 = "7"
 Range("AR1:AW1").Select
 ActiveCell.FormulaR1C1 = "8"
 Range("AX1:BC1").Select
 ActiveCell.FormulaR1C1 = "9"
 Range("BD1:BI1").Select
 ActiveCell.FormulaR1C1 = "10"
 Range("A1:A3").Select
 With Selection
.HorizontalAlignment = xlCenter
.VerticalAlignment = xlBottom
.WrapText = False
.Orientation = 0
.AddIndent = False
.ShrinkToFit = False
.MergeCells = False
End With
Selection.Merge
ActiveCell.FormulaR1C1 = "Wavenumber (nm)"

Range("E53").Select
ActiveCell.FormulaR1C1 = "=AVERAGE(R[-49]C[-3]:RC[-3])"
Range("E53").Select
Selection.Copy
Range("D8").Select
Selection.End(xlDown).Select
Range("E53:E2816").Select
Range("E2816").Activate
ActiveSheet.Paste
Application.CutCopyMode = False
Selection.Copy
Range("F53").Select
ActiveSheet.Paste
Range("G53").Select
ActiveSheet.Paste
Range("K53").Select
ActiveSheet.Paste
Range("L53").Select
ActiveSheet.Paste
Range("M53").Select
ActiveSheet.Paste
Range("Q53").Select
ActiveSheet.Paste
Range("R53").Select
ActiveSheet.Paste
Range("S53").Select
ActiveSheet.Paste
Range("W53").Select
ActiveSheet.Paste
Range("X53").Select
ActiveSheet.Paste
Range("Y53").Select
ActiveSheet.Paste
Range("AC53").Select
ActiveSheet.Paste
Range("AD53").Select
ActiveSheet.Paste
Range("AE53").Select
ActiveSheet.Paste
Range("AI53").Select
ActiveSheet.Paste
Range("AJ53").Select
ActiveSheet.Paste
Range("AK53").Select
ActiveSheet.Paste
Range("AO53").Select
ActiveSheet.Paste
Range("AP53").Select
ActiveSheet.Paste
Range("AQ53").Select
ActiveSheet.Paste
Range("AU53").Select
ActiveSheet.Paste
Range("AV53").Select
ActiveSheet.Paste
Range("AW53").Select
ActiveSheet.Paste
Range("BA53").Select
ActiveSheet.Paste
Range("BB53").Select
ActiveSheet.Paste
Range("BC53").Select
ActiveSheet.Paste
Range("BG53").Select
ActiveSheet.Paste
Range("BH53").Select
ActiveSheet.Paste
Range("BI53").Select
ActiveSheet.Paste
Range("BD1:BI1").Select
Selection.End(xlToLeft).Select
Range("BD1:BI1").Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
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Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Selection.End(xlToLeft).Select
Range("BD1:BI1","H1:BI1") Select
Range("B1:G1,H1:M1").Select
Range("B1:G1,H1:M1").Activate
With Selection
  .HorizontalAlignment = xlCenter
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = False
End With
Selection.Merge
Range("B1:G1,H1:M1,N1:S1,T1:Y1,Z1:AE1,AF1:AK1,AL1:AQ1,AR1:AW1,AX1:BC1,BD1:BI1") _
  .Select
'Final formatting is going to go before dataselect. This just seems to make sense to me

Range("B1:G1,H1:M1").Select
Range("H1").Activate
With Selection
  "HorizontalAlign = xlCenter"
  "VerticalAlign = xlBottom"
  "WrapText = False"
  "Orientation = 0"
  "AddIndent = False"
  "ShrinkToFit = False"
  "MergeCells = False"
End With
Selection.Merge
Range("B1:G1,H1:M1,N1:S1,T1:Y1,Z1:AE1,AF1:AK1,AL1:AQ1,AR1:AW1,AX1:BC1,BD1:BI1") _
  .Select
Range("BD1").Activate
With Selection
    .HorizontalAlignment = xlGeneral
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .ShrinkToFit = False
    .MergeCells = True
End With
With Selection
    .HorizontalAlignment = xlGeneral
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .ShrinkToFit = False
    .MergeCells = True
End With
Selection.Merge True
With Selection
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .ShrinkToFit = False
    .MergeCells = False
End With
Selection.Merge
Range( _
    "B1:G1,H1:M1,N1:S1,T1:Y1,Z1:AE1,AF1:AK1,AL1:AQ1,AR1:AW1,AX1:BC1,BD1:BI1,B2:D2") _
.Select
Range("B2").Activate
With Selection
    .HorizontalAlignment = xlGeneral
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .ShrinkToFit = False
    .MergeCells = True
End With
With Selection
    .HorizontalAlignment = xlGeneral
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .ShrinkToFit = False
    .MergeCells = True
End With
With Selection
    .HorizontalAlignment = xlGeneral
    .VerticalAlignment = xlBottom
    .WrapText = False
.Orientation = 0
.AddIndent = False
.ShrinkToFit = False
.MergeCells = True
End With
With Selection
  .HorizontalAlignment = xlGeneral
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = True
End With
With Selection
  .HorizontalAlignment = xlGeneral
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = True
End With
With Selection
  .HorizontalAlignment = xlGeneral
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = True
End With
With Selection
  .HorizontalAlignment = xlGeneral
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = True
End With
With Selection
  .HorizontalAlignment = xlGeneral
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .ShrinkToFit = False
  .MergeCells = True
End With
.ShrinkToFit = False
.MergeCells = True
End With
With Selection
.HorizontalAlignment = xlGeneral
.VerticalAlignment = xlBottom
.WrapText = False
.Orientation = 0
.AddIndent = False
.ShrinkToFit = False
.MergeCells = True
End With
Selection.Merge True
With Selection
.HorizontalAlignment = xlCenter
.VerticalAlignment = xlBottom
.WrapText = False
.Orientation = 0
.AddIndent = False
.ShrinkToFit = False
.MergeCells = False
End With
Selection.Merge
Range("BD2").Activate
With Selection
.HorizontalAlignment = xlCenter
.VerticalAlignment = xlBottom
.WrapText = False
.Orientation = 0
.AddIndent = False
.ShrinkToFit = False
.MergeCells = False
End With
Selection.Merge
Range("F6").Select
Rows("1:1").Select
Range("BJ1").Activate
Selection.Insert Shift:=xlDown, CopyOrigin:=xlFormatFromLeftOrAbove
Range("A1").Select
ActiveCell.FormulaR1C1 = "Maximum Intensity for Si"
Range("E1").Select
ActiveCell.FormulaR1C1 = "=max(R[5]C:R[1000]C)"
Range("E1").Select
Selection.Copy
Range("F1:G1,K1:M1,Q1:S1,W1:Y1,AC1:AE1,AK1:AQ1,AU1:AW1,BA1:BC1,BG1:BI1").Select
ActiveSheet.Paste
Range("A2").Select
ActiveCell.FormulaR1C1 = "Average Intensity for Si"
Range("A3").Select
ActiveCell.FormulaR1C1 = "Standard Error for Max Intensity"
'Average Calculations
Range("E2"), Select
Range("F2"), Select
Range("G2"), Select

'Standard Deviation Calculations
Range("E3"), Select
Range("F3"), Select
Range("G3"), Select

ActiveWindow.LargeScroll ToRight:=1
ActiveWindow.SmallScroll ToRight:=90
ActiveWindow.SmallScroll Down:=45
ActiveSheet.Shapes.AddChart2(240, xlXYScatterSmoothNoMarkers).Select
ActiveChart.ChartObjects("Chart 1"). Activate
ActiveChart.Axes(xlCategory). Select
ActiveChart.ChartTitle. Select
ActiveChart.ChartArea. Select
ActiveChart.Axes(xlCategory). Select
ActiveChart.Axes(xlCategory).MinimumScale = 450
ActiveChart.Axes(xlCategory).MaximumScale = 750
Application.CommandBars("Format Object").Visible = False
ActiveChart.ChartArea. Select
With ActiveChart
Axes(xlCategory, xlPrimary). HasTitle = True
Axes(xlCategory, xlPrimary). AxisTitle.Characters.Text = "Wavenumber (nm)"
Axes(xlValue, xlPrimary). HasTitle = True
Axes(xlValue, xlPrimary).AxisTitle.Characters.Text = "Intensity (Counts)"
End With
ActiveChart.ChartArea. Select
ActiveSheet.Shapes("Chart 1"). IncrementLeft -105
ActiveSheet.Shapes("Chart 1"). IncrementTop -351.75

36
ActiveWindow.SmallScroll Down:=-24
ActiveSheet.Shapes("Chart 1").IncrementLeft 141
ActiveSheet.Shapes("Chart 1").IncrementTop -142.5
ActiveWindow.SmallScroll Down:=-9
End Sub
B.2: Matlab Script

```
answer = input('Are you sure you want to run this program. Previous graphs will be closed. (Y/N) ', 's')
if answer == 'N'
    return
else

clear all, format compact, close all, clc

name = input('What is the name of the file of interest? ', 's');
folder = name(1:end-5)
mkdir(folder)

tabs = {'00 mg per L', '10 mg per L' '20 mg per L' '30 mg per L' '40 mg per L'};
%tabs = [ 2 3 4 5 6]
cells = {'E', 'K', 'Q', 'W', 'AC', 'AI', 'AO', 'AU', 'BA', 'BG', 'F',
        'AE', 'AK', 'AQ', 'AW', 'BC', 'BI'};
start = '59:';
stop = '2822';
c = strcat( cells,start,cells,stop)
average = [ 20 40 60];
standarddeviation = [20 40 60];
guess = [500 75 675 50]
x = xlsread(name, '00 mg per L', 'A59:A2822')

file_id = strcat( '_processed','.xlsx')
new_filename = strrep(name, '.xlsx', file_id)
for j = 1:5;
    for i = 1:30;
        %Performs peakfitting
        s = c{i};
        I = num2str(i)
        y = xlsread( name, tabs(j), s);
        fig = figure
        peakfit([x,y], 600, 300, 2, 1, 0, 1, guess, 0);
        string = strcat(pwd,'/',folder,'/','tabs{j}','_', I , '.jpeg')
        saveas(fig, string)

        %Determines what the wavelength of the fitted peak maximum is.
        if ans(1,2) < 0;
            fit_peaks(i+1,1) = ans(2,2);
        else
            fit_peaks(i+1,1) = ans(1,2);
        end

        %Determines what the intensity ofthe fitted peak is at the
```

%determined wavelength.
fit_peaks(i+1,2) = ans(1,3);

%Determines what the experimental intensity of the actual
%spectra is at the determined wavelength.
ind = find( abs(x - ans(1,2)) < 0.5,1)
TF = isempty(ind);
if TF == 1
    ind = 1;
    fit_peaks(i+1,3) = y(ind)
else
    fit_peaks(i+1,3) = y(ind)
end
end
xlswrite(new_filename, fit_peaks, j)
end
end
Appendix D: Additional Stern-Volmer Plots

Figure 21. Stern-Volmer plot for the cell experiment for averaged intensity ratios. Error bars represent one standard deviation. Orange linear fit and blue 2nd order polynomial fit lines are included.

Figure 22. Stern-Volmer plot for the cell experiment at a lasing time of 20 seconds.
Figure 23. Stern-Volmer plot for the cell experiment at a lasing time of 40 seconds.

Figure 24. Stern-Volmer plot for the cell experiment at a lasing time of 60 seconds.
Figure 25. Stern-Volmer plot for the frustule experiment for averaged intensity ratios. Error bars represent one standard deviation. Orange linear fit and blue 2nd order polynomial fit lines are included.

Figure 26. Stern-Volmer plot for the frustule experiment at a lasing time of 20 seconds.
Figure 27. Stern-Volmer plot for the frustule experiment at a lasing time of 40 seconds.

Figure 28. Stern-Volmer plot for the frustule experiment at a lasing time of 60 seconds.