Report experiment 10

Laser induced fluorescence

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Summary

Several measurements are made around laser induced fluorescence (LIF). First the system was calibrated. The resolution of the spectrograph was found to be 0.85 ± 0.2 nm. The theoretical resolution $\Delta\lambda = 0.54$ nm is smaller, because only a part of the error sources were part of the computation.

Then the fluorescence, absorbtion and transmission spectrum of a rhodamine 6G solution were measured. The fluorescence peak wavelength is 570 nm, which corresponds to the orange color of the solution. Also the spectrum was measured using a yellow instead of a green laser. This showed that the absorbtion of yellow was much lower than that of green and the energy of fluorescing photons were lower.

By translation the solution perpendicular to the laser-beam and forcing the fluorescing photons to travel through the solution longer, the concentration of rhodamine 6G could be computed: $n = 0.141 \pm 0.005$ mmol l^{-1}.

The fluorescence efficiency was computed roughly by combining several measurements. This resulted in $F = 0.17$. The expected value was $F \approx 1$.

Finally some LIF measurements were done on a green leave. The qualitative result was that photoinhibition occurs, but there are also other processes causing fluorescence, albeit at other wavelengths.

Contents

1 Introduction

Laser induced fluorescence (LIF) is the optical emission from molecules that have been excited to higher energy leves by absorption of photons. One of the most frequently used applications of LIF is measuring a biological preperation with a dye. A fluorescing dye is chosen which binds to specific structures inside a cell, so that when the preperation is lit by a laser, an image of the structures can be made. Sometimes not even a dye is needed but already part of an organism, for example photosynthesis.

2   Theory

2.1   Fluorescence

In an atom or molecule are many different energy levels in which an electron can be. Electrons tend to take positions with the lowest energy, this is called the ground state of the atom or molecule (S0). When a photon is absorbed, an electron can be excited to a higher energy level (S1). Inside an energy level are smaller energy differences caused by rotation (J) and vibration (V).

Figure 1: Energy levels, LIF

Laser induced fluorescence (LIF) is a process of (at least) two steps. First an electron is excited by absorbstion of a photon (upward arrow in figure 1). The energy of the electron is the previous energy of the electron plus the energy of the photon. Then the electron falls back to the ground state, emitting a photon of energy $h\nu_{fluor.}$ (downward arrow in figure 1). However, after step one the electron can undergo rotation and vibration transitions inside S1, before falling back to S0.
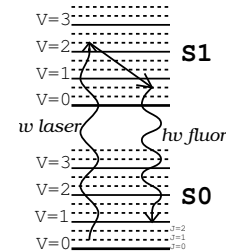
2.2   Absorbtion

When a lightwave travels through a solution, the energy of the wave is gradually dissipated as the wave advances through it, so that the light intensity is a function of the thickness:

$$I(x) = I_0 e^{-n\varepsilon x}, \tag{1}$$

where I_0 is the intensity at $x = 0$, n is the concentration and ε is the molar extinction coefficient, which is a measure for the absorbtion of a certain molecule in the solution.

2.3   CCD

A lineair CCD consist of a row of light-sensitive elements (pixels). When photons are absorbed in the vicinity of the junction, the electron-hole pairs are seperated by a built-in electric field (obtained by doping a semiconductor), causing a change in voltage, the photovoltaic effect. The efficiency of this proces is called the quantum efficiency Q. Pixels of a CCD are connected in series, so the whole CCD can be read out sequentially like a shift register.

The number of measured counts N_e consists of three components:

$$N_e = QN_f + I_d t + N_{read}, \tag{2}$$

where N_f is the number of photons that reached the pixel, I_d is the dark current (spontaneous charging of a pixel), t is the measurement interval and N_{read} is the error caused by reading the CCD.

2.4 Spectrometer

A spectrometer is a device for measuring spectral lines of light. The central element of the spectrometer is the grating, causing light of different wavelengths to reflect on a different angle. Light from the entrance slit is directed to a first concave mirror with focal distance f, which collimates the light incident on the grating (see figure 2). The diffracted light is incident on a second concave mirror, which then focuses the spectrum at the CCD, resulting in a 1-to-1 image of the entrance slit. As the grating is rotated, the dispersed spectrum moves across the CCD.[2]
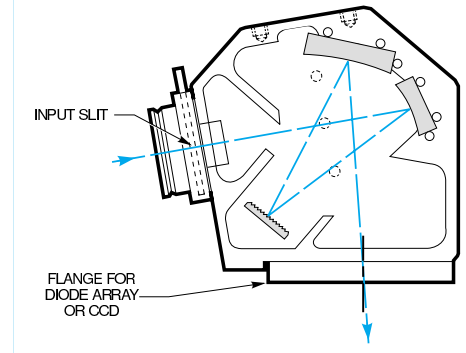

Figure 2: Optical configuration of the MS125 spectrograph

The resolution of the spectrometer is dependent on the linedistance of the slit a, the focal distance f of the first mirror, the width d_{pixel} of a CCD pixel, the width d_{slit} of the entrance slit and the angle of reflection between light beam and grating:

$$\Delta \lambda = \frac{(d_{slit} + d_{pixel})a \cos \theta}{f}. \tag{3}$$

The total bandwidth is given by formula 4, where W is the width of the CCD:

$$\lambda_{max} - \lambda_{min} = \frac{W a \cos \theta}{f}. \tag{4}$$

2.5 Photosynthesis and fluorescence

Photosynthesis is a process used by plants to obtain energy from light. In so called antennae molecules are excited by incoming photons and transported to a reaction centre, where further reactions occur. There are two types of reaction centres: PS I and PS II. In both types does fluorescence occur, but on different wavelengths.

When a plant is lit excessively, photoinhibition [3] (also called photoprotection [4]) occurs, which obstructs photosynthetic electron transport between antennae and reaction centers, and slows down the photosynthesis. The energy is converted to heat and other chemical reactions, including decreasing the pH. This results in oxidative damage of the cells, so the reaction centers don't get overloaded.

3 Experimental setup

Some calibration measurements on the ccd and spectrometer are done first. The dark current is measured by reading the CCD when all light sources are turned off. This is done with different integration times (measurement intervals).

A neon lamp is put in front of the spectrometer and the orientation of the grating inside the spectrometer is adjusted so that wavelengths between 500 and 800 nm are being projected on the CCD. The CCD can then be calibrated by comparing a measurement of the neon spectrum with known spectral lines.

For the measurement of the absorbtion of rhodamine 6G the setup of figure 3 is build. By turning off the halogen lamp the background signal is measured. Then the halogen lamp is turned on, but no sample is placed in the cuvet. After measuring this, the cuvet with a rhodamine 6G solution is placed and the spectrum is measured again. The absorbtion spectrum can be derived from those three spectra.
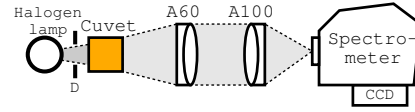
Figure 3: Setup for absorbtion measurements. A=achromatic lens, D=diafragm.

The setup of figure 4 is build to do LIF measurements. The laser has a wavelength of 543.5 nm. The fluorescence spectrum of rhodamine 6G is measured. Also the effect of horizontal and vertical translation of the cuvet is measured. Then the spectrum is measured with a yellow ($\lambda = 594.1$ nm) laser.
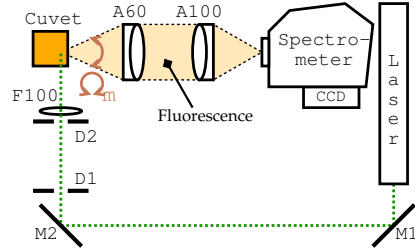
A green leave is used to do experiments on photosynthesis. The cuvet in in figure 4 is substituted for a sample of a leave. It is aligned such, that only light from fluorescence is caught by the lenses and led into the spectrometer. In this setup the leave sample is moved so a certain spot will be lit by the laser. Then the laser is turned on and after waiting for t_l seconds the spectrum is measured. This is done for different values of t_l, each measurement at a different spot on the leave.

Figure 4: Setup for LIF measurements. M=mirror, F=lens, A=achromatic lens, D=diafragm.

3.1 Efficiency

Not every photon that is absorbed emits a fluorescing photon that is detected. The total detected photons N_m are related to the laser intensity I_0 as in the following formula:

$$N_m = \frac{\Omega_m I_0 \, n\varepsilon \, F f_m}{4\pi h\nu} V_m e^{-n\varepsilon x_0}, \tag{5}$$

where Ω_m is the spacial angle in figure 4, F is the probability that fluorescence photon is emitted when a laser photon is absorbed, $f_m = QT\frac{h_{slit}}{h_{pixel}}$ with the quantumefficiency Q, temperature T, slit and ccd pixel height respectively h_{slit} and h_{pixel}, and $V_m = \frac{f_{A60}}{f_{A100}} d_{slit}$ where d_{slit} is the width of the slit and f_L is the focal distance of lens L. [1] Gathering the energy-related variables results in:

$$\int_\lambda \frac{n_m(\lambda) \, h\nu}{Q} \, d\lambda = F \cdot \Omega_m I_0 \, n\varepsilon T d_{slit} r_{laser}^2 \frac{h_{pixel}}{h_{slit}} \frac{f_{A60}}{f_{A100}} \, e^{-n\varepsilon x_0}, \tag{6}$$

with $n_m(\lambda)$ the measured counts at each wavelength.

4 Results

4.1 Calibration

The dark current is measured with different measurement intervals, which is plotted in figure 5. Fitting results in $N_e = (0.24 \pm 0.08) + (7.7 \pm 0.6)t$ (see formula 2). A sample spectrum is shown in appendix B.
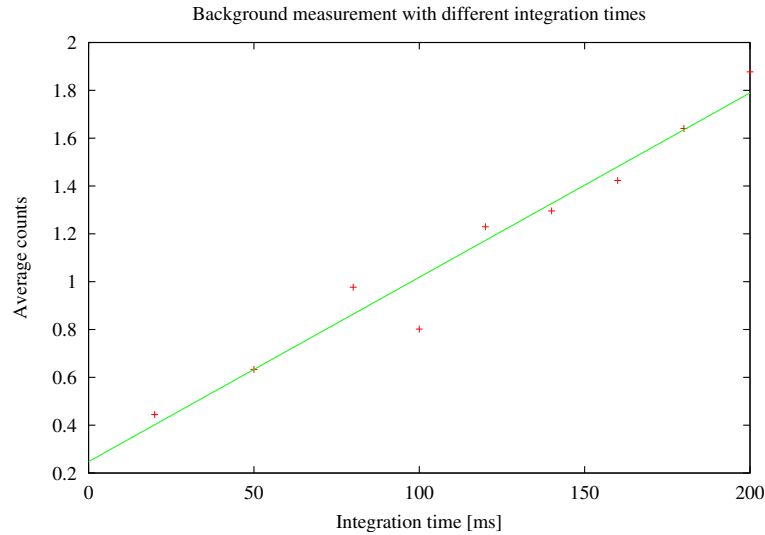

Figure 5: Dark current measurement, the average is taken over the measured spectrum.

The measured spectrum of Ne can be found in appendix A. Spectral lines are matched with literature values [1] in in figure 6. A second order polynomial fit gives the relation $\lambda = (463.4 \pm 0.2) + (0.1891 \pm 0.0005) \times Ch + (6.2 \pm 0.2) \cdot 10^{-6} \times Ch^2$ nm.
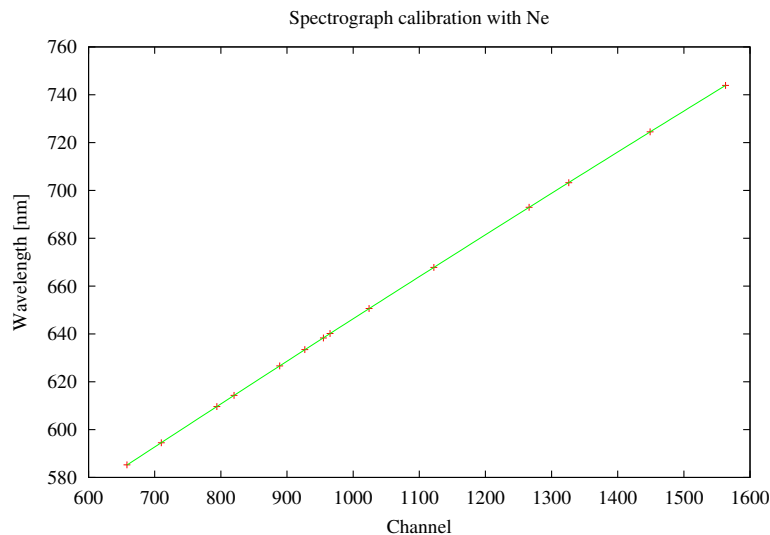

Figure 6: Calibration of the spectrograph using a neon lamp.

4.2   Absorbtion, transmission and LIF

The absorbtion, transmission and LIF spectrum of rhodamine 6G can be seen in figure 7.
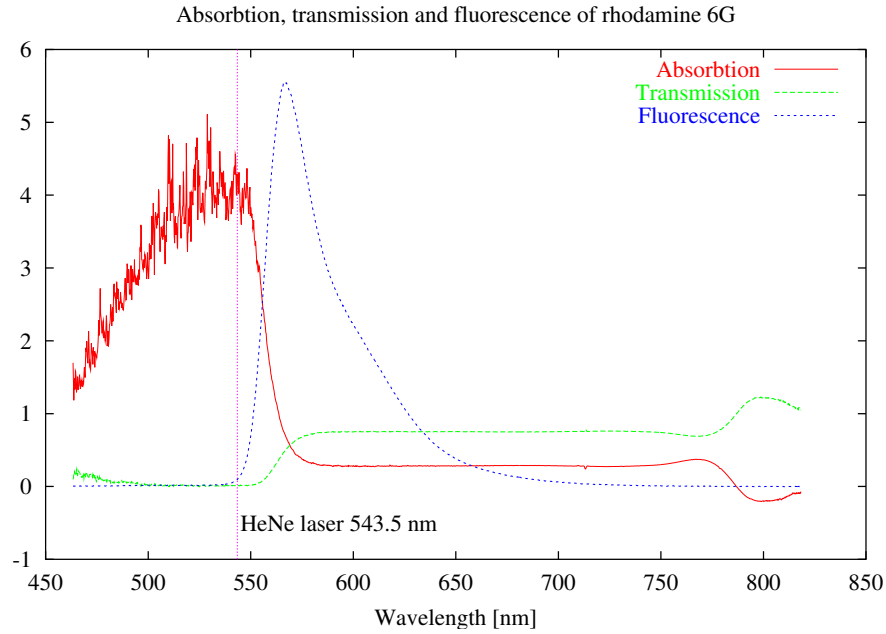
Figure 7: Absorbtion, transmission and LIF spectrum of rhodamine 6G.

The cuvet was translated perpendicular to the laser-beam. The spectra at different positions can be found in figure 8.
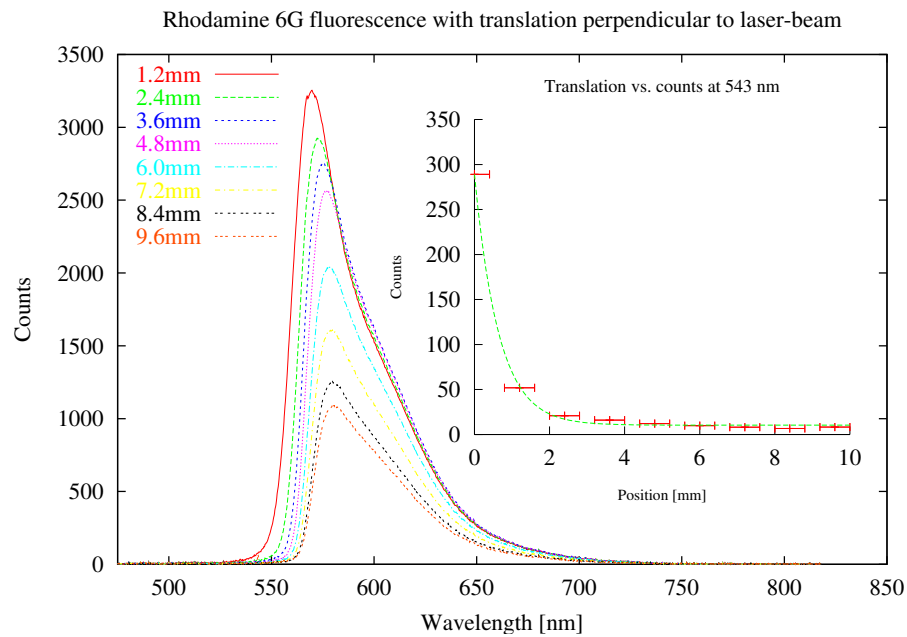
Figure 8: Spectra at different cuvet positions.

Also LIF was measured using a green (543.5 nm) and yellow (594.1 nm) laser beam. Both spectra are plotted in figure 9.
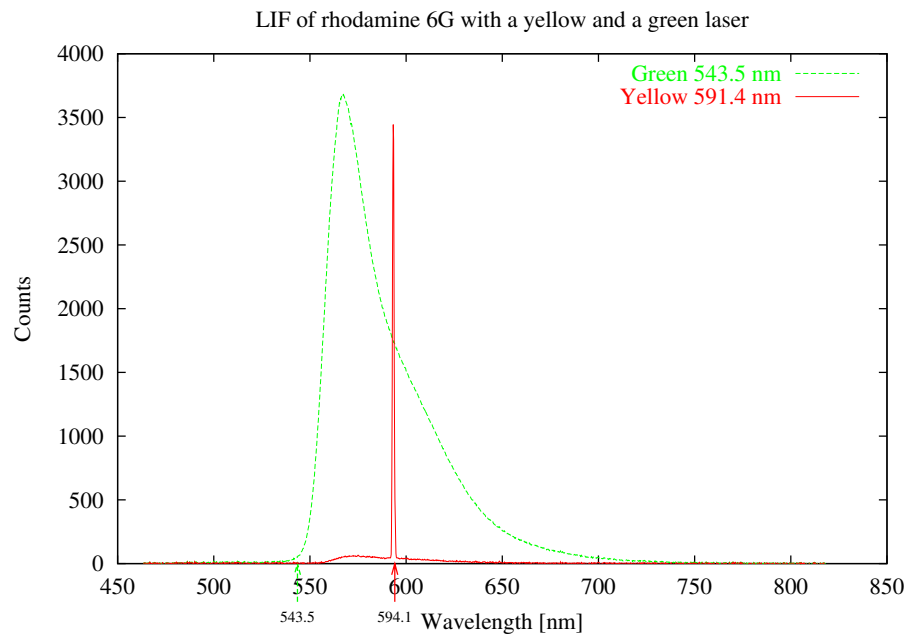

Figure 9: Rhodamine 6G spectrum with a green and yellow HeNe laser. The yellow spectrum was measured using a higher filter.

The measurement on photosynthesis of a leave can be seen in figure 10. The spectrum is measured for different times between turning on the laser and reading out the CCD.
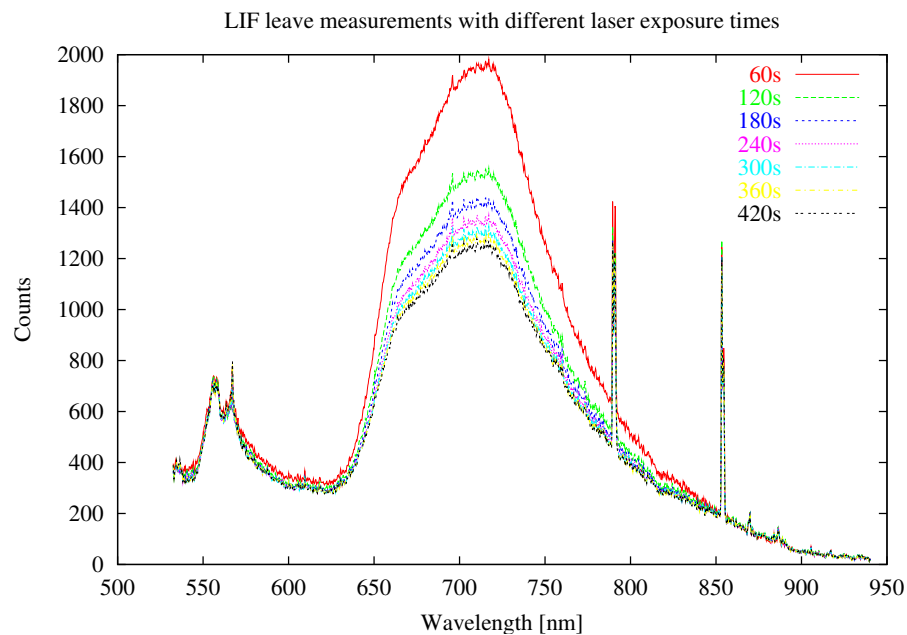

Figure 10: LIF measurement on a green leave. The number of seconds in the legend is the time between turning on the laser and reading out the CCD.

5   Discussion

5.1   Calibration

The dark current of the CCD was measured 7.7 ± 0.6 electrons/s. This is clearly not consistent with the CCD specification, 218 e^-/pixels/s. Also $N_{read} = 122$ electrons according to the CCD documentation, but it is 0.24 ± 0.08 according to the measurements.

Looking at figure 13 in appendix B it is striking that there is a quite a number of negative counts. This could be caused by a background correction of the measurement software (it should obviously be turned off during this measurement), rendering the dark current measurements useless.

The resolution of the spectrograph can be read from figure 12 in appendix A. The average resolution (FWHM) of the shown lines is 0.85 ± 0.2 nm. Using formula 3 combined with formula 4 to compute $a \cos \theta$ results in the theoretical value $\Delta\lambda \approx 0.54$ nm. The measured resolution is bigger than the computed value. This can be caused by additional errors introduced by cables and other equipment.

5.2   Absorbtion, transmission and LIF

Looking at figure 7 one can see that when absorbtion is high, transmission is low and vice versa. This is of course what is to be expected. The fluorescence peak is around 570 nm, which is yellow/orange light. That is in agreement with observations of the eye: it looks orange.

When closely looking at the fluorescence around 545 nm, one can see that there is fluorescence of wavelengths below the excitation (laser) wavelength, i.e. with a higher energy than the excitation energy. This is possible because electrons have thermal energy.

The spectrum with the yellow laser in figure 9 shows a peak at the laser wavelength, while the spectrum with the green doesn't. This is because the absorbtion of the green 543.5 nm laser is very low, while the absorbtion of the yellow 594.1 nm laser isn't (see figure 7). The shape of both spectra are very much the same; the yellow spectrum looks weaker, but that's because a filter was used to be able to measure it correctly. But one can see that the yellow spectrum is moved to the right with respect to the spectrum of the green laser. This is because the yellow laser-beam has a lower energy.

To compute the concentration of the rhodamine 6G solution, the inset of figure 8 was made with the relation of the counts at $\lambda = 543$ nm and the distance, which should obey equation 1. The fit results in $n\varepsilon = (1.55 \pm 0.07) \cdot 10^3$ m^{-1}. With the known extinction coefficient of $\varepsilon = 1.1 \cdot 10^5$ l mol^{-1} cm^{-1} this results in $n = 0.141 \pm 0.005$ mmol l^{-1}.

In figure 8 the left side of the peak 'crawls' to the right as the distance increases. This is because the fluorescing photons have to travel longer through the rhodamine 6G dye and there is more chance to collide. Especially higher energy photons have more chance to be absorbed (see figure 7).

The fluorescence efficiency is computed using formula 6: $\int_\lambda \frac{n_m(\lambda)h\nu}{Q} \, d\lambda = F \cdot 1.43 \cdot 10^{-5}$. The lhs for a LIF measurement with the green laser has been computed at $2.497 \cdot 10^{-6}$, so $F = 0.17$. This seems a bit low for a dye that optimally has an $F \approx 1$, but the whole computation is far from precise.

The fluorescence spectrum of the green leave (figure 10) is divided into two parts: the curve at the center $(650 - 750$ nm) which decays as the times increases, and some peaks $(550 - 575, 790,$ and 854 nm) which are more or less constant. The decaying curve in the center is due to photoinhibition. The brown color of the spot that was lit by the laser after a measurement also indicates that oxydation has taken place. The spikes don't change as time increases, so these are probabely reactions either not part of photosynthesis or where fluorescence takes place before electron transport.

6 Conclusions

The dark current was measured 7.7 ± 0.6 electrons/s. The deviation from the specification of 218 electrons/s is caused by a measuring mistake.

The resolution of the spectrograph was measured 0.85 ± 0.2 nm, whereas the theoretical value is $\Delta\lambda \approx 0.54$ nm. The additional error is caused by other measurement equipment.

The fluorescence of rhodamine 6G has a peak wavelength of 570 nm, which corresponds to the orange color of the solution. Excitation with a yellow laser shows a peak at the laser wavelength that doesn't occur when using a green laser. This agrees with the high absorbtion in green and low in yellow.

The concentration of the rhodamine 6G solution was $n = 0.141 \pm 0.005$ mmol l^{-1}.

The fluorescence efficiency is computed $F = 0.17$, while $F \approx 1$ is expected.

LIF on a green leave shows that photoinhibition occurs, but not all processes which procude fluorescence are effected by it.

References

[1] *Handleiding Natuurkunde Praktikum N 4/5 (3B063)*, Technische Universiteit Eindhoven, faculteit Natuurkunde, september 2000.

[2] *Introduction to Optics, second edition*, Frank L. Pedrotti and Leno S. Pedrotti, Prentice-Hall, ISBN 0-13-016973-0, Chapter 17.

[3] *An Introduction to Photosynthesis and Its Applications*, Wim Vermaas, Arizona State University, http://photoscience.la.asu.edu/photosyn/education/photointro.html.

[4] *Biophysics 354, Biological Energy Conversion*, Antony Crofts, University of Urbana-Champaign, http://www.life.uiuc.edu/crofts/bioph354/, Lecture 20, 21.

A   Measured spectrum of Ne

Measured spectrum of Ne

Figure 11: Measurement of the spectrum of neon.

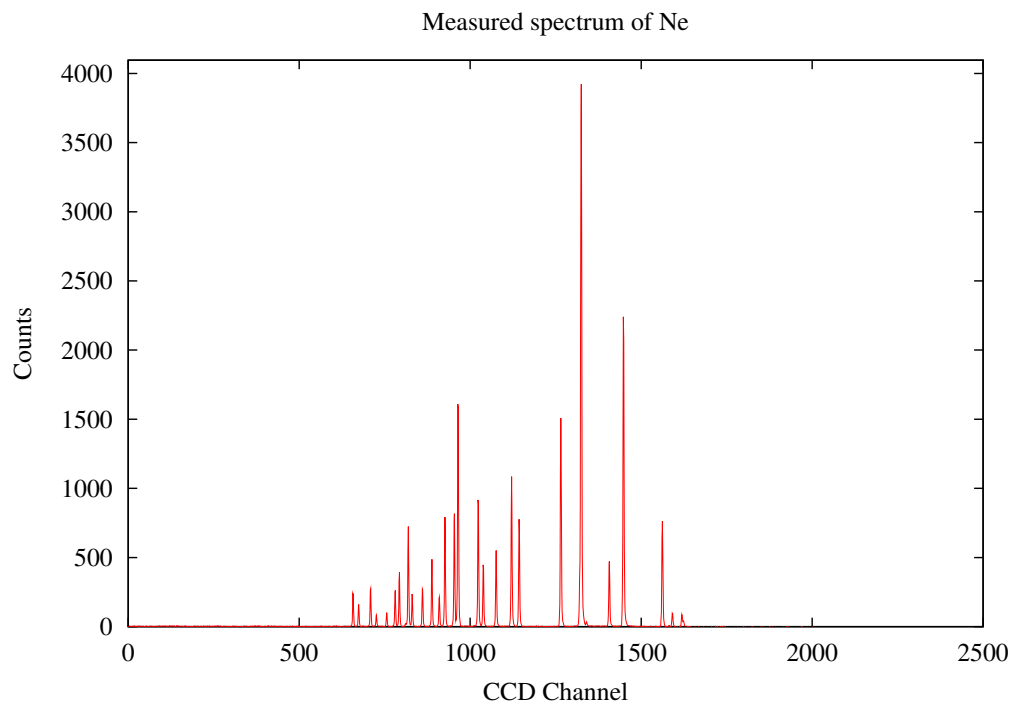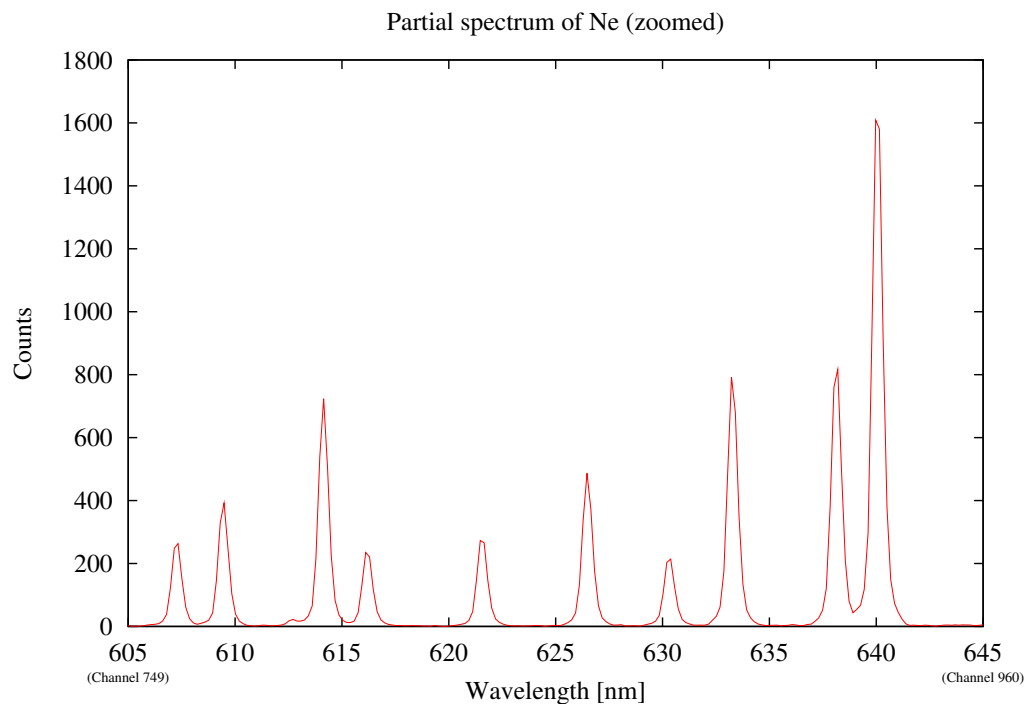Partial spectrum of Ne (zoomed)

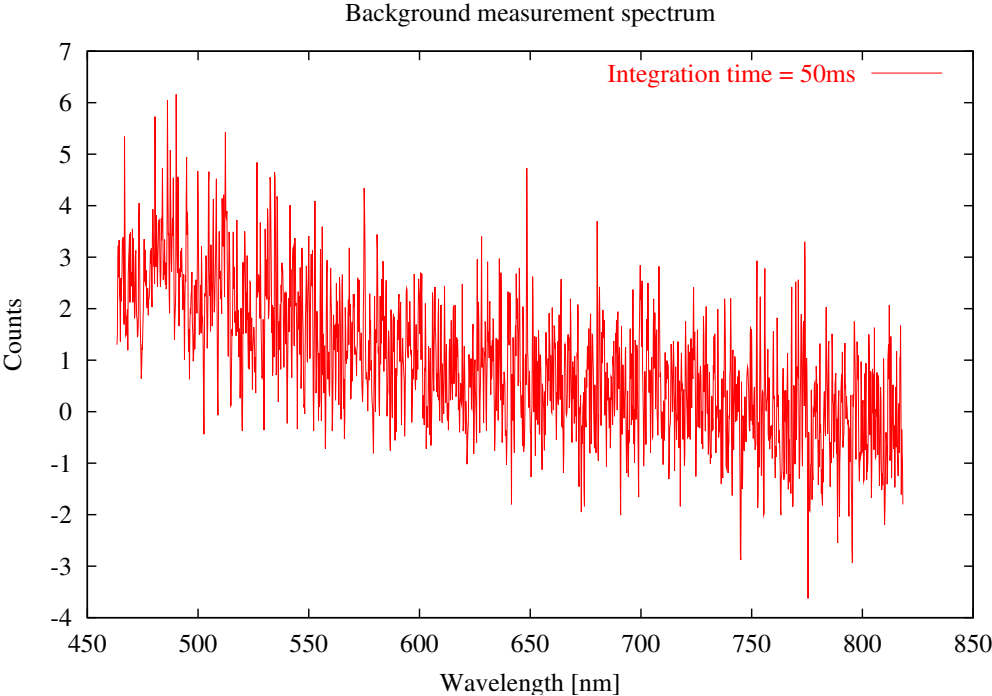

Figure 12: Zoomed in on Ne spectrum.

B   Background measurement spectrum

Figure 13: A spectrum of a background measurement.