Using Low Cost Components To Determine Chlorophyll Concentration By Measuring Fluorescence Intensity

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Submitted to the Faculty of Engineering, University of Cape Town, in fulfilment of the requirements for the Degree of Master of Science

Acknowledgements

I would like to thank the following people for their contribution towards this thesis:

- Dr Fred Nicolls, my supervisor, for his willingness to accept me in his student group. His patience and guidance over a long period was very encouraging when some of the practical measurements and data did not look promising.

- Dr Clive Garcin for going out of his way to help me by providing me with algae and chlorophyll samples, without which I would have been unable to make any useful measurements during the development of the fluorometer.

- Dr Stewart Bernard for all the valuable time he set aside to provide me with information and guidance on chlorophyll fluorescence and the requirements of the low cost fluorometer.

- Trevor Probyn for allowing me to join them during their instrument calibration in a laboratory. He then also scanned the chlorophyll calibration standard to provide me with a wavelength profile which was invaluable to interpret the measurement data.

- Emma Bone for her help during the laboratory measurements of chlorophyll a fluorescence that was required to determine the chlorophyll a measurement range of the fluorometer system.

- Rick Collins of Arius Inc. (www.arius.com) for helping me to find a better digital signal processing method that could be used to recover the modulated fluorescence signal amidst noise.

Declaration

I, Johannes P. Truter, declare that this dissertation is my own work except where otherwise stated. It is being submitted for the degree of Master of Science in Engineering at the University of Cape Town. It has not been submitted before for any degree or examination at this or any other university.

Signature of Author: 

April 2015 Cape Town

Abstract

This dissertation describes the development of a low cost fluorometer with the aim of using it as an algae and phytoplankton concentration sensor. As it forms the core of this fluorometer's functionality, chlorophyll's fluorescence characteristics and origins are discussed. Special attention is given to the variability of chlorophyll fluorescence as it has a big influence on measurements. Experimental procedures and data are provided to show why each component was finally selected for use in the fluorometer. An analogue front end device with programmable gain on each 24-bit ADC channel forms the interface between the high sensitivity TSL257 light-to-voltage light sensors and the 32-bit ARM microcontroller that controls the system. The microcontroller software controls the 470 nm LED current to create a 75 ms light pulse that has a 63 Hz sine wave modulated on it. The low cost light sensors proved to be sensitive enough to detect the low light intensities of chlorophyll fluorescence. The challenges of measuring the low level voltages from these light sensors are discussed. The amount of noise on the light sensor voltages at low chlorophyll concentrations make it difficult to accurately measure the fluorescence signal. Different light modulation and digital signal processing techniques were investigated to compare the effective recovery of the fluorescence signal. Sine wave modulation along with sample averaging provided good results. The results of laboratory experiments with pure chlorophyll α and extracted chlorophyll are discussed to give an overview of the capabilities and limitations of the developed fluorometer that is able to measure the fluorescent light from extracted chlorophyll concentrations as low as 0.01 µg/l.

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1 Introduction

This chapter starts with the background to why this thesis project was started before presenting the basic concept design of the system that was used for the laboratory experiments to confirm the system's functionality. The last section of the chapter introduces the layout of the rest of the thesis. It also provides some high level detail about the different topics that are discussed in each chapter.

1.1 The Thesis Project History

The idea for the project started when two research groups required a low cost sensor to measure algae concentrations in water. These groups were the Centre for Bioprocess Engineering Research (CeBER), at the University of Cape Town, and the Earth Observation group, at the Council for Scientific and Industrial Research (CSIR).

CeBER grows algae in the laboratories for research in different areas. These include production of oil for biodiesel as well as research into valuable pigments (phycocyanin, astaxanthin) and other biological products. In all these research areas it is necessary to know the algae concentration. Low cost algae concentration sensors would free up money for the main research topics.

The CSIR group required a low cost sensor to measure algae concentrations in dams in South Africa as well as phytoplankton concentrations in the ocean along South Africa's coast line. Algae in oceans form part of the phytoplankton group, which also contain other organisms, that makes use of photosynthesis for survival. Algae and phytoplankton have enough similar characteristics to make it possible to measure their concentrations in water with the same instruments. This is discussed in much more detail later in the thesis.

The CSIR group uses data from remote sensing equipment such as satellites. At the time they needed a sensor that could be fitted to a submersible float for several months to measure and log the algae or phytoplankton concentrations. The logged concentrations could then be transmitted on a regular basis if the system had the capability to transmit data or it could be manually downloaded when the float was visited or retrieved.

For both these groups the algae concentrations had to be measured in a way that would be possible on site (in situ) and provide immediate measurements. The measurements had to be

done without influencing the physiological state of the algae or phytoplankton or disturbing the environment around them. The trend of the algae or phytoplankton concentration over time can then be investigated. This trend provides valuable information, like the overall conditions of the water around the algae or phytoplankton, and can indicate events like pollution since population growth has been shown to be susceptible to toxic pollutants [1].

Algae and phytoplankton both contain chlorophyll that they use to turn light into energy. Chlorophyll fluoresces (emits light) when light is shining upon it. This is discussed in much more detail in Chapter 2. According to literature [2],[3],[4] the fluorescence intensity of chlorophyll is proportional to its concentration in the water. Fluorescence meters (fluorometers) that measure chlorophyll fluorescence and can determine chlorophyll concentration have been built by [5],[6],[7],[8] and [9]. Our hypothesis was that it would be possible to build a fluorometer from low cost components that could be used as a chlorophyll concentration sensor. Measuring the chlorophyll concentration would then enable the calculation of the algae or phytoplankton concentration when the chlorophyll concentration of each species is known. It was not intended to add other functionality to the fluorometer, like measuring quantum yield (number of photons emitted/number of photos absorbed) or performing biomass calculation.

A fluorometer induces fluorescence by shining an excitation light on an area or object. In the case of this project the fluorometer excites fluorescence by shining the light into a fluid containing the algae or phytoplankton. The fluorometer then measures certain fluorescence parameters, like fluorescence intensity, to calculate the chlorophyll concentration that in turn can be used to determine the algae or phytoplankton concentration. Beutler [10] did groundbreaking work on concentration measurements and developed a research fluorometer in 2003 that could determine algae and phytoplankton species composition and concentration from chlorophyll fluorescence.

Schreiber has developed commercial fluorometers for photosynthesis research since 1986 and has published many articles about chlorophyll fluorescence and its measurement [11]–[18]. Currently there are many commercial fluorometers available that make use of expensive and sometimes specialised components that drive up the price of the product. These fluorometers usually also have added features that were not required by the CSIR or CeBER for the intended use as an algae or phytoplankton concentration sensor. The development of these added features also drives up the cost of such fluorometers.

The fluorometer developed during the thesis project is called the FICC (Fluorescence Intensity

Chlorophyll Concentration). A few requirements were decided upon at the start of the design phase of the FICC. As the project mainly started with the aim of developing a low cost fluorometer, the target was to keep the total cost of the system below $1500. Only low cost commercial components that are freely available would be used in the design. This would provide a fluorometer with a cost that is a tenth of the cheapest commercial product currently being used by the CSIR. Such a low cost would make it possible to deploy several of the fluorometers on the submersible floats for long periods in dams and in the ocean along the coastline without a major financial risk of the fluorometer getting lost or damaged.

To measure the phytoplankton concentrations in the ocean, the fluorometer had to be able to measure phytoplankton concentrations as low as 0.1 µg/l. These submersible floats would run on batteries. Consequently the fluorometer had to be designed with low power consumption as a high priority. The size of the fluorometer also had to be kept as small as possible as there was little space available on the submersible float. The target shape of the fluorometer housing was a cylinder with a diameter of 30 mm and length of 150 mm. The fluorescence measurement section ideally had to have a flat contact surface with the fluid containing the algae or phytoplankton. This would make it easier to clean with a wiping mechanism while in use on the float. The housing had to be waterproof down to a depth of at least 5 m as the submersible float it was going to be fitted to could dive down to this depth.

1.2 The Concept Design

During the development of the FICC, many prototypes of the subsystems were built to investigate the comparative performance of components as well as the response of chlorophyll to various light conditions. Some of these subsystem prototypes are discussed in the thesis chapters that cover the different components and lighting methods that were tested. Even though the requirements of the FICC indicated a final product with a flat face that can be placed in the fluid containing the chlorophyll, almost all the prototypes were built around the concept of having a cuvette holder in a “front end” subsystem where some components were mounted to the cuvette holder. This ensured that there was no change in location of sensors between measurements. A cuvette containing different chlorophyll fluid could then be easily placed and removed in the cuvette holder. This was originally done since it did not require a waterproof housing, which was not available.

This front end that was designed around the cuvette had other advantages during the development period. The front end could be enclosed to keep out all light, other than the excitation light. This

removed the influence of external light, which can distort measurements. Placing the components around the cuvette holder enabled the testing of different design ideas without requiring a redesign of the housing. Making use of a cuvette also allowed the preparation of several different chlorophyll concentrations, consisting of about 5 ml each in different cuvettes, that could quickly be swapped to compare measurements. A flat faced unit would have required larger quantities of chlorophyll fluid to place the FICC in. It would also have required a good cleaning procedure between measurements to prevent cross contamination of samples. This would have taken extra time and introduced the added risk of cross contamination.

The diagram below shows the final FICC system design that was used. It also shows the front end components that are located directly around the cuvette holder.

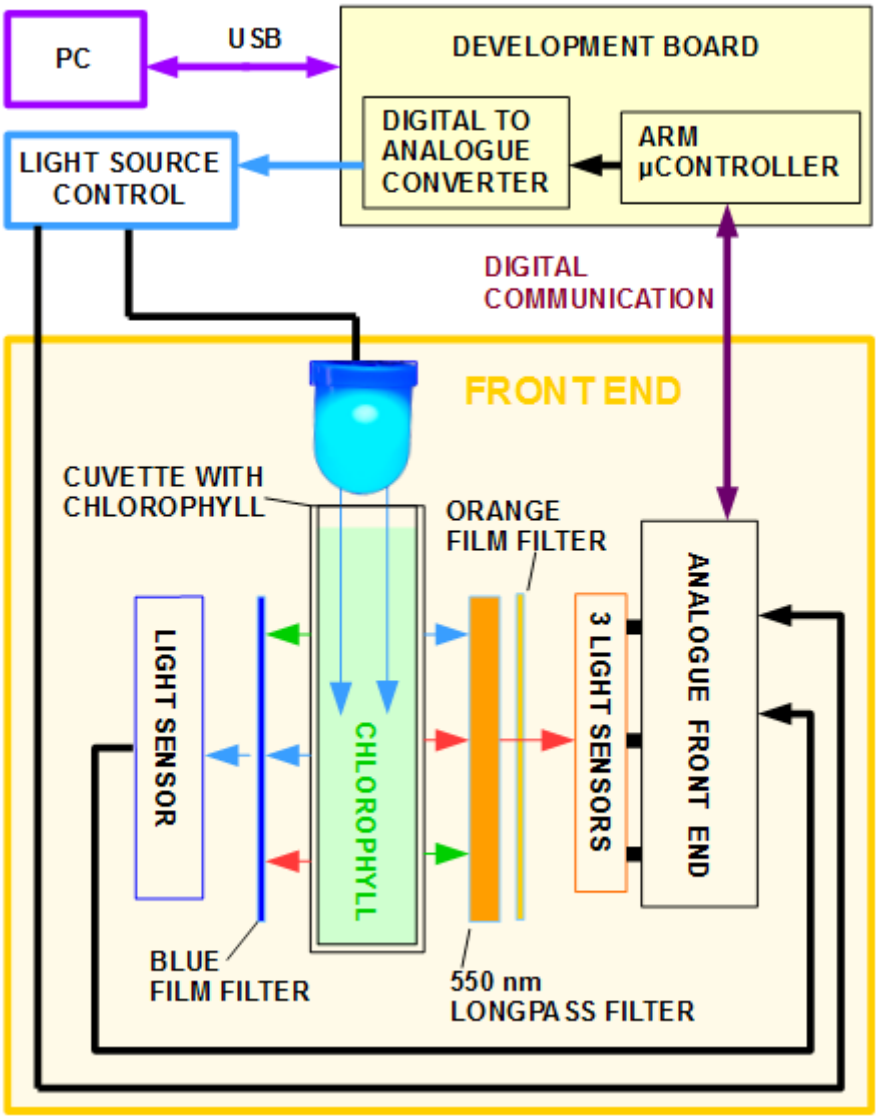


Figure 1: FICC system diagram

When a PC and USB cable are available, the FICC user can view and log the measured fluorescence data on the PC in real time. This can be done with either the user interface program that was developed for the FICC, or a communication terminal program, like Terra Term. The graphical user interface (GUI) provides oscilloscope traces of the measured data as it is being received from the FICC. Being able to see the measurement data immediately provides quick and easy confirmation that the system is functioning correctly and can be very helpful to quickly compare different sequences of excitation light intensities.

The FICC functionality is contained in software on a microcontroller development board. Two different development boards, the mbed and LPCXpresso, can be used as they share the same footprint and pin functionality for the pins that are used on the FICC. These development boards use an ARM microcontroller along with several different interface components. The FICC software makes use of the USB, serial peripheral interface (SPI) and digital to analogue (DAC) components to control and monitor the rest of the hardware.

The FICC software is used to implement the different types of fluorescence tests by controlling the excitation light intensity and reading the measured fluorescence. As is discussed later in the thesis, there are many different ways to measure fluorescence response. They all require that the excitation light intensity be controlled in different ways.

The USB connection between the PC and FICC also enables the user to quickly load different software on the FICC by simply copying it with the PC operating system. Measurements made with the same chlorophyll sample, with different test software, can then quickly be compared to determine the best software performance.

The DAC on the development board is used by the FICC software to send an analogue control voltage to the light source control unit. The software changes the control voltage according to the calculated required excitation intensity at that moment. The control voltage is then converted into a controlled current by the light source control unit. The changing current then drives the light source to achieve different excitation intensities. This light source happens to be a LED on the FICC.

The LED emits blue light (470 nm wavelength) that shines down the length of the cuvette. This causes fluorescence light to be emitted by the chlorophyll while some of the blue light is scattered by the chlorophyll particles or reflected by the cuvette sides and the enclosure walls around the cuvette. The intensity of the scattered and reflected blue excitation light is measured by a TSL250

light sensor that is situated on one side of the cuvette. It has a blue film filter in front of it that blocks out the fluorescence and other light with wavelengths longer than 570 nm. The TSL250 measurement data provides the excitation light intensity that was present in the cuvette at the time the measurement was made.

Chapter 2 discusses in detail how the fluorescence light is generated from the blue light by the chlorophyll. The fluorescence light intensity is measured by three TSL257 light sensors located on the side of the cuvette directly opposite the TSL250. These sensors have an orange film filter and a longpass fused silica filter in front of them to block out the high intensity excitation light as well as other light with wavelengths shorter than 550 nm. The reasons for having three light sensors and two light filters are discussed in more detail later in the introduction during the layout discussion of chapter 5.

A MCP3903 analogue front end (AFE) device with 6 channels measures the voltage outputs of all the light sensors. It also measures a voltage in the light source control unit that represents the current going through the LED. Each analogue channel of the AFE has a programmable gain amplifier (PGA) whose gain can be set from 1 to 32. After the input voltage of each channel has passed through the PGA, it goes to a 24-bit analogue to digital converter (ADC). The AFE has a digital communication port that is used by the development board to read the ADC data from it and also to set up the control registers of the AFE. The AFE control registers are used to set various different functions of the AFE. This is discussed in more detail in Section 6.5.

1.3 Layout of This Thesis

Chapter 2 aims to explain chlorophyll fluorescence. It starts with an overview of fluorescence history before going into some detail on the fluorescence mechanisms in phytoplankton and algae. The energy flow from the incoming light back to emitted light is explained to give an understanding of the intricate processes behind fluorescence. The difference between variable and constant fluorescence is explained along with their sources in the algae or phytoplankton.

It is essential to understand the chlorophyll fluorescence phenomenon and especially to know about its variability to be able to design a fluorometer that will excite chlorophyll fluorescence and measure it correctly. Fluorescence measurement data can get very confusing and sometimes even seem improbable if the actual meaning of measured parameters are not fully understood or when they are misinterpreted. Chapter 3 starts with a look at the Kautsky fluorescence curve that was a

milestone in the field and forms the basis of most chlorophyll fluorescence measurement parameters. It then discusses the most commonly used parameters of chlorophyll fluorescence measurements and how they should be interpreted. It also goes in much more detail than Chapter 2 regarding the factors causing variability in fluorescence intensity. The variability of chlorophyll fluorescence is one of the biggest challenges facing the correct use of a fluorometer as well as correctly interpreting the measurement data.

Chapter 3 continues the discussion of fluorescence measurement with a look into the validity of using fluorescence measurements to determine chlorophyll concentration. It shows that even though it is often stated that there is a direct relationship between fluorescence intensity and chlorophyll concentration, this is only true under specific conditions as set out by [19].

A fluorometer usually consists of two main subsystems. An excitation light source system is required to induce the fluorescence, and a sensing system is required to detect and measure the fluorescence parameters. The excitation light source system of current fluorometers have one or more light sources and usually a system that controls the excitation light intensity. Chapter 4 sets out the requirements of the excitation light system. It indicates with practical investigations why a 10 mm, 470 nm LED was finally selected for the FICC over smaller SMD or 5 mm LED options due to its higher light intensity and more consistent excitation of fluorescence. The results show that the 470 nm wavelength works well enough for this fluorometer application but if an affordable LED with a wavelength in the range of 430-440 nm could be found, it would perform better to measure chlorophyll α and b fluorescence. It is also shown that the fluorescence excitation light needs to emit at selected wavelengths to cause fluorescence in different algae and plankton species.

Chapter 4 then looks at the changes in fluorescence intensity due to variations in excitation wavelength and intensity during a measurement. The reasons for using a current source rather than a voltage source to drive the excitation LED of the FICC, are given. The author then explains why it is believed that accurate current control of the excitation LED is essential in the FICC design. The benefits of using specific intensities and wavelengths of light are also discussed, for instance, that the fluorescence signal can be recovered amidst noise. The discussion then covers some of the many excitation light modulation wavelengths and pulsing frequencies that have been used by researchers to measure different fluorescence parameters.

The second main subsystem of a fluorometer performs the fluorescence measurement function. It requires at least one light detector as well as a circuit that converts the light intensity signal to a

format that can be stored and analysed. Chapter 5 looks at the different aspects related to the measurement of the low intensity fluorescent light. It starts with a discussion of the traditional photo sensors that have been used to measure the fluorescence intensity. Section 5.1 contains an overview of the investigations that were performed when different low cost light sensors were initially compared for suitability to be used in the FICC. This shows how the TSL257 light-to-voltage sensor provided the highest sensitivity along with a fast enough response time. Chapter 5 also discusses the advantages of an increased measurement surface area and improved signal to noise ratio when three TSL257 sensors are used rather than one.

Light filters form an important part of fluorometers as they are used to provide specific wavelengths of light for excitation and also to block unwanted light wavelengths from reaching the fluorescence measurement light sensors. Section 5.2 explains why the Edmund Optics (EO) 550 nm longpass filter is combined with a Lee Filters Orange filter to enable measurement of the fluorescent light intensity while blocking the excitation light. It also shows why the Lee Filters Bright Blue filter is used to enable consistent measurements of the excitation light intensity by blocking out the fluorescent light from reaching the excitation light sensor.

Section 5.3 contains discussions of the investigations that were done to find the optimal location of the light sensors of the FICC. It shows that the final location of the light sensors are mostly dependant on practical packaging requirements since the fluorescent light is emitted equally in all directions.

The fluorescent light intensity does not always show the expected linear relationship to the excitation light intensity. Section 5.4 shows the results of practical measurements that confirmed the non-linear relationship when high chlorophyll concentrations are measured.

The benefits obtained in measuring the fluorescent light intensity with an integrated analogue front end (AFE) device, is discussed in Section 5.5. The AFE provides channels with individual settings, like programmable gain and addition of dithering noise to improve the accuracy. This section also explains that three light sensors are used in parallel to increase the signal to noise ratio. It ends with an explanation of why the non-inverting summing amplifier is not a good design to use in the FICC.

Due to the low output voltages of the fluorescent light sensors, noise makes out a fair amount of the final signal. Section 5.6 provides a description of some of the methods that were tested to filter

out noise from the fluorescence measurement data. The described methods benefit from using light modulation in the recovery of the small fluorescence signal. The actual results of the methods are discussed in Chapter 7.

Chapter 6 contains detailed information about the functional units of the FICC. It starts with a description of the mbed development board that contains the 32 bit ARM microcontroller and all the interfaces it has to the rest of the FICC subsystems. The section includes a discussion of the different software compilers that were used for various versions of the FICC software. One of the main functions of the software is to control the excitation LED intensity by sending a control signal to the LED current control unit. Section 6.2 provides a detailed explanation of how the control signal from the software is converted to a specific LED current with an accuracy of 98%. The control of the LED current is used to modulate the excitation light. Section 6.3 discusses the various light modulation methods and sequences that were tested before it was decided to use a 75 ms light pulse with sine wave modulation for use in the final tests with the FICC.

The layout and components of the FICC front end assembly is provided in Section 6.4. It shows the enclosed housing and flat faced prototype layouts that were used for the two sets of laboratory measurements. The aim of this section is to provide an idea of the relative location of all the components. These include the light sensors, light filters, cuvette and AFE. Section 6.5 contains a much more detailed discussion of the MCP3903 AFE than the one given in Section 1.2.

The LabVIEW user interface is discussed in Section 6.6. This program was specially developed for the FICC to provide real time feedback of the measurement data. The immediate display of measurement data was very helpful during comparative testing of components and light modulation methods.

Chapter 7 gives details of the methods used to prepare a chlorophyll extract and a chlorophyll calibration standard for accurate laboratory experiments to determine the FICC's performance. It also provides some of the measurement results and an analysis of them. The main goals were to determine if the FICC was sensitive enough to measure the fluorescence of the lowest chlorophyll concentrations in its requirements and if it was accurate enough to be used as a chlorophyll concentration sensor.

The process used to extract chlorophyll from Swiss chard spinach is provided in Section 7.1.1. There is a detailed description how the chlorophyll was dissolved in acetone before filtered and

freeze dried. It continues with an explanation how the chlorophyll powder was diluted in acetone to make concentrations of 1 g/l and 0.1 g/l of chlorophyll in acetone. Section 7.1.2 then describes how the chlorophyll powder was also used to make a series of chlorophyll and acetone dilutions ranging from 0.1 mg/l down to 0.01 µg/l. These dilutions were then used for performance measurements with the FICC in a laboratory. The results of these measurements are provided along with an analysis that shows that the FICC can measure the fluorescence from a 0.01 µg/l chlorophyll concentration. The results also show that the chlorophyll concentrations affects the intensity of the blue excitation light that reaches the measurement area by blocking and absorbing more of it as the concentration increases.

Section 7.2 covers the preparation and measurements made with a chlorophyll α calibration standard. It starts with an explanation of the CSIR procedure that was used to prepare the different chlorophyll concentrations. It then provides details of the measurement methods used, like the75 ms sine wave modulated light pulses. The measurement results of the different concentrations are presented. They show that there is a linear relationship between chlorophyll concentration and fluorescence intensity when the measurement samples are dilutions of one sample and all dilutions have been exposed to the same conditions in the laboratory. They also show that the FICC is not well suited to measure the fluorescence of pure chlorophyll α.

Section 7.2 contains a discussion of the results achieved when different techniques were used to reduce the noise on the original laboratory measurement data. It shows why the “brick wall” FFT filter was found to be an unsuitable technique for filtering out noise. Making use of a moving average along with the averaging of data from different sensors and light pulses, proved to be a useful technique to reduce the noise. The signal to noise ratio could be improved with a 5 data points moving average filter. A plot of the relationship between fluorescent and excitation light intensity provides an almost linear line that could be used as a calibration reference for unknown concentrations.

Chapter 8 provides the conclusions drawn from all the measurements made during the development of the FICC. It starts with a discussion of the current status of the FICC and presents the conclusions drawn from the literature and the practical investigations. The big challenge of coping with variable fluorescence is investigated by comparing sometimes contradictory published results with experiments made by the author. The limitations on the usability of the developed fluorometer system as an algae or phytoplankton concentration sensor are also discussed. Although the developed fluorometer has a high enough sensitivity to measure extracted chlorophyll

concentrations as low as 0.01 µg/l in the laboratory, its accuracy in measuring algae or phytoplankton concentrations was not determined. The last part of Chapter 8 looks at topics that could be investigated to further improve the FICC functionality and clarify some uncertainties. These include improving the stability of the excitation LED intensity and cost reduction by removing expensive light filters. The influence of the physiological state of chlorophyll on concentration measurements should, for example, be investigated.

2 What is Chlorophyll Fluorescence?

When designing a fluorometer to measure chlorophyll fluorescence is important to understand how it originates and what characteristics it has. It is even more important to know how to use these characteristics correctly to induce the type of fluorescence required for the measurement that needs to be done. This chapter starts with the history of how chlorophyll fluorescence was identified. It then gives an overview of the mechanisms involved in the photosynthesis systems that generate chlorophyll fluorescence. This will provide the required background for the fluorescence parameter discussion of Chapter 3.

At first we need to determine what fluorescence is. In 1834 Sir David Brewster mentioned that he saw red light when he passed strong sunlight through a green fluid that consisted of an alcohol extract from Laurel leaves. Govindjee [20] believes that this was likely the discovery of chlorophyll fluorescence though the word “fluorescence” did not exist then. Although it is not part of the fluorescence process it is important to know that Brewster also described how the amount of red light became less as the fluid concentration increased. It appears as if the fluorescence intensity decreases but this phenomenon is typically due to re-absorption of the red fluorescent light in thick chlorophyll examples and must be kept in mind during the development of a fluorometer to measure chlorophyll fluorescence. This is discussed in more detail in Chapter 3.

In 1852 Professor GG Stokes from Cambridge University coined the phrase “fluorescence” [21]. He was the first one to recognise that the phenomenon was due to light emittance and not filtering. This important fact means that the resulting fluorescing light intensity and wavelength are not always consistent for a specific light source intensity that is causing the fluorescence. The factors influencing the variability of chlorophyll fluorescence are discussed in more detail in Chapter 3.

Fluorescence in algae and phytoplankton is a product of a very intricate photosynthesis process that starts when light is absorbed to use its energy to synthesize carbohydrates from CO_2 and water [22]. Chlorophyll α is one of the most important molecules that absorbs this light energy for photosynthesis [22]. The front end of the photosynthesis apparatus consists mostly of chlorophyll antennae with a smaller number of chlorophyll reaction centres [23]. The light photons are absorbed by the antenna molecules that causes excited chlorophylls [24]. The absorbed light energy is transferred to the reaction centres via electron excitation [23]. Here the energy is converted to chemical energy (photochemistry) and heat (atom movement) [22],[25]. A detailed breakdown of the energy transfer process in the reaction centre is provided by [25]. More than 90%

of the absorbed energy is used by photosynthesis [25]. A varying amount of the remaining absorbed energy is given off again in the form of transmitted light (fluorescence).

Figure 2 is a modified Jablonski energy level diagram for chlorophyll in the photosynthesis system. It shows the different energy states of chlorophyll electrons after energy transfer from incoming photons of different wavelengths of light. The photons contain more energy as the light wavelength gets shorter. Plants (including algae and phytoplankton) have evolved to maximise absorption of the high energy blue light. The left side of the figure shows the different energy states of the electron after being energised by a photon from the corresponding wavelength on the right-hand side. The right-hand side of the figure also indicates typical amounts of absorption of light photons at that wavelength (see Figure 46 for an accurate absorption spectrum of chlorophyll α). The amount of absorption at the shorter wavelengths (blue) is usually higher than at the long wavelengths (red).

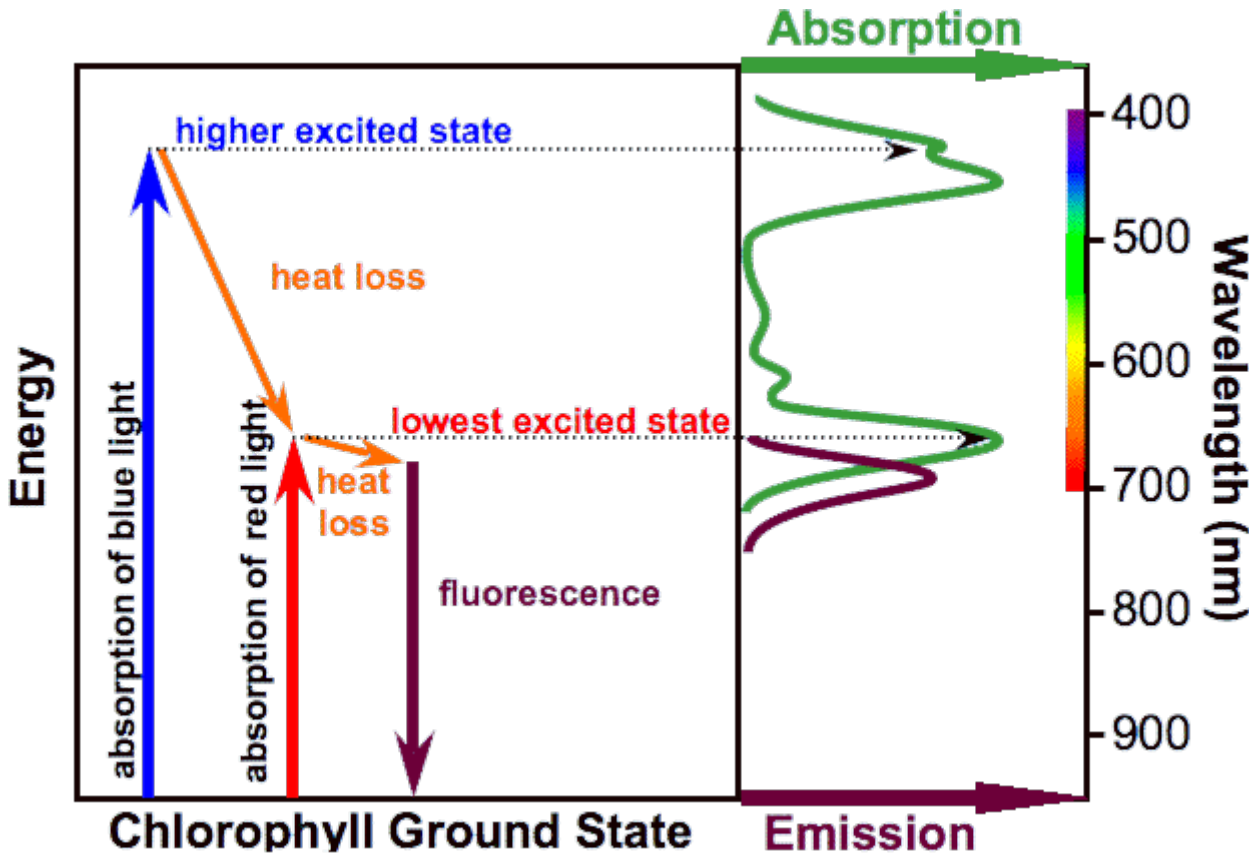


Figure 2: Chlorophyll energy levels from light absorption.

Figure above modified from [26].

The energy drop of the electrons due to fluorescence emission is also indicated in Figure 2 with a

purple arrow. The figure also shows the typical fluorescence wavelength spectrum around 700 nm on the right-hand side. A very detailed overview of the energy states involved in chlorophyll fluorescence is provided by [27]. The authors even break the fluorescence down into delayed fluorescence (DF) and prompt fluorescence (PF). This further breakdown of fluorescence is not of interest to our application of the fluorometer as a chlorophyll concentration sensor.

The chlorophyll used for photosynthesis is found in two sub-systems of the photosynthesis system, namely photosystem 1 (PSI) and photosystem 2 (PSII). One important difference between the PSI and PSII systems is that the PSII fluorescence varies in intensity while the PSI intensity is constant [28]. The change in PSII fluorescence intensity is caused by the opening and closure of its reaction centres. The reaction centres can only transfer energy at a certain rate. When this rate is exceeded by too much energy coming from absorbed light, the reaction centres start to close. As the reaction centres keep on closing more and more electrons have to return to their ground energy state by emitting photons as fluorescence. A formula that shows the relationship between the absorbed energy flux (I_a) and the rate of fluorescence emission (F) is provided by [25]. It shows how all the different energy reactions compete with fluorescence. This formula is not applicable for the application of the FICC. It is however important to know that there are several competing reactions in the algae and phytoplankton that will cause changes in the amount of fluorescence over time from a specific sample. The amount of fluorescent light will then change in proportion to the amount of energy available for fluorescence. Even if the energy-providing light stays constant, the amount of fluorescence energy will change if any other energy consuming process inside the algae or phytoplankton increases or decreases its energy consumption.

The difference between the fluorescence emission when all the reaction centres are open and closed is called the variable fluorescence (F_v). Variable fluorescence and its measurement parameters are discussed in detail in Section 3.2.

The study of the mechanisms and energy flow in the photosystems of algae and phytoplankton is a research field of its own. For the purposes of this fluorometer project it is just important to know that there are complex systems at work to control the amount of light energy being absorbed by the algae or phytoplankton, which then also controls the amount of fluorescence being emitted. The chlorophyll fluorescence measurement methods are discussed in the next chapter. This discussion will at times also touch on the responses and control mechanisms of the photosystems but only to explain certain phenomenon that could affect the use of the fluorometer as a chlorophyll concentration sensor or the interpretation of measurement data.

3 Chlorophyll Fluorescence Measurement

The changes in the amount of fluorescence emission under a constant excitation light, as discussed in Chapter 2, can make the interpretation of fluorescence measurements difficult. This variable fluorescence does have certain consistent characteristics that have been identified over several decades. These characteristics influence fluorescence measurements in specific ways. It is necessary to fully understand these effects on measurement data to prevent invalid interpretations [19]. This chapter provides an initial overview in Section 3.1 of the most basic fluorescence parameters before discussing the multitude of modern parameters that have been defined in Section 3.2. Some of the most common fluorescence measurement methods will be covered as they are related to many of the parameters.

The many factors influencing the variability of chlorophyll fluorescence are then investigated in Section 3.3 to show how this has an impact on the design of a fluorometer as well as its application. Even though the fluorometer for the thesis project does not directly make use of most of the fluorescence parameters listed, they are discussed to enable comparison of measurement data with data from commercial sensors that use them. It should also clarify references to literature that mention these parameters.

In Section 3.4 the determination of chlorophyll concentration from fluorescence measurements is investigated. This investigation will show the core requirements of a fluorometer design that is aimed to calculate chlorophyll concentration from the fluorescence data.

3.1 The Kautsky or OJIP Curve

In 1931 Kautsky and Hirsch [29] published a paper describing how chlorophyll α fluorescence intensity changes over time for leaves due to various photosynthesis processes. These experiments were done with dark-adapted leaves (that were left in complete darkness for about 30 minutes). The characteristic transient described by them is therefore commonly known as the Kautsky effect. A plot of this fluorescence intensity change over time is sometimes also called the OJIP curve after the four characteristic points (O, J, I, P) that were defined on it. These four points are discussed in detail in this chapter. Figure 3 shows three examples of OJIP curves. Several variations of these characteristic points on this curve and their terminology have been developed over the years [19] but they all provide information about the change in the amount of fluorescence emission over time. Section 3.2 provides more detail about many of these other reference points

and parameters.

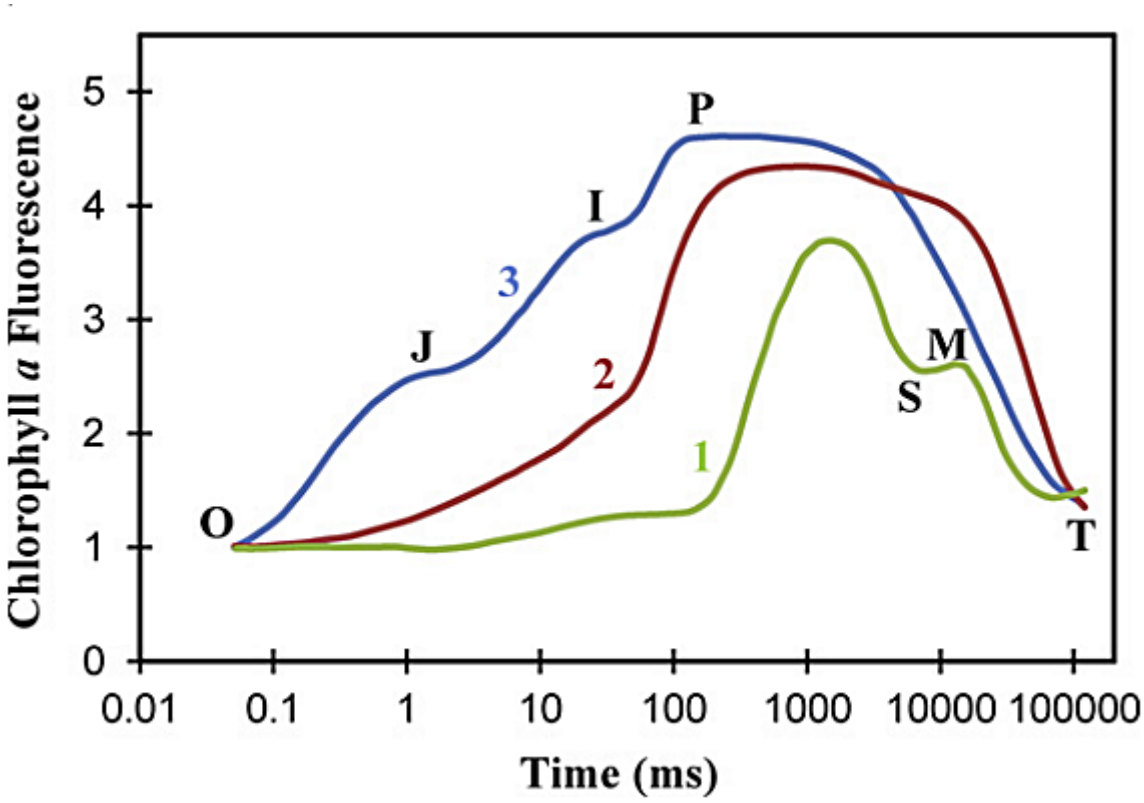


Figure 3: Kautsky / OJIP curve of a pea leaf.
Graph above taken from [24].

The shape of the OJIP curve and the fluorescence values reached are not fixed for a specific measurement sample [30]. Figure 3 above shows 3 curves for one pea leaf where different excitation light intensities were used for each curve measurement. The O, J, I and P inflection points have different fluorescence values on the three curves that are also reached after different amounts of time since the start of the measurement. The fluorescence value at a specific reference point, as well as the time to reach it, change along with the state and physiology of the sample [24]. The O, J, I and P points on the Kautsky or OJIP curve therefore provide information about the chlorophyll state and physiology when used correctly [19],[31],[32]. The factors influencing the shape of the curve are now discussed in more detail along with the meaning of the O, J, I, and P points.

The O point is the fluorescence emission immediately after the chlorophyll is exposed to the excitation light [24],[33],[18]. At this point all the chlorophyll light receptors are open [28] if the sample has been dark-adapted. The fluorescence emission value at the O point is usually referred to as F_O. If the measurement sample has not been dark-adapted the starting fluorescence value is

not a true F_O value and should be referred to differently, as is discussed in Section 3.2.

There are several different theories regarding the exact processes behind the different phases of the OJIP curve. These are discussed in detail in [30]. The author of the thesis assumes that for the application of the fluorometer as a chlorophyll concentration sensor it does not matter what the exact processes behind the shape of the curve are. It only matters to know that there are different characteristic responses that can be measured.

The fluorescence emission rising phase from O to J is a photochemical phase that is strongly influenced by the intensity of the excitation light [24],[34],[11],[30]. Figure 3 shows how the excitation light intensity changes the slope of the initial rise. During measurement 1, with the lowest intensity light, the initial rise is slow. The initial rise then gets faster for the second and third measurements as the excitation light intensity increases. The fluorescence rises from the O value to the J value as the light receptors start to close down. The light receptors close to limit the electron flow into the photochemical system since it cannot quench the large number of electrons fast enough. The percentage of light receptors that close down and the amount by which they close down differ between the various theories but they all agree that the receptors close down in response to the excitation light [30].

The fact that the OJ phase of the OJIP curve is a photochemical phase means that control of the excitation light intensity can be used as a tool during this phase to cause a correlational fluorescence emission response from the chlorophyll. As the excitation light intensity is increased or decreased the rate of change in the fluorescence emission will change accordingly. This direct relationship between the excitation light intensity and the fluorescence response can be used as an indication of the chlorophyll concentration. This is discussed in detail in Section 3.4. The rise from O to J can take from 2 ms to 10 ms [35] depending on the excitation light intensity. This time period must be kept in mind when designing a fluorometer that uses the characteristics of this photochemical phase to make measurements. If the measurements are made after the OJ phase of the Kautsky curve has ended, the fluorescence response will not be as expected.

The following two phases of the curve from J to I and I to P, are slower phases. In 2012 the most widely accepted theories stated that these phases are mostly influenced by temperature [30]. The control of the excitation light intensity is therefore not a very useful tool to induce specific fluorescence responses during these phases. The rise from J to I on the Kautsky curve takes 20-100 ms [11]. Some authors, like Schreiber et al. [11], use different terminology but refer to the

same inflection point J on the OJIP curve as I_1 and to the I point as I_2.

After reaching the I point the fluorescence emission rise then slows down until it reaches a maximum at value P in about a second from the start of illumination [36]. This time period is also dependent on the excitation light intensity. Even under high intensity saturating light this point cannot be reached in less than 200 ms [25].

After the highest (P) fluorescence emission has been reached, the transient curve goes through three other phases identified as SMT by [24],[35]. These slower phases are not discussed in this document as they are not relevant to the FICC as it does not measure any parameters during these phases.

The changes in fluorescence emission happens much faster at the start of the Kautsky transient than towards the end. The logarithmic time scale used on the horizontal axis of Figure 3 gives a clearer indication of the rate changes during the very quick initial rise than a linear time scale would. On a linear time scale the initial transients just look like a very steep jump. This logarithmic time scale is proposed by [24] as created by Strasser et al. [37]. The vertical axis of Figure 3 has no unit assigned to it since the fluorescence emission usually has no unit on these graphs. It is usually just indicated as a relative value that can be any measured unit, like an analogue to digital conversion number for the amount of light measured by a light sensor.

In 2014 Stirbet et al. [35] provided a very detailed examination of all the processes involved in the photosynthesis apparatus during the fluorescence transient curve. This amount of detail is not relevant to the thesis application of the fluorometer as a concentration sensor but will be useful for the development of a fluorometer that is designed for use in photosynthesis research.

3.2 Chlorophyll Fluorescence Nomenclature and Measurement Analysis

The previous chapter provided an overview of the most common terminology used for the characteristic points on the Kautsky curve. Different terminology have however been allocated to similar or the same fluorescence parameters by different authors. This can easily lead to confusion regarding the meaning of a measurement value. The most commonly used nomenclature is now discussed to provide an overview of the link between them and how they should be interpreted.

The fluorescence emission of a dark-adapted sample at point O of the Kautsky induction curve is

identified by F_O. When the sample has not been dark-adapted, like when the excitation light was only briefly switched off, the fluorescence emission is identified by F_O' [38]. Parameters of light-adapted samples are usually identified with the superscript ' character. The difference between F_O and F_O' is normally negligible [34].

Some fluorescence measurement techniques do not actually measure F_O but calculate it by various methods, like fitting a polynomial curve to the data. It is important to know how F_O was determined when F_O is used in calculating other fluorescence parameters since an estimated F_O could cause incorrect calculations. Some techniques that calculate F_O use different terminology to refer to the value, for instance, the pulse frequency modulation (PFM) technique calculates a F_O value but also uses Fα to indicate that it is not a true F_O value [39]. The designer of a fluorometer should clearly indicate how F_O was determined to enable users to interpret measurement parameters correctly.

Stirbet and Govindjee [24] give a very detailed overview of the chlorophyll fluorescence nomenclature used in the JIP test that they describe. The JIP test is the method they used at the time to measure the fluorescence parameters. These include minimum fluorescence (F_O), maximum fluorescence (F_M) and variable fluorescence F_v ($F_v = F_M – F_O$). It is shown that there are different F_O and F_M parameters for dark-adapted samples (F_O, F_M) and samples kept in light (F_O', F_M') [24],[18]. These parameters must be used correctly when the chlorophyll physiology is analysed and discussed [19].

When the excitation light is intense enough to cause the internal mechanisms (PSII reaction centres) of the chlorophyll to close down and emit maximum fluorescence, the excitation light is in the “saturating” range [32]. This light intensity is sometimes applied in pulses, referred to as saturation pulses. These saturating pulses are used in some chlorophyll fluorescence analysis techniques, like the pulse amplitude modulation (PAM) and saturation pulse method [14] to measure maximum fluorescence. Saturation light pulses do not substantially affect the state of the chlorophyll sample [32],[18] if it is short enough. A saturating pulse of a few hundred millisecond duration (600 ms example shown by [40]) can be used to measure maximum fluorescence without influencing steady state fluorescence [40]. This multi turnover process of the primary light acceptor Q_A can take from 50-1000 ms [40].

The measurement of maximum fluorescence (F_M) is a controversial topic [18]. Koblizek et al. [41] provide a long list of influences on the F_M value which is a key factor used to calculate many photosynthetic processes. Without taking these influences into account F_M is indeterminate.

Absolute measurement of fluorescence parameters, like maximal and effective quantum yield, is complicated by many factors as indicated in 2012 [13]. In most cases a relative indication of the changes in fluorescence parameters is good enough [13]. This is also assumed to be true for the intended application of the FICC as photosynthetic processes are not measured or calculated.

According to [25] F_M is reached when primary acceptor Q_A is fully reduced. This might however not be the case for the F_M measured by all fluorescence measurement techniques. Many modern techniques do not measure the Kautsky curve to determine the maximum fluorescence. They use many different methods to excite the fluorescence up to a maximum emission that is usually referred to as F_M [38] or a variation of it. In the PAM technique, for instance, F_M is the maximum fluorescence yield reached during a saturation pulse, of a dark-adapted sample. The maximal fluorescence yield during a saturation pulse that is reached by an illuminated sample is referred to as F_M**'** [18]. The pump and probe (PP) technique reaches a maximum fluorescence (F_{SAT}) by adding a constant light to a series of “pumping flashes”. The fast repetition rate (FRR) technique reaches F_M by continuing the series of actinic light (facilitates photosynthesis) flashes for longer periods until a maximum value is reached.

Although the fluorescence theory is that F_M' cannot be higher than F_M there has been several documented experiments where F_M' was higher than F_M [42]. These rare occurrences of F_M' being higher than F_M were not related to one specific measurement method or species but is rarely found in literature [42]. During development of a new type of fluorometer it is not impossible that such an unexpected phenomenon might be found. It should not be immediately assumed that the new fluorometer is not working. According to [42] the phenomenon of F_M' being higher than F_M appears in specific circumstances where one of the causing factors could be that the fluorometer that was used made use of very low levels of actinic light.

Different maximum fluorescence levels can also be reached by means of light pulses that are designed to cause different physiological changes (referred to as single and multiple turnovers) in the primary light acceptor Q_A [38]. Single turnover of the light acceptor happens when the flash of saturating excitation light is so short that it ends before the start of the thermal phase of the induction curve. This provides a maximum fluorescence ($F_{M(ST)}$) value that is roughly the same as F_J (the fluorescence emission at the J point of the Kautsky curve) [38],[43]. This technique requires a very short measurement period that depends on the excitation light intensity [44]. It can be 10 ms or less. Times of 2-3 ms were measured by [44] with a 5000 µmol photons m^{-2} s^{-1} light.

A multiple turnover flash of saturating excitation light lasts up to 1 second. It saturates all light acceptors while going through the photochemical and thermal phases to reach the P point on the Kautsky curve [43]. It is suggested by [43] that ST and MT should be placed in brackets to indicate single ($F_{M(ST)}$) and multiple turnover ($F_{M(MT)}$) maximum fluorescence. Most of the practical measurements made with the FICC made use of saturating light pulses to measure the fluorescence response. Section 6.3 provides details of all the different forms of light and light pulses that were tested with the FICC. None of these techniques aimed to specifically induce single or multiple turnovers but rather intended to find a repeatable fluorescence intensity measurement technique.

The F_M values measured at different times with the same instrument should provide results that can be compared and analysed. We are however warned by [43] that direct comparison of results from different fluorometers should be done with caution even if they use the same measurement technique, since factors, like the system geometry, can influence results. The user therefore needs to fully understand the limits of measurement techniques as well as the meaning of terminology used to indicate measured parameters if results from different techniques are to be compared. The differences in results between fluorometers and measurement techniques must be kept in mind during the development of a new fluorometer if its measurements are to be compared with those of another fluorometer. Differences between the two fluorometer measurements could be caused by many factors, like the position of the detector relative to the sample holder [43]. Section 5.1 investigates the effect of the light detector position on measurements.

Using the herbicide DCMU is generally accepted as an accurate method to measure F_M of a sample as it blocks electron transport inside the chlorophyll [32] that would normally diminish the maximum fluorescence emission. This causes the fluorescence rise to almost reach the maximum F_M level of a sample [45],[18],[46]. Figure 4 shows the fluorescence rise of pea leaves with and without DCMU as measured by [44]. DCMU can however only be used for in vitro testing and calibration of fluorometers as it permanently modifies the chlorophyll with the consequence that it eventually kills the host.

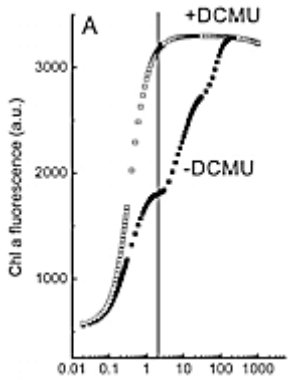


Figure 4: Fluorescence rise with and without DCMU.

Figure 4 taken from [44].

The variable fluorescence (F_v) is calculated by subtracting the minimum fluorescence from the maximum fluorescence (F_M-F_O). Accurate determination of F_v is then dependent on accurate measurement of F_O and F_M. It is often assumed that the fluorescing chlorophyll is coupled to PSII and that the fluorescence intensity is an indication of the fluorescence yield [40]. This assumption is rarely true and that PSI can have a significant contribution to the F_O fluorescence [40]. This often leads to overestimation of fluorescence parameters due to the influence of an incorrect F_O value on calculation of other parameters, like F_v.

It is generally believed that the variable fluorescence (F_v) comes from PSII [24],[45] while PSI emits constant fluorescence. In 2013 Lazár [47] however found that simulations of a PSI model show that up to 17% of the variable fluorescence could be originating in PSI. He suggests that this should be further investigated to confirm if the model is correct. If a fluorometer is designed for photosynthesis research, this finding by [47] should be kept in mind when developing algorithms to determine photosynthesis parameters. It is assumed that for the application of the FICC it does not make a difference what the source of the variable fluorescence is as long as the variations in fluorescence intensity are according to the known behaviour that will be discussed in Section 3.3.

F_v is used by some fluorometers to determine the maximum photochemical efficiency of PSII. The maximum photochemical efficiency is related to the often used ratio of F_v/F_M [38]. The accuracy of this ratio is dependent on the accurate measurement of F_M and F_O by the fluorometer since F_v=F_M-F_O. The difference in light levels just before and during measurements must be taken into account to make valid interpretations of the F_v/F_M ratios when comparing data. The F_v/F_M ratio changes when a sample is exposed to strong light or even low levels of far-red actinic light [48].

Although the quantum yield and photochemical efficiency measurements are often used for research of the photosynthesis process, these calculations are not implemented as functions of the FICC as it was not deemed to be a requirement for measuring chlorophyll concentration. It is possible that the measurement data of the FICC might seem to indicate a F_O level that can be used to calculate some parameters but this will almost certainly not be a true F_O value as no software or hardware was specifically implemented to accurately measure F_O.

The fluorescence emission at time t (F_t) can be used to calculate the relative fluorescence ($V_t = (F_t - F_O)/(F_M - F_O)$) at time t. This allows comparison of transients measured at different times as it compares the relative contribution of the absolute fluorescence at a moment in time to the variable fluorescence [24],[34]. The relative fluorescence calculation is again dependent on an accurate F_O measurement.

There has been decades of ongoing debate about the accuracy of information that can be gained from analysing chlorophyll fluorescence in phytoplankton [32],[49],[50]. Examples of fluorescence parameters that get skewed when the plants are put under stress conditions, are provided by [28]. In 1995 Govindjee [20] stated that the Kautsky effect is a much used but also much abused tool due to the fact that the important influencing parameters are often neglected during experiments that look at the relationship between photosynthesis and fluorescence. When used correctly, fluorescence measurements can provide valuable non-invasive measurement methods of photosynthesis processes in vivo [45],[19],[18],[46]. This makes it possible to gather research information faster and also do it in situ with some fluorometers. Fluorescence measurements have gained acceptance as a valid way to monitor changes in phytoplankton biomass and other similar characteristics [20],[18]. Absolute measurements are difficult to achieve and require controlled environments and calibrated equipment.

Lazár [28] warns against presenting fluorescence parameters that were measured during the fluorescence rise using the mean and standard deviation (or standard error) when comparing measurements made at different times under different conditions. The distribution of any data presented in this way should be Gaussian. This is generally not the case with the (F_O, F_M, F_v, F_v/F_M) fluorescence parameters.

We should be careful to trust fluorescence parameter data [19]. Kruskopf et al. state that “Chl α content and fluorescence parameters do not deserve the unquestioned status they usually enjoy as indicators of biomass and physiological status”. They show that different phytoplankton species

produce different fluorescence results for the same tests with the same instruments. Their research shows that chlorophyll fluorescence analysis can hold immense value if the researcher fully understands and explains what is being analysed. Research documents often give wrong meaning to measurement data, like presenting chlorophyll concentration as biomass. The results of [19] show differences in measured parameters of the same samples between the two fluorometers used. Their research article is based on data generated with the Waltz PHYTO-PAM-E and Hansatech Handy-Photosynthesis Efficiency Analyzer. These two fluorometers use different methods to determine one of the important fluorescence parameters, F_O (minimum fluorescence). This causes differences in the calculated F_v/F_M ratio. This again points out one of the problems with fluorescence parameter data where different fluorometers determine parameters, with the same name, differently.

Single turnover (ST) and multiple turnover (MT) fluorescence results are compared by [38] to indicate that the same fluorescence parameters have different values for each method used. They suggest that researchers clearly indicate which method was used when using the fluorescence terminology. Fluorometer users must have a clear understanding of what they are measuring with the specific instrument and how the instrument is measuring it. Designers of fluorometers also need to clearly understand which fluorescence parameters they intend to measure and which technique will provide the best results with the planned hardware.

The importance of understanding the difference between fluorescence intensity and fluorescence yield is emphasised by [18]. This difference is important for designers of fluorometers to keep in mind. Fluorescence intensity can vary in orders of magnitude depending on the light conditions while fluorescence yield carries photosynthesis information and usually varies by a factor of 5 to 6 [28],[18]. The fluorescence yield as measured by the PAM method (discussed in [12], [15]–[17]) is determined by the difference between maximum intensity when a saturating light pulse is applied and the fluorescence intensity shortly after the light pulse has stopped.

There are many fluorometry techniques, like fast repetition rate (FRR), pulse amplitude modulation (PAM), plant efficiency analyser (PEA), pump and probe (P&P), pump during probe (PDP), fluorescence induction and relaxation (FIRe) and advanced laser fluorometry (ALF) [24], that can be used to measure photosynthesis parameters. Each method has some limitations and advantages over the others, for example, [51] show that the measurement of F_o and F_M can be affected by using different light duration and intensity settings of a PAM fluorometer. These techniques are much more complex than the techniques used during the practical investigations of

the thesis project, which mostly aimed to measure chlorophyll concentration by means of interpreting differences in measured fluorescence intensity. Some techniques use a saturation pulse [3],[18] to measure fluorescence parameters. Saturation pulses also form the basis of the techniques used by the FICC as is discussed in detail in Section 6.3. When using a saturation pulse method in the fluorometer design, it should not be assumed that all algal species will saturate with a specific excitation light intensity [51]. This is due to the differences of PSI and PSII systems between species. The fluorometer design should ideally make provision to change the saturation pulse light intensity to suit the requirements of the species under test.

3.3 Chlorophyll Fluorescence Variability

The well documented variability of chlorophyll fluorescence intensity [40],[52] and wavelength is now covered. The variability in fluorescence intensity referred to here is not a reference to the intensity changes as found in the Kautsky curve, but the variance in measured intensity when the same test is performed at different times. This variability can make interpretation of fluorescence measurement data very difficult if the factors influencing it are not controlled or at least noted during measurements.

The variability in fluorescence intensity as well as fluorescence wavelength can complicate the development of a fluorometer when measurements with different components or measurement techniques are compared to determine the most suitable component or technique. The fluorescence intensity variability caused the biggest challenges during the development of the FICC. Due to this variability, most fluorometer suppliers suggest frequent calibration of fluorometers used in the field to compensate for changes in fluorescence responses of algae and phytoplankton.

A specific chlorophyll sample will fluoresce with different intensities that depend to a large extent on the elapsed time since the last light was received, and the intensity of that light [38]. This is described as the light-adapted state of the chlorophyll. When higher plants have been kept in the dark for about 10-30 minutes they are in a dark-adapted state. It can take 90 minutes and longer for some algae to reach this state [53]. This state is often used as a starting point for fluorescence measurements. Clear differences were found by [10] in fluorescence intensity between measurements made on the same algal cultures after different amounts of light adaption. Figure 5 below shows how the maximum fluorescence during a saturating pulse changes over a period of 35 minutes for a specific sample. During this 35 minute period far-red (FR, >700 nm) and actinic

lights (AL) were switched on and off to measure their effects on the maximum fluorescence. The arrows below the x-axis indicate where light sources were switched on (up) and off (down). The changing value of maximum fluorescence during the saturating pulses can be used by photosynthesis researchers to understand the state of the sample but it can definitely make it difficult to compare measurements made with different configurations when a new fluorometer is being developed.

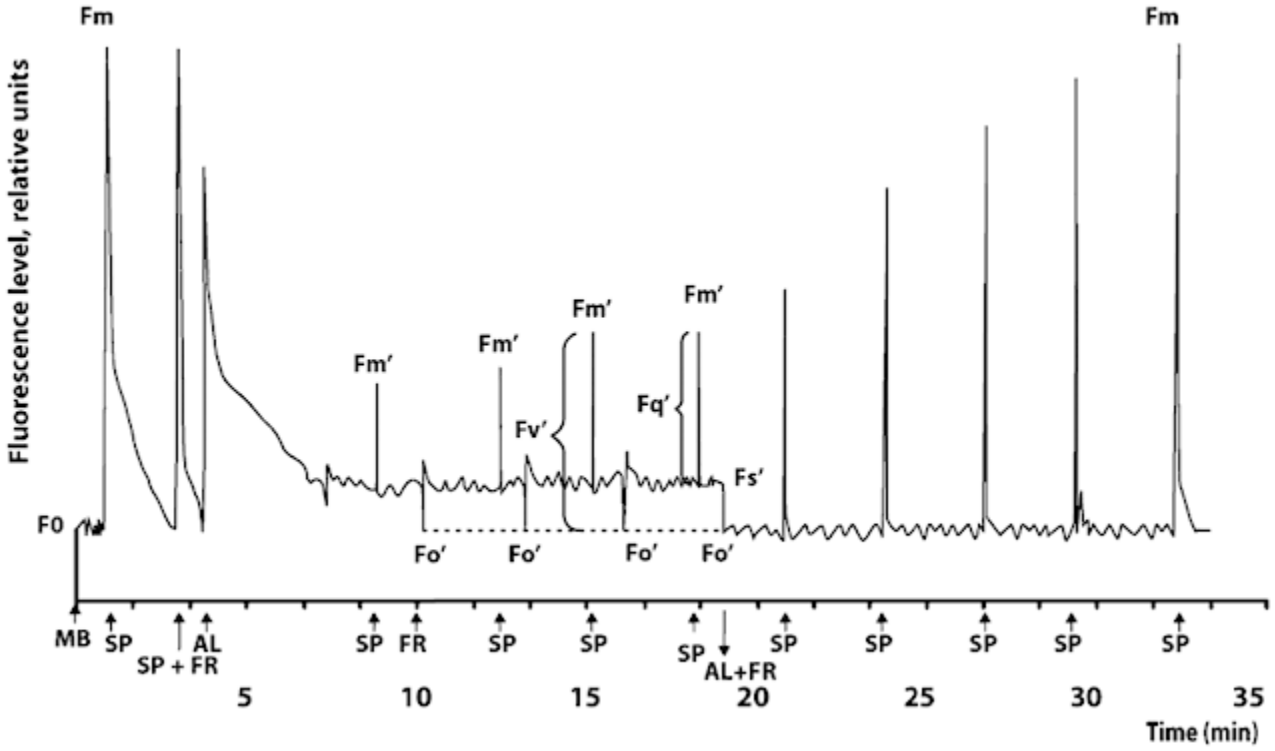


Figure 5: Maximum fluorescence variations.
Graph above modified from [54].

Under a saturating light the total amount of fluorescent light emitted during the first second of illumination (O to P rise of the Kautsky curve) is made up of the variable fluorescence (F_v) and a small amount of constant fluorescence from PSI [25]. The percentage of contribution from PSI to the total amount of fluorescence varies depending on the PSI/PSII ratio in the algae or phytoplankton as well as the wavelength at which the fluorescence is measured [35]. Different PSI/PSII ratios will therefore have different F_M to F_O ratios. This could cause changes in F_M to F_O ratio measurements if the PSI to PSII ratio has changed between measurements due to photosystem composition changes in the algae or phytoplankton. If a measurement is being made in an area where different species are present, a species composition change between measurements could also have a different PSI/PSII and F_M to F_O ratio.

As Figure 5 shows, chlorophyll fluorescence intensity for a specific measurement sample is very variable over time. This must definitely be taken into consideration when analysing fluorescence measurements to determine chlorophyll concentration. Several sources are listed by [55], who state that fluorescence intensity of chlorophyll varies due to several influences, like species, light exposure and nutrient availability. During a temperature increase from 0º C to 20º C, [56] found that the relative fluorescence decreased. They also found that chlorophyll α in 80% acetone (often used in fluorometer calibration and testing) showed a slowly decreasing relative fluorescence from 5º C to 30º C.

Extracted chlorophyll is often used with solvents in different concentrations for testing and calibrating fluorometers. When using such dilutions it must be kept in mind that chlorophyll α fluoresces more strongly when it is not biologically active than when it is functional in light harvesting or photochemistry [57],[53] in the algae or phytoplankton. The fluorescence in a solution would therefore be much higher than when it is functional in phytoplankton or algae. The measured fluorescence intensity of an extracted chlorophyll concentration can therefore not be used as a calibration value for the same concentration of biologically active chlorophyll in algae or phytoplankton.

The fluorescence peak wavelength of chlorophyll (extracted from spinach and blue-green algae) dissolved in different concentrations of water and acetone, shifts down in wavelength as the acetone concentration increases [58]. For extracts containing 10% and higher concentrations of acetone, [58] found a shift from 675 nm to 660 nm. This shift in fluorescence wavelength with dilutant concentration changes must be kept in mind if the fluorometer has any light filters (discussed in Section 5.2) or light sensors that have a wavelength dependent response. If a fluorometer makes use of a narrow bandpass filter, for example, to measure the fluorescence intensity while blocking other light wavelengths, this shift in wavelength could move the fluorescent light outside of the bandpass filter range as different concentrations are tested. This would provide fluorescence intensity measurements that show an incorrect relationship between the chlorophyll concentration and fluorescence intensity.

The amount and the colour of light that is available while the algae or phytoplankton is growing, will have an effect on their fluorescence response. The fluorescence and absorption spectra of phytoplankton species change along with the amount of light under which they grow [59]. Variations in absorption and fluorescence of up to 10 times were found in cultures grown in

different amounts of limited light [59]. The biochemical composition of microalgae also change depending on the colour of light present [60]. The composition of the photosystem develops to optimize the use of available light for photosynthesis [60]. The influence of different wavelengths of light on algal growth was compared by [61]. They found that red light enhanced the biomass production in both strains of microalgae they investigated. The chlorophyll content of Chlorella vulgaris increased 1% more over a 14 day period when grown in green light compared to the other light wavelengths. The combination of all the biological changes in the algae and phytoplankton under different light conditions will have an effect on fluorescence measurements made with the same fluorometer at different times if the time between measurements is long enough for the changes to take place. The fluorometer user and designer need to keep this in mind when comparing measurements.

During an investigation by this author into the effect of light pulse duration on maximum fluorescence intensity, it was found that the maximum fluorescence achieved by the specific algae sample was not constant over 90 minutes even though the applied light was the same for every measurement. Figure 6 shows how the maximum fluorescence changed. The Chlorella vulgaris algae sample, used in most of the measurements for the thesis, was kept in the dark for 4 hours before the first fluorescence measurement was made. During each measurement a series of light pulses was applied for about 10 seconds and the maximum fluorescence intensity measured. This was repeated at intervals ranging from 5 to 10 minutes during which the algae was kept in the dark. After about 95 minutes the sample was stirred and the maximum fluorescence suddenly jumped to the highest value of all, as can be seen on the right-hand side of Figure 6. It would appear that over time the algae slowly sank down to the bottom of the cuvette, which caused a lower concentration in front of the sensor and therefore reduced fluorescence intensity. When the sample was stirred the fluorescence intensity increased along with the increased concentration in front of the sensor. The movement of algae in relation to the light sources and sensors must be kept in mind as it could influence measurements made over long durations.

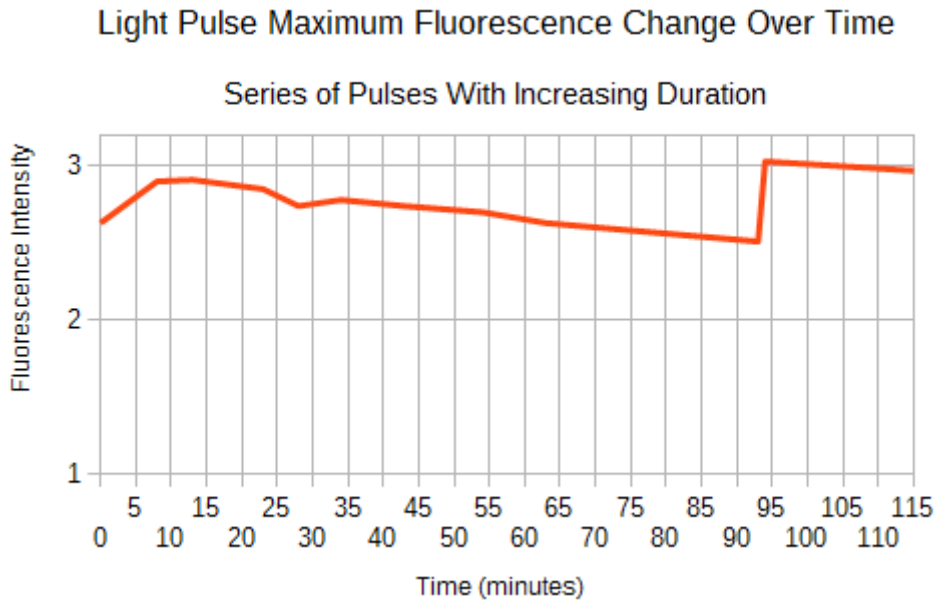


Figure 6: Effect on fluorescence of Chlorella vulgaris sinking.

After the jump in maximum fluorescence after stirring, there was again a steady decrease in fluorescence intensity similar to before the stirring. The steady decrease after stirring is believed to be due to the algae sinking again. Constantly stirring samples might seem to be an easy solution to this problem but it could also affect the fluorescence intensity when cells move in and out of the light of the measurement beam [62]. Cells that move into the measurement beam from a dark area might emit much more fluorescence than cells that were in the light beam for some time and have already gone past the P point on the Kautsky curve. Figure 7 below shows this effect of stirring on the measured fluorescence of Nannochloropsis oculata samples that were dark-adapted for 10 minutes. The numbers on the graphs indicate the increasing actinic irradiance levels in µmol quanta m^{-2} s^{-1}. The actinic light was active for 10 seconds between saturating light pulses. With the stirrer activated (graph b) the fluorescence reaches higher levels than with the stirrer inactive (graph a), due to new cells entering the light.

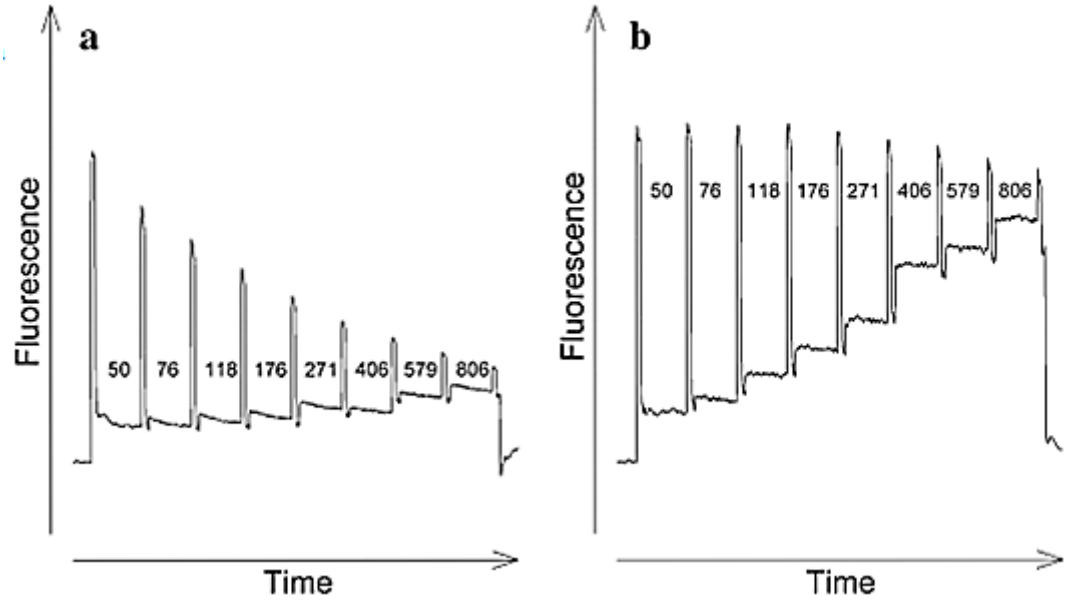


Figure 7: Effect of stirring on fluorescence measurement.
Graphs above taken from [62].

In situ (on site) measurements in dams or the oceans have many added varying influences on measured fluorescence intensity besides the factors already mentioned which could affect laboratory measurements. One of the most common challenges is that there are other substances besides chlorophyll in natural water, like chromophoric dissolved organic matter (CDOM), that also fluoresces. CDOM samples from several European sites fluoresced at wavelengths ranging from 280 nm up to 520 nm when [63] excited them with wavelengths from 200 nm to 450 nm. A fluorometer user or designer needs to keep the fluorometer excitation wavelength/s, as well as the measurement spectrum, in mind to determine how much substances like CDOM could influence measurements.

Fluorescence measurements made at different times could also vary due to the changing of the species composition between measurements since there will be more than one phytoplankton group present in the sample area being analysed. Measurements of fluorescence intensities for different phytoplankton species at several excitation light wavelengths are presented by [10]. These show definite differences between species in fluorescence intensity for each specific excitation wavelength. The differences were so distinct that it was used to present models to identify species from their fluorescence data for the various wavelengths. When [64] used a fluorometer with 9 excitation wavelengths, they also found that they could use the differences in fluorescence intensities to identify phytoplankton species. Due to the fact that the chlorophyll

fluorescence response differs between phytoplankton groups [10], the fluorescence intensity from a specific measured volume will change when the species composition changes. This phenomenon is due to the physiological differences in the different light harvesting antenna pigment systems of the phytoplankton [10],[13]. Among other things, like β carotene, the chlorophyll content differs between the different species. If the fluorometer is used as a chlorophyll concentration sensor only, the change in fluorescence intensity during a species composition change might not be a big problem as long as the user knows that it is only chlorophyll concentration that is being measured and not the concentration of a specific species.

If only one excitation wavelength is used, as in the FICC, the fluorescence intensity will change during a species composition change but there will be no way to detect if it was due to a species change or chlorophyll concentration change. A fluorometer designer needs to decide if the higher cost and complexity of multiple excitation wavelengths are worth the added ability to detect species composition changes which will lead to a better understanding of fluorescence intensity changes, as shown by [10],[64].

3.4 Chlorophyll Concentration Measurement

It is often stated that chlorophyll α fluorescence can be used as a measurement tool for chlorophyll α concentration [10],[65],[50],[8]. A fairly direct relationship was found by [42], between chlorophyll α concentration and F_M when using concentrations of 0.5 to 2.5 µg/ml. In 2012 [4] presented a conference paper that stated that the relationship between fluorescence and chlorophyll α concentration is quite linear. The final graph of concentration to fluorescence ratio was however drawn from only one set of measurements that fitted the requirements of a linear relationship between concentration and fluorescence. Four sets of other measurement data were left out. At least one of these does not show a linear relationship. The concentrations used by [4] were high (2 mg/l to 10 mg/l), which might explain the nonlinear relationship found at the highest concentration tested. A high correlation ($r = 0.95$, $n = 96$) was shown by [66] between chlorophyll α concentrations measured with the Fluoroprobe and laboratory methods. The specific fluorometer was found to be a good instrument for indicating algal blooms [66] where the chlorophyll concentration changes are in orders of magnitude.

The chlorophyll α concentrations measured with a LED-based fluorometer is compared with the chlorophyll α concentration determined in a laboratory process by [8]. His graphs show a direct relationship ($R^2 = 0.97$) between the chlorophyll concentrations measured by the two different

methods. A drop of 0.45% per second in fluorescence intensity was seen by [8] after 7 to 10 seconds under high excitation light intensities. The variation of fluorescence intensity is usually found with chlorophyll and can negatively or positively influence comparisons of fluorometer concentration measurements with other concentration measurement methods. The procedure to measure the maximum fluorescence needs to be repeatable and accurate to make comparisons with other methods useful.

The usability of fluorescence as a measurement tool of chlorophyll concentration seems to come down to how accurate the chlorophyll concentration measurement needs to be. The multiple excitation wavelength Algae Online Analyser (AOA) fluorometer overestimated chlorophyll concentration between 1.2 to 3.4 times compared to the High Performance Liquid Chromatography (HPLC) derived values when it was compared by [55]. It was however still found to be a useful tool for detecting algal blooms.

The chlorophyll concentration of a measurement sample has an impact on the measured fluorescence intensity. Concentrations higher than 10 mg/l should be avoided as their measurements show decreases in the maximum fluorescence over minimum fluorescence ratio (F_M/F_O) for such high concentrations instead of the expected constant ratio [51]. In concentrations higher than 400 µg/l, the re-absorption of fluorescent light affects the fluorescence versus concentration gradient [10]. Chlorophyll absorbs light in the wavelength range from 640 nm to 680 nm [67]. The fluorescence intensity that is detected in high concentrations of chlorophyll will be reduced due to this absorption of the fluorescent light emitted around 685 nm. Some chlorophyll in a dense suspension could also physically block the excitation light from reaching other chlorophyll [51] to induce fluorescence and also block fluorescent light from reaching the light sensor.

Chlorella vulgaris was cultivated by [68] in growth media with different chemical compositions. The results show that different chlorophyll concentrations and biomass were achieved for the same species after fixed time periods. This emphasises that fluorescence from chlorophyll concentration cannot be used to accurately determine species concentration if the chlorophyll concentration of the sampled species is not known. The results of [68] also show how the biomass in the N8 growth medium continued to increase while the chlorophyll concentration stayed fairly constant. In the one growth medium Chlorella vulgaris showed a decrease in chlorophyll content while the biomass was increasing. Biomass can therefore also not be accurately determined from chlorophyll fluorescence unless the sampled species biomass chlorophyll relationship is determined regularly. These examples show that fluorometer users and designers should not assume direct or constant

relationships between chlorophyll fluorescence and any concentration measurement other than chlorophyll. Chlorophyll fluorescence is only an indicator of chlorophyll concentration if no calibration was done to determine the relationship to another substance.

4 The Excitation Light System

Since fluorometers usually don't use ambient light to measure fluorescence, they require a built in light source system to cause the fluorescence that is measured. This chapter provides an overview of the requirements that were followed to design the light source system of the FICC. A light source of this system will be referred to as the excitation light from here on. The excitation light system switches the excitation light on and off for specific time intervals to excite the chlorophyll fluorescence in specific ways according to the measurement technique being used. The excitation light intensity is usually also controlled by this system to enable specific measurements, like measuring the fluorescence response curve.

The excitation light can have one or more dominant wavelengths. The first section of the chapter discusses some of the most common wavelengths used in fluorometers. The wavelengths are provided for reference as a starting point for the design of a new fluorometer. The discussion shows why 470 nm was selected for the FICC.

The limitations and advantages of different excitation light sources, like LEDs, are investigated next. The reasons for selecting a 10 mm LED as the FICC excitation light source are provided along with the results of some investigations into the suitability of other LEDs as light sources.

The need for accurate excitation light intensity control to achieve accurate fluorescence parameter measurements, is shown. Different ways to control the light intensity are investigated. Accurate excitation light intensity control also enables accurate modulation of the excitation light. Modulation of the excitation light is discussed to show its applications, like improving fluorescence signal recovery amidst noise.

4.1 Excitation Light Wavelengths

As mentioned in Chapter 2, fluorescence is a by-product of photosynthesis and specific wavelengths of light are absorbed more than others. The fluorometer excitation light source should provide a light spectrum that is wide enough to cover the light absorption ranges of the PSII systems of all the phytoplankton groups that will be analysed [3]. Figure 8 below shows the light absorption and emissions spectra of chlorophyll α and b that is found in PSII of algae and phytoplankton. The left hand curves show the absorption spectra and the right hand curves the

fluorescence emission spectra. Maximum absorption occurs at the wavelength that causes the highest fluorescence emission.

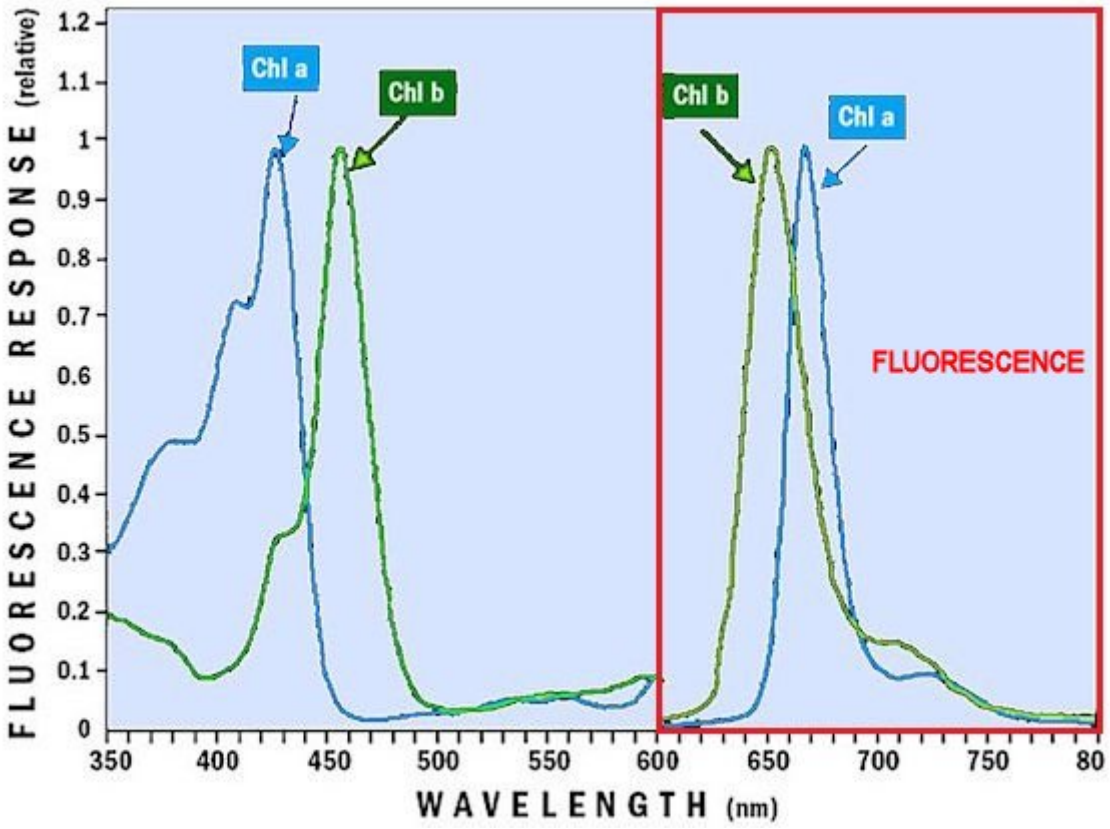


Figure 8: Chlorophyll excitation and emission spectra.

Figure above modified from [69].

Chlorophyll is usually targeted as a fluorescence source in algae or phytoplankton during the design of fluorometers since it is almost always present. If only chlorophyll α or b fluorescence is to be measured, a specific wavelength suited to that type of chlorophyll can be selected for the excitation source. The maximum absorbance wavelength of chlorophyll α is around 425 nm. To excite maximum chlorophyll α fluorescence the light source should then have its maximum intensity at 425 nm, as used by [5] in their low cost fluorometer. The absorption peak wavelength of chlorophyll b, in 90% acetone, is at 457 nm [67]. If a device is then designed to measure chlorophyll b fluorescence, the optimal excitation light source would have a wavelength around 457 nm to generate the maximum fluorescence.

Figure 8 shows that if the excitation light system is intended to cause fluorescence in both chlorophyll α and b it will have to emit light with a spectrum ranging from about 400 nm to 470 nm

to be most effective. This can be achieved with a white light source and a bandpass light filter that covers this spectrum range. Due to factors that will be discussed in Section 4.2, most modern fluorometers do not use white light sources but are designed with LEDs as excitation light sources. LEDs do not normally emit light over a wide enough spectrum to cover 400-470 nm. This basically leaves the fluorometer designer with two options. If the fluorometer is designed to have more than one LED excitation light source, the two wavelengths of maximum absorption would cause optimal fluorescence in both types of chlorophyll. Two LEDs with dominant wavelengths at these two spectrum points would provide optimal fluorescence. If the fluorometer can only have one excitation light source, a LED with a wavelength around 435 nm can however be used to measure both types of chlorophyll fluorescence since they both still absorb a fair amount of light at this wavelength. The fluorescence intensity will not be as high as when the optimal excitation light wavelengths are selected but it could still be useful. It is very likely that the optimal wavelength will not coincide with the wavelength of production LEDs. The designer then needs to find the LED with the closest wavelength to the ideal value.

One of the challenges in designing a fluorometer is to decide how many light sources should be used in the excitation light system and what their wavelengths should be. Additional excitation light sources increase the fluorometer size, complexity and cost while the additional wavelengths provide more functionality, like the ability to differentiate between species. Having only one light source causes problems when a fluorometer is used in an environment where multiple species from different taxonomic classes are present [70]. The different taxonomic classes provide different fluorescence responses at a specific wavelength [10],[70]. The fluorescence intensity measured at the single wavelength could change a lot if the concentration of one species with a big response at that wavelength, changes. The fluorescence intensity changes are then a much less accurate indication of algae concentration if the fluorometer was calibrated with a less responsive species. If more than one excitation wavelength is to be used to enable species differentiation along with fluorescence measurements, the excitation wavelengths must be selected carefully. Optimal selection of the excitation wavelengths will ensure that the different species will have identifiable differences in their fluorescence responses at the different wavelengths [71]. The selected wavelengths should target fluorescing pigments found in the algae or phytoplankton that differ in concentration between the species.

The following are some examples of fluorometer designs with multiple excitation light sources along with the wavelengths they use. The concentrations of different algae species was measured by [71] by means of 5 wavelengths (450, 525, 570, 590 and 610 nm). Jakob et al. [50] describe

how 5 wavelengths (470, 520, 645 and 665 nm) could be used to differentiate between green algae, diatoms and cyanobacteria despite the fact that the excitation wavelengths were not at the peak absorption wavelengths of the specific pigments that were targeted in the different species. The species could be identified due to the pronounced differences in their fluorescence responses at the selected wavelengths. A new multi-colour fluorometer with 13 independent light sources that use 7 wavelengths of light (400, 440, 480, 540, 590, 625 and 725 nm), is described by [13]. The light sources are used to generate light with different combinations of wavelengths. The combination of wavelengths depend on the fluorescence measurement. The light sources can provide actinic light during continuous illumination or various types of light pulses. Zhang et al. [72] used 12 excitation wavelengths (400, 430, 450, 460, 470, 490, 500, 510, 525, 550, 570, and 590 nm) to differentiate between 43 phytoplankton species. The FluoroProbe in [66] uses 6 LEDs (370, 450, 525, 570, 590 and 610 nm) to differentiate between 4 classes of phytoplankton.

Commercial LEDs with the ideal wavelengths of 425 nm and 457 nm for chlorophyll fluorescence, are currently very scarce, and are also much more expensive than LEDs with wavelengths of 465 nm and higher. LEDs of 465-470 nm are therefore often used in fluorometers [8],[12],[73] even though they will cause very little chlorophyll α and b fluorescence for the amount of excitation light applied. This will require that the light sensing system be more sensitive to measure the low levels of fluorescence compared to when a more suitable excitation wavelength is used. Figure 9 shows how much less fluorescence is induced in chlorophyll by an excitation wavelength of 470 nm compared to 430 nm. In the experiment by [6] the total chlorophyll concentration was kept constant at 2 nmol/ml. They used samples of Liriodendron tulipifera with different concentrations of chlorophyll α and b.

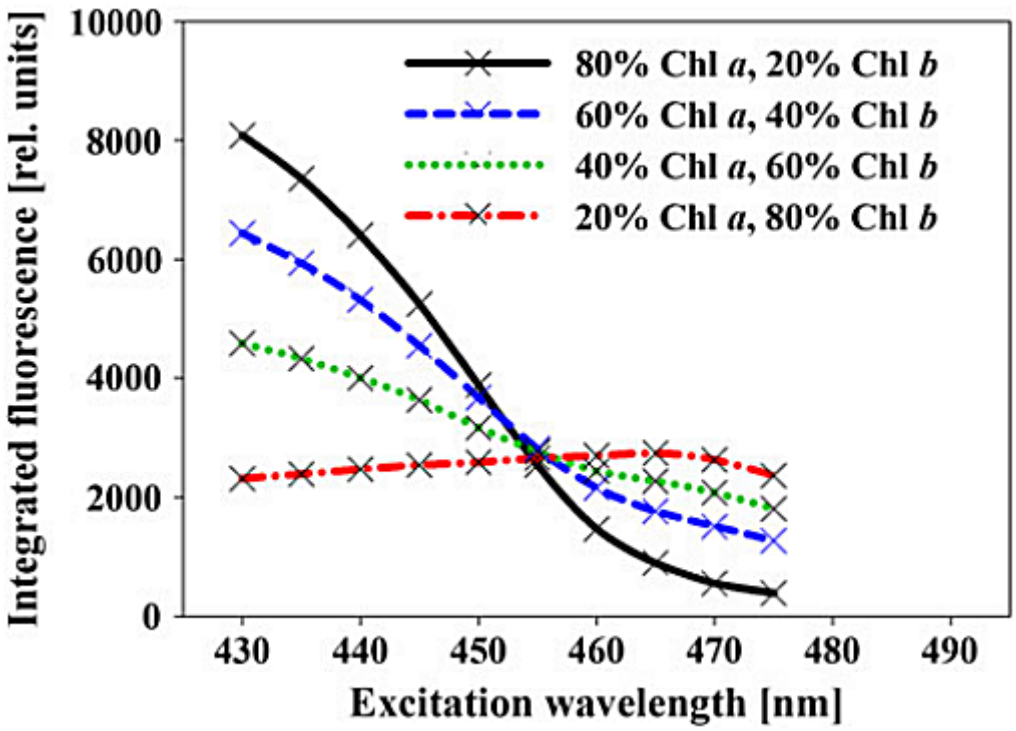


Figure 9: Chlorophyll α and b excitation wavelength responses.

Graph above from [6].

Older designs of fluorometers used light sources like Xenon or Halogen lamps [15], which provide a white light that covers a wide spectrum. Bandpass light filters were often used with these light sources to only allow a specific wavelength band of light through to the sample [74],[75]. The modern trend is to provide multiple LED light sources that cover a wide light bandwidth range [13], [64],[72] when the increased size, complexity and cost is not a limitation. The individual light sources provide advantages, like new measurement techniques [13] with accurate control of the light intensity and wavelength.

If F_O needs to be measured accurately, a far-red light source could help to ensure more consistent F_O values. In the PAM fluorometer described by [18], the minimum fluorescence (F_O) is measured by means of a low intensity far-red light source before the maximum fluorescence (F_M) is measured with the high intensity saturation pulse in the 400-700 nm wavelength range. The far-red light source is usually in the wavelength range of 720-735 nm [76]. The far-red light source has the added function of oxidising the Q_A light acceptors [76] to ensure maximum dark adaption and minimum influence of closed light acceptors on the intensity response of the next excitation light [77]. The advantages and processes involved in using far-red light to measure F_O and F_O', is explained in detail by [76]. It should be noted that [78] believes that for cyanobacteria specifically,

PAM fluorometers using a blue LED as actinic light source provides more consistent results than fluorometers using red LEDs.

If the light source is not consistent in intensity and wavelength it will affect measurements. When [42] used the Osram 64255 Halogen lamp, it had a change in colour spectrum during intensity changes due to the fact that the supply voltage was used to change the intensity. This had a measurable effect on the steady state fluorescence (F_s) value. The LED that [79] tested had a shift of 11.7 nm in wavelength when the temperature was changed from 0ºC to 45ºC. This could have a measurable effect if the LED wavelength is on a steep slope of the fluorescence emission versus wavelength curve. A fluorometer designer needs to ensure that the excitation wavelength stays as constant as possible by minimising external factors. The light source can, for instance, be placed on a heat sink to keep the temperature more constant.

4.2 Excitation Light Sources

The most common light sources for modern fluorometers seem to be LEDs [6],[5],[8],[13],[9]. Xenon [15],[80] and halogen [17] lamps have also been used successfully. In the past xenon and halogen lamps had the advantage over LEDs of being able to provide much higher light intensities, which is required to provide saturating light for fluorometers. LEDs have however been constantly developed to provide higher light intensities every year. The range of LEDs that provide enough light to be used in fluorometers is increasing all the time.

The white light of xenon and halogen lamps covers a wide spectrum. Fluorometer designers can extract specific sections of the spectrum for excitation by means of light filters. These filters are unfortunately often quite expensive. The narrow light spectrum emitted by a LED allows the fluorometer designer to provide required excitation wavelengths without the need for expensive bandpass light filters. There are however currently still wavelengths for which no commercial LEDs exist. Unless a specific wavelength of excitation light is very crucial for a measurement, a LED with the closest wavelength will have to be chosen. It should be noted that many LEDs that are sold as “white light” LEDs do not actually cover the whole spectrum from 350 nm to 800 nm involved in the photosynthesis process. Many of these “white” LEDs will, for instance, excite very little chlorophyll α fluorescence since they emit little light around 425 nm. Figure 10 shows an emission spectrum of an Osram LCW E6SG white LED. The solid line shows how much the relative illuminance varies over the spectrum. The dotted V(λ) line on the graph is the standard eye response curve.

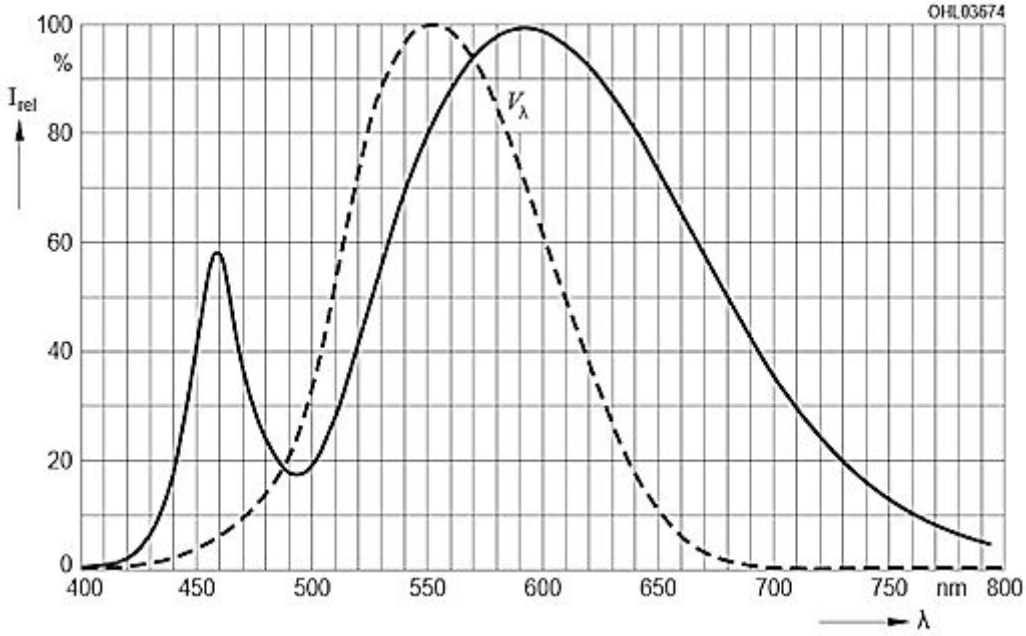


Figure 10: White LED emission spectrum.
Spectrum graph above taken from [81].

One of the problems with lamp light sources is the heat they generate. The excitation light source should be carefully selected and should generate as little heat as possible to prevent damage to the subject under test [34]. A combination of heat and fluorescence measurement can damage the plant. When heat treatment was applied by [82] to tobacco leaves before the fluorescence measurement, it was found that leaves could be damaged by Reactive Oxygen Species (ROS) formation during fluorescence measurements. The heat of the light source can also influence its wavelength. This is a disadvantage, as was discussed in Section 4.1. LED light sources have the advantage that they generally do not create a lot of heat as long as the supplier's current limits are adhered to. This is discussed in more detail in Section 4.3

LEDs require comparatively little current for the light intensity they generate. This is an advantage for the design of portable fluorometers that run on batteries. Since it is intended to use the FICC in a remote system running on batteries, the low current consumption of LEDs was one of the main reasons for selecting a LED as the light source. The current consumption of the LED at maximum intensity (20 mA) makes up a significant amount of the total consumption of the electronics in the FICC. This was one of the considerations when it was decided to use only one excitation light source.

A 470 nm LED (Microtec MT-1003UBC/W20/A9) is used in the FICC. The 470 nm wavelength was the closest to the absorption peaks of chlorophyll α and b that was available in low cost LEDs at

the time when the choice had to be made. This wavelength has been successfully used in other fluorometers [12] [42],[73],[74]. The 10 mm diameter LED has the same width as the 10 mm wide cuvette that was used in all investigations and experiments. The LED light beam therefore illuminates the whole area inside the cuvette. This provides more stable fluorescence measurements than a narrow light beam. It reduces the possibility that algae or phytoplankton would move into and out of the light beam to cause fluorescence variations due to its light-adapted state (discussed in Section 3.3).

The Microtec MT-1003UBC/W20/A9 LED was selected after the performance of several LEDs were tested with measurements on algae samples. All these LEDs had a peak wavelength of 470 nm ± 5nm. A few of the LEDs that looked promising are discussed next to show why they were not selected.

The performance of a high intensity 5 mm blue LED (Microtec MT-333UBC/W20/465-5/U1U2) was tested to see if it could match that of the 10 mm LED. The 5mm LED was considered since it would have allowed a smaller FICC device due to its smaller size. Due to the close proximity of the light source to the cuvette, the 5 mm LED had a narrow light beam inside the cuvette that could not cover the whole 10 mm width. This meant that not all of the algae in the cuvette could be illuminated at all times to cause fluorescence. This can cause undesirable variations in fluorescence measurements due to the different light-adapted states of the algae in and outside of the light.

The Osram LBN91E SMD LED was chosen for its small size (3.2 mm x 1.6 mm) while still providing a fairly high luminous intensity of 5600 mcd. It would have been the easiest to package in the FICC housing due to its small dimensions. Testing of the LBN91E showed that it did not excite enough fluorescence to be usable even with the high sensitivity TSL257 light sensor. The measured fluorescence intensity already reached a minimum analogue to digital converter (ADC) value of 55 at a 10 mg/l algae concentration. This concentration was still much higher than what was planned to be measured in the ocean. The left-hand plot in Figure 11 shows the fluorescence intensity induced by the LBN91E, plotted for different algae concentrations.

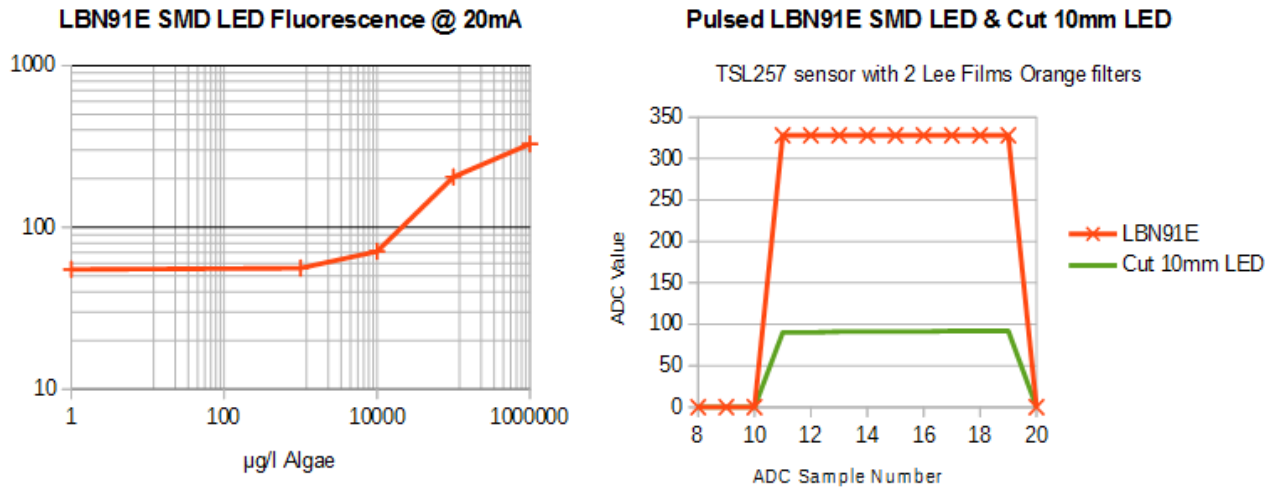


Figure 11: 1206 Size SMD LED fluorescence.

The right-hand side of Figure 11 contains plots of the measured fluorescence intensities induced by the LBN91E and a modified Microtec MT-1003UBC/W20/A9 10 mm LED. It shows the ADC values of the light intensity during a light pulse. The fluorescence excited by the modified 10 mm LED was much lower than that of the LBN91E. It was barely measurable even with high algae concentrations. It could therefore not be used in the FICC.

The 10 mm LED was modified to get a shorter length and a flat front end since the FICC needs to be as small as possible and should ideally have a flat front end. It would seem that the destruction of the LED housing optics caused the light to disperse to such an extent that very little excitation light reached the light sensor target area inside the cuvette. The fluorescence intensity achieved was much lower than that of the standard 10 mm LED.

The two LEDs were fitted next to each other (Figure 12) and were alternately switched on to measure the excited fluorescence with the same algae concentration. The front part of the 10 mm LED was ground away at an angle of 45 degrees to provide a flush mating surface with the cuvette. The angle of 45 degrees was chosen with the idea that the light beam would pass through the algae in front of the light sensors which were fitted lower down next to the cuvette.

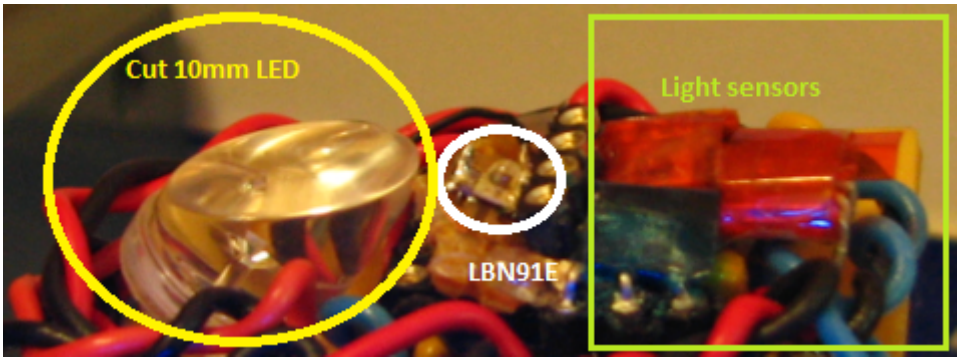


Figure 12: Cut LED & SMD LED layout.

4.3 Controlling the Excitation Light Intensity

This section looks at the reasons why the excitation light intensity should be controlled and how it was done for the FICC. It therefore only considers controlling the light intensity of LEDs as it is the only type of light source used in the FICC. Variations in each LEDs brightness and forward voltages are caused by manufacturing tolerances [83]. The impact of these tolerances on the excitation intensity control is also looked at. Changing the excitation light intensity has some unwanted consequences that must be kept in mind. These are mentioned during the discussion about different types of LED intensity control, like pulse width modulation (PWM).

Modern fluorometers are usually designed to modulate the light intensity, as is discussed in Section 4.4. This requires accurate control of the excitation light intensity to achieve the required modulation. The fluorometer should not be able to provide a high light intensity due to inaccurate control. High amounts of light can cause stress in a plant [22]. The photosynthesis system can be damaged irreversibly by high levels of light [84]. Due to the differences in fluorescence intensity between high and low chlorophyll concentrations it is also convenient to be able to reduce the excitation light intensity if the fluorescent light sensor is saturating.

In principle the light intensity of a LED is controlled by controlling the current through it. An accurate LED current control system is therefore a necessity in the design of a fluorometer that will be changing its excitation light intensity. Without accurate excitation intensity control it will be difficult to make sense of a change in fluorescence intensity since it will be difficult to determine if it was due to a change in excitation intensity or due to other changes, like the chlorophyll concentration.

There are two main approaches to control the LED current: either use a voltage source or a current source to drive the LED. The voltage source method controls the LED supply voltage to indirectly control the LED current since the circuit resistance stays constant. The current source method changes the circuit resistance or the supply voltage to control the LED current directly. It was decided to use the current source option in the FICC. It was believed that this would minimise the possible error in the control of the excitation light intensity. This required the design of a current source system that measures and controls the actual LED current. The circuit diagram and functional description of the system is provided in Section 6.3.

In the case where a voltage source is used, fixed resistors are normally placed in series with the LED to limit the current. The assumption is that the LED current, and therefore excitation intensity, can be calculated since the supply voltage, circuit resistance and LED forward voltage are all known. The calculated current will however have some percentage of error since the forward voltage of each LED is different and it also changes with the LED junction temperature (Osram application note [83]). For example, the datasheet of the LBN91E LED shows that its forward voltage can vary from 2.8 V to 3.8 V due to production tolerances. This can have a big impact on the LED current if the supply voltage is 5 V. The fluorometer designer needs to calculate the current error range (based on the LED supplier's tolerance data) and decide if this will cause a big enough error in the light intensity control to impact the fluorescence measurements. It should be kept in mind that the actual excitation light intensity error will be even bigger than the LED current error at certain points since the light intensity does not have a linear relationship with the current.

The datasheet of the Microtec 10 mm LED (used in the FICC) indicates that its luminous intensity is not directly proportional to the forward current through it. An investigation was performed by the thesis author to confirm this nonlinearity. A sawtooth signal was applied to the current control circuit of the LED to increase the LED current at a constant rate from 0 mA up to 20 mA. It would then switch off the LED and repeat the cycle. LED current and excitation light intensity measurements were made at 19 different points of each cycle. These measurements were made for several cycles to confirm if the LED current and excitation light intensity relationship stayed the same.

The LED light intensity was plotted along with the LED current. This plot (Figure 13) shows a similar relationship between light intensity and forward current as the graph in the LED datasheet. The blue plot (LED light intensity) shows a slightly curved line with a fall in gradient as the intensity increases. The red line is the voltage over a 120 Ω series resistor that was used to sense the LED current. It keeps a fairly constant slope that is very close to the steadily increasing current control

setting of the sawtooth signal. Both plots show the ADC measured voltage for that channel. Different scales were used for the two channels to present the excitation light intensity curve close to the current sense curve.

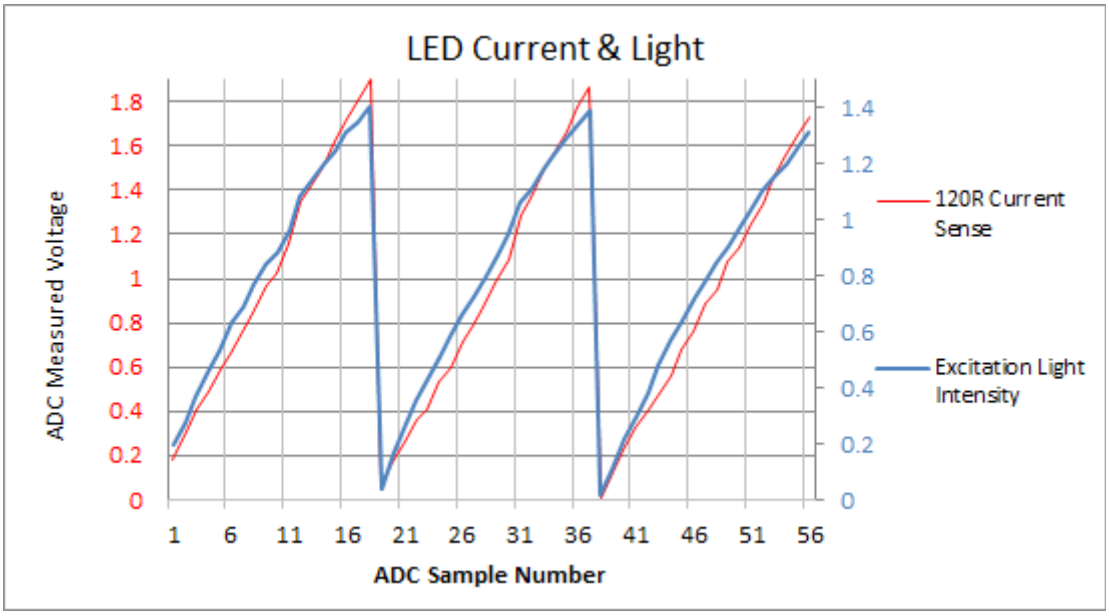


Figure 13: LED current vs LED light intensity.

For absolutely accurate control of the LED intensity this nonlinear relationship between the LED current and light intensity can be taken into consideration to calculate the required current to achieve a specific excitation intensity. The nonlinear relationship is however ignored in the implementation of the FICC. The software controls the LED current as if it has a direct relationship to the excitation intensity. As the FICC measures the actual excitation light intensity along with the fluorescence intensity all the time, the true excitation intensity can be accessed if required during analysis of the measurement data.

The FICC uses sine wave modulated light as an excitation source. The reasons for this and the way it is implemented are discussed in detail in Section 4.4. Since the nonlinear current to light intensity relationship would cause some distortion to the sine wave, some of the FICC measurement data was analysed to determine its impact.

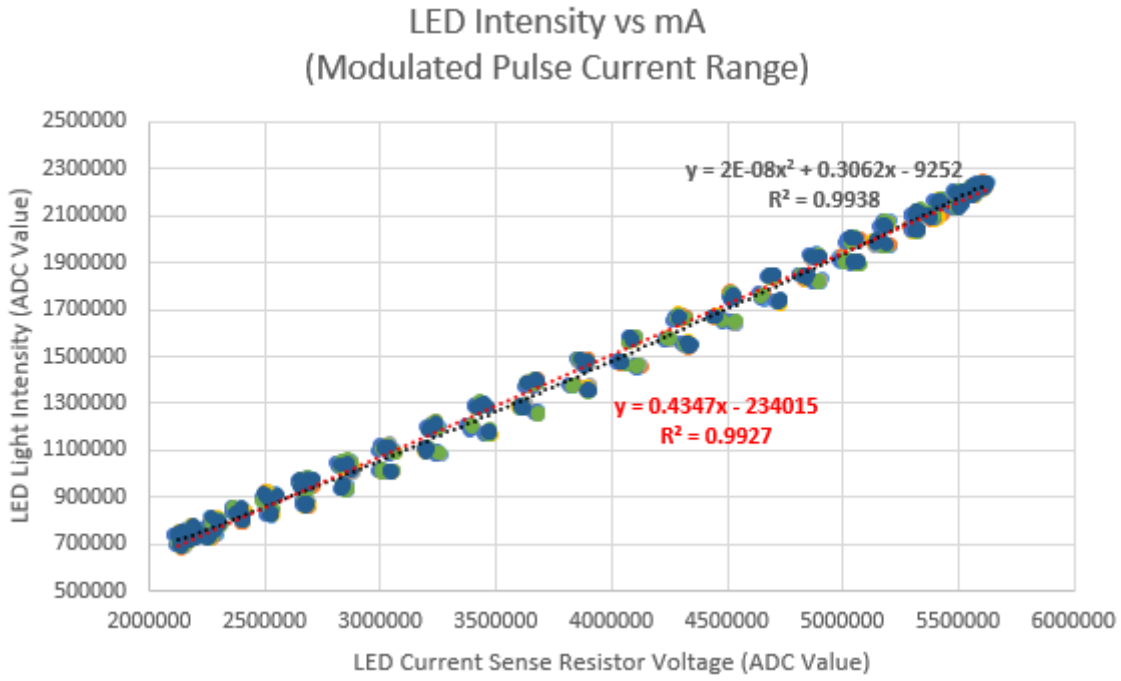


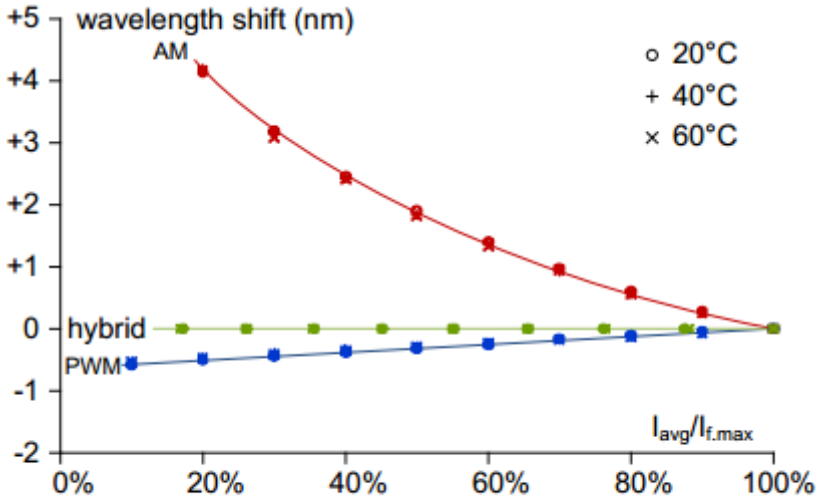
Figure 14: LED intensity vs current relationship during sine modulation.

Figure 14 shows the measured relationship between the current through the LED and the blue excitation light intensity in the range where the light is modulated with the sine wave. There is a fairly linear relationship between the LED current and light intensity. The linear and polynomial trend lines have very similar coefficients of determination (R^2). The red trend line shows a linear fit to the data while the blue trend line shows the exponential fit. It is believed that there is not enough distortion of the sine wave to motivate the complication of the FICC design by trying to compensate with software for the nonlinear relationship between the LED light intensity and current.

The different coloured dots in Figure 14 are measurement points from different series of light pulses that were modulated with sine waves. The sine waves are not visible as the graph has no time axis. As the LED current increased and decreased to create the sine wave, the LED intensity also increased and decreased synchronously.

There is one more fact that should be mentioned regarding Figure 14: the different ADC channels measuring the light sensor voltage and current sense voltage were not sampling at the same moment in time (there were a few microseconds offset between them), which might have affected the results. It is however believed that the slow modulation frequency of about 65 Hz would mean that the time offset between ADC channels would not have a big influence.

The two most popular methods to control the LED light intensity are pulse width modulation (PWM) and continuous current reduction (CCR) [85],[86]. Figure 15 shows the results of [87] when they tested different current control methods on a blue LED. The top line shows the increase in wavelength when they reduced the LED intensity by means of reducing a constant current. Beczkowski et al. call this amplitude modulation (AM) but mention that it is also sometimes called CCR. This is the method used by the FICC to modulate the intensity of its blue LED. Different colour LEDs have different amounts of wavelength increase when the forward current is reduced [88]. The dominant wavelength of a blue LED increases slightly when a constant current is reduced [87],[85].



Peak wavelength shifts of blue diode when dimmed with PWM, AM and hybrid dimming strategies at different heatsink temperatures.

Figure 15: LED wavelength shift for different forward currents.

Figure above taken from [87].

This slight wavelength shift during LED current changes might influence measurements if the excitation wavelength is on the edge of the chlorophyll response curve. In these special circumstances fluorometers, like the FICC, that make use of LED current modulation could then possibly provide measurements where the fluorescence intensity does not show the expected linear response to the excitation light intensity when the minimum and maximum values are compared. Figure 16 shows a small part of the absorption spectrum measurement that was made on the chlorophyll α calibration standard discussed in Section 7.2. The red lines show more or less the worst case scenario of how much the absorption can change for a 4 nm change in wavelength. The change in fluorescence intensity due to the change in absorption for this calibration standard is not known. It is however assumed by the thesis author that this change in fluorescence intensity,

due to the LED's wavelength shift, will be constant for all concentrations of the chlorophyll and should therefore not negatively affect concentration measurements of the FICC.

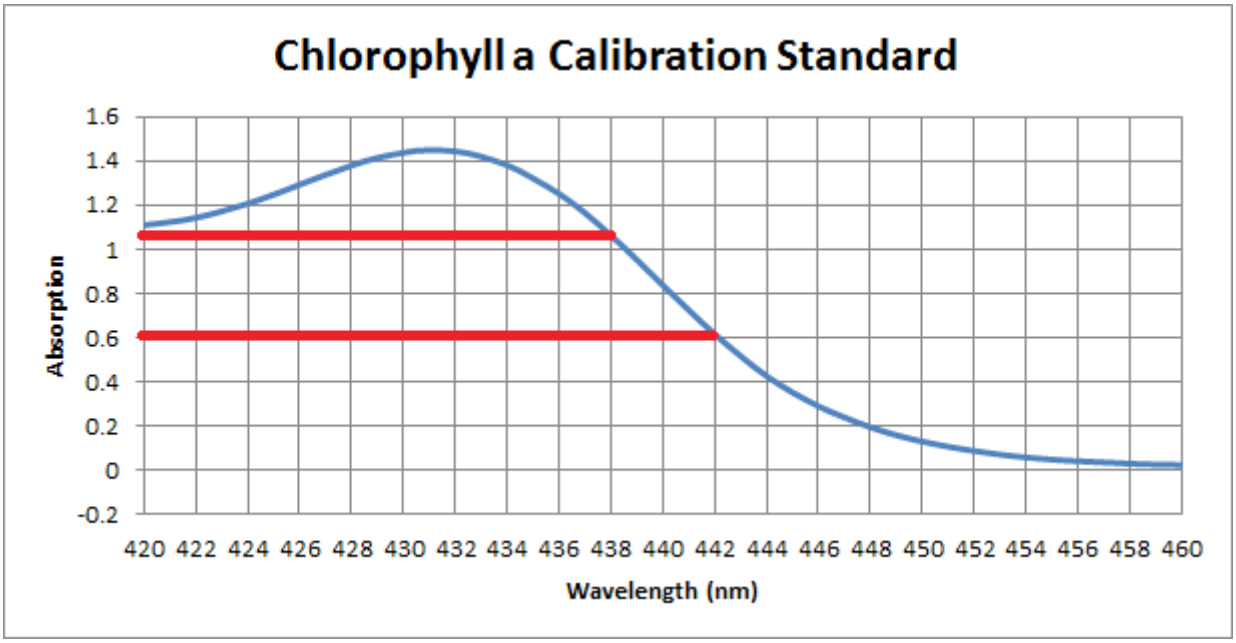


Figure 16: Effect of LED wavelength shift.

PWM also changes the LED dominant wavelength but decreases it [87]. A hybrid scheme of PWM and AM is proposed by [87] to achieve a quite stable wavelength at different intensities for LED lighting applications. This scheme was however not attempted for the FICC since it is believed the PWM pulses would cause photosynthetic responses that will complicate the determination of chlorophyll concentration from the fluorescence measurements.

Since the LED light intensity and forward voltage are affected by the LED junction temperature [83], changes in the junction temperature could be compensated for when a LED is used as the excitation light source in a fluorometer. Dasgupta et al. [79] found that a constant voltage source, along with a thermistor and resistor, was the best way to keep the LED light intensity constant for temperature changes. When a thermistor is selected from measurements according to the formula [79] supply, it will compensate for the decreased light output efficiency of the LED at higher temperature by increasing the LED current. They further indicate how a system with photometric feedback should provide very stable light intensity control. Such a system was not tested for the FICC since the components, like the Peltier cooler, seemed too expensive.

One more reason to have accurate excitation light intensity control is that the F_V'/F_M' value is light intensity dependant due to more non-photochemical quenching under higher light intensities [76].

The non-photochemical quenching affects the variable fluorescence more than the minimum fluorescence. Accurate intensity control will provide more repeatable measurements of F_V'/F_M'.

4.4 Modulating the Fluorescence Excitation Light

This section starts with an explanation why light modulation is used in the FICC. It then looks at some of the many different light modulation techniques that are used in fluorometers.

When the sample of algae or phytoplankton is kept in an enclosed area where no light, other than the fluorometer excitation light, can reach it, all measured fluorescence is a response to the fluorometer light. If other light sources can cause fluorescence in the sample it can be quite difficult to determine how much of the measured fluorescence is in response to the fluorometer light. To get around this problem the fluorometer excitation light can be modulated with a sine wave to make it possible to distinguish the fluorometer induced fluorescence signal from the fluorescence caused by other light sources [32],[18]. The fluorometer designer can make use of analogue or digital signal processing (DSP) techniques to filter out the fluorescence signals that are not at the modulation frequency.

The modulation of the excitation light can also include changes to the average intensity. The average amplitude of the sine wave is moved by changing a DC offset, as is shown in Figure 17. This can be used to adapt the excitation intensity to the chlorophyll concentration when the induced fluorescence is too much or too little for the light sensor. The average excitation intensity can also be changed to create specific photosynthesis responses.

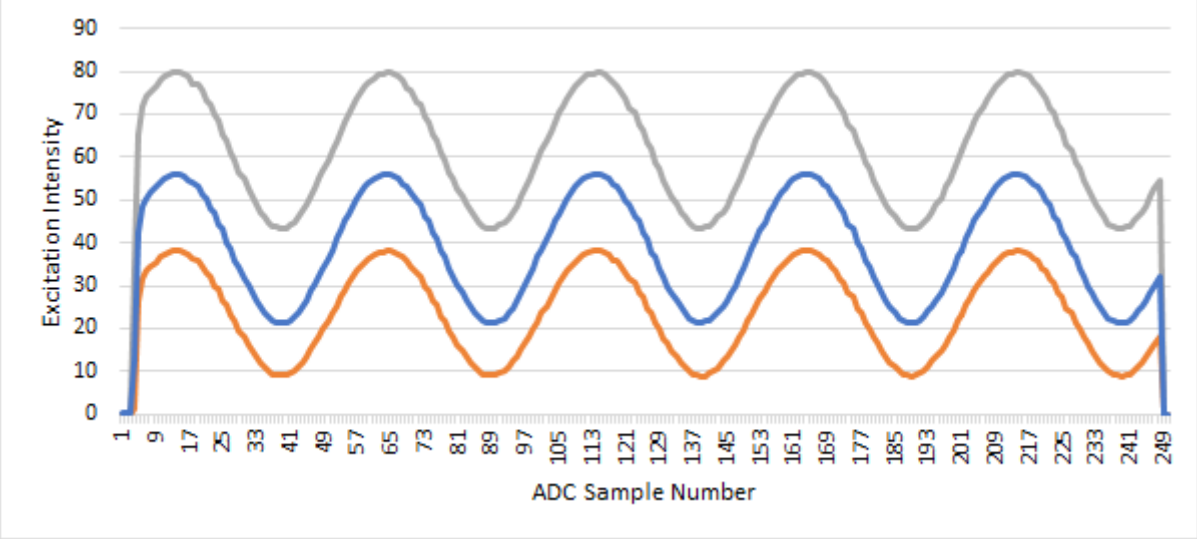


Figure 17: Modulated light pulses with different DC offsets.

As mentioned in Chapter 3, many fluorometry techniques have been developed for different research areas of photosynthesis. They all make use of different light intensities applied in pulses or for certain periods. Some of these techniques apply modulation of the light when it is switched on. Schreiber [18] refers to a design where all fluorometer light sources, including the saturation pulses, are modulated to provide very high sensitivity. This design can however only be used in laboratory conditions where no external light sources are present. A pseudo-random sequence (PRS) modulation of the excitation light was used by [89] to develop a fluorometer that can measure chlorophyll concentrations as low as 0.0103 µg/l. The excitation LED intensity is modulated by [90] to determine the phase shift between the excitation light and the fluorescence. This enables the measurement of the fluorescence lifetime response which provides information on absolute quantum yield but requires very accurate timing of measurements.

The effect of the light modulation on the sample state and physiology must be known when analysing measurements to ensure that they are interpreted correctly. Ideally the modulation of the light should have no effect or cause a very specific required effect. In 1963, [91] found that a constant intensity actinic light and a 50 Hz switched light with the same average intensity, had the same effect on all the species they investigated (Chlorella, spinach chloroplast, Porphyra and Anacystis). Various other light modulation frequencies have been documented to be successful in specific fluorescence measurement techniques. There is quite a wide range of frequencies, for example 1 Hz [65], 50 Hz [91] and 1 kHz [74]. Beutler [10] used 100 µs light pulses with five LEDs, of different wavelengths switched sequentially at 5 kHz, to successfully characterise micro-algae

species from their fluorescence responses. Schreiber [12] used 10 µs light pulses at four different wavelengths (470, 535, 620 and 650 nm) to develop a new generation PAM fluorometer. It is one of the challenges for the fluorometer designer to select an excitation light modulation technique and frequency that will provide the required photosynthetic response from the measurement sample without changing its state in an unknown way.

Even though the light intensity and duration of light pulses have a big impact on the fluorescence response, very few authors seem to provide detailed information about it when reporting their findings when comparing a newly developed fluorometer with proven devices or proven laboratory methods. The reason behind this might be to protect intellectual property but this makes it difficult to find a light modulation technique that produces repeatable results for a new fluorometer.

Section 6.3 provides details of the light modulation technique that is used in the FICC, as well as results from various investigations that were done to compare different techniques in the search for one that will deliver repeatable results.

5 The Fluorescent Light Measurement

One of the FICC's design requirements is that it should be able to detect phytoplankton concentrations as low as 0.1 µg/l. Since the chlorophyll is contained inside the phytoplankton, its concentration depends on the species composition but is definitely lower than 0.1 µg/l. It was decided to aim for a sensor sensitivity that could detect 0.01 µg/l of chlorophyll. At these low chlorophyll concentrations there is very little fluorescent light emitted. This chapter starts by looking at the light sensors that can be used to measure the low intensity fluorescent light. The light sensors that are used in the FICC are discussed before the results of investigations into different light sensors are presented. This is followed by a discussion about light filters. Even at maximum fluorescence only about 3% of the light absorbed by chlorophyll is re-emitted as fluorescence [25]. It is therefore usually not easy to measure the low levels of fluorescent light without putting measures in place to isolate it from other higher intensity light sources. This is usually done by means of light filters. Section 5.2 presents information that must be kept in mind when a fluorometer designer wants to select a light filter. It also discusses the light filters used in the FICC. This includes investigations that were done to find the optimal combination of light filters that would allow the fluorescent light measurement without increasing the fluorometer cost too much.

Several different light sensor locations were investigated to determine locations for the sensors that would be practical to implement while still providing consistent measurements. These investigation results are presented in Section 5.3.

When chlorophyll concentrations get high it causes re-absorption of the fluorescent light. This causes a non-linear relationship between the chlorophyll concentration and measured fluorescence light. Section 5.4 covers this phenomenon.

Due to the very low levels of fluorescent light at low chlorophyll concentrations, the voltage outputs of the voltage-to-light sensors are quite low. It became clear that it would be necessary to amplify these low voltages. Section 5.5 explains how this is done while also discussing the benefits of using the MCP3903 AFE to measure these low voltages. It also explains why a non-inverting summing amplifier should not be used to combine light sensor signals.

The last part of Chapter 5 discusses the different methods that were investigated to remove as much noise as possible from the measured data in an effort to make it possible to differentiate between lower chlorophyll concentrations. When the noise is not removed it forms such a large

part of the measured fluorescence intensity that many of the lower chlorophyll concentrations have intensity peaks that have the same value as higher chlorophyll concentrations and low intensities that are the same as that of lower chlorophyll concentrations. When the noise is removed there are no fluorescence intensity values that can belong to different chlorophyll concentrations.

5.1 Finding the Right Light Sensor

Any light that needs to be measured with a fluorometer must be converted to an electrical signal. This can be done in various ways. Highly sensitive photodetectors, like photomultiplier tubes, are often used to detect the weak fluorescent light of chlorophyll α [92]. These devices were deemed too expensive to be used in the development of the FICC. Several cheap commercial light sensors (less than $3) were investigated to confirm if they were sensitive enough to be used as fluorescence sensors. This section provides details of the two sensors that were finally selected for use in the FICC before it covers the investigations that were done to compare the performance of all three sensors that were considered.

The high sensitivity TSL257 light-to-voltage sensor was finally selected for the FICC to measure the fluorescent light as its low cost and good performance seemed to meet the requirements. The 330 µs response time of the TSL257 is good enough as it can measure the initial fluorescence rise fairly accurately during the OJ section (2-10 ms [36]) if the OJIP curve is being measured with a saturating pulse. The response time should not affect the measurement of the maximum fluorescence as it takes at least 200 ms to reach the F_M/P point of the fluorescence curve in saturating light [11]. As is discussed later, this sensor was successfully used several times to measure OJIP fluorescence curves when the FICC functionality was tested. During the laboratory experiments discussed in Section 7.1, the TSL257 proved to be sensitive enough to detect the emitted fluorescent light of 0.01 µg/l chlorophyll.

The TSL250 light-to-voltage sensor was selected to measure the intensity of the FICC excitation LED. It has a lower sensitivity than the TSL257. This makes it better suited to measure the much higher intensity excitation light that could saturate the TSL257.

Three light-to-voltage sensors were initially selected for comparative testing due to their high sensitivity compared to other low cost commercial light sensors. These were the TAOS (now ams) TSL257 and TSL250 as well as the Texas Instruments OPT101. The OPT101 was successfully used by [6] in a fluorometer. All three sensors have a photodiode and a transimpedance amplifier

on a single monolithic integrated circuit. The TSL257 and TSL250 have fixed gains while the OPT101 gain can be adjusted by means of an external resistor.

During the comparison tests of the light sensors, only one sensor layout was used. The sensors were placed next to each other to face the algae-containing cuvette from the same side. The 470 nm blue LED was located in a lid of the cuvette enclosure to shine down the length of the cuvette containing the Chlorella vulgaris algae. A Lee filters Orange 105 filter was put in front of all the sensors to block out the blue excitation light. The sensors' output voltages were measured with the 12 bit on-board ADC of the mbed development board that forms the core of the FICC.

The TSL250 was initially compared with the OPT101 that had its programmable gain set to 5 times the normal gain (5 MΩ external resistor added). The OPT101 provided an output voltage that was about 10% of the TSL250 for the same fluorescence intensity from an algae concentration. The algae concentrations were not measured but were calculated from rough dilutions made with a plastic pipette that had 0.5 ml increments. The exact concentrations were not important to know as the main aim was to compare the sensors' output voltages for the same dilutions. The 100% level was a high algae concentration and 0% was when the cuvette was filled with tap water only.

Algae Dilution	TSL250	OPT101 (5MΩ)
100%	210	21
75%	195	21
56%	175	19
42%	142	17
32%	108	13
24%	76	10
18%	54	7
13%	42	5
10%	28	3
8%	20	3
6%	13	2
4%	10	1
0%	2	1

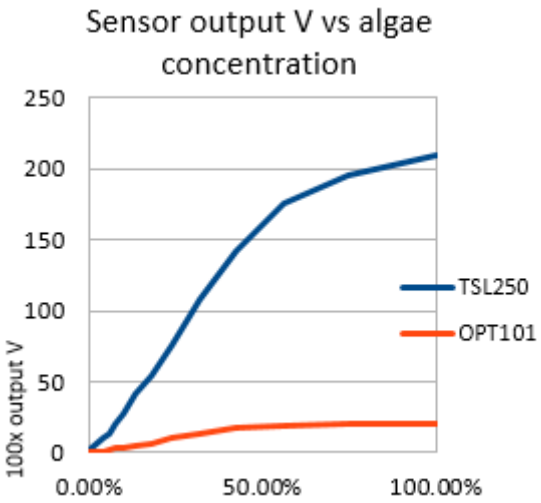


Table 1: TSL250 & OPT101 comparison.

With its standard gain the OPT101 sensor provided very low output voltages (< 0.4 V) even for high algae concentrations. When the OPT101 gain was increased to 5 and 10 times the normal

gain, the output voltages reached levels that could be used to easily differentiate between algae concentrations when there was a 20% difference between them. The increased gains did however cause delays in the fall time of the fluorescent light intensity measurements. The graph below shows the delay in the fall of the output voltage of the OPT101, compared to the TSL250, after a 10 ms light pulse.

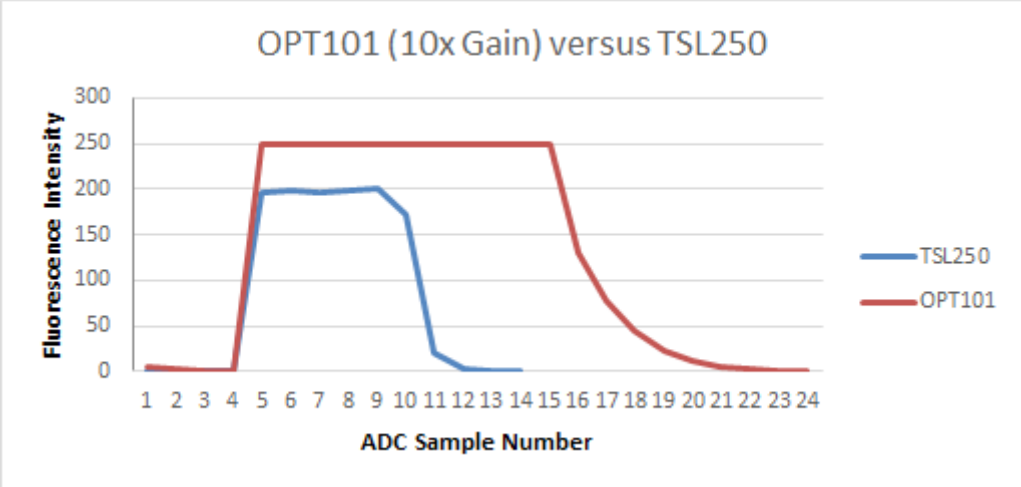


Figure 18: OPT101 (10x gain) delay versus TSL250.

This slower discharge of the OPT101 output voltage was most likely due to the high impedance of the ADC input. It was decided not to spend time to develop an ADC input circuit for the OPT101 sensor that might provide a better discharge response since the TSL250 was performing well enough to be used. The OPT101 has its maximum light response around 850 nm, which is also not ideal. The maximum response of the TSL250 is at 635 nm, which is much closer to the fluorescence wavelengths of chlorophyll.

The two TAOS sensors were then compared. Their datasheets indicate that the irradiance responsitivity of the TSL250 and the TSL257 are both more than 80% of the maximum around 630-680 nm. This is the peak fluorescence wavelength range of chlorophyll α and b indicated by [69]. Their responsitivity is even higher around 684 nm. This is the in vivo fluorescence peaks indicated by [22],[30],[93]. These sensors are therefore very well suited to measuring fluorescence light whereas many other light sensors are optimised to measure infrared wavelengths.

Measurements with different concentrations of chlorophyll confirmed that the TSL257 provided an output voltage more than 6 times that of the TSL250 for the same concentrations. The TSL257 was able to still provide measurable differences in its output voltage for the fluorescent light from

concentrations lower than the estimated 5 mg/l while the TSL250 voltage output had almost no change at such low concentrations. Figure 19 below shows the results of comparing the two sensors. The table on the left-hand shows the measured light intensity values for the estimated chlorophyll concentrations. The graph shows the plots of all measurements except for 0 µg/l chlorophyll.

Chlorophyll ug/l	TSL250	TSL257
160000	30	182
80000	25	163
40000	20	136
20000	16	108
10000	12	81
5000	9	61
2500	8	51
1250	6	42
625	6	35
0	5	35

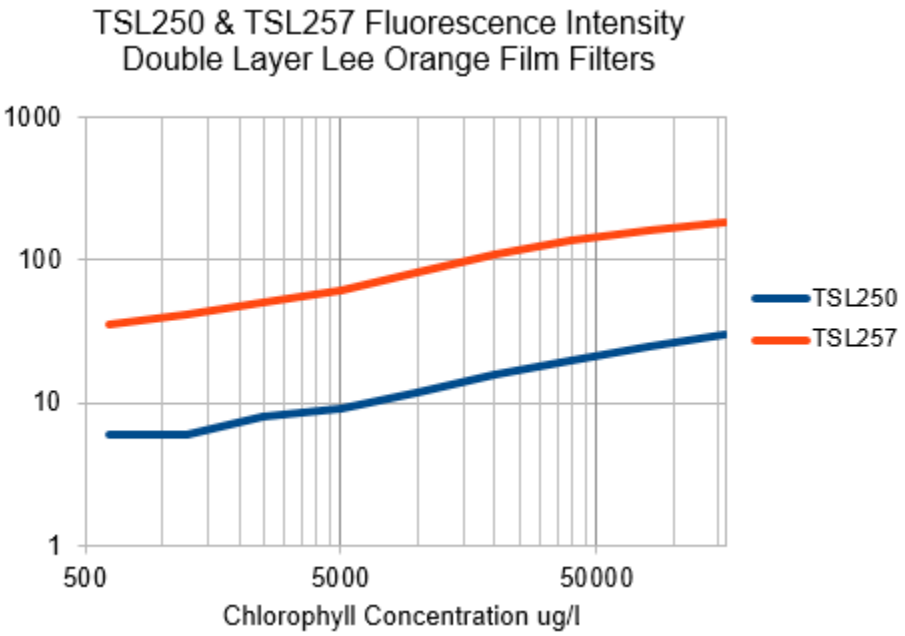


Figure 19: TSL250 & TSL257 comparison.

The 50% dilution of each chlorophyll sample was performed on the dilution that was used in the previous measurement. The dilutions could not be done accurately with the cheap plastic pipette that was available. As the main aim was to compare the two sensors for the same chlorophyll dilutions the actual concentrations did not matter much at this point.

The measured fluorescence intensity dropped along with the reduction in concentration. This showed that the sensors could be used in the FICC for measuring chlorophyll concentrations in the range of 625 µg/l to 160 mg/l. The usefulness in other ranges were tested later, as is discussed in Section 7.1. The author believes that the measured fluorescence intensity levelled off at the low concentrations instead of going down to 0 due to a small amount of excitation light passing through the double layer of film filter that was used. The next section discusses the importance of the light filters in the FICC.

5.2 Using Light Filters

The FICC uses two sets of light filters to ensure that the light sensors measure as much as possible light from the targeted light source and as little as possible from any other source. The fluorescent light is isolated with an Edmund Optics (EO) 550 nm longpass fused silica filter (#49027) that is combined with a Lee Filters 105 (Orange) film filter. The excitation light is isolated by means of an EO 550 nm shortpass fused silica filter (#49826) in series with a Lee Filters 141 (Bright Blue) film filter. The theory behind using these light filters in the FICC is now discussed along with the investigations that were performed to select these specific filters.

Light filters are used in fluorometers to remove unwanted light wavelengths. This enables measurement of specific wavelengths, like the fluorescence spectrum of chlorophyll, or shining specific wavelengths of light onto the measurement area. Longpass filters are used to block light with wavelengths shorter than the cut-off wavelength. Shortpass filters are used to block light with wavelengths longer than the cut-off wavelength. Bandpass filters are used to block most light outside a very narrow wavelength range. The fluorometer designer needs to choose the right filter to either block or transmit specific wavelengths.

The selection of the light filters must be done carefully after considering the intended use and the environment where the fluorometer will be used. The light filter should block or attenuate the unwanted wavelengths without affecting the desired wavelengths so much that it affects the performance of the fluorometer. The lower cost light filters do not normally have as big a difference in attenuation between the blocked and transmitted wavelengths as the more expensive filters. The lower cost filters therefore usually also attenuate a fair amount of the light in the wanted wavelength range or transmit an amount of light that should be blocked. This is not a problem if the amount of transmitted light from the unwanted wavelengths does not interfere with the measurement of the ideal wavelengths.

As fluorescence happens at a higher wavelength than absorption, light filters can be used to block out the unwanted light of either the fluorescence or the excitation light source. The bright excitation light wavelengths can be blocked from saturating the highly sensitive fluorescent light sensor [10], [18]. If very accurate measurement of the excitation light intensity is required a bandpass filter can be used to block other light from reaching the light sensor that measures the excitation light intensity. If its intensity does not need to be measured accurately a shortpass filter can be used if the excitation light is much brighter than any other light source around the fluorometer

measurement area.

A long pass filter can be selected such that it will allow the fluorescence wavelengths of the chlorophyll to pass while still blocking the shorter wavelengths of the excitation LED and other light sources, like DOM (Dissolved Organic Matter). DOM can fluoresce at wavelengths up to 521 nm. The long pass filter for the fluorescent light sensor should then ideally have a cut-off wavelength longer than 521nm. DOM fluorescence is excited by wavelengths of 455 nm and shorter [94],[95], [96]. It should therefore not be excited into fluorescence by the 470 nm LED of the FICC as the datasheet shows only about 5% luminous intensity for the LED at 455 nm. The DOM fluorescence wavelengths should however still be blocked where possible as any DOM in the measurement volume could be excited into fluorescence if any ambient light with the right wavelengths reaches it. Chlorophyll has in vivo fluorescence peaks with wavelengths of 683-685 nm [22],[30],[93] as well as 720-735 nm [22]. These fluorescence peaks should not be blocked by the long pass or other light filters in front of the fluorescent light sensor.

When F_O is measured around 685 nm only about 10% of the initial total fluorescence comes from PSI. This can increase up to 30% at wavelengths greater than 700 nm [35],[76]. The additional PSI fluorescence contribution causes an underestimation of the F_V'/F_M' ratio [76]. The influence of PSI on fluorescence parameters in the light-adapted state must be taken into account when light above 700 nm is also measured [76]. Using a short pass light filter to limit or remove the PSI fluorescence above 700 nm would improve the F_V'/F_M' ratio accuracy. The variable fluorescence contribution from PSII can also be improved with a bandpass light filter that will allow the PSII fluorescence around 685 nm to pass while blocking the shorter excitation wavelengths as well as the longer PSI fluorescence wavelengths. The effect of light filters on the measurement of F_O and F_V'/F_M' should be kept in mind when comparing variable fluorescence measurements of fluorometers with light filters to fluorometers without them.

The intensity of the blue excitation light used in the FICC is much more than that of the fluorescent light. To prevent the excitation light from saturating the fluorescent light sensors, three light filters were tested to find the best way to block the blue light without attenuating too much of the fluorescent light. These were an Edmund Optics (EO) 550 nm longpass fused silica filter (#49027), Edmund Optics (EO) 692 nm bandpass fused silica filter (#67024) and a Lee Filters 105 (Orange) film filter. The EO longpass filter and Lee Filters 105 filter were selected as they would block out the shorter wavelength of the excitation LED while allowing through all the different wavelengths at which chlorophyll fluoresces. The drawback of using longpass filters is that they might transmit the

fluorescence light of other substances in the sample that might fluoresce at longer wavelengths than chlorophyll. The EO 692 nm bandpass filter was selected for testing as it would not block the reported in vivo chlorophyll fluorescence peak around 685 nm [22],[30] with its bandwidth of 40 nm. The bandpass filter seemed to provide the best chance to isolate the chlorophyll fluorescence from all other light for measurement. The light transmission curves of the light filters are included in Appendix B.

The main aim of the filter investigation was to find a light filter combination that would transmit the optimal amount of fluorescent light while also blocking as much as possible of the excitation light. The filters were compared by measuring the fluorescent light intensity of different concentrations of extracted chlorophyll. The chlorophyll was placed in an enclosed cuvette to ensure that only the excitation and fluorescent light could reach the light sensor. During these comparison tests the 470 nm LED was pulsed at 50Hz with an on time of 10 ms. The maximum fluorescent light intensity reached during the pulse was used as the measured value for the concentration. The indicated concentrations in Figure 20 are estimates since the dilutions were done with a plastic pipette. The actual concentrations were not important at this stage since the main aim was to compare the effects of the filters on the emitted fluorescence when the same chlorophyll concentrations were used for all the filters. Figure 20 shows the plots of the measured fluorescence intensities for the three main filter comparisons. The ADC values were converted by the microcontroller software to have a value of 100 per volt.

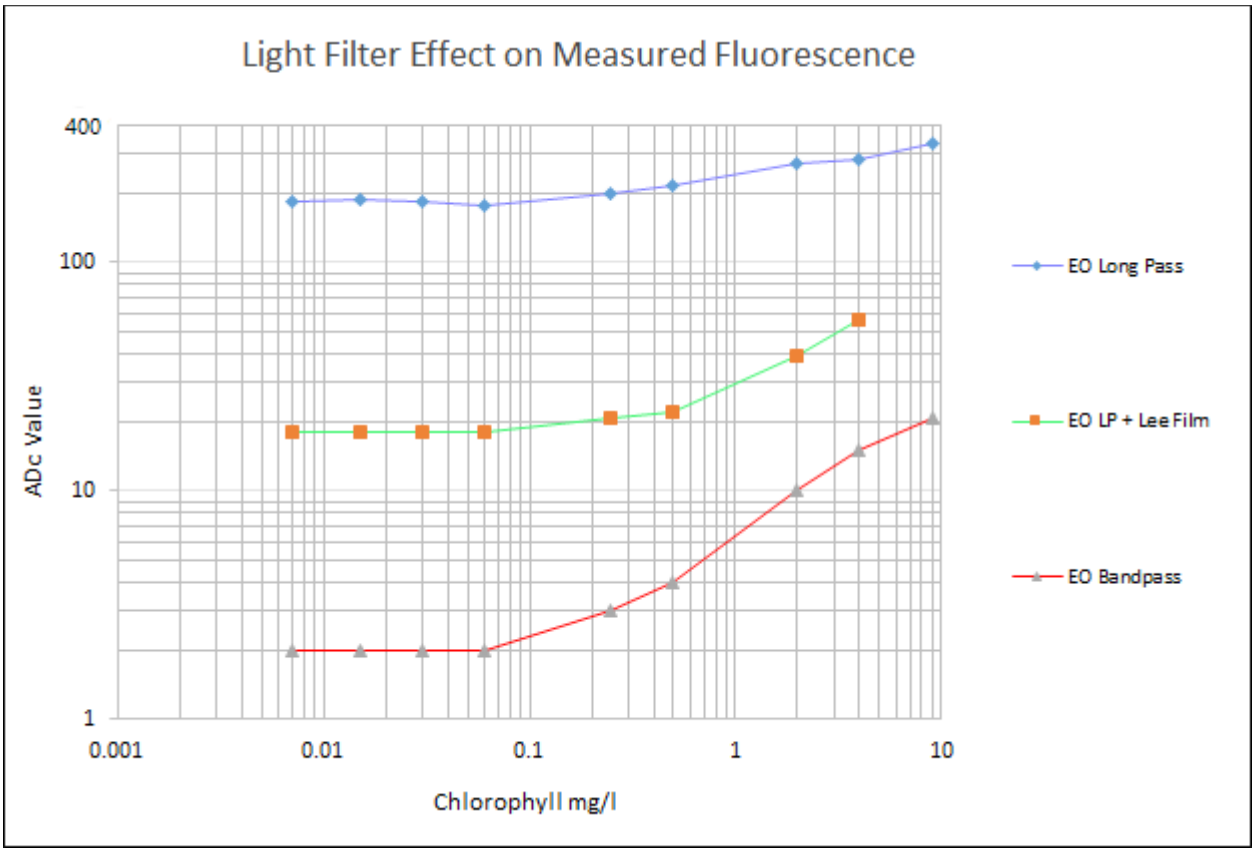


Figure 20: Effect of different filters on measured fluorescence.

The EO longpass filter on its own allowed so much fluorescent light through that the light sensor output reached the maximum voltage (3.3 V) and ADC value (330) at chlorophyll concentrations of 9 mg/l and higher. This meant that the FICC would not be able to measure chlorophyll concentrations higher than 9 mg/l if only the EO longpass filter would be used. At low chlorophyll concentrations the ADC input voltage levelled out around 1.8 V even though the light sensor output voltage could drop to 0 V when no light was detected. This author believes that this must have been due to the amount of blue excitation light that still passed through the EO longpass filter. The total span of the light sensor output voltage for the measured chlorophyll concentrations was 1.5 V, around 45% of the possible range. Even though less than half of the possible voltage span was used, it still provided a large enough voltage difference between the tested concentrations for good differentiation.

When the EO bandpass filter was tested, the light sensor output voltage at the lower chlorophyll concentrations levelled out around 0.02 V. This is about 1% of the value for the EO longpass filter alone. The maximum voltage for a concentration around 9 mg/l was 0.21 V. This is about 6% of the value for the EO longpass filter alone. The voltage difference between chlorophyll concentrations was very low and made differentiation difficult. The EO bandpass filter has a very narrow

bandwidth range of 690 nm +/- 20 nm which seemed to block out fluorescent light when it was tested by the author with an extracted chlorophyll and acetone mixture. The findings of [97] confirm that the EO bandpass filter would block out much of the fluorescent light when chlorophyll that was extracted from spinach, is used. Two chlorophyll b fluorescence peaks were found in spinach (at 641 nm and 651 nm) when [97] measured it at -196º C. Chlorophyll α peaks ranged from 662 nm to 705 nm. All measurements and theory indicated that this particular bandpass filter was not suited for use in the FICC.

The most useful results were found when the EO longpass filter was combined with a Lee Filters 105 (Orange) film filter (green line in Figure 20). With the low chlorophyll concentrations the light sensor output voltage levelled out at a lowest point (0.2 V) of around 11% of that of the EO longpass filter alone. The light sensor output voltage stayed far below the maximum at the higher chlorophyll concentrations. The total span of the light sensor output voltage was about 0.4 V for the range of concentrations tested. The compromise of this filter combination was that it caused very little voltage difference between different concentrations but it did not have the problem of limiting the FICC to measure chlorophyll concentrations lower than 9 mg/l.

The 470 nm excitation light intensity is also measured in the FICC to provide the actual excitation light intensity at any moment in case it will be helpful to determine the cause of changes in fluorescence intensity. This can be used for instance to monitor the LED functionality or as a turbidity measurement to get a rough idea of the sample concentration. The excitation intensity data can also be used in DSP methods to remove noise from the fluorescence measurements. This is discussed in more detail in Section 5.6.

During most of the FICC development tests an Edmund Optics 550 nm shortpass fused silica filter (#49826) was used in series with a Lee Filters 141 (Bright Blue) film filter. This was done since the EO filter was available and it was thought that very accurate excitation light intensity measurements would be achieved by blocking as much as possible of the fluorescent light. No tests were done to compare the performance of the two filters on their own. During some early investigations and during the laboratory experiments discussed in Section 7.2.2, the low cost Lee filter on its own did provide satisfactory results during measurements of the excitation light intensity. It is mentioned here just for information in case some measurements or graphs show excitation light intensity measurements that are slightly different from intensities provided in other sections. Different measured intensities between different investigations should not matter as long as it is not used for direct comparison.

5.3 Light Sensor Location

This section discusses the impact of the light sensor location on the measured fluorescence. It also covers the different light sensor locations that were used during different investigations. The final sensor placement of the FICC is discussed in Section 6.4.

During the requirements determination phase of the FICC it was determined that the most practical housing for the FICC would have a flat sensor face that could be cleaned easily when it was on site for long durations. The light sensors and excitation light would then have to be next to each other and face towards the measurement area. This layout was however difficult to implement during some of the tests where different components such as the light filters, were replaced for comparison purposes. It was often much easier to place the components on different sides of the cuvette inside the PVC housing that was available. Even though the literature indicated that fluorescent light is radiated equally in all directions [22],[98] (except for Dunaliela tertiolecta) it was decided to test different sensor locations to determine the effect of other factors relating to the sensor location.

Fluorescence measurements were made with TAOS TSL257 light sensors positioned at 90 degrees to the axis of an excitation light beam, but directly opposing each other on either side of the beam (Figure 21). There was no measurable difference in the fluorescence intensity measured on either side. This confirmed that the fluorescence emittance was the same to both sides of the cuvette.

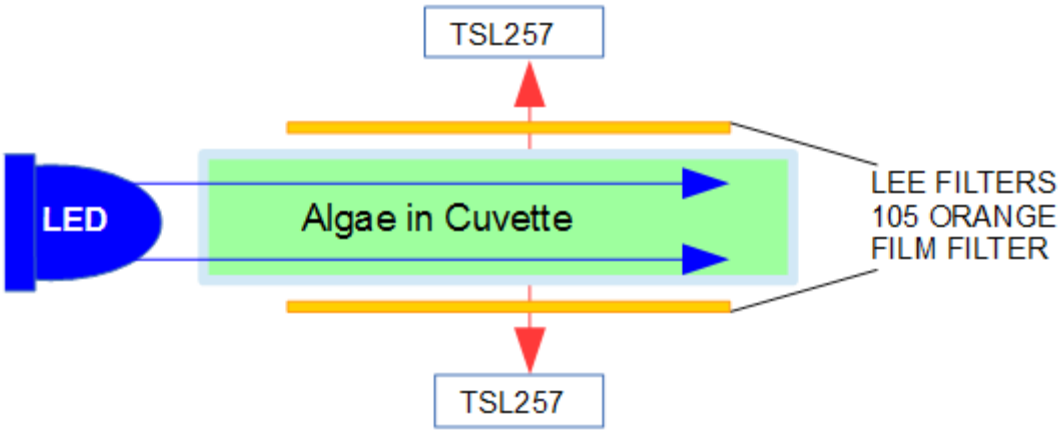


Figure 21: Fluorescence on opposite sides.

In theory it should be possible to place the light sensor anywhere at a fixed radius from the measuring point and still measure the same amount of fluorescent light, if the fluorescence intensity from the algae is the only factor that needs to be considered for the light sensor location. In reality it is not possible to place the sensor anywhere due to factors like packaging of components in the fluorometer housing. Several light sensor locations were tested to determine the optimal location for sensitivity as well as practical packaging in the housing.

During the tests of the first prototype TOAS TSL257 light sensor unit, the design used one light sensor for measuring the excitation light intensity and one for the fluorescence intensity. The sensors were placed next to each other facing the side of the cuvette while having a 90 degree angle to the excitation light beam that was shining down the length of the cuvette. The excitation light sensor had a Lee Filters Bright Blue 141 filter in front of it to block out fluorescent light. The fluorescence sensor had a Lee Filters Orange 105 filter to block out the excitation light. The main problem with this layout was that the EO longpass filter could not be used due to a lack of space. It was also very difficult to fit the film light filters around the two light sensors.

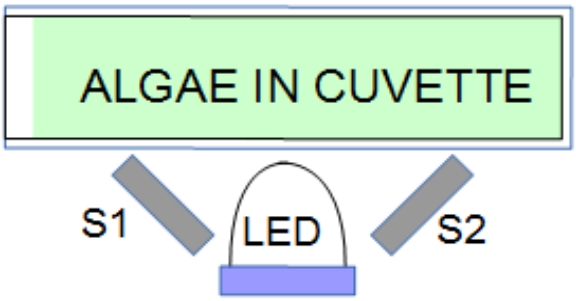


Figure 22: Light sensors next to LED.

When the LED was placed alongside the light sensors on the same side of the cuvette (Figure 22), the light sensors received enough fluorescent light to take measurements. The main challenge of this layout was to block the strong excitation light of the LED from reaching the light sensors right next it by means of light filters without also blocking the low intensity fluorescent light. Due to the lack of space it was again not possible to use the EO longpass filter and it was also very difficult to fit the film filters around the light sensors.

Placing three light sensors on the opposite side of the cuvette, directly facing the LED light source, proved not to be an ideal layout. Even though it did provide useful measurements for one sensor directly across from the LED when high algae concentrations were tested, the other two light

sensors detected little fluorescent light. In low algae concentrations the sensor directly opposite the LED could not detect fluorescent light as the amount of blue light still coming through the orange filter was much more than the fluorescent light.

5.4 Light Intensity Changes for Different Chlorophyll Concentrations

This section highlights a few things that must be kept in mind by the fluorometer designer and user when the fluorescence of high chlorophyll concentrations will be measured. High chlorophyll concentrations respond differently to low concentrations.

During the comparison testing of the light sensors, described in Section 5.1, some of the measurements made with high algae concentrations provided results that did not have the expected straight line when plotting fluorescence intensity against chlorophyll concentration. It was decided to investigate this further. The acetone dissolved chlorophyll (discussed in Section 7.1.2) was used to make a series of measurements to determine the effect of chlorophyll concentration on the maximum fluorescence intensity. The first measurement was made with a high chlorophyll concentration. The actual concentration was not measured. It was a visibly thick and dark green concentration. The sample in the cuvette was diluted with pure acetone by about 30% each time before the next measurement was made. The dilutions were done with a plastic pipette and could not be done very accurately. This resulted in the graph not having a smooth curve but it did not affect the overall shape of the chlorophyll concentration to fluorescence intensity curve. Figure 23 shows the results of the measurements.

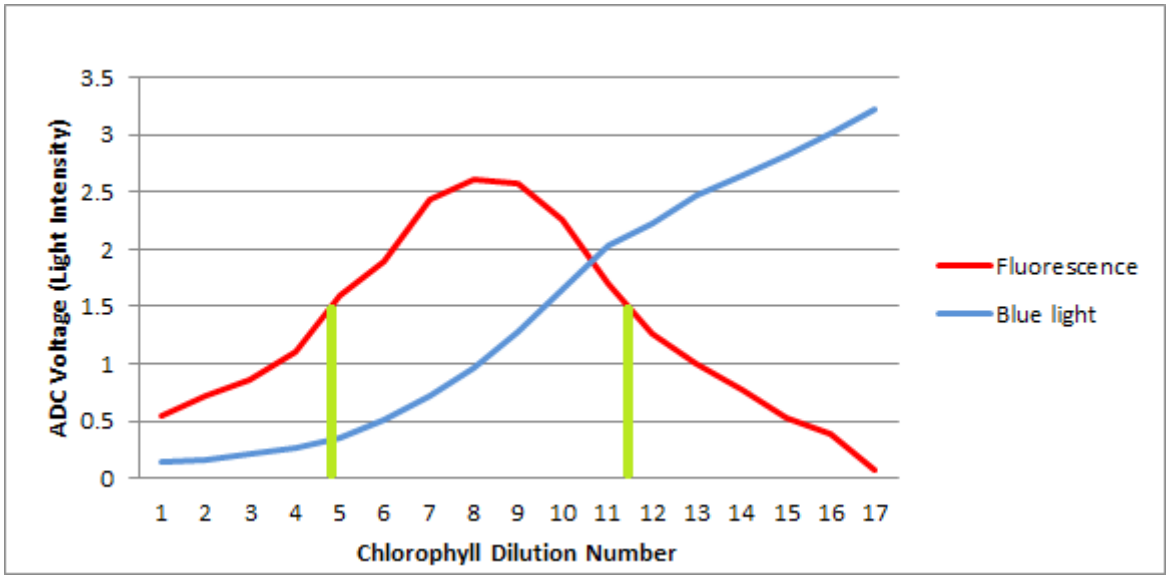


Figure 23: Light intensity versus concentration.

In the initial high chlorophyll concentration a low amount of blue excitation light could reach the light sensor that had a blue light filter in front of it. The low intensity of the blue light reaching the sensor showed that a lot of the blue excitation light was blocked from reaching the area in front of the sensor. The chlorophyll in that area could then also not cause much fluorescence. The fluorescence intensity detected by the light sensor with the orange filter was very low. Some of the fluorescent light would also have been re-absorbed by the chlorophyll [51]. As the mixtures were diluted for the following measurements more blue excitation light could reach the blue light sensor and chlorophyll. The fluorescence intensity therefore showed a rising trend which is not normally expected for a decrease in chlorophyll concentration. A decrease in chlorophyll concentration should normally have a decrease in fluorescence since there is less chlorophyll to emit fluorescence. After a maximum fluorescence intensity was reached there was the expected downward slope with the decrease in chlorophyll concentration. This slope showed a fairly proportional relationship between fluorescence intensity and chlorophyll concentration. The excitation light intensity continued to increase as less of the light was blocked or absorbed in the lower chlorophyll concentrations.

Combining the excitation and fluorescence intensity curves generated a graph (Figure 23) where there was only one chlorophyll concentration where a specific fluorescence and excitation light intensity combination was possible. The FICC therefore always measures the excitation light intensity as well. If only fluorescence intensity is measured there will be two very different chlorophyll concentrations that would have the same fluorescence intensity when high concentrations are present since. It will therefore not be possible to determine the chlorophyll

concentration with confidence if the excitation intensity is not also measured. This is indicated in Figure 23 by the two vertical green lines that correspond to two very different chlorophyll concentrations for the same fluorescence intensity ADC voltage of 1.5V. Even though the actual starting concentration of the algae is not known it is believed that such a high concentration is unlikely to be found outside of a laboratory.

Figure 24 shows a 3 axis presentation of the same data that was used for Figure 23. The third axis shows the LED current for a specific measurement. The graph shows how the fluorescence intensity increased with an almost direct relationship to the increase in excitation LED current for each sample. The fluorescence intensity to LED current relationship stayed linear regardless of the chlorophyll concentration. Since the excitation intensity has an almost linear relationship to the LED current of the FICC (Section 4.3), this data indicates that the excitation and fluorescence intensities have a linear relationship regardless of the chlorophyll concentration.

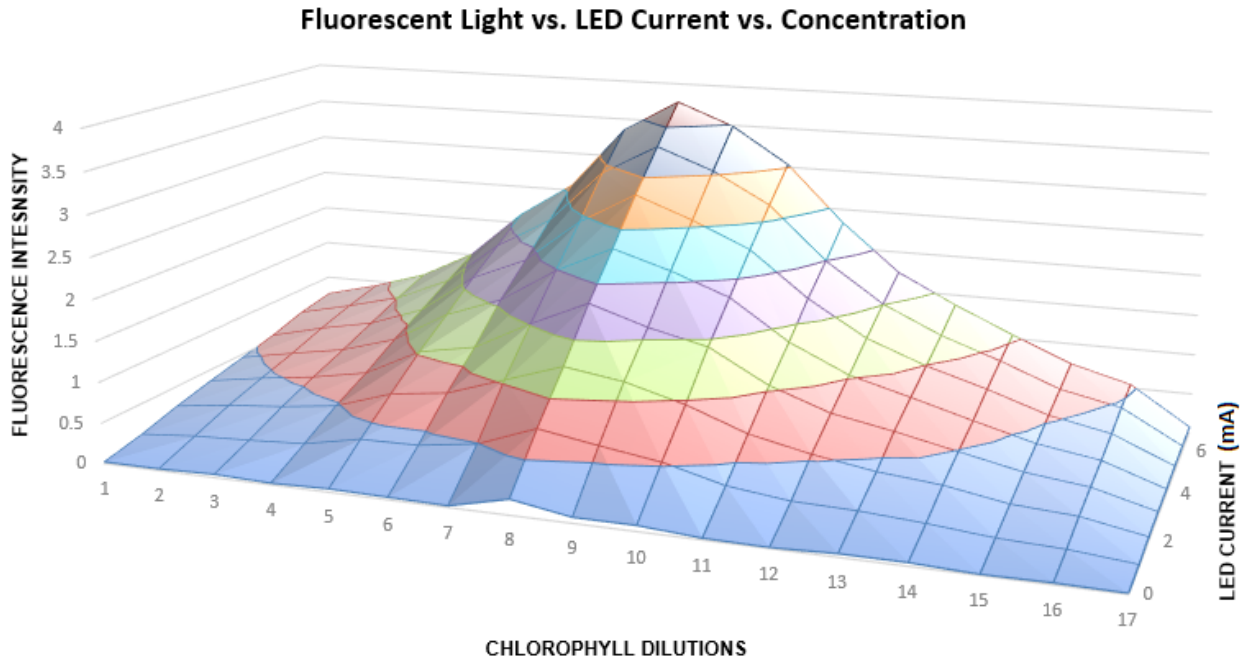


Figure 24: Fluorescence intensity for different concentrations.

When light sensors were placed next to the cuvette at different distances along the excitation light axis, as shown in Figure 25, the different sensors provided similar intensity measurements in medium to low algae concentrations. In high algae concentrations the measured intensity decreased along with the distance of the light sensor from the LED. Some of the excitation light was physically blocked by the chlorophyll particles from reaching the chlorophyll in front of the farther sensors. This then also reduced the fluorescence measured by the sensors as the distance

to the LED increased.

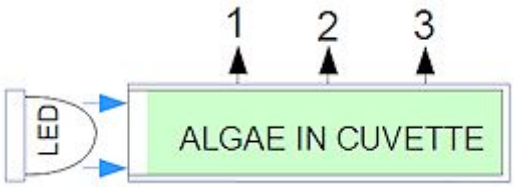


Figure 25: Effect of concentration and distance on light intensity.

The fluorometer designer should try to design the fluorometer with minimal distance between the LED and fluorescence sensors to limit the effect of chlorophyll concentration on the amount of excitation light that reaches the chlorophyll in front of the fluorescence sensors.

5.5 Improving the Fluorescence Light Sensor Signal Quality

This section explains how three light sensors and an analogue front end device were used to improve the quality of the measured fluorescence signal.

The FICC uses three TSL257 light-to-voltage sensors to measure the fluorescent light, which reaches very low intensities at low chlorophyll concentrations. It was decided to use three sensors next to each other as it increased the signal to noise ratio as well as the detection surface area. The hypothesis was that since all three sensors would be measuring the same fluorescent light intensity the average value of the three sensors would have a smaller noise component than a single sensor. The noise in the three signal channels would be random. Adding the three sensor outputs provides three times the actual signal value at any moment while the random noise will not be three times bigger.

In the final design of the FICC, the three light sensor output voltages are separately amplified and measured with separate ADC channels to minimise adding and amplifying common noise on the signals. This is implemented by means of the MCP3903 AFE device that provides a programmable gain of 1 to 32 times for each channel before each channel is digitised by its own 24-bit ADC.

An extra feature of the AFE is that it adds dithering noise to the measured signal when it is small. The dithering noise is added before the ADC and causes the voltage to cross more of the least significant bit (LSB) boundaries. Since the dithering noise is random the quantization noise loses

its coherence with the original signal and increases the signal to noise ratio [99]. According to the Microchip MCP3903 datasheet the dithering noise improves the accuracy of the measured signal as it suppresses the idle tones of the ADC. The dithering noise can be removed from the measurement data with digital signal processing (DSP) techniques. Section 6.5 provides more technical details of the MCP3903 AFE.

The first implementation of the idea to use three light sensors made use of a summing amplifier to add up the three weak fluorescence signals and thus create a higher voltage on which external electrical noise would have a much smaller impact. The fluorescence signal (output voltage) of one of the TSL257 light sensors was compared with the combined signal of the three TSL257 sensors generated by the summing amplifier. The output voltage of the summing amplifier circuit did not show the expected summed values of around three times that of the single sensor circuit, during all measurements. This circuit design made use of a non-inverting summing amplifier that was chosen because it requires only a positive power supply. Further investigation found that the non-inverting summing amplifier has the bad characteristic that its input impedance changes along with the input voltages and therefore rarely works as expected from an ideal summing amplifier. The tests did however confirm that combining the output of three light sensors improved the system sensitivity to enable much lower chlorophyll concentration detection than what was possible with the single sensor design.

5.6 Recovering the Fluorescence Signal

The fluorescence measurement data will always have some noise in it. This can be due to the electrical noise in the fluorometer subsystems or external factors like electromagnetic interference from other electrical systems. If the noise to signal ratio gets so large that it interferes with the accuracy of the measurements, it has to be reduced before analysing the data. With the amount of processing power available nowadays this can be done fairly easily with DSP methods.

The main focus of the FICC project was to use low cost hardware to develop the fluorometer. These investigations to find methods that could improve the quality of the existing measurement data with post processing were done to prove the concept. It is very likely that there are more suitable methods that were not investigated. Some of the methods that were investigated will now be discussed to give an overview of how they improve the measurement data. The actual results obtained with these methods when applied to the FICC measurement data, are discussed in detail in Section 7.2.2.

One of the easiest ways to get rid of high frequency noise on a signal is to use a moving average filter. It is implemented by replacing a data point with the calculated average of it and a number of neighbouring values. When applied to the data of a signal it becomes a low pass filter [100]. In the formula below, *m* is the number of data points that are used and Z_t is the unfiltered value at time t.

$$Y_t = \frac{1}{m} \sum_{j=0}^{m-1} Z_{t-j}$$

Table 2 contains fictional data and times to illustrate how the moving averages were calculated on the measurement data of the FICC. There is also a verbal description of the process below the table.

	Time	0 us	100 us	200us	300 us	400 us	500 us
Pulse 1	Sensor 1	3	4	3	2	1	2
	Sensor 2	3.2	4.2	3.2	2.2	1.2	2.2
	Sensor 3	3.1	4.1	3.1	2.1	1.1	2.1
	Average 1	***3.1***	***4.1***	***3.1***	***2.1***	***1.1***	***2.1***
Pulse 2	Sensor 1	3.1	4.1	3.1	2.1	1.1	2.1
	Sensor 2	3.3	4.3	3.3	2.3	1.3	2.3
	Sensor 3	3.2	4.2	3.2	2.2	1.2	2.2
	Average 2	***3.2***	***4.2***	***3.2***	***2.2***	***1.2***	***2.2***
Pulse 3	Sensor 1	2.9	3.9	2.9	1.9	1	1.9
	Sensor 2	3.1	4.1	3.1	2.1	1	2.1
	Sensor 3	3	4	3	2	1	2
	Average 3	***3***	***4***	***3***	***2***	***1***	***2***
	Average 4	*3.1*	*4.1*	*3.1*	*2.1*	*1.1*	*2.1*
	Mov. Avg.		3.433333	3.1	2.1	1.766667	

Table 2: Calculation of moving averages

The first average is obtained for a specific light pulse measurement by calculating the average value of the three light sensors at time t after the light pulse started. The Average 1, 2 and 3 rows in Table 2 contain the average values of the three sensors for the specific light pulses. The three sensor average values at time t of the different light pulses are then used to calculate the total average (Average 4) of all the light pulses at time t. Lastly, a three value moving average filter is used to calculate the final values (Mov. Avg.) in the bottom row. The yellow and brown areas in Table 2 indicate the three values that were used to calculate the specific moving average.

Another method investigated made use of a “brick wall FFT filter”. It was selected because it was easy to implement with the Excel spreadsheet that was available and examples of its use seemed

to provide very good noise filtering. The process starts with a fast Fourier transform (FFT) of the time domain data to get it into the frequency domain where the data is put into rows representing frequency bins. There is a column where the user can select which frequencies will be used in an inverse fast Fourier transform (IFFT) to get the data back in the time domain. Any frequencies not selected will be filtered out.

For filtering the FICC data, only the frequency bin corresponding to the excitation light modulation frequency of the FICC is then selected before the IFFT is performed. All other frequencies of noise are then removed. Appendix C has a screen capture of part of the Excel sheet that was used for this calculation. The Excel sheet is a modified version of the one originally created by Daniel S Merrick (College of Engineering, San Jose State University). It can be downloaded from http://www.engr.sjsu.edu/dmerrick/fftFilter.xls

6 Technical Details of the FICC

This chapter gives a detailed breakdown of the subsystems of the FICC as indicated in Figure 26. The figure shows the main components of the final system design. The technical details of each of the subsystems are discussed in the following sections.

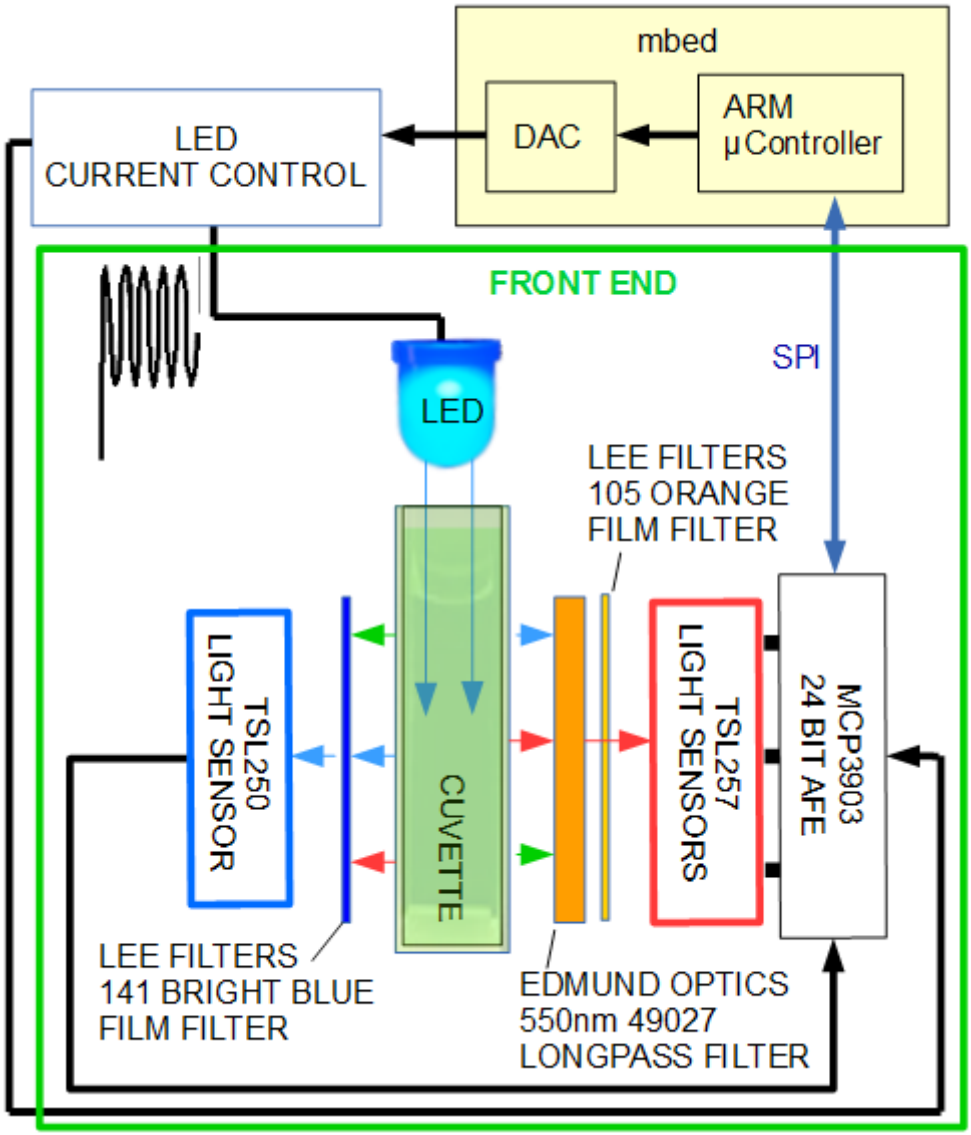


Figure 26: FICC system diagram.

6.1 The mbed Development Board

The mbed development board was chosen to control the system due to the vast amount of information and software that is available for it. This saved time and money by not having to

develop a microcontroller system for control and logging of the data. Proven software functions could also be used for standard functionalities, like serial communication between the FICC and a PC. Programming time was therefore spent on adapting the existing software to the needs of the FICC project rather than creating basic functions from scratch.

The mbed online compiler was used for creating most of the software. The online compiler enables quick import of example programs on the website and is free. The available libraries were all fully compatible with the online compiler. There was therefore no time lost modifying existing software libraries to comply with the peculiarities of the compiler. Some of the prototype software was compiled and tested with the µvision package from Keil. The size limitation on the software that could be compiled meant that only parts of the software could be compiled and tested. This option was useful when internet connection to the mbed online compiler was not available or difficult.

The mbed LPC1786 has a NXP 32-bit ARM Cortex-M3 microcontroller with all required peripherals to easily connect to a PC through a USB connection. The FICC software running on the mbed, calculates the required intensity of the excitation light for that specific moment of the measurement. Depending on the type of measurement that is being made, this can be a constant or rapidly changing intensity. The mbed's on-board digital to analogue (DAC) converter is used to send the excitation intensity control signal to the LED current control circuit. Once the excitation intensity is set, the measured fluorescence intensity is read from the external MCP3903 Analogue Front End (AFE). The mbed's on-board SPI ports are used for communicating with it. Before any measurements are made, the FICC software sets up the AFE control registers.

The mbed plugs into a motherboard that was designed specifically to house it. The motherboard provides power and other interface connections to the rest of the system. The mbed is pin to pin compatible with the LPCXpresso development board. The LPCXpresso board has the same microcontroller and can use the same software as the mbed. It does however have additional pins with additional functionality. The motherboard was designed to accommodate the additional pins of the LPCXpresso to enable future expansion of the FICC functionality.

6.2 LED Current Control

The excitation light intensity control circuit (Figure 27) is basically a voltage to current converter. The mbed DAC supplies a control voltage ranging from 0 to 3.0 V to the current control circuit. This is then converted to a LED current ranging from 0 to 20 mA. As simulations for 2 slightly different

designs of the circuit did not provide conclusive proof of which one was best, the current design has selector links to switch between the optional circuits. Two selector links can be used to send the LED current through either a small 15 Ω or larger 120 Ω current sense resistor. The current sensing voltage over the 15 Ω resistor is then amplified by an operational amplifier with fixed gain. This optional amplification circuit can be used if the LED currents and sensing voltages will be small. If the 120 Ω resistor is used the current sensing voltage is fed back directly to the input operational amplifier of the current control circuit. There is another removable link (JP4 in Figure 27) that can be used to provide LED current control through a transistor along with the BSS138 FET. This option was included in case the FET had problems delivering enough current on its own without overheating.

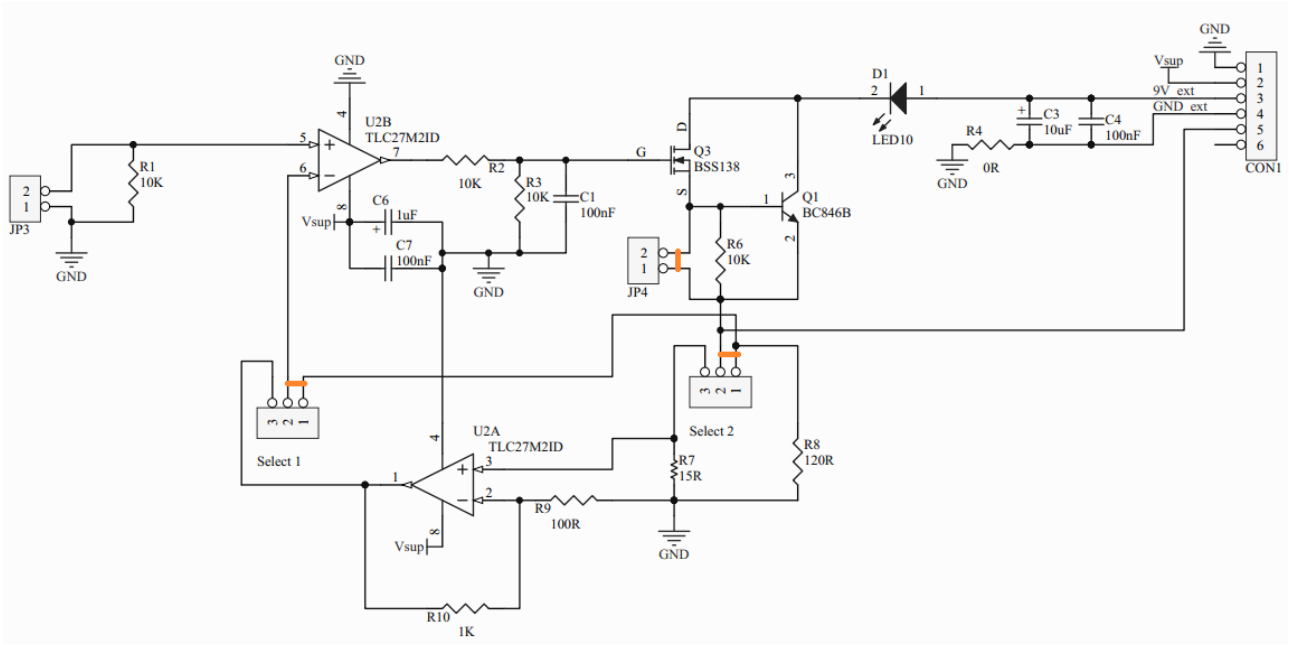


Figure 27: LED current control circuit.

This design enables the microcontroller software to switch the LED on and off by setting the current control circuit input voltages to 0 V or 3.0 V or to control the LED current as required to generate the required light modulation. The modulation capability provides flexibility to the FICC to use different modulation waveforms and test new measurement techniques. Sine, sawtooth, triangle and square wave modulations are some of the possibilities that were implemented in software during different investigations.

This voltage controlled current source provides satisfactory performance in the FICC. Figure 28 shows how the circuit allows accurate LED current control with an error percentage around 2%.

The green line indicates the control voltage from the DAC into the circuit while the red line shows the voltage over the 120 Ω current sensing resistor. The error percentage between the control voltage and actual current sense voltage is indicated by the blue line.

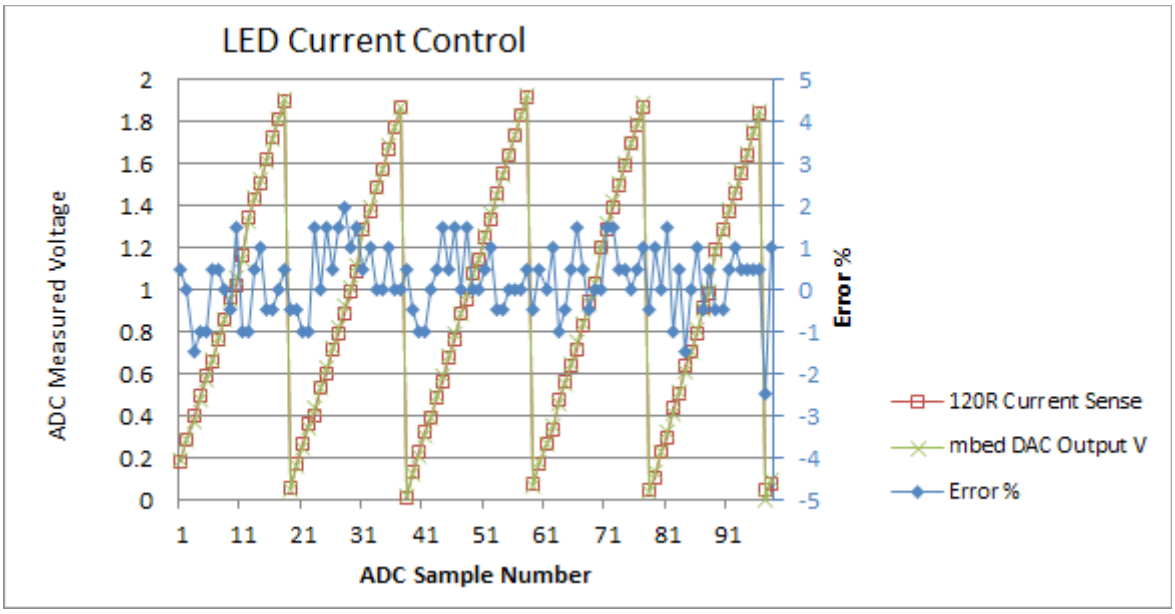


Figure 28: Current control accuracy

The first prototype circuits of the FICC did not have such accurate current and light control as it used a fixed voltage source that could be switched on and off to provide current to the excitation LED with a current limiting resistor in series. As a consequence of the fact that LEDs have a negative temperature coefficient, the LED current and light intensity increases with increasing temperature for the same voltage. The LED light intensity could therefore not be kept absolutely constant by that circuit. This design also did not allow software intensity control or modulation of the excitation light. This led to the decision to change to the current FICC design discussed above, where the current would be controlled by the software on the mbed development board rather than switching the fixed supply voltage of the LED.

6.3 Modulation of the Light Source

This section starts with an overview of how a Kautsky fluorescence curve can be measured with the FICC. It is the most basic fluorescence measurement that the FICC can perform as it only requires that the excitation light should be switched on at constant intensity for about 10 minutes. The FICC must also be able to make fluorescence measurements in much shorter time frames.

This requires that different fluorescence measurement methods be used that make use of light modulation. Several different light modulation methods were investigated to confirm the FICC functionality and to find one that would provide repeatable fluorescence measurements. These investigations are presented here.

Due to the variability of chlorophyll's response to the excitation light, it is difficult to find a lighting method that provides repeatable fluorescence parameter measurements without also analysing the physiological state of the chlorophyll. It did not make sense to develop software for the FICC that could perform onboard analysis of the chlorophyll's state to be used in calculating fluorescence parameters. Researchers like Schreiber [11]–[17] and Govindjee [24], [30], [58], [67] have spent decades trying to find optimal fluorescence analysis methods. Each different method has advantages over others when a specific photosynthesis characteristic is investigated. The FICC therefore provides the raw measurement data in response to the light modulation method programmed in the software at the time. The data can then later be analysed with the selected method to determine the required fluorescence parameters. For its intended use at the CSIR as a chlorophyll concentration sensor that can detect algae blooms, it would be good enough to detect changes in the fluorescence intensity that are different by orders of magnitude.

The simplest method to measure fluorescence response is to keep the sample in a container that is blocked from any light sources other than the system excitation light, and then switch on a constant light source for several minutes. All three red graphs in Figure 29 show the fluorescence intensity change for a Chlorella vulgaris sample during such a measurement with the FICC. The blue graphs show the excitation light intensity and are included to show that the changes in measured fluorescence was not caused by similar changes in excitation intensity.

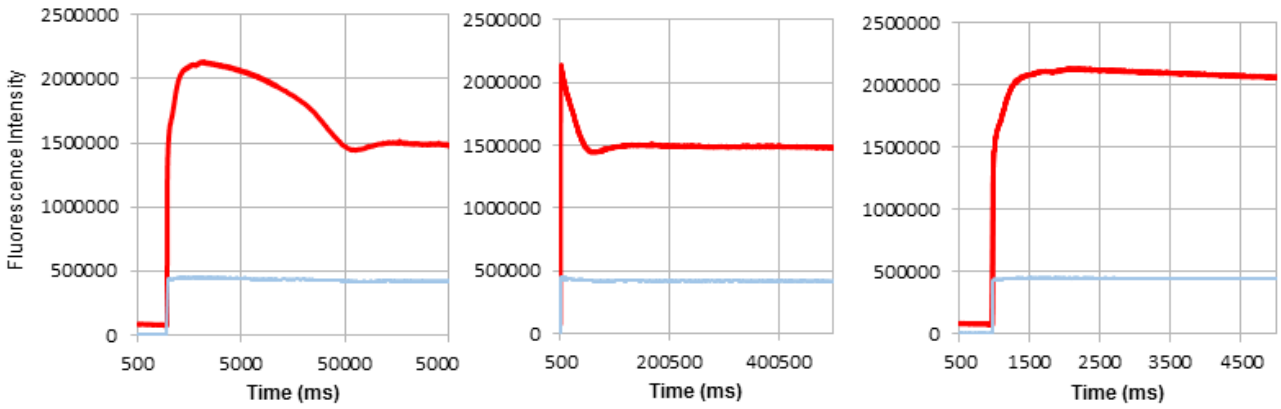


Figure 29: Constant light fluorescence curve.

A typical Kautsky curve was measured over the period of about 8 minutes. It has a rapid initial rise and a slow drop-off after the maximum fluorescence intensity is reached. A logarithmic scale is used for the time axis of the graph on the left in Figure 29 to make the shape of the initial rise more visible. When exactly the same data is plotted on a linear time scale the initial rise looks like a vertical line if the total time of the measurement is several minutes. This can be seen in the middle graph of Figure 29. The right-hand graph in Figure 29 uses a linear time scale but shows only the first five seconds of the same measurement data. These three graphs show how much the time scale can influence the appearance of the Kautsky curve.

Several fluorescence curve measurements were made with the FICC. The measurements show that the FICC functions as expected when a Kautsky curve is measured. It can measure the changing low intensity chlorophyll fluorescence response (red line) as well as the bright excitation light intensity (blue line) that stayed fairly constant during the measurement in Figure 29. Figure 30 below shows three different durations (20 ms, 250 ms and 600 ms) of the same fluorescence curve that was used for Figure 29, plotted on linear time scales.

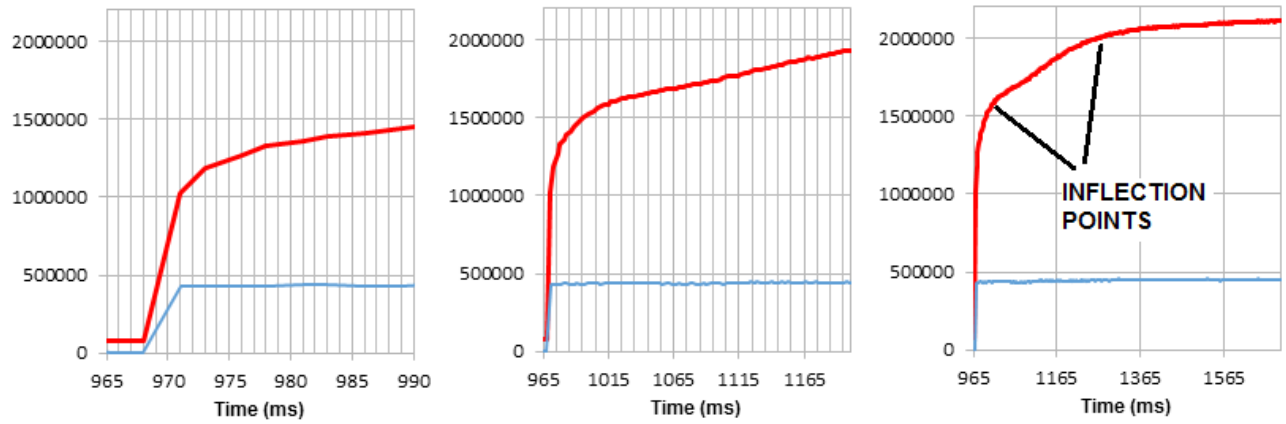


Figure 30: Kautsky curve seen over different durations.

These three graphs make it easier to see the two inflection points on the curve that are not clearly visible on the longer time scales. They also again show how much the appearance of the Kautsky curve changes with different durations. During this measurement there was a very rapid initial rise of the fluorescence intensity during the first 4 ms before the rate of intensity change slowed down. This corresponds to the expected I inflection point of the OJIP nomenclature used on the Kautsky curve. The rate of intensity change is much slower from about 12 ms after the blue excitation light was switched on at the 968 ms mark in Figure 30. Around 400 ms after the blue light was switched on, the J inflection point was reached and the intensity change slowed down even more.

The measurement of Kautsky curves over periods of about 10 minutes will consume too much energy if the FICC is to be used as a remote sensor running on batteries. From an energy saving point of view the better solution would be to use very short excitation light flashes. Although the complete Kautsky curves are useful for determining photosynthetic information it is not essential for measuring the chlorophyll concentration, which is the main function of the FICC.

For the last series of FICC laboratory measurements, the excitation light was modulated with a 65 Hz sine wave. This frequency was used because it was the fastest frequency that the microcontroller software could drive with the on-board DAC while still having enough time to read all the ADC values from the AFE. A single fluorescence measurement consists of five 75 ms modulated light pulses. Figure 31 shows one of these light pulses with its 65 Hz sine wave modulation. The 5 modulated light pulses had a dark period of about 2 seconds between them.

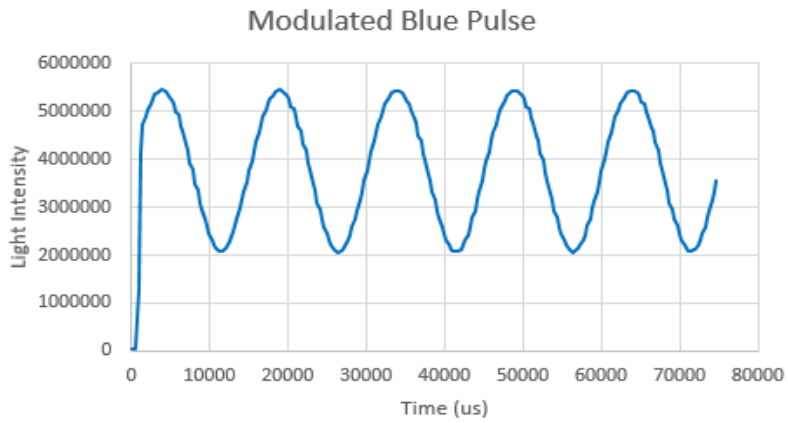


Figure 31: Modulated excitation light pulse.

The duration, amplitude and wave type of the modulated pulse in Figure 31 was not selected at random. Many different excitation light intensities, pulse durations and modulation wave shapes were investigated to find the most suitable combination. The most interesting results are now presented as a background to how the final modulated pulse was developed.

The discussion of the various investigations will cover many different pulse durations that were tested. It will however start with some results that make the author believe that the 75 ms light pulse of the FICC allows enough time to provide accurate fluorescence measurements without wasting electrical energy.

The effect of varying the duration of the excitation pulse on maximum fluorescence was

investigated by repeatedly applying a series of light pulses with increasing pulse duration to a Chlorella vulgaris algae sample. The maximum fluorescence intensity reached for each pulse kept on increasing as the pulse width increased. The fluorescence intensity increase was not caused by a chlorophyll status change as the intensity returned to its initial low value when a new series of pulses started with a very short pulse almost immediately after the longest pulse of 10 ms. Figure 32 below shows the fluorescence intensity change for one such series of measurements as measured with the TSL250 sensor. The blue square waves are the excitation light intensity that was also measured at the time. It is not the reducing time between pulses that caused the rising fluorescence intensity. The black circled area shows how the first short pulse after the longest pulse has a drop in intensity even though it is almost immediately after the longest pulse.

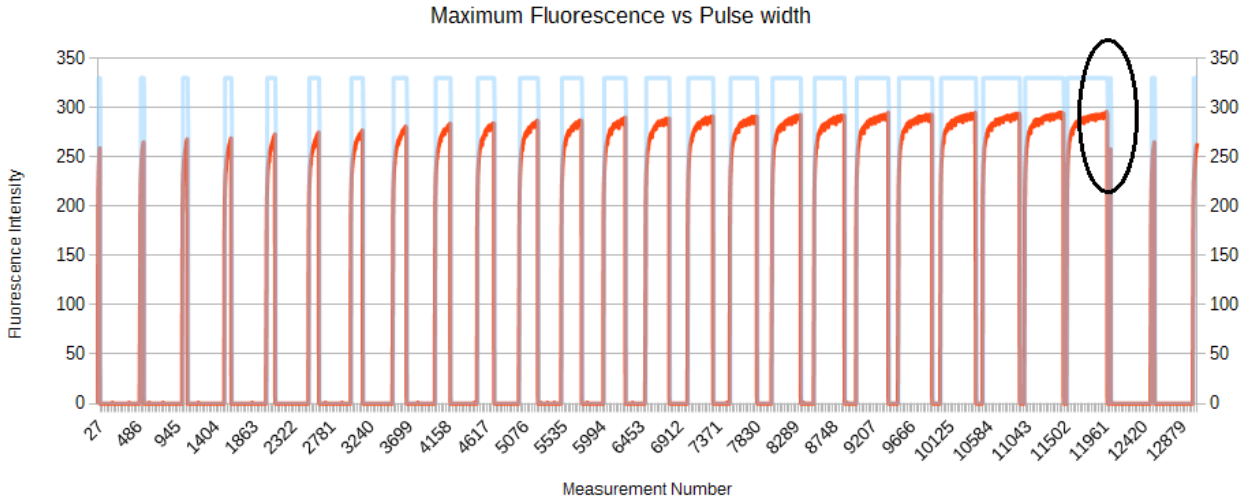


Figure 32: Maximum fluorescence for pulse width.

The optimal duration of the excitation light pulse to induce maximum fluorescence intensity had to be determined. Since low power consumption is one of the main requirements of the FICC, the LED should be switched on for as short as possible but long enough to provide useful and repeatable measurements. When a series of light pulses with increasing duration at maximum LED intensity (20 mA) were applied to an algae concentration, it was found that the fluorescence intensity had a much slower rate of increase after about 10 ms. The longer duration pulses reached virtually the same maximum intensity but probably wasted electrical energy after 10 ms. Figure 33 shows the measured fluorescence intensity for pulses up to about 35 ms. For minimal energy consumption 10 ms pulses seemed to provide a possible solution.

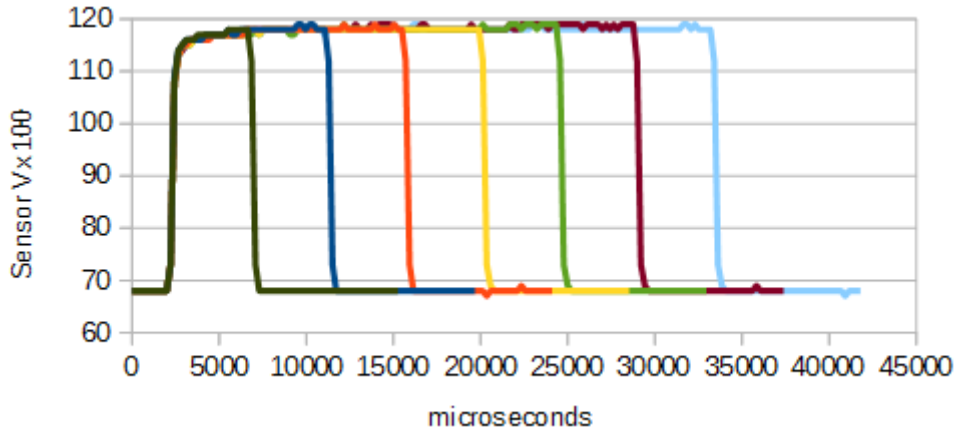


Figure 33: Light pulse duration effect.

The results presented in Figure 33 looked like it could have been caused by a circuit slowly charging up to its maximum value. The measurement data of other measurements were investigated to confirm if it provided the same shape. When only the first 75 ms of a few pulses presented in Figure 35 are plotted on a linear time scale, it provides a curve similar to Figure 33. Figure 34 shows that there is not much to gain in terms of a higher initial measured fluorescence intensity by extending the pulse duration past 10 ms. A shorter duration pulse can be used to save electrical energy but extending the light pulse duration does provide more measurements at the "high" fluorescence value which will help to counter the effect of noise on the measurement by having more samples from which to calculate an average. Measurement data over the JIP section of the OJIP transient could also be used for some photosynthesis analysis if it was required.

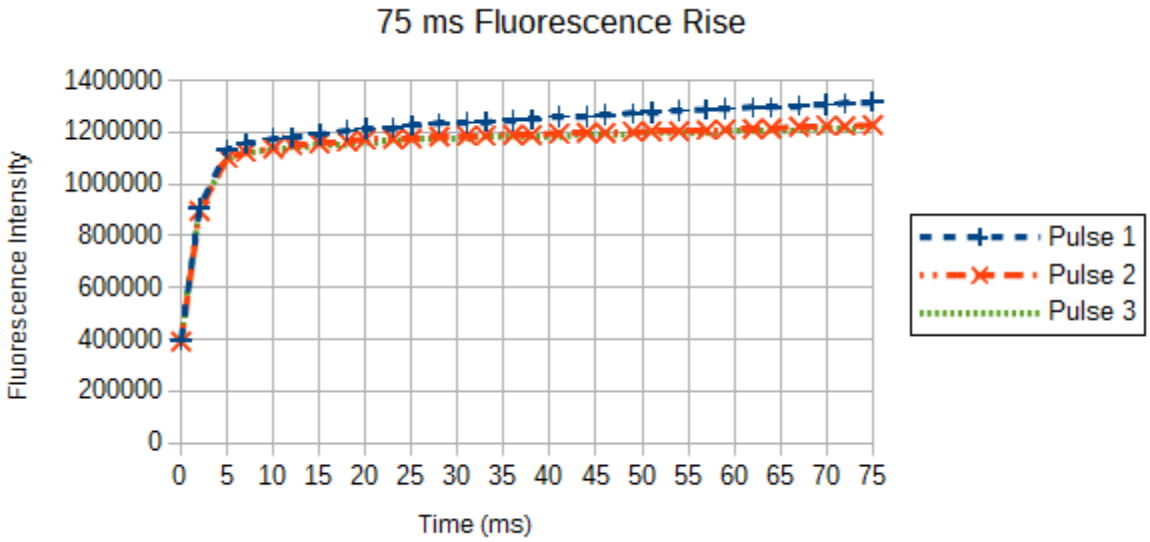


Figure 34: Fluorescence rise over 75 ms.

An investigation was done to see if a saturation pulse combined with a low intensity actinic light would provide more consistent maximum fluorescence measurements when longer duration pulses were used. The hypothesis was that the constant low intensity actinic light would remove the effect of the initial fluorescence rise that normally causes the fluorescence pulses to start with a high value and then drop in maximum intensity on consequent pulses. Figure 35 shows that this illumination method did not succeed in providing fluorescence pulses with similar maximum fluorescence values. Over the period of about 11 minutes the maximum fluorescence intensity reached during each pulse kept on falling. This result is very similar to what [12] found when saturation pulses were applied with a PAM fluorometer.

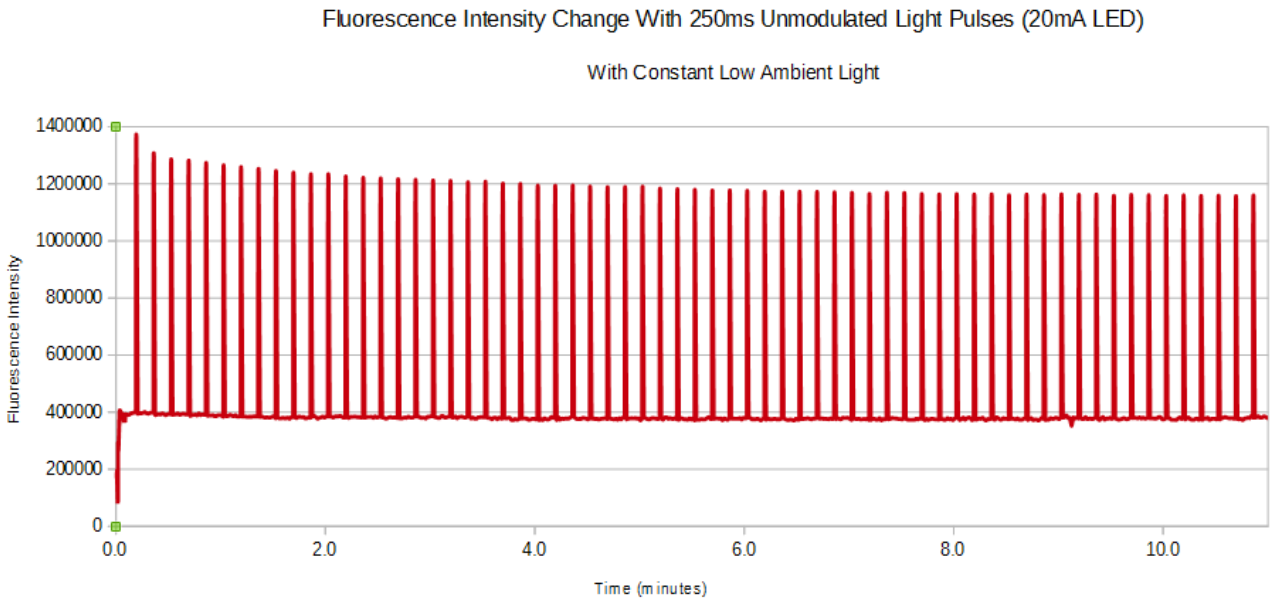


Figure 35: Fluorescence change with unmodulated 250 ms pulses.

Many fluorometers use far red light as a low intensity ambient/actinic light. Blue light is sometimes used [78]. Since the FICC only has the single 470 nm blue LED light source, it was used as the ambient as well as excitation light sources. The ambient light indicated in Figure 35 was created by not switching the LED off completely between saturating pulses.

Several investigations were done to try to get an understanding of the variability of the chlorophyll fluorescence during different exposures to light. The Chlorella vulgaris that was used for most investigations provided different maximum fluorescence intensity measurements for the same sample when it was made minutes or hours apart. The influence of the excitation light intensity, duration and off time on the emitted fluorescence had to be determined.

An investigation was done where the light pulse duration was extended to 5.4 seconds with 6.4 seconds of no light between pulses. The aim of the investigation was to see if there was any consistent fluorescence pattern that could be used to make more repeatable fluorescence measurements with the FICC. The first pulse again caused the biggest fluorescence response while each following pulse caused a lower maximum fluorescence value. The fluorescence intensity change over time also got less with every following pulse. Figure 36 shows the 5 pulses on a logarithmic time scale with all pulses starting at the same point on the graph to provide a comparison of how the fluorescence intensity changed over time. Even though these pulses were made a few seconds apart on the same sample, the fluorescence response was slightly different for each pulse. This again shows the variability in chlorophyll fluorescence response to exactly the same light exposure. It was interesting to note that the slope of the first 100 ms of the fluorescence intensity was fairly consistent from the second pulse onwards. This corresponds with the measurements for the first 75 ms of a light pulse, presented in Figure 34.

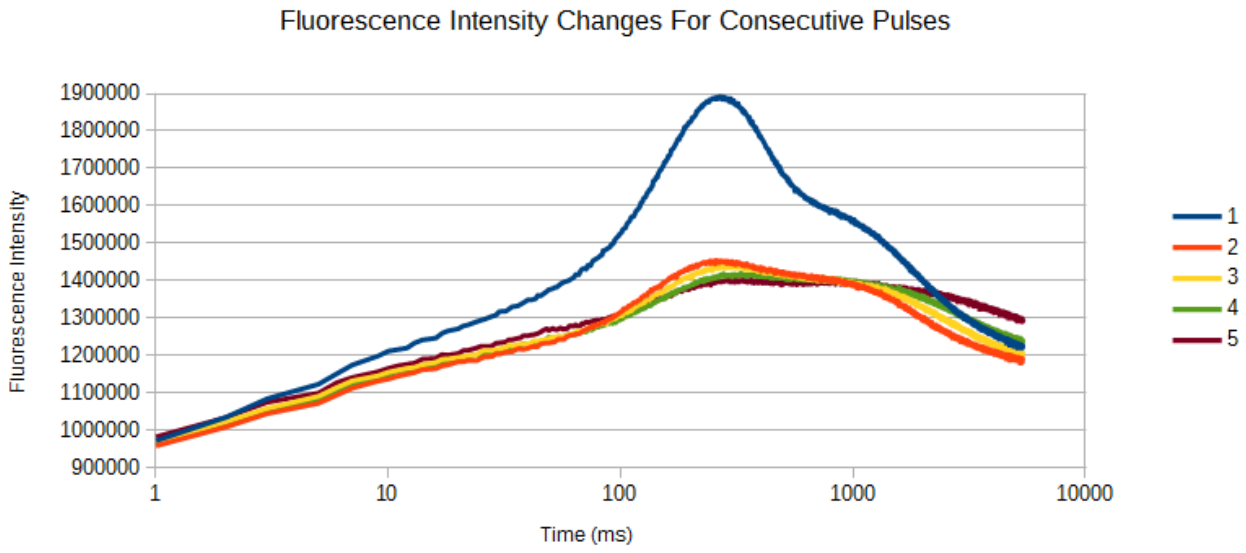


Figure 36: Fluorescence intensity change for consecutive pulses.

As is discussed in Section 5.6, it was decided to modulate the excitation light pulses with a sine wave to improve the recovery of the fluorescence signal amidst noise. Several investigations were done to determine the effect of the sine wave modulation on the measured fluorescence. Figure 37 shows the measured fluorescence intensity when the LED light was modulated with a sine wave while it was switched on for 2 seconds. During the time that the light was on, the fluorescence peak values again showed the expected Kautsky curve trend that was previously presented in Figure 29.

The lowest values of the sine wave modulation showed the same trend although the change in intensity was not as big in terms of actual voltage. Note that there was 2 seconds of darkness for dark-adaption of the chlorophyll between each period of modulated light that is only visible when looking at the time axis labels.

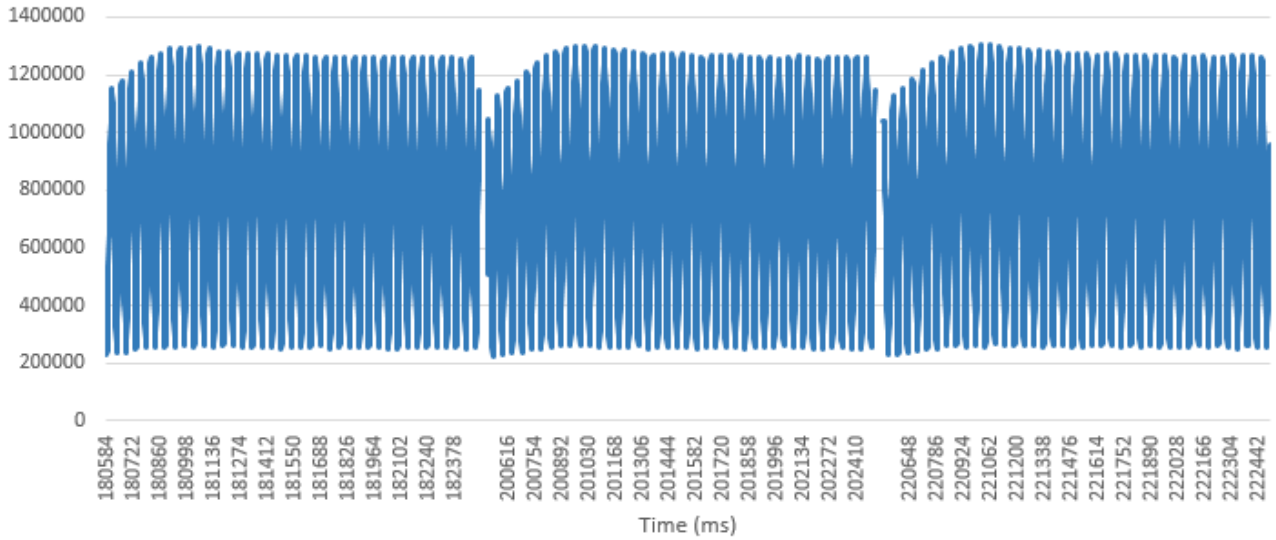


Figure 37: Fluorescence change with 2 s sine wave modulation.

Having confirmed that modulating the light with the sine wave did not have a substantial influence on the fluorescence response it was decided to compare the effect of different modulated pulse durations on the fluorescence response.

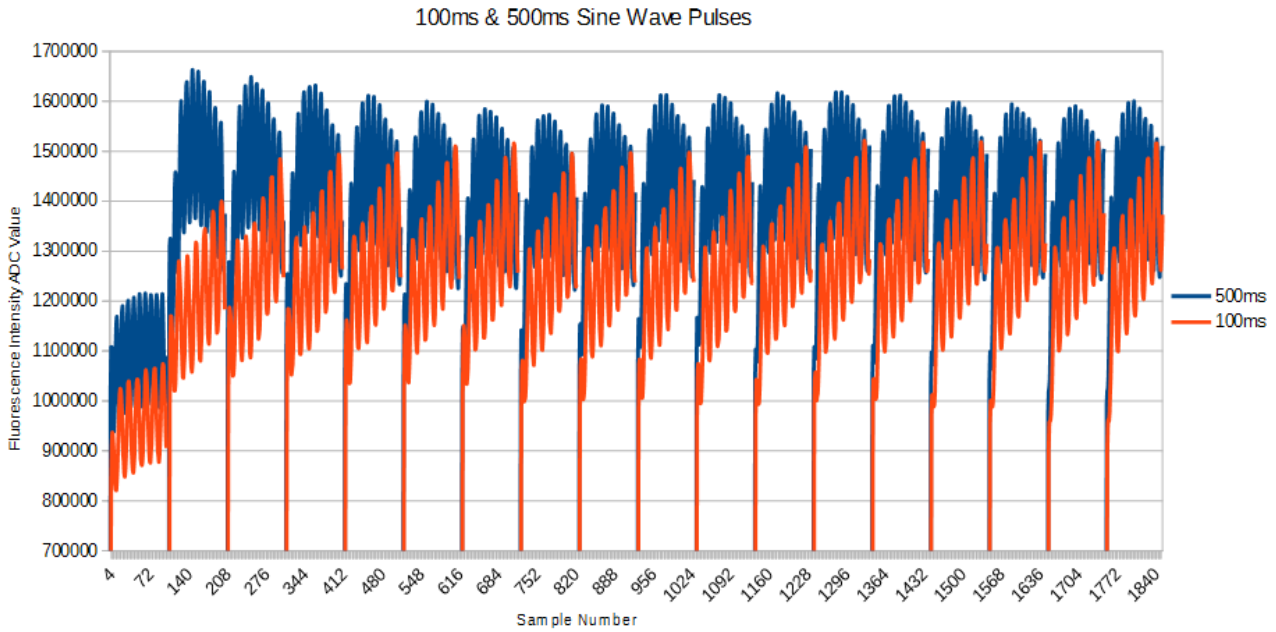


Figure 38: 100 ms & 500 ms sine wave pulses effect.

Figure 38 indicates the differences in fluorescence response that was found when Chlorella vulgaris algae was exposed to 100 ms and 500 ms light pulses that were modulated with a sine wave. There was 40 seconds of darkness between every consecutive pulse to allow the chlorophyll to return somewhat to a dark-adapted state. Note that the horizontal axis makes use of the ADC sample number and therefore represents different time scales between the measurements. It does however make it easy to clearly see the different intensity responses to the different pulse durations. It is clear that the 500 ms pulses caused a rise and fall in fluorescence intensity during each pulse while the 100 ms pulses just showed a rising intensity during each pulse. The 500 ms light pulses was long enough for the chlorophyll response to go past the peak fluorescence point of the curve during every pulse. The author does not have the required biochemistry knowledge to make an academic analysis of the mechanisms behind these fluorescence responses but suspects that the 500 ms pulses must have caused some physiological state change to the chlorophyll. This might explain the inconsistent fluorescence intensities of consecutive pulses. The shorter 100 ms pulses might be short enough to induce a fluorescence response without changing the physiological state of the chlorophyll so much that it could not recover during the 40 seconds of darkness.

The maximum fluorescence intensity of the 500 ms pulses changed by about 5% over the series while that of the 100 ms pulses changed very little from the 3rd pulse onwards. The 100 ms pulses provided more consistency and was therefore seen as being useful for implementation in the FICC.

All these investigations pointed to using a pulse duration of 100 ms or less to get fluorescence measurement data that was as consistent as possible while also providing some potential data for photosynthesis analysis. At the sampling rate of the AFE (discussed in Section 6.5), it took 75 ms to take 256 24-bit measurements on each channel. Extending the pulse duration to more than 75 ms would have required more RAM from the microcontroller, used more electrical energy and not provided much more accurate measurements. It was decided to implement the 75 ms pulse duration on the FICC as a good all round compromise between these factors.

6.4 The FICC Front End Assembly

The FICC front end assembly consists of several different components, as is indicated in Figure 26. Figure 39 shows a photo of an early FICC prototype where most of the front end components can be seen. This layout was used for all tests and laboratory experiments, except for the last one with the chlorophyll calibration standard (discussed in Chapter 7.2). The layout used for the last

experiment is presented later in this section.

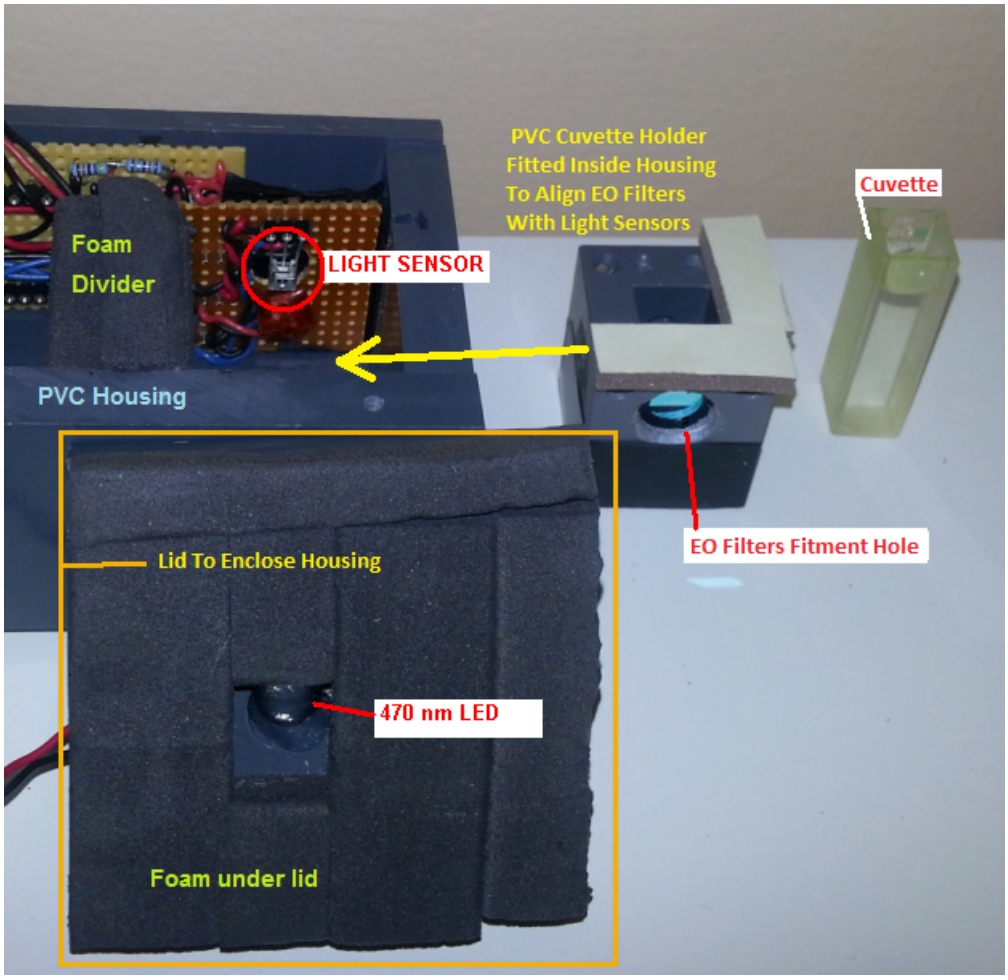


Figure 39: Prototype housing and layout.

The cuvette was always placed inside the PVC cuvette holder, which was placed inside the PVC housing. In the photo the cuvette holder is outside the housing to show it and the other prototype components more clearly. The photo shows some of the first light sensor and control electronics prototypes as they were mounted. This layout of the electronic components around the cuvette holder was kept for all consequent versions of the FICC. The cuvette holder and other components were mounted to prevent movement during or between measurements. This ensured that the cuvette position would not change in relation to the sensors, light filters and other components for different measurements. Foam was used to fill any gaps to ensure that no ambient light could reach the cuvette or light sensors.

The cuvette holder had 12 mm holes on opposite sides for the fitting of the Edmund Optics light filters between the cuvette and the light sensors. These holes lined up with the position of the

TAOS TSL250 and TSL257 light sensors. This ensured that the filters would be in the same position for all measurements.

The 10 mm 470 nm LED was fitted in a lid that covered the top of the cuvette holder and the sensor area to block out external light. A recess in the foam under the lid fitted over the top of the cuvette. This ensured that the LED was always directly above the cuvette and shone down its central axis.

Figure 40 shows the flat face prototype front end that was used during the last laboratory experiments with the chlorophyll calibration standard, which is discussed in Section 7.2.2. Due to a lack of funds and access to manufacturing facilities this front end was made by hand. The angles of holes drilled into the PVC mounting block could not be made very accurately. The location of the light sensors and LED differ from the proposed design for a flat faced unit in Figure 55 but was the closest that could be achieved under the circumstances.

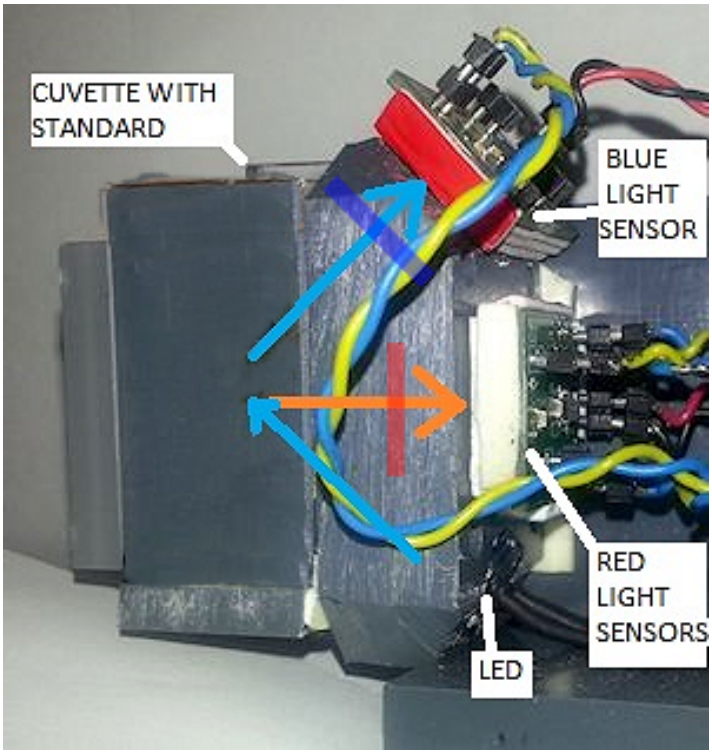


Figure 40: FICC flat face prototype.

The 10 mm 470 nm LED shone towards the cuvette at an upward angle of about 45 degrees to the cuvette's vertical axis. The fluorescence light passed through the EO longpass and Lee orange filters to the three TSL257 light sensors that were located at a 90 degree angle to the vertical axis of the cuvette. The TSL250 light sensor was located at an angle of about 90 degrees to that of the

LED light beam. The Lee bright blue film filter was fitted in front of the TSL250 light sensor. There was a tight sealing foam lid that was placed over the top of the cuvette to block all ambient light from entering there.

One of the problems with this layout was only discovered when the measurement data of the experiments were analysed. White ambient light passed through the LED and influenced the measurements of the low intensity fluorescence light sensors. This shows that the back end of a LED in a fluorometer should also be covered to block light from passing through the LED to the measurement area.

6.5 The MCP3903 Analogue Front End

As one of the requirements of the FICC is to minimise its size, the number of components had to be kept to a minimum. It was decided to look for a single device solution that would provide programmable gain as well as a high ADC resolution. The MCP3903 analogue front end (AFE) that was chosen provides one package with 24-bit ADC resolution and programmable gain.

The AFE device has 6 separate channels with a series programmable gain amplifier (PGA) on each channel. The gain of the PGA can be set from 1 to 32 by software on the mbed by means of a serial peripheral interface (SPI). This makes it possible to reduce the gain if the ADC is saturating or increase it when the signal at the ADC is getting too small.

Each AFE channel also has a 24-bit analogue to digital converter (ADC) which provides a 0.3 μV resolution. This is a big improvement from the 12 bit ADC (1.2 mV resolution) that was available during the first laboratory experiment as well as most of the initial measurements of the project that was done with the 12 bit on-board ADC of the mbed. The ADC values are read from the AFE through the same SPI interface that is used to control it.

The AFE has control registers that can be set up by the development board microcontroller. The control registers determine which of the many selectable features on the AFE are activated and have status bits that indicate the current status of the device. In the FICC application the control registers are used mainly for the following:

- To activate the 24-bit option of the ADC rather than 16 bits.
- To set the individual gain of each analogue channel from 1 to 32.
- Reading the status of the current ADC conversion.

- Resetting the AFE.

6.6 The LabVIEW User Interface and Data Storage

A graphical user interface (GUI) and data logging program was developed in LabVIEW. This provided an interface on the PC to plot measurement data live while it can also save the data to the hard disk along with user comments. Being able to see the ADC data live was especially helpful in the early stages of the project when it was not clear yet if the system and light sensors were working as expected. The immediate visual feedback helped in developing the excitation light modulation software.

Figure 41 shows the main screen of the LabVIEW GUI. The top oscilloscope trace has its own time and amplitude settings while the bottom 4 oscilloscope traces share the same time and amplitude settings. Each oscilloscope trace has a control to select the data channel to display. The path where the data file is stored is displayed on screen above the area where the user can add a comment before pressing the button to start or stop logging the data.

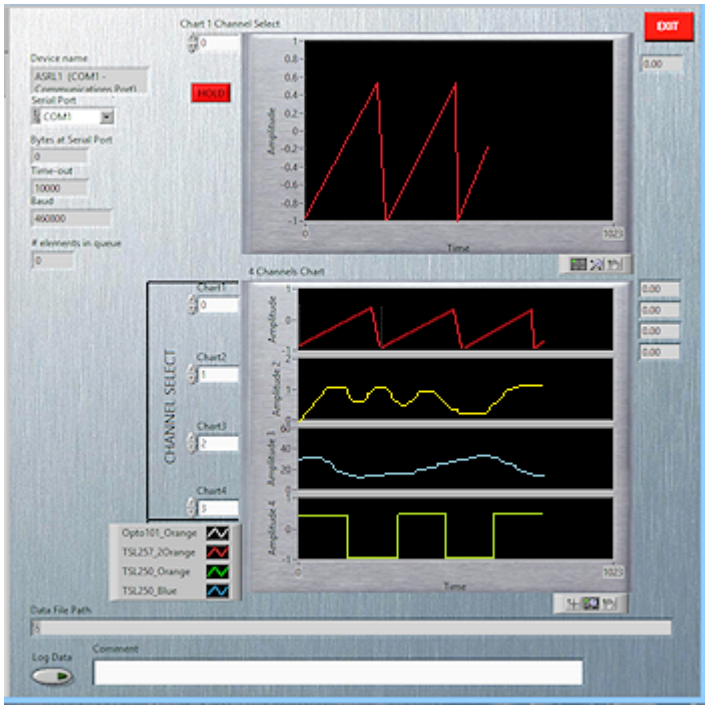


Figure 41: LabVIEW GUI.

7 FICC Practical Evaluations

While many investigations were done to determine the suitability of components and measurement methods to be used in the FICC, there were two main experiments done in laboratories to determine the functionality and measurement range of the complete prototype systems as they were at that time.

The chlorophyll extraction process that was used to prepare the chlorophyll concentration for the first laboratory experiment is explained in detail in Section 7.1 before the measurement results are presented and discussed. The final laboratory experiment is discussed in detail in Section 7.2. It starts with the detailed preparation procedure of the chlorophyll calibration standard before presenting the results and discussing their relevance on the aimed application of the FICC as a chlorophyll concentration sensor.

7.1 Measurements with a Chlorophyll Extract

Due to the inconsistent chlorophyll fluorescence intensities measured with live algae it was decided to use extracted chlorophyll, dissolved in acetone, for some of the system and component investigations to minimise the fluorescence variability due to biological activity. The following is a description of the exact processes that were followed to make the two sets of extracted chlorophyll solutions.

7.1.1 First Spinach Chlorophyll Extraction

Chlorophyll was extracted from spinach and dissolved in acetone. This made it possible to keep the solution in a freezer to enable sets of measurements to be made over several weeks. The following is a description of the process that was followed:

- 300g Swiss chard spinach was shredded in a commercial liquidizer.
- The shredded plant matter was then put in 500 ml acetone.
- The mixture was then stirred with a magnetic stirrer for 30 minutes.
- The solution was then filtered twice with filter paper.
- A rotary evaporator, set to 50ºC, was then used to dry the solution for about two hours.
- It was further dried to powder form by leaving it overnight in a freeze drier.
- The powder was weighed on a laboratory scale.
- A new solution of some of the powder and acetone was made with a concentration of 10 g/l.

The exact chlorophyll concentration was unknown since the powder also contained other plant material.

- Two new concentrations of 1 g/l and 0.1 g/l were made up to be analysed with a Unicam Heλios α.
- The dilutions were scanned from 400 nm to 900 nm.
- The absorbance measured at 680 nm were 0.562 for a 1 g/l concentration and 0.066 for a 0.1 g/l concentration.

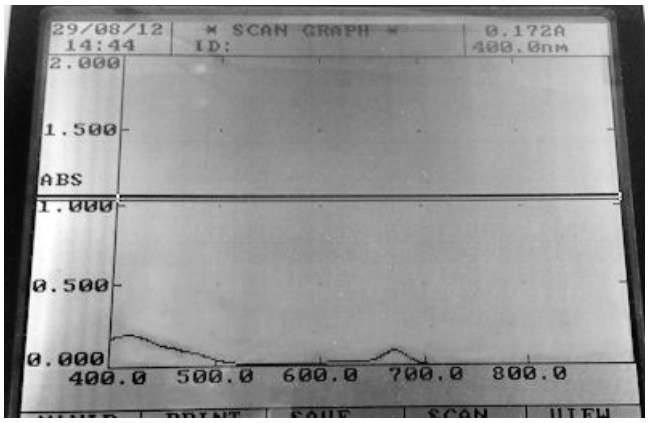


Figure 42: Spinach extracted chlorophyll absorbance spectrum.

Figure 42 contains a photo of the absorbance spectrum as measured by the Unicam Heλios α. According to this measurement the optimal excitation wavelength for this solution would have been around 430nm at the maximum absorption point. This was 40 nm from the FICC excitation wavelength of 470 nm. There was however still a fair amount of absorption around 470 nm that would have caused fluorescence during the investigations. This was also confirmed with fluorescence measurements during the investigations. The second absorption peak around 680 nm was not targeted with an excitation light source during any of the investigations.

7.1.2 Second Chlorophyll Extraction

The main aim behind preparing a second batch of extracted chlorophyll was to determine the fluorometer performance over a range of chlorophyll concentrations from 0.1 mg/l down to 0.01 µg/l. It had to be confirmed if the low cost components could measure the low intensity fluorescence at the lowest chlorophyll concentrations of the FICC requirements. A secondary aim was to look for patterns in measurement data that could be used in future to extract additional or more accurate information from measurements.

Chlorophyll powder, extracted from spinach two months before (described in Section 7.1.1), was dissolved in acetone. About 90 ml of this mixture was put in small bottles for later use in investigation measurements during development of the FICC. Its concentration was unknown. The remaining mixture was diluted with acetone until the dilution gave an absorbance reading of 0.562 @ 680 nm in a Unicam Heλios α absorbance meter. This was the equivalent value measured two months before for a 1 g/l concentration of chlorophyll. A 0.1 mg/l dilution was made with acetone from which five further dilutions were made where each dilution had a tenth of the concentration of the previous. The dilutions were made with highly accurate laboratory pipettes.

Each of the five chlorophyll concentrations, as well as a pure acetone sample, were put into cuvettes. The cuvettes were placed in the prototype FICC cuvette holder with the TSL257 light sensors and EO and Lee Filters light filters on each side. The outputs of the three TSL257 sensors were added with a non-inverting summing amplifier (discussed in Section 5.5) and measured as one channel along with channels for LED current, excitation light intensity and the output voltage of one of the TSL257 fluorescence sensors. The illuminating LED was placed on top to shine the blue light down into the length of the cuvette. A series of measurements were made on the decreasing concentrations of extracted chlorophyll down to pure acetone. An Eagle µDaq datalogger was used on this day to log all the measurement channels since it could measure several channels synchronously and had less noise on its ADC measurements than the ADC of the mbed development board.

The excitation light intensity was controlled by software to generate two types of light modulation for the measurements. Five cycles of a sine wave were alternated with five cycles of a sawtooth wave. Two types of light modulation were used as it was not clear at this point of the FICC development what type of modulation would provide the best way to recover the fluorescence signal from noise. The sine wave was used as it was believed that DSP methods would work well on a pure sine wave. The sawtooth modulation was used to test a hypothesis that it should be easy to recover the fluorescence response amidst noise as it would have a direct relationship to the excitation light intensity. The excitation light intensity was increased at a constant rate to generate the sawtooth signal. The fluorescence signal should then also have had a constantly increasing intensity. The noise on the fluorescence signal would be random and should then cancel out when the average fluorescence slope was calculated. If the hypothesis was proven correct, the different chlorophyll concentrations would have different slopes on their fluorescence sawtooth signals.

When the laboratory measurement data was analysed later it was found that the Eagle µDaq

datalogger did not write to disk all the data that was displayed on the laptop screen during the day of the measurements. The stored data contained a very small amount of the overall measurements. The shape of the modulated signals were barely recognisable. There was still enough data to plot the excitation to fluorescent light intensity relationships of all the measurements. Figure 43 shows a plot of the fluorescent light intensity against the excitation light intensity at that moment as measured for the different chlorophyll concentrations. These are the combined measurements for both types of light modulation.

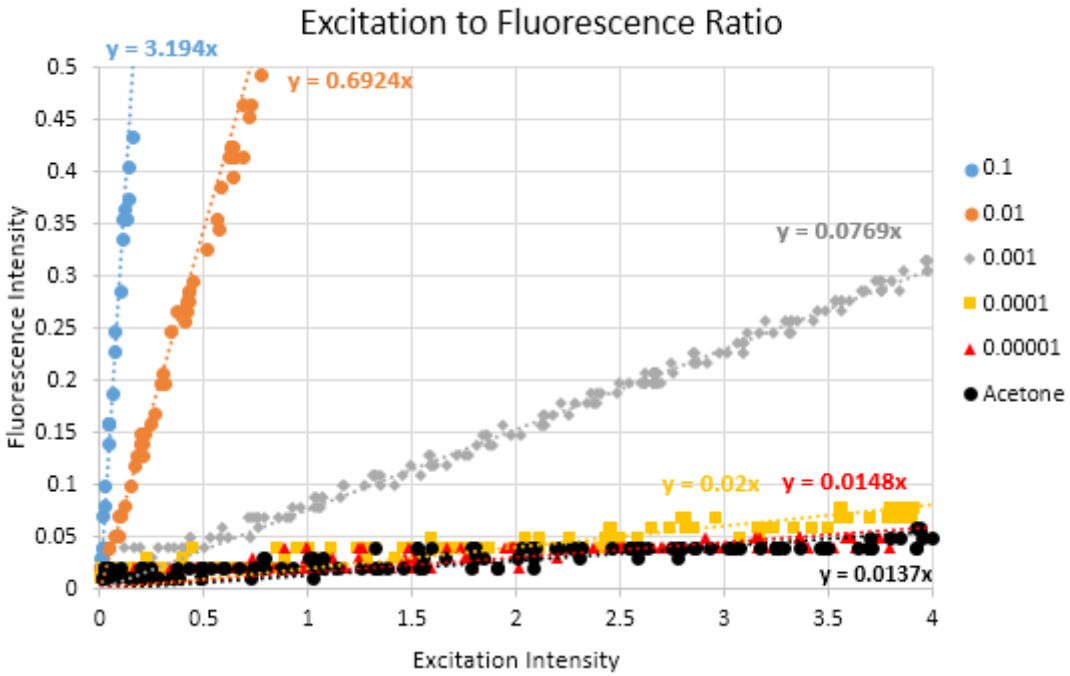


Figure 43: Fluorescent to excitation light ratio.

Figure 43 shows that the excitation to fluorescent light intensity relationship is linear for each set of measurements on a specific chlorophyll concentration. The plot for each concentration has a specific slope independent of the light modulation. This corresponds with the literature that the fluorescence intensity has a direct relationship to excitation intensity and chlorophyll concentration. It also shows that the FICC components were performing consistently and was sensitive enough to measure differences in the fluorescent light intensity of the different chlorophyll concentrations.

Trend lines were added to the plots in Figure 43 to show the different slopes of the chlorophyll concentration measurements. The different slopes of pure acetone and a chlorophyll concentration of 0.01 µg/l indicate that the FICC can differentiate between them with enough measurement points even though it looks like a lot of the values overlap. The graph and data show that the FICC

can definitely differentiate between concentrations higher than 0.01 µg/l.

Figure 44 shows the slopes of the trend lines in Figure 43 plotted against the different chlorophyll concentrations.

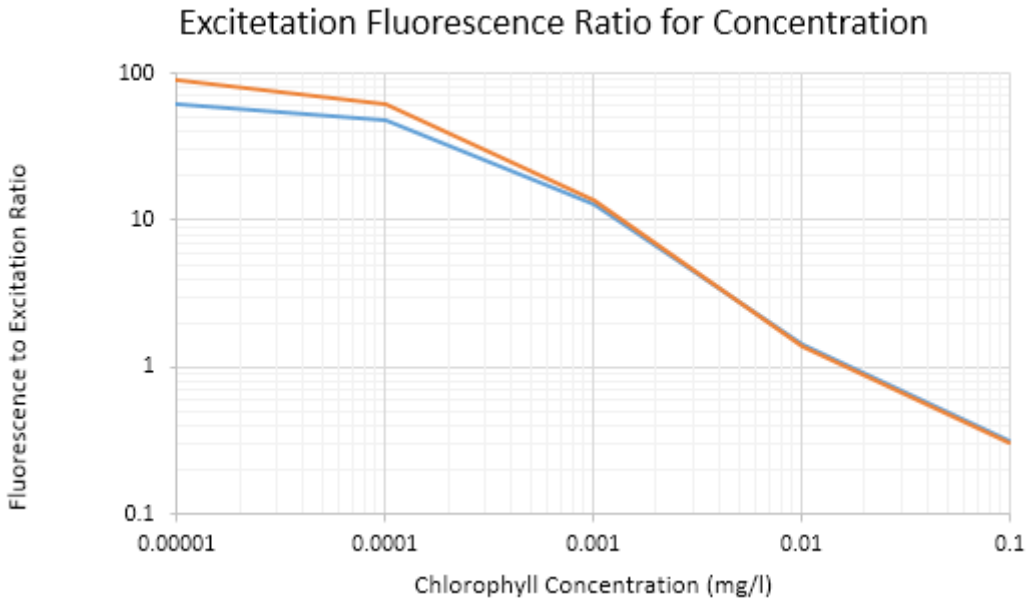


Figure 44: Excitation to fluorescence ratio for concentrations.

The upper line shows the slopes when the trend lines are forced to go through 0. These are the correct trend lines since there is a direct relationship between the excitation and fluorescent light and there will be no fluorescent light when there is no excitation light. The lower line in Figure 44 shows the trend line slopes when they are not forced to go through 0. It is only at the lowest chlorophyll concentrations where the two lines in Figure 44 diverge due to the effect of noise on the small signals. When the trend lines of the lower chlorophyll concentrations are not forced through 0, the noise on the fluorescence signal at low excitation intensities causes the trend line to indicate that there will be some fluorescence with no excitation light present. That is not possible.

All the fluorescence measurements discussed here are the output voltages of a single TSL257 light sensor without any amplification. Combining the output voltages of 3 sensors and then amplifying the signal should have provided a much bigger difference between acetone and the low concentrations. Unfortunately this was not possible to confirm due to the non-inverting summing amplifier that provided inconsistent performance (discussed in Section 5.5). The experiment discussed in Section 7.2.2 did eventually provide proof of the benefit to combine the output voltages of three sensors as well as amplifying the voltages.

Figure 45 shows the measured excitation light intensity for different excitation LED currents during the laboratory measurements. The results confirm the findings discussed in Section 5.4, which were not made under laboratory conditions. It shows that a large percentage of the blue excitation light was absorbed in the higher chlorophyll concentrations. The measured excitation intensity increased up to four times for the same LED current at low concentrations. This can be seen by comparing the excitation light sensor voltages of the 0.1 mg/l and 0.01 µg/l concentrations. The graph flattens out at the top because the maximum output voltage of the light sensor was 4 V.

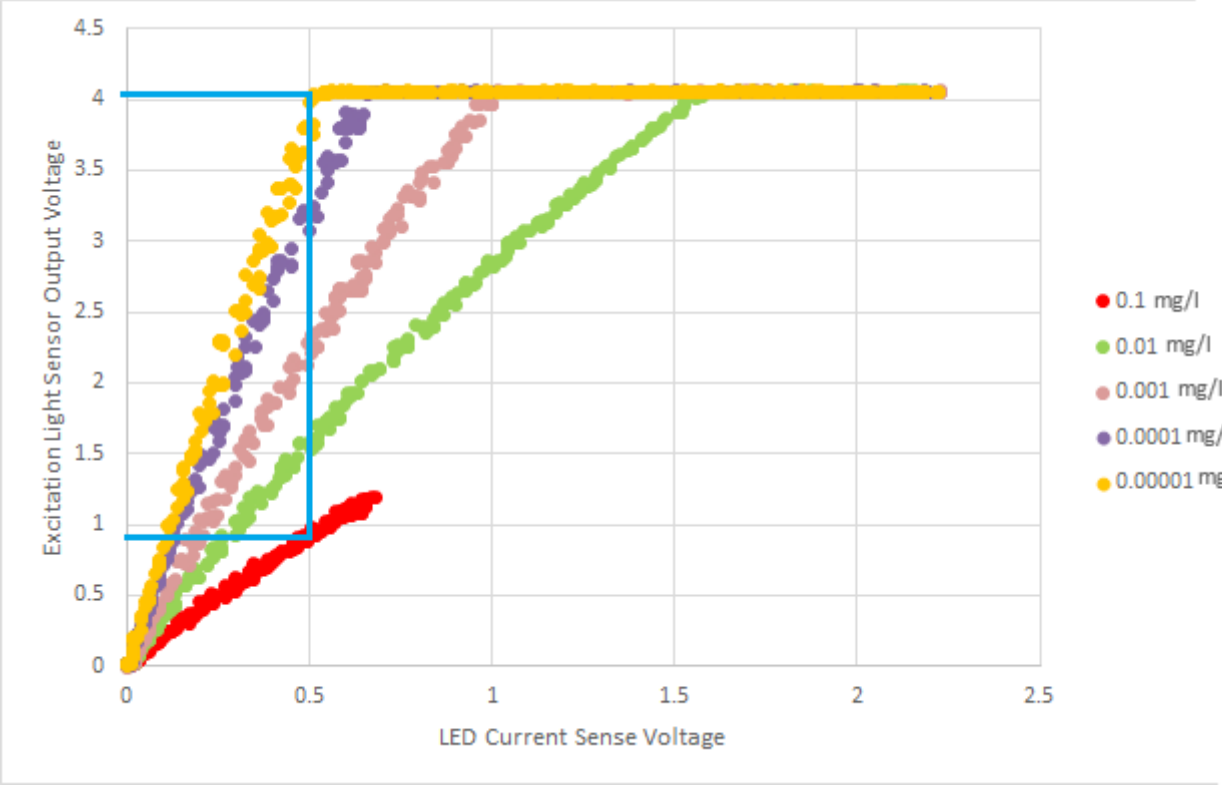


Figure 45: Excitation light changes for concentration and current.

Figure 45 shows an advantage of measuring and logging the LED current along with the excitation intensity. The measured excitation intensity for a specific LED current also changes with the change in chlorophyll concentration. When the LED current is measured along with the excitation light intensity, the combination of the data can be used to confirm a change in chlorophyll concentration that was determined from the fluorescence intensity measurement.

7.2 Measurements with a Calibration Standard

After the last front end unit of the FICC was developed to include the AFE, the functionality and performance of the final prototype had to be confirmed with measurements on accurate chlorophyll concentrations. The author did not have the equipment or skills to prepare such chlorophyll concentrations. The CSIR frequently calibrate their research fluorometers with very accurate chlorophyll concentrations prepared in a laboratory. The author was allowed to join such a calibration session to make measurements on their chlorophyll calibration dilutions with the FICC. On this occasion the Sigma Aldrich calibration standard was used to calibrate two of the CSIR Trilogy® laboratory fluorometers from Turner Designs. This was done with a series of laboratory measurements on different concentrations of a chlorophyll calibration standard. The calibration standard (Sigma Aldrich product code: C6144-1MG) is described as chlorophyll α from Anacystis nidulans algae. The calibration standard contains only chlorophyll α. The FICC is not suited to make fluorescence measurements on chlorophyll α but there were no other options at the time.

The discussion of the experiment starts with an explanation of the procedure that was used to prepare and measure the different chlorophyll concentrations. The measurement results are then presented along with some investigations that were done into methods to reduce noise from the measurement data.

7.2.1 Chlorophyll α Calibration Standard Preparation and Measurement

The calibration standard dilutions were prepared by qualified personnel with very accurate instruments. To minimise the effect of ambient light on the chlorophyll, all lights in the laboratory were switched off for the duration of the preparation and measurements. Only a small amount of ambient sunlight was present in the laboratory. The chlorophyll dilutions were also kept in a dark drawer when they were not being used.

The following is the calibration standard preparation procedure that is always followed by the CSIR group:

- Turned on the Shimadzu UV-2501 spectrophotometer and let it warm up for 10 minutes.
- Ran the spectrophotometer utilities software to perform the system checks.
- Performed a baseline check with no cuvette inside.
- Measured the acetone blank in a 10 mm cuvette in the spectrophotometer.
- Measured the reconstituted 10 mg stock as above.
- Made a dilute working stock (20 ml 10 mg chlorophyll + 180 ml 90% acetone = 1 mg).

- Measured the 1 mg chlorophyll working stock and the subsequent dilution series.

The table below contains the results of the dilution series measurements.

	Working stock (ml)	**90% acetone (ml)**
1.	40	10
2.	30	20
3.	20	30
4.	15	35
5.	10	40
6.	5	45
7.	2.5	47.5
8.	1	49
9.	0.2	49.8

Using the 10 mm cuvettes, a baseline blank (nothing but the manifold), a 'reference' and 'sample' 90% acetone blank were measured.

The absorbance of the 10 mg, 1 mg and entire dilution series was them measured at 664 nm and 750 nm (using the 'Go To WL' function on the spectrophotometer software). As the dilutions were made to calibrate the Trilogy fluorometers for use in the field, they had chlorophyll α concentrations that were expected to be found in the ocean. There were no concentrations aimed at finding the lowest functional limit of the FICC.

The absorbance values are as follows:

Concentration Chl	664 nm	750 nm
90% acetone blank	0	0
10mg*	0.787	0.003
1mg*	0.814	0.008
1.	0.654	0.007
2.	0.492	0.009
3.	0.328	0.012
4.	0.246	0.009
5.	0.161	0.005
6.	0.084	0.005
7.	0.036	-0.002
8.	0.015	-0.005
9.	0.005	0

* The 10 mg and 1 mg values are very close. The person who made the measurements suspects that the dilute working stock (1 mg) was measured twice. It is believed that the rest of the measurements were valid since the calibration measurements of the two Trilogy laboratory fluorometers were very similar to measurements during previous calibration sessions.

Small amounts of each chlorophyll concentration were poured into three different cuvettes before fluorescence measurements were made with the FICC and two Trilogy fluorometers. The lowest concentration was measured first. The cuvettes were rinsed with pure acetone every time before the next highest concentration was measured. This was done to minimise the risk of contamination of lower concentrations by chlorophyll that remained in the cuvette from the previous sample.

The FICC made use of the 75 ms sine wave modulated light pulses described in Section 6.3. A sequence of five light pulses were applied while the AFE gain for the fluorescence intensity channels were set to 1. The gain for the fluorescence intensity channels were doubled before another five modulated light pulses were applied. This process was repeated another four times until a measurement with a gain of 16 was completed. The whole process then started again with five pulses at a gain of 1.

There were 250 24-bit ADC values for every AFE channel during each 75 ms light pulse. The AFE channels measured the blue excitation light intensity, the three fluorescence light intensities, the LED current control set point voltage and the LED current sensing voltage.

7.2.2 Chlorophyll a Calibration Standard Measurement Results

This section provides the results of the measurements that were made on the chlorophyll calibration standard dilutions. It starts with a general discussion about the overall functionality of the FICC. It then discusses the effectiveness of implementing fluorescence signal recovery methods on the measurement data from this experiment.

Figure 46 shows the absorption spectrum of the C6144-1MG chlorophyll α calibration standard that was used. This measurement was performed with the Shimadzu UV-2501 spectrophotometer.

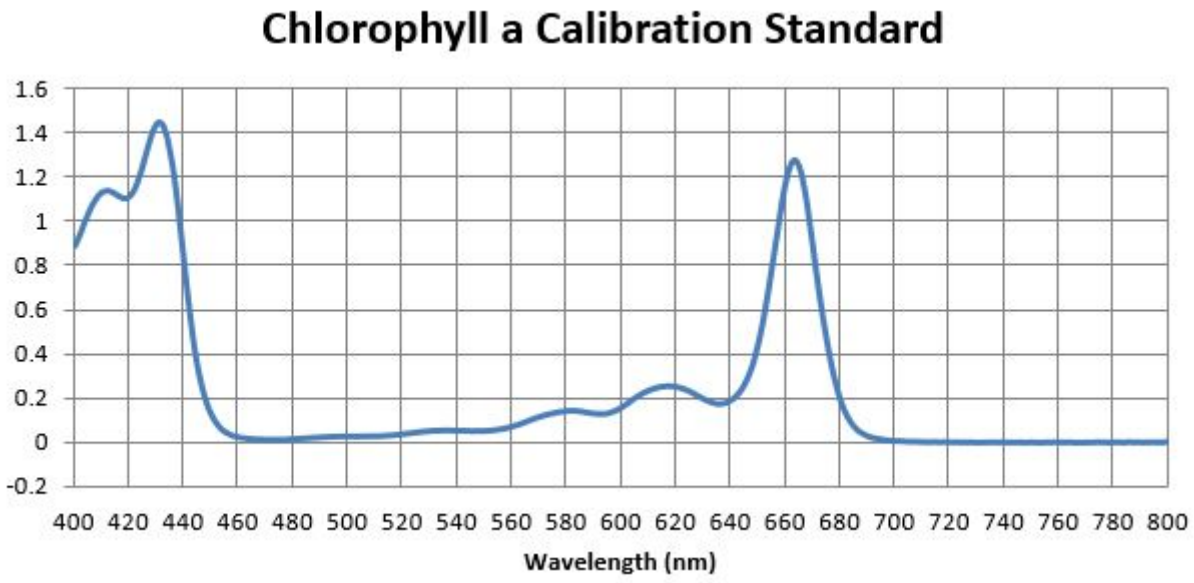


Figure 46: Absorption spectrum of calibration standard.

Figure 46 shows almost no absorption at the FICC excitation wavelength of 470nm. Consequently there was very little fluorescence induced by the FICC excitation LED. The maximum ADC value did not even reach half of the ADC range at the highest chlorophyll concentration of 738 µg/l when the AFE channel gain was set to 16. This was a much smaller fluorescence signal than what was expected. When the FICC was tested the previous day with a sample of sea water, the ADC saturated when the channel gain was set to 32. The saturation was not caused purely by the high gain since tests at the same time with tap water did not cause any ADC saturation. The author believes that the sea water sample must have contained some chlorophyll b. The peak absorption wavelength of Chlorophyll b is closer to 470 nm and would cause more fluorescence emission than chlorophyll α.

Despite the low fluorescent light intensities induced by the excitation wavelength, the FICC was able to generate measurement data with definite differences between the fluorescent light intensities of most chlorophyll concentrations. At the lowest concentrations the noise on the fluorescence signal makes up a large part of the logged values. It is therefore difficult to differentiate between the lowest concentrations when the raw measurement data is looked at.

A few methods (discussed in Section 5.6) were investigated to recover a better fluorescence signal from the noisy measurement data. The aim was to show that there would be ways to improve the data to better differentiate between the measurement data of very low chlorophyll concentrations. It is highly likely that there are methods that would provide even better results than the ones that are discussed here. The main aim of this thesis is not to find the ultimate method to filter and analyse the measurement data of the FICC. It is to show that the FICC system provides the functionality to provide the measurement data that can then be filtered and analysed to determine the chlorophyll concentration.

In the FICC, the LED current and intensity are measured and logged along with the other data. These LED values represent the actual excitation light modulation at any moment and can be used as synchronising signals to recover fluorescence intensity from the data amidst noise. The LED current signal is a reliable source to recover the light modulation signal when the actual excitation intensity is not required. Its value is not influenced by chlorophyll or other material concentrations in the measurement area, as is the case with the LED light intensity signals. The LED current and excitation intensity are however not used by the methods that are discussed here. The discussion starts with the averaging of measurements method before the results of the "brick wall" FFT filter is discussed.

The moving average filter removed most of the high frequency noise from the measurement data. This provided good results for the higher chlorophyll concentrations. When the power spectrum density is used to calculate the signal to noise ratio, there is an improvement of at least 3 dB when a moving average of 5 samples are used on the measurement data of one light pulse. Figure 47 shows the combined results after applying a moving average filter to the laboratory measurements that were made with the chlorophyll calibration standard dilutions. It shows the different fluorescence intensities of the different chlorophyll concentrations for the duration of one light pulse with sine wave modulation.

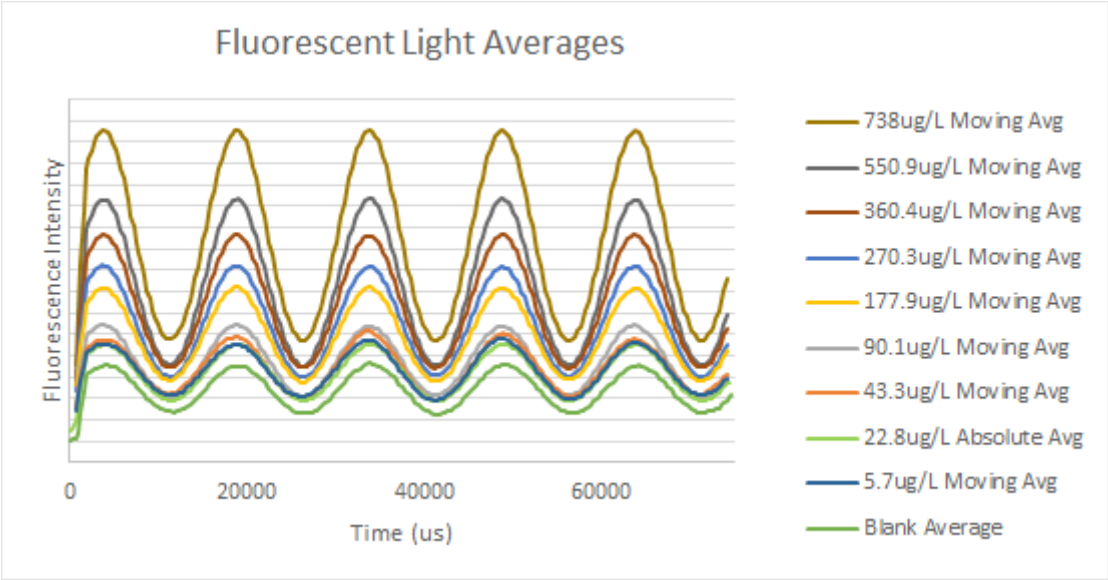


Figure 47: Averaged fluorescent light intensities.

The data used to calculate the moving averages consists of 250 measurements per light sensor for every 75 ms light pulse. The average value of the three light sensors were calculated for time x. Figure 47 shows the final data after applying a moving average for 5 samples around a specific moment in time. It provides much better differentiation between the lowest chlorophyll concentrations than the raw data did. An example of a plot of the raw data can be seen in Figure 50. There is a range between the pure acetone and 5.7 µg/l samples where the FICC could potentially be able to measure the fluorescence of chlorophyll α concentrations lower than 5.7 µg/l.

Figure 48 shows two different ways that were used to analyse how useful moving averages of measurement data were to determine chlorophyll concentration.

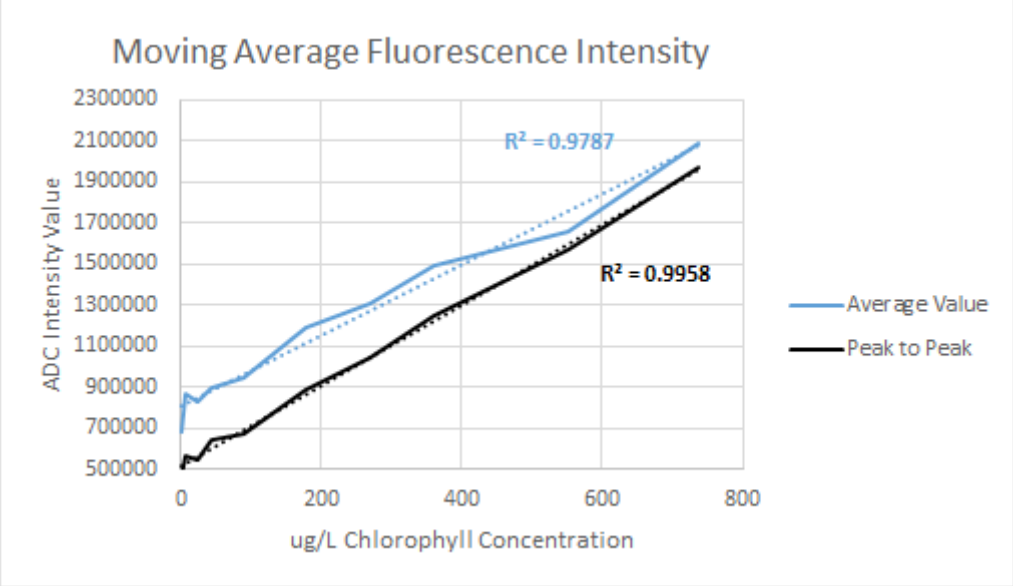


Figure 48: Moving average fluorescence vs chlorophyll concentration.

When the average fluorescence intensity value of all the data for a specific chlorophyll concentration was calculated and plotted against chlorophyll concentration, the linear trend line of the plot had a R^2 of 0.9787. When the maximum peak to peak value of the fluorescence sine wave was plotted for each concentration the R^2 value of the trend line was 0.9958. The peak to peak fluorescence intensity measurements provide a more accurate method to determine chlorophyll concentration. The author believes that the peak to peak fluorescence response is not affected as much as the average fluorescence value by external light entering the measurement area. This was not investigated with any further practical measurements to confirm.

Since these plots and R^2 values are based on a limited data set of around 1250 data points per concentration that were all made within a few minutes, it is possible that an analysis on bigger datasets over a longer time period will show different results.

The author believes that the influence of external light can be seen in the reduced accuracy of the average value trend line. Any external light entering the measurement area would cause a DC offset in the modulated sine wave. Figure 49 shows an example of one of the modulated sine waves that was influenced by ambient light entering the measurement area. The red trend line shows how the moving average of the last 151 measurements reduced in intensity over time. This would affect the average light intensity calculated for the sample while the chlorophyll concentration did not change.

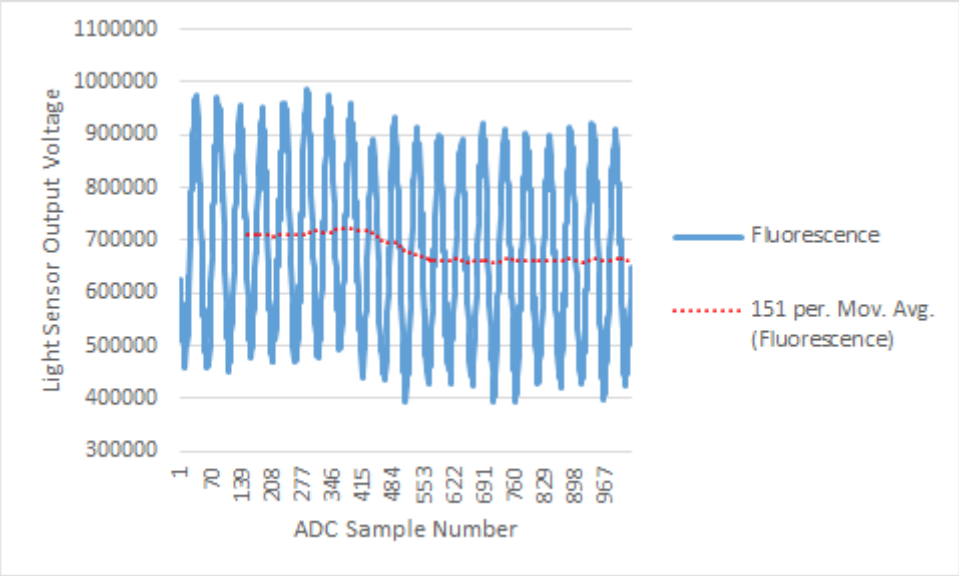


Figure 49: Modulated sine wave DC offset change.

The “brick wall” FFT filter was implemented on the measurement data with an Excel spreadsheet after the laboratory experiments were completed.

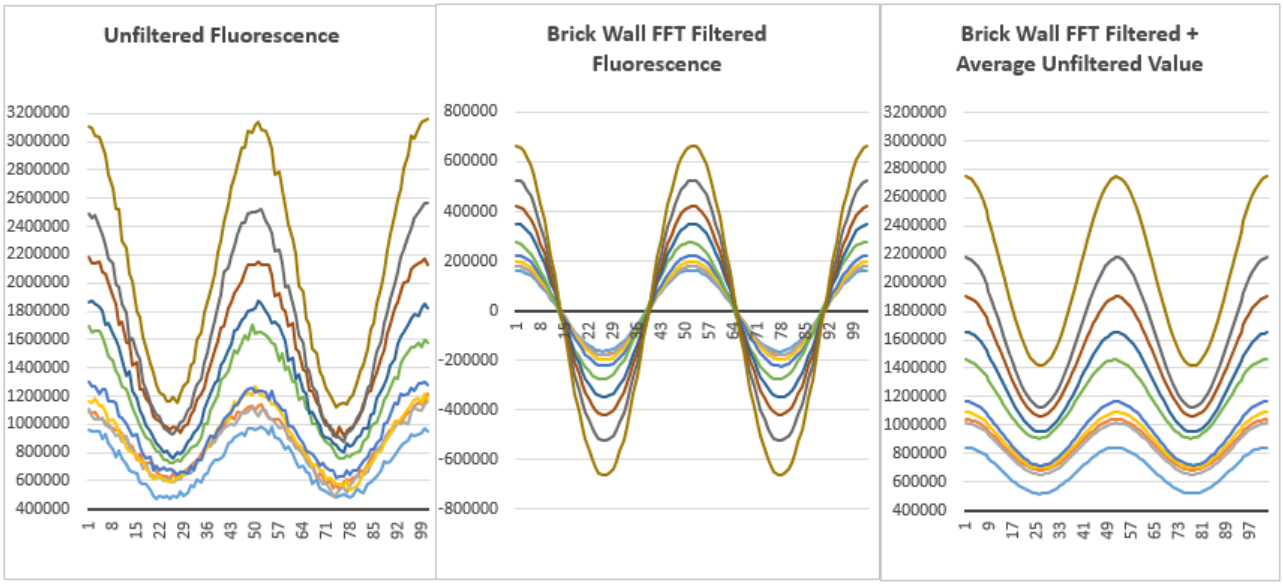


Figure 50: FFT "brick wall” filtered signals & unfiltered signals.

The left-hand side of Figure 50 shows the original fluorescence signals of the different chlorophyll concentrations. The centre graph shows the same signals after they were put through the “brick wall FFT filter”. The filtered signals are clean sine waves that have lost their offsets on the light intensity axis. There is also a definite loss of information if the amplitudes are compared with those of the raw data on the left. The data for the graph on the right-hand side of Figure 50 was created

by adding the average value of the unfiltered data for a specific concentration to the filtered sine wave data of that concentration. The aim of this was just to provide a visual presentation of the amplitude loss that was seen for each concentration that looked similar on the graph to the original data. There was no theory that suggested that this would be a valid way to present the "brick wall" filtered data.

It was decided to investigate if the "brick wall" FFT filtered data did have any useful relationship to the tested chlorophyll concentrations. The peak to peak amplitude of the different chlorophyll concentrations did have a fairly direct relationship with the concentrations. There was also enough of a difference between the peak to peak fluorescent light intensities to make it possible to differentiate between concentrations. The blue graph in Figure 51 shows the chlorophyll concentration to fluorescence intensity relationship when the peak to peak amplitudes of the filtered sine waves are used. The R^2 value of the trend line is 0.9978. This is more accurate than the curve generated from the moving averages method.

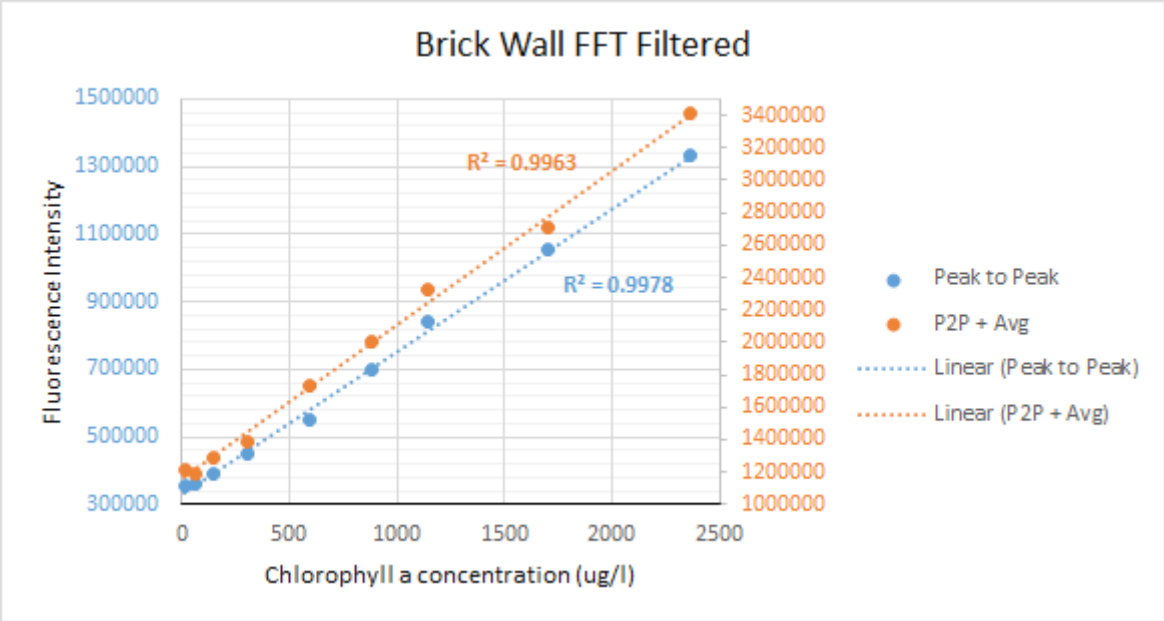


Figure 51: "Brick wall" FFT filtered fluorescence relationship to concentration.

The orange graph shows the relationship when the average values of the unfiltered sine waves are added to the filtered data. In this case the R^2 value of the trend line is 0.9963 and shows that this step reduced the accuracy. These are the signals on the right-hand side of Figure 50. This reduced accuracy is more reason not to use this method to create a chlorophyll to fluorescence calibration curve.

The “brick wall” FFT filter has many problems, as discussed on the forum [101]. The one that became most obvious to this author was the Gibbs effect that causes distortions even when the input data of the filter is a pure sine wave without noise. Figure 52 shows the distorted output signal (blue line) along with the pure sine wave signal (orange line) that was put through the “brick wall” FFT filter. In this case several frequency bins next to the modulation frequency were also selected before the inverse FFT was performed. This shows the limitation that the only time the filter does not distort the output signal is when only the modulation frequency bin is selected for the IFFT.

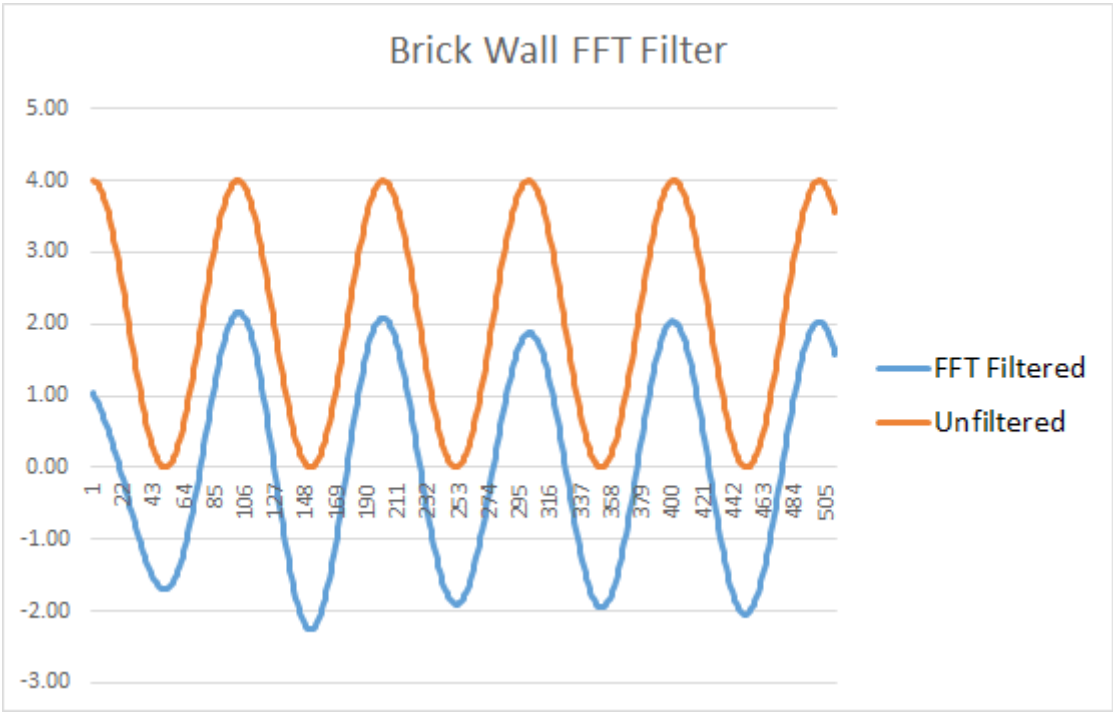


Figure 52: Gibbs effect due to “brick wall” FFT Filter.

The author decided not to use the “brick wall” FFT filter for any other analysis of measurement data. There are too many known problems with the method and there are likely also some that have not been seen yet during the investigation.

7.3 FICC Calibration

The calibration of the FICC is used to determine the relationship between the fluorescent light intensity and the concentration of the species or substance that will eventually be measured. Chlorophyll calibration standards are normally used to calibrate commercial fluorometers to create

a reference for measuring changes in algae or plankton species concentration in the field. Such a calibration can not be used for making absolute species concentration measurements unless the exact chlorophyll content of the species samples in the field is also determined. Fluorometers can also be calibrated with algae or plankton in the field as long as there is a way to accurately measure the concentration of the samples used for the calibration.

Several different concentrations of the chlorophyll or algae need to be prepared as accurately as possible. Spectrophotometers are normally used to make accurate measurements of the prepared concentrations. Any inaccuracies in the calibration sample preparation will directly affect the accuracy of the final measurements. According to application note number 998-0048 of Turner Designs, a bigger number of chlorophyll concentrations will provide more accuracy in the final measurements of the unknown concentrations.

The fluorescent light intensity of the different chlorophyll concentrations are measured and plotted against the concentration. A linear trend line is then fitted to the measured data to create a calibration line that can be used to determine the chlorophyll concentration for a specific fluorescent light intensity. Graphs like Figure 44, 48 and 51 can be used as calibration curves for the FICC.

8 Thesis Conclusions

This chapter starts with a look at the current status of the FICC. The overall performance and remaining challenges in the existing subsystems is discussed. This is then followed by an overview of possible solutions to the remaining challenges as well as suggestions on how the FICC can possibly be improved by different approaches to the general challenges of building an accurate chlorophyll concentration sensor.

8.1 Current Status of the FICC

To excite the fluorescence that will be measured the fluorometer needs to provide enough light energy at a wavelength which will be absorbed by pigments in the sample before the excess energy is emitted as fluorescence. The 470 nm excitation wavelength of the FICC is not well suited to pure chlorophyll α but has a high enough intensity to still excite enough fluorescence that it can be measured if the chlorophyll concentration is not too low. The excitation wavelength is better suited to chlorophyll b. The FICC will therefore be able to detect lower concentrations of algae or phytoplankton if it contains chlorophyll b.

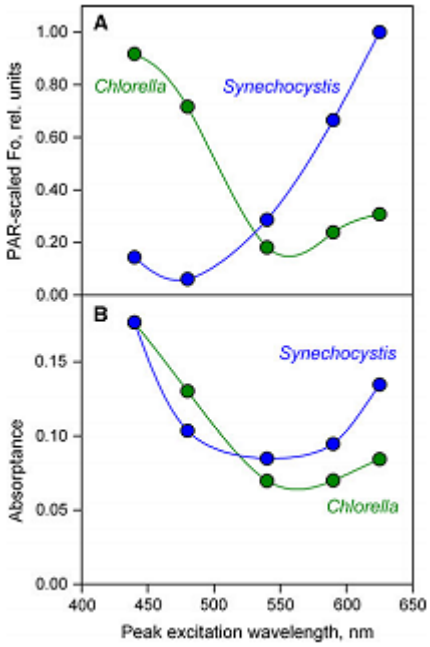


Figure 53: Chlorella vulgaris absorption and fluorescence.

Figure 53 taken from [13].

Figure 53 shows the absorption and fluorescence curves of Chlorella vulgaris as measured by [13]. It shows that Chlorella vulgaris, an algae species that was used in many of the FICC measurements, has a useful response to the 470 nm excitation wavelength of the FICC. The performance of the FICC with algae or phytoplankton that does not contain chlorophyll b is not known since there was no opportunity to test it.

The ratio of chlorophyll α and b content in Chlorella vulgaris changes according to environmental conditions [102],[103]. Such changes in chlorophyll content in algae or phytoplankton will impact fluorescence measurements. As the FICC mainly measures chlorophyll b fluorescence with the 470 nm excitation light, the fluorescence measurement will mostly give an indication of chlorophyll b concentration. An accurate chlorophyll α concentration can only be determined once the chlorophyll α and b ratio of the measured sample is known.

The laboratory experiments with the chlorophyll extracted from spinach showed that the FICC could measure a difference between concentrations as low as 0.01 µg/l and acetone. The proportion of the different types of chlorophylls in this extract is unfortunately not known. The lowest pure chlorophyll α concentration that could be identified from the unfiltered measurement data was 43 µg/l. During these experiments the FICC had a hand made flat face prototype front end that would not have provided optimum excitation light illumination. The light sensors could also not be placed optimally to receive the most fluorescence light. It is believed that the FICC will be able to deliver improved performance with an accurately machined front end.

Chlorophyll that is biologically active in algae or phytoplankton will fluoresce less than the extracted chlorophyll used in the FICC laboratory tests. The lowest chlorophyll concentration that the FICC can measure in living algae or phytoplankton will therefore be higher than the laboratory concentrations. It is unfortunately not known what the lowest concentration of algae or phytoplankton is that the FICC can detect and identify accurately enough as there was no opportunity to measure such samples and compare the results with any proven device or other concentration measurement method.

The commercial fluorometers that can measure fluorescence from very low chlorophyll concentrations usually make use of very expensive photomultiplier tubes or more modern components, like PIN photodiodes and silicon photomultipliers. The TSL257 light-to-voltage sensors used in the FICC have a much lower cost (around $2) than these components. This has

contributed hugely to the low overall cost of the FICC. The low cost has come at the price of reduced sensitivity compared to the other photo sensors mentioned. The TSL257 sensors still provided good enough performance in the ranges of chlorophyll concentrations that were tested. The TSL257 sensors have higher sensitivity than the OPT101 sensors that were successfully used in the development of a low cost fluorometer by [6]. It should then be possible to develop the FICC from a prototype laboratory fluorometer into a portable field fluorometer. The success of the low cost sensors would seem to indicate that highly sensitive and costly photo sensors are not an absolute requirement for the development of a fluorometer if it is not going to be used for very accurate photosynthesis research.

The high sensitivity of the TSL257 light sensors require that the high intensity excitation light must be prevented from reaching the sensors and saturating it. Light filters are definitely required for this purpose. The prices of light filters can vary considerably depending on the supplier and characteristics of the filter. It provides another challenge to the fluorometer designer to find the right compromise between cost and performance. The EO #49027 longpass filter is the most expensive single component of the FICC ($90). Its performance until now has warranted its continued use in the FICC as it blocks out enough of the excitation light without blocking the fluorescent light. The detected light intensity with a blank sample is far enough below the lowest measured chlorophyll concentrations that it allows for a possible increase in measurement range with better noise removal.

It is not known by this author if the percentage of fluorescence contribution by PSI, as discussed in Chapter 2, could have an important effect on the total variable fluorescence measured by the FICC. The variable chlorophyll fluorescence is often used to analyse the PSII system [45],[104]. For such measurements it would be important to know the percentage of fluorescence originating in PSII. Since the FICC is not used to analyse chlorophyll fluorescence from a specific photosystem it is assumed that the exact source will not matter as long as the total fluorescence intensity is measured.

Chlorophyll quantification with fluorometers are often wrong due to fluorescence variation caused by factors like the physiological state of cells, the species composition and environmental conditions [105],[106]. The contradicting literature about the validity of using fluorescence intensity measurements to determine chlorophyll concentration seem to depend on the required accuracy of the concentration measurement, the range of concentrations being measured as well as the methods used in the comparisons. When the fluorometer will be used only as an indicator of big

changes in chlorophyll concentration of a single algae or phytoplankton species, like during a bloom, fluorescence intensity can provide useful measurements. When accurate measurements of chlorophyll concentration are required it must be done very carefully while all the influencing factors, as discussed by [50], are taken into account.

From the laboratory experiments and other measurements this author concludes that when a specific sample is diluted several times into different concentrations and the fluorescence intensity is immediately measured, there is a linear relationship between fluorescence intensity and concentration. When any sample has been dark-adapted or has had time to change state, the fluorescence intensity to concentration relationship of different concentrations starts to vary increasingly as time goes by.

All the analysis methods used by this author on the measurement data do again indicate that there is a linear relationship between fluorescence intensity and chlorophyll concentration. This author believes that a lot of the deviation from the trend lines are caused by the variability of chlorophyll fluorescence since the data shows clear fluorescence intensity changes, without excitation intensity changes, during measurements of the chlorophyll dilution.

Unfortunately there was no money available for the development of the required waterproof housing to build a final production prototype.

8.2 Further Investigations for the FICC

The stability of the LED light intensity over time has not been measured. As the LED light intensity was being measured and logged along with all the other variables it was not considered to be something that should be known beforehand and compensated for. Casual inspections of measurement data seemed to show that the LED intensity was stable enough not to have a negative influence. If the chlorophyll concentration range of the FICC is to be increased the stability of the LED intensity might become more important. The stability should then be measured to confirm if it influences low concentration chlorophyll measurements.

The stability and accuracy of the current control circuit of the LED can very likely be improved to optimise its performance at the frequency of the modulation sine wave. The RC filter at the gate of the FET can for instance be designed to block frequencies higher than the modulation frequency. The RC filter currently has a cut-off frequency of 159 Hz. This is more than double the 63 Hz

modulation frequency that is currently being used.

The current cost of the FICC system can be reduced a lot by replacing the high quality EO filters with cheaper alternatives that will perform well enough for the requirements of the FICC. Accurate testing will have to be done with known chlorophyll concentrations to confirm if a filter like the Lee Filters 105 Orange film filter, that also blocks around 15% of the passband light, performs well enough with low chlorophyll concentrations to replace the EO longpass filter.

The Lee Filters 141 Bright Blue film filter was successfully used in the chlorophyll α experiments on its own to measure the excitation light intensity since the fluorescent light that must be blocked has a very low intensity while the excitation light is so bright that it does not matter much if 20% of it is also blocked by the filter. It must be confirmed if it blocks enough light at wavelengths longer than 700 nm to be used with chlorophylls and other substances that can be excited by the 470 nm of the FICC. This would reduce the cost of the FICC by removing the expensive EO shortpass filter that was used in some experiments.

LEDs can be used to measure light intensity when it is bright enough [8]. It might be possible to measure the excitation light intensity with a SMD LED of the same wavelength. This would remove the need for the blue light filter and blue light sensor. This could provide enough space for one or more additional excitation LEDs with different wavelengths to enable detecting changes in species composition.

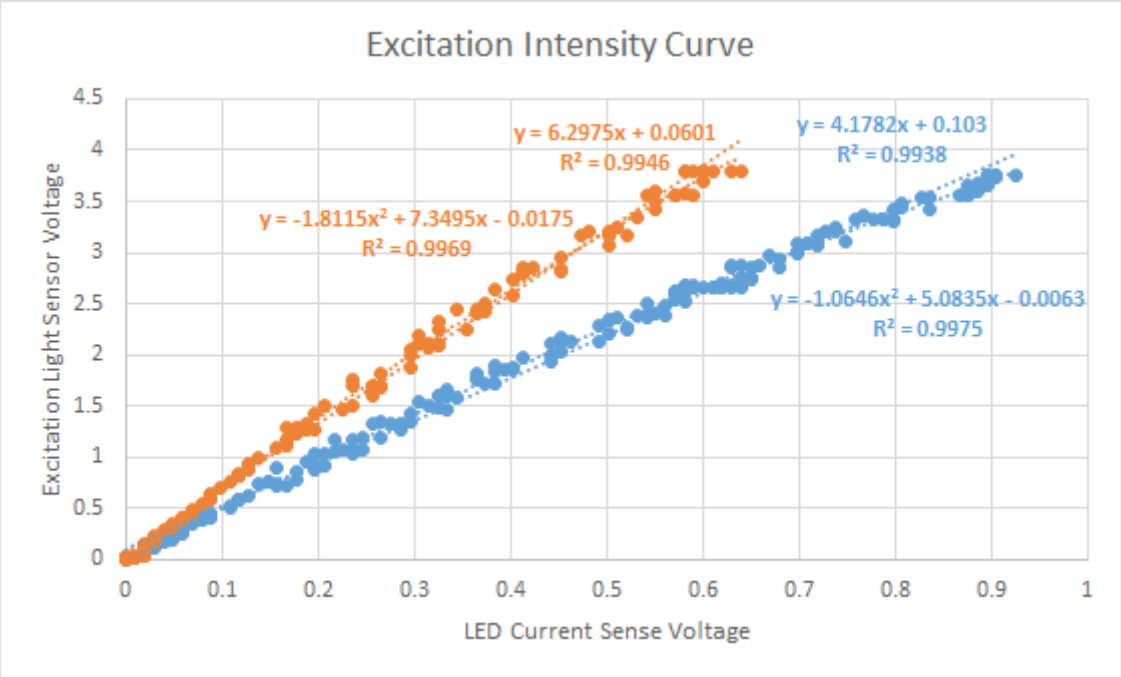


Figure 54: Excitation intensity curve.

It should be investigated if and by how much the FICC accuracy will improve if the slightly nonlinear relationship between excitation intensity and LED current is taken into account in the light modulation. Figure 54 shows two series of data from different chlorophyll concentrations that were discussed in Section 7.1.2. Two trend lines were fitted to each series of data to show the difference between a linear fit and a second order polynomial fit. It shows that the polynomial fit is more accurate but there is not a massive difference. It is however not known if these small differences can add up to cause inaccurate chlorophyll concentration measurements at very low concentrations where the differences in light levels are small.

As the measured fluorescence intensity is only used as an indicator of chlorophyll concentration in the FICC, the PSII state is not analysed during measurements. The physiological state of PSII does however have a big influence on the amount of fluorescent light emitted in response to the incoming excitation light intensity [19],[46]. It should be investigated how much the physiological state of PSII influences concentration measurements when the current 75 ms pulsing scheme of the FICC is used. It might also be possible to determine some of the physiological state information from the measurement data of the rising edge of the light pulse.

This author believes that the 300 µs response time of the TSL257 sensor makes it too slow to measure the F_O fluorescence during an excitation light pulse. This is not currently seen as a problem since F_O is not used to determine the chlorophyll concentration. If F_O needs to be

measured in future, a different light sensor with faster response might be needed if extrapolation of the fluorescence rise curve cannot calculate the F_O value closely enough.

The SMD version of the TSL257 light-to-voltage sensor (3.8 x 2.6 mm) is smaller than the through hole version (5.6 x 4.6 mm) that is currently in use in the FICC. Using three SMD sensors would reduce the size of the optical front end which would make it possible to design a smaller fluorometer that can more easily be fitted to measurement platforms like the one designed by the CSIR.

The last concept design of a flat face version of the FICC (Figure 55) includes the EO longpass light filter as well as the EO shortpass filter. This does unfortunately increase the diameter of the FICC beyond the ideal 30mm if the current components are used. The diameter of this design can most likely be reduced by making use of smaller light filters and SMD light sensors. The fact that the returning fluorescent light also changes angle when it goes from the water to the air inside the FICC (Snell's law) means that the light sensors have to be located quite a distance to the sides of the FICC to measure the fluorescent light. This bending of the light is not shown in Figure 55. This design has not been tested yet as there were no funds to manufacture it. A similar layout was however tested successfully, as is described in Section 6.4.

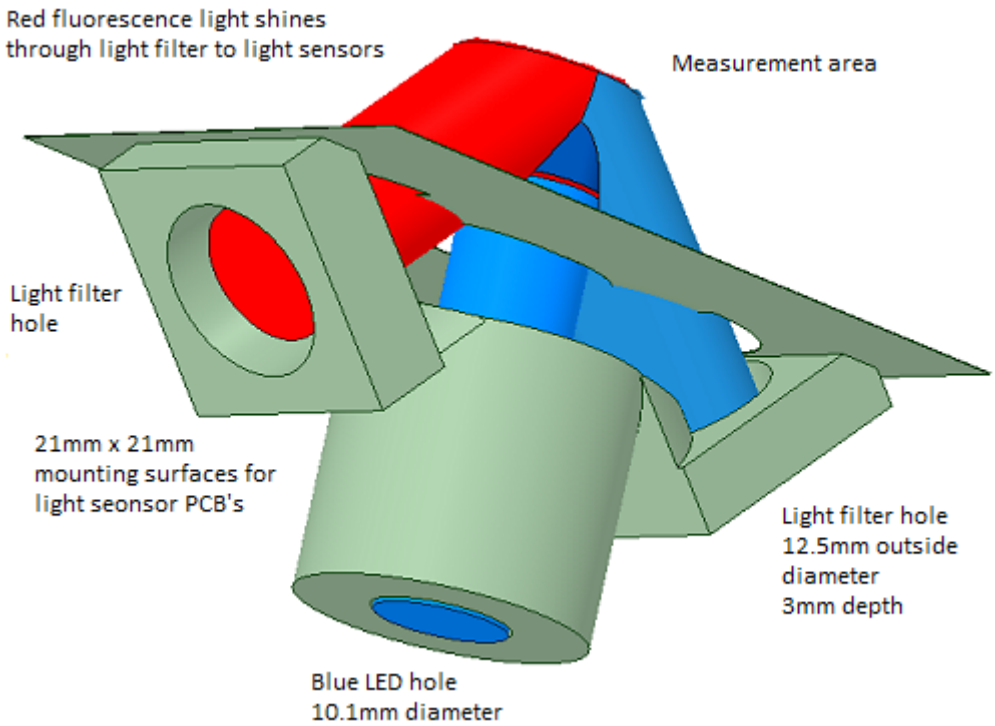


Figure 55: Flat faced FICC prototype light sensor locations.

Appendix A

Beutler et al. [10] gives the following table of fluorescence parameters:

F	Fluorescence intensity	Actual fluorescence intensity at any time
F_O	Minimal fluorescence (dark)	Fluorescence intensity with PS II reaction centres open while the photosynthetic membrane is in a non-energised state i.e. dark or low light-adapted qP= 1 and qN= 1. It can also be used for the O level in the O-I-D-P-T nomenclature but it should be clearly described how it is determined.
FI	fluorescence at I level	Fluorescence intensity at I level (O-I-D-PT nomenclature).
FP	fluorescence at P level	Fluorescence intensity at P level (O-I-D-PT nomenclature).
FS	fluorescence in steady state	Fluorescence intensity at the steady state. i.e., T-level in O-I-D-P-T nomenclature. Steady state is defined as a period within which the fluorescence intensity does not change while the external parameters remain constant.
F_M	maximal fluorescence (dark)	Fluorescence intensity with all PS II reaction centres closed (i.e. qP= 0) all non-photochemical quenching processes are at a minimum (i.e. qN= 0) (this is the classical maximum fluorescence level in the dark or low light-adapted state).
F_M'	maximal fluorescence (light)	Fluorescence intensity with all PS II reaction centres closed in any light-adapted state i.e. qP= 0 and qN ≥0.
F'_O	minimal fluorescence (light)	Fluorescence intensity with PS II reaction centres open in any light-adapted state i.e.. qP= 1 and qN ≥0.
F_v	variable fluorescence (dark)	Maximum variable fluorescence in the state when all non-photochemical processes are at a minimum, i.e. (F_M-F_O).
F'_v	variable fluorescence (light)	Maximum variable fluorescence in any light-adapted state i.e. (F_M'-F_O').

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Appendix B

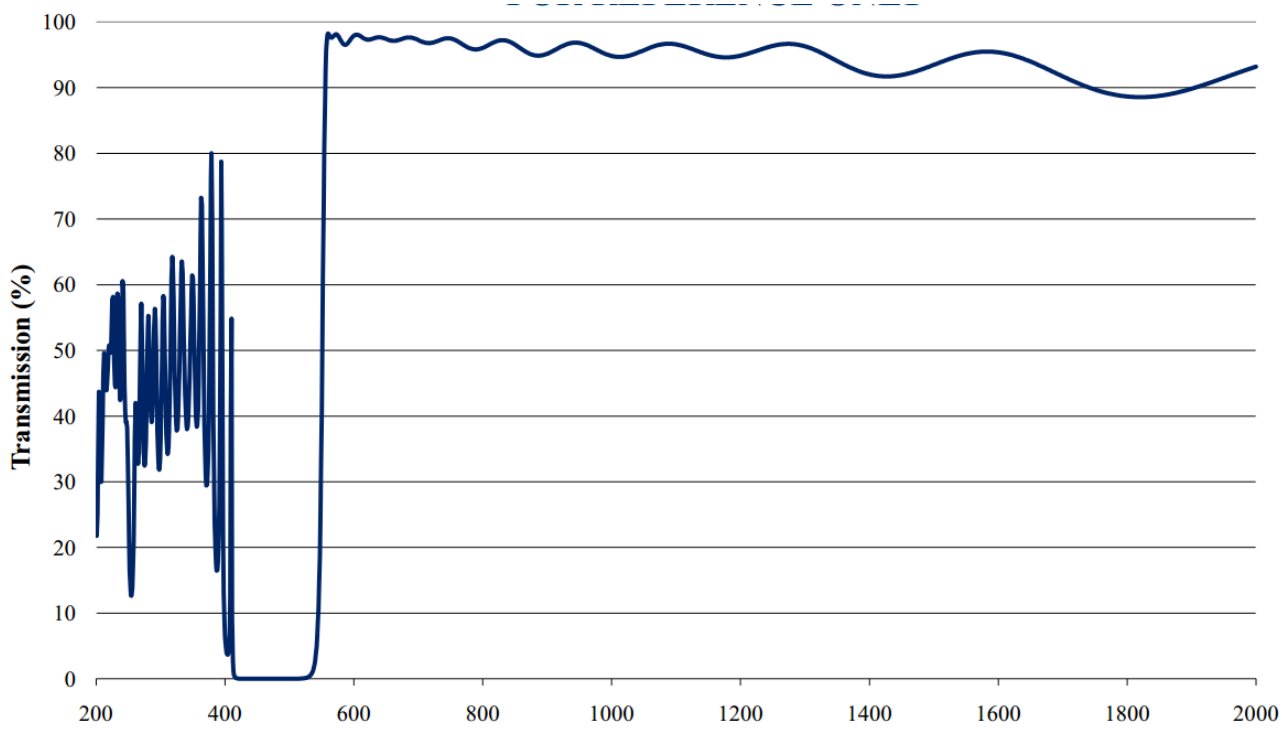


Figure 56: EO longpass 49027.

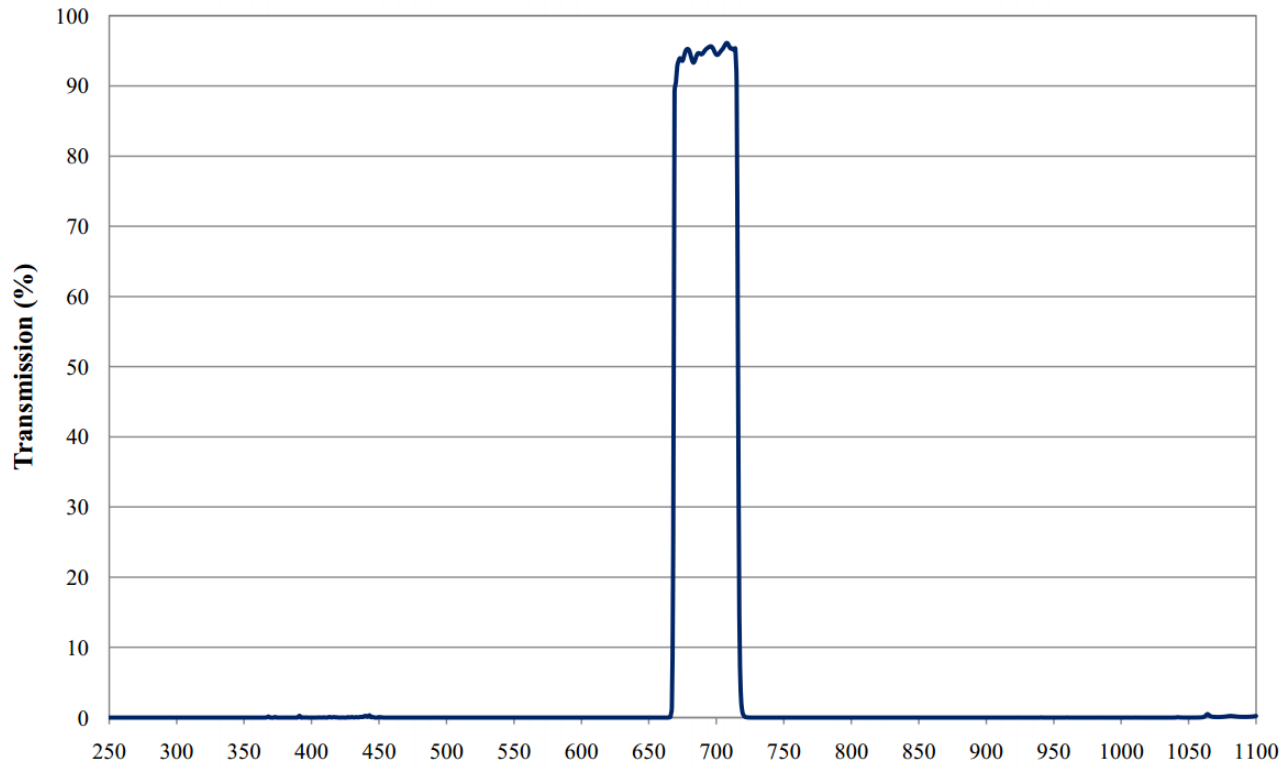


Figure 57: EO 692nm bandpass 67024.

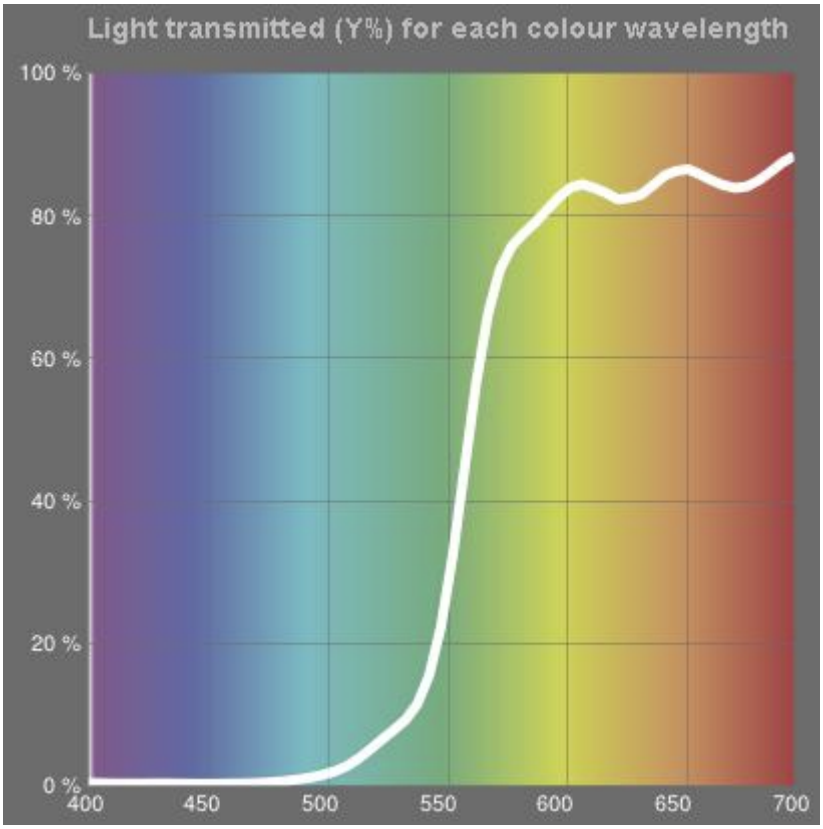


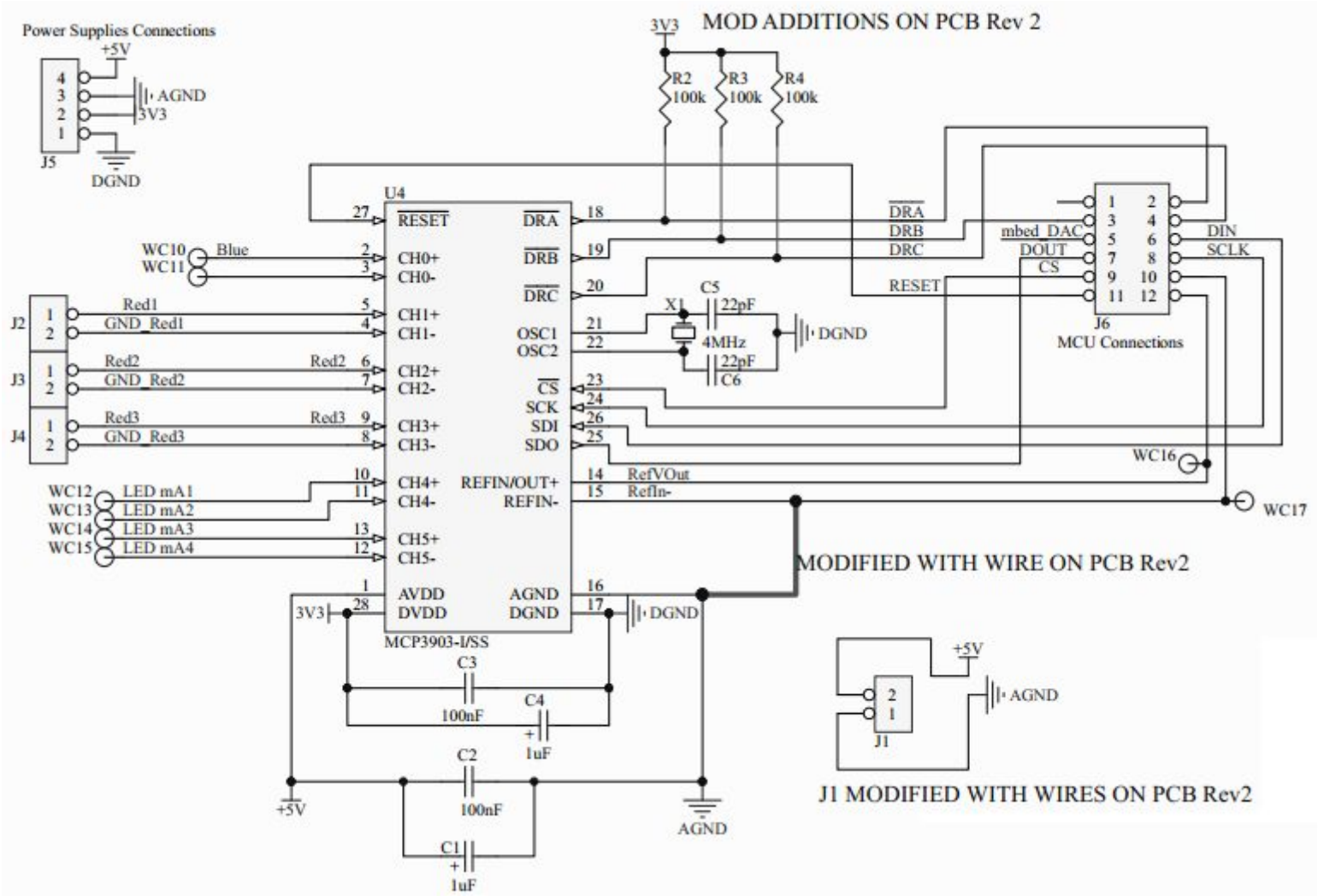
Figure 58: Lee filters Orange 105.

Appendix C

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P
1	Dan Merrick 01/10/04		Time	Signal		Result of forward FFT (spectrum)	Hz	magnitude		amplitude		FFT filter	Filtered forward FFT (spectum)	Complex result of inverse FFT (time base)	magnitude
2		0.0003	0	4		2080.69493216856	0	2081		4.06		0	0	1.32939136089303	1.33
3			0.0003	3.9960535		33.000814211199+2.9423172743641i	3.255208333	33		0.06		0	0	1.27169095220831	1.27
4			0.0006	3.9842294		33.9548540752298+6.059615441182961	6.510416667	34		0.07		0	0	1.21036581831084	1.21
5			0.0009	3.9645745		35.6776229069721+9.56338390040327i	9.765625	37		0.07		0	0	1.14557362012016	1.15
6	# of data points	1024	0.0012	3.9371663		38.4152172650541+13.7553854116561i	13.02083333	41		0.08		0	0	1.07748119525474	1.08
7	Edit macro if # of points is changed		0.0015	3.902113		42.6401896121599+19.1314920408269i	16.27604167	47		0.09		0	0	1.00626424871173	1.01
8			0.0018	3.859553		49.3039098089417+26.6243783098037i	19.53125	56		0.11		0	0	0.932107024495401	0.93
9	wave 1 amplitude	4	0.0021	3.8096541		60.5629511870704+38.2893652596753i	22.78645833	72		0.14		1	60.5629511870704+38.2893652596753i	0.855201957925615	0.86
10			0.0024	3.7526134		82.4571153751335+59.8217486419076i	26.04166667	102		0.20		1	82.4571153751335+59.8217486419076i	0.775749308373422	0.78
11			0.0027	3.6886559		140.552494795406+115.248315403678i	29.296875	182		0.36		1	140.552494795406+115.248315403678i	0.693956772196009	0.69
12			0.003	3.618034		686.684537768958+628.885295130514i	32.55208333	931		1.82		1	686.684537768958+628.885295130514i	0.610039075676056	0.61
13	Low Cutoff Freq in Hz	20	0.0033	3.5410265		-205.432512088375-208.155903197296i	35.80729167	292		0.57		1	-205.432512088375-208.155903197296i	0.524217547812664	0.52
14			0.0036	3.4579373		-84.1762102609566-93.6411312677542i	39.0625	126		0.25		1	-84.1762102609566-93.6411312677542i	0.436719672861201	0.44
15			0.0039	3.3690942		-51.0066695968294-61.9008962489026i	42.31770833	80		0.16		0	0	0.347778622578569	0.35
16	High cutoff Freq	40	0.0042	3.274848		-35.6219276080151-46.9104458954969i	45.57291667	59		0.12		0	0	0.257632768197524	0.26
17			0.0045	3.1755705		-26.7997996683328-38.1256627597918i	48.828125	47		0.09		0	0	0.166525172229318	0.17
18			0.0048	3.0716536		-21.1129574071863-32.3231621622095i	52.08333333	39		0.08		0	0	7.47030602771476E-002	0.07
19			0.0051	2.9635074		-17.1632410189065-28.1857948321939i	55.33854167	33		0.06		0	0	-1.758272686618E-002	-0.02

Frequency domain bin selection column

Appendix D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3V3
DGND
J5
MOD ADDITIONS ON PCB Rev 2
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R2 100k
R3 100k
R4 100k
U4
RESET
DRA
DRB
DRC
CH0+
CH0-
CH1+
CH1-
CH2+
CH2-
CH3+
CH3-
CH4+
CH4-
CH5+
CH5-
OSC1
OSC2
CS
SCK
SDI
SDO
REFIN/OUT+
REFIN-
AVDD
DVDD
AGND
DGND
MCP3903-I/SS
WC10
WC11
Blue
Red1
GND_Red1
Red2
GND_Red2
Red3
GND_Red3
WC12
WC13
WC14
WC15
LED mA1
LED mA2
LED mA3
LED mA4
J2
J3
J4
C5
22pF
X1
4MHz
C6
22pF
DGND
RefVOut
RefIn-
WC16
WC17
mbed_DAC
DOUT
CS
DIN
SCLK
J6
MCU Connections
MODIFIED WITH WIRE ON PCB Rev2
C3
100nF
C4
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C2
100nF
C1
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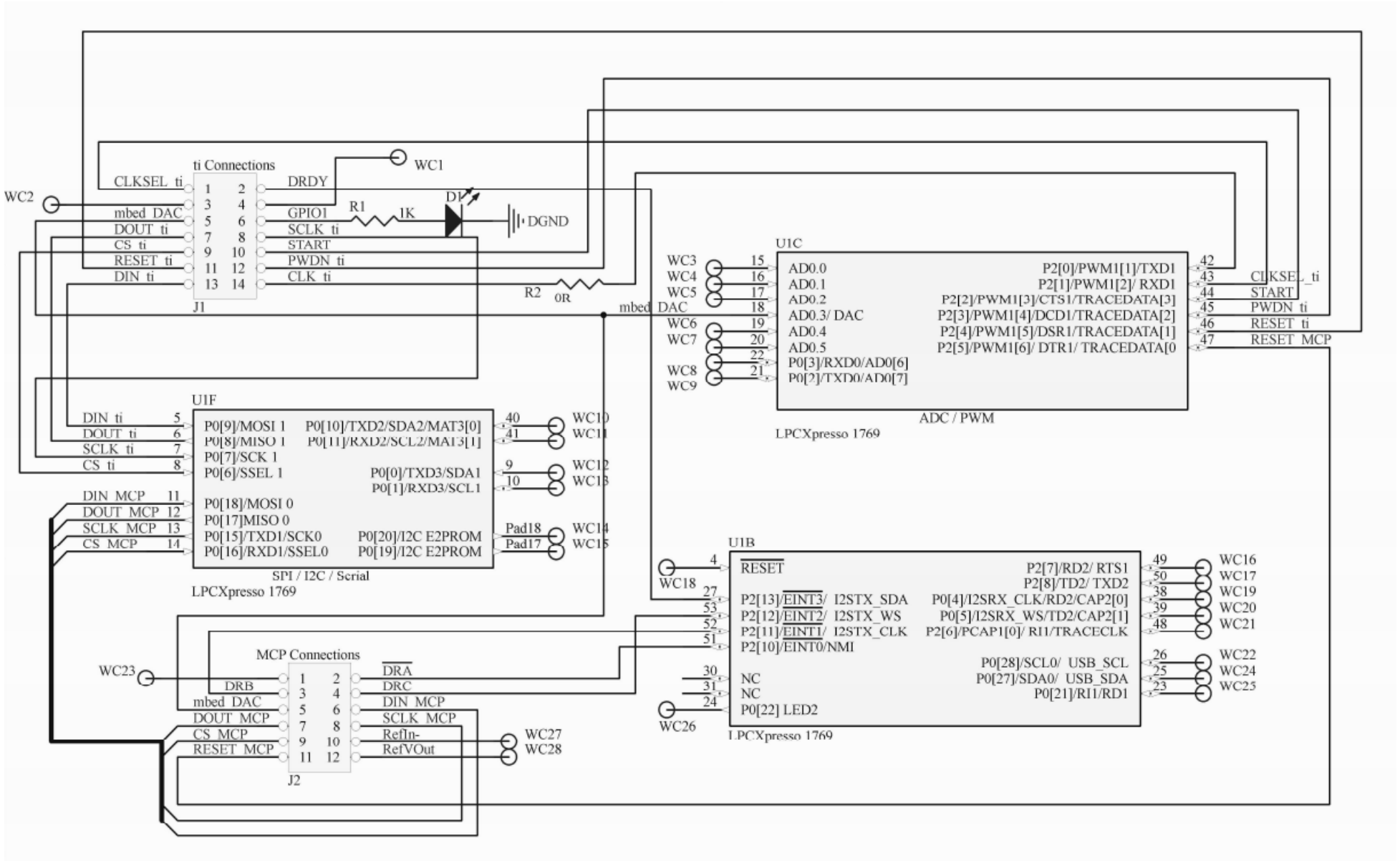
+5V
AGND
J1
J1 MODIFIED WITH WIRES ON PCB Rev2


ti Connections
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CS_ti
RESET_ti
DIN_ti
DRDY
GPIO1
SCLK_ti
START
PWDN_ti
CLK_ti
J1
WC1
WC2
R1
1K
D1
DGND
R2
0R
mbed_DAC
U1C
AD0.0
AD0.1
AD0.2
AD0.3/ DAC
AD0.4
AD0.5
P0[3]/RXD0/AD0[6]
P0[?]/TXD0/AD0[7]
P2[0]/PWM1[1]/TXD1
P2[1]/PWM1[2]/ RXD1
P2[2]/PWM1[3]/CTS1/TRACEDATA[3]
P2[3]/PWM1[4]/DCD1/TRACEDATA[2]
P2[4]/PWM1[5]/DSR1/TRACEDATA[1]
P2[5]/PWM1[6]/ DTR1/ TRACEDATA[0
ADC / PWM
LPCXpresso 1769
U1F
P0[9]/MOSI 1
P0[8]/MISO 1
P0[7]/SCK 1
P0[6]/SSEL 1
P0[10]/TXD2/SDA2/MAT3[0]
P0[11]/RXD2/SCL2/MAT3[1]
P0[0]/TXD3/SDA1
P0[1]/RXD3/SCL1
P0[18]/MOSI 0
P0[17]MISO 0
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P0[16]/RXD1/SSEL0
P0[20]/I2C E2PROM
P0[19]/I2C E2PROM
SPI / I2C / Serial
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RESET
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P2[12]/EINT2/ I2STX_WS
P2[11]/EINT1/ I2STX_CLK
P2[10]/EINT0/NMI
NC
NC
P0[22] LED2
P2[7]/RD2/ RTS1
P2[8]/TD2/ TXD2
P0[4]/I2SRX_CLK/RD2/CAP2[0]
P0[5]/I2SRX_WS/TD2/CAP2[1]
P2[6]/PCAP1[0]/ RI1/TRACECLK
P0[28]/SCL0/ USB_SCL
P0[27]/SDA0/ USB_SDA
P0[21]/RI1/RD1
LPCXpresso 1769
MCP Connections
DRA
DRB
DRC
mbed_DAC
DIN_MCP
DOUT_MCP
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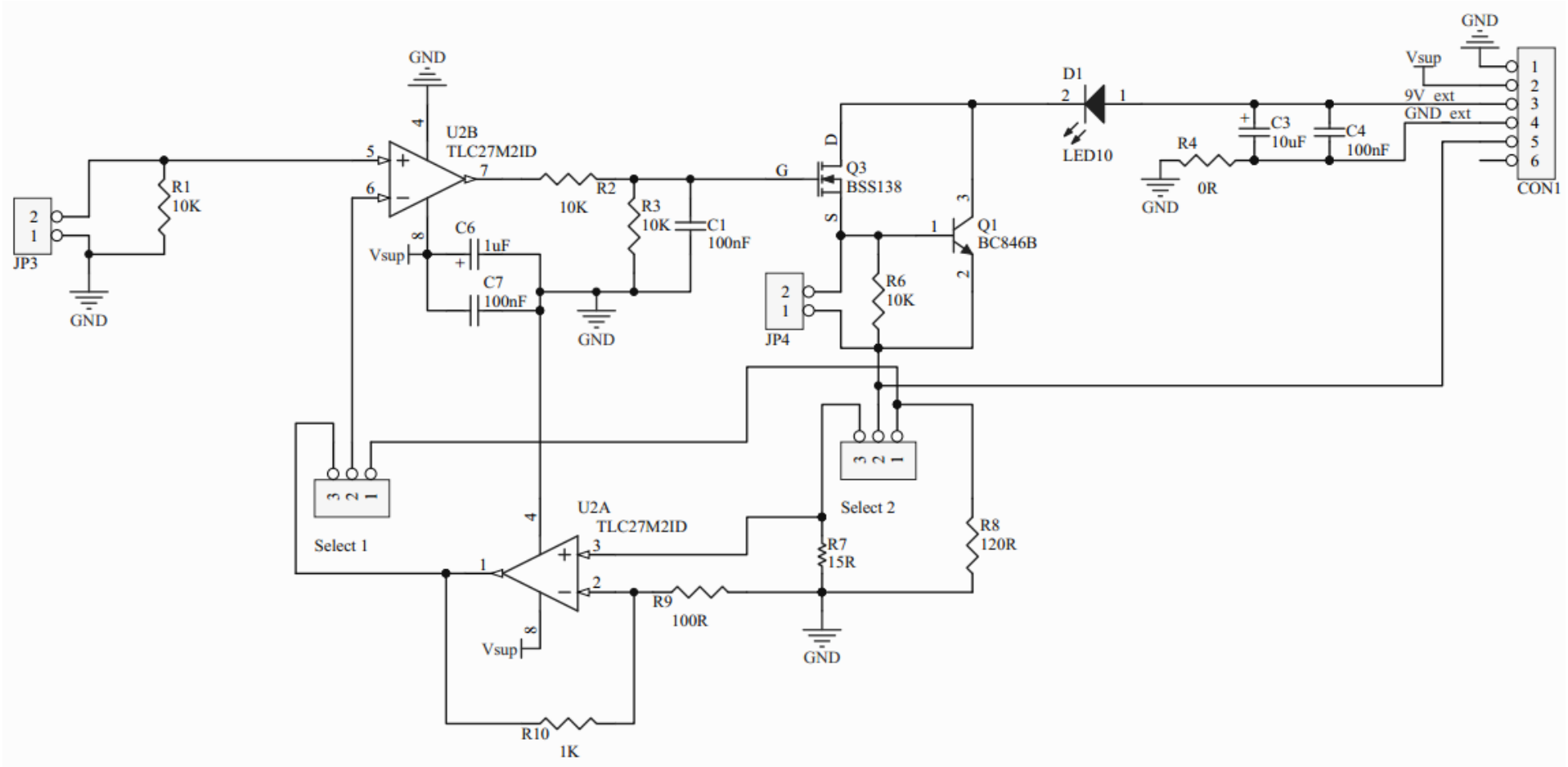
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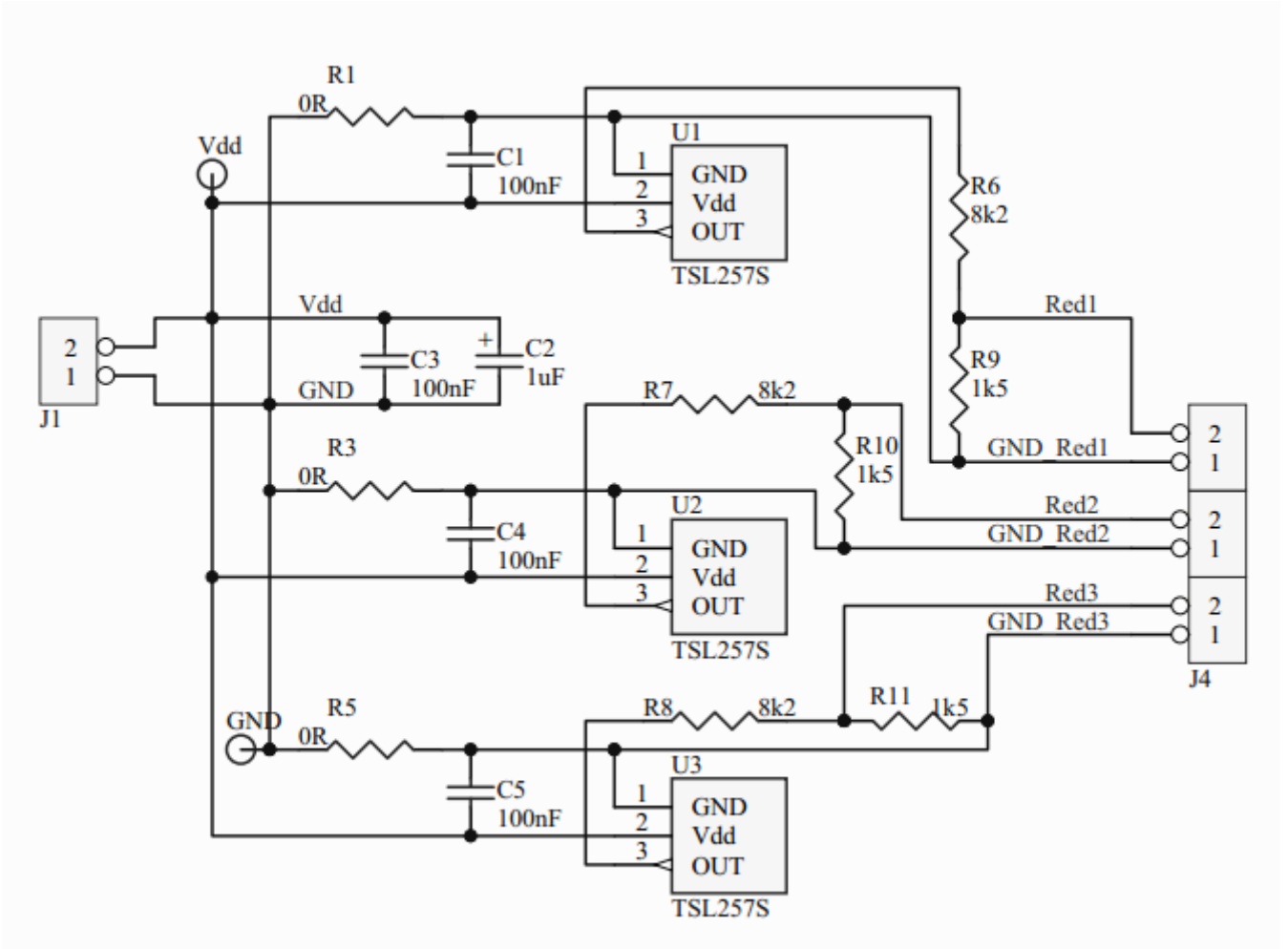
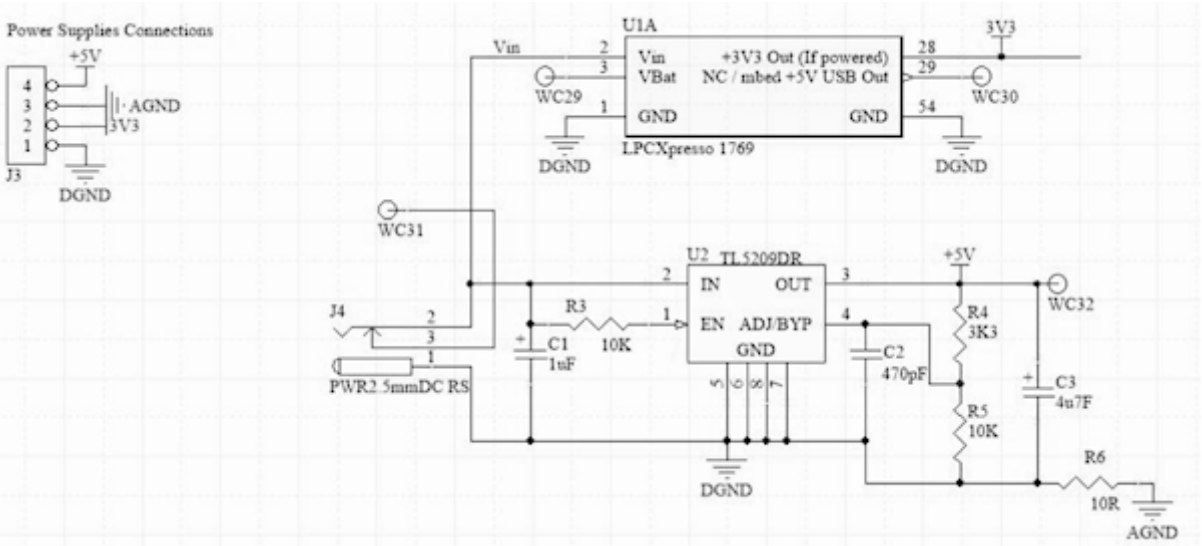
10K
JP3
R2
10K
R3
10K
C1
100nF
C6
1uF
C7
100nF
Vsup
Q3
BSS138
Q1
BC846B
R6
10K
JP4
D1
LED10
R4
0R
C3
10uF
C4
100nF
9V_ext
GND_ext
CON1
Select 1
Select 2
U2A
TLC27M2ID
R7
15R
R8
120R
R9
100R
R10
1K


R1
0R
Vdd
C1
100nF
U1
1
2
3
GND
Vdd
OUT
TSL257S
R6
8k2
Red1
R9
1k5
Vdd
C3
100nF
C2
1uF
GND
2
1
J1
R7
8k2
R10
1k5
GND_Red1
R3
0R
C4
100nF
U2
GND
Vdd
OUT
TSL257S
Red2
GND_Red2
Red3
GND_Red3
J4
GND
R5
0R
R8
8k2
R11
1k5
C5
100nF
U3
GND
Vdd
OUT
TSL257S


Power Supplies Connections
+5V
4
3
2
1
AGND
3V3
J3
DGND
U1A
Vin
2
3
1
Vin
VBat
GND
+3V3 Out (If powered)
NC / mbed +5V USB Out
GND
28
29
54
3V3
WC29
WC30
DGND
DGND
LPCXpresso 1769
WC31
U2 TL5209DR
IN
OUT
EN
ADJ/BYP
GND
+5V
WC32
J4
R3
10K
C1
1uF
PWR2.5mmDC RS
C2
470pF
R4
3K3
R5
10K
C3
4u7F
R6
10R
DGND
AGND