Department of Physics, Chemistry and Biology

Master Thesis

Flow Cytometry Sensor System Targeting Escherichia Coli as an Indicator of Faecal Contamination of Water Sources

Tobias Benselfelt

Performed at Acreo Swedish ICT AB

2014-06-24

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Författare
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Sammanfattning
Abstract

Poor water quality is a global health concern affecting one billion people around the world. It is important to monitor water sources in order to maintain the quality of our drinking water and to avoid disease outbreaks. Targeting Escherichia coli as a faecal indicator is a widely used procedure, but the current methods are time consuming and not adequate to prevent spreading of faecal influence.

This Master thesis demonstrates the development of a near infrared fluorescence flow cytometer sensor system targeting Escherichia coli, using fluorescently labeled chicken IgY antibodies. The near infrared light was chosen to avoid fluorescence from blue-green algae that are present in the water source.

The hardware was developed with a 785 nm laser line to detect Alexa Fluor 790 labeled antibodies, using a photomultiplier tube or two different CMOS cameras. The antibodies were labeled using a commercial labeling kit, and evaluated using antibody binding assays and the developed hardware.

The IgY antibodies were successfully labeled with Alexa Fluor 790 and the function was maintained after the labeling process. The result demonstrates the principles of the sensor system and how it solved to the problem with fluorescence from blue-green algae. An aperture was used to overcome the suboptimal laser and filter setup, and to increase the sensitivity of the system. However, only a small fraction of the cells could be detected, due to challenges with the focal depth and loss of sensitivity in the photomultiplier tube at near infrared wavelengths. Further development is required to create a working product.

Nyckelord
Keyword
Sensor system, Water quality, Flow cytometry, Faecal contamination, Faecal indicator, Escherichia Coli, Untreated water, Near infrared light, NIR, Antibody, Immunoglobulin, IgY, Alexa Fluor 790, Photomultiplier tube, CMOS, camera.

Abstract

Poor water quality is a global health concern affecting one billion people around the world. It is important to monitor water sources in order to maintain the quality of our drinking water and to avoid disease outbreaks. Targeting *Escherichia coli* as a faecal indicator is a widely used procedure, but the current methods are time consuming and not adequate to prevent spreading of faecal influence.

This Master thesis demonstrates the development of a near infrared fluorescence flow cytometer sensor system targeting *Escherichia coli*, using fluorescently labeled chicken IgY antibodies. The near infrared light was chosen to avoid fluorescence from blue-green algae that are present in the water source.

The hardware was developed with a 785 nm laser line to detect Alexa Fluor 790 labeled antibodies, using a photomultiplier tube or two different CMOS cameras. The antibodies were labeled using a commercial labeling kit, and evaluated using antibody binding assays and the developed hardware.

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Sammanfattning

Bristande vettenkvalitet är en global hälsorisk som påverkar en miljard människor runt om i världen. Det är viktigt övervaka våra vattenresurser för att bibehålla en good vattenkvalitet och för att hindra spridning av vattenburna sjukdomar. *Escherichia coli* används ofta som en indikator på fekal smitta i vatten, men de befintliga detektionsmetoderna är tidskrävande och inte tillräckliga för att förhindra spridning av sjukdomar.

Detta examensarbete innehåller utvecklingen av en flödescytometer baserad på nära infrarött ljus för att detektera *Escherichia coli*, genom användandet av fluorescentinmärkta IgY-antikroppar extraherade från äggulan i hönsägg. Nära infrarött ljus används för att undvika fluorescence från blå-gröna alger som förekommer i vattentäkter.

Hårdvaran utvecklades med en laser vid 785 *nm* för att detektera antikroppar märkta med Alexa Fluor 790, med hjälp av en photomuliplikator eller två olika CMOS kameror. Antikropparna märkets med hjälp av ett kommersiellt inmärkningskit och utvärderades via bindningsanalys och med den utvecklade hårdvaran.

IgY antikropparna kunde effektivt märkas med Alexa Fluor 790 och behöll sin funktion efter processen. Examensarbetet demonstrerar principerna bakom sensorsystemet och en lösning till problemet med fluorescence från blå-gröna alger. En bländare användes för att kompensera för en icke-optimal laser- och filteruppsättning, och ökade känsligheten hos systemet. Dock kunde bara ett fåtal celler detekteras på grund av utmaningar med fokaldjupet och försämrad känslighet hos photomultiplikatorn för nära infraröda våglängder. Vidare utveckling krävs för att skapa en fungerande produkt.

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Common Abbreviations

$[X]$	Concentration of X (M)
$\alpha - Y$	Antibody targeting Y
ε	Extinction coefficient $[cm^{-1}M^{-1}]$
λ	Wavelength
ϕ	Quantum yield
A_λ	Absorbance at wavelength λ
Ab	Antibody
Ag	Antigen
APD	Avalanche photodiode
AR	Anti reflective
BSA	Bovine serum albumin
c	Concentration $[M]$
CCD	Charge coupled device
CFU	Colony forming unit
$CMOS$	Complementary metal–oxide–semiconductor
Da	Dalton, molecular weight $[g/mol]$
$EMCCD$	Electron multiplying CCD
F_λ	Fluorescence intensity for wavelength λ
F/P	Degree of labeling or fluorphores per protein
FIB	Fecal indicator bacteria
$FITC$	Fluorescein isothiocyanate
FP	Fluorescence polarisation
$FRET$	Fluorescence resonance energy transfer
h	Planck's constant
I_0	Intensity of incident light
I	Intensity of transmitted light
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
kDa	Kilo Dalton
K_A	Association constant $[M^{-1}]$
K_D	Dissociation constant $[M]$
l	Path length [cm]
M	Molar
MAP	Mussel adhesive protein
$MPCC$	Multipixel photon counter

MPN	Most probable number
NIR	Near infrared light
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMT	Photomultiplier tube
QE	Quantum efficiency. A detectors ability to detect photons.
r	Relative antibody binding
UV	Ultra violet light
$USEPA$	United state environmental protection agency
ν	Frequency $[Hz]$
VIS	Visible light
WHO	World health organization

1 Introduction

Poor water quality is estimated to cause 4% of the total annual disease outbreaks [1]. Lack of access to safe water supplies is a global health concern affecting one billion people around the world. Poor rural areas with inefficient water treatment plans are highly affected, and it is estimated that 34% of the world's population live in areas with inadequate water sanitation facilities [2]. Not only poor areas are affected by water contamination, and the public health authority in Sweden (Folkhälsomyndigheten) reported 142 cases of water related pathogenic outbreaks between the years 1980 and 2004, with the largest outbreak affecting 11,000 people [3].

The increasing population and accumulation of people in larger cities increases the risk and the potential damage of pathogenic outbreaks. It is of great importance to monitor the surface drinking water sources, such as rivers and lakes, in order to maintain the quality of the drinking water. The world health organisation (WHO) states that "better tools and procedures to improve and protect drinking-water quality at the community and urban level, for example through Water Safety Plans" are required in order to implement a sustainable water practice around the world. One fairly simple and effective strategy is to monitor the water source for contaminating agents, such as faecal influence. This information can be used for selective closure of the untreated water intake, or to take other actions, to reduce the impact of the contamination [4–6]. This strategy takes approximately 20 hours from sampling to result, in the best scenario[1], and new sensor systems are needed to be able to react in time.

Sensation is a project with the goal to create a complete solution for water quality management based on new sensor systems. Approximately 20 actors in the Swedish water industry, including partners from academia, collaborate in this project to create novel demonstrations and evaluations of sensor technologies that can be used in water quality monitoring. The aim is to dedicate research in this area and increase the knowledge for further development of new applications. This master thesis is a part of the research done by Acreo Swedish ICT AB in Göteborg, Sweden, as a subproject within the main project Sensation.

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[1] Information from Johanna Hilding, Process Engineer at Trollhättan Energi AB, personal communication, May 2014

1.1 Project Background

Acreo Swedish ICT AB is developing a fluorescent single channel flow cytometry sensor system targeting *Escherichia coli* as an early warning for faecal contamination in the river Göta Älv. The project is organised by Trollhättan Energi in cooperation with Göteborg stad (Kretsloppskontoret and Göteborg Vatten), Norrvatten and Vivab. The aim is to evaluate whether this system can be used as a substitute to the currently used methods, in order to reduce the time between sampling and result. The sensor system, described in Figure 1.1, is designed with an optical hardware setup to detect *Escherichia coli* in a flow channel by specific targeting using fluorescently labeled antibodies. The fluorescent marker was initially chosen in the visible range and fluorescent molecules, Quantum dots and fluorospheres were tested. Problems regarding affinity, when conjugating larger particles to the antibodies, were detected and the untreated water tests showed high levels of fluorescence from chlorophyll in algae and cyanobacteria. The project group decided to move towards near infrared (NIR) wavelengths, in order to decrease the fluorescent background. The decision to work with fluorescent molecules instead of fluorescent particles, was made in order to avoid affinity loss of the labeled antibodies previously encountered.

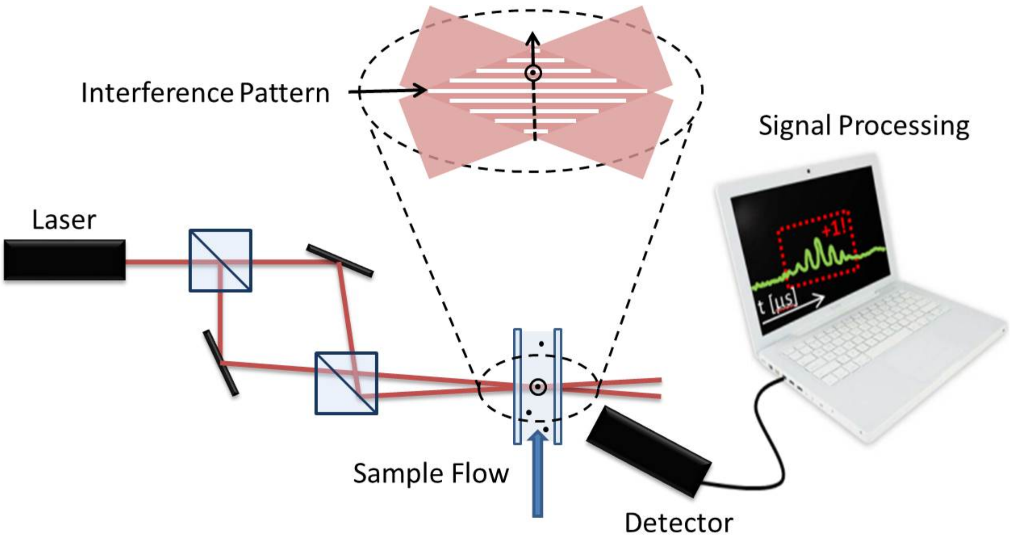


Figure 1.1: Schematic of the optical system setup.

The system was designed with larger dimensions and a more robust structure, compared to conventional flow cytometry (Figure 4.2), in order match the industrial environment and to make the hardware economically sustainable for actors in the field of water production. The main alteration was to increase the size of the channel to make it more durable, and to have a higher throughput to match the pressures in a water treatment facility.

The interference pattern was introduced to deal with the larger channel, and the idea was to analyse the frequency of the pulse characteristics created when a stained bacterium flows through the interference pattern. This would give a signal pattern that could be separated from the random noise in measurements with low signal-to-noise ratio.

The detection was limited to a single fluorescent channel and a simple circular flow cell was used. A relatively cheap avalanche photodiode was initially used, but showed lack of sensitivity and a photomultiplier had to be installed. This was a setback for the sensor system due to the high cost of photomultiplier tubes, which was not planned to be the solution in the final system.

2 Aim

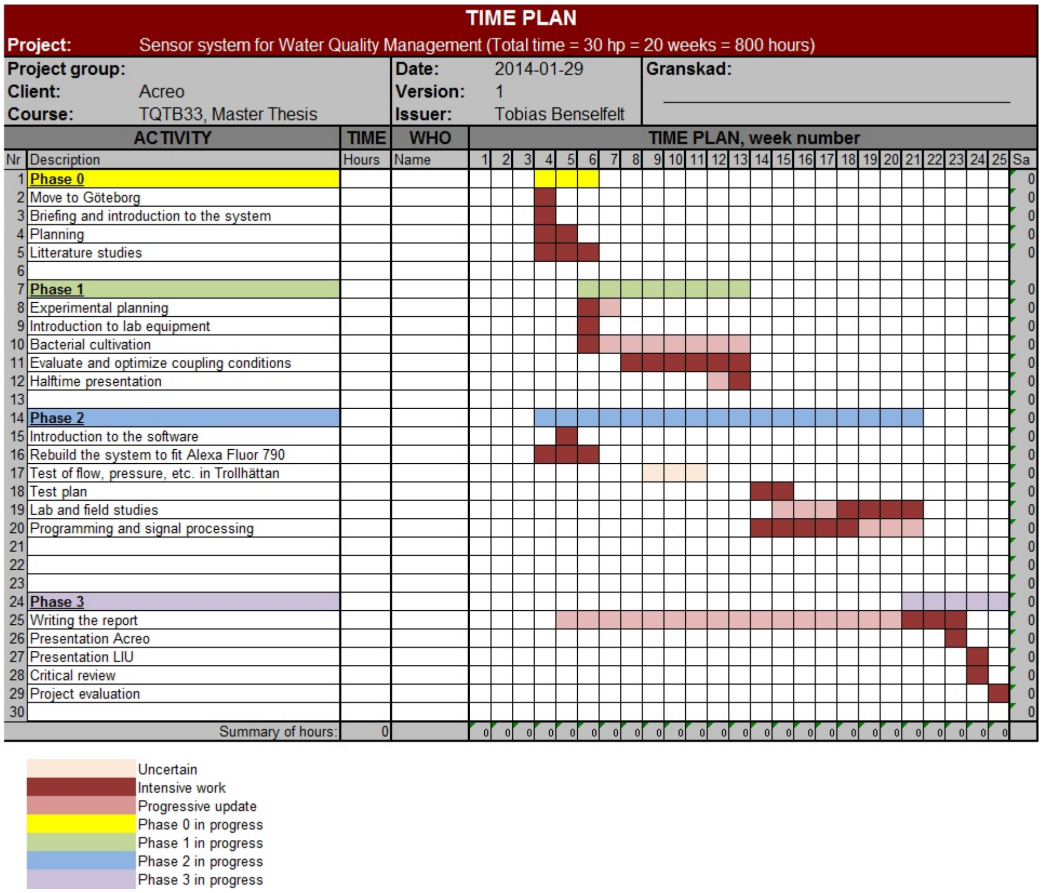
The aim of the project was to rebuild the flow cytometer system for near infrared light, in order to avoid fluorescence from chlorophyll. The system would be evaluated and compared to current methods to detect faecal contamination, if development lead to comparable system. To do this there were two phases to focus on:

1. Design and evaluation of the antibody-fluorophore conjugation for specific staining of *Escherichia coli*
2. Design and evaluation of the flow cytometry hardware to detect specifically stained *Escherichia coli*

3 Project Process

3.1 Timeplan

A timeplan was created during the planning of this project (Figure 3.1), and a short report was written to describe the methodical choices and the reading frame. The report explained the basic strategy and some examples of how it could be done, rather than detailed information. The timeplan was a rough estimation of the different parts of the project.



TIME PLAN

Project: Sensor system for Water Quality Management (Total time = 30 hp = 20 weeks = 800 hours)

Project group:
Client: Acreo
Course: TQTB33, Master Thesis

Date: 2014-01-29
Version: 1
Issuer: Tobias Benselfelt

Granskad: ______________________

ACTIVITY		TIME	WHO	TIME PLAN, week number																									
Nr	Description	Hours	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Sa
1	**Phase 0**						🟨	🟨	🟨																				0
2	Move to Göteborg						🟥																						0
3	Briefing and introduction to the system						🟥																						0
4	Planning						🟥	🟥																					0
5	Litterature studies						🟥	🟥	🟥																				0
6																													
7	**Phase 1**								🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩													0
8	Experimental planning								🟥	▨																			0
9	Introduction to lab equipment								🟥																				0
10	Bacterial cultivation								🟥	▨	▨	▨	▨	▨	▨	▨													0
11	Evaluate and optimize coupling conditions										🟥	🟥	🟥	🟥	🟥	🟥													0
12	Halftime presentation														▨	🟥													0
13																													0
14	**Phase 2**						🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦					0
15	Introduction to the software							🟥																					0
16	Rebuild the system to fit Alexa Fluor 790						🟥	🟥	🟥																				0
17	Test of flow, pressure, etc. in Trollhättan											░	░	░															0
18	Test plan																🟥	🟥											0
19	Lab and field studies																	▨	▨	▨	🟥	🟥	🟥	🟥					0
20	Programming and signal processing																🟥	🟥	🟥	🟥	🟥	▨	▨	▨					0
21																													0
22																													0
23																													0
24	**Phase 3**																							🟪	🟪	🟪	🟪	🟪	0
25	Writing the report							▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	🟥	🟥	🟥			0
26	Presentation Acreo																									🟥			0
27	Presentation LIU																										🟥		0
28	Critical review																										🟥		0
29	Project evaluation																											🟥	0
30																													0
	Summary of hours:	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

░ Uncertain
🟥 Intensive work
▨ Progressive update
🟨 Phase 0 in progress
🟩 Phase 1 in progress
🟦 Phase 2 in progress
🟪 Phase 3 in progress

Figure 3.1: Timeplan created in the beginning of the project

3.2 Systematic Follow-up

Follow-up meetings were held with the supervisor group every Monday for month 1-2, and every other Monday when the project was in motion. Short personal meetings were held with the supervisors of the different parts to discuss simple matters. The current status and problems were discussed during the meetings, and a brief plan of how to move on was created. The Follow-up workflow can be summarised in Figure 3.2.

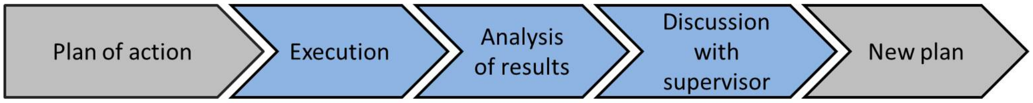

Plan of action
Execution
Analysis of results
Discussion with supervisor
New plan

Figure 3.2: Schematic of the workflow used to reach the goal.

4 Theoretical Background

4.1 Faecal Contamination

Faecal contamination is a common source for microbial pathogens (Table 4.1) in surface water [7, 8]. Faecal contamination can derive from sources like manure runoff from agricultural areas, runoff from livestock or wild animals, sewage overflow, discharge of municipal or industrial wastewater, and in some cases from rare accidents [4–6]. The majority of these contamination sources are in their nature sensitive to rainfall, which can be correlated to elevated levels of detected microbes in surface water due to increased manure runoff[2] [5] .

Table 4.1: Examples of Faecal Pathogens

Name	Type	Risk Class
Campylobacter spp.	bacterium	2
Pathogenic *Escherichia coli*	bacterium	2
Salmonella spp.	bacterium	2
Shigella spp.	bacterium	2
Vibrio cholera	bacterium	2
Yersinia enterocolitica	bacterium	2
Hepatitits A	virus	2
Hepatitits E	virus	3
Adenoviruses	virus	2
Enteroviruses	virus	-
Norwalk/noro-virus	virus	2
Astrovirus	virus	2
Rotavirus	virus	2
Entamoeba histolytica	protozoa	2
Giardia intestinalis	protozoa	2
Cryptosporodium pavrum	protozoa	2

The cost of detecting multiple organisms in surface water is too high in comparison to the benefits, and instead a faecal indicator bacteria (FIB) is used in practice [4–6, 9]. The United States Environmental Protection Agency (US EPA) declare the following criteria for an optimal indicator organism [10]:

---

[2] Monitoring of precipitation in the surrounding environment can be used as a complementary technique to contamination monitoring.

- The organism should be present whenever enteric (intestinal) pathogens are present
- The organism should be useful for all types of water
- The organism should have longer survival time than the hardiest enteric pathogens
- The organism should not grow in water
- The organism should be found in warm blooded animals' intestine
- The testing method should be easy to perform
- The density of the indicator organism should have some direct relationship to the degree of faecal pollution

No organism will perfectly fit these descriptions and these statements are used as an aiming point.

4.1.1 Escherichia coli as an Indicator

Escherichia coli (E. coli) is a highly abundant bacterium in faeces. E. coli has been proposed as the most advantageous indicator for faecal contamination [11], and is recommended by US EPA [10] as a FIB. However, there is not always a direct quantitative correlation between E. coli detection and faecal contamination [4]. Another discussed problem is the shorter survivability of E. coli in surface water compared to other pathogens [11, 12]. When targeting bacteria as a pathogen the number of living cells is the only interesting value. However, the viable count method is flawed when using E. coli as a bacteria to indicate presence of other pathogens that might survive longer in harsh conditions. To that end viable count of E. coli can show misleading information regarding the degree of faecal contamination. This error can be reduced by detecting non colony forming or intact dead bacteria as well[3], but will possibly lead to scenarios where E. coli is detected without prescence of pathogens. If overestimation is better can be discussed and depend on the cost of false positives versus the risk of not knowing at all.

---

[3] Intact dead cells or cells unable to grow into a colony will be detected in the proposed flow cytometer.

4.2 Biosensors for Bacterial Detection

Detection of waterborne pathogens is an area of extensive research and many different biosensor techniques are used [12, 13]. Some category examples are found in Table 4.2.

Table 4.2: Examples of Popular Biosensor Techniques

Technique
Bioluminescence expression induced by bacteriophages
Impedance biosensors
Surface plasmon resonance
Piezoelectric biosensors
Immunosensors based on optical detection
Immunosensors based electrochemical detection
Genosensors by PCR amplification of unique sequences
Metabolism based biosensors
Electronic noses and electronic tongues

All these categories can be divided into several techniques with more or less involvement of advanced technology. However, the most commonly used biosensor technique[4] is an automated version of basic viable count in combination with metabolism based biosensing [14–17]. This technique is based on target specific enzymatic degradation of a known metabolite that results in fluorescent or coloured products that can be monitored as explained in section 4.4. All the above techniques have pros and cons regarding sensitivity, specificity, functionality in harsh conditions, cost and speed. Metabolism based biosensors have good properties in most of these areas but are slow [15]. Since the technique is based on bacterial growth, it will only produce a detectable signal when proliferation has reached a certain stage.

4.3 On-line Monitoring

On-line monitoring, described in Figure 4.1, is the optimal contamination detection technique where the detector system is connected to the untreated water intake and acquires data in a real time fashion [7]. On-line monitoring requires fast and accurate detection methods that can be made when the sample flows through the detection chamber. On-line monitoring can give feedback to close the water intake or to take other actions in order to avoid contamination of the water purification plant.

---

[4] Most commonly used for water monitoring in the river Göta älv in Sweden.

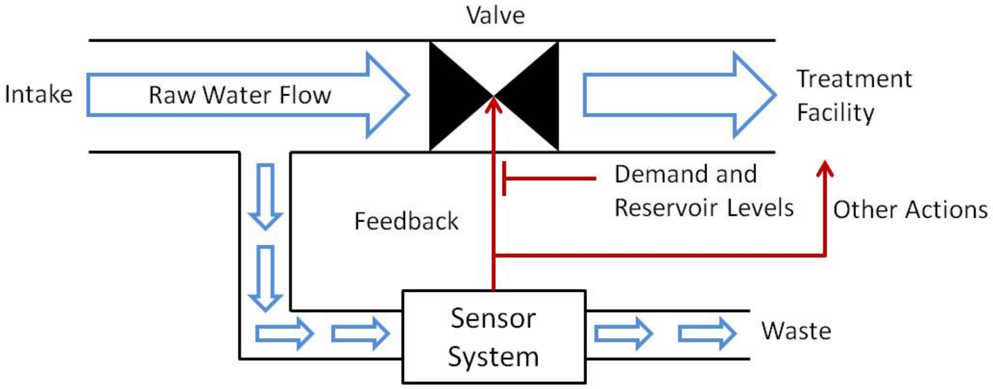


Figure 4.1: Schematic of on-line monitoring with a feedback system to close the water intake or to take other actions in the treatment facility. The closing of the water intake has to be regulated with fresh water demand and the reservoir levels in mind.

4.4 Bacterial Enumeration

The bacterial cells must be enumerated in order to evaluate the presence of faecal contamination. The standard method used is viable count by counting colonies after growth on a plate medium [18]. The concentration of the undiluted sample can be calculated (CFU/volume) by creating dilution series and counting the colony forming units (CFU). Another used method is the most probable number technique (MPN), which is similar to viable count with the difference that the bacteria are grown in broth [18]. The sample is diluted to the point that only a fraction of a set number of broth tubes show bacterial growth. The growth can be detected by turbidity or more advanced colorimetric methods, and the result can be compared with standardised tables to extract the MPN value. These techniques only detect the number of cells that are able to proliferate and are rather time consuming. More advanced detection techniques are required in order to reduce the risk of infection during the sample processing time.

In environmental samples with a mixture of different bacteria these simple approaches cannot be used. A defined substrate can be used for target specific growth of a bacterium. The target expresses a unique enzyme in order to metabolize the defined substrate, which will allow the bacterium to grow where other bacteria cannot. The method using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) for total coliforms and 4-methyl-umbilliferyl-β-D-glucuronide (MUG) for E.coli, was developed to detect urinary tract infections but was later used for water monitoring [19]. The specific bacteria can grow in this medium and will create coloured or fluorescent products that can be detected. The concentration is determined using the MPN technique.

4.4.1 ColiLert® and Colifast®

ColiLert® and Colifast® are two sensor systems for detection of E. coli and the total amount of coliform bacteria in water. Both systems are metabolism based sensors and are able to send alerts upon detection of critical levels of E. coli or other coliform bacteria.

The ColiLert®3000 system has been available since 1999 and is based on the bacterial enzymes β-galactosidase and β-glucoronidase, which will give a yellow and a blue fluorescent colour when exposed to ONPG and MUG [19]. The system runs 4×100 mL samples each day and has a detection limit of 1 coliform [CFU]/100 mL. The detection time depends on the bacterial concentration and higher concentrations give a lowered detection time. The time needed to detect the lowest detectable concentration is 17 hours [17].

The Colifast® at-line monitoring system (CALM) is based on the enzyme β-galactosidase and the molecule 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) that will fluoresce when cleaved by the enzyme. The system can detect 1-100 coliforms [CFU]/100 mL in 6-11 hours and high bacterial concentrations can be detected in less than 1 hour. The systems can give an earlier indication within 4-6 hours after the initiation of the process [15].

Trollhättan Energi is using the ColiLert technique, and Göteborg Vatten is using ColiLert as well as the automated ColiFast system. The time from sampling to result is approximately 20 hours, including sample transport and result evaluation, but methods like these are not common in this field. Many purification facilities send untreated water tests to lab and get results in 2-3 days, and it is possible that some facilities do not test untreated water at all since it is not demanded by the Swedish Food Agency[5].

Even though these methods are faster than standard enumeration methods, the detection time can still be an issue. Extensive amounts of water can still pass through the water treatment facilities during the time it takes to receive the warning. The systems are functional for on-line monitoring but they are not fast enough to be fully functional as effective feedback systems.

---

[5] Information from Johanna Hilding, Process Engineer at Trollhättan Energi AB, personal communication, May 2014

4.5 Flow Cytometry

Flow cytometry, depicted in Figure 4.2, is an optical detection and sorting method for particles, usually cells, in a capillary micro flow channel. A small sample volume in combination with flow mechanics, allows detection and sorting of single cells. Flow cytometry can be used by detecting different light scattering properties of different cell types and shapes, as well as specific targeting by fluorescent staining [20, 21]. Flow cytometry in the latter case often fall under the immunosensor category, when using antibody-dye conjugates to stain different cell types in different colours. Flow cytometry has a wide range of applications from medicine to environmental microbiology [21, 22]. Detection and sorting of E. coli and other bacteria from lake and sewage water has been presented elsewhere [23].

Flow cytometers are mainly used with wavelengths in the visible spectral region. Recently, there is interest in extending flow cytometers into the NIR region to increase the range of available staining methods and to reduce background fluorescence in biological samples [24, 25].

Advances in detector and laser technology has made it possible to create relatively inexpensive and simple flow cytometers in smaller sizes. The production of a very simple cell counter using micro fluidics has been presented elsewhere [26], and is an indication of the future direction of this field. The flow cytometry principle is well suited for on-line monitoring, since it is already based on flow through detection mechanics.

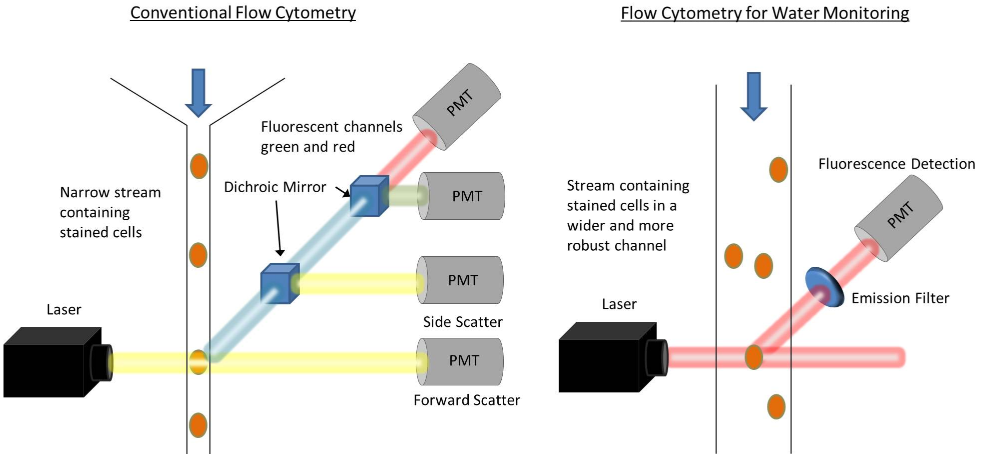

Conventional Flow Cytometry
Flow Cytometry for Water Monitoring
PMT
Fluorescent channels green and red
Dichroic Mirror
PMT
Narrow stream containing stained cells
PMT
Laser
Side Scatter
PMT
Forward Scatter
Fluorescence Detection
Stream containing stained cells in a wider and more robust channel
PMT
Emission Filter
Laser

Figure 4.2: Schematic of a conventional flow cytometry setup (Left), to detect scattering properties as well as fluorescence, and the cytometry setup proposed to use for water monitoring (Right). The cytometer for water monitoring is more basic and have larger dimensions to fit the industrial environment, water pressure and water throughput.

4.6 Fluorescence

Fluorescence is the process in which a molecule reaches an excited electron state by absorbing electromagnetic radiation, and the subsequent relaxation to the ground state resulting in emission of electromagnetic radiation. This process does not occur for the majority of the molecules found in nature, and is typically present in molecules with large amounts of π-bonds. This feature is highly usable to detect specific molecules with high sensitivity due to the low background noise [27,28]. The process can be seen in Figure 4.3 showing several available excitation paths and several relaxation paths.

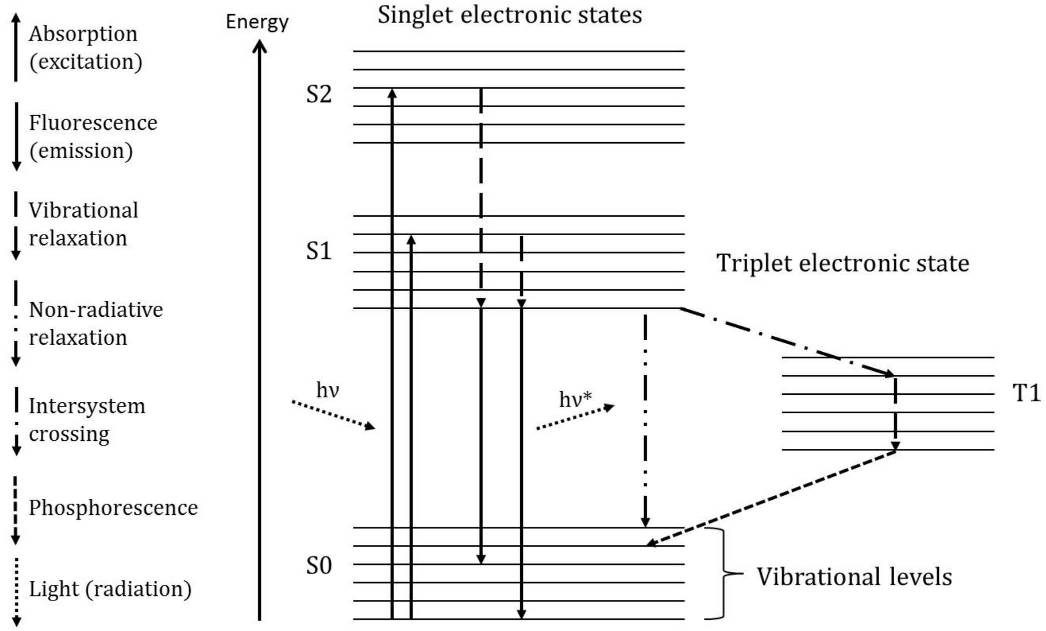

Absorption (excitation)
Fluorescence (emission)
Vibrational relaxation
Non-radiative relaxation
Intersystem crossing
Phosphorescence
Light (radiation)
Energy
Singlet electronic states
S2
S1
S0
hν
hν*
Triplet electronic state
T1
Vibrational levels

Figure 4.3: Jablonski diagram of of the absorption/emission process. Fluorescence S1-S0, Phosphorescence T1-S0 and non-radiative relaxation are the three available relaxation paths. $h\nu > h\nu^*$ mainly due to vibrational relaxation.

Quantum yield (ϕ) [28] (4.1) is the ratio of the absorbed light and the emitted light, and is a measure of the efficiency of a fluorescent molecule. As the molecule can reach the ground state in several non-radiative relaxation processes, the quatum yeld of most fluorophores is < 1.

$$\phi = \frac{Number\ of\ Photons\ Emitted}{Number\ of\ Photons\ Absorbed} \tag{4.1}$$

The fluorescence intensity (F_λ) is the signal detected, and should be as high as possible in order to separate it from the background and hardware noise. The fluorescence brightness is a measure of the theoretical fluorescence intensity of a dye, and is defined as the Quantum yield times the absorbed light. The absorbed light is generally calculated by the Beer-Lambert law described in equation 4.2 [27, 28].

$$I = I_0 e^{-ln(10)\varepsilon_\lambda cl} \tag{4.2}$$

Where I_0 is the the incident light intensity, I is the transmitted light intensity, ε_λ is the extinction coefficient of the fluorescent molecule, c is the concentration of the fluorescent molecule and l is the path length of the incident light within the sample. The extinction coefficient is the constant that will decide the ratio between the incident light and the transmitted light, and has the unit $M^{-1}cm^{-1}$. The fluorescent intensity (F_λ) measured at a specific wavelength can be calculated using equation 4.3 [27].

$$F_\lambda = I_0\ ln(10)\ \varepsilon_{\lambda^*}\ c\ l\ \phi f_\lambda\ [j] \tag{4.3}$$

Where f_λ is the fraction of emitted light at wavelength λ and j is the portion of the light detected by the detector also known as Quantum Efficiency (QE). j is written in brackets to emphasize that it does not affect the real intensity but rather the intensity that can be detected, depending on the detector of choice. The wavelength λ^* is to emphasize that the excitation and the emission wavelength are generally not the same. Note that the linear expression is true for relatively low concentrations of dye and might also be affected by the intensity of the light due to bleaching effects. The typical appearance of the emission (F_λ) spectrum and the excitation spectrum can be seen in Figure 4.4.

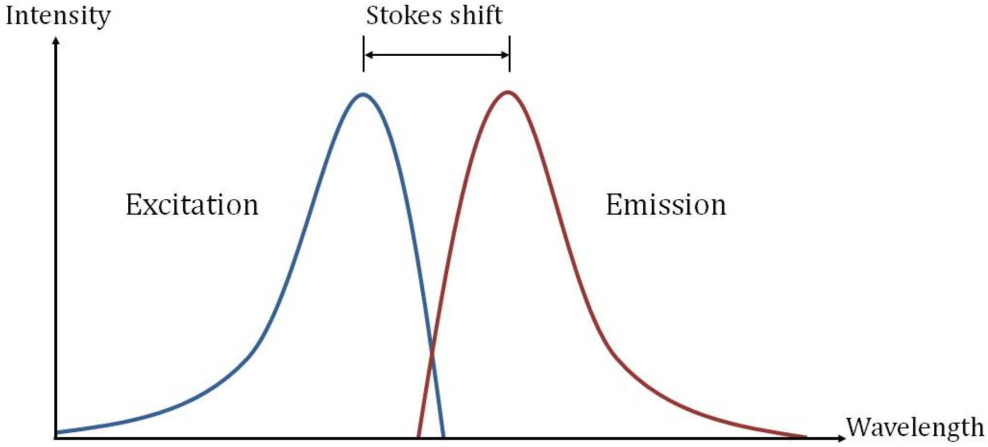


Figure 4.4: Illustration of the excitation and emission spectrum separated by the stokes shift.

The fluorescent signal is in most cases significantly lower than the incident light, which makes it hard to separate the fluorescent signal from the illuminating source. Two facts are used to enable separation. The fluorescent intensity is uniform in all directions, which makes it possible to detect the fluorescent signal at a 90 degrees angle to the light source beam. There is also a red shift between the excitation and emission maxima called Stokes shift (Figure 4.4), which makes it possible to use filtering to separate the excitation and emission [27]. The Stokes shift can derive from several physical phenomena, one being the loss of energy due to the vibrational relaxation described in Figure 4.3 [28]. The energy is inversely proportional to the wavelength, according to the Planck relation in equation 4.4 where E is energy, h is the Planck constant, c is the speed of light and λ is the wavelength. The fact that $h\nu^* < h\nu$ (Figure 4.3) is associated with the emission being shifted towards higher wavelengths.

$$E = h\nu = \frac{hc}{\lambda} \tag{4.4}$$

4.6.1 Auto Fluorescence

Auto fluorescence is an expression for the fluorescence from biomolecules in cells or tissue. Auto fluorescence is regarded as negative when staining samples, since it will create a higher background level that will make it harder to resolve the stained areas. Auto fluorescence must always be taken into consideration when working in the UV-VIS range of light and with biological samples. Fluorescence from chlorophyll in algae will be present in water samples and will interfere with the measurement. Chlorophyll fluoresce in a very broad peak in the 600 - 700 nm range, and has a very long tail into the NIR region [29]. Other types of algae are also present and will emit light over a wide spectrum [30]. When working with untreated water samples there will always be a risk that the sample contains algae, and therefore preferable to move the excitation and emission above the range of chlorophyll fluorescence, to NIR light.

4.6.2 NIR Fluorescence

NIR fluorescence is commonly used when staining biological samples, in order to avoid auto fluorescence. NIR light penetrates cells and tissue, which is used for immuno-fluorescent imaging of for example tumors in small animals [31]. NIR dyes are generally larger than visible dyes, with a molecular weight of 1000-3000 Da, which creates some practical issues. NIR dyes have lower solubility, tend to aggregate and have a lower Quantum yield resulting in a lower intensity compared to visible dyes. The general solution to these problems is to introduce negatively charged sulfonic groups that will increase the solubility and the Quantum yield [32]. The introduction of negative charges might alter the isoelectric point of the antibody. The isoelectric point is important to maintain the function of the antibody, and disturbing the charge distribution can therefore cause loss of function or increase nonspecific binding. However, NIR dyes might be hard to use in water based systems if the modifications of the dye are not made.

4.7 Antibodies

Antibodies are large proteins, typically around 150 kDa for IgG, that are produced in animals as a part of the immune system. The function of antibodies is to bind to a target antigen/epitope on an invading pathogen or toxin with high specificity and affinity. Antibodies make the target easier to detect by the rest of the immune system, as well as neutralising pathogens and toxins by aggregate formation or by blocking active membrane groups, that are needed for the pathogen to survive in the body. [20]

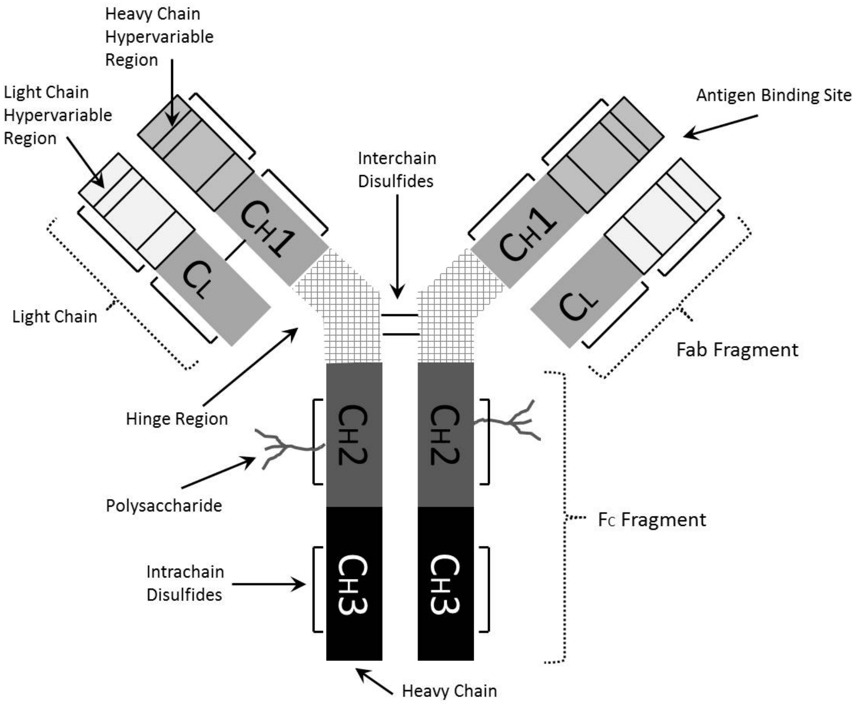

Heavy Chain Hypervariable Region
Light Chain Hypervariable Region
Antigen Binding Site
Interchain Disulfides
CH1
CH1
CL
CL
Light Chain
Fab Fragment
Hinge Region
CH2
CH2
Polysaccharide
Fc Fragment
Intrachain Disulfides
CH3
CH3
Heavy Chain

Figure 4.5: Structure of a typical IgG [33].

The structure of the antibody (Figure 4.5) is crucial for its specificity and affinity towards the target antigen, and displacement of the delicate structure can affect the antibody function [34,35]. When labeling antibodies with molecules like fluorescent dyes, there are some possible problems to be taken into consideration:

- The dye or the coupling method can cause rearrangements in the antibody so that the specificity and affinity is changed or lost
- The dye can block antigen binding site (hypervariable region, see Figure 4.5)
- The dye or the coupling method can cause aggregation of antibodies
- The dye prevents the antibody to reach the the antigen on cells surfaces due to steric hindrance, e.g. caused by the presence of lipopolysaccharides (LPS) on the cell surface

The antibody antigen interaction can be described by the equilibrium expression 4.5.

$$[AbAg] \rightleftharpoons [Ab] + [Ag] \tag{4.5}$$

Which turns into the equilibrium equation 4.6.

$$[AbAg] = K_A[Ab][Ag] \tag{4.6}$$

Where [Ab] is the free antibody concentration, [Ag] is the free antigen concentration and [AbAg] is the bound complex concentration. In order to measure the affinity of the antibody-antigen interaction the fraction of bound antigen to the total amount of antigen is introduced in equation 4.7.

$$r = \frac{[AbAg]}{[Ag]_{tot}} = \frac{[AbAg]}{[Ag] + [AbAg]} = \frac{K_A[Ab][Ag]}{[Ag] + K_A[Ab][Ag]} \tag{4.7}$$

Which is further simplifed to equation 4.8.

$$r = \frac{K_A[Ab]}{1 + K_A[Ab]} \tag{4.8}$$

Multiplying both sides in equation 4.8 with $1 + K_A[Ab]$ gives equation 4.9.

$$r + rK_A[Ab] = K_A[Ab] \tag{4.9}$$

With further rearrangement 4.9 turns into equation 4.10.

$$\frac{r}{[Ab]} = K_A - rK_A \tag{4.10}$$

Which is the 1:1 form of the commonly known Scatchard equation [20], with the difference that the Scatchard equation is mainly used with the antigen as the varying concentration. Results can be plotted in a Scatchard plot as $\frac{r}{[Ab]}$ versus r which will be a straight line with slope $-K_A$.

Equation 4.11 is used to calculate the dissociation constant K_D which is commonly used to describe the antibody-antigen interaction. K_D is the free antibody concentration of which the binding event is 50% saturated. This method has been summarised and evaluated elsewhere [36] and is referred to as indirect ELISA determination of dissociation constants.

$$K_A = \frac{1}{K_D} \tag{4.11}$$

Antibodies usually have two binding sites, which will alter these equations. This can be neglected to simplify the measurement and calculations, and the model is based on the assumption that one antibody binds to one site at the target bacteria.

It is not possible to determine the affinity when working with polyclonal antibodies, since it is a mixture of antibodies targeting different sites on the antigen. In that case it is only possible to measure the mean affinity as a measure of the total strength of the interaction. The antibody used in this project is not affinity separated and contain high amounts nonspecific antibodies. The term affinity is used to describe the average ability of this mixture to stain E. coli. The dissociation constant presented in this project is not the real value, and is mainly used to compare unlabeled and labeled antibodies to see if there is loss of affinity due to labeling. To make this clear K_D will be stated as $\overline{K_D}$ further on.

4.7.1 IgY

IgY is an immunoglobulin found in birds that is usually extracted from chicken egg yolk. The fact that antibodies from the immunised chickens are transfered to the egg yolk has been known for a long time. There are indications that the amount found in egg yolk is even higher than the amount found in the serum. This method of antibody production does not require bleeding of animals and produces larger amounts of antibodies, approximately 1500 $mg/month$ for avian IgY compared to 200 $mg/month$ for mammalian IgG [37].

There are several proposed advantages using IgY, rather than the traditional mammalian IgG. IgY recognises more epitopes on mammalian proteins, and the amount of bound mammalian secondary antibody will be three to five times higher if a primary IgY is used. This makes IgY preferable when working with immunoassays. More advantages can be found in the fact that IgY does not activate the complement system, and that IgY does not bind to human or bacterial Fc-receptors. Fc-receptors are regions that can bind the constant Fc part of the antibody (Figure 4.5), which will increase non-specific binding to bacteria. The latter fact makes IgY highly useful for microbiological assays. IgY is recommended for use in both research and industry applications due to the advantages, the reduced production cost and increased animal wellbeing [37].

4.8 Coupling Chemistry

One widely used coupling procedure is the reaction of succinimidyl esters and primary amines to form an amide bond, seen in Figure 4.6. Proteins such as antibodies have several free primary amines, which makes this a suitable technique that is used in most commercial antibody labeling kits [38]. Succinimidyl ester coupling is the best approach in terms of cost, yield and simplicity of the labeling process. It is also known that coupling to primary amines is the most gentle technique and has lower effect on the antibody function compared to other techniques [34]. The kit used in this project is based on a succinimidyl ester conjugation technique.

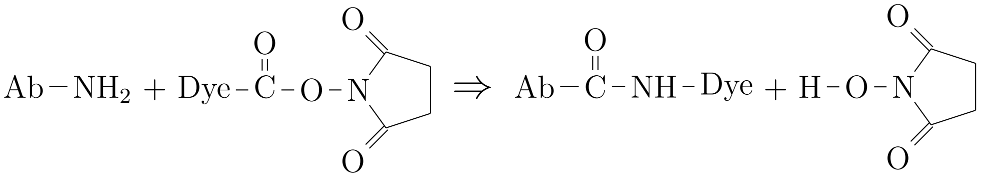


Figure 4.6: Illustration of the reaction of succinimidyl ester functionalised dye and primary amines, to form an amide bond.

The reaction is favored by alkalinity in order to keep the primary amines deprotonated. Deprotonated amines will have a slightly negative net charge that can react with the slightly positive carbon in the link between the dye and the succinimidyl ester. To that end, a bit of sodium bicarbonate is used to increase the alkalinity. It is important to keep the antibody solution free from ammonium ions or primary amines, such as BSA containing buffers or Tris buffers, for an efficient labeling.

4.9 Mussel Adhesive Protein

Mussel adhesive protein (MAP) is a protein that is used by marine organisms to attach to a variety of substrates, e.g. mineral, metal, plastic and wood surfaces. The protein is highly effective in creating a cross-link between biological and synthetic materials. Attachment of E. coli to ELISA plate wells using MAP has been demonstrated elsewhere [39].

4.10 Optical Hardware for NIR

Most optical components are optimised for the visible region of light. Working in the NIR region will create challenges with either higher prices of NIR compatible components, or loss of signal with the available equipment for visible light. This is problematic when working with sensor systems, where lack of sensitivity is a common problem. The fact that NIR dyes tends to produce lower signal [40] will increase the challenge even more. This Subsection will present some theory of the different components and what problems to expect.

4.10.1 The Detector

There are basically three different kinds of optical detectors used in this area of research; photomultiplier tubes (PMT), charge coupled devices or complementary metal–oxide–semiconductor devices (CCD or CMOS cameras) and avalanche photodiodes (APD), which are semiconductive detectors with an integrated electron multiplication. PMTs are generally the most sensitive detectors due to the integrated amplification via electron multiplication, but will lose sensitivity in the NIR region (Figure 5.2B). CCD cameras are suitable for the NIR region but cameras with the sensitivity needed are quite expensive. APDs have integrated amplification, are less sensitive in the visible region but extend further into the NIR region. There are indications that PMTs are not sensitive enough for NIR flow cytometry, and that an APD with high amplification is the suitable choice [24, 25].

4.10.2 Optical Components

Optical components are usually optimised for a band of wavelengths and an anti-reflective (AR) coating is used to match these wavelengths. The AR coating used in the visible range loses its function as the light gets closer to infrared. Optical components, such as objectives and focusing lenses, might have increased reflection that can cause strange optical effects, when used for NIR light. AR coatings for NIR-IR is available and entirely new equipment is recommended to achieve the best results. However, high quality optics in fluorescence microscopy can still be used this close to the visible spectrum. Further information can be found through Thorlabs or Olympus Corporation.

5 Materials

5.1 E.coli strains

The E. coli genotypes used in this project include a K12 strain and two standard laboratory strains XL1 Blue and XL10 Gold. These strains were used by Acreo to produce the antibodies for this project. The idea behind the choice of E. coli genotypes was that these basic strains would present a standard minimum antigen expression, with surface structures that are crucial for all E. coli types that can be found in nature. The strain used for basic affinity measurement was the wildtype K12 strain.

5.2 Immunoglobulins

IgY antibodies were produced by the company Agrisera from hen egg yolk. IgY is slightly heavier than the most commonly used IgG antibodies with a molecular weight of 167250 Da [41], and has an extinction coefficient of approximately $210,000\ M^{-1}cm^{-1}$ at 280 nm [42,43]. The IgY mixture was not affinity separated and contained an unknown ratio of antibodies specific to E. coli and other antibodies. Agrisera states that there is usually 0.5-10 % specific antibodies targeting E.coli. These antibodies were obtained in order to perform a simple and inexpensive test to indicate the properties of the sensor system.

5.3 Alexa Fluor 790 Antibody Labeling Kit

The IgY antibodies were labeled using the Alexa Fluor 790 Labeling kit, which is a standardised procedure in order to label small amounts antibodies for research applications. The kit contains dye and purification columns to separate the labeled antibodies from free dye. Further information can be found at the web page of Life Technologies$^{\text{TM}}$ and the protocol can be found in Appendix C. The dye has a molecular weight of approximately 1750 g/mol, and the extinction coefficient is $260,000\ M^{-1}cm^{-1}$ at 785 nm, which is rather high compared to other dyes in the Alexa Fluor series. The Quantum yield is not stated in the product sheet and could not be obtained from the company, but there are some indications that NIR-dyes have lower Quantum yield than visible dyes [40]. However, a lower Quantum yield might not be an issue due to the high absorption of the molecule. An indication that Alexa Fluor 790 is one of the brightest NIR dyes have been presented elsewhere [44]. The excitation/emission spectra of Alexa Fluor 790 can be seen in Figure 5.1

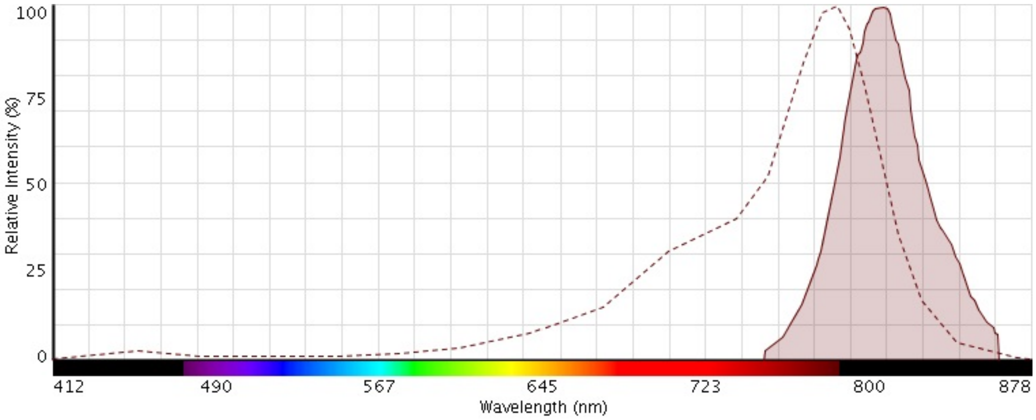


Figure 5.1: Excitation/emission spectra of Alexa Fluor 790. The graph is from the Fluorescence SpectraViewer function at Life Technologies^{TM} homepage.

5.4 Buffers and Reagents

The buffers and reagents used can be found in Table 5.1.

Table 5.1: Buffers and reagents

Type	Function
1M PBS	Standard buffer for biochemical procedures
MAP	Immobilise E.coli on microtiter plates
BSA	Stabiliser and blocking
Tween 20	Minimises nonspecific binding
Sodium Azide	Preservative to avoid bacterial growth
Sodium Bicarbonate	Change pH to increase the reactivity of primary amines
Coating Buffer	Used to coat the microtiter plates with MAP
Rabbit α-chicken FITC	Secondary antibody
Chicken α-E. coli	Primary antibody

Phosphate buffered saline (PBS) was used as the standard buffer for antibodies and cell suspensions. Mussel adhesive protein (MAP) was used to attach E. coli to microtiter plates during immunoassay experiments. Bovine serum albumin (BSA) was used as a stabilizer for antibody solutions and cell suspensions, as well as blocking agent during immunoassays and to block the flow channel. Proteins can bind to glass or plastic via hydrophobic or dipole attraction and BSA was used to block the available binding sites. This will stop antibodies or cells to bind to surfaces, which is important since nonspecific binding can produce errors at low protein concentrations. BSA will also bind to cells and antibodies to block nonspecific sites in order for the specific staining to be more accurate. Tween 20 is a detergent that reduces nonspecific binding during immunoassays. Sodium azide is toxic and is used to prevent bacterial growth in cell suspensions or antibody solutions. Sodium bicarbonate was used to increase the reactivity of antibody labeling process. The coating buffer, containing 0.3 % sodium carbonate and 0.6 % sodium bicarbonate in Milli-Q water, was used to increase the coating efficiency of MAP. The secondary antibody was used to detect bound IgY during immunoassays.

5.5 Optical setup

The optical components used in the sensor system can be found in Table 5.2.

Table 5.2: Optical Components

Component	Reference Number	Supplier
800 nm long pass filter	FEL0800	Thorlabs
780 nm band pass filter	FL780-10	Thorlabs
760 nm band pass filter	FB760-10	Thorlabs
785 nm Laser	L785P090	Thorlabs
760 nm Laser	QLD-760-10S	QPhotonics
CMOS Monochrome USB camera	83-769	Edmund Optics
CMOS microscope camera	ZYLA 3-tap	Andor
PMT	R928	Hamamatsu
10x and 20x Objectives	UPlanFl	Olympus
Circular flow channel chromatography tube	160-2530-10	Scantec Lab
Square flow channel	131.050-QS	Hellma Analytics

The filter setup in Figure 5.2A was the most suitable configuration available at Thorlabs. Note that the setup has a small overlap at $792 - 793\ nm$ that was known to be a limitation of the function, especially when the laser was not wavelength tested and could deviate 10 nm from the mean position at 785 nm. There are not many available lasers between 730 nm and 780 nm due to technical and material problems when designing laser in this range. The laser for this application had to be chosen close to the emission.

The CMOS camera was used to align the system since NIR light is outside the range of human vision. CCD or CMOS cameras are sensitive in the NIR region [25]. A high sensitivity CMOS microscope camera was used as a best possible scenario indication to test the system.

The Photomultiplier tube (PMT) was first acquired for the 600 nm range and has lower sensitivity at 800 nm (Figure 5.2B). Due to high S/N-ratio in the initial system setup, this was thought to be a minor problem. The Equivalent Noise Input, which is a measure of the sensitivity in the detector, will increase towards 800 nm, meaning that a higher intensity has to be detected to give the same S/N as before. The company Hamamatsu states that the variation between tubes is high above 800 nm so that the functionality of individual PMTs is unknown.

The microscope objective, used to magnify the flow channel, had to be changed to the objective used in a fluorescence microscope, due to insufficient transmission in the NIR region of the simple objective used in the initial system setup. The UPlanFl objectives used in the fluorescence microscope has a 10 % reduced transmission at 800 nm which is acceptable. The lack of suitable AR coating for NIR light can possibly create some strange optical effects.

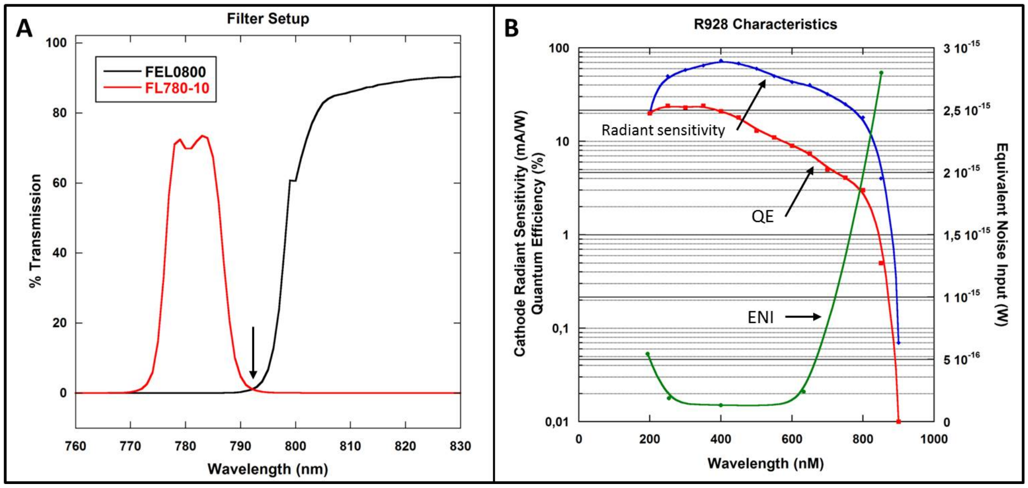


Figure 5.2: (A) The filter setup used for the NIR system. The arrow indicate the critical region where the filters overlap. (B) Properties of PMT R928. Data points are extracted from information from Hamamatsu and fitted using a smooth curve fit. The equivalent noise input (ENI) describes the intensity needed to produce a signal equal to the noise and is a measure of the detector sensitivity. Further information can be found at the homepage of Hamamatsu. There is uncertainty regarding the slopes due to large distance between discrete data points. Note that the left Y-axis is in logarithmic scale and that the right Y-axis is in linear scale.

5.5.1 Aperture

The aperture is a hole with adjustable size in front of the sensor area in cameras. The function is to regulate the amount of light that falls on the sensor area. Using a wider aperture gives a brighter picture with less focal depth and contrast. Using a smaller aperture gives a darker image with increased contrast and focal depth [25]. The light will be limited to the light that can pass through the center of the objective where the highest quality can be found, and this will remove unwanted optical effects, reflections and distortions. The aperture was used to enhance the functionality of the PMT since the optics used was not optimal for NIR wavelengths.

5.6 Equipment

The equipment used can be found in Table 5.3.

Table 5.3: Equipment

Equipment	Description
Incubator	Bacterial growth environment
Spectrafuge 16M	Table top centrifuge
ElmerPerkin Lambda 25	Spectrophotometer
Fluoromax-P	Spectrofluorometer
Fluoroskan Ascent FL	Plate reader
TI DAQ	Data acquisition card
Micro flow pump	Older model from Ismatec
Nunc Immuno Wash 8	Semiautomatic plate washer

The incubator was used for cultivation of E. coli on agar plates. The spectrafuge 16M was used to wash cells and as driving force for the column separation during the antibody labeling. The spectrophotometer was used to determine antibody concentration and fluorophore to antibody ratio of labeled antibodies. The Fluoromax-P was used for early saturation tests and to measure the excitation and emission spectra of antibodies labeled with Alexa Fluor 790. The Fluoroskan Acent FL was used to measure FITC during immunoassay experiments. The Data acquisition card was used to convert the analog PMT signal into a digital signal used in LabView. The micro flow pump was used to test the hardware. The Nunc Immuno Wash 8 was used to wash microtiter plates between different immunoassay steps.

The plate reader is not usable in the NIR range and the Fluoromax-P has reduced sensitivity in the NIR range, since the detector is a PMT with loss of sensitivity above 800 nm, as explained in the previous subsection.

6 Method

6.1 Bacterial Cultivation

E. coli from the K12 strain was cultured over night on agar plates in a chamber at 37°C with an atmosphere of 5% carbon dioxide. The cells were suspended in PBS and stored at 4°C prior to use.

Direct cell count of samples with high concentration can be determined using a Petroff Hausser counting chamber. A Petroff Hausser counting chamber is a special microscope slide with a pattern of wells that are 1/50 mm deep, with an area of 1/400 mm^2, so that each small chamber has a volume of 5×10^{-5} mm^3 and the total counting chamber has a volume of 0.02 $mm^4 = 0.02$ μL. The number of cells in each chamber can be counted and the total cell content can be calculated in $cells/mL$. This approach is used in laboratory trials to get a fast estimate of the cell concentration, which is used to set up experiments where a specific cell concentration is needed.

6.2 Antibody Labeling

The IgY stock solution targeting E. coli, with a concentration of 25 mg/mL, was diluted in PBS containing 0.01% sodium azide. A tenth the volume of 1M sodium bicarbonate was added to the antibody solution to increase the alkalinity. The antibody solution (100 μL) was transfered to the vial of reactive dye and incubated for 1 hour at room temperature. The labeling solution was transfered to the separation column provided in the labeling kit. The column was centrifuged at $1100 \times g$ into a collection tube. The column allowed the larg antibody conjugates to pass while the small free dye molecules were stuck in the stationary phase. The labeled antibody solution was stored at $4°C$. The protocol can be found in Appendix C.

Three batches were prepared using different antibody concentrations, to test if this produced antibodies with different fluorophores per protein ratios (F/P). The initial concentrations used were 1 mg/mL, 1.5 mg/mL and 2 mg/mL.

6.3 Degree of Labeling

The degree of labeling or F/P was measured and calculated using absorbance measurements at 280 nm (A_{280}), to measure the protein content, and at

785 nm (A_{785}) to measure the dye content. The equations used to calculate the F/P are presented in equation 6.1 and 6.2.

$$[Ab] = \frac{[A_{280} - (A_{785} \times CF_{280})] \times dilutionfactor}{\varepsilon_{280}} \tag{6.1}$$

$$F/P = \frac{A_{785} \times dilutionfactor}{\varepsilon_{785} \times [Ab]} \tag{6.2}$$

ε is the molar extinction coefficient and CF_{280} is the correction factor for the contribution from the dye at the wavelength 280 nm.

Labeled IgY (2 μL) was diluted in 150 μL PBS in a 100 μL quartz cuvette with a lightpath of 10 mm from Hellma Analytics. PBS was used as a reference. The sample was measured at 280 nm and 785 nm and the F/P was calculated. The emission spectra was measured as a control and was normalised to the antibody concentration to confirm the calculations.

6.4 Affinity

An immunoassay (Figure 6.1) was designed to test the functionality of the labeled antibodies and to evaluate the effect of the labeling process. A fixed amount of E. coli was immobilised on a microtiter plate, and the cells were incubated with different antibody concentrations in a dilution series. A secondary antibody labeled with fluorescein isothiocyanate (FITC) was used to target the specifically bound IgY to allow measurement in the plate reader. This data can be plotted as a saturation curve or as a Scatchard plot seen in Figure 6.2. Note that concentration of antibodies binding specifically to E. coli is unknown, and that the antibody concentration stated is the sum of the specific and other antibodies. The data will indicate the function of the antibody solution, but cannot be used to draw any conclusions regarding individual E. coli specific antibodies. The protocol used can be found in appendix B.

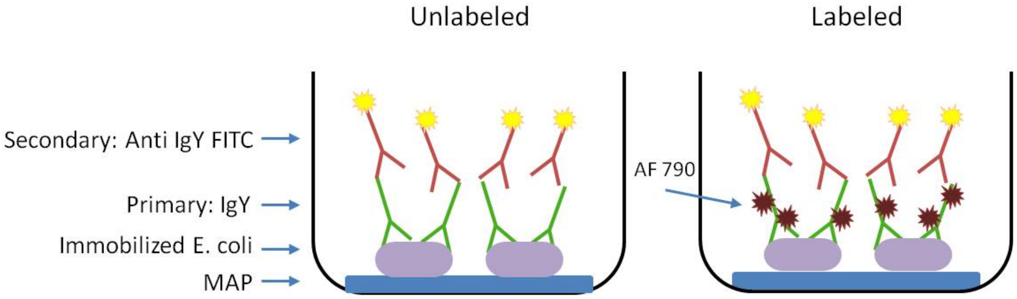


Figure 6.1: Procedure used to measure the affinity by a two-step assay with secondary antibody labeled with (FITC).

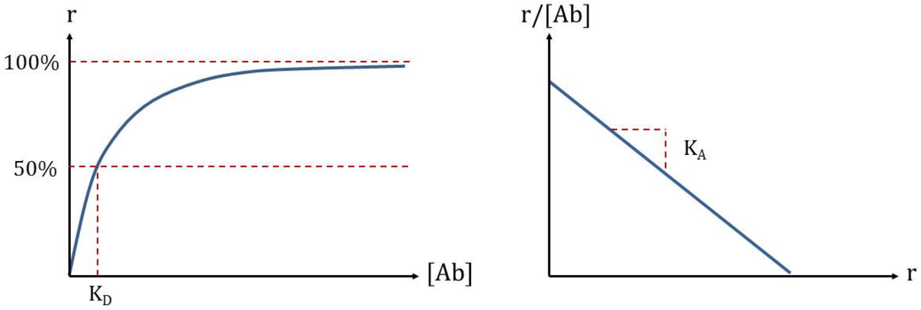


Figure 6.2: Theoretical graph appearances and how the association constant and dissociation constant can be calculated. The left graph is a saturation curve and the right graph is a Scathcard plot.

The saturation assay method was simplified using an semiautomated plate washer setup depicted in Figure 6.3

Matlab simulations were made to get an idea of what to expect from the experimental trials. The code can be found in Appendix D.

KaleidaGraph was used for curve fitting of the data and the linear regression function LINEST in Excel was used to produce statistical data of the slope constants. A student's t-test in Excel was used to indicate if the difference between labeled and unlabeled antibodies was statistically significant.

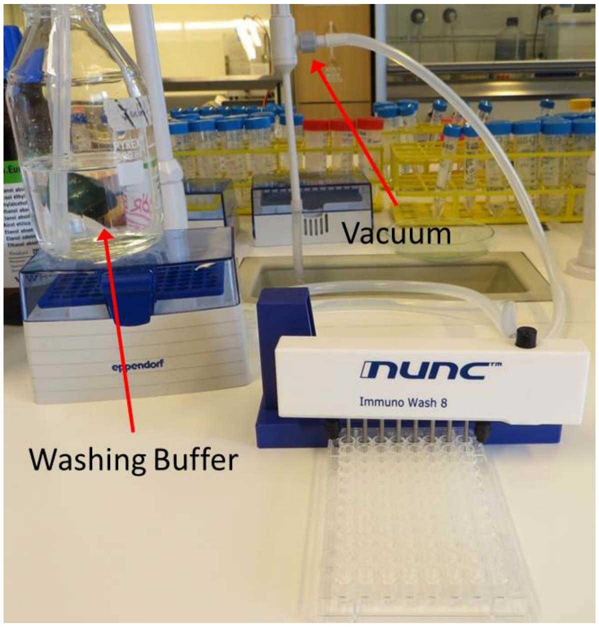


Figure 6.3: Picture of the semiautomated plate washer setup.

6.5 E. coli Immobilization

E. coli was immobilised in microtiter plates in order to perform the binding assay, developed to evaluate the affinity of the labeled antibodies. Mussel adhesive protein (MAP) was used for the immobilisation and the protocol can be found in Appendix A. A capture antibody immoblised in microtiter plates were used as a reference to evaluate the MAP efficiency. A small drop of unlabeled IgY was added to the center of the well to demonstrate the capture antibody principle. Some results regarding the immobilisation are also presented as a side project that was of interest to Acreo Swedish ICT, and to demonstrate the assay design.

6.6 Sensor System Evaluation

Fixed E. coli were labeled, using the same labeling kit as for the antibodies, to attach Alexa Fluor 790 directly onto membrane proteins, and the bacteria were washed to remove unbound dye. This was done to create a reference sample with high intensity and low background, explained in Figure 6.4, to be used to test the hardware. Samples containing different amount of cells were prepared and pumped into the measurement chamber using a micro flow pump. The performance of the system was evaluated using a high sensitivity CMOS microscope camera from Andor.

Antibody-mediated staining was done by mixing E. coli fresh from agar plates in PBS containing 0.5-1 % BSA. The concentration of labeled antibody used was in the range of $1-10\ \mu g/mL$. The samples were diluted in water prior to use to increase the signal to noise ratio by decreasing the background fluorescence. The dilution was a simple procedure to reduce the effect of unbound and nospecific antibodies in the sample.

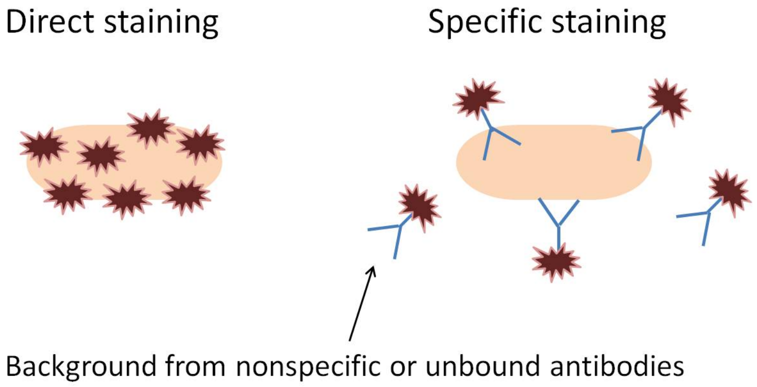


Figure 6.4: The different staining methods to evaluate the system.

Staining of environmental samples were done using no blocking or blocking by a 1:1 mixture of environmental sample and PBS buffer with 1 % BSA. The staining were done by adding approximately $1\ \mu g/mL$ antibodies to the unblocked or blocked sample.

Testing was conducted at Trollhättan Energi to demonstrate the performance of the system in the future environment, including the future pump system. This was done by adding directly stained cells to fresh untreated water taken from a valve in the water treatment facility. The sample was measured using a simple CMOS camera and a PMT.

6.6.1 Background Fluorescence Evaluation

Untreated water and treated sewage water samples were tested, without modification, to make sure that the fluorescence from chlorophyll in algae containing samples was low at this wavelength. The samples were examined in the fluorescence microscope to confirm the algae content, using a filter setup with excitation between 530 and 550 nm and emission above 590 nm, which had previously been shown to produce fluorescence from chlorophyll in algae cells.

A reference sample containing E. coli in PBS was tested to evaluate if the scattering from cells could affect the measured signal.

7 Results

7.1 Final Results Antibody Labeling

Similar F/P values were measured for the three batches of labeled antibodies (Table 7.1). The values were controlled by measuring the emission spectra and normalising the intensity to antibody concentration (Figure 7.1). The peak of the emission was shifted to 800 nm, instead of the expected 810-815 nm peak position stated in the product sheet. However, shifts like these are reasonable according to the customer service at Life Technologies. The final antibody concentration was approximately 70 % of the initial antibody concentration, but appears to decrease with increasing initial antibody concentrations. The resulting batch volume was slightly larger than the added antibody volume, and is the main reason for the reduced concentration of the labeled antibody solution.

Table 7.1: F/P and antibody concentration before and after separation column.

Batch	$[ab]_{Before}$	$[ab]_{After}$	F/P
1	1 mg/mL	0.756 mg/mL	3.51
2	1.5 mg/mL	1.07 mg/mL	3.47
3	2 mg/mL	1.404 mg/mL	3.29

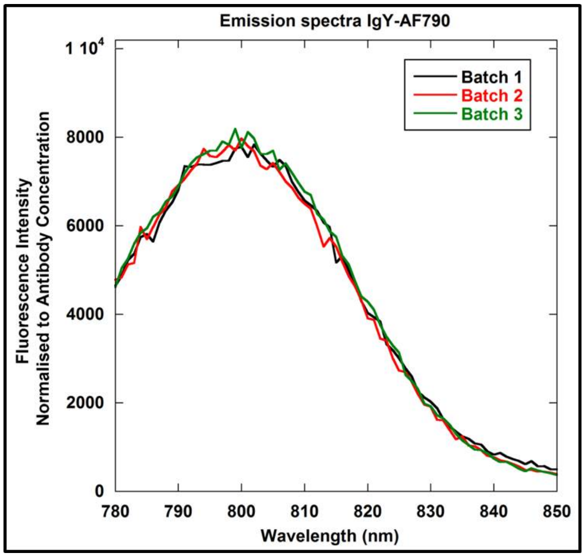


Figure 7.1: Emission profile of labeled antibodies normalised to antibody concentration.

7.1.1 E. coli Immobilization

The immobilization using MAP was comparable to immobilization using a capture antibody, and is demonstrated in Figure 7.2. E. coli immobilisation without any immobilization agent showed minimal attachment of E. coli to the microtiter plate. The use of a coating buffer with sodium carbonate and sodium bicarbonate (pH 9.6) gave the best result when coating the microtiter plates with MAP. The coating buffer increases the alkalinity, which will improve the cross-linking between MAP and the microtiter plate. Increasing the cell concentration from $\sim 10^8$ to $\sim 10^9$ $cells/mL$ resulted in a higher and more homogeneous E. coli immobilization (Figure 7.2 C-D).

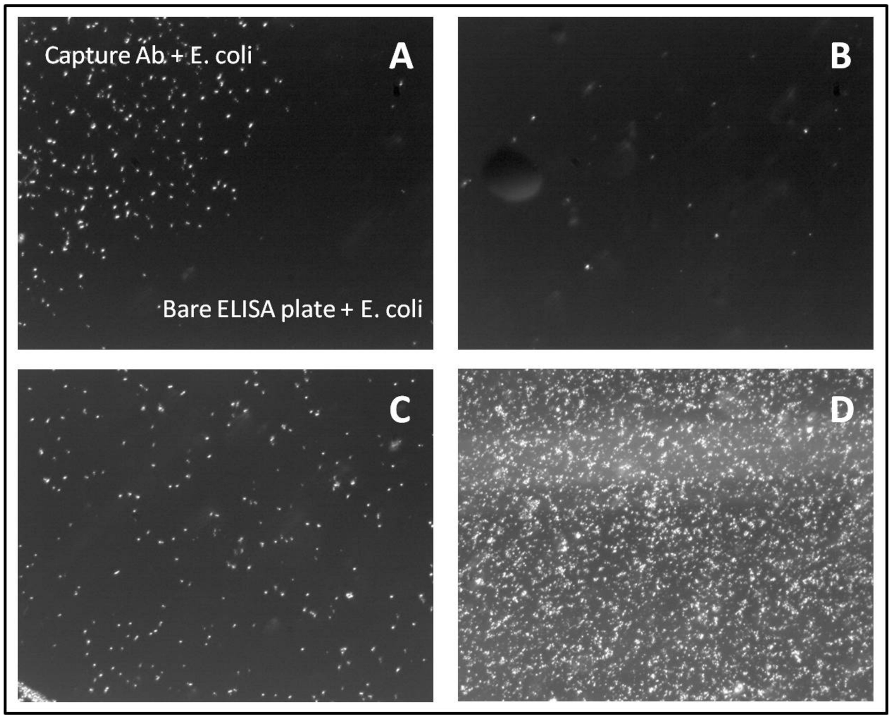


Figure 7.2: Phase contrast microscopy pictures showing the E. coli immobilization (one white dot per E. coli). (A) A drop of IgY was added in the center of the well prior to addition of $\sim 10^8$ $cells/mL$, which demonstrates the difference between a capture antibody and a bare surface. (B) E. coli immobilization in bare well ($\sim 10^8$ $cells/mL$). (C-D) Coating with 50 $\mu g/mL$ MAP in coating buffer and immobilization of E. coli from solutions containing (C) $\sim 10^8$ $cells/mL$ and (B) $\sim 10^9$ $cells/mL$. The brighter appearance of (D) is due to camera brightness setup.

7.1.2 Affinity

Simulated saturation curves (Figure 7.3) of antibodies different affinities were created using Matlab. This gave an idea of what to expect from experimental trials. Labeled and unlabeled IgY were tested (Figure 7.4A) and linear regression (Figure 7.4B) was used to calculate the $\overline{K_D}$ values.

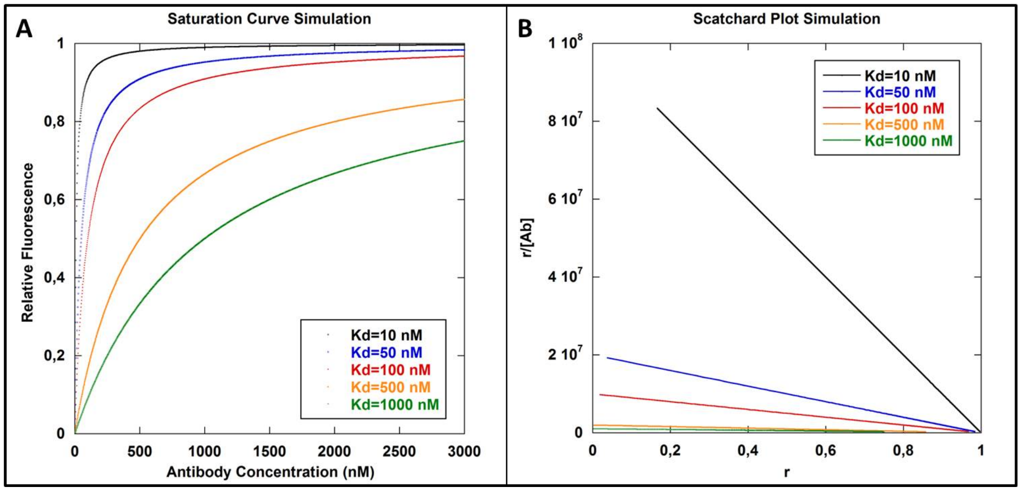


Figure 7.3: (A) Simulated saturation curves for antibody-antigen interactions of different affinities. (B) Simulated Scathard plots for different affinities.

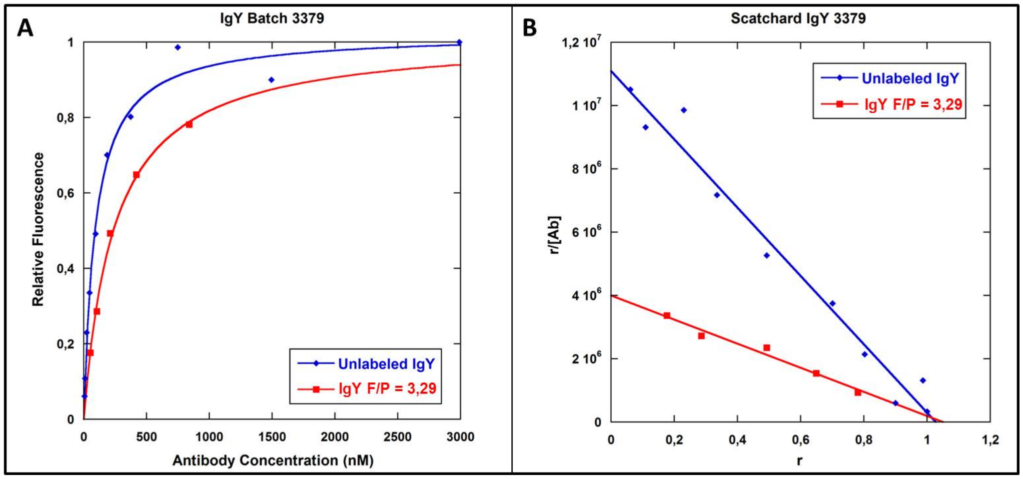


Figure 7.4: Binding characteristics for antibody antigen interaction. (A) Saturation curve for unlabeled and labeled IgY Batch 3379. (B) Scatchard plot IgY batch 3379. Note that this data is presented with the total antibody concentration, both specific and other antibodies.

The resulting saturation characteristics of the antibodies can be seen in Table 7.2, indicating the function of the IgY antibody solution. The unlabeled antibodies gave a 50 % saturation at 93 nM and the labeled antibodies at 263 nM. The labeling induces a loss of function with a factor of approximately 2.8 and the difference is significant ($P = 0.5$) with a student's t-test and duplicate samples.

Table 7.2: Affinity Results

Type	$\overline{K_D}$	Standard deviation
IgY 3379	93 nM	5.9 %
IgY 3379-Alexa Fluor 790	263 nM	9.4 %

7.2 The Hardware Development Process

Several challenges were identified during the modification of the sensor system.

1. The interferometer, used to create the interference pattern, was not optimised for NIR wavelengths (minor challenge)
2. The circular channel reflected a great proportion of the laser light which caused high background levels (medium challenge)
3. The availability of affordable lasers was limited in the $730 - 780$ nm range due to technical difficulties or material limitations (major challenge)
4. The available filters were not optimal for the lasers used (major challenge)
5. Small Stokes shift, of approximately 15 nm, of Alexa Fluor 790 labeled antibodies which made filtering even harder (medium challenge)
6. Theoretical indications of inadequate sensitivity of the PMT around 800 nm (most difficult challenge)
7. Reduced transmission for the objective at higher wavelengths (minor challenge)

The interferometer was removed to test the new setup with direct laser light, as an easy evaluation of the system before the interferometer was needed.

A square flow channel was acquired and modified with connectors to micro flow pumps seen in Figure 7.5A. The water filled square channel showed no reflections compared to the circular channel seen in Figure 7.5B. This can intuitively be explained by the shape difference and reflection theory depicted in Figure 7.6A. There is a great difference in the reflected light when the channel is filled with air and water due to refractive index configuration (Figure 7.7). The demonstration of how the channel is detected can be seen in Figure 7.6B.

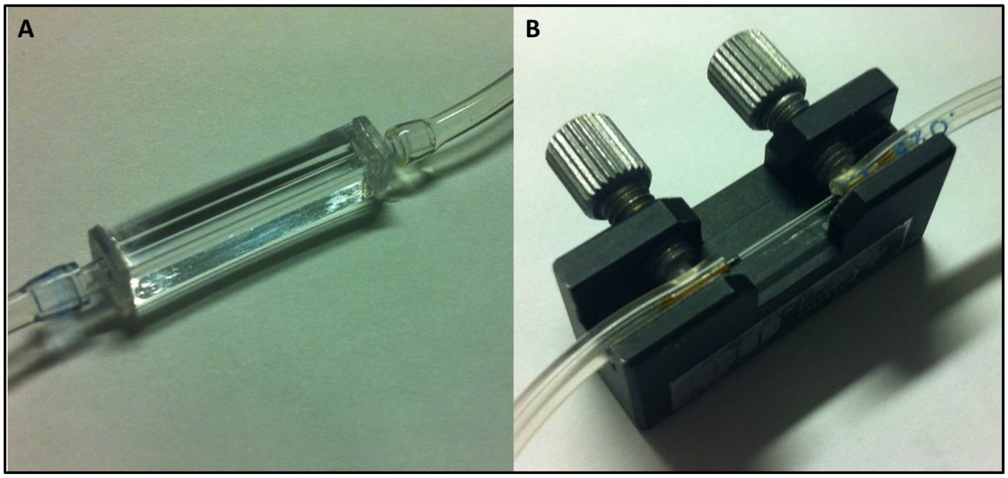


Figure 7.5: (A) The custom made flow channel connected to micro flow tubes. (B) The initial circular flow channel.

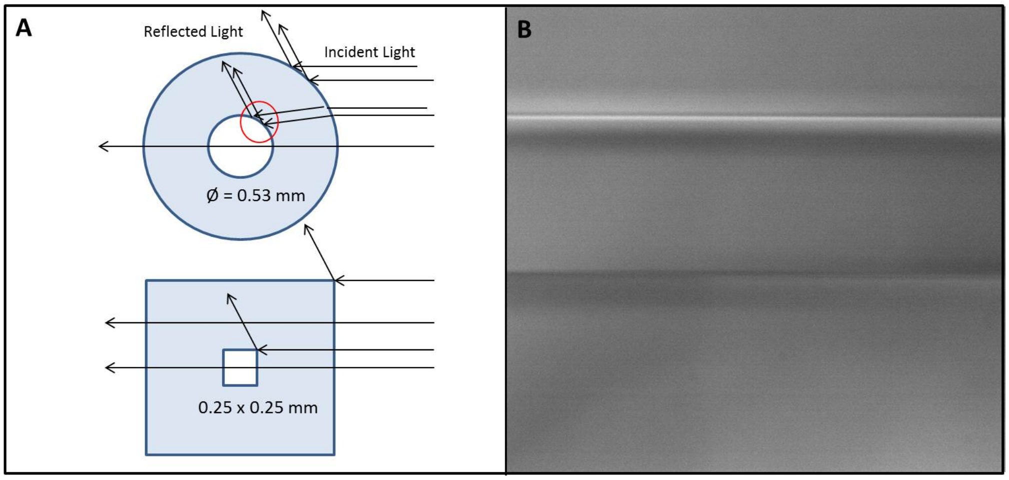


Figure 7.6: (A) Demonstration of the probable reason for higher reflections from the circular channel. The red circle indicates the interface where total internal reflection might occur. The reflection arrows indicate regions rather than specific points. (B) Picture of the detection area by side illumination (white light) of the square channel and no emission filters. This is a picture of the channel filled with air without laser illumination.

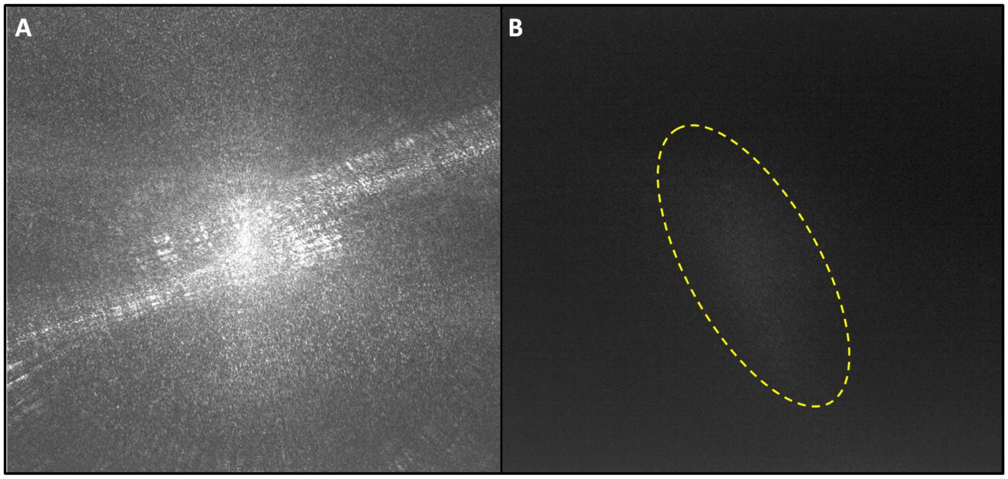


Figure 7.7: Laser reflection from the circular channel filled with air (A) and water (B). Detected at a 90 degree angle relative the laser beam. The yellow ellipse emphasises the reduced reflection due to the change of refractive index inside the channel.

E. coli directly stained with Alexa Fluor 790 (high fluorescence and high concentration) could be detected in the flow channel (Figure 7.8A) using a relatively simple CMOS camera. However, no signal could be detected using the PMT. The explanation to this was believed to be the spatial resolution in the camera, in combination with a reasonably high background due to the sub-optimal laser and filter configuration. The idea behind this is explained in Figure 7.8B.

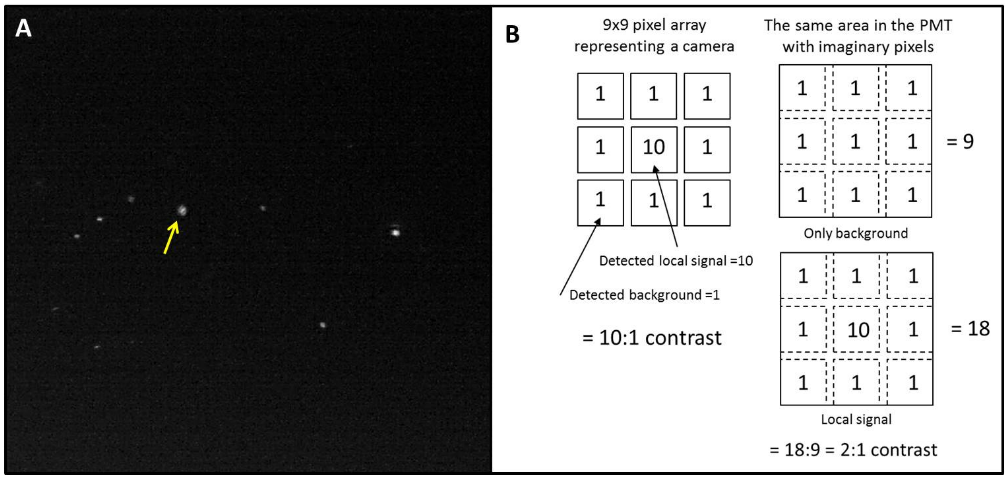

A
B
9x9 pixel array representing a camera
1 1 1
1 10 1
1 1 1
Detected local signal =10
Detected background =1
= 10:1 contrast
The same area in the PMT with imaginary pixels
1 1 1
1 1 1
1 1 1
= 9
Only background
1 1 1
1 10 1
1 1 1
= 18
Local signal
= 18:9 = 2:1 contrast

Figure 7.8: (A) Picture of cells in the channel taken with a simple CMOS USB camera. The shape of the cells could almost be detected for the brightest E. coli (yellow arrow). (B) Schematic to explain the idea behind spatial resolution effect on the detectable contrast.

The performance of this camera was hard to evaluate, and further results could not be obtained until Acreo Swedish ICT received the newly ordered high sensitivity CMOS microscope camera. This camera was known to be very sensitive and previous work with cameras like this made it possible to get some idea of the intensity level. However, the results obtained were not satisfying and indicated that the dye had low brightness. This was concluded by the fact that long exposure times were needed in order to detect the directly or specifically stained E. coli. The result is displayed in Figure 7.9 with exposure times marked.

A new laser with a bandpass filter at 760 nm was obtained to investigate if a more suitable setup could decrease the background noise. The laser was ordered from a company in the USA, which was the the only provider of lasers in this region of the spectrum that could match the budget of the project. The new camera was tested with the 760 nm laser setup, but no signal could be detected. The lower intensity of the laser (10 mW compared to 90 mW

with the 785 nm laser) and the position away from the absorption peak, in combination with low brightness of Alexa Fluor 790, was believed to be the explanation.

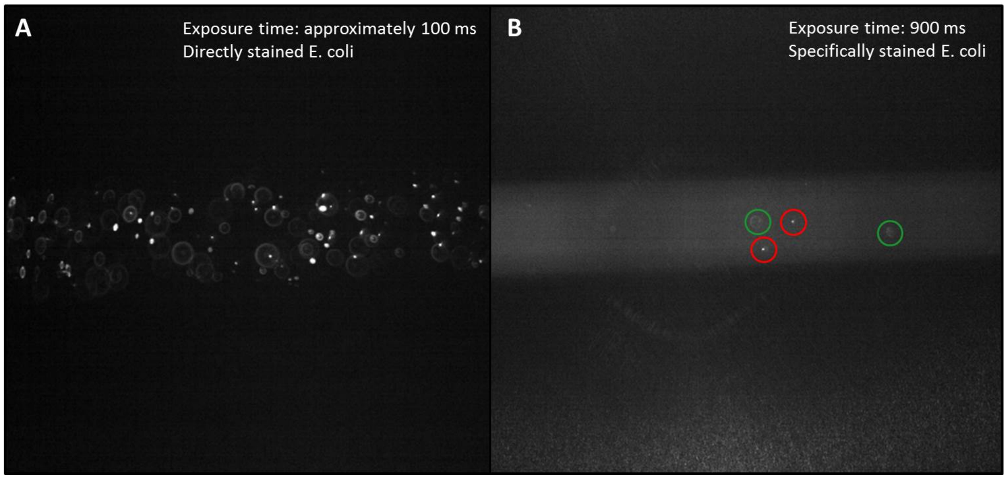

A
Exposure time: approximately 100 ms
Directly stained E. coli
B
Exposure time: 900 ms
Specifically stained E. coli

Figure 7.9: (A) Picture of high amounts of directly stained E. coli in the channel taken with a high sensitivity CMOS microscope camera. The cells are stationary in the channel. (B) Specific staining of E. coli in a high background from unbound labeled antibodies. The cells are stationary in the channel. Red rings are stained cells in focus. Green rings are stained cells out of focus. The laser reflection could also be detected with high exposure times.

The system was modified with a different focus lens. The lens used until this point would produce a line rather than a spot, in order to create the interference pattern. The new lens generated a small spot in the channel to reduce the illuminated area, focus the light and maybe reduce the laser reflections. This setup showed better results and reduced the exposure times needed by 8-10 times. It was thought that the wider laser line induced bleaching before the sample reached the detection point. Relatively fast bleaching could be detected with the direct laser light.

The laser output power was changed and an optimum was detected using a current of 60 mA. Increased current generated higher laser output and increased bleaching of the sample, but not significantly higher fluorescence. Lower current lead to lower fluorescence intensity. This setup was was further tested with real samples and specific staining, to get the final results in order to evaluate the function of the system at this point.

An adjustable pinhole or aperture was mounted in front of the detector to test if this could remove the background noise and increase the contrast. This was a great improvement and directly stained cells could be detected using the PMT. This confirmed that the contrast between the background and stained cells was a problem using the PMT. The best S/N ratio was achieved with the aperture set to 2 *mm* in diameter.

7.3 Final Results Hardware

The final hardware setup is schematically described in Figure 7.10.

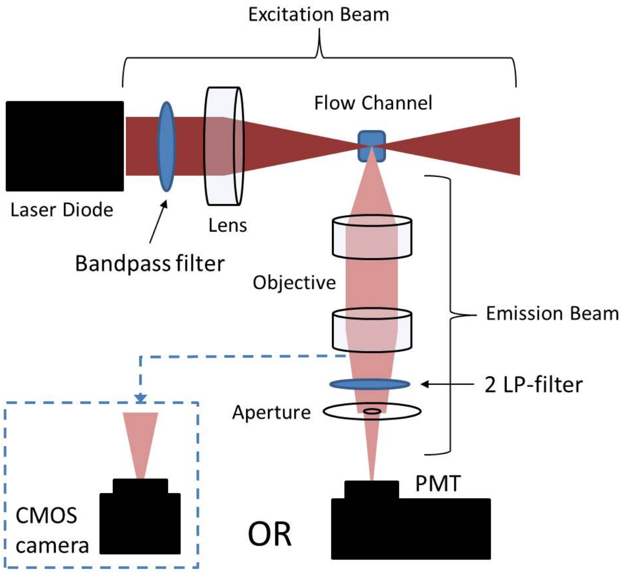

Excitation Beam
Flow Channel
Laser Diode
Lens
Bandpass filter
Objective
Emission Beam
2 LP-filter
Aperture
PMT
CMOS
camera
OR

Figure 7.10: Schematic showing the optical setup. The final system was designed for the 785 *nm* laser. A CMOS camera and a PMT were used to evaluate the system.

7.3.1 Environmental Samples

The hardware setup showed low levels of fluorescence from algae containing untreated water. Some indication of algae could be detected with high exposure times. There were no indication of algae particles in treated sewage water samples. This demonstrate that the shift of wavelength successfully removed the previous problem, where chlorophyll from algae was detected by the sensor system.

Specific staining of environmental samples, using no blocking or blocking by a 1:1 mixture of environmental sample and PBS buffer containing 1% BSA, showed different results. A lot of particles of different shapes and sizes could be detected in unblocked samples. The blocked samples showed reduced amounts of detected particles in more similar shapes and sizes. This indicated that nonspecific binding is a prominent factor and that blocking is needed for the application to work.

7.3.2 Using Alexa Fluor 790 Labeled Antibodies

The specific staining of E. coli strain K12 in PBS with 1% BSA can be summarised with Figure 7.9B, resulting in a high background from unbound and nonspecific antibodies. E. coli was detected in different planes of focus, which demonstrated the challenge with the focal depth. This picture was taken when the sample was diluted to reduce the background. It was hard to detect stained cells in the undiluted sample due to extremely high background.

7.3.3 Field Trials in Trollhättan

The typical signal in the PMT using the 2 mm aperture is demonstrated in Figure 7.11. Calculations of the peak frequency and flow rate, concluded that the peaks detected represented only a small fraction of the total number of E. coli that should have passed the detection area. The measured flow speed was approximately 2 $mL/55\ s$. A rough estimate of the number of peaks was 100, and the fact that the cells that should have passed was in the order of 10^5 indicate the future challenge. Weakly stained cells and cells outside the plane of focus were not detected. The highly variable peak intensity was thought to be generated when multiple E. coli were detected simultaneously, and that detected cells just outside the plane of focus produced lower signals.

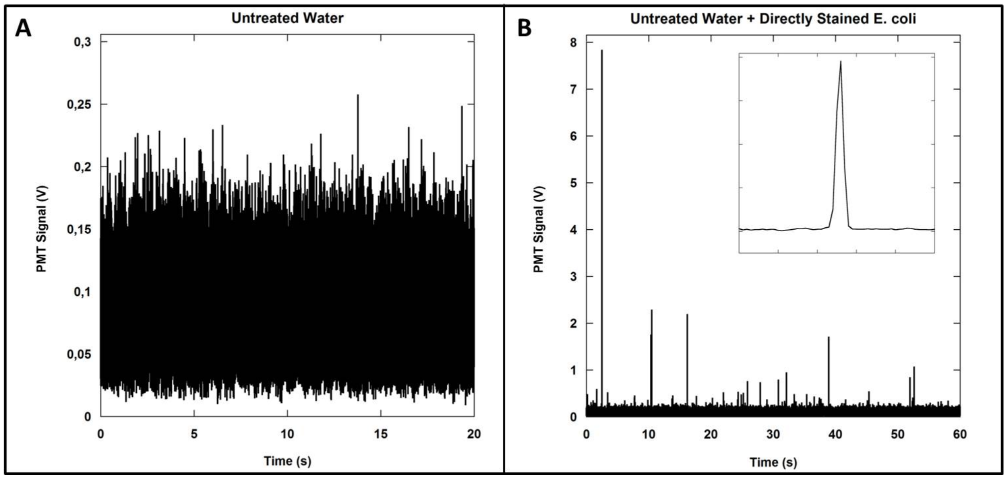


Figure 7.11: PMT signal of untreated water (A) and untreated water with addition of roughly 10^5 directly stained cells/mL (B). Insert is the zoomed peak appearance of the largest peak.

8 Discussion

8.1 Antibody Labeling

The labeling process resulted in sufficient labeling of the antibodies. The suggested F/P ratio is $1 - 4$ according to the product specification and the F/P achieved was within this range. The labeling was not affected by the suggested conditions and it would be interesting to decrease the F/P, by increasing the initial antibody concentration even more or by reducing the incubation time. A lower F/P might reduce the loss of binding function of the labeled antibodies, but would also reduce the fluorescence intensity for each antibody. An optimal condition might be possible to achieve but other parts of the system were prioritised.

The small Stokes shift of Alexa Fluor 790 makes it harder to separate the excitation and emission. The deviation from the stated peak position ($810 - 815$ *nm*) might be due to measurements in another solvent compared to the reference solvent in the product documentation, or due to other effects when the dye is attached to the antibody.

There might be free dye molecules in the antibody solution after the purification column, according to the product specification, which would have lead to some kind of difference between the labeled batches. For example, the batch with lower antibody amount would have had more free dye that could pass through the column, and the F/P difference between the batches would probably have been larger. During the labeling procedure it was possible to detect the coloured dye in the column, and it was mainly located in the first $2-3$ *mm* of the approximately 2 *cm* thick stationary phase. If dye was able to pass the column, it would have been expected to be a more evenly distributed colour in the column. It was concluded that the dye was mainly stuck in the upper part of the column, and that low levels of free dye molecules can be found in the labeled antibody solution. Dialysis could be used in the future to clarify this.

8.1.1 Affinity

The most optimal way to test the binding ability would be if a single binding event could be monitored, by using radio labeling to avoid optical effects like scattering. Another more simple approach would have been to stain cells and get a qualitative evaluation using a fluorescent microscope. However, no plate reader or no camera, with the appropriate filter setup for the fluorescent microscope, was available in the NIR range of light, and therefore a dual binding assay using a secondary antibody had to be developed. The secondary antibody was labeled with FITC and was usable with the plate reader.

The dual binding could affect the saturation in two ways. First, the dual binding even can shift the saturation curve, depending on the antibody concentration and the linearity of the binding event for the secondary antibody. Second, the secondary antibody might have different affinity towards labeled and unlabeled primary antibodies. The result was used as a qualitative comparison between labeled and unlabeled antibodies, when a more suitable technique could not be used. This was an indication that the antibody was functional, and that it could be used to test the hardware. The indirect assay method or similar methods have been used in scientific papers [34, 36] which indicates that the effect of the dual binding might be negligible, and that the result can used as a quantitative $\overline{K_D}$ value, if the concentration of the E. coli specific antibodies is known.

The fact that the antibody solution contained high levels of antibodies not recognising E. coli, would probably affect the binding event through nonspecific binding. This might lead to some errors of the saturation curve appearance. It would be preferable to affinity purify the antibodies and repeat the measurement, to make sure that other antibodies do not affect the saturation curve. The fact that the antibody is created for three different E. coli strains, might reduce the number of antibodies specific for the K12 strain, being the strain used for laboratory tests.

The presence of antibodies not recognising E. coli lead to higher antibody concentrations being necessary to reach saturation. The saturation data and specific staining, detected with the hardware, indicated that the antibodies are functioning well and that the solution can be further improved with affinity separation of the antibody.

8.1.2 Specificity

Affinity is not the only interesting property of the antibody function. Specificity is as important in practice. It was discussed so test the specificity but this was not addressed since Acreo had no suitable bacterial strains, since there was lack of time and since the hardware was not in a state were specificity was a problem. The aim was to demonstrate the principle rather than quantitative measurements, where specificity would have been a problem. It is reasonable to assume that the antibodies will lack in specificity and target related coliform strains, but the severity of this problem will remain unknown until it is important to test. The specificity is important for future development of this application. It would be interesting to evaluate if it is possible to create an antibody mixture, that can target a wide range of E. coli strains and still maintain the specificity, with respect to this bacterial species. However, cross-reactivity with closely related bacteria would not really be an issue, since the idea is to detect intestinal bacteria in an environment where they should not be present.

It is also recommended to separate the antibodies according to specificity in the future, when the development reaches further into a working product.

8.2 Hardware Development

There are some limitations and unknown factors when working with NIR light. The main limitation is that these wavelengths are outside the visible spectrum, which makes it hard to align an optical configuration in the NIR range. It is possible to directly see the laser beam and the size and shape of the spot when working with lasers in the visible range. With NIR this has to be done using a camera. The appearance of these reflections are hard to interpret and thus makes the process challenging. One method used to circumvent this problem was to fill the channel with highly fluorescent matter and align to the highest fluorescent signal.

The methodology used to test the hardware was dependent on the available equipment. It was clear that the new microscope camera, that arrived towards the end of the project, was the best option. It is hard to evaluate function of a hardware system based on wavelengths that are not commonly used, and it is very hard to make intensity comparison between different wavelengths, due to different wavelength dependent properties of hardware components. It was reasonable to assume that the system would work for the NIR wavelengths even though the new setup had a lowered sensitivity.

The use of directly stained cells, was preferred in order to start with the best result and to tune the instrument for lower intensities. The directly stained cells were measured to be at least 4-6 times brighter than specifically stained cells. The directly stained cells had no background fluorescence from free dye molecules and could provide an increased contrast. The fact that the directly stained cells were thought to give high intensity lead to the assumption that the filter setup was the main problem, when in fact the low intensity of Alexa Fluor 790 might have been the larger problem for the application.

8.2.1 Environmental Samples

The fact that there were only low intensity indications of particles in untreated water and no indication at all in treated sewage water, demonstrate that the change of wavelength from the previous system was successful in reducing the fluorescence from blue green algae. The observed indication can either be scattering from particles or weak fluorescence from e.g. chlorophyll. Untreated water showed higher background scattering or fluorescence than treated sewage water. The explanation to this might be fluorescence from different type of algae, and that untreated water contains higher amounts of algae that fluoresce at higher wavelengths. Another explanation can be that there are increased amounts of nutrients in sewage water, and that the algae are in a different growth phases in such samples, briefly discussed elsewhere [29]. However, the intensity level is weak compared to the direct or specifically stained cells and will not be a problem in the future.

8.2.2 Specific Staining

The specific staining in Figure 7.9B demonstrated a future challenge of the system, with background fluorescence from free antibody molecules. The S/N ratio can be greatly improved by using solution containing 100 % antibodies targeting E. coli. A reasonable scenario in the future is to have a mixture of monoclonal antibodies towards different kind of E. coli strains. This will sacrifice a bit of S/N ratio for a better targeting of a wide range of E. coli types. It is reasonable to assume that the diversity of E. coli strains in a sample is low compared to the diversity within antibody mixture, resulting in a lot of free antibodies in the sample.

One simple way to increase the S/N ratio was to dilute the sample in order to decrease the surrounding antibody concentration. The bound antibodies will encounter a new equilibrium, but the rate of which antibodies dissociate from E. coli is slow compared to the association rate, and the intensity of stained cells will remain during the time of the run if the duration of the measurement is short enough. Dissociation rate of IgY or IgG is normally in the range of $10^{-3} - 10^{-4}\ s^{-1}$ [45]. This method might not be usable in practice when the E. coli concentration is low and high volume is required to get a correct estimate of the E. coli concentration. Dilution of untreated water sample will significantly increase the runtime.

Re-introduce the interference pattern might increase the ability to detect stained cells in moderate backgrounds. The current system is not able to resolve specifically stained cells, without removal of unbound antibodies.

8.2.3 Alexa Fluor 790

It was believed that Alexa Fluor 790 had too low brightness to be useful for a flow-through system, since high exposure times were needed to detect specifically stained E. coli. To use the high sensitivity CMOS camera is not an option if a system like this should be affordable in practice. Further modification of the system enhanced the function and lowered the exposure times needed. The belief that the dye had low brightness was maintained, and the conclusion was that a brighter staining is needed if a less sensitive detector is to be used in the future.

8.2.4 Focal depth

The microscope camera highlighted a challenge that must be addressed for the system to work in practice. The focal depth of the method must be large enough to fill the entire flow channel. E. coli out of focus will have lower intensity or a different optical appearance in an imaging method, and a different peak characteristic in the detected signal (Figure 7.9A). A channel with a small depth and larger width might be a solution to this problem. This will on the other hand increase the chances of cells to pass the detector simultaneously, and give some challenges with the focal point of the laser instead.

The E. coli levels in untreated water is usually $1 - 100$ cells/100 mL. It is believed that the greatest challenge for this application is to have enough throughput to make the measurement duration reasonable and still be able to optically detect all the cells. However, it might be possible to create a new standard by detecting a fraction of the E. coli, and correlate this to known contamination levels.

It is possible that the total intensity of the light from out of focus E. coli is maintained but spread over a larger amount of pixels. In that case the camera has limitations compared to a PMT, since the total amount of light is more interesting than the spatial position of the light. The higher sensitivity from the PMT and an interference pattern might be more beneficial in dealing with the focal depth.

An increased magnification in the objective will give better signals, due to the fact that the stained E. coli is larger in contrast to the background area, but will reduce the focal depth. It is reasonable to assume that the PMT is less dependent on the magnification in the objective, compared to the camera, and would work better with lower magnifications.

8.2.5 Aperture

One last effort was to test the effect of an aperture in front of the detector. The aperture greatly increased the performance when detecting directly stained cells using the PMT. This was an indication of that the earlier theories, of why the PMT could not resolve the signal, were true. The exact theory behind this can be further investigated and there is probably some optimization to be done. The system might be further improved by positioning a pinhole directly in front of the detector or even in front of the flow channel to remove unwanted reflections.

The aperture used will not only increase the contrast in the PMT, but will also increase the focal depth. If this technique is enough to detect the entire volume, has to be evaluated.

8.3 PMT versus Imaging

A PMT was initially used in the project but was later substituted by a CMOS camera, since no signal could be detected by the PMT. The PMT is very sensitive and requires a flawless setup with low background, in order for a small stained E. coli to be comparable to the noise from a large area. Working with a 3-dimensional flow channel makes the background more dominant, compared to a conventional flow cytometer where the detection area is a point where single cells pass the laser. The camera might lack some sensitivity compared to the PMT, but have the spatial resolution with many pixels, compared to one large pixel in the PMT earlier explained in Figure 7.9. The camera made it possible to detect weak signals even though the background was rather high. It appears to be better to use an imaging technique when working with a larger scale flow cytometer. There are different type of cameras available that can enhance the signal. The only consequence is the matter of high cost, but a PMT is expensive as well and is not optimal for this application either.

Other available camera technologies are high dynamic range cameras which tend to be more light sensitive[6]. There are cameras with electron amplification similar to the PMT that are called electron multiplying CCD (EMCCD), and have the benefits from the spatial resolution in the camera and high sensitivity from the PMT [46]. Linescan cameras are another alternative used in industry to detect moving objects like in flow cytometry applications. The sensor area is constructed as a small line which gives benefits when monitoring movable objects, providing a better frame rate and contrast [47].

Using APDs was discussed during the project since some literature indicate higher sensitivity in the NIR range, but this kind of detector would have the same problem as a PMT. This might however be a cheaper alternative if future research makes a sensor without pixels a suitable choice. Hamamatsu are developing APD sensor arrays with photo counting which increase the sensitivity. They are known as multi-pixel photon counter (MPPC) and might be usable in future applications [48].

There are also PMTs with better performance in the NIR range (Hamamatsu R3890 or R3896) and also photon counting PMTs with higher sensitivity, but the cost is, again, the main issue.

8.4 Evaluation of the system

The system solves the problem with the fluorescence from blue-green algae, which was the main limitation with the previous system setup, which is a great step towards a working product. However, during the modification other challenges were identified, especially regarding the sensor sensitivity at higher wavelengths and the function of the optical equipment. The detector has to be sensitive enough at a reasonable price, which is not the case in this project. There are also some challenges regarding the staining procedure. To have high affinity and specificity for a wide range of naturally abundant E. coli is challenging.

The main limitation of this technique, is to have enough throughput to get an estimate of the E. coli concentration within a reasonable period of time. The people working at Trollhättan Energi were concerned about the throughput of this system at an early stage. For the system to detect all the cells the channel dimensions might have to be decreased, but to fit the practical use the dimensions might have to be increased, which creates a dilemma for the future.

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[6] Information from Michael Johansson, BergmanLabora AB, personal communication, May 2014

The discussed challenges show that the system is not in a state where it could be quantitatively compared to conventional detection methods. However, this work provides a qualitative evaluation to discuss advantages and disadvantages regarding the different methods.

The NIR modifications turned out to be more challenging than expected, and more work has to be put into the system. The final evaluation is that it is interesting to see how well this system can perform, when all the different parts show acceptable performance. To invest in further developments can be justified with the results obtained in this Master Thesis.

8.5 Error Sources

One error source is the determination of the antibody concentrations. The extinction coefficient was, in the literature, found in the range from $210,000$ $M^{-1}cm^{-1}$ to $240,000$ $M^{-1}cm^{-1}$, which will affect the calculated antibody concentration. The antibody stock solution was diluted and measured, which indicated an extinction coefficient closer to $210,000$ $M^{-1}cm^{-1}$ also found in most of the literature. The use of $210,000$ $M^{-1}cm^{-1}$ as the extinction coefficient made the comparison between the stock solution and the labeled antibodies more reliable. This error is larger than the measured deviation of antibody concentration and that is why the standard deviation was left out.

An error when measuring the saturation curve, was the lack of labeled antibody to be able to reach saturation. It appears that the last 2-3 points of the saturation curve are important to determine the maximal binding, and failure to reach saturation can influence the result. The last data points are also in the region with the largest errors due to high antibody concentration and some deviation within the washing procedure. The Scatchard plot will reduce some of this effect and the linear fit should give an accurate $\overline{K_D}$ value, even though the binding is not fully saturated.

An error and a problem when working with the optical hardware, was the ability to compare different optical setups. The alignment was sensitive to small changes and it was hard to produce exactly the same setup after a minor modification. Minor changes could increase or decrease the sensitivity and contrast, making it hard to know if the optimal conditions were reached.

8.6 Outlook

It is easy to see the benefits with this system, with reduced test time, in order to avoid pathogenic outbreaks. Even countries with advanced purification facilities will have problems with faecal contamination. The risk will increase with an increasing population and increasing population density in larger cities. The risk must be kept low in order to protect our most important natural resource.

A future hardware can be used for a variety of applications, and not only to detect E. coli. Modification of the staining procedure with aggregate formation, might be used to detect smaller particles like viruses or even toxins.

8.7 Project Process

Research and development is hard to plan in advance. It was mentioned that the timeplan was just an early outline, and that problems would be taken into consideration as they appeared. The actual need for signal processing and hardware modification was rather unknown at the project start.

The early plan of the project was soon disrupted due to challenges with the hardware. The idea to test a finished setup and to work with signal processing, turned into a struggle to get the system to work. However, the timeplan was maintained in structure; to initially work with the dye and the coupling chemistry, and to move on to the hardware for the second half of the project. The difference was that the hardware turned out to need further development, before signal processing or field testing could be applied as planned.

A lot of time was spent on understanding how the system worked and to discuss challenges with the supervisors. Information on detectors, filters and lasers had to be gained and the theoretical work was dominant in some periods, which was the reason the project did not reach as far as I had hoped.

The project was hampered by the challenges to work with NIR and lack of equipment for this wavelength. NIR applications are still in a research stage and it was clear that few research groups are working with NIR fluorescence. Attempts to contact external facilities to use their fluorescent microscope were made, but gave no results since they also lacked the suitable equipment.

The only way to confirm the staining was to use the flow cytometer setup, which was associated with considerable uncertainty.

The fact that the interference pattern was set aside might have been a bad choice, since the system was different and the knowledge gained from the old system was hard to use. With hindsight it would have been better to keep the initial design of the system, in order to limit the different factors that might affect the result. A lot of questions arose regarding the difference between the using and omitting the interference pattern, and if the removal of the interference pattern was the main reason to why the PMT was unable to resolve the signal. However, to align the interference pattern could take most of a day, which would have made the process to test different setups even more time consuming.

More work should have been put on the hardware from the start, and less on evaluations of the antibodies, as it turned out that the antibody mixture might not be fully functional in practice, due to low amounts of E. coli specific antibodies stained with a dye of low brightness. However, a lot of the antibody evaluation was done waiting for delivery of parts to the hardware. The best way to evaluate the antibodies and Alexa Fluor 790 was to use the flow cytometer in the end of the project.

A microscope with a filter setup for Alexa Fluor 790 and a sensitive microscope camera would have made things a lot easier, especially early in the project, since it would have allowed for fast qualitative evaluations of the labeled antibodies.

Almost all the ideas and commitments resulted in enhanced performance of the system, and the theories about the system turned out to be quite accurate. This shows that the collaboration and the discussion lead to good ideas, and that the future direction of the development is interesting.

9 Conclusions

This thesis demonstrates the development of a NIR flow cytometer application to detect faecal contamination of water sources. The final system was not in a state for a quantitative comparison with conventional methods, but some interesting conclusions were made as stated in the following list.

- IgY was successfully and efficiently labeled with Alexa Fluor 790 using succinimidyl ester coupling to primary amines.
- The labeling process reduced the binding ability of the antibody solution by a factor of 2.8. However, this is not critical to the antibody function.
- Lack of suitable lasers and filters made the optical configuration to target Alexa Fluor 790 suboptimal. High background from laser leakage reduced the signal to noise ratio.
- Alexa Fluor 790 produced low intensity fluorescence. This signal might be enough for stationary applications such as fluorescent microscopy, where a high exposure time can be used. Flow cytometry applications, where the time resolution is of importance, probably requires a brighter dye.
- The NIR optical setup indicated little or no autofluorescence from algae, which solved the main problem for the application.
- There are limitations regarding detectors in the NIR region. Semiconductor based detectors such as APDs, CCDs, CMOS cameras are suggested the literature. The sensitivity of the PMT used was not enough due to a relatively high background.
- The lack of sensitivity in the PMT can partly be explained by the lack of spatial resolution. The interference pattern used earlier might be crucial for the PMT to work when detecting particles in a larger volume.
- The contrast offered by the PMT was increased by mounting an aperture in front of the sensor area, to reduce interfering light from the background. However, only a small fraction of the directly stained cells generated a signal higher than the noise floor.
- The main future challenge is to meet the throughput requirements and to detect cells from the entire flow volume.

10 Future Aspects

Flow cytometry quantification of bacteria has recently been accepted, by the Federal Office of Public Health (FOPH) in Switzerland, as a method to determine the bacterial content in drinking water [49]. There are products available for total bacterial cell count that are portable and easy to use [50]. The company Partec, that produce flow cytometry systems, believe that this will be a standard in Europe in a couple of years. Trollhättan Energi, the main funder of this project, is currently looking into on-line systems based on flow cytometry. This clearly indicates that the future development for this technology is interesting, and that development towards specific detection and more basic instruments is valuable. The instrument can be a very powerful tool if it is possible to overcome the difficulties and if the cost can be reduced.

10.1 Hardware

An interesting future sapect might be to look at the aperture, and to evaluate the function and the positioning of this technique. It would be interesting to test if the optical interference pattern is needed, or if a pattern with small slits in front of the channel or in front of the detector can give the same benefits. This might be a lot cheaper and would make the instrument easier to align.

The design of a wider and thinner channel to have a smaller depth and to still maintain the needed volume flow can be an improvement in the future.

All available detectors benefit from cooling which will decrease the noise floor. The sensor system will be located in an environment with flowing water and it might be possible to use the water to cool the detector. This will provide a low energy consuming cooling technique. However, the condense from this type cooling might be a problem.

10.2 Ratio of Antibody and Escherichia Coli

Another future aspect, is how to set the amount of needed antibody based on the E. coli concentration in untreated water. Using a fixed antibody concentration to detect a highly alternating E. coli concentration, will probably produce issues with the contrast and signal processing since the intensity will be dependent on this relationship. The majority of the measurements will hopefully show low levels of contamination, and to use excessive amounts of reagents is another issue with respect to increased cost. Some kind of closed feedback system could be useful to optimise the signal and to decrease the cost.

One solution to the high background, might be to run the small sample through a dialysis tube inside a surrounding flow of water. This can be a simple and cost effective solution to lower the background noise. However, the flow rate might be large in comparison to the diffusion rate.

Another solution to the high background might be to use Fluorescence Resonance Energy Transfer (FRET) [28], which is the transfer of energy between two fluorophores when they are adjacent. The fluorescence from separated dyes in the solution can thus be ignored, by using staining chemistry that will position the fluorophores close in space so that FRET can occur. The question is if this is possible with fluorophores in the NIR range.

10.3 Fluorescence Polarization

Fluorescence Polarization (FP) [38] is a technique that was discussed as a possible technique to enhance the S/N ratio of the system. A fraction of the antibodies used to label the cells will be free in the sample volume, which will decrease the S/N ratio. FP use the fact that fluorophores will emit light with the same polarization as the light they absorb. However, the polarization will change relative to the detector if the molecule rotates during the lifetime of the excited state. Larger molecules have a slower angular rotation in a solution, and it might be possible to separate the fluorescence of the fast rotating unbound antibodies from the fluorescence of the stationary antibodies bound to E. coli. However, this is used for small molecule systems and the rotational difference between E.coli and IgY might not be enough. This method is easy and cheap to test and might enhance the function of the system in the future.

10.4 Qdot 800

The last step in this project was to order and test a Quantum dot (Qdot 800) antibody labeling kit, in order to increase the intensity of the fluorescent label. The labeling kit was based on click chemistry on modified carbohydrate groups on the Fc part of the antibody, seen in Figure 4.5, and could possibly reduce the loss of antibody function that had been a problem when labeling antibodies with Qdots in the earlier system setup. These tests were not conducted in time to be a part of this report, but show the future direction of the development.

Qdot 800 can possibly provide a higher brightness and are less sensitive to bleaching. Qdot 800 will also make the laser and filter setup easier to design, since Qdot 800 can absorb light over the entire visible spectrum, with the absorbance peak at UV light, and emit light above 750 *nm*. Another benefit is the structure of the probe towards E.coli. Fluorescent dyes will attach to a single antibody, making the antibody into a probe. Many antibodies can bind to a single Qdot particle, making this a better choice for a solution containing E. coli specific antibodies and other antibodies. There is always a chance that one specific antibody is bound to the Qdot, so that the Qdot can bind to the target E. coli. This might reduce the background fluorescence when all the probes have reasonable affinity and specificity.

10.5 New Applications

Setbacks are not always bad and the fact that the earlier system was highly effective in detecting different kinds of algae has produced some interest from other areas. To further develop small, portable and cheap algae detectors could be of interest in for example marine biology applications[7].

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[7] Information from Dag Ilver, Acreo Swedish ICT, personal communication, May 2014. He discussed this option with an old friend working with marine biology.

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Appendix

A E. coli Immobilization

1. The MAP stock solution (60 mg/mL) was diluted 60 times in 1% citric acid.
2. Prior to use the MAP solution containing 1 mg/mL was diluted in coating buffer to a final concentration of 50 $\mu g/mL$
3. 100 μL of the solution was added to each well and the microtiter plate was incubated at 37°C for 1 h under slow phase rocker stirring.
4. The wells were washed 3 times with PBS using an automatic plate washer.
5. 100 μL cell suspension was added to each well and the microtiter plate was incubated at 37°C for 1 h under slow phase rocker stirring.
6. The wells were washed 3 times with PBS.
7. 100 μL 8% formaldehyde was added to each well to fix the immobilised cells for 10 minutes.
8. The wells were washed 3 times with PBS.
9. Microtiter plates were stored at 4°C until further use.

B Saturation Curve

1. Microtiter plates with immobilised E. coli was blocked with blocking buffer containing 1% BSA in PBS at 37°C for 1 h. 100 μL blocking buffer was added to each well.
2. 100 μL high concentration primary antibody (unlabeled or labeled IgY) was added to the first well.
3. 100 μL from well 1 was transfered and mixed with well 2, $2 \rightarrow 3$ and so on to create a serial dilution with the factor of 0.5 to the previous wells.
4. The microtiter plate was incubated at 37°C for 1 h.
5. The wells were washed 5 times with washing buffer containing 0.05% tween 20 in PBS to lower nonspecific binding.
6. The secondary antibody, rabbit anti chicken - FITC 1.5 mg/mL, was diluted 500 times in blocking buffer to a final concentration of 3 $\mu g/mL$.
7. The microtiter plate was incubated at 37°C for 30 min.
8. The wells were washed 5 times with washing buffer.
9. A blank with only immobilised E.coli and a control incubated with the secondary antibody was used as reference.
10. The plate was read in a Fluoroskan Ascent FL plate reader.

C Antibody Labeling Kit

- 1.1Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionised water (dH2O) to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. The bicarbonate solution, which will have a pH $8-9$, can be stored at $2-8°$ C for up to two weeks.
- 1.2 If the antibody to be labeled has a concentration of .1 mg/mL and is in an appropriate buffer (see Preparing the Protein, above), dilute it to 1 mg/mL and then add 1/10th volume of 1 M sodium bicarbonate buffer (prepared in step 1.1). If the protein is a powder lyophilised from an appropriate buffer, prepare a 1 mg/mL solution of the antibody by adding an appropriate amount of 0.1 M sodium bicarbonate buffer to the protein. Prepare 0.1 M sodium bicarbonate buffer by diluting the 1 M solution 10-fold with dH2O.

  Note: Bicarbonate, pH 8.9, is added to raise the pH of the reaction mixture, since succinimidyl esters and TFP esters react efficiently at alkaline pH.
- 1.3 Transfer 100 μL of the protein solution (from step 1.2) to the vial of reactive dye. Cap the vial and gently invert it a few times to fully dissolve the dye. Violent agitation of the protein solution can result in protein denaturation. Note: To visually confirm that the dye has fully dissolved, it may help to peel the label off the vial of reactive dye.
- 1.4 Incubate the solution for 1 hour at room temperature. Every 10.15 minutes, gently invert the vial several times in order to mix the two reactants and increase the labeling efficiency. Note: During the incubation period, proceed to steps 2.1.2.4, below, to prepare a spin column for the purification of the labeled protein. This will take 15 minutes.

  The purification step removes the unbound dye from the dye-conjugated protein. In applications that utilize repeated wash steps after labeling with the dye-conjugated antibody, purification may not be necessary.
- 2.1 Place a spin column in a 13 x 100-mm glass tube.

  Note: The enclosed spin column should have two frits inserted at the bottom. If the two frits are not present, one or both of them may be in the plastic bag. Insert the second or both frits into the column and push them down to the bottom of the column with a glass or plastic stir rod. If any resin from the column get past the frits and ends up in the collection tube with the conjugate, it will do no harm and removing it is optional.

- 2.2 Stir the purification resin (Component C), then add 1.0 mL of the suspension into the column and allow it to settle by gravity.
- 2.3 Continue to add more of the suspension until the resin bed volume is 1.5 mL.
- 2.4 Allow the column buffer to drain from the column by gravity. Initially, some pressure may be required to cause the first few drops of buffer to elute. Place the spin column in one of the provided collection tubes and centrifuge the column for 3 minutes at 1100 × g using a swinging bucket rotor. To convert revolutions per minute (rpm) into relative centrifugal force (g-force), either consult the conversion chart provided by the centrifuge manufacturer or use the following equation:

  Relative centrifugal force = (1.12 x 10–5) $(\text{rpm})^2(\text{radius})$

  where radius = radius in centimeters measured from the center of the centrifuge spindle to the bottom of the rotor bucket. Discard the buffer, but save the collection tube. The spin column is now ready for purifying the conjugated antibody.

  Note: A fixed angle rotor will suffice if a swinging bucket rotor is not available.
- 2.5 Load the 100 μL reaction volume (from step 1.4, page 3) dropwise onto the center of the spin column. Allow the solution to absorb into the resin bed.
- 2.6 Place the spin column into the empty collection tube and centrifuge for 5 minutes at 1100 × g.
- 2.7 After centrifugation, the collection tube will contain labeled protein in approximately 100 μL of PBS, pH 7.2, with 2 mM sodium azide; free dye will remain in the column bed. Discard the spin column.

D Matlab Code

```
ab=[0:2:3000]; %antibody concentration (nM)
m1=1; %Bmax
m2=10; %KD (nM)
y=(ab*m1)./(m2+ab);

figure(1);
plot(ab,y);
xlabel('Antibody Concentration (nM)');
ylabel('Relative Binding');
hold on

s=y./(ab*1e-9); % r/[ab] for scatchard plot.

figure(2);
plot(y,s) % The scatchard plot
```