Title: Novel Glyco-arrays

Submitted by: Seng Peiwen, Lynette

U1636195

Supervisor: Professor Matthew I. Gibson

(University of Warwick)

Table of Contents

Table of Contents 1

List of Figures, Tables and Graphs 2

Acknowledgements 3

Abstract 4

Abbreviations 5

1. Introduction 6

1.1 Biological importance of glycomic interactions 6

1.2 Microarrays and methods of printing 7

1.3 Current area of application and interest 9

2. Project Aims 11

3. Results and Discussion 12

3.1 Printing controls 12

3.2 Determination of a suitable blocking agent 15

3.3 Testing of microarray strategy using Con A 19

3.4 Incubation of rest of lectins (RCA, UEA I, WGA) 22

4. Conclusion and Future Work 26

5. Instruments 27

5.1 Microarray contact printer 27

5.2 Microarray Fluorescence Scanner 27

6. Experimental Methods 28

6.1 Control Printing 28

6.2 Dilution of Lectins 29

6.3 Blocking Tests 29

6.4 Lectin Incubations of Microarrays 30

6.5 Analysis of Results using Agilent Feature Extraction software and ImageJ 30

7. Safety Considerations 31

8. References 32

9. Appendices 35

List of Figures, Tables and Graphs

Figure 1. Illustration of contact printing……9
Figure 2. Pattern of print of first dip……12
Figure 3. Pattern of print of second dip ……...……12
Figure 4. Template of microarray to be produced……14
Figure 5. Scanned pattern of microarray (left), original pattern on slide (right)……..15
Figure 6. Background noise of PBST + BSA blocking agent in comparison with array spots……17
Figure 7. Structure of glycopolymer and role of ethanolamine……...18
Figure 8. Examples of the delamination of slides……19
Figure 9. Example of result analysis using a mask……..20

Table 1. Library of glycopolymers for testing……13
Table 2. Proportion of combination of blocking agents……...16
Table 3. Images of microarrays before and after incubation with blocking agents….17
Table 4. Arrays after incubation with alternative blocking agents……..18
Table 5. Result Tabulation of Con A Lectin……20
Table 6. Result Tabulation of RCA Lectin……..22
Table 7. Result Tabulation of UEA I Lectin……23
Table 8. Result Tabulation of WGA Lectin……24
Table 9. List of reagents and their suppliers……28
Table 10. Pipetting pattern on 384-well microplate……35

Graph 1. Average Fluorescence Intensity of Glycan-Terminated Polymers with Con A Incubation……..…21
Graph 2. Average Fluorescence Intensity of Glycan-Terminated Polymers with RCA Incubation……...…22
Graph 3. Average Fluorescence Intensity of Glycan-Terminated Polymers with UEA I Incubation……23
Graph 4. Average Fluorescence Intensity of Glycan-Terminated Polymers with WGA Incubation……..24

Acknowledgements

I would like to express my deepest appreciation for all who have contributed towards this research project and report. I would like to especially thank Professor Matthew Gibson from the University of Warwick for the opportunity to be part of his thriving research group, for the extremely current and engaging project as well as for his incredible input and ideas whenever challenges arose. I would also like to express my utmost gratitude to Caroline Biggs for her invaluable guidance and support throughout this project, which without, would have not been possible. Lastly, I would like to give special thanks to Nanyang Technological University and the School of Physical and Mathematical Sciences for granting me the opportunity to experience research abroad.

Abstract

The field of glycomics has been challenging to study due to the complexity in the nature and behaviour of glycans. Glycomic interactions are the frontier of biological processes but have been poorly understood. However, in recent years, microarray technology has played a revolutionising role in facilitating our understanding in the binding activities between carbohydrates and proteins.

In this project, not only a cost effective, but more importantly, an efficient method in printing reproducible glycan-terminated polymer microarrys in the lab is discussed. This will contribute to research in the field of glycomics by using simple but effective microarray technology and strategies.

Abbreviations

BSA - Bovine serum albumin

Con A – Concanavalin A

dH_2O – Distilled water

DP – Degree of polymerisation

FUC - Fucose

GAL - Galactose

GLU – Glucose

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MAN - Mannose

NSB – Non-specific binding

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline with Tween-20

pHEA - poly-2-hydroxyethyl aspartamide

RCA – Ricinus communis agglutinin

UEA I – Ulex europaeus agglutinin I

WGA – Wheat germ agglutinin

1. Introduction

1.1 Biological importance of glycomic interactions

Glycans, more commonly known as carbohydrates, represent one of the four fundamental building blocks of life, and are the most abundant biological molecules on our planet.[1] They are chains of monosaccharides, which exist in various chain lengths[2] that play a significant role in the biological processes that happen in the body.[3] Glycoproteins are proteins with a sugar attached to them, and have diverse functions such as the immunology and protection of the body and communication between cells.[4] The diverse carbohydrate structures displayed on cell surfaces are well suited to serve as interaction sites between cells and their environments.[5]

Lectins comprise a family of widely occurring proteins that bind specifically to glycan structures.[5] Lectins and carbohydrates are linked by a number of relatively weak interactions that ensure specificity yet permit unlinking as needed, resembling a Velcro, where each interaction is relatively weak but the composite is strong.[6] Since individual protein-carbohydrate interactions are usually weak, multivalency is often required to achieve biologically relevant binding affinities and selectivities,[7] as well as to enhance the affinity of the interactions.[8]

The ubiquitous presence of lectins on cell surfaces and their exquisite specificity for carbohydrates on the membrane of glycoproteins make them eminently suitable candidates for being the mediator of cell recognition.[9] In the recent years, lectin research has grown in both size and scope, with interest garnering in the biological significance of their interactions with carbohydrates.[10] Because of how protein-carbohydrate interactions (also known as glycomes) are involved in a multitude of biological processes and responses, such as pathogen-cell adhesion and cell migration, the study of these interactions is crucial in improving current understanding of pathogen-host interactions, especially in this age of decreased antibiotic discovery and increased antibiotic resistance.[11]

However, carbohydrates and their modifications are extremely difficult to study. The biosynthesis of glycans is not template-driven, and the fact that it is also regulated by

many factors including the variations in linkage stereochemistry, linkage regiochemistry and branching generate natural glycans of enormous structural diversity. Adding on to this diversity, modifications of at various sites of the glycan yield additional structures that can change dynamically, resulting in an array of glycoforms.[12] This results in the difficulty of the prediction and control of glycan expression.[13] Furthermore, as the majority of lectins usually contain more than two shallow binding pockets for carbohydrates, their binding profiles are usually highly complex.[14] These factors have caused the detection and characterisation of glycomes to be challenging.

1.2 Microarrays and methods of printing

Microarrays are ordered arrays of DNA, nucleic acids or any material of interest, gridded out onto small solid supports, typically microscope slides. Microarrays have set foot in the medical field, as well as other industries such as forensics, and have greatly impacted biological research and drug discovery.[15] High quality arrays, alongside standardised hybridization protocols, accurate scanning technologies and robust computational methods have allowed DNA microarray technologies to be perhaps the most successful and mature methodologies for high-throughput and large-scale genomic analyses.[16]

These current advances in the area of genomics and what microarray technologies can offer have led to the interest of using microarray technologies in glycomics. Just as with the concept of DNA microarray technologies, glycomics can be studied using carbohydrate microarray technologies. Glycoarray, also known as carbohydrate microarray, is another type of microarray in the medical field. It is a new generation technology that has recently emerged as a high-throughput tool for studying carbohydrate-binding proteins, as well as for furthering knowledge in this area of study.

There are many advantages in using microarrays. Besides the minimal use of precious carbohydrate material due to miniaturisation, the main advantage of any microarrays would be how it can be mass analysed.[5] This is as a wide range of glycans can be

placed on one static array in large quantities. This can dramatically increase the output of data, allowing for extensive study of glycomes. This array technique acts as a close approximation of the happenings on a cell's surface, resulting in a more accurate study of glycomics.

The printing of the microarrays occur in two methods: contact and non-contact printing. Both methods are capable of producing arrays consisting of thousands to tens of thousands of spots per standard microscope slide.

Non-contact printing techniques are more modern and varied than contact printing. The most common method involved in this technique is utilising inkjet technologies.[17] It generally involves piezoelectric dispensing that controls the delivery of sample solutions through a glass capillary via electrical signals.[18] Non-contact printing offers very precise printing results with well distributed size and morphology of depositions. However, due to the high level of precision involved, nozzle constraints might arise as a limitation. Any alteration of spot size is accomplished by altering the size of the nozzle or by altering the pressure pulse through precise control of pulse duration and voltage.[19] Furthermore, it is extremely sensitive to environmental factors such as temperature and humidity, as they have significant effects on the printability of materials and final spot shape and size.[20] Hence, printing of just a couple of slides might require several hours when using the non-contact method.

Contact printing refers to microarrays formed through pin-printing, which involves the direct contact between printing pins, on an array robot, and the substrate (slide). The printing pins have been etched delicately to pick up tiny amounts of a sample precisely. Generally, the microscale dimension pins are first dipped into the sample solutions from a multi-well source plate to extract the samples. They will then pre-blot the samples on a 'pre-print' surface to achieve a consistent morphology, before spotting the directly onto the substrate surface.[21]

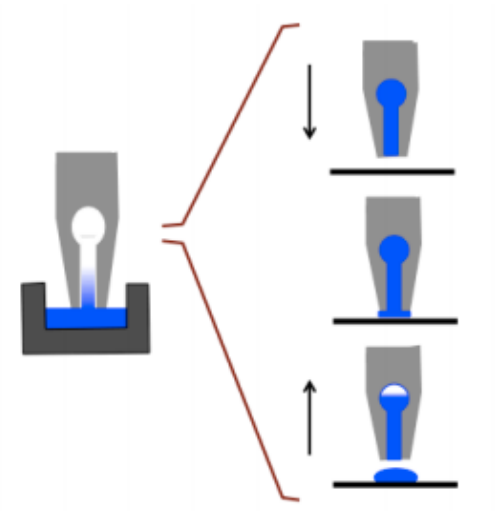


Figure 1. Illustration of contact printing

Unlike non-contact printing, the total volumes of sample delivered using contact printing is dependent directly on the contact time between the pins and the substrate surface. During the pre-blotting stage, approximately 0.5nL of the sample is retained and the excess is removed. This is to ensure consistency in the sizes of the spots on the substrate and to maintain the reproducibility and rapidness during the printing process. The final results are immobilised arrays of 100-200 micron spots. This feature makes microarrays a very appealing technique as it is an accurate imitation of the existence of carbohydrates on an actual cell-cell interface. This allows for the parallel investigation and analysis of a diverse range of interactions between proteins and carbohydrates.

1.3 Current area of application and interest

There are many attractions in using glycopolymer microarrays as a technique to study the biological activities on the surface of cells. Pathogens have evolved to recognise glycans on their host cell surface, which they initially bind to and subsequently infect. Glycopolymer microarrays act as a biomimetic surface in probing how pathogens interact with their hosts. There exist the potential for the development of anti-adhesion therapy, a process whereby the adhesion of pathogenic organisms is prevented.[22] The usage of microarray technologies can lead to the determination of the binding profiles of the array spots, allowing for the study and development of synthetic mimics such as glycopolymers.[23]

Another attraction would be to utilising the glycopolymer microarrays at the nano level as a platform that can be used as a biosensor. This is crucial for combating the spread of infectious diseases or to detect biological warfare agents.[24] Some previous work has shown that using monosaccharide coated surfaces has compared the relative binding of various lectins in generating an algorithm that enables the identification of the bound lectins.[25]

2. Project Aims

The objective of this project is to develop a technique in mimicking the interactions that occur on a cell's surface by using glycan microarrays and fluorescently labelled lectins, in an attempt to improve the research field of glycomics.

The project involves the understanding of the techniques used to produce glycan-terminated polymers microarrays that serves great importance in the field of glycomics. Glycopolymers were chosen to be used in this project as they play a critical role in various biological recognition events like cell-cell adhesion, cell growth regulation and immune reponse.[26] It thus allows for a more accurate depiction of activities on a cell's surface as compared to using regular glycans.

An efficient high-throughput technique is to be adopted in the printing of stable and reproducible microarrays. Secondly, finding a suitable agent in blocking the microarrays in order to reduce unnecessary background fluorescence. Finally, using fluorescently labelled lectins to observe the behaviour of the glycan-terminated polymers bounded to the surface of the slide.

3. Results and Discussion

3.1 Printing controls

Printing conditions and strategies needed to be determined and tuned in order to produce microarrays that are efficient for analysis. The mechanics of the microarray printer had to be understood in order for constructive and systematic printing to be adapted, as well as for accurate data analysis. Once the schematics of the microarray printer was fully understood, a range of microarray substrates could be produced, with the tailored features that depended on the necessity of print.

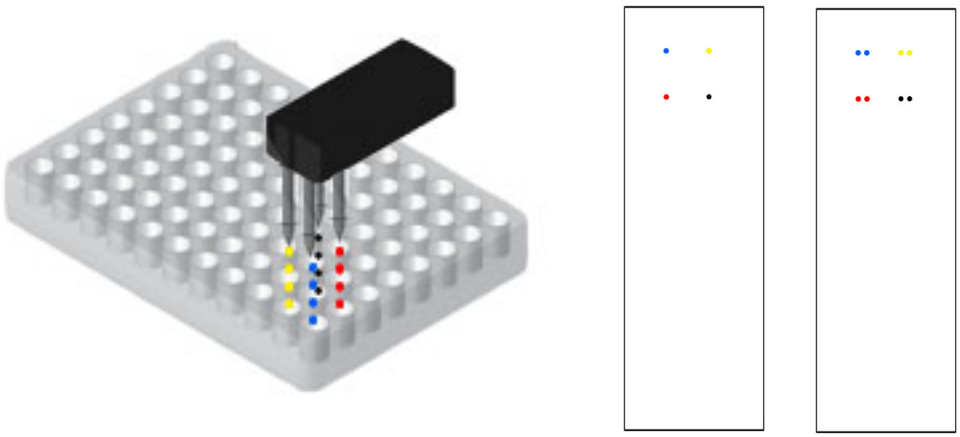


Figure 2. Pattern of print of first dip (same concentration across each block)

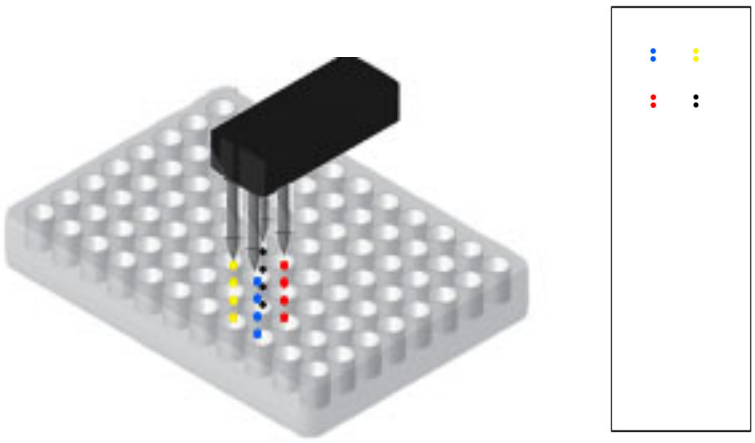


Figure 3. Pattern of print of second dip (decreasing concentration down each block)

Figure 2 and 3 explains the basis of print that the microarray printer adapts. In figure 2, it was observed that repeated spotting of the first dip happens across the slide. The

second dip's spotting pattern is represented in figure 3, with the spotting happening in the next row after the first. With this deeper understanding, systematic pipetting of sample solutions can be performed in order to get microarrays that are easy to interpret.

Sample Code	Name of Glycopolymer
Glc 25	Glucose-terminated Poly(*N*-hydroxyethylacrylamide) with 25 repeated units
Glc 50	Glucose-terminated Poly(*N*-hydroxyethylacrylamide) with 50 repeated units
Glc 100	Glucose-terminated Poly(*N*-hydroxyethylacrylamide) with 100 repeated units
Fuc 25	Fucose-terminated Poly(*N*-hydroxyethylacrylamide) with 25 repeated units
Fuc 50	Fucose-terminated Poly(*N*-hydroxyethylacrylamide) with 50 repeated units
Fuc 100	Fucose-terminated Poly(*N*-hydroxyethylacrylamide) with 100 repeated units
Man 25	Mannose-terminated Poly(*N*-hydroxyethylacrylamide) with 25 repeated units
Man 50	Mannose-terminated Poly(*N*-hydroxyethylacrylamide) with 50 repeated units
Man 100	Mannose-terminated Poly(*N*-hydroxyethylacrylamide) with 100 repeated units
Gal 25	Galactose-terminated Poly(*N*-hydroxyethylacrylamide) with 25 repeated units
Gal 50	Galactose-terminated Poly(*N*-hydroxyethylacrylamide) with 50 repeated units
Gal 100	Galactose-terminated Poly(*N*-hydroxyethylacrylamide) with 100 repeated units

Table 1. Library of glycopolymers for testing

There are four types of glycan-terminated polymers, with three degree of polymerization each, involved in this project. With a total of 12 samples to work with, systematic arrays needed to be produced.

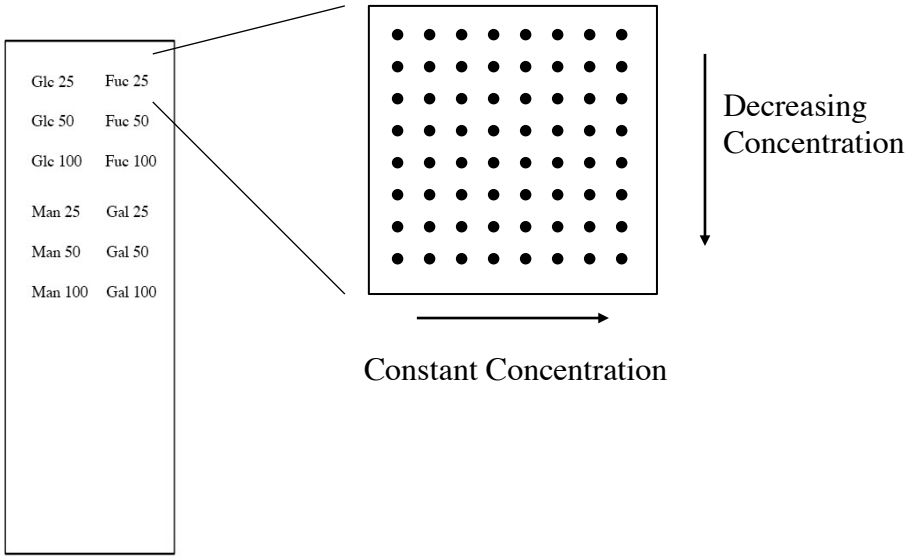


Figure 4. Template of microarray to be produced

The template in figure 4 was to be adopted in all the microarrays produced on epoxide slides. The same group of glycans were grouped together, with their different degrees of polymerisation increasing down the slide. Epoxide slides were chosen to be printed on due to its ability to bind with the thiol group that is present on the glycopolymers.

Besides testing the various glycan-terminated polymers, it is also paramount to measure the highest concentration of solubility, before that particular glycan-terminated polymer becomes insoluble. The 12 samples were diluted, by half, 7 times from the original concentration of 20 mg/ml, resulting in each sample having a total of 8 concentrations ranging from 20 mg/ml down to 0.156 mg/ml.

In order to achieve this particular template, a systematic printing order in the pipetting of the samples into the microplate needed to be strategised. With reference to the pattern of print of the microarray printer explained in figures 2 and 3, the first dip of the set of four printing pins was observed to be into the A1, A2, B1 and B2 well, as represented by the four coloured boxes. The second dip was then observed to be A3, A4, B3 and B4, and the pattern continues for the subsequent dips. The samples were hence pipetted into a 384-well microplate in the order shown in the appendix.

The next step would be to interpret the microarray scans. In order to do so, a colour test was conducted using 4 colour dyes to get a clearer picture of the projected results

in comparison to the spots on the glass slide. On a plain glass microscope slide, the colour dyes were manually spotted in different, distinct patterns.

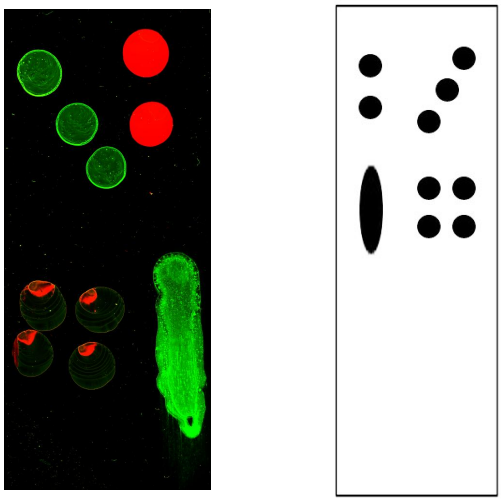


Figure 5. Scanned pattern of microarray (left), original pattern on slide (right)

In figure 5, the right image shows the original spotting pattern and the left shows the scanned results. From these two images, it was concluded that the microarray scanner produces results that were mirror images of the original microarray. Accurate analysis of result tabulation can be performed once the method of interpretation was laid out.

After all preparation had been tuned, the microarrays were printed on purchased epoxide slides using the microarray printer. Each dip was repeated 8 times. The printed slides were left in the printer to incubate for an hour at 21°C and 72% humidity. This is to ensure that the spots were well dried, but not completely evaporated, before further testing.

3.2 Determination of a suitable blocking agent

Unfortunately, there is still high potential for non-specific binding (NSB) to occur on the surface of the microarrays, contributing largely to background noise. Non-specific binding is most commonly dealt with the use of surface blocking strategies. Surface blocking is typically a post-print step in which regions between the arrayed spots are masked with a surface-active blocking agent that adsorbs irreversibly to the off-spot

array features to prevent undesired adsorption.[27] This blocking agent does not take part in any array reaction, minimising any interference in the interactions between the lectins and glycans. Should the microarrays be directly incubated with the lectins, the free primary amine groups on them would covalently bind to the surface of the slide, causing the poor ratio of signal to noise.

There are two main classes of blocking agent, proteins and detergents. Protein blockers block unoccupied sites on the surface and stabilise biomolecules that are bound to the surface, while detergent blockers block ionic and hydrophobic biomolecules bonded to the surface. For this project, a common protein blocking agent, bovine serum albumin (BSA), as well as a very successful non-ionic detergent blocking agent, Tween-20,[28] were tested.

Blocking Agent	Components	Weight Percentage
PBS + BSA	PBS	-
	BSA	0.05
PBST + BSA	PBS	-
	BSA	2
	Tween-20	0.05

Table 2. Proportion of combination of blocking agents

As seen in Table 1 above, the first blocking agent was made with PBS and BSA, and the second had Tween-20 added. The concentrations used to make the combinations were referred from ELISA technical guide to surface blocking.[29]

The microarrays were dip coated in the two blocking agents for an hour. The dip coating process was carried out at 40°C and 50% humidity so as to minimise the evaporation of the blocking agents. Images were taken before and after every step using the microarray scanner.

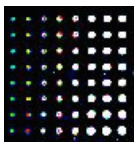
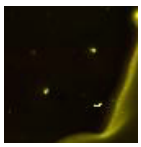
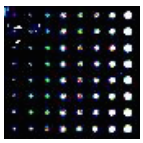
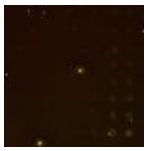
	Pre-block	Post-block
PBS + BSA		
PBST + BSA		

Table 3. Images of microarrays before and after incubation with blocking agents
(Note that only part of the arrays are shown)

After incubation with the BSA and BSA + Tween-20 blocking agent, the respective microarrays were dip washed in PBS and PBST respectively, and finally with dH_2O. This step is necessary as it is to ensure the removal of any excess blocking agent present on the surface of the slide. The microarrays were then scanned and the results obtained is shown in table 2. In both tests, the background fluorescence was kept at a minimum. However, the array spots were all washed off the slide that was incubated with PBS + BSA. For the slide that was dip washed with PBST + BSA, the spots remained and hence was chosen as the blocking agent to be used.

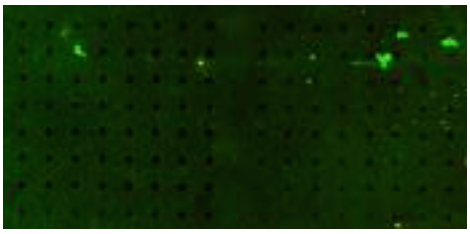


Figure 6. Background noise of PBST + BSA blocking agent in comparison with array spots

Following the determination of PBST + BSA being the more suitable blocking agent, the microarray was incubated with fluorescently labelled Concanavalin A (Con A) lectin for 1 hour at 40°C and 50% humidity. In addition, the slide was wrapped with aluminium foil in order to minimise the loss of fluorescence function of the lectin when exposed to light. Thereafter, the slide was dip washed in PBST then dH_2O once again to remove any excess lectin. The microarray was proceeded to be scanned and the result is shown in figure 6. Though the array spots were clearly observed, they were not fluorescing but instead, were reflected as black spots. The background was

interpreted to be too noisy (too bright), overshadowing the fluorescence by the array spots.

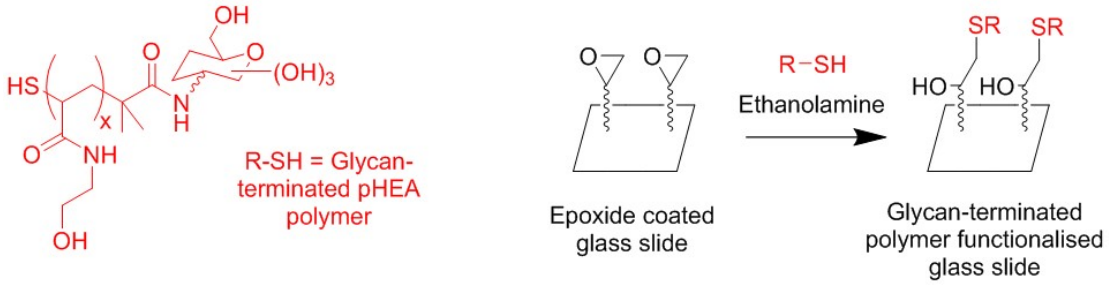


Figure 7. Structure of glycopolymer and role of ethanolamine

An alternative blocking agent needed to be tested for better results to be obtained. 1mg/ml of synthesised poly-2-hydroxyethyl aspartamide (pHEA, DP 10) was diluted in dH_2O, PBST, and PBST + BSA separately. A drop of 2-aminoethanol was added to each solution in order to deprotonate the glycopolymers to allow for their binding to the epoxide surface, as seen in figure 7. Plain glass slides and microarrays on epoxide slides were then incubated with the three solutions separately and the background fluorescence were observed.

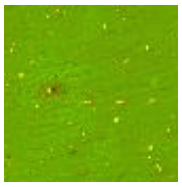
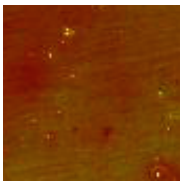
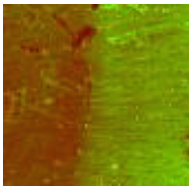
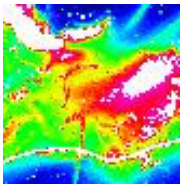
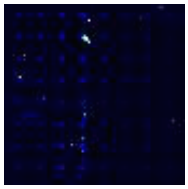
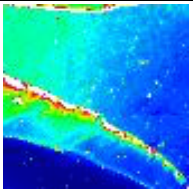
	pHEA in dH_2O	pHEA in PBST	pHEA in PBST + BSA
Plain glass slide			
Microarray on epoxide slide			

Table 4. Arrays after incubation with alternative blocking agents

After the slides were scanned, it was observed that both the solutions with pHEA in dH_2O and pHEA in PBST + BSA not only produced arrays that were high in background fluorescence, but also arrays in which the array spots were washed out.

The pHEA in PBST combination managed to eliminate majority of the background noise, as well as to keep the microarray unchanged, and hence, was chosen to be the new blocking agent to be used henceforth.

3.3 Testing of microarray strategy using Con A

After determining that the best suited blocking agent is pHEA in PBST with a drop of ethanolamine, the blocking process for the microarrays were carried out for an hour at 40°C and 50% humidity. They were then dip washed in PBST, followed by dH_2O for five seconds each before being left to be air dried. Thereafter, the slides were incubated with the Concanavalin A (Con A) lectin for an hour at the same temperature and humidity as the blocking process in a dark humidity chamber. The same post-incubation dip washing was carried out and the slide was left to be air dried. The microarrays were then ran in the microarray scanner and the results were obtained. The results were viewed using the 'Feature Extraction' software.

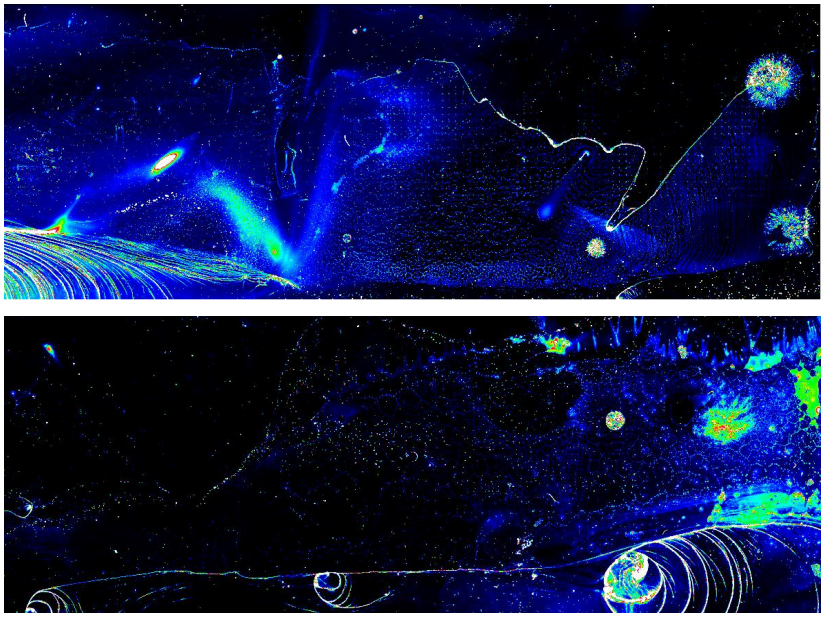


Figure 8. Examples of the delamination of slides

However, it was observed from the software that the array spots were washed out completely after reaction with the lectins, leaving only background fluorescence to be picked up by the scanner. Furthermore, a consistent 'tearing' pattern of the slide was

present. It was suspected that it was due to the unstable nature of the hydrogel underneath the epoxide layer that caused the deformation of the surface. When in contact with any liquid, swelling of the hydrogel is induced, increasing the instability of the hydrogel and finally resulting in the lifting of the surface.[30] This was thought to be the cause of delamination of the surface of the microarrays. Working with this hypothesis, the incubation time for both the blocking process and reaction with were reduced to a maximum of 15 minutes.

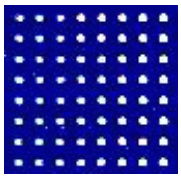
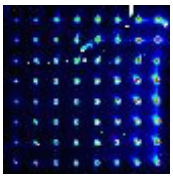
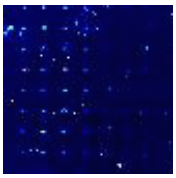
Original Array	Post Block	Con A
		

Table 5. Result Tabulation of Con A Lectin
(Note that only part of the arrays are shown)

The final results were tabulated and shown in table 5. The array spots were visible after reaction with Con A, with the background fluorescence kept low, as well as the absence of delamination of the surface. This allows for the further processing of data and data extraction.

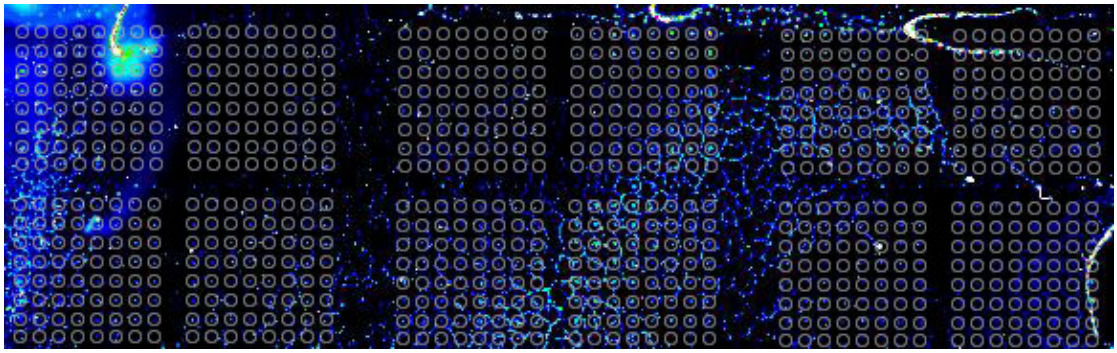
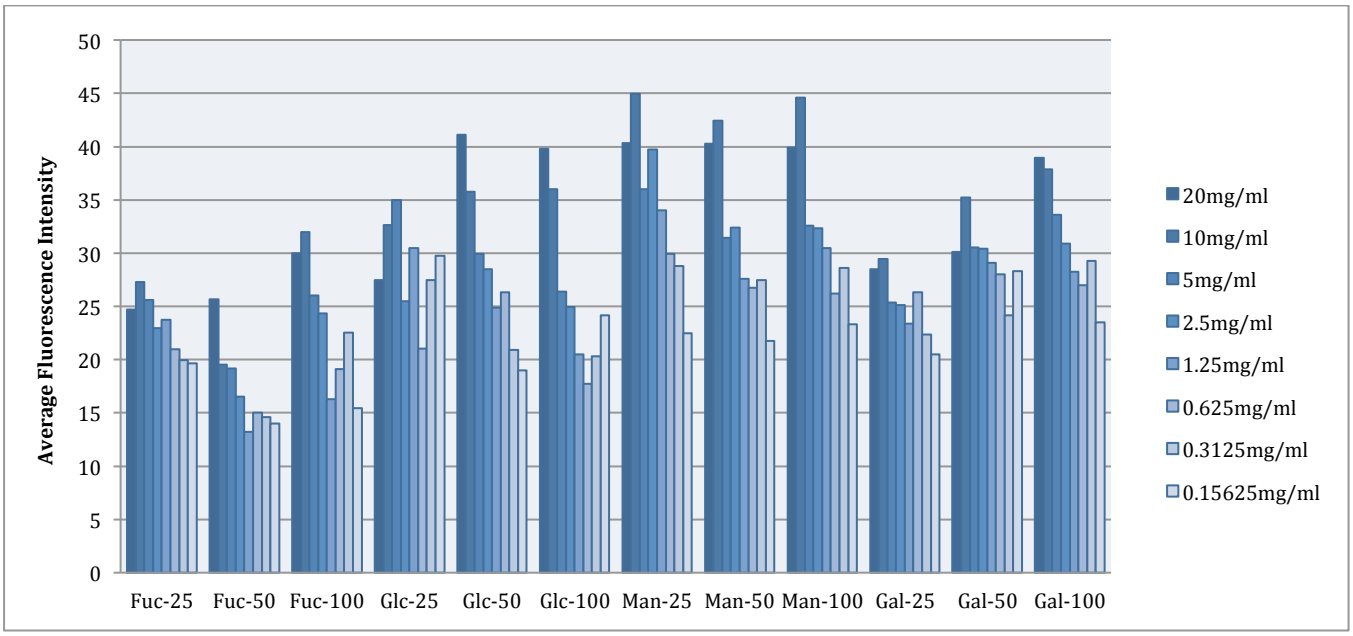


Figure 9. Example of result analysis using a mask

In the Feature Extraction software, it was not possible to mask out the array spots to calculate their fluorescence intensity directly. Since the data extraction functions of it is fairly limited, an alternative software had to be utilised to ensure the maximal retrieval of results data. ImageJ allowed for the direct calculation of fluorescence

intensity and hence the results were processed using it. A mask was laid over the array spots to ensure a more accurate calculation.



Graph 1. Average Fluorescence Intensity of Glycan-Terminated Polymers with Con A Incubation

After the average fluorescence intensity of each array spot was obtained, the overall average fluorescence intensity of each concentration of every sample was further calculated. The graph above reflects the comparison of intensity of the samples.

It should be noted that lectins generally bind with many sugars, and the only basis for comparison between the different samples would be the relative difference in binding affinity. According to literature, it is expected that Con A has the highest binding affinity to mannose[31] as compared to the other three lectins. With reference to graph 1, the results corresponds to the literature. A general trend observed with each sample is the increasing fluorescence intensity with increasing concentration of the glycan-terminated polymer. This positive correlation points to the successful binding of lectin to each substrate.

3.4 Incubation of rest of lectins (RCA, UEA I, WGA)

Continuing, further tests with the same strategy used for the testing of Con A lectin were performed on three more fluorescently labelled lectins; Ricinus Communis Agglutinin (RCA), Ulex Europaeus Agglutinin I (UEA I) and Wheat Germ Agglutinin (WGA).

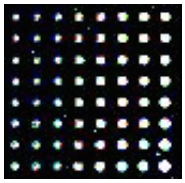
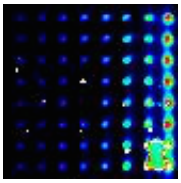
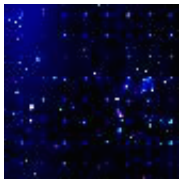
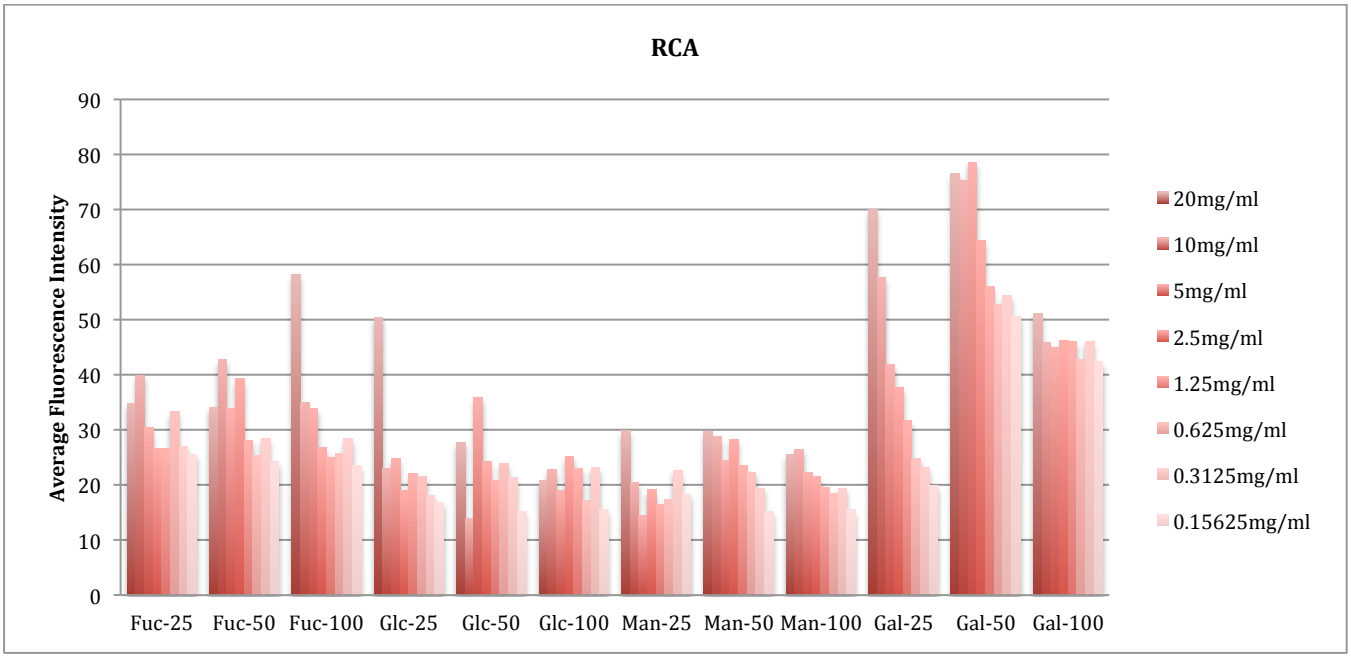
Original Array	Post Block	RCA
		

Table 6. Result Tabulation of RCA Lectin
(Note that only part of the arrays are shown)



Graph 2. Average Fluorescence Intensity of Glycan-Terminated Polymers with RCA Incubation

Similarly, literature has stated that RCA has a high binding affinity for galactose.[32] This was once again successfully observed from graph 2, with the peak of intensities for galactose being higher than the rest. The relationship between the average fluorescence intensity, which reflects the binding affinity, and concentration of the glycan-terminated polymers continued to share a good correlation.

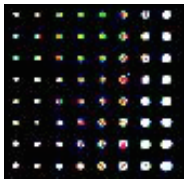
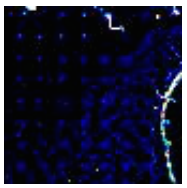
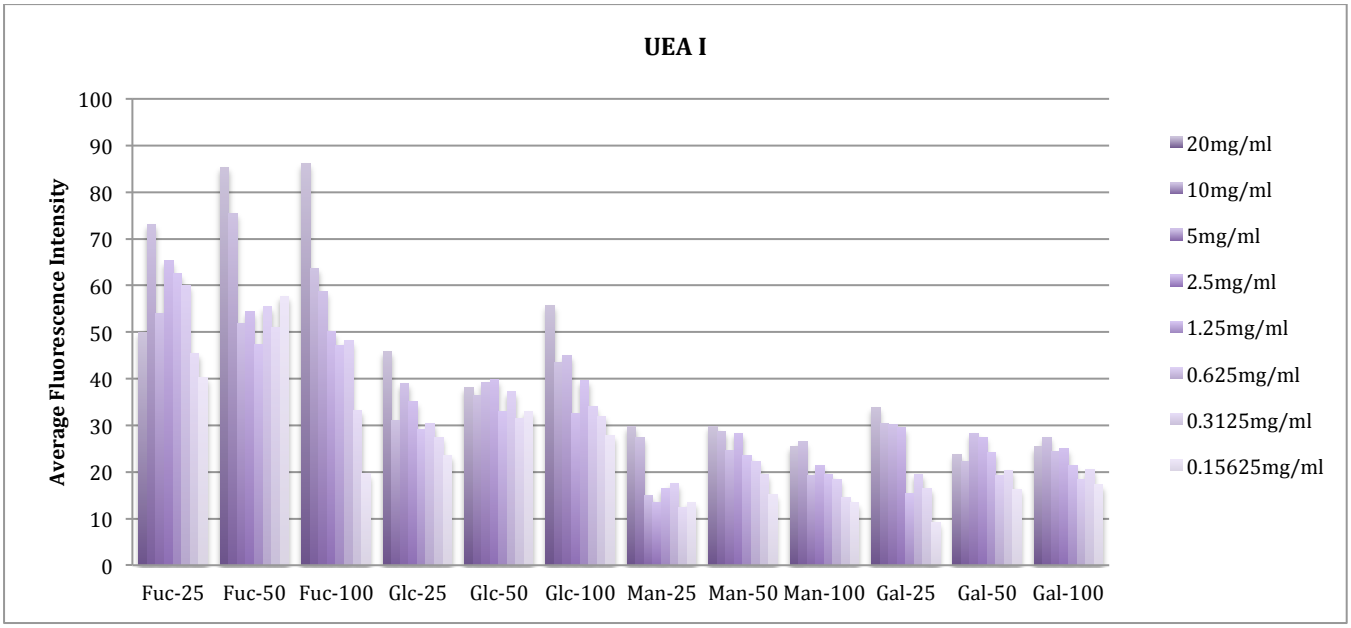
Original Array	Post Block	UEA I
		

Table 7. Result Tabulation of UEA I Lectin
(Note that only part of the arrays are shown)



Graph 3. Average Fluorescence Intensity of Glycan-Terminated Polymers with UEA I Incubation

The UEA I lectin has a preference for fucose.[33] It is also stated that there is a high possibility of glycans that is lacking fucose binding to UEA I as well.[34] That, however, is not shown as obviously in the graph above. It is reflected still, that amongst the glycan-terminated polymers, fucose bound the most to UEA I.

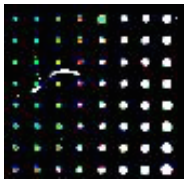
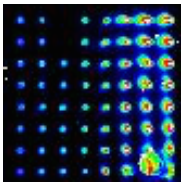
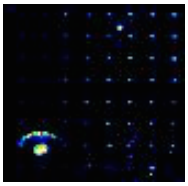
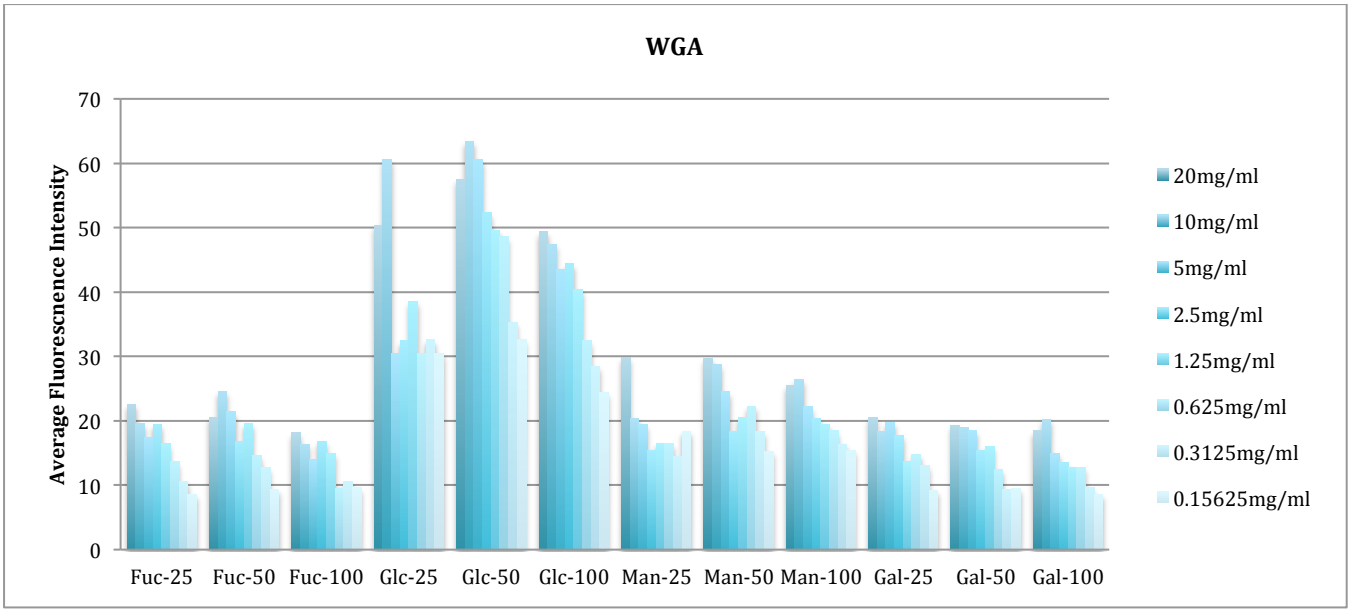
Original Array	Post Block	WGA
		

Table 8. Result Tabulation of WGA Lectin
(Note that only part of the arrays are shown)



Graph 4. Average Fluorescence Intensity of Glycan-Terminated Polymers with WGA Incubation

Lastly, for WGA, it is stated that it has a strong binding affinity for glucose.[35] Once again, it is reflected in the above graph in which the experimental results correspond to the literature. The general relationship between the concentrations of the polymers and average fluorescence intensity is also kept.

Majority of the processed and tabulated results successfully coincided with what previous findings have stated. The relationship between the binding affinity and polymer concentration also generally showed a positive correlation. However, upon closer look at the trend in each polymer, there were a few anomalies. This could be explain by the post dip-washing processes not be thorough enough, which caused the amount of samples on the array spots to differ. The higher the amount of samples on a

spot, the greater the binding affinity. However, it should also be noted that the longer the dip-washing process, the higher the chances of the array spots being washed out. Hence, a balance between the thoroughness of the dip-washing processes and the consistency in the amount of sample on the array spots has to be further experimented and struck.

4. Conclusion and Future Work

The great potential that the area of microarray technologies possess has allowed for great developments of this project. The work done utilised the advantages of the efficiency and reproducibility of microarrays to gain a greater knowledge of the interactions that happens on the surface of cells. It was paramount in understanding how the microarray machine functions during the printing process in order to produce systematic microarrays efficiently.

It was observed that a combination of protein and detergent blockers were able to successfully block the microarrays. However, it was not able to lower the background fluorescence enough for the array spots to be fluorescent. An alternative blocking agent was subsequently made and was successful not only in the blocking of the microarray, but also in lowering the signal-to-noise ratio as compared to the previous blocking agent. Both the blocking techniques minimised the non-specific binding interactions of the fluorescently labelled lectins to the surface and as such, the binding intensities of the lectins to the microarrays were able to be measured.

Most of the results that were tabulated tallied with the findings stated in the literatures. Binding affinities were able to be graphed out with the helped of various data extraction softwares. The technique still requires further development and tuning to increase the sensitivity of the microarrays in order to produce more accurate and detailed binding profiles. However, general profiling images were still successfully produced and the different binding specificities were still able to be observed. A clear distinction was seen between each binding profile.

The aim of the project was to achieve an efficient and reproducible glycan-terminated polymer microarray technique of analysis that mimics the happenings on a cell's surface. The final results obtained have shown strong promise and potential in the development into this area. It has also been proven that it is highly possible to use a simplified method in understanding how the interactions on the surface of cells work. However, follow up studies have to be conducted in order to get a deeper understanding and a more wholesome picture of the complex interactions that take place on the surface of cells.

5. Instruments

5.1 Microarray contact printer

To construct the glycan-terminated polymer microarrays, a 4-pin Array-IT SpotBot® 3 microarray contact printer was used. SpotBot® 3 SPOCLE Generator was used to create the printing routines and the SpotBot® 3 SpotApp software was used to carry out the printing of the microarrays.

The loading deck accommodates a 384-well microplate (for the samples to be spotted). The slide substrate deck accommodates 14 substrates of standard size (25mm x 76mm). The printing chamber offers humidity control between 10-85%.

5.2 Microarray Fluorescence Scanner

The primary method of analysis in this project was via fluorescence imaging. Using fluorescein isothiocyanate (FITC) labelling, the lectins were directly labelled by the supplier. This method of analysis allows for real-time as well as sensitive observation of even fairly weak interactions between the carbohydrates and lectins. [36] The images were taken using an Agilent G2565CA scanner (2μM resolution). The scanner utilizes a standard two colouring scanning technique of the SHG-YAG laser (532nm) and a helium-neon laser (63nm). Slides were contained in holders and placed into the carousel for scanning to take place.

6. Experimental Methods

Reagent	Supplier
BSA	Sigma Aldrich
Ethanolamine	Sigma Aldrich
Expoxide slides	CORNING®
HEPES beffer	Sigma Aldrich
Lectins	Vector Laboratories Inc.
Microarray Printer Wash Buffer	Arrayit SpotBot ® 3 Wash Buffer
PBS tablets	Sigma Aldrich
Plain microscopic slides	J. Melvin Freed Inc.
Tween 20	Sigma Aldrich

Table 9. List of reagents and their suppliers

The glycan-terminated polymers used were previously synthesised by Joshua Parkin under the supervision of Dr. Sarah-Jane Richards from the University of Warwick in January 2016.[24] They have been kindly donated to this project for further research in this field.

6.1 Control Printing

Serial dilutions of the 12 different glycan-terminated polymers were made up in a 384-well microplate (refer to table in appendix). The starting concentration was at 20mg/ml. Eight dilutions, with a factor of 2, were conducted. The overall concentrations ranged from 20mg/ml down to 0.156 mg/ml. The 4-pin Array-IT SpotBot® 3 microarray contact printing chamber was left to reach a humidity level of above 75%. Once it was reached, the 384-well microplate containing the glycan-terminated polymers was placed in the loading deck. 14 purchased CORNING® epoxide slides were loaded in the slide substrate deck.

After the slides were printed, they were left to incubate in the printing chamber for 1 hour at the same level of humidity as the printing process. The slides were carefully removed using tweezers and place into holders for the scanning process to take place. Fluorescence images of the original microarrays were taken. All slides were dip washed in dH_2O for five seconds and then left to air dry until completely dry. The

slides were scanned again. Unused slides were stored in a dark, dry and sealed container until needed again.

6.2 Dilution of Lectins

Four lectins (Con A, RCA, UEA I and WGA) were diluted from the original concentration of 2mg/ml down to 0.1mg/ml. This was done by adding 1 part of the lectins to 20 parts of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The 0.1mg/ml lectins were the used for incubating the microarrays.

6.3 Blocking Tests

Starting out, two solutions were prepared; BSA (0.4g, 2% weight percentage) in PBS (10mM), as well as BSA (0.4g, 2% weight percentage) and Tween-20 (10µL, 0.05% weight percentage) in PBS (10mM). The microarray slides were submerged in each solution in a sealed incubation chamber at 40°C and 50% humidity for an hour. After incubation, the slides were removed and washed in PBS solution and PBST solution respectively for five seconds, followed by the dip washing in dH_2O for another five seconds. They were left to air dry until complete dryness was achieved. The microarrays were scanned once again and their fluorescence images were taken. It was concluded that the PBST + BSA solution was a better fit. However, after further test with Con A, an alternative blocking agent needed to be made.

Three solutions were made by diluting 1mg/ml of synthesised poly-2-hydroxyethyl aspartamide (pHEA, DP 10) in dH_2O, PBST, and PBST + BSA separately. A drop of 2-aminoethanol was added to each solution. The same method of incubation was carried out on purchased standard plain J. Melvin Freed Inc. microscopic glass slides as well as previously made microarrays on purchased CORNING® epoxide slides. The results were compared and pHEA in PBST was concluded to be the best blocking agent out of the three. It was hence chosen to be used for all blocking processes of the microarrays.

6.4 Lectin Incubations of Microarrays

A layer of each of the four 0.1mg/ml lectins (Con A, RCA, UEA I and WGA) that were previously prepared was placed onto the surface of the blocked microarrays using a glass pipette. The lectin incubated microarrays were placed back into the sealed incubator chamber for an hour at 40°C and 50% humidity. After the incubation process, the slides were dip washed in PBST for five seconds, followed by dH_2O for another five seconds to wash off any excess lectins on the surface of the substrate. The microarrays were left to air dry until completely dry.

However, further testing showed that the delamination of the surface of the microarrays were consistently happening. It was concluded that the long incubation time was responsible for this. It was then shortened from one hour to a maximum of 15 minutes. The results of the short incubation period improved significantly. Hence, the incubation time was finalised to be a maximum of 15 minutes for any incubation processes henceforth.

6.5 Analysis of Results using Agilent Feature Extraction software and ImageJ

Two pieces of software were used in the result tabulation for this project. The Agilent Feature Extraction software was used to view the corresponding Tagged Image File (TIF) output files directly from the Agilent G2565CA scanner. However, it was only capable in calculating the fluorescence intensity values of the array spots by manually picking the peaks from a generated intensity graph. This will potentially result in large human error and inaccuracy. Its vague calculation method pushed for a secondary software to be used.

Image J is able to split the image of the scanned microarrays into discrete colour channels (red, green and blue). The green channel was used as it is also used in FITC labelling., showing only intensities from the green channel. ImageJ also allowed for the average fluorescence intensity of each array spot to be calculated directly with the help of a mask. The minimised any human error involved and was able to tabulate results in a short amount of time.

7. Safety Considerations

There are no specific hazards associated with any of the reagents used in the course of the project, except for ethanolamine. Safety gear (goggles, lab coat and gloves) were worn at all times in the lab.

Ethanolamine is a corrosive chemical and contact with it can severely irritate and burn the skin and eyes. Breathing in ethanolamine can irritate the nose, throat and lungs, causing coughing, wheezing and shortness of breath. High exposure of it may affect the nervous system.[37] Ethanolamine was handled in the fumehood due to its corrosive nature.

8. References

[1]*Hart, G. W. (2013). Thematic Minireview Series on Glycobiology and Extracellular Matrices: Glycan Functions Pervade Biology at All Levels. Journal of Biological Chemistry, 288(10), 6903.*

[2] *G. W. Hart, (2013). J. Biol. Chem., 10, 288–298.*

[3] *A. Varki, R. Cummings, J. Esko and et al., (2009). Essentials Glycobiol, 2, Chapter 6.*

[4] *T. Muramatsu, Nagoya J. (1994). Med. Sci., 57, 95–108.*

[5] *Laurent, N., Voglmeir, J., & Flitsch, S. L. (2008). Glycoarrays—tools for determining protein–carbohydrate interactions and glycoenzyme specificity. Chemical Communications, (37), 4400.*

[6] *Berg, J., Tymoczko, J., & Stryer, L. (2002). Biochemistry (1st ed.). New York: W.H. Freeman and Co.*

[7]*Wittmann, V., & Pieters, R. J. (2013). Bridging lectin binding sites by multivalent carbohydrates. Chemical Society Reviews, 42(10), 4492.*

[8] *Piotukh K, Serra V, Borriss R, Planas A. (1999). Protein-carbohydrate interactions defining substrate specificity in Bacillus 1,3-1,4-beta-D-glucan-4-glucanohydrolases as dissected by mutational analysis. Biochemistry. 8, 16092–16104.*

[9] *Lectin-carbohydrate interactions. (n.d.). Retrieved January 19, 2017, from http://www.sciencedirect.com/science/article/pii/0959440X9190173Q*

[10] *Sharon, N. & Lis, H. (1989). Lectins as cell recognition molecules. Science, 246(4927), 227-234.*

[11] *Biggs, C. I. (2015). Bio-Integrative Polymer Surfaces.*

[12] *Rademacher TW, Parekh RB, Dwek RA. (1988). Glycobiology. Annu Rev Biochem. 57, 785–838.*

[13] *Muthana, S. M., Campbell, C. T., & Gildersleeve, J. C. (2012). Modifications of Glycans: Biological Significance and Therapeutic Opportunities. ACS Chemical Biology, 7(1), 31-43.*

[14] *Fernandez-Alonso, M. D., Diaz, D., Berbis, M. A., Marcelo, F., Canada, J., & Jimenez-Barbero, J. (2012). Protein-Carbohydrate Interactions Studied by NMR: From Molecular Recognition to Drug Design. Current Protein and Peptide Science, 13(8), 816-830.*

[15] *A. Carnero (2006), Clin. Transl. Oncol., 8, 482–490.*

[16] *Trevino, V., Falciani, F., & Barrera-Saldaña, H. A. (2007). DNA Microarrays: a Powerful Genomic Tool for Biomedical and Clinical Research. Molecular Medicine, 13(9-10), 527–541.*

[17] *Barbulovic-Nad, I., Lucente, M., Sun, Y., Zhang, M., Wheeler, A. R., & Bussmann, M. (2006). Bio-Microarray Fabrication Techniques—A Review. Critical Reviews in Biotechnology, 26(4), 237-259.*

[18] *I. Barbulovic-Nad, M. Lucente, Y. Sun, M. Zhang, A. R. Wheeler and M. Bussmann (2006), Crit. Rev. Biotechnol., 26, 237–259.*

[19] *de Gans, B.J.; Schubert, U. S. Langmuir (2004), 20, 7789.*

[20] *Calvert, P. (2001). Chemistry of Materials, 13, 3299.*

[21] *Brennan, J. D., Capretta, A., & Biology, C. (2013). Surface Characterization and Comparison of Contact vs. Non-Contact Printed Sol-Gel Derived Material Microarrays. Retrieved January 19, 2017, from http://macsphere.mcmaster.ca/handle/11375/15293*

[22] *I. Ofek, D. L. Hasty and N. Sharon. (2003) FEMS Immunol. Med. Microbiol., 38, 181– 191.*

[23] *Muñoz-Bonilla and M. Fernández-García. (2015) Materials (Basel)., 8, 2276–2296.*

[24] *Richards, S., & Gibson, M. I. (2014). Optimization of the Polymer Coating for Glycosylated Gold Nanoparticle Biosensors to Ensure Stability and Rapid Optical Readouts. ACS Macro Letters, 3(10), 1004-1008.*

[25] *L. Otten and M. I. Gibson. (2015) RSC Adv., 5, 53911–53914.*

[26] *Narain, R. (2011). Engineered carbohydrate-based materials for biomedical applications: polymers, surfaces, dendrimers, nanoparticles, and hydrogels. Hoboken, NJ: Wiley.*

[27] *Rampal, J. B. (2007). Microarrays, Second Edition: Volume I, Synthesis Methods. Totowa, NJ: Humana Press Inc.*

[28] *J. Chambers and A. Brajter-toth (2002), in Electroanalytical Methods of Biological Materials, 344–346.*

[29] *J. Gibbs (2001), Effective Blocking Procedures.*

[30] *Kang, M. K. (2010). Swelling induced deformation and instability of hydrogels.*

[31] *Ofek I, Mirelman D, Sharon N (1977). "Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors". Nature. 26 (5595): 623–5.*

[32] *Okada, T., & Minoura, N. (2011). Fluorescence emission and polarization analyses for evaluating binding of ruthenium metalloglycoclusters to lectins and tetanus toxin C-fragment. Journal of Biomedical Optics, 16(3), 037001.*

[33] *GALAB technologies (2016), Tools Glycosci.*

[34] *Molin, K., Fredman, P., & Svennerholm, L. (1986). Binding specificities of the lectins PNA, WGA and UEA I to polyvinylchloride-adsorbed glycosphingolipids. FEBS Letters, 205(1), 51-55.*

[35] *Horton, D., & Tipson, R. S. (1978). Advances in carbohydrate chemistry and biochemistry. New York: Academic Press.*

[36] *I. Sigma-Aldrich (2010), SIGMA Fluorescein Isothiocyanate, 2–4.*

[37] *New Jersey Department of Health and Senior Services. (2016). Hazardous Substance Fact Sheet. Retrieved January 19, 2017, from http://nj.gov/health/eoh/rtkweb/documents/fs/0835.pdf*

9. Appendices

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	Glc-25 20 mg/ml	Fuc-25 20 mg/ml	Glc-25 10 mg/ml	Fuc-25 10 mg/ml	Glc-25 5 mg/ml	Fuc-25 5 mg/ml	Glc-25 2.5 mg/ml	Fuc-25 2.5 mg/ml	Glc-25 1.25 mg/ml	Fuc-25 1.25 mg/ml	Glc-25 0.625 mg/ml	Fuc-25 0.625 mg/ml	Glc-25 0.3125 mg/ml	Fuc-25 0.3125 mg/ml	Glc-25 0.15625 mg/ml	Fuc-25 0.15625 mg/ml
B	Glc-50 20 mg/ml	Fuc-50 20 mg/ml	Glc-50 10 mg/ml	Fuc-50 10 mg/ml	Glc-50 5 mg/ml	Fuc-50 5 mg/ml	Glc-50 2.5 mg/ml	Fuc-50 2.5 mg/ml	Glc-50 1.25 mg/ml	Fuc-50 1.25 mg/ml	Glc-50 0.625 mg/ml	Fuc-50 0.625 mg/ml	Glc-50 0.3125 mg/ml	Fuc-50 0.3125 mg/ml	Glc-50 0.15625 mg/ml	Fuc-50 0.15625 mg/ml
C	Glc-100 20 mg/ml	Fuc-100 20 mg/ml	Glc-100 10 mg/ml	Fuc-100 10 mg/ml	Glc-100 5 mg/ml	Fuc-100 5 mg/ml	Glc-100 2.5 mg/ml	Fuc-100 2.5 mg/ml	Glc-100 1.25 mg/ml	Fuc-100 1.25 mg/ml	Glc-100 0.625 mg/ml	Fuc-100 0.625 mg/ml	Glc-100 0.3125 mg/ml	Fuc-100 0.3125 mg/ml	Glc-100 0.15625 mg/ml	Fuc-100 0.15625 mg/ml
D	Man-25 20 mg/ml	Gal-25 20 mg/ml	Man-25 10 mg/ml	Gal-25 10 mg/ml	Man-25 5 mg/ml	Gal-25 5 mg/ml	Man-25 2.5 mg/ml	Gal-25 2.5 mg/ml	Man-25 1.25 mg/ml	Gal-25 1.25 mg/ml	Man-25 0.625 mg/ml	Gal-25 0.625 mg/ml	Man-25 0.3125 mg/ml	Gal-25 0.3125 mg/ml	Man-25 0.15625 mg/ml	Gal-25 0.15625 mg/ml
E	Man-50 20 mg/ml	Gal-50 20 mg/ml	Man-50 10 mg/ml	Gal-50 10 mg/ml	Man-50 5 mg/ml	Gal-50 5 mg/ml	Man-50 2.5 mg/ml	Gal-50 2.5 mg/ml	Man-50 1.25 mg/ml	Gal-50 1.25 mg/ml	Man-50 0.625 mg/ml	Gal-50 0.625 mg/ml	Man-50 0.3125 mg/ml	Gal-50 0.3125 mg/ml	Man-50 0.15625 mg/ml	Gal-50 0.15625 mg/ml
F	Man-100 20 mg/ml	Gal-100 20 mg/ml	Man-100 10 mg/ml	Gal-100 10 mg/ml	Man-100 5 mg/ml	Gal-100 5 mg/ml	Man-100 2.5 mg/ml	Gal-100 2.5 mg/ml	Man-100 1.25 mg/ml	Gal-100 1.25 mg/ml	Man-100 0.625 mg/ml	Gal-100 0.625 mg/ml	Man-100 0.3125 mg/ml	Gal-100 0.3125 mg/ml	Man-100 0.15625 mg/ml	Gal-100 0.15625 mg/ml

Table 10. Pipetting pattern on 384-well microplate