DEVELOPING AND UTILIZING A ROBUST MASS SPECTROMETRY-BASED APPROACH TO STUDY GLUCOSE METABOLISM AND TRANSPORT IN THE LENS

Ali Zahraei

Department of Physiology
Faculty of Medical and Health Sciences
University of Auckland
New Zealand

ABSTRACT

The lens is an essential component of the ocular system and has a high metabolic demand. However, the delivery of energy sources such as glucose and waste removal cannot occur via the vasculature as this would obstruct the path of light through the lens. Thus, the lens requires a highly developed glucose delivery system to maintain its transparency. This thesis not only examines glucose uptake in the lens and the microcirculation that can transport glucose within the lens, but also develops a highly spatially resolved methodology to be able to study this. Firstly, I optimised a paradigm that would allow Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) analysis of glucose uptake and metabolism in ocular lenses. Specifically, the matrix choice, tissue preparation steps and normalisation strategies were carefully optimised to glucose and its metabolites using MALDI-IMS applied to organ cultured bovine lens tissue. Next, this optimised method was applied in combination with MALDI-IMS and gas chromatography mass spectrometry (GC-MS) to understand the mechanisms of glucose uptake, transport and metabolism of glucose in the bovine lens under physiological normoglycemic conditions. The results revealed that glucose uptake first occurs in the equatorial region, followed by the anterior and posterior poles. Additionally, I spatially correlated the pattern of glucose uptake to the expression of glucose transporters (GLUTs) in the bovine lens. Using spatial proteomics, I discovered that GLUT1 is the predominant isoform expressed in the lens fibres, and GLUT3 is predominantly expressed in the lens epithelium. The location of GLUT1 was further confirmed using immunohistochemistry. Subsequently, MALDI-IMS demonstrated that, once glucose is taken up, it can be metabolised in the epithelium, outer cortex and even the lens nucleus. In addition, I attempted to investigate whether glucose delivery to deeper regions of the lens occurs via an intracellular or extracellular pathway. Unfortunately, MALDI-IMS and GC-MS methods did not have the required resolution to visualize extracellular delivery using current stable isotopically-labelled tracers. However, GC-MS showed that glucose delivery to the nucleus could be reduced by inhibiting the microcirculation using the Na⁺/K⁺ ATPase inhibitor ouabain, a result that provided indirect support of the hypothesis that

II
an extracellular pathway is used to deliver glucose to the lens nucleus. Further research will be needed to confirm this hypothesis.

Overall, this thesis provides high-resolution information about the pattern of glucose uptake, its correlations with the expression of glucose transporters, and the distribution of the glucose metabolites, thereby enhancing our understanding of lens metabolism. The tools developed in this thesis to study lens tissue in physiological conditions can be applied to advance our understanding of lens pathologies such as diabetic and age-related cataracts in which glucose metabolism in the lens is perturbed. Therefore, this thesis establishes a consolidated research pipeline to study metabolomics (the dynamics of nutrient uptake, transport and metabolic flux) and proteomics in the lens to enable future spatially-resolved screening of metabolic changes in pathological models.
DEDICATION TO MY FAMILY
ACKNOWLEDGEMENTS

These three and a half years of my doctoral journey have been some of the most challenging and rewarding years of my life, and despite the many obstacles (including a global pandemic), I would have not enjoyed this journey nearly as much without the support of my incredible mentors, friends and family. To each of you, close and afar, I am eternally thankful! Thank you for your part in my journey!

First and foremost, I am deeply grateful for my primary supervisor Dr Gus Grey for all the guidance, support, outstanding feedback and consistent encouragement throughout the course of my PhD. You have allowed me to build on the skills I brought to the lab, and guided me to accomplish so much more than I could have imagined at the beginning of my PhD. Gus, I have really valued our long discussions about experiments and results. Thank you for being so approachable and collaborative!

I also express my sincerest gratitude to my co-supervisors Professor Paul Donaldson and Dr Nicholas Demarais. Paul, your ability to not only keep track of, but also successfully guide and manage, so many projects and people, both in the Molecular Vision Lab (MVL) and within the School of Medical Sciences in your role as Head of School, is absolutely amazing and inspiring. Thank you for your unwavering support and guidance in all matters from the academic and intellectual, logistical, and financial. Your passion for your work is very infectious! Thank you for the opportunity to be a part of your team! And Nick, thank you for all your help and encouragement during the process of optimising the MALDI-IMS methodology. Thank you very much for sharing with me your knowledge and expertise with SolariX, your thorough teachings have enabled me to now count that among my skills.

A special thanks to Dr George Guo for your expertise and mentorship. You were always there for discussions about anything that I was unsure on, and I could not have finished this project without your knowledge and guidance. And also, a great thanks to Dr Kyriakos Varnava for your help and support with the LC-MS analysis. Thank you for all the invaluable expertise you brought to my PhD project. In addition, I would like to extend my thanks to Ms Sara Green, Dr Erica Zarate and Dr Jin Wang for your incredible service in obtaining my GC-MS data – you have been a strong technical pillar for me.
I am very grateful to the extended MVL for your comradery, your insightful comments and suggestions during lab meetings, and cheerful lunchtime chats. Your friendship over these years has made my time in this lab absolutely amazing. Associate Professor Julie Lim, thank you for always being willing to answer my questions and for your input and expertise on the lens hyperglycaemia parts of my project. I would also like to say a huge thanks to my colleague and friend Dr Rosica Petrova for your invaluable advice that will benefit me beyond my PhD, and also for helping me during the optimisation of the immunohistochemistry protocol for my project. In addition, I thank Dr Renita Martis and Ms Ivy Bo Li for your time and efforts in teaching me many different techniques – your kindness was really appreciated. I extend my thanks to all other members of the MVL for making the lab such an enjoyable place to work.

I am incredibly grateful for The University of Auckland (UoA), the UoA Mass Spectrometry Hub, and the UoA Biomedical Imaging Research Unit for providing me with the resources necessary to make my research possible. Thank you to the Health Research Council of New Zealand for your generous funding towards my project; thank you to the Faculty of Medical and Health Sciences Postgraduate Student Association and the Nico Nibbering Student Travel Award for supporting my travel to The International Mass Spectrometry Conference in Netherlands in 2022 – it allowed me to share the findings in this thesis with the international research community and gain their valuable feedback on my work.

I want to give my deepest appreciation to Dr Yukti Vyas for your unwavering encouragement, support, and unconditional guidance during my PhD journey. You have been my anchor these last few years, and I am so grateful for your love, humour, and friendship – it means the absolute world to me. I am also very thankful for my friends in New Zealand – you became my extended family, especially when I was far from mine during the pandemic, and I am so grateful for you for keeping me sane! Finally, but the most importantly, I thank my incredible family for being my champions! Mum, Dad, and my siblings, Mohammad, Fatima and Mohadeseh thank you so much for your blessings, loving support, and patience! I am forever grateful! And a huge thank you to my dear daughter, Luna, for continuously motivating me to do my best and unconditionally loving me – you have made me stronger, better and more fulfilled than I could have ever imagined. I cannot possibly express to you all how grateful I am.
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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1,5-DAN HCl</td>
<td>1,5-naphthalenediamine hydrochloride</td>
</tr>
<tr>
<td>3-OMG</td>
<td>3-O-methyl-D-glucose</td>
</tr>
<tr>
<td>9-AA</td>
<td>9-Aminoacridine</td>
</tr>
<tr>
<td>AAH</td>
<td>Artificial aqueous humour</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>AR</td>
<td>Aldose reductase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CySSG</td>
<td>Cysteine glutathione disulphide</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (known as an atomic mass unit)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier-transform ion cyclotron resonance</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography–mass spectrometry</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GRIN</td>
<td>Gradient index</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>GSSG</td>
<td>Glutathione disulphide</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>IC</td>
<td>Inner cortex</td>
</tr>
<tr>
<td>IMS</td>
<td>Imaging mass spectrometry</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mm Hg</td>
<td>Millimetres of mercury</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MVRC</td>
<td>Molecular vision research cluster</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Central nucleus</td>
</tr>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>Sodium-potassium adenosine triphosphatase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium-doped yttrium aluminium garnet</td>
</tr>
<tr>
<td>NEDC</td>
<td>N-(1-naphthyl) ethylenediamine dihydrochloride</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>OC</td>
<td>Outer cortex</td>
</tr>
<tr>
<td>Osm</td>
<td>Osmolarity</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PTMs</td>
<td>Posttranslational modifications</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rmp</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>Sorbitol Dehydrogenase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-glucose Cotransporter</td>
</tr>
<tr>
<td>SIL</td>
<td>Stable isotopically-labelled</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilyl</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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Chapter 1 || INTRODUCTION

Maintaining the transparency and refractive properties of the lens is critical to preserving our vision as we age (Donaldson et al., 2017). The age-dependent failure to maintain lens transparency results in lens cataract, a clouding of the lens that obstructs light transmission and results in light scattering that initially affects image quality and, if untreated, can lead to blindness (Truscott & Friedrich, 2019). Surgical intervention is currently the only therapeutic option for cataract. It is the most common ophthalmic operation in the world and an efficient cure for cataract (Allen & Vasavada, 2006). However, the treatment is very expensive, placing an increasing economic burden on the healthcare system in every country. For example, in New Zealand alone, approximately 16,000 cataract surgeries are performed each year, and more than $30 million was spent on public inpatient and day stay services for cataract surgery between 2009 and 2010 (Economics, 2020). Moreover, people of Māori, Pacific, and Indian descent in New Zealand exhibit a higher incidence of cataract and develop cataract earlier than the general population, making cataract a significant health issue in this country (Yoon et al., 2016). In developing countries, where cataract surgery is less available, cataracts are an even more significant health and economic burden (Tabin et al., 2008). While increasing access to cataract surgeries is one possible solution, since cataract is primarily a disease of old age, a purely surgical solution is unlikely to keep pace with the world’s growing aging population. Hence, the absolute number of people worldwide blinded by cataract is anticipated to grow to 61 million by 2050 (Burton et al., 2021).

There are three major types of lens cataracts defined by the site of initial cataract formation: subcapsular cataract, cortical cataract and age-related nuclear (ARN) cataract, with diabetic cortical cataract and ARN cataract being the most frequent (Braakhuis et al., 2019). Oxidative stress from both endogenous and external causes is the leading cause of ARN cataract and develops in the lens nucleus (Costello et al., 1992). Cortical cataract, which is primarily observed in diabetic patients is characterised by the formation of wedge-shaped or radial spoke opacifications in the lens cortex (Bron et al., 1993; Obrosova et al., 2010). Hence to reduce the need for and costs associated with cataract surgery, a greater
understanding of the different cellular mechanisms that manifest as the specific types of cataract is required. However, to guide such studies, we first need to know how the normal lens maintains its transparency and refractive properties.

In this thesis, I have attempted to add to this body of knowledge by developing novel imaging mass spectrometry methodologies that enable the delivery, uptake and metabolism of glucose in different regions of the normal lens to be studied. This basic information is required if we are to determine how the dysfunction of glucose metabolism in the diabetic lens results in diabetic cortical cataract so that we can in the future develop alternative therapies to delay or prevent cataract formation in diabetic patients. To provide context for the studies conducted in my thesis, I will first review the role of the lens in the eye and the structure and function of the lens with an emphasis on our current understanding of the metabolism of glucose in the lens, before outlining the aims of my PhD project.

### 1.1. Anatomical structures of the eye

The eye is a well-structured and essential organ in the optical system (Figure 1.1). It is made up of three fundamental layers; the corneoscleral layer, the uveal layer (composed of choroid, ciliary body and iris) and the neural layer (retina), organised to optimise functionality of the eye (Forrester et al., 2020). The transparent cornea, the opaque sclera, and the translucent conjunctiva are all fibrous structures. The cornea and lens are the two primary optical structures. To enable clear vision, these tissues are organised in a particular order and must all operate optimally. Light enters the eye through the cornea, which functions as the principal barrier between the external environment and the ocular tissues. The curved structure of the cornea also allows it to refract light into the eye. Light then passes through the pupil, which is a circular hole in the pigmented iris underneath the cornea. In combination with the eyelids, the pupil regulates the light levels entering the eye. After the pupil, light passes through the lens, where there is an additional refraction to ensure that light is effectively focused onto the retina. Light then traverses the vitreous humour in the posterior chamber before reaching the retina (Donaldson et al., 2017). When light hits the retina, it initiates a chemical reaction known as the 'phototransduction cascade'. Activation
of the phototransduction cascade produces action potentials that travel via the optic nerve to the visual cortex in the brain, where visual processing occurs (Levin et al., 2011; Sefton et al., 2015).
Figure 1.1: The Anatomy of the human eye.

The eye is composed of three main layers: the corneoscleral layer (blue), the uveal layer (red) and the neural layer (yellow). Incident light passes through the cornea, aqueous humour, iris, lens and vitreous humour. The cornea and lens focus light on the retina. Adapted from “Anatomy of the Human eye”, by BioRender.com (2022).
1.1.1. The fibrous tunic: Cornea and sclera

The cornea and sclera (Figure 1.1) form the outermost layer which functions as the primary barrier against external pathogens (Meek & Knupp, 2015). They cover and protect the eyeball's spherical shape. This tough fibrous envelope preserves the ocular tissues while also providing a structural support for intraocular contents and enables extraocular muscle attachment. The cornea, with four layers, is the transparent anterior surface of the eye and it is responsible for approximately two-thirds of the total optical power of the eye (Maurice, 1957; Patel et al., 1995).

The epithelium, the cornea's outermost layer, where incident light meets the eye, is continually exposed to physical stress, microbial invasion, external chemical assaults, and ultraviolet radiation. It is only partially permeable to some compounds including polar or hydrophilic chemicals, water, or ions. Therefore, producing topical medication formulations that are able to pass across the cornea with acceptable bioavailability for the treatment of intraocular ophthalmic disorders is a major challenge (Malhotra & Majumdar, 2001).

The inner stromal layer is critical for maintaining transparency, representing about 90% of the corneal thickness (Besharse et al., 2010; Riordan-Eva & Cunningham, 2011). The stroma is mainly composed of extracellular matrix components such as collagen fibres and extremely hydrophilic proteoglycans, which allow water to enter the stroma (Besharse et al., 2010). The endothelium comprises a single layer of mitochondria-rich epithelial cells that actively transport ions across their cell membrane via different electrolyte pumps that establish an osmotic gradient to draw water from the stroma and into the aqueous humour (Diecke et al., 2011; Noske et al., 1994). The sclera is a layered elastin and collagen bundle structure that stretches from the limbus anteriorly to the lamina cribrosa posteriorly. The canal of Schlemm near the limbus drains the aqueous humour from the eye. Through the sclera's lamina cribrosa, the optic nerve and blood vessels exit the eye posteriorly (Davis, 1929).

1.1.2. The retina
The retina covers roughly 65 percent of the eye's inner surface, and it consists of multiple layers of neural cells and tissue that detect light and transduce it into electrical signals (Forrester et al., 2020). Anteriorly, the retina is connected to the ciliary body, and to the optic nerve in the posterior eye. When light rays reach the retina, they pass through the inner retina's light-insensitive components before reaching the outer photoreceptor-containing nuclear layer. Photopigments, which absorb photons of light, are found in the outer segments of photoreceptors. Light absorption activates a G-protein-linked signalling cascade, which changes the membrane potential of the photoreceptor and therefore the amount of neurotransmitter released into the synaptic cleft. Neurotransmitters stimulate or inhibit bipolar cells, which synapse onto ganglion cells whose axons constitute the optic nerve. The optic nerve transmits ganglion cell action potentials to the primary visual cortex, which interprets and processes the information into a visual image (Levin et al., 2011).

1.1.3. The uveal tract

The uveal tract, also known as vascular tunic, is made up of three main components: (1) the choroid; (2) the ciliary body, and (3) the iris. This highly vascularised central region of the eye facilitates the ocular tissue’s metabolic demands, regulates light entry, and absorbs reflected light.

The choroid is the uveal tract’s posterior portion. It is a thin, pigmented, vascular tissue between the sclera and the retina. The choroid in the posterior region of the eye supplies blood to the retina’s deeper layers (Bassnett et al., 2011), and keeps the intraocular temperature stable at 37°C (Forrester et al., 2020). The ciliary body is a ring-shaped structure located behind the iris and comprises two parts: the ciliary epithelium and the ciliary stroma, which house the ciliary vasculature and the ciliary muscles (Oyster, 1999; Talks et al., 1997). The epithelium consists of a two-layered structure with a pigmented epithelium facing the stroma and vasculature and a non-pigmented epithelium facing the aqueous humour. The ciliary body has two primary responsibilities. Firstly, the ciliary epithelial cells secrete the aqueous humour, a plasma filtrate enriched in nutrients and devoid of red blood cells, which acts as the source of nutrition for the avascular lens and cornea (Do & Civan, 2004; Kiel et al., 2011; Link & Nishi,
Secondly, the ciliary muscle regulates the curvature and therefore optical power of the lens to maintain focus during changes in viewing distance (Wisely et al., 2017). The iris is located between the cornea and the lens, and it determines pupil size by contracting a pair of antagonistic muscles called the sphincter and dilator pupillae. As part of the lens accommodation reflex, the pupil size contracts to restrict off-angle light entering the eye, and hence it controls the amount of light entering the eye (Forrester et al., 2020).

1.1.4. Aqueous humour

While in most areas of the body the blood supply provides nutrients and removes metabolic waste, for the avascular tissues of the eye (lens, cornea and vitreous humour) this function is performed by the aqueous humour (Snytnikova et al., 2017). Hence the aqueous humour has two main functions: to nourish the avascular ocular tissues (lens and cornea), and to regulate intraocular pressure by inflating the eyeball.

It is a transparent fluid that resembles the plasma, but has a low protein concentration. It is slightly alkaline, with a pH in the anterior chamber of 7.2-7.4. It consists of 99.9% water, with the remaining 0.1% made up of glucose, vitamins, amino acids and other nutrients such as glutathione (GSH). The glucose concentration in the aqueous humour is roughly the same as that in blood serum (Chylack Jr, 1971). Most of the aqueous humour is discharged by the ciliary body and then drained from the posterior chamber into the anterior chamber via the pupil (Figure 1.2). The majority of the aqueous fluid then exits the eye via the trabecular meshwork with a smaller proportion using the uveoscleral route (Kiel et al., 2011).
Figure 1.2: Diagram of aqueous humour flow.

Normal direction of flow of the aqueous humour from the posterior chamber, through the pupil, and into the anterior chamber. Aqueous humour drains the eye primary through two pathways: the trabecular meshwork and the uveoscleral pathway. Adapted from American Academy of Ophthalmology, illustration by Mark Miller, (https://www.aao.org/image/aqueous-humor-flow-).
1.1.5. Vitreous humour

The vitreous humour is a transparent gel that permits light to reach the retina. It is located within the posterior chamber, the largest cavity of the eye, and is limited anteriorly by the lens and posteriorly by the retina and optic nerve head. Vitreous humour plays an important part in eye protection. Most significantly, it supports the maintenance of the eyeball’s spherical shape. The pressure of the vitreous humour helps in the retention of the retina. It is mainly composed of water, although it is highly viscous and has a gel-like consistency. This consistency is due to its non-water constituents, namely hyaluronan (hyaluronic acid), opticin, vitrosin, fibronectin, fibrillin as well as a network of collagen type II and IX (Filas et al., 2013; Sebag & Balazs, 1989). Organic compounds including ascorbate, glucose, lactate, amino acids, and proteins can all be found in the water component. For the majority of the investigated animals, including rats, pigs (DiMattio, 1989), and rabbits (Henke & Demarais, 1992), the glucose content was shown to be half that found in plasma, while the solutes in the vitreous humour are similar to those in the blood plasma and aqueous humour, and are derived from the retina's and ciliary body's vasculature (Levin et al., 2011).

1.2. The Lens

The ocular lens is a biconvex, transparent cellular tissue suspended in the anterior segment of the eye that functions to focus light onto the retina. The transparency of the lens is established by a tissue architecture that minimises light scattering, while its refractive properties are the result of its curved surfaces and refractive index gradient (GRIN) that focuses light onto the retina (Donaldson et al., 2017).

1.2.1. Lens tissue architecture

The lens is surrounded by the capsule, which is a robust yet porous acellular thickening of the basal lamina (Figure 1.3). Underneath this capsule an epithelial cell monolayer covers the anterior surface of the lens, with the remainder of the lens consisting of highly elongated secondary lens fibre
cells at different stages of differentiation (Donaldson et al., 2017; Forrester et al., 2020). The lens anterior epithelium is further divided into different cellular zone (Figure 1.3D). The germinative zone is a subpopulation of these cells located near the lens equator which exhibit a higher proliferation rate (Bassnett & Shi, 2010), than other cells located in the central zone of the epithelium (Wu et al., 2015). The epithelial cells of the germinative region divide and initiate a process of cell differentiation in the transition zone to form highly elongated secondary lens fibre cells (Martinez & De Iongh, 2010; Wang et al., 2003). These differentiated fibre cells elongate towards the anterior and posterior poles of the lens (Figure 1.3A). A cell maturation gradient is created in the lens, where the differentiating fibre cells continue to be laid down throughout life such that older fibre cells are located deeper within the lens (Augusteyn, 2007). These sheets of differentiating and mature fibre cells that have elongated towards the anterior and posterior poles appear to be stacked like layers of an onion from an axial view, covered by the epithelial layer on the anterior surface (Figure 1.3A). This gradient of differentiation allows the lens to be divided into distinct regions known as the outer cortex (OC), the inner cortex (IC), and the core or lens nucleus (N) (Figure 1.3A-B). The very centre of the lens core contains primary fibre cells that were laid down in the lens vesicle during the earliest stages of development of the embryonic lens (Wride, 2011).
Figure 1.3: Structure of the mammalian lens.

(A) Axial and (B) coronal diagram of lens, show both lens’s poles (anterior and posterior), the suture, the capsule (light blue), the epithelium (yellow), the outer cortex (pink), the inner cortex (magenta) and the nucleus/core (purple). (C) A photograph of a bovine lens displaying the lens’s anterior and posterior surface and the location of the anterior pole, indicated with a red circle. (D) A schematic diagram to show the dissection of a lens epithelial layer model indicating the pole (red circle) and the different zones; Transitional zone (blue), Germinative zone (yellow) and Central zone (orange).
This well-organised cellular structure of the lens is necessary for lens transparency, and this is crucial for healthy vision. This is more obvious in coronal cross-sections taken through the lens equator which reveal the flattened hexagonal profile adopted by differentiating fibre cells in the outer cortex (Figure 1.3B), that allows packing into an orderly array. This organised cellular structure is maintained by cytoskeletal proteins (Bassnett et al., 2011). Each cell consists of two broad sides and four narrow side membrane domains. Since the extracellular space between fibre cells is smaller than the wavelength of light, light scattering is minimised and tissue transparency is promoted (Bassnett et al., 2011; Donaldson et al., 2017). According to diffraction theory, the regular spatial arrangement in the outer cortex decreases light dispersion caused by regional refractive index changes between lens fibre cell cytoplasm and membranes (Michael et al., 2003). However, in the deeper layers of the inner cortex and the core, the cross-sectional profile of mature fibre cells and primary fibre cells (Figure 1.3B) becomes gradually spherical and irregular because of the loss of cytoskeletal components that regulate cell structure and cytoplasm compaction. In these deeper regions light scattering is minimised due to refractive index matching between the cell membranes and cytoplasm (Fagerholm et al., 1981). Fibre cells continue to elongate until both ends of a fibre cell meet and interdigitate with fibre cells from the opposing hemisphere of the lens at the anterior and posterior poles. This stitching together of anterior or posterior tips of fibre cells from the opposite sides of the lens at the anterior and posterior poles, respectively, results in patterns of cell association known as the lens sutures (Michael et al., 2003; Zampighi et al., 2000). In the young human lens fibre cells combine with each other to form Y-shaped sutures at both the anterior and posterior poles (Figure 1.4), however, as the lens grows and develops more complex star shaped sutures are formed (Fan et al., 2018). In all mammalian lenses, sutures are proposed to provide an extracellular route that directs nutrition and antioxidants to the lens centre (Zampighi et al., 2000). In human lenses the development of a more complex star-shaped suture as the lens grows is thought to improve the accessibility of nutrients to the lens core and compensate for the continued addition of lens fibre cells that occurs throughout life (Wu et al., 2017).
Figure 1.4: Schematic diagram of suture in the lens.

The apical (A) and basal (B) membrane domains of elongated fibre cells from different lens hemispheres meet at the anterior and posterior poles, respectively to form the Y-shaped sutures that are orientated at 180° to each other. Adapted from a research article (Ruan et al., 2020).
1.2.2. Establishment of lens transparency

The transparency and refractive properties of the lens are established by its unique tissue architecture that minimises light scattering, and its geometry and refractive index gradient (GRIN) that contributes to the correct focussing of light onto the retina (Donaldson et al., 2017). During the early postnatal phases of development, the lens vasculature degrades, eliminating the vasculature as a source of light scattering. To further minimise light scattering fibre cells within the inner cortex are devoid of nuclei and cellular organelles (Bassnett, 1995, 2002; Bassnett & Beebe, 1992). This elimination of mitochondria means that aerobic metabolism only occurs in the epithelium and differentiating fibre cells that retain functional mitochondria and the majority of the lens relies on anaerobic metabolism to provide its energy needs (Farnsworth et al., 1989).

To improve its optical performance the lens also generates a GRIN. The GRIN is established by spatial differences in the protein-to-water ratio across the lens, due to the variable expression of crystallin proteins, which changes with age (Jones et al., 2005), and the active removal of water from the lens core (Gao et al., 2015). As epithelial cells differentiate into fibre cells they initiate the over expression of different crystallin protein subtypes (α, β, and γ), which contribute to the formation of the GRIN (Uhlhorn et al., 2008). Whilst β- and γ-crystallins are primarily structural proteins, α-crystallins also have chaperone-like characteristics (Augusteyn, 2007; Bloemendal et al., 2004). In general, α-crystallin is predominantly found in the lens cortex. In contrast, truncated forms of α-crystallin, plus β- and γ-crystallin have been localised in central lens regions (Anderson et al., 2020; Debois et al., 2010; Schey, Anderson, et al., 2013). The crystallin proteins contribute more than 90% of the lens dry weight and range in concentration from 240 mg/mL in the outer cortex to 400-600 mg/mL in the lens core (Slingsby et al., 2013). Together, they are densely packed in a short-range spatial order that creates a smooth radial refractive index gradient from the periphery to the core (Delaye & Tardieu, 1983). Despite a high level of crystallin proteins in the lens core, their expression correlates inversely with the water content of the lens core (Bloemendal et al., 2004). This cellular architecture guarantees that inherent spherical
aberration is corrected and transparency in the lens is preserved (Besharse et al., 2010; Donaldson et al., 2001).

1.3. Lens function

While it is the structural organisation of the lens that establishes the transparent and refractive properties of the lens, the lens is not a passive optical element (Donaldson et al., 2017), but a biological tissue that requires the input of energy to drive the structural and functional processes that actively maintain its optical properties over many decades of life. The primary source of energy for the lens is glucose, which in the absence of a direct blood supply into the lens, must be sourced from the surrounding ocular humours and then delivered to the different regions of the lens where it is subsequently metabolised. To deliver glucose and other nutrients, and then remove the resultant metabolic waste products, it has been proposed that the lens utilizes circulating ionic and fluid fluxes to generate an internal microcirculation system (Donaldson et al., 2017; Mathias et al., 2007).

Mathias et al. originally proposed the concept of an internal microcirculation based on the characteristics and distribution of ion pumps and channels observed in the lens (Mathias et al., 1997). They proposed that this distribution of ion pumps and channels generates an inward influx of ions and water at the anterior and posterior poles of the lens and an outward flux at the equator (Figure 1.5A). It is this circulating flux of water that was initially proposed to deliver solutes to the lens core more efficiently than predicted by passive diffusion alone. More recently, existence of this microcirculation system and its role in actively maintaining the optical properties of the lens has been experimentally validated through a variety of different techniques by various groups (Candia et al., 2012; Gao et al., 2011; Vaghefi & Donaldson, 2018; Vaghefi et al., 2015; Vaghefi et al., 2011). In the microcirculation $\text{Na}^+$ first enters the lens at the anterior and posterior poles via an extracellular pathway associated with the lens sutures (Figure 1.5A). As the extracellular $\text{Na}^+$ enters deeper into the lens towards the lens core, it moves down its electrochemical gradient into fibre cells (Figure 1.5B, top panel).
Figure 1.5: Lens Microcirculation Overview.

(A) The microcirculation model is shown in three dimensions in this image, with ion and fluid fluxes entering the lens from both poles via the extracellular space (influx), passing through fibre cell membranes, and then leaving the lens through an intercellular outflow pathway mediated by gap junctions, which leads the fluxes to the equatorial efflux zone, where they exit the lens. Adapted from Shi et al., 2009. (B) Equatorial cross-sections demonstrating the differential spatial distribution of ion channels and transporters between the epithelium fibre cells (E), differentiating fibre cells (DF), and mature fibre cells (MF) that generate the circulating Na⁺ ion current (top), drive isotonic fluid fluxes (middle), and in turn supply nutrients and exchange the metabolic waste from the MF cells (bottom). Adapted from a research article (Braakhuis et al., 2019).
The Na\(^+\) then travels from the core to the lens periphery via an intracellular outflow pathway formed from the gap junction proteins Cx46 and Cx50 (Figure 1.5B, top panel). This outflow pathway directs the flow of Na\(^+\) towards the lens equator where the Na\(^+\)/K\(^+\) ATPase is concentrated in epithelial and peripheral fibre cells, which then actively remove Na\(^+\) from the lens (Candia & Zamudio, 2002; Delamere & Dean, 1993). The information that the ion current is driven mainly by Na\(^+\) is supported by research on the conductance, selectivity, and driving force of Na\(^+\) crossing the lens core fibre cell membranes, which predict the amount of Na\(^+\) influx is comparable to the amount of Na\(^+\) efflux that happens at the lens periphery (Mathias et al., 1997). Since the deeper fibre cells lack Na\(^+\)/K\(^+\) ATPase it is their connection to the surface cells via gap junctions which generates the negative membrane potential in fibre cells that contributes to the electrochemical gradients required to maintain osmotic equilibrium and the diffusion of Na\(^+\) into the deeper fibre cell (Candia & Zamudio, 2002; Mathias et al., 1997; Mathias et al., 2007; Schey et al., 2017).

This circulating Na\(^+\) current also generates an osmotic gradient, which in turn produces a circulating isotonic fluid flux (Figure 1.5B, middle panel). Like the Na\(^+\) current, this water flux is proposed to also enter the lens preferentially at both poles via the extracellular space before entering deeper fibre cells via aquaporin (AQP) water channels. Water then returns to the lens surface via an intracellular outflow pathway mediated by gap junctions before exiting the lens again via AQPs in the membranes of peripheral cells located at the lens equator. The movement of water through gap junction channels was subsequently shown to generate a substantial hydrostatic pressure gradient (0 to 330 mm Hg) that was maximal in the core of the lens (Gao et al., 2011). This gradient was shown to be conserved across species that exhibited lenses of remarkably different lens sizes (Gao et al., 2013) and has been shown to be subject to dual feedback regulation (Gao et al., 2011; Gao et al., 2015; Gletten et al., 2022). This pressure gradient is thought to drive the removal of water and metabolic wastes from the lens creating a well stirred intracellular space.

This circulating flux of water has in turn been hypothesised to convect nutrients towards the deeper lying mature fibre (MF) cells via an extracellular pathway that delivers the nutrients to the deeper
MF cells faster than would occur by passive diffusion alone (Figure 1.5, bottom panel). Consistent with this prediction these MF cells have been shown to contain an array of nutrient and antioxidant transporters (Lim et al., 2020), many of which are secondary active transporters that utilise the energy contained in the Na\(^+\) electrochemical gradient, which is generated by the activity of Na\(^+\)/K\(^+\) ATPase located at the lens surface, to actively accumulate nutrients from the extracellular space (Mathias et al., 2007).

A number of different approaches have been taken to study these fluid and nutrient fluxes in the lens. The fluid flow has been specifically measured in bovine lenses by modified Ussing chambers that use thin O-rings to physically separate the anterior and posterior influx and equatorial efflux regions of the lens (Candia et al., 2012). Using this approach, Candia et al., showed that water does indeed enter the lens via both poles and exits at the equator compartment. To more directly visualise these water fluxes, Vaghefi et al., have performed a series of experiments on organ cultured bovine lenses that used a combination of MRI imaging modalities to show that water fluxes preferentially enter the lens at both poles and that this movement of water is generated by the lens microcirculation system (Vaghefi et al., 2011). They also went on to show that while inhibiting water fluxes had no effect on overall lens transparency, it did alter the refractive power of the lens (Vaghefi et al., 2015).

More recently, Vaghefi et al have used MRI to visualise the rate of penetration of extracellular contrast agents (GadoSpin: GDF and FeraSpin-XS: FXS) into the bovine lens (Figure 1.6A) and have shown that solute delivery to the lens nucleus does indeed occur faster than would be expected by passive diffusion alone (Figure 1.6B-C), and that this delivery was abolished by inhibiting the microcirculation system (Vaghefi & Donaldson, 2018). These contrast agents were utilised as analogues of lens nutrients and small molecules, which presumably remained in the extracellular space. (Vaghefi & Donaldson, 2018).
Figure 1.6: Spatial and temporal penetration of contrast agents into the bovine lens.

A temporal two-dimensional MRI images showing the pattern of penetration into the lens of GDF and FXS (A) This figure also illustrates a comparison between the average observed penetration rates of two utilised MRI contrast agents in the OC (B) and N(C) of the bovine lens, and the theoretical penetration rate (red) calculated for passive diffusion. The figure is adapted from Vaghefi’s publication (Vaghefi & Donaldson, 2018).
While these MRI experiments have confirmed that the microcirculation system is essential for solute delivery to the lens nucleus and suggest that the extracellular delivery of solutes occurs via the lens sutures, these studies did not specifically study the delivery of physiologically relevant molecules nor their subsequent uptake via the transporters known to be expressed in these MF cells, or their subsequent metabolism in the lens nucleus. Several approaches have been trialled to study the differences in metabolites in different lens regions, in different lens ages and lens pathology. Typically, a microdissection approach is employed, where lenses are manually divided into 2–5 anatomical regions based on tissue consistency or physical distances (Tamara et al., 2016; Tsentalovich et al., 2015; Yanshole et al., 2014). While this information has been important for defining significant changes that may underlie lens cataract formation, spatial information is limited. Hence to provide this next level of validation of the lens microcirculation system new approaches with higher spatial and temporal resolution are required that can visualise the delivery of small metabolically relevant molecules to the different regions of the lens and follow their regional specific metabolism. One technique that has the potential to achieve this is imaging mass spectrometry (IMS).

1.4. Metabolite imaging in the lens using IMS

Imaging mass spectrometry (IMS) is a technique which combines mass spectrometry with microscopic imaging. Using this analytical approach, molecular tissue maps can be created. IMS is an imaging approach that requires no prior information of the individual molecules present in the sample because it detects and can putatively identify a large number of molecules using their exact mass and isotopic pattern directly from tissue (van Hove et al., 2010). Further verification of the identity can be achieved by employing a complementary method such as LC-MS, utilizing tandem MS and/or comparison with mixed pure standards of the compound. Due to specialised sample preparation methods which bias the detected molecules towards specific classes (Metabolites, Lipids, Peptides, or Proteins), the only knowledge required is which species of interest to investigate. IMS maps the distribution of many molecules simultaneously and is therefore highly multiplexed. It accomplishes this by scanning
tissue sections pixel-by-pixel with an ionisation source and acquiring a mass spectrum for each pixel (Figure 1.7) (Watrous et al., 2011).

By selecting a mass-to-charge (m/z) ratio from the resulting dataset, chemical distributions are rebuilt in two dimensions, with signal intensity in each pixel typically encoded by a colour scale. The molecular make-up of the tissue surface is mapped and elucidated in this way. Because different histological tissue features can have their own unique molecular profile, this chemical make-up is frequently complicated. As a result, IMS is a useful analytical tool for probing further into these local chemical complexities. An ionisation source, a mass analyser, and a detector make up a mass spectrometer (McDonnell & Heeren, 2007). To achieve spatially resolved analysis, IMS employs a local desorption and ionisation technique. A desorption/ionisation beam aimed at the sample surface generates ions locally in this type of ionisation source. Following that, the ions are directed into the mass analyser. The ions are sorted here depending on their m/z ratio and transferred to the detector for measurement. A peak in the mass spectrum is recorded when enough ions with a specific mass-to-charge value contact the detector.
Figure 1.7: General IMS Workflow.

An ionisation source scans the tissue section pixel by pixel. An average mass spectrum is generated for each pixel, reporting the intensities of thousands of m/z ratios related to specific molecular species. Following acquisition, the intensity of a single m/z in each pixel can be used to visualise that signal.
As a result, every measured m/z signal can represent either one particular or several molecules with the same molecular mass. A mass analyser with a high resolving power can distinguish between molecules with very similar masses, while molecules with the same molecular formula cannot be separated by mass alone, and other separation techniques (e.g., ion mobility, chromatography) may be required. In the IMS data, the height of the peak is determined by the concentration of the molecule, affinity of ionisation, and ion suppression throughout the experiment.

Secondary ion mass spectrometry (SIMS), matrix-assisted laser/desorption ionisation (MALDI), and desorption ionisation (DESI) IMS are the most widely used mass spectrometry imaging techniques in biomedical research (Vickerman, 2011). These technologies exploit different ionisation mechanisms to capture the information in biological systems.

Our group has utilised MALDI-IMS to visualise the distribution of GSH in the bovine (Nye-Wood et al., 2016) and human lens (Grey et al., 2019). In the bovine lens the distribution of GSH in different regions of the bovine lens was found to be the highest in the lens cortex and decreased towards the nucleus (Figure 1.8). While treating the bovine lens with hyperbaric nitrogen (HBN) had no affect on the distribution of GSH, treatment with hyperbaric oxygen (HBO) resulted in a decrease in GSH levels and an increase in the oxidised form of GSH, glutathione disulphide (GSSG) in the outer cortex. In addition, consistent with this result NADPH, which regenerates GSH from GSSG, was present in the lens cortex and periphery in normal and HBN lenses, but was reduced in HBO-treated lenses (Nye-Wood et al., 2016).

In the human lens MALDI-IMS has been used by Grey et al., to investigate how levels of GSH change in the human lens with age (Grey et al., 2019). This study found that while the absolute levels of GSH decreased slightly in the lens cortex with increasing age, a more pronounced decrease was observed in the nucleus (Figure 1.9). These MALDI-IMS results are consistent with earlier studies that suggested an age-dependent decrease in GSH delivery to the lens core is an initiating event in the onset of lens cataract (Truscott, 2000a).
Figure 1.8: Molecular imaging of GSH, GSSG and NADPH in bovine lens.

Visualization of GSH, GSSG, and NADPH in axial sections of normal, HBN-treated, and HBO-treated bovine lenses using MALDI FTICR imaging. The images depict the distribution of GSH (top), GSSG (middle), and NADPH (bottom) at a spatial resolution of 150 μm. The figure is adapted from a research article (Nye-Wood et al., 2016).
Figure 1.9: Quantification of GSH in the aging human lens using MALDI-IMS.

Three-dimensional surface plots were used to display absolute quantitative GSH images in human lenses, covering an age range from 29 to 82. The figure is adapted from a research article (Grey et al., 2019).
In this thesis, my goal was to optimise the MALDI-IMS approach developed in our lab to image regional differences in GSH to study the delivery, uptake and metabolism of glucose in the different regions of the lens. To provide context for these studies in the next section, I will review glucose metabolism pathways in the morphologically distinct regions of the lens.

1.5. Glucose metabolism pathways

Glucose is an essential energy source and a metabolic intermediate for most living organisms, from bacteria to human. It is a key carbohydrate substrate for protein and lipid synthesis (Ostrowska et al., 2015). The energy metabolism of the lens is entirely reliant on glucose, and the programmed organelle degradation observed in the lens has a dramatic effect on the metabolism of glucose in the different lens regions. The lens epithelium has direct access to the surrounding ocular humours that bathe the lens and have the highest levels of GSH and NADPH, which detoxifies the lens and protects it from oxidative damage (Bhat, 2001). The outer cortex of the lens is characterised by intact crystallin proteins, membrane proteins, abundant enzymes, and enzymatic activity. The DF cells in this region of the lens still contain functional mitochondria so aerobic metabolism dominates in this region (Van Heyningen, 1977), along with a range of biochemical processes that are similar to many other nucleated cells in other tissues (Grey & Schey, 2008, 2009; Stella et al., 2010). In the lens nucleus, anucleate MF cells are characterised at the protein level by crystallin and membrane proteins that have undergone extensive posttranslational modifications, have a lower enzyme activity and in the absence of mitochondria rely on anaerobic pathways to process glucose, a complement of biochemical processes that are markedly different from the epithelial cells and cortical DF cells (Gonen et al., 2001; Schey, Grey, et al., 2013).

1.5.1. Glucose delivery

In lieu of a blood supply, glucose is taken up from its surrounding humours, where it is present in human aqueous and vitreous humour at concentrations of ~3.2mM (Davies et al., 1984) and 3.0mM (Kokavec et al., 2016), respectively. While the normal concentration of glucose within the lens itself
varies between species, it is in the order of 10mg glucose in 100g tissue (Kuck, 1965). The mechanism of how glucose is delivered and taken up from the surrounding humours has attracted significant research, and our understanding of these mechanisms has developed over time. Early studies suggested that glucose was taken up by epithelial cells at the anterior surface of the lens, and then transported to deeper lying fibre cells through an intracellular pathway mediated by gap junction channels (Goodenough, 1992). However, more recent research has indicated that this theory may not be completely accurate. Specifically, a study on the movement of cysteine in monkey lenses showed that in the outer cortex, the uptake of nutrients is concentrated at the equator of the lens (Figure 1.10), rather than across the entire epithelial surface as previously believed (Sweeney et al., 2003).

However, calculations have shown that peripheral uptake followed by passive diffusion through such an intracellular pathway mediated by gap junctions would be insufficient to deliver adequate glucose to the lens nucleus (Vaghefi & Donaldson, 2018). Hence, the idea that glucose is delivered by an extracellular route via the internal circulatory system has been proposed (Mathias et al., 2007). However, the actual delivery of glucose to the lens core has yet to be confirmed. These findings raise important questions about the mechanisms of nutrient transport in the lens and the need to develop new methods to visualize glucose delivery and uptake. As such, further research is needed to better understand the complexities of nutrient transport and metabolism in the lens, with the potential to uncover new insights into the development and treatment of lens-related pathologies.
Figure 1.10: Potential mechanism of cysteine uptake in primate lenses.

This model shows inward movement in the equatorial plane, but with little movement through the polar axis (indicated with arrows). However, this model does not provide information on the outward movement of cysteine from the lens. Adapted from Truscott's group paper (Sweeney et al., 2003).
1.5.2. Glucose uptake in lens

Glucose uptake is mediated by two major protein families: the GLUT family, also known as facilitative-glucose-transporters that mediate the facilitative diffusion of glucose; and the SGLT family that utilises the energy stored in the Na⁺ electrochemical gradient to actively accumulate intracellular glucose (Wright et al., 2011a). GLUT and SGLT isoforms have different affinities for glucose, allowing it to be taken up from extracellular environments with varying glucose levels. GLUT transporters are structurally analogous and consist of 12 transmembrane domains with both amino and carboxyl termini facing the cytoplasmic side (Figure 1.11) (Mueckler et al., 1985).

There are 13 functioning facilitative glucose transporters in humans, known as GLUT1-12 and the H⁺/myo-inositol cotransporter (HMIT) (Joost & Thorens, 2001; Wood & Trayhurn, 2003). The GLUT family has been divided into three classes (Long & Cheeseman, 2015). GLUT1-4 belong to Class I (Figure 1.11) and have in addition to glucose been shown to transport galactose and oxidised ascorbic acid (Liu et al., 2001; Rumsey et al., 1997). GLUT 5, 7, 9 and 11 make up Class II (Figure 1.11) and transport urate and fructose along with glucose. Class III (Figure not shown) is comprised of GLUT6, GLUT8, GLUT10, GLUT12, and HMIT.

The SGLT family comprises proteins with a size of 60 to 80 kDa, containing 580 to 718 amino acids and are composed of 14 transmembrane helices, with both the COOH and NH₂ terminals facing the extracellular space (Navale & Paranjape, 2016). These transporters work by cotransporting glucose with sodium ions, and they utilise the sodium electrochemical gradient generated by the Na⁺/K⁺ ATPase to actively accumulate intracellular glucose (Figure 1.12). SGLT1 and SGLT2 are two members of the SGLT family that are expressed in the kidney (Wright et al., 2011b) and intestine (Crane, 2005; Kimmich & Randles, 1984). SGLT transporters have a higher affinity for glucose than GLUTs, which enables SGLTs to be more effective than GLUTs at accumulating glucose when extracellular levels of the metabolite are low.
Figure 1.11: Schematic membrane topology of GLUTs from glucose transporters.

The transmembrane domains are presented in figure from 1 to 12. The glycosylation (N), amine (-NH₂) and carboxylic acids (-COOH) sites are indicated. Gradient colouring highlights the transmembrane domains predicted to form the glucose transport channel, and regions that interact with hexose substrates and the GLUT inhibitor cytochalasin B are indicated for class I and II transporters. The figure is adapted from a publication (Macheda et al., 2005).
Figure 1.12: A model that describes the mechanism of glucose uptake coupled with sodium.

This is a mechanical model consisting of 6 states, based on Schultz's proposal (Schultz, 1986) that explains the process of glucose uptake in conjunction with Na⁺. States 1-3 face outward, while states 4-6 face inward. The carrier initially has low affinity for external sugar before sodium binds. After external Na⁺ and sugar binding, the carrier undergoes a conformational change to release the ligands, either Na⁺ or glucose first. The carrier then undergoes another conformational change to re-expose the binding sites to the external surface of the membrane. The voltage dependence of Na⁺/sugar cotransport could arise from the electro-diffusion of Na⁺ or the reorientation of the charged form of the carrier from one surface to the other. This figure is adapted from a research article from Wright’s group (Wright et al., 2011a).
The presence and spatial distributions of GLUTs and SGLTs in the lens have been investigated at the mRNA and protein level in a number of species, including mice and rats (Merriman-Smith et al., 1999; Merriman-Smith et al., 2003), in bovine tissue, and in humans (Lim et al., 2017). Glucose uptake in the lens seems to be mainly mediated by the GLUT1 & 3 isoforms of facilitated glucose transport family (Merriman-Smith et al., 1999). GLUT1 was localised in the rat lens epithelium by immunohistochemistry, while notable levels of GLUT3 were expressed throughout the cortex (Cholkar et al., 2013). While both GLUT1 and GLUT3 have been found in rodent and human lenses, in the bovine lens, only GLUT1 has been found by western blotting (Lim et al., 2017). Nevertheless, it is clear that GLUTs are essential for lens transparency since the knockout of GLUT1 in a mouse model led to lens cataract formation (Swarup et al., 2018).

GLUT3 also has a significant affinity for glucose and a greater maximal turnover number for glucose than of GLUT1 (Liang et al., 2018; Thorens, 1993), so it allows the fibre cells to continue to take up glucose effectively even when supplies are constrained (Gould & Holman, 1993; Manolescu et al., 2007). The presence of a broad immunoreactive band in immunoblot analysis indicates that the GLUT3 protein may be subjected to varied degrees of post-translational modification, such as glycosylation (Watanabe et al., 1996). This difference is consistent with the general understanding that GLUT isoforms vary in their tissue specificity and affinity (Gould & Holman, 1993).

1.5.3. Metabolism of glucose in the lens

The presence of glucose transporters in fibre cell membranes throughout the lens suggests that lens fibre cells can directly take up extracellular glucose in all regions of the lens (Lim et al., 2017; Merriman-Smith et al., 1999; Merriman-Smith et al., 2003). Once inside the cell, glucose can be utilised in many metabolic processes to release energy in the form of ATP, which is required to maintain the structural integrity and, therefore, transparency of the lens (Donaldson et al., 2017). In the lens, it is primarily metabolised by three pathways (Figure 1.13): glycolysis (Kinoshita, 1955), the pentose
Glycolysis is essential to maintain lens physiological function and tissue transparency over many decades of life since perturbations to major enzyme-mediated steps in glycolysis result in lens swelling and cataract (Hejtmancik et al., 2015; Kinoshita, 1955). The lens has a similar glycolytic pathway to other tissues, and within the lens, it was traditionally believed that the primary location of glucose metabolism was the epithelium (Swarup et al., 2018). For example, aerobic glycolysis and metabolism via the citric acid cycle are possible only in the lens epithelium and peripheral fibre cells that contain cell nuclei, mitochondria, and other cellular organelles.

However, the aerobic metabolism performed in the lens only accounts for ~20–30% of the total lens ATP production, while only consuming ~3% of the glucose supplied to the lens (Hockwin et al., 1971; Trayhurn & Van Heyningen, 1972). The remaining 70% of lens glucose metabolism is carried out under anaerobic conditions (Bron et al., 1993), which produces lactate that is thought to contribute to a measurable pH gradient from the periphery to the centre of the lens (Bassnett & Duncan, 1986).
Figure 1.13: Major pathway of glucose metabolism in the lens.

Glucose is taken up from its surrounding AH by GLUTs and can be metabolised via several metabolic pathways: polyol, glycolysis, pentose phosphate and glycogen synthesis.
The environment in which the lens sits also plays a role in determining metabolic function and lens transparency. The lens sits in a mildly hypoxic environment in the normal eye, yet it can actively control its ion balance, maintain ATP levels, and synthesise proteins. However, exposure of cultured lenses to oxidative stress induced by exposure to hydrogen peroxide (Giblin et al., 1985) or hyperbaric oxygen (F.J. et al., 1988) stimulates the pentose phosphate pathway by increasing hexokinase activity (Hejtmancik et al., 2015). Despite not generating a large amount of ATP, the pentose phosphate pathway is essential because it uses the enzyme glucose-6-phosphate dehydrogenase to synthesize substantial amounts of NADPH (Figure 1.13) that is used by glutathione reductase to maintain redox balance in the lens by regenerating GSH from oxidised GSSG (Giblin et al., 1981). In the pentose phosphate pathway glucose-6-phosphate is metabolised to ribulose-5-phosphate through the oxidative phase (Figure 1.8). In this process, a number of reducing equivalents (NADPH) are produced, which help protect against oxidative stress by reducing oxidised glutathione. Ribulose-5-phosphate also is metabolised in one of two ways to sedoheptulose-7-phosphate (S7P) in the nonoxidative phase (Lal et al., 1995; Szwergold et al., 1995). S7P is then cycled back via fructose-6-phosphate to undergo glycolysis or can be further metabolised to erythrose 4-phosphate to be used in the synthesis of aromatic amino acids.

In addition, NAPDH is used by the third glucose metabolic pathway in the lens, the polyol (sorbitol) pathway. This route was initially described by van Heyningen in 1959, who observed the accumulation of polyols in the lens (Dvornik et al., 1973). In this pathway, sorbitol is formed by aldose reductase using NADPH as a cofactor, which is then converted to fructose by a second enzyme, polyol (sorbitol) dehydrogenase, using nicotinamide adenine dinucleotide (NAD+) as a coenzyme (Figure 1.13). Under normal physiological conditions, almost one-third of the glucose entering the human lens is metabolised through this sorbitol pathway (Jedziniak et al., 1981). In the human lens, the majority of aldose reductase, and its activity, is thought to be present in the epithelium (Jedziniak et al., 1981).

Overactivation of the polyol pathway has been implicated in the pathogenesis of diabetic cataract (Bron et al., 1993; Chung et al., 2003). The critical significance of the AR pathway as the starting mechanism in diabetic cataract development has been the topic of extensive investigation (Figure 1.14).
It has been shown that intracellular sorbitol accumulation causes osmotic alterations, leading to the degeneration of swollen cortical fibres of the lens and the formation of sugar cataracts (Kinoshita, 1974; Kinoshita et al., 1979). Poorly controlled levels of blood glucose can lead to elevated glucose uptake in the lens and conversion to the metabolite sorbitol.

This osmolyte cannot cross the cell membrane via diffusion because of the polar nature of sorbitol. Therefore, the elevated sorbitol accumulation causes a hyperosmotic impact, which drives a fluid infusion to counteract the osmotic gradient, and then causes osmotic cell swelling and cell damage, specifically in the lens cortex. (Merriman-Smith et al., 1999). Furthermore, this pathway also leads to decreased levels of the abundant lens antioxidant GSH, increased reactive oxygen species, and therefore, oxidative stress in the lens that damages lens protein function, leading to cataract formation (Lee & Chung, 1999).

In summary, glucose plays very important roles in the different regions of the normal and cataractous lens, yet our ability to study glucose uptake, transport and metabolism with sufficient spatial and temporal resolution in these different regions with current techniques is limited. Thus, the general goal of this thesis was to develop imaging mass spectrometry as a technique that could fill this gap in our knowledge to shed new light on the function of the normal lens and ultimately the cataractous lens.
Molecular pathways involved in the development of diabetic cortical cataract. An increase in glucose causes a decrease in GSH and an increase in reactive oxygen species (ROS). The cumulative effects of induced osmotic and oxidative stress limit the capacity of fibre cells to regulate their volume. This causes cell swelling, depolarisation, and sodium and calcium ion influx. Calcium ion accumulation triggers calcium-dependent proteases, which target cytoskeletal and crystallin proteins. Moreover, proteins are altered by the creation of advanced glycation end products (AGEs), which have been shown to modify the structure and function of crystallins, leading to growth in insoluble proteins, the development of aggregation, and finally this leading to cataract. Figure is adapted from a research article (Braakhuis et al., 2019)
1.6. Thesis objectives

While previous work in the MVRC (Grey, 2016; Nye-Wood et al., 2016) and other groups (Boughton et al., 2020) had used MALDI-IMS to map a variety of other lens metabolites, and MALDI imaging had been used to image glucose metabolites in other tissues (Dekker et al., 2015; Yang et al., 2018), MALDI-IMS has not yet been applied to study glucose metabolism pathways in the lens. Thus, the overarching goal of my thesis was to develop a suite of techniques based around MALDI-IMS that would allow me to study the delivery, uptake and metabolism of glucose in the different regions of the bovine lens. I have chosen the bovine lens as a model system because it is physically comparable to human lenses, it is widely available, it is large, and it has a plethora of previously obtained data on metabolites, proteins (Han & Schey, 2006), water transport (Vaghefi et al., 2011), and optics (Vaghefi et al., 2015). To achieve this overarching goal, I have pursued the following specific aims.

1) To optimise and validate MALDI-IMS as a method to study regional difference in glucose metabolism (Chapter 3)

In this chapter, I have evaluated and optimised a sample preparation method for matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI-IMS) analysis of ocular lens glucose uptake and metabolism. Matrix choice, tissue preparation and data normalisation strategy were determined using negative ion mode MALDI-Fourier transform-ion cyclotron resonance MS of bovine lens tissue and validation was performed using gas chromatography-MS. Finally, I evaluated the ability of the optimised method to detect exogenous lens nutrient uptake in ex vivo cultured normal bovine lenses. In the following result chapters (4 and 5), this method was applied to study the dynamics of glucose uptake, transport and metabolic flux to aid in understanding the lens function. This work was published under the title ‘Mapping Glucose Metabolites in the Normal Bovine Lens: Evaluation and Optimization of a MALDI Imaging Mass Spectrometry Method’ by Ali Zahraei et al., *J Mass Spectrom*, 2021. See Appendix B.
2) To spatially correlate the pattern of glucose uptake to glucose transporter distributions in cultured lenses and map glucose metabolism in different lens regions. (Chapter 4)

To spatially correlate the pattern of glucose uptake to glucose transporter distributions in cultured lenses and map glucose metabolism in different lens regions, I incubated bovine lenses ex vivo in artificial aqueous humour containing normoglycaemic stable isotopically-labelled (SIL) glucose (5mM) for 5 min-20 h. Next, I investigated the glucose uptake pattern and metabolism across the lens section by subsequent matrix-assisted laser desorption/ionisation (MALDI) imaging mass spectrometry (IMS) analysis using high resolution mass spectrometry. In addition, I identified the GLUTs in epithelium flat mounts and fibre cells. First, I manually dissected normal, unincubated lenses and then analysed them by gel-based proteomic technique (GeLC/MS) to detect GLUTs. Afterwards, I have done indirect immunofluorescence and confocal microscopy of axial lens sections from unincubated fixed lenses labelled with primary antibodies to localise specifically GLUT1 and GLUT3. Using these experimental approaches, I spatially correlated the pattern of glucose uptake to glucose transporter distributions in cultured lenses. This work establishes a consolidated research pipeline to study metabolomics and proteomics in the lens to enable its use in studying pathological models such as cataracts. These results have been published under the title 'Mapping glucose uptake, transport and metabolism in the bovine lens cortex' by Ali Zahraei et al., Front Physiol., 2022." See Appendix B.

3) To study the role of lens microcirculation in the delivery of glucose to the lens nucleus. (Chapter 5)

This chapter aimed to establish evidence of the extracellular/sutural delivery pathway of small molecules in the lens nucleus. In this approach, I investigated how glucose and sorbitol are delivered to the lens nucleus by incorporating protocols to perturb the microcirculation using Ouabain. SIL glucose/sorbitol-incubated ± Ouabain lenses were subjected first to MALDI-IMS and then to gas chromatography-mass spectrometry (GC-MS) to verify the identity of metabolites detected by MALDI-
IMS. By implementing two mass spectrometry techniques, I attempted to investigate the role of microcirculation in the delivery, uptake and metabolism of glucose in the normal lens. However, the results are not conclusive as MALDI-IMS and GC-MS do not have enough sensitivity to answer the question. In the future, a higher concentration of SIL Sorbitol or even other extracellular markers such as Mannitol could be tested. More importantly, utilising higher resolution mass spectrometers could help gain clarity in revealing if the microcirculation is involved in the delivery of glucose in the lens in physiological conditions.

Use of the protocols developed in this thesis to gain new insights into regional differences in lens nutrient uptake, transport and metabolism in the lens has provided the necessary basic information on glucose homeostasis to develop in the future anti-cataract therapies designed to delay the onset of cataract and reduce the need for cataract surgery.
This chapter covers the materials and methods used to prepare control and treated tissue and the essential technical details about the processes utilised in the experiments. The experimental protocols described in this chapter are the final, optimised protocols established throughout this thesis. If relevant the details on the optimisation of a particular method are presented in the appropriate results chapters. All experimental approaches followed the University of Auckland's Health and Safety requirements.

2.1. General chemicals and solutions

All solvents used were HPLC grade, and all solutions were dissolved in Milli-Q® water at 25°C (Merck Millipore, Massachusetts, USA). Where noted, the pH of solutions was measured by an S20 SevenEasy™ pH meter (Mettler Toledo, Ohio, USA) and adjusted using NaOH or HCl (1 M). Osmolarity was measured by an osmometer (Wescor AC061, Utah, USA) and adjusted using NaCl and Milli-Q® water. In addition, the Faculty of Medical and Health Sciences Central Sterilising Services cleansed and autoclaved all glassware and plastic items.

A range of Artificial Aqueous Humour (AAH) was used in this research based on the designed experiments. Osmolarity and components were adjusted to more closely resemble the natural composition of the aqueous humour (Table 2.1). In addition, all the AAH solutions used for lens incubation were pre-heated to 37°C.

2.2. Eye Tissue and lens dissection

Fresh bovine eyes were collected from a local abattoir (Auckland Meat Processors, Ltd, Auckland, NZ) within 2-3 h after the death of the animals. Donor animals were up to 24 months old, and from both genders (specific gender information was unknown to us). Once in the lab, to reduce microbial contamination, whole bovine eyes were soaked in 70% ethanol for a few seconds before being rinsed with Milli-Q® water. To extract the bovine lenses, the sclera in the posterior hemisphere was incised
with a single edge razor blade (American Line 66-0089). The vitreous humour was gently removed to access the posterior side of the lens and the zonules which connect the lens to ciliary muscle. The zonules were cut carefully with the razor blade without damaging the lens capsule, and the lens removed anteriorly. The isolated lenses were washed in 1x phosphate-buffered saline (1xPBS) and then placed into a 6-well culture plate (Thermo Scientific™ Waltham, MA, USA) with the posterior side facing upwards. Extracted lenses were used immediately for incubation experiments or were either fixed for immunohistochemistry or frozen immediately and stored at -80 °C until required.

2.3.  *Ex vivo* lens incubations

2.3.1. Lens Incubation in Artificial Aqueous Humour (AAH) Solution

Freshly dissected bovine lenses were incubated in 12 ml of a variety of pre-heated AAH solutions (Table 2.1). Lenses were incubated for times ranging from 5 min to 20 h at 37 °C in 5% CO₂ inside a tissue incubator (Heracell VIOS 160i CO₂ incubator, ThermoFisher Scientific, Waltham, MA, USA). Following incubation, samples were rinsed three times with 20 ml isotonic AAH to remove any residual incubation solution from the lens tissue surface and then stored at -80 °C.
Table 2.1: Artificial aqueous humour (AAH) recipes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Isotonic AAH</th>
<th>Isotonic AAH with Ouabain</th>
<th>Isotonic AAH with SIL Glucose</th>
<th>Isotonic AAH with SIL Glucose Ouabain</th>
<th>Isotonic AAH with SIL Sorbitol</th>
<th>Isotonic AAH with SIL Sorbitol Ouabain</th>
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<tbody>
<tr>
<td>NaCl (mM)</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>117.5</td>
<td>120</td>
</tr>
<tr>
<td>KCl (mM)</td>
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<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
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<tr>
<td>NaHCO3 (mM)</td>
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<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
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<td>NaH2PO4 (mM)</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MgSO4 (mM)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl2 (mM)</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>SIL Glucose (mM)</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SIL Sorbitol (mM)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Ouabain Octahydrate (mM)</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HEPES (mM)</td>
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<td>10</td>
<td>10</td>
<td>10</td>
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<td>10</td>
</tr>
<tr>
<td>Osmolarity (mOsm)</td>
<td>290±5</td>
<td>290±5</td>
<td>290±5</td>
<td>290±5</td>
<td>290±5</td>
<td>340±5</td>
</tr>
</tbody>
</table>
2.3.2. *In situ* Lens Incubation with SIL Glucose

To advance our knowledge about glucose uptake in the lens, I established a semi-*ex vivo* experiment to culture whole bovine eye tissue. Since the fresh bovine eyes are delivered to the laboratory are at room temperature (RT), they were initially incubated for 1 h at 37 °C in 5% CO₂ inside the tissue incubator to return them to body temperature. Eyes were then injected with 40 µl of 1 M SIL glucose into the aqueous humour in the anterior chamber; (Figure 2.1), to yield a concentration of SIL glucose in AH of ~40 mM. Following injection, eyes were returned to the incubator for times that ranged from 30 min to 4 h. Following incubation, lenses were dissected (see section 2.2) and rinsed three times in 20 ml isotonic AAH to remove any residual AH from the lens tissue surface before being stored at -80 °C until required for further analysis.
Figure 2.1: SIL glucose injection to the whole bovine eye.

This figure demonstrates the location of injection of the SIL glucose into the aqueous humour in the anterior chamber of the eye. The SIL glucose was delivered to the anterior chamber only and could then mix with the aqueous humour. Figure adapted from Biorender.com.
2.4. MALDI mass spectrometry

2.4.1. Matrix spot preparation to optimise matrix choice for SIL glucose analysis

The MALDI matrices (Table 2.2), including 9-Aminoacridine (9-AA), 1,5-diaminonaphthalene hydrochloride (1,5-DAN HCl), 2,5-dihydroxybenzoic acid (DHB) and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDC) were dissolved in methanol (MeOH): water (90:10 v/v) to a final concentration of 7 mg/ml. 1 mg/ml SIL glucose was dissolved in the same solvent and then mixed by pipetting with the different matrices at a ratio of 1:1 (v/v). A volume of 1 μl of the final samples was pipetted onto a stainless steel MALDI target immediately after mixing and allowed to dry at room temperature.

Table 2.2: MALDI Chemical list

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Abbreviations</th>
<th>Formula</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(1-naphthyl) ethylenediamine dihydrochloride</td>
<td>NEDC</td>
<td>C_{10}H_{7}NCH_{2}CH_{2}NH_{2}.2HCl</td>
<td>259.17</td>
</tr>
<tr>
<td>2,5-dihydroxybenzoic acid</td>
<td>DHB</td>
<td>(HO)<em>{2}C</em>{6}H_{5}CO_{2}H</td>
<td>154.12</td>
</tr>
<tr>
<td>1,5-diaminonaphthalene</td>
<td>1,5-DAN</td>
<td>C_{10}H_{6}(NH_{2})_{2}</td>
<td>158.20</td>
</tr>
<tr>
<td>9-Aminoacridine</td>
<td>9-AA</td>
<td>C_{13}H_{10}N_{2}</td>
<td>194.23</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
<td>3-OMG</td>
<td>C_{7}H_{14}O_{6}</td>
<td>194.18</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose</td>
<td>C_{6}H_{12}O_{6}</td>
<td>180.16</td>
</tr>
<tr>
<td>Stable isotopically-labelled Glucose</td>
<td>SIL Glucose</td>
<td>13C_{6}H_{12}O_{6}</td>
<td>186.11</td>
</tr>
<tr>
<td>Stable isotopically-labelled Sorbitol</td>
<td>SIL Sorbitol</td>
<td>13C_{6}H_{14}O_{6}</td>
<td>188.13</td>
</tr>
</tbody>
</table>
2.4.2. Tissue preparation for MALDI-IMS

Frozen lenses were mounted on cold chucks using CryomatrixTM compound (Thermo Scientific). A cryostat (Leica CM3050S, Leica Microsystems GmbH, Wetzlar, Germany) was used to cut either axial or coronal sections with a thickness of 20 µm (Figure 2.2). The axial orientation allowed for the visualisation of the anterior pole, the posterior pole and epithelium, while serial sectioning in the coronal plane enabled sections from both surfaces (anterior and posterior sutures) and the centre of the lens to be collected. Next, lens sections were immediately mounted on cooled double-sided carbon tape (ProSciTech, Kirwan, Australia) attached to non-conductive glass slides (PINK COLORFROST, LabServ, NZ). The collected sections were subsequently stored in a vacuum desiccator for at least 1 hour immediately before the matrix application step. These prepared sections were also used in scanning electron microscopy to evaluate the section morphology (see section 2.8).
Figure 2.2: Axial and Coronal orientations of lens sections

This schematic demonstrates the sections taken through the lens equator resulting in (A) coronal sections, and the section taken through the anterior and posterior poles, resulting in (B) axial sections.
2.4.3. Matrix and IS application for MALDI-IMS

A TM-Sprayer (HTX Technologies, Carrboro, NC) was used to apply internal standard (IS) and matrix solutions to the tissue sections. 3-OMG (500 μM) was used as the IS due to its chemical similarity to glucose and because it has a molecular weight (C_7H_14O_6, Mw = 194.079) that is different to both endogenous glucose (C_6H_{12}O_6, Mw = 180.063) and SIL glucose (^{13}C_6H_{12}O_6, Mw = 186.110). NEDC (7 mg/ml in 90% MeOH) was applied using the following spray settings: solvent flow rate of 0.15 ml per minute, nozzle speed of 700 mm per minute, nozzle temperature of 70°C, track spacing of 2 mm, dry time of 0.5 min, for four passes. Following the NEDC deposition, samples were stored in a vacuum desiccator until data collection.

2.4.4. MALDI data acquisition

All MALDI data acquisition was carried out using a Bruker SolariX XR 7T FT-ICR mass spectrometer using FTMS Control v2.2.0 and flexImaging v5.0 (Bruker Daltonics, Billerica, MA). Prior to the spectra acquisition, an external calibration was employed using red phosphorus in MALDI-MS negative ion mode. To record a MALDI spot spectrum, 10 spectra were accumulated in negative ion mode with 100 laser shots. MALDI-IMS datasets were acquired in negative ion mode from m/z 100 to 1000 with a resolving power (m/Δm) of 66,000 at m/z 400. A Smartbeam II Nd:YAG laser at 355 nm was applied for MALDI-IMS, with 200 laser shots accumulated per position at a repetition rate of 1 kHz. The laser beam size was set to medium for images collected at 150 μm sampling size, while for the high spatial resolution images (30 μm sampling size), minimum beam size was used (Figure 2.3, General metabolomics workflow).

Sections from each lens were collected and analysed in the same imaging dataset so that intensity levels of detected analytes could be compared directly between samples. Lens sections were sampled in a randomised order to avoid sampling order artefacts. Following MALDI-IMS, datasets were imported into SCiLS Lab (version 2022a Pro, SCiLS GmbH, Bremen, Germany), whereas MALDI spot data
processing was conducted using the DataAnalysis software package (Bruker Daltonics; v5.0). MALDI-IMS of metabolites in tissue sections were normalised using either the root mean square (RMS) method or to the 3-OMG ([M + Cl]^- m/z 229.0473) IS signal, and MALDI images plotted at the theoretical m/z +/-0.005 Da with weak denoising on. Signals detected in the MALDI-IMS datasets were identified using database interpretation, HMDB and METASPACE (Palmer et al., 2017), of accurate masses and on-tissue MS/MS where possible, and comparison to spectra collected by GC-MS analysis of equivalent lens samples (see Appendix A).

2.5. GC-MS analysis

2.5.1. Sample preparation for GC–MS analysis

Whole bovine lenses underwent identical organ culture in the AAH solutions (see section 2.3.1) and were then micro-dissected into three lens regions with tweezers. The capsule, containing the lens epithelium, was peeled from the underlying fibre cell mass and discarded. Then the outer cortex (OC) was separated from the denser inner cortex (IC) and the hard, central nucleus (N). Each sample (OC, IC, N) was placed in a separate Eppendorf tube containing 1 ml of 90% MeOH (Figure 2.3). Samples were agitated and homogenised using a handheld homogeniser (Ultra-Turrax, Ika, Germany) in the extraction solvent for 45 min at 4°C. The remaining cell debris was removed via centrifugation at 16,000 g for 20 min at 4°C in a centrifuge (Eppendorf® Microcentrifuge, Model 5415 R), and the supernatants containing the extracted small molecules were stored at −80°C until analysed. Samples were then dried in a SpeedVac Concentrator (Thermo SPD121P, ThermoFisher Scientific, Waltham, MA, USA) and underwent automated trimethylsilyl (TMS) derivatisation (Zarate et al., 2017) in preparation for analysis via GC–MS.
Figure 2.3: Metabolomics Workflow, integration of MALDI-IMS and GC-MS.

Lenses are dissected from fresh bovine eyes (A), which is followed by incubation in artificial aqueous humour (AAH) at 37 °C for times ranging from 5 min to 20 h (B). Then, the lenses are sectioned using a cryostat (C) and NECD matrix is sprayed using a TM sprayer (D) to facilitate the ionization of the metabolites so that it can be detected by the mass spectrometer (E). Slides are scanned, and data is acquired by the MALDI-IMS (F) and then exported to SCiLS Lab software for analysis. To verify the identification of metabolites the incubated and control lenses (B) are micro-dissected into three regions (G: outer cortex [OC], inner cortex [IC], and nucleus [N]). Following this, the metabolites are extracted (H) and then metabolites are derivatised with TMS (I) so that it can be detectable by the GC-MS (J). Recorded MS data is then exported to R-package for further analysis (K).
2.5.2. GC–MS analysis

The derivatisation process and GC-MS analysis were conducted by the Faculty of Science Mass Spectrometry Centre (part of the University of Auckland Mass Spectrometry Hub (MaSH)) on a fee-for-service basis. Instrument parameters were based on published literature by Villas-Boas et al. (Villas-Bôas et al., 2006). An Agilent 7890B gas chromatograph coupled to a 5977A inert mass spectrometer with a split/splitless inlet was used. 1 µl of the sample was injected using an Agilent autosampler into a glass split/splitless 4 mm ID straight inlet liner packed with deactivated glass wool. To optimise the signal level, multiple split ratios were applied and accounted for in subsequent data analysis steps. The inlet was set to 230°C with a pressure of 99.26 kPa, and a column flow of 1.3 ml per minute constant flow mode, giving a calculated average initial linear velocity of 39 cm/s. The column was a fused silica ZB-1701 30 m long, 0.25 mm ID, 0.15 µm stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex). The carrier gas was ultra-high purity grade helium (99.9999%, BOC). The GC oven temperature programming started isothermally at 70°C for 5 minute, increased 10°C per minute to 179°C; increased 0.5°C per minute to 180°C, held 2 minute; increased 10°C per minute to 220°C, held 1 minute; increased 2.5°C per minute to 265°C, held 1 minute; increased 10°C per minute to 280°C, held 1 minute; increased 1°C per minute to 290°C and held for 0.6 minute. The transfer line to the Mass Selective Detector (MSD) was maintained at 250°C, the source at 230°C and the quadrupole at 150°C. The detector was turned on 4.5 minute into the run. The detector was run in positive-ion, electron-impact ionisation mode, at 70 eV electron energy, with an electron multiplier set with no additional voltage relative to the autotune value. Chloroform blanks were run for every 10 – 12 samples to monitor instrument carryover. Mass spectra were acquired in scan mode from 40 to 650 amu with a detection threshold of 100 ion counts.

2.6. Proteomics analysis

2.6.1. Protein preparation
Protein fractions from the epithelium were prepared by peeling the capsule off from six freshly collected bovine lenses. First, the lenses are dissected from fresh eyes, washed with 1×PBS in a petri dish and then oriented with the anterior lens capsule down towards the bottom of a glass slide. A cross-pattern of radial cuts was made into the posterior lens capsule with two pairs of fine tweezers. Using very fine tweezers, the capsule petals were peeled towards the lens equator (Figure 2.4).

Epithelial tissue regions then were micro-dissected using an 8mm surgical punch from the central, peripheral and equatorial regions (Figure 2.5A). Samples from two lens fibre regions (Cx and N) were also collected (Figure 2.5B). Tissue from these regions were homogenised using a handheld homogeniser (Ultra-Turrax, Ika, Germany) in 0.5 mL homogenising solution (Table 2.3) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Following centrifugation at 15 000 g for 30 minutes at 4 °C, the pellet was separated from the supernatant and used for the next step of the proteomics workflow, SDS-polyacrylamide gel electrophoresis.
Figure 2.4: Flat mounting of lens epithelial cell monolayer.

This schematic demonstrates how flat mounts were created for microdissection and proteomic analysis. (A) After dissecting the bovine lens is washed with 1xPBS, (B) the lens capsule is gently peeled from the posterior side in a flower pattern on a glass slide, (C) the fibre cell mass is removed, and (D) the epithelial tissue is flat mounted to be ready for sampling with a surgical punch. Figure modified from (Parreno et al., 2022).
Table 2.3: Buffers and solutions used in proteomics analysis

<table>
<thead>
<tr>
<th>Buffers/Solution</th>
<th>Components</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenising Solution</td>
<td>5mM Tris (pH 8.0), 2 mM EDTA, 2 mM EGTA</td>
<td>Aliquoted and stored at -20°C</td>
</tr>
<tr>
<td>Storage Solution</td>
<td>5mM Tris (pH 8.0), 2mM EDTA, 2mM EGTA, 100mM NaCl</td>
<td>Aliquoted and stored at -20°C</td>
</tr>
<tr>
<td>SDS PAGE Running Buffer (1x)</td>
<td>200mM glycine, 25mM Tris, 3.5mM SDS (0.1%w/v) – diluted from 10x stock, pH adjusted to 8.3</td>
<td>Autoclaved glassware at RT for a few months</td>
</tr>
<tr>
<td>Loading Dye (1x)</td>
<td>63.5mM Tris (pH 6.8), 2% SDS, 10%v/v glycerol, 0.02%w/v bromophenol blue, 5% β-mercaptoethanol</td>
<td>Aliquoted and stored at -20°C</td>
</tr>
<tr>
<td>Blue Coomassie Stain Solution</td>
<td>0.2%w/v Coomassie brilliant blue, R-250, MeOH (50%v/v, Glacial acidic acid (10%v/v), H₂O (40%)</td>
<td>Stored at room temperature</td>
</tr>
<tr>
<td>Destain Solution</td>
<td>30% MeOH</td>
<td>Stored at room temperature</td>
</tr>
</tbody>
</table>
Figure 2.5: Schematic diagram of the lens regions selected for proteomics.

This diagram illustrates an epithelial cell flat mount (A) and an axial section (B) of the bovine lens indicating regions (red circles) where the tissue is collected for subsequent proteomic analysis. Central epithelium (blue), peripheral epithelium (yellow), equatorial epithelium (grey), peripheral fibre cells (red), lens cortex (Cx) and nucleus (N).
2.6.2. SDS-polyacrylamide gel electrophoresis

In proteomics, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for sample preparation prior to mass spectrometry to separate the proteins based on the differences in their molecular weights. At the first step, both the pellet and supernatant were loaded on a commercial SDS PAGE (Figure 2.6). Based on the collection of equivalent areas of tissue using the tissue punch approach, similar protein concentrations were present in each sample. Lens’s proteins were separated using a 10% Mini-PROTEAN® TGX™ precast gradient polyacrylamide gel (#456-8033 Bio-Rad Laboratories Inc., Hercules, California, USA). Prior to loading the samples into their corresponding lanes, 15 μl of the protein samples were mixed with 5 μl of loading dye (Table 2.3) and incubated at RT for 10 min. The molecular weights of the protein bands were evaluated by running a 10 μL Precision Plus Protein™ Standards Dual Colour ladder (Bio-Rad Laboratories) alongside the samples. 10 μl of the protein samples mixed with loading dye were then deposited in electrophoresis chambers, loaded with SDS PAGE running buffer (Table 2.3), and run at 100V until the dye front migrated from stacking gel into the running gel (approximately 30 min). The voltage was then increased to 150 V until the dye front reached the bottom of the gel (approximately 45 min), using the Bio-Rad Laboratories PowerPac™ HC High-Current Power Supply.

Following electrophoresis, gels were stained with Coomassie solution (Table 2.3) for 30 min with gentle shaking. After the staining step, the gels were washed three times with 5 ml destain solution (Table 2.3) and 1 min gentle shaking. The gel was then covered with destain solution and incubated at RT while rocking for 3 h until the protein bands were seen without background staining.
Figure 2.6: General proteomics workflow.

This diagram shows the general workflow that is performed to detect GLUTs in the normal unincubated bovine lenses. From the dissected bovine lens (A), the micro-dissected regions are collected, homogenised and centrifuged to get the pellet in the bottom of tube (B). The pellet is then run on SDS-PAGE by electrophoresis to separate the proteins (C), the gel stained, and the gel band corresponding to the molecular weight of GLUTs is cut (a prominent band of 53KDa) from the SDS-PAGE (D). This band is washed and de-stained, the proteins are enzymatically digested by trypsin into peptides (E), and then they are ionised and fractionated by LC-MS/MS (G). Spectra acquired by mass spectrometer are searched in extensive databases for protein identification (H), followed by further bioinformatic analysis.
2.6.3. Gel-based proteomics GeLC/MS

Proteomics analysis to identify glucose transporters (GLUTs) in the lens samples was performed as a fee-for-service at MaSH in collaboration with Dr Kyriakos Varnava. A prominent band at 53kDa, shown in Figure 2.7, corresponding to the molecular weight of GLUTs was cut using a 6x2 mm punch. The gel bands were placed in different 1.5 mL Eppendorf tubes and diced into cubes. The gel cubes were then prepared for LC/MS analysis following standard protocols as follows. Briefly, the cubes were washed and de-stained with 50% acetonitrile in a 50 mM ammonium bicarbonate buffer. Following de-staining, the cubes were dehydrated with acetonitrile, reduced using dithiothreitol and concomitantly alkylated with iodoacetamide. Subsequently, the cubes were dehydrated again and were finally subjected to an enzymatic digestion using 12.5 ng/µl porcine trypsin (Promega Corp., Madison, Wisconsin) in a temperature-controlled microwave at 45°C for 1 hour. The digest was acidified to halt digestion, and peptides were injected onto a 0.3 x 10 mm trap column packed with Reprosil C18 media (Dr. Maisch HPLC GmbH, Ammerbuch, Germany) and desalted for 5 min at 10 µl/min before being separated on a 0.075 x 200 mm picofrit column (New Objective, Inc., Littleton, Massachusetts, USA) packed in-house with 3 µm Reprosil C18 media. A 30-50% solvent B gradient was applied at 300 nl/min using a NanoLC 400 UPLC system (Eksigent Technologies, Redwood City, California), where solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile.

The picofrit spray was directed into a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (AB Sciex LLC, Framingham, Massachusetts) scanning from 300-2000 m/z for 200 ms, followed by 40 ms MS/MS scans on the 35 most abundant multiply-charged peptides (m/z 80-1600). The mass spectrometer and HPLC system were controlled by the Analyst TF 1.7 software package (AB Sciex LLC, Framingham, Massachusetts, USA). The resulting MS/MS data were searched against a database comprising Uniprot bovine entries appended with a set of common contaminant sequences using ProteinPilot version 5.0 (AB Sciex LLC, Framingham, Massachusetts, USA).
Search parameters were as follows: Sample Type = Identification; Search Effort = Thorough; Cys Alkylation = Iodoacetamide; Digestion = Trypsin. The peptide summary exported from ProteinPilot was further processed in Microsoft Excel using a custom macro to a) remove proteins with Unused Scores below 1.3, b) eliminate inferior or redundant peptide spectral matches, and c) sum the intensities for all unique peptides from each protein.
Figure 2.7: SDS-PAGE analysis of proteins in the lens samples.

Image of a representative gel showing the lens protein fractions. At the end of the electrophoretic separation, lens proteins are sorted by size. The red box indicates the band at the molecular weight of 50kDa corresponding to the molecular weight of GLUTs (53kDa). This band is cut from samples of each collected region using a 6x2 mm punch for proteomic analysis. In the left column, a molecular weight marker is run and used to estimate the size of the proteins.
2.7. Immunohistochemistry

Immunohistochemistry was used to localise GLUT proteins in normal bovine lenses at the cellular level. Commercially available anti-GLUT primary antibodies that were applied for IHC (Table 2.4). Goat anti-rabbit IgG- Alexa Fluor® 488 used as a secondary antibody (Cat # 35552, Thermofisher), DAPI as cell nuclei marker (Cat # 62248, Thermofisher), and WGA-Alexa Fluor 594 to mark the cell membranes (Cat # W11262, Thermofisher).

Table 2.4: Glucose transporter primary antibodies.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Manufacture</th>
<th>Antibody dilution</th>
<th>Epitope Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Transporter GLUT1 peptide (ab202335)</td>
<td>Abcam</td>
<td>1:200</td>
<td>C-terminus</td>
</tr>
<tr>
<td>Rabbit Anti-mouse GLUT-1 (GT11)</td>
<td>Alpha Diagnostic International Inc, San, Antonio, TX, USA</td>
<td>1: 200</td>
<td>C-terminus, cytoplasmic domain</td>
</tr>
<tr>
<td>Rabbit Anti-Mouse GLUT-3 (GT31)</td>
<td>Alpha Diagnostic International Inc, San, Antonio, TX, USA</td>
<td>1: 200</td>
<td>C-terminus, cytoplasmic domain</td>
</tr>
<tr>
<td>Goat Anti-Mouse GLUT-3 (sc-31838)</td>
<td>Santa Cruz Biotechnology, Inc, Dallas, TX, USA</td>
<td>1: 200</td>
<td>N-terminus, extracellular domain</td>
</tr>
<tr>
<td>Rabbit Anti-Human GLUT-3 (GT32)</td>
<td>Alpha Diagnostic International Inc, San, Antonio, TX, USA</td>
<td>1: 200</td>
<td>C-terminus, cytoplasmic domain</td>
</tr>
<tr>
<td>Rabbit Anti-Human GLUT-3 (GT32)</td>
<td>Alpha Diagnostic International Inc, San, Antonio, TX, USA</td>
<td>1: 200</td>
<td>N-terminus, extracellular domain</td>
</tr>
</tbody>
</table>
2.7.1. Fixation, cryoprotection and cryosectioning of the bovine lenses

Whole bovine lenses were fixed, cryoprotected, and cryosectioned in the axial orientation using standard protocols (Gletten et al., 2022). Briefly, bovine lenses were fixed in 2% paraformaldehyde (PFA) and 0.01% glutaraldehyde, pH 7.2-7.4 for 72 h at RT followed by three 10 min PBS washes and subsequent cryoprotection in 10% and 20% sucrose for two days at 4°C and one day at RT respectively, then overnight at 4°C in 30% sucrose. Lenses were stored in 30% sucrose at 4°C for up to two weeks before cryosectioning.

2.7.2. Immunohistochemistry and Confocal Microscopy

The fixed lenses were positioned in the axial orientation on chucks (see Figure 2.2) and coated with Tissue-Tek Optimal Cutting Temperature (OCT, Sakura Finetek, California, USA). OCT-coated lenses were snap-frozen in liquid nitrogen for 25-35 seconds. Sections were cut at 16 μm thickness with a cryostat at -20°C and mounted onto blank microscope slides (76x26 mm, extra white, Thermo Scientific). Axial lens sections were collected and examined under a light microscope to confirm they were intact. Sections were then washed three times in 1xPBS to remove OCT before storing them in a humidity box at 4°C for up to one month. Prior to immunolabelling, sections were permeabilised in 0.5% Tween 20® for 20 min and then incubated in the blocking solution (3% BSA, 3% Normal Goat Serum, in 1xPBS) for one hour at RT. Sections were incubated overnight in GLUT1 or GLUT3 primary antibodies (Table 2.4) diluted 1:200 in the blocking solution to minimise non-specific binding of antibodies to the tissue. The following day, sections were washed three times in 1xPBS and incubated with secondary antibody conjugated to Alexa Fluor-488. Sections were then washed and incubated in a mixture of Alexa Fluor WGA-594 and DAPI diluted at 1:100 and 1:1000, respectively, in 1xPBS for 1h at RT to visualise the lens cell morphology (Lim et al., 2017). Following a series of washes in 1xPBS, sections were mounted in Vectashield (Vector Laboratories, CA, USA), and coverslips were placed over
the sections gently and then sealed with nail polish. All prepared bovine slides were stored in a slide holder at 4°C until imaged by fluorescence confocal microscopy (Figure 2.8).

Labelled sections were imaged using a 60x magnification and 1.35 numerical aperture objective lens on an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) with FluoView 2.0c software at the University of Auckland Biomedical Imaging Research Unit (BIRU). Digital images (2048 x 2048 pixels) were collected using optimised gain and offset settings that captured the entire dynamic range of the signal at each tissue location. Raw images obtained from separate channels were pseudo-coloured and then overlaid using Adobe Photoshop software (v23.1.1, Adobe Inc., San Jose, California).
Figure 2.8: The immunofluorescence workflow.

Schematic diagram detailing the immunohistochemistry workflow. Lens tissue is prepared as described previously (A), and immunohistochemistry assay is performed to localise GLUTs in the lens (B). Fluorescence microscopy (C) is utilised to capture images from immuno-labelled section. Figure created using Biorender.com
2.8. Scanning electron microscopy

Following cryosectioning and matrix application onto the lens slices, the surface morphology of mounted tissue sections was characterised by scanning electron microscopy (SEM). Axial sections were coated with a 5 nm layer of gold using a sputter coater (Q150R Plus, Quorum Technologies) before SEM imaging at a magnification of ×1100. SEM morphology studies were carried out on a JEOL Benchtop Scanning Electron Microscope, NeoScope™ JCM-6000 (Jeol, Japan) using secondary electrons at 15 kV current, in a high vacuum, and the processing of the images was carried out using JEOL’s Image Software (https://www.jeolusa.com). The SEM imaging was conducted in collaboration with the Polymer Biointerface Centre at the University of Auckland.

2.9. Data Analysis

2.9.1. Principal component analysis

Principal component analysis (PCA) is a statistical approach for characterising the structure of data and identifying linear combinations of observed variables that maximise the grouping of samples into distinct classes (Wall et al., 2003). It is one of the most significant and influential techniques in chemometrics along with various other fields (Bro & Smilde, 2014), and it is generally used to visualise patterns in multivariate datasets. Its purpose is to demonstrate data points relative positions in fewer dimensions while maintaining as much information as feasible about the relationships between the data points and investigating correlations between dependent variables. In this study, PCA was used for the purpose of discriminant analysis and was performed with a custom-written R script by Dr George Guo from MaSH and the Cardinal package (Bemis et al., 2015). The imZML data were generated by SCiLS Lab. The m/z peaks were picked with 20 ppm bin size, smoothed with the ‘Gaussian’ method, one-pixel smoothing radius, and a threshold of 0.3% of maximum intensity. Sampling locations lying outside the tissue sections were removed manually from each lens section to remove off-tissue regions as a source
of variation. Each sampling location in the MALDI-IMS data was considered as an individual observation.

**2.9.2. Intensity profile analysis for MALDI images**

ImageJ 1.50i (National Institute of Health, USA) was utilised to investigate the intensity of pixels through the equatorial region of axial lens sections. MALDI images of a variety of m/z’s representing SIL metabolites were assessed. The analysed region was selected manually using the rectangular selection tool. Then, the average profile plot was produced where the x-axis displayed the horizontal distance along the tissue diameter, and the y-axis was the averaged pixel intensity.

**2.9.3. GC–MS data analysis**

GC-MS data were converted into .cdf files by the function provided in Automated Mass Spectral Deconvolution and Identification System (AMIDS). A sub-library of the spectral database was generated by searching the QC samples against the NIST20 database. The identification results were then combined with an amino acid and sugar standard reference spectral library. Spectra of SIL molecules of interest were annotated and curated manually with the assistance of the NIST20 database. Peak quantitation was performed within the MassOmics package, an in-house R-based package for metabolomics analysis (Guo et al., 2021). In addition, the identities of three primary SIL metabolites signals including glucose, sorbitol, and glucose-6-phosphate were validated through retention time matching using reference standards and the GC-MS analysis of identically incubated lenses (see the Appendix A, Figure 7.5-7). Eventually, the compound intensities were measured by peak area integration of curated quantification fragment ions with a tolerance of 0.5 Da.

**2.9.4. Statistical Analysis**

Regarding the statistical tests, pixel-level PCA was performed to detect matrix differences within data sets. Pixel-level PCA also was utilised to perform an outlier test, by ranking features and selecting
25% ~ 75% of feature values. In addition, to test if the data was normally distributed, the data were further normalised (RMS) and rescaled, then the data were evaluated via a bar plot at feature/sample level. All data in the results chapters are presented as the mean ± standard deviation of the mean (SEM). ANOVA was performed to determine the statistical significance (p-values) by using R package (R Core Team, 2021). In all analyses, a p-value < 0.05 indicated a significant difference in compared values. Further details specific to the data presented are provided in their respective chapters.
Chapter 3 || MAPPING GLUCOSE METABOLITES IN THE NORMAL BOVINE LENS: EVALUATION AND OPTIMISATION OF A MALDI IMAGING MASS SPECTROMETRY METHOD

Due to the lack of a blood supply, the lens sources glucose directly from the surrounding ocular humours and then metabolises this glucose to supply the distinct energy requirements in the different lens regions that maintain the overall transparent and refractive properties of the lens. In this chapter, I present my efforts to develop and optimise matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI-IMS) to spatially-resolve the pattern of glucose uptake, transport and metabolism in the normal bovine lens. During my research, I made several key findings. Firstly, I found that using NEDC as the MALDI matrix is the most appropriate choice for maximizing the signal level of glucose and minimizing background ions. Secondly, I discovered that the use of fresh-frozen tissue is necessary to detect glucose and its metabolites effectively as this approach preserves the metabolic state of the samples, enabling reliable detection. Additionally, I determined that the most effective protocol for applying the matrix is the simultaneous spray application of internal standard/matrix, as it reduces tissue cracking and promotes crystal formation. Furthermore, I found that normalizing the IMS data based on an internal standard (specifically 3-OMG) is the best method for accurately depicting the distribution of glucose and its metabolites within the bovine lens. Moreover, the developed MALDI-IMS methodology can detect both endogenous and SIL lens metabolites introduced through ex vivo lens culture, expanding the possibilities for studying metabolic processes. Lastly, I confirmed the identity of the mapped metabolites using GC-MS analysis, providing additional validation and ensuring the reliability of the results, which have subsequently been published (Zahraei et al., 2021). To provide some context for the need to optimise and validate this MALDI-IMS approach, I will provide an overview of imaging mass spectrometry technologies.
3.1. Imaging mass spectrometry

Imaging mass spectrometry (IMS) is a technique which combines mass spectrometry with microscopic imaging to provide molecular tissue maps of a large number of molecules from a specific class in the tissue (van Hove et al., 2010). In the IMS data, the height of the peak is determined by the concentration of the molecule, affinity of ionisation, and ion suppression throughout the experiment. A variety of mass spectrometry imaging techniques have been used in biomedical research, exploiting various ionisation mechanisms to capture the chemical and spatial complexity of biological systems, and therefore, have distinct capabilities. Secondary ion mass spectrometry (SIMS), employs an ion beam to desorb and ionise the surface molecules (Bodzon-Kulakowska & Suder, 2016). These "secondary ions" are then transported to a mass analyser, which is usually a time-of-flight (ToF) analyser. SIMS is a desired technology for single-cell imaging and other high spatial resolution imaging studies ranging from nm to μm resolution as these beams can be focused down to a minimal area of ~50 nm (Hagenhoff, 2000). The disadvantage of this high spatial resolution is that a lot of energy hits the sample in a single pixel, leading to the fragmentation of ionised molecules. Modern molecular primary ion beams have been developed that produce less fragmentation, allowing for the analysis of more intact biomolecules (Kollmer, 2004; Weibel et al., 2003).

Desorption ionisation (DESI) IMS utilises charged solvent droplets at supersonic velocities to desorb and ionise chemicals, comparable to how SIMS uses energetic molecular ions to probe surfaces (Bodzon-Kulakowska & Suder, 2016). The measurable molecular categories, which are mostly metabolites and lipids, are determined by the solvent spray's composition. The spatial resolution, which is generally ~200 μm, is also limited by the solvent spray (Ifa & Eberlin, 2016). Using charged solvent droplets has the downside of wetting the surface, which might result in delocalisation. Nevertheless, by adjusting the instrument settings, this may be addressed in a significant part. An additional advantage of DESI is that it functions at atmospheric pressure, making it suitable for direct investigations of tissues that cannot be subjected to vacuum.
Matrix-assisted laser/desorption ionisation (MALDI) uses laser desorption and ionisation to ionise samples, which requires a small organic crystal known as a matrix layer to cover the sample surface. Based on the chosen solvent/matrix mixture, this matrix crystallises on the tissue surface, incorporating molecules of interest and enabling the detection of specific molecular classes. While MALDI-IMS was first developed to map protein and peptide distributions, more recently, it has been used to map small molecules and metabolites and is particularly powerful in this area when coupled to mass analysis via Fourier transform-ion cyclotron resonance (FT-ICR) (Cornett et al., 2008).

In summary, while SIMS and DESI are ideal for analysing different tissues or when high spatial resolution is required, a trade-off between spatial and chemical information still remains. Hence, as a result, MALDI is the most commonly used ionisation approach for IMS biomedical research applications because it can provide high spatial resolution down to the single-cell level (Dueñas et al., 2017), while still reflecting the molecular complexity of a tissue. As with any technique MALDI-IMS has a number of parameters that need to be optimised for it to be successfully used to identify a particular class of molecules in different tissues. Some of these parameters are discussed below.

3.1.1. Optimising MALDI-IMS

Matrix composition: The MALDI matrix assists in absorbing laser light and the subsequent ionisation of compounds integrated into matrix crystals (Bodzon-Kulakowska & Suder, 2016). Different molecular classes, such as proteins, peptides, lipids, drugs, and metabolites (Chatterji & Pich, 2013; Greer et al., 2011; Grey & Schey, 2008; Heikkinen et al., 2019; Kotnala et al., 2021; Ly, Buck, Balluff, Sun, Gorzolka, Feuchtinger, Janssen, Kuppen, van de Velde, & Weirich, 2016), can be detected and visualised depending on the type of matrix applied. The spatial resolution that may be attained is determined by the size of these matrix crystals and the size of the laser focus spot. As a result, MALDI-IMS studies are widely carried out with a spatial resolution of 20-50 μm. Instrumentation and matrix application methods have improved recently, enabling spatial resolutions of 5 μm (Ogrine Potočnik et al., 2015) and even 1 μm, as reported by Spengler and Hubert in 2002 (Spengler & Hubert, 2002).
**Tissue preparation:** The techniques used in IMS must be compatible with biological tissues in order to be applied in biomedical research. Often human tissue will first need to be preserved and archived before being made available for analysis. In contrast, animal tissue can often be obtained as fresh samples that are immediately frozen to stop biological processes and prevent deterioration of the molecular structure of the sample (Heeren et al., 2008). While freezing does not normally change the molecular structure of biological molecules it can change the cellular morphology. However, this is not a problem for the MALDI-IMS technique as MALDI-IMS is not routinely capable of sub-cellular sampling. Hence, in order to detect and visualise the molecular complexity of a tissue with IMS, the tissues do not require extensive sample preparation.

On the other hand, clinical investigations require a focus on the morphology of samples obtained. To enable long-term storage, pathology labs fix the samples using formalin. Formalin-fixed paraffin-embedded (FFPE) samples contain protein crosslinks to prevent degradation, maintain tissue histology, and improve the sample's handleability (Thavarajah et al., 2012). Such FFPE samples can be stored at room temperature, which is much cheaper and more accessible than long-term sample storage in a deep freezer. However, the molecular modifications caused by FFPE fixation creates challenges to analysing these samples with IMS since the chemical preservation phases modify the molecular information of the tissues when compared to fresh frozen tissue. While tissue preparation techniques have been developed to make FFPE tissue compatible with MALDI-IMS, the extra tissue preparation steps can be challenging to optimise and execute routinely.

**Validation:** MALDI-IMS has several benefits over the conventional technique of biochemical identification, including being user-friendly, having high speed and accuracy, and being relatively economical (Rychert, 2019). High resolution MALDI-IMS can accurately identify very closely related species. However, it has some limitations as well. The inability to discriminate between related species can be due to the similarity of the molecular weight. For example, MALDI is currently unable to differentiate glucose from fructose. To overcome this, a number of off-line and on-line strategies, such
as pre-separation of ions using ion mobility approaches (Kanu et al., 2008; McLean et al., 2007), GC-MS (Frankfater et al., 2020) and LC-MS (Snytnikova et al., 2017), have been implemented.

### 3.1.2. Optimisation of MALDI-IMS to study glucose metabolism in the bovine lens

While a range of other lens metabolite classes have been mapped using this MALDI-FT ICR-IMS approach ( Boughton et al., 2020; Grey, 2016; Nye-Wood et al., 2016), glucose has not. However, in other tissues several glucose metabolites have been detected using a variety of different MALDI matrices, for example, 9AA and TiO₂ (Dekker et al., 2015; Yang et al., 2018), and isobaric hexose monophosphates and bisphosphates have been imaged elegantly in the brain by using MS³ on a linear ion trap (Kleinridders et al., 2018). However, the tissue preparation and MALDI matrix conditions for glucose and glucose metabolite detection in the lens have not previously been investigated. Therefore, I first needed to select an appropriate matrix, then optimise its application to lens sections to develop MALDI-IMS as a technique to study glucose metabolism in the different regions of the bovine lens.

### 3.1.3. Optimisation of MALDI matrix choice for use in the bovine lens

I first analysed a standard of glucose (SIL glucose) used in later ex vivo lens culture experiments in several different matrices to determine the optimal matrix and solvent conditions to promote desorption/ionisation via MALDI (see Figure 3.1). For all trialled matrices, the predicted and observed m/z for ions are all reported in Appendix A, Table 7.1. Signal quality was determined by monitoring ion intensity, signal-to-noise ratio and the presence of matrix-related ions and other chemical background signals. The result of this comparison is reported in Table 3.1.

Both [M-H]⁻ and [M+Cl]⁻ ions were monitored because these are the most commonly detected negative ions of primary metabolites for standards and particularly from tissue where chloride is known to be present (Zhang & Jacob, 1997). SIL glucose was analysed in DHB matrix; however, no ions for SIL glucose were detected in either polarity (data not shown), consistent with previous studies (Yang et al., 2010). DHB was not pursued further because it also lacked the ability to map metabolites of SIL glucose, which are generally detected in negative ion mode. Next, the typical negative ion mode
metabolite matrix 9-AA was trialled. While the signal for the [M+Cl]− ion at m/z 221.052 was significant, the matrix background signals were also abundant, and no [M-H]− ions were detected at m/z 185.076 (Figure 3.1A).
Figure 3.1: Matrix-assisted laser desorption/ionisation (MALDI) matrix optimisation for detection of glucose.

Negative ion mode MALDI-Fourier transform-ion cyclotron resonance (FT-ICR) mass spectra of stable isotopically-labelled glucose prepared in three different matrices: (A) 9-aminoacridine, (B) 1,5-diaminonapthalene HCl and (C) N-(1-naphthyl) ethylenediamine dihydrochloride. Left panel: full spectrum; right panel: zoomed in spectrum of the [M-H]⁻ (m/z 185.0762) (inset, blue) and [M+Cl]⁻ (m/z 221.0523) ions.
Table 3.1 Signal quality comparison for matrix optimisation of SIL glucose

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Adduct</th>
<th>Observed* m/z</th>
<th>Resolution</th>
<th>Intensity of ions</th>
<th>Relative intensity %</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>9AA</td>
<td>[M-H]^−</td>
<td>185.076</td>
<td>243284</td>
<td>247504</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>[M+Cl]^−</td>
<td>221.052</td>
<td>143543</td>
<td>44703772</td>
<td>37</td>
<td>1116</td>
</tr>
<tr>
<td>1, 5 DAN-HCl</td>
<td>[M-H]^−</td>
<td>185.076</td>
<td>72360</td>
<td>440862</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>[M+Cl]^−</td>
<td>221.053</td>
<td>98630</td>
<td>12088696</td>
<td>0.8</td>
<td>72</td>
</tr>
<tr>
<td>NEDC</td>
<td>[M-H]^−</td>
<td>185.076</td>
<td>148405</td>
<td>6738470</td>
<td>0.1</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>[M+Cl]^−</td>
<td>221.052</td>
<td>145137</td>
<td>12716825600</td>
<td>100.0</td>
<td>73311</td>
</tr>
</tbody>
</table>

* Theoretical m/z of SIL glucose: [M-H]^− = 185.0762, [M+Cl]^− = 221.0523
Then, 1,5-DAN HCl, which has also previously been used as a metabolite matrix (Calvano et al., 2018; Liu et al., 2014), was trialled in negative ion mode (Figure 3.1B). A low-intensity signal for the [M-H]⁻ ion of SIL glucose was detected at m/z 185.076, whereas the [M+Cl]⁻ ion signal, detected at m/z 221.052 was higher than the [M-H]⁻, but not abundant in relation to matrix ions. Keeping in mind that, ultimately this method would be applied to unwashed tissue sections to map endogenous and exogenous metabolite distributions, a more salt-tolerant matrix was trialled next.

Finally, I trialled NEDC, an inorganic salt that has previously been used to detect metabolites (Chen et al., 2012; Wang et al., 2015), metal ions (Hou et al., 2014), lipids (Grey, 2016) and GSH (Grey et al., 2019) in ocular lens tissue. A strong response for the [M+Cl]⁻ ion was detected at m/z 221.052 (Figure 3.1C), with a weak signal for the [M-H]⁻ ion, and no signal was detected in positive ion mode (data not shown). There was also a very limited background signal from the matrix, suggesting that NEDC may be a good candidate to detect glucose and its related molecules in lens tissue.

### 3.1.4. Optimisation of matrix application

The ocular lens is a difficult tissue to generate high-quality tissue sections from due to the different tissue hydration states of its cortical and nuclear regions. Therefore, an optimised method is required to spray the matrix on the lens tissue. Several tissue preparation methods have been developed to generate lens tissue sections for MALDI-IMS analysis, including MeOH soft-landing (Han & Schey, 2006), tape landing (Anderson et al., 2015) and sucrose treatment (Palmer et al., 2012). These approaches have utilised fresh-frozen tissue. Metabolite profiles in other tissues have also been obtained from fixed tissue (Carter et al., 2011; Ly, Buck, Balluff, Sun, Gorzolka, Feuchttinger, Janssen, Kuppen, van de Velde, Weirich, et al., 2016), which, due to the fixation process, stabilises the tissue during the sectioning process. Fixed tissues can also produce better macroscopic and microscopic morphology that is more suitable for higher spatial resolution MALDI-IMS analysis (Carter et al., 2016; Garikapati et al., 2019). Therefore, tissue sections from both fresh frozen and fixed lens tissue were generated, and matrix spray application parameters were determined. Optical images were captured to assess tissue section
morphology and SEM images were collected to determine matrix crystal morphology (Figure 3.2). Intending to ultimately focus on the accurate spatial mapping of particular classes of lens nutrients (i.e., glucose), in addition to matrix application, I also trialled combinations of matrix and IS by spray application to determine the optimal protocol.

When matrix was applied in ethanol (EtOH):water (50:50 v/v), tissue section morphology remained largely intact (Figure 3.2A); however, SEM imaging showed that matrix crystal formation was poor (Figure 3.2E), suggesting that the number and quality of mass spectral signals would be limited. Spray deposition of matrix compounds in MeOH:Water (90:10 v/v) has been successfully employed for previous MALDI-IMS analysis of the ocular lens (Demarais et al., 2019; Grey et al., 2019), and this solvent was used for three different matrix applications. First, the IS was sprayed on the bovine lens section prior to matrix application. When this routine was applied to bovine lens sections, large cracks appeared in the tissue (Figure 3.2B). This resulted in low-quality MALDI-IMS results due to tissue discontinuities coupled with uneven matrix crystal formation across the cracked tissue sections (Figure 3.2F). Next, the IS was first combined with a matrix solution before spray deposition using the same parameters as previous applications. While there was some tissue cracking, this was generally limited (Figure 3.2C), and matrix crystal formation on the tissue surface was relatively uniform (Figure 3.2G). Matrix crystal uniformity was not due to the presence of IS in the matrix solution because matrix spray alone also showed limited tissue cracking and relatively uniform crystal formation (data not shown). Last, this same spray routine was applied to sections from fixed lens tissue. While the cracking pattern (Figure 3.2D) and matrix crystal morphology (Figure 3.2H) was different on fixed tissue, the concurrent application of IS/matrix spray was deemed the optimal protocol to minimise tissue cracking and improve the morphology of matrix crystals. Both fresh frozen and fixed tissues were analysed by MALDI-IMS and data quality was assessed by considering the number of signals, signal intensity, signal-to-noise ratio and signal spatial distribution.
Figure 3.2: Optimisation of matrix solution spray conditions for bovine lens metabolite analysis.

Macroscopic tissue morphology (A–D) and matrix crystal coverage and morphology (E–H) were used to optimise matrix application for matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI IMS). (A) N-(1-naphthyl)ethylenediamine dihydrochloride (NEDC) applied in 50% ethanol (EtOH) showed good tissue morphology but poor crystal formation (E). (B) NEDC applied in 90% methanol (MeOH) following internal standard (IS) showed poor tissue morphology and uneven crystal formation (F). (C) Application of NEDC in 90% MeOH concurrent with IS showed good tissue morphology and even crystal formation (G). (D) NEDC applied in 90% MeOH concurrent with IS showed adequate morphology of fixed tissue and evidence of crystal formation (H). Scale bars A-D = 1 cm, F-I = 20 μm with a magnification of ×1100.
3.1.5. Optimisation of tissue preparation

To assess signal differences between fixed versus fresh frozen tissue, I conducted a PCA analysis (Figure 3.3). All sampling locations on tissue sections from each treatment group were compared; tissue sections were not separated into anatomically distinct regions before comparison. The two treatments clustered distinctly in PC1, indicating large differences between the treatments based on signal intensity (Figure 3.3A). A feature plot of PFA fixed and fresh frozen samples (see Appendix A, Table 7.1), showing the lens metabolites most affected by PC1 (Figure 3.3B), generated a list of m/z features that contributed to the variation between tissue fixation protocols. MALDI images of a sub-set of these m/z signals for selected metabolites are plotted for fixed (left) and frozen (right) sections in Figure 3.3C. The abundant lens antioxidant GSH (Figure 3.3C, i) showed a high signal in the cortex and low/no signal in the nucleus of the lens for both preparation methods.

However, the fixed tissue signal for GSH was lower than that of the fresh frozen. Similar results were also observed for the GSH analogue ophthalmic acid (Figure 3.3C, ii), where the signal for both preparations is predominately located in the cortex, but the overall intensity is lower in fixed tissue. Another GSH-related molecule l-cysteine-GSH disulphide (CySSG) (Figure 3-3C, iii), which has previously been detected in the bovine lens inner cortical region was again detected in this region of fresh frozen lenses (Nye-Wood et al., 2016), but no signal for CySSG was detected at all in fixed tissue.
Figure 3.3: Comparison of fixed and fresh lens tissue metabolite imaging mass spectrometry (IMS).

(A) Principal component analysis (PCA) analysis between paraformaldehyde (PFA) fixed tissue and fresh frozen tissue. (B) Feature plot showing the extent of change of m/z features resulting from PFA fixation. Features that only have high intensities in the fresh frozen sample indicate that fixation suppresses the signal of these ions. (C) Matrix-assisted laser desorption/ionisation (MALDI) images of important lens metabolites in fixed (left) and fresh frozen (right) tissue: (i) glutathione (GSH) (m/z 306.076), (ii) ophthalmic acid (m/z 288.120), (iii) Cysteine glutathione disulphide (CySSG) (m/z 425.080), (iv) ATP (m/z 505.988), (v) putative glucose (m/z 215.032) and (vi) sorbitol (m/z 217.048).
Finally, a signal assigned to ATP was high in the OC of the fresh frozen tissue but absent from the fixed lens sample (Figure 3.3C, iv). However, not all signals in the fixed tissue preparation showed decreased intensity compared with fresh frozen. The signal for putative lens glucose (Figure 3.3C, v) and sorbitol (Figure 3.3C, vi) were elevated in fixed tissue, particularly in the lens nucleus. While this putative elevation of sugar signal was interesting, together, these results showed that the relative intensities and distributions of multiple lens small molecules were affected by fixation. With our focus on spatially resolving glucose, its metabolites and other lens metabolites such as antioxidants that are involved in the maintenance of lens transparency and are linked to glucose metabolism, this suggested that fixation prior to MALDI-IMS analysis was not a suitable protocol. Moreover, because the mechanism of PFA fixation is through its reaction with primary amines to form methylene bridge crosslinks (Hopkinson et al., 2010; Wisztorski et al., 2010), glucose is not expected to be fixed through this treatment. Therefore, fresh frozen tissue was utilised for the remainder of the study.

### 3.1.6. Validation and normalisation of MALDI-IMS data

When using MALDI-IMS to map a specific nutrient or class of metabolites, it is best to utilise a data normalisation approach based on the spray application of a chemically similar standard to account for any ionisation differences that result from different anatomical regions or local tissue environments. While human lens GSH does not appear to suffer significantly from ionisation differences in different lens regions (Grey et al., 2019), the effect of lens tissue microenvironment on the ionisation of glucose and related molecules is unknown and was therefore tested in our bovine lens samples (Figure 3.4). Additionally, the method of IS application was tested, because the choice of the best method of application, either application underneath tissue section, on top of tissue section prior to matrix application or simultaneous IS/matrix application, has been shown to depend on the molecule of interest (Heikkinen et al., 2019; Pirman et al., 2013; Teearu et al., 2017). The glucose analogue 3-OMG was chosen as the sprayed IS due to its chemical similarity yet different molecular weight to both endogenous glucose and its metabolites and the targeted SIL glucose and its metabolites in ex vivo cultured lenses.
MALDI images were plotted and signal intensity plots along the optical axis (anterior to the posterior pole), were extracted following sequential IS/matrix application (Figure 3.4A, upper) and simultaneous IS/matrix application (Figure 3.4A, lower). The signal intensity of 3-OMG was non-uniform across the bovine lens sections and indicated ion suppression in the lens cortex relative to the nucleus (Figure 3.4A, red).
Figure 3.4: Validation of normalisation routine.

Matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI-IMS) and gas chromatography–mass spectrometry (GC-MS) analysis. (A) MALDI images of bovine lens sections prepared for metabolite imaging using sequential (upper) or simultaneous (lower) application of standard and matrix. The internal standard (IS), 3-O-methyl-glucose (m/z 229.048, red), showed signal suppression in the bovine lens cortex relative to the nucleus. MALDI images of m/z 215.032 with root mean square (RMS) normalisation (green), shows the signal most abundant in the lens nucleus, while m/z 215.032 with IS normalisation (blue) shows signal more abundant in the lens cortex. (B) Intensity plots from posterior to anterior pole, in a region indicated by the white box in (A), shows no difference in the resulting intensity plot of m/z 215.032 when normalised to IS. (C) (i) GC–MS analysis of relative levels of isobaric metabolites glucose (orange), fructose (red) and myo-inositol (blue) from normal bovine lenses physically dissected into the outer cortex (OC), inner cortex (IC) and nucleus (N); (ii) schematic diagram of a bovine lens digittaly dissected into OC (green), IC (blue) and N (red) regions that were used for the relative quantitation of the m/z 215.032 signal from MALDI-IMS datasets; (iii) relative quantitation of m/z 215.032 on IMS data using RMS normalisation (orange) vs relative quantitation using IS normalisation (blue).
Hence, when the distribution of putative glucose signal (m/z 215.032) was plotted with RMS normalisation, the signal was more abundant in the nucleus relative to the cortex (Figure 3.4A, green) and reversed when normalised to 3-OMG (Figure 3.4A, blue). Signal intensity plots of the MALDI images were very similar (Figure 3.4B, red and green), particularly for the putative endogenous glucose signal normalised to IS (Figure 3.4B, blue), suggesting that simultaneous spray application of IS/matrix was an appropriate method for lens nutrient mapping by MALDI-IMS.

To confirm the identity of m/z 215.032, on-tissue MS/MS could not be performed because CID of this [M+Cl]− ion yielded no structural information due to the loss of the [Cl]− adduct, and the intensity of the [M-H]− ion was too low. Therefore, GC-MS was performed on bovine lenses physically dissected into the OC, IC and nucleus (N) regions to determine both the relative abundance of the putative identities of m/z 215.032 (e.g., glucose, fructose, myo-inositol) and their abundance in the different lens regions (Figure 3.4C, i). This GC-MS analysis was then used to confirm the most suitable method of MALDI-IMS data normalisation. The analysis showed that, for endogenous compounds, myo-inositol was much more abundant in all lens regions than either glucose or fructose. Finally, to compare the two normalisation protocols, MALDI-IMS data were digitally dissected into OC, IC and N (Figure 3.4C, ii), and the relative quantitation of the signal at m/z 215.032 (glucose, fructose or myo-inositol) was plotted for each region using both RMS and IS normalisation (Figure 3.4C, iii). Together, these results confirmed that IS normalisation was appropriate to map glucose-like metabolites and that the abundant signal at m/z 215.032 in the MALDI-IMS data should be assigned to myo-inositol. Signals for other endogenous metabolites, such as glucose monophosphate(s) (m/z 259.022) and sorbitol (m/z 217.022), were also detected and were localised predominantly to the lens cortex (data not shown).

3.2. **Use of MALDI-IMS to study glucose metabolism in organ cultured bovine lenses**

Having optimised the matrix/IS composition and its application to frozen tissue sections and established an appropriate signal normalisation method I then applied these protocols to study glucose metabolism in bovine lenses incubated in normoglycaemic AAH containing SIL glucose (Figure 3.5).
Sections underwent both low spatial resolution analysis (150 μm spot-to-spot sampling, Figure 3.5B, ii) to capture the whole lens section and high spatial resolution analysis (30 μm spot-to-spot sampling, Figure 3.5C) to test the sensitivity of the established approach. In addition to the uptake of SIL glucose into the lens, evidence for at least two metabolic pathways (Figure 3.5A) was spatially detected. SIL glucose-6-phosphate/fructose-6-phosphate ([M+Cl]− = m/z 265.042), the early metabolic intermediates in a number of metabolic processes such as glycolysis and de novo myo-inositol synthesis, were detected in the MALDI-IMS data (Figure 3.5C, i).

This signal was assigned glucose-6-phosphate/fructose-6-phosphate because existing knowledge of glucose metabolism in the ocular lens suggests that glucose-6-phosphate/fructose-6-phosphate is likely to be more abundant than glucose-1-phosphate under normal conditions (Kinoshita, 1955). In addition, SIL sorbitol ([M+Cl]− = m/z 223.068) was detected and is part of the polyol pathway, which in some species plays a pathological role in diabetic lens cataract formation by converting excess glucose to sorbitol to cause osmotic cell damage (Figure 3.5C, iii). The identity of these molecules was confirmed by both GC-MS analysis of metabolites extracted from micro dissected lenses (see Appendix A, Figures 7.1-3) and the absence of signal for these metabolites in MALDI-IMS datasets of control lenses (Figure 3.6). However, this approach was unable to distinguish between glucose-6-phosphate and fructose-6-phosphate. In addition, on-tissue MS/MS was unable to confirm the identity of the ion signal assigned glucose-6-phosphate/fructose-6-phosphate due to the lack of structural information gained when performing CID on a [M + Cl]− ion.
Figure 3.5: Detection of polyol metabolic pathway in cultured bovine lenses.

(A) Schematics of common metabolites produced by the metabolism of glucose in the lens. (B) Optical scan (i) and matrix-assisted laser desorption/ionisation (MALDI) image of stable isotopically labelled (SIL) glucose (ii) in an axial section of a lens incubated for 30 min ex vivo in SIL glucose. (C) Higher resolution MALDI images from regions marked by red box in panel Bii showing signals for (i) SIL glucose-6-phosphate (G6P)/fructose-6-phosphate (F6P) (m/z 265.042), (ii) SIL glucose signal (m/z 221.052), and (iii) SIL sorbitol (m/z 223.068).
Figure 3.6: Distribution of SIL metabolites in control bovine lenses.

MALDI images of (i) SIL glucose (m/z 221.052), (ii) SIL sorbitol (m/z 223.068), and (iii) SIL glucose-6-phosphate/fructose-6-phosphate (m/z 265.042) using RMS normalization. Each panel contains an example section from an uncultured fresh frozen lens sample (‘Fresh Frozen’, left) and a lens cultured in AAH containing regular glucose for 30 mins ex vivo (‘Incubated Control’, right). This figure demonstrates that no SIL metabolites are detected in control bovine lenses. Spatial resolution = 150 μm.
The 30 min incubation was chosen based on trial studies that have shown this duration to be optimal for detecting SIL glucose and its metabolites in the outer lens cortex. In support of this, SIL glucose was detected, along with its metabolites glucose-6-phosphate/fructose-6-phosphate and sorbitol, following the 30 min incubation period. The assignment of m/z 221.052 as SIL glucose is valid for both technical and metabolic reasons. Firstly, MALDI analysis of glucose, myo-inositol and fructose standards suggests that, in the NEDC matrix, glucose ionises with greater efficiency than myo-inositol and fructose (Figure 3.7). Secondly, my analysis showed that glucose is not readily metabolised to myo-inositol in the lens because no SIL myo-inositol was detected in the GC-MS data. However, SIL fructose levels were detected by GC–MS analysis at approximately 30% of SIL glucose levels (data not shown).

These results are consistent with previous $^{14}$C$_6$ glucose uptake studies that have shown little accumulation of radioactivity in the inositol fraction, with it remaining as glucose or being converted to sorbitol or fructose (Kinoshita et al., 1963). Therefore, while SIL fructose may be present in the incubated lenses and contribute to the signal detected at m/z 221.052 in the MALDI-IMS data, the ionisation efficiency data suggest that this signal is predominantly SIL glucose.
Figure 3.7: Comparison of ionisation efficiency of standards of SIL glucose, fructose and myo-inositol in NEDC matrix in negative ion mode MALDI-FT-ICR MS.

(A) Spectrum of myo-inositol ([M+Cl]^- = m/z 215.032) and SIL glucose ([M+Cl]^- = m/z 221.052). (B) Spectrum of fructose ([M+Cl]^- = m/z 215.032) and SIL glucose ([M+Cl]^- = m/z 221.052). Each standard was reconstituted to 1 mg/mL in water and spotted 1:1 with 7 mg/mL NEDC in 90% MeOH. In both compared cases, signal for SIL glucose is higher than for fructose/myo-inositol. These standard signals were recorded on the MTP target frame III.
3.3. Discussion

In this chapter, I have developed and optimised a tissue preparation method for MALDI-IMS analysis of glucose and its metabolites in tissue sections from the bovine lens, validated it by complementary GC–MS analysis of dissected lens regions, and used this approach to detect the uptake of SIL glucose and its subsequent metabolism in cultured bovine lenses. Firstly, matrix choice was optimised using SIL glucose. Next, an appropriate method to control for ionisation differences was tested. Application of IS prior to matrix application is a typical approach to minimise ionisation differences from different tissue regions. As a result of this routine application, large tissue cracks appeared and uneven matrix crystal formation led to poor quality MALDI images, and in this respect, the sequential IS/matrix application method was rejected. The simultaneous IS/matrix spray was deemed the best matrix application protocol to limit tissue cracking and promote matrix crystal formation. Next, tissue fixation was assessed for its efficacy in preserving both tissue morphology and ion signal by MALDI in lens tissue because some studies have reported the detection of small molecules (e.g., lipids and metabolites) from fixed tissue (Garikapati et al., 2019; Kadar et al., 2014). In the present study, fixation with PFA had a significant effect on the spatial distributions and intensities of many endogenous metabolite signals. This was not surprising because the lens contains high concentrations of small molecules, such as GSH and ophthalmic acid, which are significant contributors to the lens antioxidant defence system and, therefore, long-term tissue transparency, and their chemical fixation has been demonstrated (Lim et al., 2007).

Although the PFA fixation chemical reaction is very complicated, few published studies have considered the fundamental molecular chemical reactions that underlie the fixation process (Kamps et al., 2019; Kiernan, 2000). Previous work has demonstrated the formation of methylene bridges by the reaction of PFA with amine groups; thus, the targeted components become somewhat confined in tissue samples. In the present study, signal intensity for fixable lens small molecules, such as GSH and ophthalmic acid, was lower compared with fresh frozen tissue likely due to molecular crosslinking and
the inability of the subsequent matrix application and MALDI process to ionise these molecules (see Figure 3.3C, i–iv). Interestingly, due to fixable molecules being crosslinked together, subsequent mass spectrometry analysis can generate peaks from molecules that are less involved in the fixation process (Wisztorski et al., 2010). Indeed this seems to be the case with N-glycan imaging of fixed and embedded tissue by MALDI-IMS, where protein signal is eliminated due to chemical crosslinking, whereas glycan imaging is possible following PNGase F treatment (Drake et al., 2018). Therefore, this may explain the possible enhancement of some signals of sugar-like molecules (myo-inositol and sorbitol, see Figure 3.3C, v and vi) detected in the fixed lens preparation. A second possibility is that some non-fixable small molecules are washed out of the lens during the fixation process, which would alter the complement of signals that are detected during the MALDI-IMS analysis.

Irrespective of this mechanism, the present study aimed to develop a method to detect lens glucose uptake, transport and metabolism, and because glucose contains no amine groups and is not fixable by formaldehyde-based chemicals, I would not predict chemical tissue fixation to be amenable to glucose detection. Washout of the SIL glucose signal by treatment with a chemical fixative would be particularly detrimental to studies of lens glucose uptake because cells at the surface of the lens that have taken up SIL glucose during lens culture would be exposed to the fixative solution first and for the longest time during fixation. Dehydration-based fixation was not trialled because the SIL metabolites of interest are also soluble in EtOH/MeOH and their spatial distributions and relative abundance would, therefore, be compromised if this were used. While chloroform has been used to enhance glucose metabolite detection in brain tissue (Yang et al., 2018), any washing of the fragile lens tissue would disrupt the tissue section structure and lead to poor image quality.

In the process of optimising the data normalisation for MALDI-IMS, GC-MS was used to identify the endogenous signal at m/z 215.032. In other MALDI-IMS studies of the lens and other tissues, m/z 215.032 has been assigned to glucose (Dekker et al., 2015; Grey et al., 2019; Palmer et al., 2012; Wang et al., 2015). However, this signal could also represent other isobaric metabolites such as fructose and myo-inositol, which are also commonly found in biological tissues. GC-MS analysis showed that there
was very little free endogenous glucose present in the lens, and the signal at m/z 215.032 was predominantly myo-inositol. Myo-inositol can be synthesised de novo from glucose and is an important component of inositol phosphates, which are important secondary cell signalling messengers, and phosphatidylinositol lipids, which are a small component of lens phospholipids (Deeley et al., 2008). In addition, myo-inositol is thought to be an osmo-regulator in cultured bovine lens cells (Cammarata et al., 1994). The presence of myo-inositol in the bovine lenses in the current study is consistent with previous studies reporting myo-inositol concentrations in the lens of 7-30 mM kg⁻¹ of the wet lens (KRAUSE & WEEKERS, 1938; Pirie & van Heyningen, 1964; Van Heyningen, 1957).

The detection of SIL glucose-6-phosphate/fructose-6-phosphate was expected because these are the initial metabolites in several metabolic processes, including glycolysis, which is the main energy source for the lens OC cells because they contain both nuclei and mitochondria to facilitate this process (Bassnett & Beebe, 1992). The detection of sorbitol, albeit at low levels, was also expected, because the polyol pathway is active in many animal lenses. While the activity of aldose reductase, the enzyme responsible for converting glucose into sorbitol, is high in the lens (Kim et al., 2017; Srinivasan, 2014), the relatively low levels of sorbitol may be a result of two factors. First, these lenses were only incubated in SIL glucose for 30 min, therefore limiting the time for conversion to take place. Second, these lenses were incubated under normoglycaemic conditions, where the proportion of glucose converted to sorbitol is considerably lower (3%) than under hyperglycaemic conditions (~30%) (Bagchi & Nair, 2018).

Interestingly, some cofactors involved in these first steps of glucose metabolism, such as ATP and ADP for the conversion of glucose to glucose-6-phosphate in glycolysis, were also detected using the developed tissue preparation method with spatial resolution of 150 μm (Figure 3.8) and 30 μm (Figure 3.9), whereas others, such as NADPH for the conversion of glucose to sorbitol, were not. Previously, 9-AA has been used to detect NADPH in bovine lens tissue (Nye-Wood et al., 2016), whereas other metabolic intermediates such as pyruvate and lactate have also been detected in other tissues (Dekker et al., 2015). Therefore, it appears that for a comprehensive analysis of the metabolome by MALDI-IMS, it remains necessary to use multiple MALDI matrices.
Figure 3.8: Distribution of energy molecules in cultured bovine lenses.

MALDI images of ADP ([M-H]⁻ = m/z 426.021), and ATP ([M-H]⁻ = m/z 505.987), in lenses incubated in AAH containing regular glucose (‘Incubated Control’, left) or stable isotopically-labelled glucose (‘Incubated SIL Glucose’, right) for 30 mins ex vivo (spatial resolution 150 μm). Both (A) IS normalisation and (B) RMS normalisation are shown since it may not be appropriate to normalise an energy molecule with a sugar molecule. However, despite the normalisation method used the distribution of ATP/ADP did not change significantly, and their abundance in the outer cortex is expected since outer cortical cells contain both cell nuclei and mitochondria and are metabolically active.
Nevertheless, it is encouraging that both SIL glucose-6-phosphate and SIL sorbitol were detectable in the 30 min incubation, and in the following chapter, longer incubation times, over 20 h in normoglycemia condition will be used, which will increase the amount of glucose taken up into the lens, and the time for glucose metabolism to take place, and hence the ability to detect it by MALDI-IMS.

*Conclusion:* In summary, in this chapter, a robust MALDI-IMS method to detect and map glucose and related metabolites in the bovine lens was optimised and validated by GC-MS. This methodology promises to be a powerful spatially resolved tool to map lens nutrients and metabolites. In chapter 4, I will focus on glucose uptake in the lens in lieu of a blood supply. In addition, once glucose is taken up in the cell, I will explore how it is metabolised via different metabolomic pathways in normoglycemia condition. With the potential to extend this approach, in chapter 5, I will study the lens’ nutrient delivery pathway to understand the role of sutures and microcirculation in the lens.
Figure 3.9: Higher spatial resolution MALDI-IMS of energy molecules in cultured bovine lenses.

MALDI images of ADP ([M-H]⁻ = m/z 426.021, left) and ATP ([M-H]⁻ = m/z 505.987, right) from lenses incubated in AAH containing stable isotopically-labelled glucose for 30 mins ex vivo at 30 μm spatial resolution. (A) Displays molecular distribution based on IS normalisation and (B) shows RMS normalisation.
Chapter 4 || MAPPING GLUCOSE UPTAKE, TRANSPORT AND METABOLISM IN THE NORMAL BOVINE LENS

Having in the previous chapter established a mass spectrometry-based method to map the uptake and metabolism of glucose in a normal bovine lens, in this chapter I have used this method to characterise the spatial and temporal uptake and metabolism of SIL glucose from the bathing medium in bovine lenses maintained in organ culture for up to 20 h. MALDI-IMS showed that the initial locations of SIL glucose uptake and metabolism occurred predominantly in the epithelial and DF cells located at the lens equator. However, longer incubations of up to 20 h showed that SIL metabolites were able to penetrate to other regions of the lens, including the lens nucleus. These time courses of SIL-glucose uptake and metabolism were all validated via GC-MS. To correlate SIL glucose uptake with the presence of GLUTs, a proteomics analysis approach (GeLC-MS) was first used to verify the regional expression of GLUTs in the bovine lens. This approach detected both GLUT1 and GLUT3 in the bovine lenses, but their relative abundance changed as epithelial cells differentiated into fibre cells. Finally, an immunohistochemical labelling of GLUT1 was used to correlate areas of SIL-glucose uptake with levels of GLUT1 labelling intensity. The results of this Chapter that confirms that MALDI-IMS with SIL metabolite validation performed via GC-MS can be used to map initial locations of SIL glucose uptake and map glucose utilisation by the different metabolism pathways in the bovine lens have been published (Zahraei et al., 2022).

I will start by presenting my results that examined the use of this multi-omics approach to visualise the delivery of SIL glucose to the lens before presenting results on the uptake, and metabolism of glucose in different regions of the bovine lens.

4.1. Glucose delivery
To initially assess the pattern of glucose uptake, *ex vivo* bovine lenses were incubated in AAH containing a normoglycaemic level of SIL glucose \([^{13}\text{C}_6\text{H}_{12}\text{O}_6}\), which due to its shift in mass (+6 amu) can be detected as a *m/z* signal in the mass spectrum that is distinct from the spectrum of endogenous glucose \([^{12}\text{C}_6\text{H}_{12}\text{O}_6]\) and other isobaric metabolites. Initially, incubations from 5 min to 20 h were used to assess global uptake (Figure 4.1). A signal detected at *m/z* 221.0528 in the MALDI-IMS data was assigned as SIL glucose ([M+Cl]) since it was within 2 ppm of the predicted *m/z* of SIL glucose (Table 4.1), was not present in control lenses incubated in AAH containing unlabelled glucose (see Figure 3.6).

Closer inspection of the spatial distribution of time points up to 1 h incubation showed initial uptake of SIL glucose occurred at the equator and then spread around the entire periphery of the lens. This signal was more uniformly distributed in the lens outer cortex at 2-8 h, while at 16 h and 20 h SIL glucose signal had reduced markedly in the lens anterior region, and a very low signal was also detected in the lens nucleus. Digital dissection of three replicates of each time point into cortex and nucleus (Figure 1.4B) showed signal intensity changes for *m/z* 221.0528 were most prominent in the cortex, but the signal was also detectable in the nucleus at longer time points even though it was of low intensity relative to the cortex (Figure 4.1C). When intensity plots were normalised to the maximum signal in each region, the SIL glucose signal was present in the nucleus after 16 h of incubation in SIL glucose AAH (Figure 4.1D).
Figure 4.1: Visualisation of SIL glucose uptake in incubated bovine lenses.

(A) A series of MALDI images of axial sections taken from bovine lenses organ cultured in AAH containing 5mM SIL glucose for 5 min to 20 h showing regional differences in SIL glucose (m/z 221.0528) uptake. Control lenses incubated in normoglycaemic AAH containing non-labelled glucose showed no signal at m/z 221.0528. Lenses orientated with anterior surface to the right. (B) Schematic diagram indicating the two regions (cortex (Cx) = blue, nucleus (N) = red) from which SIL glucose signals were extracted for relative quantification and comparison. Relative intensity plots of m/z 221.0528 from each lens region showing signal increase over time normalised to maximal signal in the dataset (C), and to maximal signal within each lens region (D). The error bars represent standard error of the mean.
Table 4.1: Predicted and observed SIL metabolite m/z.

<table>
<thead>
<tr>
<th>SIL Metabolite Identity</th>
<th>Adduct</th>
<th>Predicted m/z</th>
<th>Observed m/z</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>[M+Cl]⁻</td>
<td>221.0523</td>
<td>221.0528</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>[M-H]⁻</td>
<td>265.0425</td>
<td>265.0432</td>
<td>2.6</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate</td>
<td>[M-H]⁻</td>
<td>345.0089</td>
<td>345.0106</td>
<td>4.9</td>
</tr>
<tr>
<td>Sedoheptulose-7-phosphate</td>
<td>[M-H]⁻</td>
<td>295.0531</td>
<td>295.0510</td>
<td>7.1</td>
</tr>
<tr>
<td>UDP-Glucose</td>
<td>[M-H]⁻</td>
<td>571.0678</td>
<td>571.0722</td>
<td>7.7</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>[M+Cl]⁻</td>
<td>223.0680</td>
<td>223.0686</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Because of the low signal intensity of the SIL glucose signal in the deeper lens regions, I chose to focus on the initial (5 to 30 min) uptake of glucose in the epithelium and peripheral fibre cells (Figure 4.2). After a 5 min incubation, the SIL glucose signal (m/z 221.0528) was most intense around the equatorial region, with a higher signal in the epithelial and outer cortical region just anterior to the equator. The signal intensified after 15 min, extending both posteriorly and anteriorly, and continued to spread to the entire lens periphery after 30 min (Figure 4.2A). This preferential uptake of SIL glucose at different lens surface locations was quantified in the equatorial, anterior, and posterior regions (Figure 4.2B) and average changes in intensity were plotted as a function of time for three lenses (Figure 4.2C). This analysis showed SIL glucose uptake from the surrounding media was strongest in the equatorial region of the lens, before being detected in the anterior and posterior regions of the lens (Figure 4.2C). Together these results suggested a differential affinity or rate of glucose uptake occurred in the equatorial region of the lens that consisted of equatorial epithelial cells and DF cells.
Figure 4.2: Comparison of initial glucose uptake at the surface of the lens.

(A) A series of MALDI images of axial sections taken from bovine lenses organ cultured in AAH containing 5 mM SIL glucose for 5 to 30 min showing initial differences in SIL glucose (m/z 221.0528) uptake at the surface of the lens. Control lenses incubated in normoglycaemic AAH containing non-labelled glucose showed no signal at m/z 221.0528. Lenses orientated with anterior surface to the right.

(B) Schematic diagram indicating the different regions (anterior = red, equator = blue, posterior = green) of the lens surface from which SIL glucose signals were extracted for relative quantification and comparison. (C) Intensity plots from the different regions shown in B indicate that the initial uptake of SIL glucose at the surface of the lens occurs predominately in the equatorial region, which contains both equatorial epithelial cells and elongating fibre cells, relative to the anterior and posterior regions of the lens surface. The error bars represent standard error of the mean.
4.2. Glucose uptake

The time course of SIL-glucose penetration into the lens suggests hot spots of glucose uptake exist where SIL-glucose is preferentially taken up from the extracellular space presumably via glucose transporters located in the membranes of lens fibre cells. To correlate these hotspots with the cellular location of glucose transporters I have localised GLUT transporters with immunohistochemistry but first I performed a proteomic analysis of GLUT1 and GLUT3 in the bovine lens.

4.2.1. Identification of glucose transporters

To determine whether the observed pattern of SIL glucose uptake was due to the presence of GLUTs at different quantities in different lens regions, proteomics with relative quantitation was performed on membrane protein fractions prepared from the different tissue regions (see Figure 2.5). In the first instance, tissue from epithelial flatmounts (see Figure 2.5A) and the cortical and nuclear regions (see Figure 2.5B) of the lens were sampled to determine the presence or absence of different GLUT isoforms in a non-quantitative manner. Cell membrane proteins from each preparation were separated via SDS-PAGE, and the gel band corresponding to the molecular weight of GLUTs (~53kDa) was cut (see Figure 2.7), trypsinised, and peptides separated via reverse-phase HPLC and detected via MS/MS. Interestingly, this approach detected peptides from both GLUT isoforms 1 and 3 in each lens region (Figure 4.3).

Next, to focus on further understanding, the pattern of initial SIL glucose uptake revealed by MALDI-IMS in peripheral lens tissues, tissue punches from central, peripheral, and equatorial epithelium regions were collected and compared to outer cortical fibre cells harvested from the lens equatorial region (see Figure 2.5), using the above GeLC-MS approach. GLUT1 was detected in all lens epithelial regions, and in lens fibre cells, with a combined peptide number of 11 across all regions (Figure 4.3A), confirming the previous detection in the bovine lens by western blot analysis (Lim et al., 2017). Several other proteins were detected in this band, with almost complete coverage of vimentin (data not shown), an intermediate
Figure 4.3: GLUT peptides detected in different bovine lens regions.

Amino acid sequences of bovine GLUT1 (A) and GLUT3 (B). Peptides detected by proteomic analysis are indicated in Green = Epithelium, Underline = Cortex, Italic = Nucleus, and represent a combination of some 11 and 16 peptide fragments collected from all tissue regions for GLUT1 (A) and GLUT3 (B), respectively.
filament protein that forms part of the cytoskeleton which is known to be expressed in the lens epithelium and DF cells (Sandilands et al., 1995). Consistent with the less spatially resolved sampling above, GLUT3 was also detected in all lens epithelial regions and outer lens fibre cells, with 16 peptides detected across all analysed regions (Figure 4.3B). When GLUT signals were normalised to the vimentin signal to control for different cell densities that are present in different lens epithelium regions (Wu et al., 2015), the levels of GLUT1 remained relatively constant (Figure 4.4A). In contrast, the levels of GLUT3 trended down from central to the equatorial epithelium (Figure 4.4B); however, these differences were deemed not statistically significant by ANOVA. Relative quantitation of GLUT1:GLUT3 showed that GLUT1 was less abundant than GLUT3 in all regions of the epithelium. However, in outer cortical fibre cells, that ratio reversed and GLUT1 was more abundant (Figure 4.4C).

4.2.2. Localisation of GLUTs in the outer cortex

To further confirm the presence of GLUTs in the bovine lens, and determine their subcellular location, immunofluorescence confocal microscopy was utilised. Axial lens sections were triple labelled with the cell membrane marker WGA (red), DAPI to mark cell nuclei (blue), and antibodies against GLUT1 or GLUT3 (green), and representative images of the epithelium and underlying fibre cells collected from designated regions from the anterior pole to equator (Figure 4.5A). For GLUT3, using an antibody targeting the C-terminal tail of the protein, no labelling was observed (data not shown).
Figure 4.4: Spatial proteomics of GLUTs in the lens epithelium and outer cortex.

(A) Relative quantitation of GLUT1 protein levels in the different lens epithelium and outer fibre cell regions, normalised to vimentin. (B) Relative quantitation of GLUT3 protein levels in the different lens epithelium and outer fibre cell regions, normalised to vimentin. (C) GLUT1:GLUT3 ratio in different bovine lens tissue regions. CE = central epithelium (blue), PE = peripheral epithelium (yellow), EE = equatorial epithelium (grey), PF = peripheral fibre cells (red). ns = not statistically significant as tested by ANOVA.
Figure 4.5: Immunofluorescence mapping of GLUT1 in bovine lens.

(A) Schematic diagram of lens axial section with locations of imaged regions indicated (1-4). (B-E) Cell membrane marker (red) and cell nuclei (blue) signal from marked regions in panel (A) show epithelial and fibre cell morphology. (F-I) GLUT1 immunolabelling from corresponding regions. (J-M) MALDI images of SIL glucose (m/z 221.0528) uptake in bovine lenses indicating the extent of SIL glucose uptake mediated by GLUTs in each region.
This was surprising since peptides from the C-terminus of GLUT3, the epitope for the majority of trialled GLUT3 antibodies (see Appendix A, Figure 7.8), were detected by mass spectrometry (Figure 4.4A). However, this is consistent with previous Western blot analysis that was unable to detect GLUT3 in bovine lenses (Lim et al., 2017).

In contrast, GLUT1 was detected predominantly in the cell membrane of epithelial cells at the anterior pole, with a low level of labelling in underlying lens fibres (Figure 4.5B, F). A similar pattern was detected in lens epithelial cells positioned mid-way between the anterior pole and equator (Figure 4.5C, G). In epithelial cells located in the peripheral epithelial zone, GLUT1 immunolabelling was more diffuse, and some punctate labelling was also detected in the underlying lens fibres (Figure 4.5D, H). Finally, at the lens equator in the transition zone where lens epithelial cells are differentiating and elongating to form fibre cells, pronounced cell membrane labelling for GLUT1 was detected, in addition to punctate cytoplasmic labelling (Figure 4.5E, I). MALDI-IMS of sections from bovine lenses incubated for 30 min in AAH containing SIL glucose showed uptake of SIL glucose ([m/z] 221.0528), in regions where GLUT1 immunolabelling was detected (Figures 4.5J-M).

The spatial correlation of GLUT1 labelling and SIL glucose signal suggests that GLUT1 mediates the uptake of glucose in these specific regions of the normal bovine lens cortex. However, a role for GLUT3 cannot be ruled out, and genetic tools and/or pharmacological interventions could be used in the future to determine the relative roles of GLUTs, 1 and 3, in glucose uptake in the bovine lens cortex.

4.2.3. Glucose uptake in the posterior lens

While it is clear that glucose is taken up from the aqueous humour in the anterior and equatorial lens, I was not able to determine from this set of experiments the distribution and function of GLUTs in the posterior lens. That is, does the SIL glucose signal that appears in the posterior lens (from 5 min incubation but particularly apparent from 30 min incubation, see Figure 4.1) originate from uptake in the anterior/equatorial lens and intracellular transport, or is glucose taken up directly into posterior lens cells. To determine this, I developed an in-situ glucose administration and incubation system (see section
2.3.2. Briefly, 20 µl AAH containing SIL glucose (1M) was injected into the anterior chamber of the fresh bovine eye (see Figure 2.1). Injected bovine eyes were incubated at 37°C from 30 min, to 4 h. Following incubation, eyes were dissected to extract the lens, and lenses then processed for MALDI-IMS using my optimised protocol. The exposure of SIL glucose to the anterior lens only through injection of SIL glucose into the anterior chamber, was able to determine whether SIL glucose taken up into the anterior lens was then transported to the posterior lens.

MALDI images of lenses exposed to SIL glucose via aqueous injection at the anterior surface only (Figure 4.6A) showed SIL glucose at anterior surface at all time points and no signal detected in the posterior lens. This signal was more uniformly distributed in the lens epithelium at 1-2 h, while at 4 h glucose signal had reduced significantly in the lens equatorial region, while strong signal was also detected in the lens fibre cells at the anterior pole. In comparison, MALDI images of SIL glucose in isolated lenses which were exposed to SIL glucose at both the anterior and posterior surface (Figure 4.6B) showed signal in the posterior lens at all time points.

Immunolabelling with anti-GLUT1 antibodies showed a low level of signal equivalent to anterior pole labelling levels was also detected in lens fibre cells at the posterior pole (Figure 4.7). This suggested that there were indeed functional GLUT transporters in the fibre cells of the posterior lens.

Together these results from the proteomics and immunolabelling experiments suggest that GLUTs coordinate glucose uptake into the lens. How glucose is metabolised in the different regions of the lens will now be addressed.
Figure 4.6: Posterior fibre cells can take up glucose.

A series of MALDI images of axial sections taken from bovine lenses organ cultured in AAH containing 5mM SIL glucose for 30 min to 4 h showing initial differences in SIL glucose (m/z 221.0528) uptake at the surface of the lens. (A) Whole bovine eyes in which SIL glucose was injected into the aqueous chamber and (B) isolated lenses incubated in SIL glucose.
Figure 4.7: Immunofluorescence mapping of GLUT1 in posterior bovine lens.

(A) Schematic diagram of lens axial section with locations of imaged regions indicated (1-3). (B-D) Cell membrane marker (red) signal from marked regions in panel (A) show fibre cell morphology. (E-G) GLUT1 immunolabelling from corresponding regions.
4.3. Glucose metabolism

Once taken up into the cell, glucose is rapidly metabolised to produce ATP to drive other signalling and metabolic processes and reducing equivalents such as NADH and NADPH. Since lens glucose is predominantly metabolised by glycolysis (to produce ATP), the pentose phosphate shunt (to produce NADPH), and the sorbitol pathway (see Figure 1.13), analysis of the fate of SIL glucose was performed by mapping the distribution of SIL glucose metabolites in lenses incubated for up to 20 h in SIL glucose AAH (Figure 4.8). For reference, the pattern and time course of SIL glucose throughout the lens is shown again in Figure 4.8A. In addition to this SIL glucose signal, signals for SIL glucose metabolites from each of the three metabolic pathways for glucose were detected by MALDI-IMS (Figure 4.8B-F). The identities of these signals were validated through a combination of accurate mass matching, isotopic distribution comparison (see Appendix A, Figure 7.4) and GC-MS analysis of identically incubated lenses (see Appendix A, Figure 7.5). The results for each pathway are summarised in Table 4-1 and presented below.

4.3.1. The glycolytic pathway

The initial step in glycolysis, and a number of other metabolic pathways, is the phosphorylation of glucose. After 5 min a very low signal was observed at \( m/z \) 265.0432, representing a SIL hexose-6-phosphate, which is likely glucose-6-phosphate, glucose-1-phosphate, fructose-1-phosphate, or potentially a mixture of these metabolites (Figure 4.8B). Previous GC-MS analysis supported the assignment of glucose-6-phosphate (G6P) to this signal (see Appendix A, Figure 7.3). The signal at 5 min was localised to the same region of lens epithelium that takes up SIL glucose. In longer incubations, the signal for G6P distributed to the entire epithelium (by 15 min) and then throughout the entire peripheral cortex (at time points longer than 2 h).

Finally, signal intensity was observed in the entire cortex after several hours of incubation. At 20 h, a low signal level was also detected in the nucleus. Later steps in the glycolysis pathway result in the
formation of fructose-1,6-bisphosphate (F16BP). A signal that matched the predicted m/z for SIL F16BP was detected at m/z 345.0106 (Figure 4.8C). It was detected at very low levels after 5 min of incubation and was restricted to the outer cortical fibre cells even after several hours of incubation. These relatively low signal levels may indicate either a low concentration of F16BP, its relatively transient nature in the cell before being metabolised further or that the MALDI conditions used to detect it were not optimal.

Previous reports have shown that this metabolite can also be detected by the MALDI matrices 9-AA (Dekker et al., 2015; Nye-Wood et al., 2016) and 1,5-DAN (Calvano et al., 2018; Liu et al., 2014). Therefore, in the future, more detailed mapping of glycolysis pathways in the lens may require the parallel use of multiple matrices optimised for specific metabolites.
Figure 4.8: Visualising lens glucose metabolism and transport in ex vivo bovine lens.

MALDI images from axial sections taken from bovine lenses organ cultured for varying times in AAH containing 5 mM SIL glucose. Lenses orientated with anterior surface to the right. (A) m/z 221.0528 assigned to SIL glucose. (B) m/z 265.0432 assigned SIL to glucose-6-phosphate, as a marker of glycolysis. (C) m/z 345.0106 assigned to SIL fructose-1,6-bisphosphate, as a marker of glycolysis. (D) m/z 295.0510 assigned to SIL sedoheptulose-7-phosphate, as a marker of the pentose phosphate pathway. (E) m/z 571.0722 assigned to SIL UDP-glucose, as a precursor of glycogen synthesis (F) m/z 223.0686 assigned to SIL sorbitol, as a marker of the polyol pathway.
4.3.2. The pentose phosphate pathway

In the oxidative phase of the pentose phosphate pathway, glucose-6-phosphate is metabolised to ribulose-5-phosphate, and in the process, a number of reducing equivalents (NADPH) are produced, which help protect against oxidative stress by reducing oxidised glutathione. In the non-oxidative phase, ribulose-5-phosphate is metabolised in one of two ways to sedoheptulose-7-phosphate (S7P) (Lal et al., 1995; Szwergold et al., 1995). S7P is then cycled back via fructose-6-phosphate to undergo glycolysis or can be further metabolised to erythrose 4-phosphate to be used in the synthesis of aromatic amino acids. A signal at \textit{m/z} 295.0510, matching the predicted \textit{m/z} for SIL S7P, was detected in all lenses incubated in SIL glucose (Figure 4.8D). This signal was generally limited to the most peripheral tissue regions, suggesting localisation to peripheral fibre cells and epithelium, with some stronger regions of signal generally on the lens anterior surface. At longer time points, a low signal was detected in the lens cortex, in a region where signals for both SIL glucose and SIL G6P were also detected (Figure 4.8A, B).

A signal at \textit{m/z} 571.0772 was also detected, although only at time points longer than 30 min (Figure 4.8E). This signal matched the predicted \textit{m/z} for SIL UDP-glucose, a metabolite that is a precursor to glycogen and is also involved in the synthesis of glycosphingolipids. Signal for SIL UDP-glucose was relatively uniform around the entire lens, initially appearing in the peripheral cortex (at 1 h) before increasing in intensity and spreading throughout the entire lens cortex. However, no SIL glycosphingolipids were detected, potentially due to their concentration being below the limit of detection, that their biosynthesis takes longer than the 20 h incubation period used in this study, or that MALDI sampling conditions were not optimised for glycosphingolipid detection.

4.3.3. The polyol pathway

Another major metabolic pathway present in the lens is the polyol (sorbitol) pathway, which converts glucose to sorbitol through the enzyme aldose reductase. Aldose reductase is known to be present in the bovine lens (Del Corso et al., 1989), and its activity is elevated in hyperglycaemia
(Srivastava et al., 2005). Under normal glucose conditions, a signal at $m/z$ 223.0686 that matched the predicted $m/z$ for SIL sorbitol was localised to the anterior lens surface after 30 min of incubation, indicating the formation of SIL sorbitol (Figure 4.8F). Over time, signal intensity for SIL sorbitol increased and was localised to the cortex at 8 h and longer time points. In addition, this signal was also detected in the lens nucleus at 16-20 h.

To more clearly display the SIL metabolite signals detected in the lens nucleus, a subset of the two-dimensional MALDI images presented in Figure 4.9 were re-plotted as three-dimensional surface plots, where both the colour and height of the signal represents the signal intensity. MALDI images of the six SIL metabolites detected in both 4 h and 20 h incubations were plotted (Figure 4.9A-L), and intensity plots of each signal through the lens equator were generated (Figure 4.9M, N). While it is clear the MALDI-IMS did not detect SIL metabolite signals in the lens nucleus after only 4 h of incubation, signals for SIL glucose, and SIL sorbitol in particular, were evident in the nucleus after 20 h of incubation. These results tend to suggest that SIL glucose is being delivered to the nucleus, but the levels detected are reduced due to the local metabolism of glucose to sorbitol which appears to be accumulated in the lens.
Figure 4.9: Signal intensity plots of SIL glucose and metabolites showing presence in nucleus over time.

3D intensity plots of SIL metabolite signals obtained in bovine lenses organ cultured for 4 h (A-F) and 20 h (G-L) in SIL glucose AAH. Lenses orientated with anterior surface to the right. (A, G) SIL glucose; \( m/z \) 221.0528, (B, H) SIL G6P; \( m/z \) 265.0432. (C, I) SIL F16BP; \( m/z \) 345.0166, (D, J) SIL S7P; \( m/z \) 295.0510, (E, K) SIL UDPG; \( m/z \) 571.0722, and (F, L) SIL sorbitol; \( m/z \) 223.0686. Dashed lines indicate regions used to generate signal intensity plots used to compare spatial differences in SIL metabolite accumulation after 4 h (M) and 20 h (N) of incubation in SIL glucose. Blue = SIL glucose, red = SIL sorbitol, purple = SIL G6P, yellow = SIL S7P, grey = SIL F16BP, green = SIL UDPG.
4.4. Discussion

In this study, I have used MALDI-IMS to visualise regional differences in the delivery, uptake and metabolism of SIL glucose in the normal bovine lens. Additionally, I have employed proteomic analysis (LC-MS/MS) and immunohistochemistry to validate my findings, confirming the presence of GLUT3 in the bovine lens, a result not reported previously (Lim et al., 2017). Using this multi-pronged approach, I have shown that extracellular SIL glucose initially preferentially enters the lens at the equator (Figure 4.1) in a region where the abundance of GLUT1 relative to GLUT 3 increases (Figure 4.4). Furthermore, the SIL glucose taken up from the extracellular space in these peripheral regions of the lens was available for use in the three main glucose metabolism pathways as indicated by the presence of metabolites of SIL glucose metabolism (Figures 4.8 and 4.9). At longer time points, SIL glucose and a subset of SIL glucose metabolites were detected in the deeper regions of the lens, albeit at a lower level than what was observed in the outer cortex of the lens (Figures 4.8 and 4.9), a result that is consistent with the identification of GLUT1 transporters in the nucleus of the lens (Figure 4.5) and (Lim et al., 2017). These results show that my novel approach can be used to map the delivery, uptake, and metabolism of glucose in the bovine lens and that there are differences in how glucose is metabolised in the different regions of the lens.

The results obtained in this study using MALDI-IMS are consistent with those obtained using Raman spectroscopy (Hu et al., 2015), and NMR approaches (Cheng et al., 1991; Nakamura et al., 2003; Sawada et al., 2003). In these previous studies, glucose uptake and its metabolism to sorbitol were mapped in bovine and rabbit lenses organ cultured under hyperglycaemic conditions. In bovine lenses exposed to 50 mM glucose-1-13C for up to 4 days, glucose and sorbitol were restricted to the outer cortex, with glucose levels decreasing over time following initial uptake and a corresponding increase in cortical sorbitol levels (Sawada et al., 2003). In rabbit lenses incubated in 35.5mM glucose for 28h, glucose was found throughout the lens, but was more abundant in the outer cortex, while sorbitol was abundant in the lens nucleus, and lactate was also detected (Cheng et al., 1991). While valuable, these studies suffered
from poor spatial resolution, poor time resolution, and a limited number of metabolites were detected. Using our higher resolution MALDI-IMS approach, we have also shown that glucose and sorbitol were also present predominantly in the lens outer cortex, with the accumulation of sorbitol following the depletion of glucose signal as it was metabolised by the polyol pathway, albeit in a normoglycemic model.

From the early time points of SIL glucose uptake in the peripheral epithelium and equatorial region (Figure 4.2) it appears that initial uptake occurs preferentially in a location associated with the lens equator. A similar equatorial region of enhanced uptake of radioactive cysteine was found in monkey lenses (Sweeney et al., 2003). The enhanced SIL glucose signal in this region does not appear to be due to a higher density of cells, which is a known feature of the peripheral epithelial region (Wu et al., 2015), since the proteomic data was normalised to vimentin signal to account for this. These results suggest that in this region of the lens, where epithelial cells differentiate into fibre cells that undergo extensive elongation, increased levels of glucose are required to meet the high energy demands of DNA replication and protein and lipid synthesis. The observed initial preferential uptake of SIL glucose in this equatorial region can be explained by enhanced penetration of SIL glucose into the lens due to the relative lack of tight junctions between epithelial cells in this region which has been observed for the mouse lens (Goodenough et al., 1980). Alternatively, the observed uptake distribution could be due to an increasing cell membrane area in elongating cells, which has been proposed to explain the enhanced Na⁺/K⁺ ATPase channel current observed at the lens equator relative to the central epithelium (Gao et al., 2000).

Interestingly in the present study, both GLUT1 and GLUT3 were detected by my proteomic approach, a result that was consistent with a previous study that analysed samples collected from the bovine lens using laser micro-dissection (Wang et al., 2008). In contrast, antibody-based approaches to detect GLUT3 in the current study by immunofluorescence and previously by Western blot (Lim et al., 2017) failed to detect the protein in the bovine lens. While the precise epitopes of the GLUT3 antibodies used in this study are not known, a large portion of the C-terminal tail of GLUT3, which is the region targeted by most antibodies and two of the antibodies trialled here (see Table 2.4), was detected by LC-
MS/MS (see Figure 4.3). Possible reasons that an antibody will not bind to a target sequence include inaccessibility, either through protein structure or interaction with other proteins, post-translational modification (PTM) to amino acid(s) in the epitope, such as phosphorylation, or low homology between the sequences used to raise the antibody and the target protein in the tissue. Since unmodified peptides of GLUT3 were detected via LC-MS/MS, PTM is unlikely to explain the inability of the antibody to detect GLUT3. In addition, SDS-PAGE and Western blotting, as used previously, breaks protein-protein interactions yet was unable to detect GLUT3. This suggests that the GLUT3 antibody epitope is not masked by protein binding. Finally, and most likely is that the mouse antibody may not bind with enough affinity to bovine GLUT3 due to a lack of homology between the murine and bovine sequences (see Appendix A, Figure 7.8). In comparison to GLUT1, which has significant sequence homology between mouse, human and bovine (Appendix A, Figure 7.8A), the GLUT3 sequence appears more varied between species (Appendix A, Figure 7.8B). Nevertheless, while the present study reports GLUT3 is indeed present throughout the bovine lens, GLUT1 appears to be the major isoform and it is present in both epithelial and fibre cells. This is in contrast to the rat lens, where GLUT3 was determined to be the major isoform present in fibre cells (Merriman-Smith et al., 1999; Merriman-Smith et al., 2003), but is consistent with previous studies by Lim et al., on the bovine lens (Lim et al., 2017).

Once taken up into cells, I found that SIL glucose was able to be consumed by the three main glycolytic pathways in the lens since SIL glucose metabolites from each pathway could be detected by MALDI-IMS (Figure 4.8). Unfortunately, a higher number of SIL metabolites could not be detected, possibly because of the transient nature of some metabolites and their rapid metabolism to other metabolic intermediates. Alternatively, the failure to detect other metabolites could be due to the MALDI matrix conditions used, which were optimised for the detection of glucose and its immediate metabolites, and may lack the sensitivity to detect other common endogenous metabolites in glycolysis and the citric acid cycle that could be detected by employing other MALDI matrices (Dekker et al., 2015).

While the highest signal intensities for SIL glucose were detected predominantly in the metabolically more active outer cortical regions of the lens, weak signals were also present in the lens nucleus, at longer
incubation times (Figures 4.1, 4.8 and 4.9). Furthermore, more intense signals for the metabolites SIL G6P and SIL sorbitol, relative to SIL glucose, were also detected in the lens nucleus at these longer incubation times. While the observed SIL glucose uptake pattern may represent uptake via GLUTs in the lens epithelium and outer cortical fibres, followed by passive diffusion via an intracellular pathway mediated by gap junctions (Goodenough et al., 1980), the appearance of SIL glucose and its metabolites in the nucleus within 20 h is too rapid to occur via passive diffusion alone. It has been predicted that the time required for molecules such as glucose to move into the centre of a bovine lens via simple diffusion to be more on the order of several days (Mathias et al., 1997). Thus while the current experiments cannot rule out the possibility that the observed accumulation of SIL glucose and its metabolites in the lens nucleus occurs via an intracellular pathway, the time course is more consistent with a delivery via an extracellular route as has been shown for MRI contrast agents (Vaghefi et al., 2011). In these experiments, MRI contrast agents were used as an extracellular tracer molecule to show that solutes could be delivered to the nucleus faster than is predicted via passive diffusion (Vaghefi & Donaldson, 2018) via a pathway that was localised to the lens sutures (Vaghefi et al., 2012). If indeed SIL glucose was also delivered to the lens nucleus via this extracellular sutural pathway, then the GLUTs, shown to be present in the bovine lens nucleus (Figure 4.4), would then be able to transport it into nuclear lens fibres, where the local metabolism of this SIL glucose would produce the signals for SIL G6P, and SIL sorbitol observed in our MALDI images (Figures 4.8 & 4.9).

In summary, I have developed and validated an approach to map the spatial and temporal delivery, uptake, and metabolism of glucose to different regions of bovine lens. In the next chapter I use this methodology to focus on how glucose is delivered to the lens nucleus in an attempt to distinguish between the intracellular and extracellular pathways that have been proposed to account for its delivery to the deeper regions of the lens.
Chapter 5 || THE ROLE OF LENS MICROCIRCULATION IN THE DELIVERY OF GLUCOSE TO THE LENS NUCLEUS

In the previous chapters I have shown that MALDI-IMS in combination with GC-MS analysis of micro-dissected regions of lenses incubated in SIL tracers is capable of mapping SIL glucose uptake, delivery, and metabolism in all regions of the bovine lens, including the lens nucleus. While signals for SIL glucose and SIL metabolites detected in the lens nucleus were of lower intensity and required longer incubation times to detect than those observed in the more metabolically active outer cortex, my current protocols do not provide any information on how SIL glucose was delivered to the lens nucleus. Hence, in this chapter I have investigated whether MALDI-IMS or GC-MS analysis of micro-dissected regions of lenses incubated in SIL tracers can be used to distinguish between the intracellular and extracellular pathways that have been proposed to account for nutrient delivery to the deeper regions of the lens. Despite either using lens sections orientated to visualise extracellular delivery preferentially via the lens sutures, or lenses incubated in SIL sorbitol, which acts as an extracellular tracer molecule, I was unable to visualise extracellular delivery of SIL tracers using MALDI-IMS. Nor was I able to use GC-MS methods to detect the delivery of the extracellular tracer SIL sorbitol to the lens nucleus. However, I was able to show using GC-MS that the delivery of glucose to the lens nucleus was able to be reduced by incubating lenses in the Na⁺/K⁺ ATPase inhibitor ouabain, which has been shown to inhibit the microcirculation system. Although not yet definitive these results support the hypothesis that glucose delivery to the lens nucleus occurs via an extracellular pathway and highlights the areas of further study required to fully test this hypothesis. To provide context for the results presented in this chapter a brief review of the two possible pathways that could account for the observed delivery of SIL-labelled metabolites to the lens nucleus is first provided.

5.1. Proposed pathways for delivery of solutes to the lens core
Two pathways for the delivery of glucose and other metabolites such as GSH to the lens nucleus have been proposed. One view proposed by Truscott and colleagues is based on the observation that age related nuclear cataract is initiated by a depletion of GSH levels in the nucleus, but not the lens cortex (Figure 5.1A). To explain this phenomenon Truscott proposed that the transport of GSH to the nucleus occurs by passive diffusion of GSH from the cortex via an intracellular pathway mediated by gap junction channels (Sweeney & Truscott, 1998a; Truscott, 2000b, 2005a). In support of this model, Slavi et al have showed that the lens gap junctional proteins Cx46 and Cx50 when exogenously expressed in cell lines form gap junction channels that were permeable to GSH, albeit at low levels. Furthermore, in Cx46 and Cx50 knockout mice, GSH levels in the nucleus were markedly reduced in Cx46 KO, but unaffected in the Cx50 KO, suggesting that GSH diffuses from cortical fibre cells to the nucleus via gap junction channels formed by Cx46 (Slavi et al., 2014). Truscott has proposed that with advancing age, an intracellular diffusion barrier becomes apparent in middle age, which acts to impede the intracellular diffusion of small molecules into the nucleus and that this barrier contributes to the lowered concentration of GSH observed in the lens nucleus with age (Sweeney & Truscott, 1998a; Truscott, 2000b). In this model, the network of gap junction channels supplies reduced GSH to the lens nucleus, where under conditions of oxidative stress it is oxidized to GSSG, the oxidised form of GSH. GSSG is then thought to diffuse from the nucleus to the cortex via the same gap junction pathway where it is reduced to GSH. However, GSSG has been shown not to be permeable through lens gap junction channels (Slavi et al., 2014), suggesting that the regeneration of GSH from GSSG has to occur locally in the lens nucleus.

The alternative view is that the lens uses the internal microcirculation system to deliver nutrients such as glucose and GSH via an extracellular pathway to the lens nucleus (Figure 5.1B). From here, transporters specific for GSH or glucose will uptake these substrates into the fibre cells, and in the case of glucose, supply reducing equivalents such as NADPH for GSH regeneration. In support of this model, the Molecular Vision Research Cluster (MVRC) have shown that the microcirculation can deliver solutes to the lens nucleus faster than would be expected by passive diffusion alone (Vaghefi & Donaldson, 2018), and have shown that mature fibre cells in the lens nucleus contain nutrient transporters that can
uptake the nutrients delivered via an extracellular route to the lens nucleus (Lim et al., 2017). With increasing age, it is proposed that the ability of the circulation system to deliver sufficient GSH and glucose is reduced with age, and/or that the transporters in the lens nucleus are modified leading to a reduction of GSH delivery and uptake into the lens nucleus.

As presented earlier (see Figure 1.6) the MVRC used MRI to image the penetration of MRI contrast agents (GadoSpin: GDF and FeraSpin-XS: FXS) into the lens nucleus and have shown that delivery of these extracellular space tracers occurs faster than would be expected by passive diffusion alone (Vaghefi & Donaldson, 2018). They have also shown that this delivery is driven by the microcirculation system since pre-incubating lenses in either high extracellular K⁺ (HIK) to depolarise the lens potential, or Ouabain (OUA) to inhibit the Na⁺/K⁺ ATPase inhibited the delivery of the extracellular tracer molecules to the lens nucleus (Figure 5.2). These experiments confirm that a functional microcirculation system drives solute delivery to the lens nucleus and suggest that the extracellular delivery of those solutes is mediated by the lens sutures. However, these experiments did not use physiologically relevant molecules such as glucose. Hence, I wanted to determine whether the MALDI-IMS and GC-MS based methodologies I have developed in this thesis could be used to visualise extracellular delivery of glucose to the lens nucleus and whether this delivery is driven by the microcirculation system.
Figure 5.1: Alternative views to the delivery of GSH to the lens nucleus.

A natural gradient for GSH exists in the lens with the highest (warm colours) and lowest (cool colours) concentrations being found in the outer cortex and nucleus, respectively. GSH levels in the outer cortex are maintained in young (left panels) and old lenses (right panels) by a balance of direct GSH uptake and the uptake of precursor amino acids (green arrows) for the subsequent synthesis of GSH. In the absence of GSH synthesis in the nucleus, two alternative models have been proposed to explain how GSH is delivered to the nucleus. (A) In the first model, GSH passively diffuses to the nucleus via an intracellular route mediated by gap junctions (black arrow in young lens). However, in older lenses, it is proposed that a barrier to intercellular diffusion develops (blue dotted line in old lens) which impedes the movement of GSH from its site of synthesis and regeneration in the cortex to produce the observed localised depletion of GSH in the lens nucleus. (B) In the second model, the
extracellular movement of GSH into the lens nucleus is prevented by an extracellular diffusion barrier (red dots). However, this barrier can be bypassed via the lens microcirculation system (continuous black arrows in the young lens) which acts to direct ion and water fluxes into the lens nucleus mediated via the sutures. The inflow of water along the extracellular spaces within the lens carries nutrients and antioxidants such as GSH deep into the lens and also removes waste products towards the equator via an intracellular outflow pathway mediated by gap junctions. With increasing age, the microcirculation systems declines (dotted black arrows in older lens) resulting in reduced delivery of GSH to the lens nucleus (Lim et al., 2020).
Figure 5.2: The penetration of contrast agents into the centre of the bovine lenses is reduced when the lens microcirculation system is inhibited.

A temporal two-dimensional MRI images showing the pattern of penetration into the lens of FXS (A) and GDF (B) obtained from bovine lenses pre-incubated in artificial aqueous humour (AAH), and perturbed conditions: AAH with higher K (HIK) and AAH + Ouabain (OUA) for 4 h. The figure is adapted from Vaghefi’s publication (Vaghefi & Donaldson, 2018).

5.2. Visualising extracellular delivery pathways for SIL tracers using MALDI-IMS
In an attempt to visualise extracellular delivery of more physiologically relevant molecules to the lens nucleus, I investigated two approaches. The first involved visualising SIL glucose penetration into the lens using a serial coronal sectioning approach that provided a view of the lens sutures that was different to previously presented axial sectioning and MALDI-IMS (see Chapters 3 and 4). The second involved utilising SIL sorbitol as an extracellular space tracer that has a similar size to glucose but does not cross cell membranes. The results from these two approaches to visualise the extracellular delivery route are presented in turn.

5.2.1. Visualisation of extracellular delivery of SIL glucose by MALDI-IMS

To initially assess the pattern of glucose delivery via sutures at the anterior pole, *ex vivo* bovine lenses were incubated in AAH containing a normoglycaemic level of SIL glucose. Coronal sections (Figure 5.3) were then taken from lenses incubated at three time points: 20 h, 1 h, and 30 min. I used a multiple section approach to maximise the chances that any patterning in the MALDI-IMS data that was associated with lens sutures (Figure 5.3C) would be captured. I investigated SIL glucose patterning at 20 h initially, since the results from Chapter 4 showed that SIL glucose is present in the lens nucleus at this time point. Then coronal sections were taken at 1 h and 30 min to assess the initial glucose influx at the sutures at earlier time points. Lens sections were collected through the anterior pole (Figure 5.4A-C) at 400 µm intervals. SIL glucose signal (m/z 221.0528) in the MALDI-IMS data was plotted.
Figure 5.3: Orientations of lens sections and the suture pattern in the lens poles.

This schematic demonstrates the sections taken through the lens equator resulting in (A) coronal sections, and the section taken through the anterior and posterior poles, resulting in (B) axial sections. (C) Schematic of Y-shaped sutures at the anterior pole (AP) and posterior pole (PP) of the lens.
After 20 h of incubation, SIL glucose had spread throughout the entirety of each coronal section (Figure 5.4A), although the signal was markedly reduced in the central region of sections closer to the lens nucleus. At 1 h of incubation, no signal for SIL glucose was detected in the centre of sections deeper than 1.2 mm (Figure 5.4B). The signal was more uniformly distributed throughout the periphery of each lens section. At 30 min intense signal for SIL glucose was detected in the periphery of each lens section. At this time point, only the most superficial section showed SIL glucose in its centre (Figure 5.4C, 0.4mm depth).
Figure 5.4: Exploring glucose delivery to the lens nucleus via anterior sutures.
A series of MALDI images of coronal sections taken from bovine lenses organ cultured in AAH containing 5 mM SIL glucose for 30 min to 20 h showing delivery of SIL glucose (m/z 221.0528) in different depths from the lens anterior pole. (A) 20 h, (B) 1 h, (C) 30 min with the serial sections orientated with the anterior pole on the top towards the lens nucleus on the bottom of the figure (0.4 - 2.8 mm depth), (D) 1 h, a partial MALDI image of the single section from with higher spatial resolution with a depth of 3 mm. MALDI-IMS spatial resolution of A-C: 150 μm, and D: 30 μm.
Interestingly no evidence for Y-shaped or linear patterning of the SIL glucose signal was observed. This is something that would be expected if SIL glucose signal were localised to the sutural extracellular space, which has previously been shown to accumulate gadolinium-containing contrast agent (Vaghefi & Donaldson, 2018). However, these data were collected at 150 µm spatial resolution, which may miss the relatively small sutural structures. Therefore, I analysed a section from 3 mm depth from the anterior pole of the lens, incubated for 1 h in SIL glucose AAH, at a high spatial resolution of 30 µm, to increase the chance of capturing any sutural patterning (Figure 5.4D). Still, this high-quality image did not detect SIL glucose signal in the centre of the section where the sutures are expected to be present.

Together, these data do not show evidence of preferential localisation of SIL glucose in the sutural space. This suggests that either SIL glucose is not travelling preferentially via the lens sutures, or that the spatial resolution and sensitivity of the MALDI-IMS methodology utilised is not sufficient to detect such a signal in the narrow extracellular space, despite the two different spatial resolutions used. Furthermore, the lack of sensitivity for SIL glucose in the sutural space could be due to a large proportion of SIL glucose being taken up into cells, leaving a low effective SIL glucose concentration in the extracellular space. Therefore, I decided to utilise SIL sorbitol as an alternative extracellular marker to further evaluate the ability of my MALDI IMS/GC-MS techniques to detect molecules in the extracellular space.

5.2.2. Visualisation of extracellular delivery of SIL sorbitol by MALDI-IMS

The ideal extracellular space marker should not be able to cross lens cell membranes or be transported into the cell by specific membrane proteins. Sorbitol, as a primary polyol, is relatively impermeable to lens fibres (Srivastava et al., 1982), and has a similar molecular weight (C₆H₁₂O₆ with MW of 182.17 g/mol) and structure to glucose. This made SIL sorbitol a good candidate to use as an extracellular space marker, and I hypothesised that its utilisation would allow me to determine whether SIL glucose found in the nucleus region was delivered by an intracellular or extracellular route.

To achieve this a similar experiment to that in section 5.2.2 was performed. Ex vivo lenses were incubated in normoglycaemic AAH containing 5 mM SIL sorbitol for up to 4 h. This incubation would
allow for movement of SIL sorbitol over the relatively large distances in the bovine lens (5-8 mm), through the narrow extracellular space.

A signal detected at m/z 223.068 in the MALDI-IMS, shows that the SIL sorbitol level is very low in all SIL-sorbitol incubated lenses (Figure 5.5A). The lenses exhibit low signal, with a non-symmetrical and somewhat random SIL sorbitol signal distribution which could indicate localisation to the extracellular space (Figure 5.5A, arrows). However, this level of signal could also indicate non-specific uptake into peripheral fibre cells due to cell damage induced during dissection or incubation, since the uptake pattern is not particularly symmetrical. In this figure, only one dataset is presented as an example, despite the fact that the experiment was performed with 5 replicates.

In order to determine the tissue compartment origin of the SIL sorbitol signal in periphery region in the lens, I analysed the metabolic products of sorbitol within the cells. This analysis allowed me to differentiate between the presence of SIL sorbitol due to cell membrane damage or localisation in the extracellular space. Upon uptake into the cell, sorbitol is converted to fructose through the polyol pathway using sorbitol dehydrogenase and a reducing equivalent such as NAD⁺ (see Figure 1.13). MALDI-IMS data can be used to assess whether substantial amounts of SIL sorbitol have entered the lens cells by mapping the distribution of SIL fructose at m/z 221.052 (Figure 5.5B). The presence of this signal would indicate damage to the fibre cell membrane. However, no signal for SIL fructose was observed in any of the lenses across all time points (Figure 5.5B), suggesting that the SIL sorbitol signal observed in the 1-4 h lenses is from the extracellular space.
Figure 5.5: Visualisation of SIL sorbitol uptake and its metabolism in the lens.

Two series of MALDI images of axial sections taken from bovine lenses organ cultured in AAH containing SIL sorbitol from 1 h to 4 h. (A) A signal detected for SIL sorbitol at m/z 223.068 in the MALDI-IMS, showing that the SIL sorbitol pattern from the control cultured lenses is random and non-uniform. Arrows indicate the non-symmetrical and random distribution of SIL sorbitol uptake. (B) MALDI-IMS images taken from the same lenses to show that the relative lack of the SIL sorbitol signal is not due to its intracellular conversion into SIL fructose (m/z 221.052).
In summary, the SIL tracer MALDI-IMS results presented in this chapter show that MALDI-IMS does not have the resolution to determine whether the SIL glucose signal subsequently detected in lens nucleus originates from an extracellular or intracellular delivery route. Because of the relative volumes of the two compartments, I believe that the majority of the SIL glucose signal I detect in any given region is predominately intracellular SIL glucose. Thus, while our MALDI-IMS approach can detect where in the lens SIL glucose accumulates, it cannot resolve how that SIL glucose was delivered to a specific region of the lens. To resolve extracellular delivery of SIL glucose, new generation MALDI instruments or alternative higher spatial resolution IMS approaches such as secondary ion mass spectrometry will be required (Niehaus et al., 2019; Spivey et al., 2019).

5.3. Use of GC-MS to monitor SIL tracer delivery to the lens nucleus

Since I could not visualise the route of SIL glucose delivery using MALDI-IMS I decided to see if I could use GC-MS to detect the delivery of SIL glucose to the lens nucleus at earlier time points. To achieve this, lenses were incubated in SIL tracers for varying periods of time and then micro-dissected in OC, IC, and N fractions before being subjected to a targeted GC-MS approach (selected ion monitoring) that affords higher molecular specificity and, potentially, sensitivity for the detection of the delivery of SIL tracers to the lens nucleus at earlier time points than could be obtained using MALDI-IMS. This GC-MS based approach was first utilised to assess whether the extracellular tracer SIL sorbitol and its metabolite SIL fructose could be detected in the different lens regions.

5.3.1. Delivery of SIL sorbitol to the lens nucleus

While the use of MALDI-IMS detected no SIL sorbitol or it’s metabolite SIL fructose in the nucleus region after 4 h of incubation in the extracellular tracer (Figures 5.5), I first wanted to check whether these two molecules could be detected using the GC-MS approach I developed to analyse micro-dissected regions of the lens (see Chapter 2, section 2.5). The confirmation of retention time (RT) and fragmentation spectrum in GC-MS analysis was conducted by employing the standard reference of both labelled and unlabelled sorbitol and fructose (see the Appendix A, Figure 7.6). The obtained information
was utilized to validate the identification of the SIL sorbitol and SIL fructose GC-MS signals in the incubated samples, thus ensuring the accuracy of the experimental outcomes. A signal was detected at m/z 310 (Figure 5.6A) in the GC-MS spectrum that represented SIL sorbitol. In addition to confirming the identity of the SIL sorbitol signal, signals for SIL fructose were detected by GC-MS analysis. Two chromatogram peaks were detected, representing SIL fructose, and these two peaks were merged to produce an average intensity peak to show the SIL fructose trend over time (Figure 5.6B).

Overall, when comparing the results of GC-MS with those of MALDI-IMS, both methods appear to yield similar results. Specifically, with respect to the OC region, there was low level of SIL sorbitol signal in incubated lenses at 1 and 4 h. Additionally, my GC-MS analysis detected a trace amount of SIL fructose in the OC region over 4 h, indicating the possibility of intracellular SIL sorbitol conversion to fructose in this region. However, it should be noted that the signals for both SIL sorbitol and SIL fructose were found to be very low and variable in this region, indicating the potential challenges in detecting these molecules in lens tissues.

On the other hand, neither SIL sorbitol nor SIL fructose signals were detected in the IC and N regions during the entire incubation period from 1-4 h using either MS technique. This suggests that either a) there is no extracellular delivery of lens nutrients to the nucleus, or that b) neither MALDI-IMS nor GC-MS is sensitive enough to detect a lens small molecule exclusively localised to the extracellular space. My interpretation is that these techniques lack sufficient sensitivity to detect an extracellular small molecule. Hence, in the last section I have utilised GC-MS to analyse intracellular SIL glucose accumulation in the nucleus and tested whether its delivery and accumulation can be modulated by inhibiting the microcirculation system with ouabain.
Figure 5.6: GC-MS trend of SIL sorbitol and SIL fructose signals.

The GC-MS data were taken from bovine lenses cultured in AAH containing SIL sorbitol from 1-4 h in these different regions: OC, IC, and N. The (A) upper and (B) lower panels of the figure display the intensity of GC-MS responses of SIL sorbitol and SIL fructose, respectively. The error bars represent standard error of the mean.
5.3.2. Delivery of SIL glucose to the lens nucleus

*Ex vivo* bovine lenses were incubated in AAH containing a normoglycaemic level of SIL glucose ($^{13}$C$_6$H$_{12}$O$_6$) for up to 4 h. This timepoint was chosen as an end timepoint since it would allow the assessment of the initial uptake pattern, and rate. Additionally, previous MRI-based experiments (Vaghefi & Donaldson, 2018) showed that MRI contrast agents were able to reach the bovine lens nucleus within 1 h under normal physiological conditions (see Figure 5.2). Incubations from 30 min to 4 h were used to assess uptake.

Incubated lenses were manually dissected into OC, IC and N regions. Following GC-MS sample preparation and analysis, a signal was detected at m/z 162 in the GC-MS spectrum, representing SIL glucose. The confirmation of retention time (RT) and fragmentation spectrum in GC-MS analysis was carried out using the standard reference of both labelled and unlabelled glucose (see the Appendix A, Figure 7.5). This information was then utilized to validate the identification of the GC-MS signal for SIL glucose in incubated samples.

The results obtained from the GC-MS analysis reveal that there was a marked increase in the SIL glucose signal in all three regions, from 0 to 1 h. The increase was particularly noticeable in the OC (Figure 5.7A) and IC (Figure 5.7B), while the nucleus showed a comparatively lower increase (Figure 5.7C). To better compare the rates of SIL glucose delivery to each lens region, data from each region were plotted to their own maxima (Figure 5.7D). The initial detection of SIL glucose in OC and IC regions was similar, while its detection in the nucleus was slower (shown by asterisk), as would be expected. At later time points, each region reached a peak value of SIL glucose signal, which was around 2 h. Subsequently, there was a small drop in the OC SIL glucose signal, although this decrease was not found to be statistically significant, but could reflect metabolism of the SIL glucose to downstream metabolites. Similarly, the IC and N regions also showed a decrease in SIL glucose signal, albeit to a lesser extent. Importantly, the statistical analysis results showed a significant difference in the pattern of glucose uptake in SIL-incubated lenses compared to the control lenses cultured in AAH containing...
unlabelled glucose under similar incubation timescale, as indicated by a t-test, (see Appendix A, Table 7.2).

**Figure 5.7: Delivery of SIL glucose to the different region of lens (m/z 162).**

GC-MS data were collected from bovine lenses cultured in AAH containing SIL glucose for 30 min to 4 h in different lens regions. (A) OC, (B) IC, and (C) N. (D) Comparison of SIL glucose delivery to each region, shown as lines representing the intensity of GC-MS response for SIL glucose detected at m/z 162. The red, blue, and green lines represent the SIL glucose signal in the OC, IC, and N regions, respectively. The brown line shows the response of SIL glucose in control samples incubated with non-labelled glucose. The y-axis scales in each panel (A, B, and C) are different. The number of samples for SIL glucose/Ouabain is 5, and for SIL glucose and control is 3. The error bars represent standard error of the mean.
If the bovine lens is modelled as a 3D sphere then it would take a minimum of 7 h for a molecule like glucose to reach the centre of the sphere (Vaghefi & Donaldson, 2018). However, in my experiment a signal for SIL glucose was observed in the nucleus region within 1-2 h of placing lenses in a media containing SIL glucose. This rapid early detection of SIL glucose by GC-MS indicates that the pathway delivering SIL glucose to the lens nucleus does not rely on passive diffusion. This observation supports the results obtained by Vaghefi et al., as depicted in Figure 5.2, who used MRI contrast agents to show that the internal microcirculation system in the lens delivers extracellular solutes to the lens nucleus (Vaghefi & Donaldson, 2018). Furthermore, Vaghefi et al were able to block MRI contrast agent delivery to the lens nucleus by incubating lenses in ouabain to block the Na\(^+/\)K\(^+\)ATPase that generates the lens microcirculation system.

Therefore, I performed further experiments to assess the effect that ouabain treatment would have on SIL glucose delivery to the lens nucleus. Lenses were first pre-incubated with unlabelled AAH containing 1 mM of ouabain (see Table 2.1) for 4 h, which would allow ouabain to inhibit the Na\(^+/\)K\(^+\) ATPase, and therefore stop the lens microcirculation. Following this initial perturbation, lenses were incubated in AAH containing a normoglycaemic level of SIL glucose (\(^{13}\)C\(_6\)H\(_{12}\)O\(_6\)) for up to 4 h, in the presence of 1 mM ouabain to maintain microcirculation blockade.

Results from GC-MS analysis of the microdissected lens nuclear regions showed signal for SIL glucose was above background (Figure 5.8, green) after just one hour of incubation, suggesting that SIL glucose reached the bovine lens nucleus in this timeframe (Figure 5.8, blue). Interestingly, I observed a small decrease in the rate of SIL glucose delivery into the nucleus region upon treatment with ouabain (Figure 5.8, red). Although the observed differences between normal and ouabain-treated lenses were not statistically significant (see Appendix A, Table 7.3), this small change suggests that blocking Na\(^+/\)K\(^+\) ATPase partially disrupts the delivery of SIL glucose to the lens nucleus.
Figure 5.8: Delivery of SIL glucose to the lens nucleus (m/z 162).

Two series of GC-MS data were taken from bovine lenses cultured in AAH containing SIL glucose from 30 min to 4 h in the nucleus region. Red line: SIL glucose/ouabain (lenses were perturbed with 1 mM Ouabain for 4 h), blue line: SIL glucose, and green line: control samples (incubated with non-labelled glucose). This data shows the intensity of GC-MS response of SIL glucose detected at m/z 162. Number of samples in SIL glucose/Ouabain = 5 and in SIL glucose and control= 3. The error bars represent standard error of the mean.
5.4. Discussion

In this chapter, I investigated the structural and functional aspects of the lens microcirculation to test the roles that these features play in delivery of glucose to the lens nucleus. First, MALDI-IMS was applied to evaluate the delivery of glucose to the lens nucleus via sutures at the anterior pole (see Figure 5.4). MALDI-IMS mapped SIL glucose through serial coronal sections at both low (150 µm) and high spatial resolution (30 µm). However, no suture structures were detected in sections (at any depth), despite the two different spatial resolutions applied (Figure 5.4A-D). While this suggests that SIL glucose does not travel via the sutures, I then showed that my MALDI-IMS approach was unable to detect an exclusively extracellular space tracer molecule, such as SIL sorbitol, due to a lack of sensitivity and/or spatial resolution. Hence, our inability to detect SIL glucose or sorbitol in sutural structures does not exclude the possibility that glucose and other nutrients are delivered to the nucleus via an extracellular pathway associated with the sutures. To resolve this, different approaches and technologies that increase the sensitivity in my MS methods will be required and these possibilities are discussed in Chapter 6.

Using GC-MS, I demonstrated that SIL glucose could be first detected in the lens nucleus after 1-2 h, much faster than the 16 h detected by MALDI-IMS (see Chapter 4). The speed at which SIL glucose could be detected in the nucleus tends to rule out the possibility that it is being delivered by purely passive diffusion whether it be via an extracellular or intracellular delivery route. Instead, the time course of detection of SIL glucose in the nucleus is more similar to the delivery of MRI contrast agents to the nucleus, whose delivery to this region of the lens were shown to be driven by the microcirculation system (Vaghefi & Donaldson, 2018).

Moreover, my GC-MS results suggest that the SIL glucose detected in the lens nucleus was located in the cytoplasm of mature fibre cells since my GC-MS analysis of SIL sorbitol-incubated lenses did not detect this extracellular marker in the lens nucleus. This suggests that the glucose delivered via the extracellular space is taken up by the GLUTs shown to be expressed in the mature fibre cells of the lens nucleus (Figures 4.5 and 4.7). A role for the microcirculation in the delivery of SIL glucose to the
nucleus is also supported by the finding that ouabain partially blocked the delivery of SIL glucose (Figure 5.8). Interestingly, the delivery of SIL glucose was not completely abolished by ouabain. While this could be due to an incomplete block of the Na\(^+\)/K\(^+\) ATPase activity, the concentration of ouabain used was the same as that used to completely abolish the delivery of extracellular MRI contrast agents to the nucleus (Vaghefi & Donaldson, 2018). This suggests that there may be a component of this delivery mechanism that is not sensitive to ouabain, and may be driven by an osmotic component due to the high concentration of crystallin proteins found in the lens nucleus. Further experiments would be required to test this hypothesis.

In summary, while the results of this chapter do not definitively prove that the delivery of glucose to the lens nucleus occurs via the extracellular space, they show that the delivery occurs at a rate that is consistent with the involvement of the microcirculation system and set the stage for further methodological improvements to visualise nutrient delivery to the lens nucleus. These improvements and the wider implications of my results are discussed in the next chapter.
The overall goal of my PhD project was to develop methods to study glucose delivery, uptake, and metabolism in the different regions of the lens. To achieve this, in Chapter 3 I first developed and optimised MALDI-IMS as a tool to identify areas in the lens where glucose and its metabolites are accumulated when bovine lenses were incubated in SIL glucose, before validating the identity of these signals using GC-MS. This combined approach of MALDI-IMS and GC-MS was then used in Chapter 4 to localise glucose uptake, transport, and metabolism in different regions of cultured bovine lens. Finally, in Chapter 5, I investigated whether my new methods to study glucose delivery, uptake and metabolism could be used to distinguish between the current models of intracellular and extracellular delivery of glucose to the lens nucleus (Figure 5.1). While the results presented in Chapters 3 (Zahraei et al., 2021) and Chapter 4 (Zahraei et al., 2022) have been published, the results obtained in Chapter 5 were not able to provide sufficient resolution to distinguish between the two delivery pathways proposed to deliver glucose to the nucleus of the lens. Hence, in this final Chapter, I will not only discuss the significance of the results obtained by the novel methods I have developed, but also potential improvements to these methods which, in combination with new experimental protocols, could be used to confirm how glucose and other metabolites are delivered to the lens nucleus. However, I will start this discussion by first providing a summary of the key findings to date of my PhD project.

6.1. Summary of key findings


In this chapter I documented the development and optimisation of the MALDI-IMS approach to spatially detect glucose and its metabolites in lens incubated in SIL glucose. I found that:
• NEDC is the most appropriate choice of MALDI matrix to maximise glucose signal level and minimise background ions

• The developed MALDI-IMS methodology can detect both endogenous and SIL lens metabolites introduced by *ex vivo* lens culture.

• To detect glucose and glucose metabolites, non-fixed, fresh-frozen tissue should be used.

• The simultaneous spray application of an internal standard/matrix is the most effective matrix application protocol for limiting tissue cracking and increasing matrix crystal formation.

• Normalisation based on an internal standard (3-OMG) is the best IMS data normalisation method to accurately depict glucose and glucose metabolite distributions in the bovine lens.

• The identity of metabolites mapped with MALDI-IMS was confirmed by performing GC-MS.

This work has been published in *Journal of Mass Spectrometry* (Zahraei et al., 2021).

### 6.1.2. Summary of Chapter 4: Mapping glucose uptake, transport and metabolism in the normal bovine lens

In this chapter I used the approaches developed in Chapter 3 to map the delivery, uptake and metabolism of glucose and its metabolites in different regions of the bovine lenses incubated in SIL glucose. I found that:

• The major site of glucose uptake in the lens is the germinative and equatorial regions.

• Glucose metabolism takes place mainly in the epithelial and cortical fibre cells in comparison to the central fibres.

• Glucose uptake is mainly performed by GLUT1 and GLUT3 isoforms.

• GLUT1 is the predominant isoform in lens fibres, GLUT3 is the predominant form in the lens epithelium.

• The pattern of glucose uptake is spatially correlated to GLUT distributions in the bovine lens.

• Posterior lens fibre cells can also take up glucose directly.

• The main metabolic pathways, glycolysis, polyol pathway and pentose phosphate pathway, were spatially mapped.
In this Chapter I wanted to determine if the protocols I have developed could be used to determine the pathway(s) that delivered glucose and its metabolites to the nucleus of the lens. I found that:

- Glucose is delivered to the lens nucleus faster than expected by passive diffusion.
- Ouabain treatment has a small effect on the delivery of glucose to the nucleus of the lens.
- MALDI-IMS of SIL sorbitol is not effective in providing the necessary level of detail to visualize the lens extracellular space.
- The current methodology cannot differentiate between extracellular and intracellular glucose delivery.
- It is challenging to determine if the rapid glucose delivery is mediated by the extracellular space/water microcirculation.

Overall, the work in this chapter can be considered as preliminary data that suggests further investigation is needed to advance our knowledge about the role of microcirculation in the delivery of glucose to the lens nucleus. While not definitive in determining the pathway used to deliver glucose to the lens nucleus, this set of experiments confirmed that the appearance of SIL glucose in the nucleus occurs faster than would be expected by passive diffusion alone and that its delivery was reduced by the inhibition of the lens microcirculation with ouabain.

In summary, during my PhD project I have developed a suite of methods and protocols that monitor the delivery, uptake, and metabolism of glucose in the different regions of the lens. In the next sections I first discuss the general significance of my results to knowledge of how glucose is utilised in different regions of the lens to maintain the transparent and refractive properties of the normal lens,
before discussing how my methods and protocols can be used and modified to further increase our understanding of how those properties are compromised in diabetic and age-related nuclear cataract.

6.2. Regional differences in glucose delivery, uptake and metabolism in the bovine lens.

My use of spatially-resolved mass spectrometry techniques, MALDI-IMS, GC-MS, and LC-MS, in combination with immunohistochemistry have allowed me to map glucose and its downstream metabolites in the normal lens and their localization in different regions of the organ cultured bovine lens. This approach revealed regional differences in the delivery, uptake, and metabolism of glucose within the outer cortex itself and between the outer cortex and the deeper nuclear regions. Based on my results, I propose a new model for glucose delivery, uptake and metabolism (Figure 6.1). In the following sections I will discuss how my model compares and contrasts with known lens biology, physiology and function.
Figure 6.1: A model for glucose delivery to the outer cortex and nucleus of the lens.

Glucose concentration in the lens varies naturally, with the highest concentration in the outer cortex (warm colours) and the lowest concentration in the nucleus. (A) In the outer cortex glucose is actively taken up (solid green arrows) by GLUTs in the epithelial layer and outer cortical fibre cells. Initial uptake occurs in the germinative region and lens equator. (B) Glucose is delivered to the nucleus via the sutures (gradient green arrow) through extracellular movement facilitated by the lens microcirculation system (continuous black arrows). It is then taken up into cells via functional GLUTs in the nuclear fibres. Extracellular movement can be impeded by an extracellular diffusion barrier (red dots), which is bypassed by the lens sutures.
6.2.1. Glucose delivery, uptake and metabolism in the lens outer cortex

In Chapter 4, I showed that SIL glucose first enters lens cells located in the equatorial region and germinative region of the epithelium (Figure 6.1A, *large green arrow*). Several factors are likely to determine this pattern of uptake, such as the extracellular delivery route and presence of tight junctions in different lens regions, the density of glucose transporters in lens cells, the localisation and activity of enzymes that metabolise glucose, and the metabolic demand of different lens cell populations. The uptake pattern I observed is consistent with the known biology of the lens, in which the central epithelium contains relatively quiescent cells, while the more peripheral epithelium contains a population of cells that proliferates, migrates, and elongates into fibre cells at the lens equator. The metabolic demand of these cells is high in order to provide sufficient energy to support the high level of protein and lipid synthesis that drives fibre cell elongation. Both the epithelial cell population and the outer cortical fibre cells maintain cell nuclei and mitochondria and are therefore capable of aerobic metabolism and the efficient production of ATP from glucose via the mitochondrially-localised TCA cycle. While I was able to correlate the localisation of GLUTs to glucose uptake, the role that tight junctions may play in directing cortical lens glucose uptake, and the activity levels of different enzymes are potential areas for further investigation. MALDI-IMS could be trialled to investigate enzyme level activity (Hamilton et al., 2020), while tight junction distributions would likely require alternative approaches such as proteomics and immunohistochemistry.

My current approach could also be used to further investigate glucose uptake in different lens cortical regions and compare to any intra- and intercellular glucose movement. For example, when I exposed the anterior lens only to SIL glucose, via injection of SIL glucose specifically into the anterior chamber (see Chapter 4), I observed uptake in the anterior lens and no evidence of transport into the posterior lens, suggesting that GLUTs in the posterior lens fibres were also functional. This study could be extended by utilising the 3-chamber Ussing chamber design introduced by Candia et al (Candia et al., 2012), which separates the lens into anterior, posterior and equatorial compartments, to sequentially
expose each isolated lens region to SIL glucose and then monitor its uptake and metabolism in the different lens regions.

6.2.2. Glucose delivery, uptake, and metabolism in the lens nucleus

As outlined in Chapter 5 (5.1) the transport of GSH and other metabolites like glucose to the lens nucleus has been a topic of debate in the field. On one hand, Truscott et al., propose that the transport occurs by passive diffusion of GSH from the cortex to the nucleus via an intracellular pathway mediated by the gap junctional proteins Cx46 and Cx50 (Sweeney & Truscott, 1998b; Truscott, 2005b). However, others have pointed out that due to the large distances over which diffusion has to occur that passive diffusion would not be fast enough to deliver adequate levels of metabolites to the lens nucleus to maintain the reduced oxidative state required to maintain the solubility of the high concentration of crystallin proteins in the lens nucleus (Donaldson et al., 2010).

In support of this notion, I have shown that SIL glucose and SIL metabolites can be first detected in the lens nucleus within 2 h of being placed in bathing solution that contains SIL glucose (Figure 5.7C). Based on models of diffusion into a sphere the size of a bovine lens it has been predicted that it would take glucose 7 h (see Figure 1.6C) to reach the lens nucleus (Vaghefi & Donaldson, 2018). This simple model is also probably an underestimate as it does not consider the consumption of glucose by peripheral metabolically active DF cells. Hence my detection of SIL glucose and SIL metabolites in the lens nucleus after 2 h shows that the delivery of metabolites to the nucleus occurs a lot faster than what would be predicted by passive diffusion alone. Furthermore, the detection of SIL labelled metabolites (Figure 4.8) tends to suggest that once delivered to the nucleus glucose can be locally metabolised. This is consistent with known lens function, where the metabolic activity in the lens nucleus is low but not zero (Hejtmancik et al., 2015). My results suggest that once glucose reaches the lens nucleus, GLUTs in the fibre cell membranes can take up glucose (Figure 4.4), which is then metabolised locally via anaerobic metabolism pathways (Lim et al., 2017). The energy produced can then be used to replenish, for example, co-factors such as NADPH that are utilised in the local regeneration of the antioxidant GSH.
However, while my data suggests SIL glucose is delivered to the nucleus faster than is predicted by a model of passive diffusion my results on the mechanism driving the delivery and whether this delivery occurs via an intracellular or extracellular route is less definitive. I was able to show that the rate of delivery of SIL glucose to the nucleus could be slowed but not completely inhibited by pre-treatment of lenses with the Na⁺/K⁺ ATPase inhibitor ouabain (Figure 5.8), which has been shown to abolish the extracellular delivery of solutes driven by the lens microcirculation system (Vaghefi & Donaldson, 2018). This result tended to indicate that glucose delivery to the lens nucleus is facilitated by the lens microcirculation and that glucose travels via the extracellular sutural space (Figure 6.1B, gradient arrows). However, I was unable to definitively confirm this working model as I was unable to confirm using my imaging mass spectrometry approaches whether SIL glucose and its metabolites are delivered to the nucleus via an intracellular or extracellular route. Future experiments designed to distinguish between these two pathways to validate my model of glucose delivery to the nucleus are discussed in the next section.

6.3. Proposed improvements to experimental approach to resolve intra- and extracellular delivery pathways

A major limitation of this work was the lack of spatial resolution and sensitivity to detect extracellular molecules in incubated lenses. To overcome the limitations of the current approach, either changes to the experimental protocol or other advanced MS-based technologies could be utilised in this study, such as SIMS and MALDI-2 (Niehaus et al., 2019; Soltwisch et al., 2015), which provide improved detection limit and higher spatial resolution. In the first instance, SIL mannitol (Diecke et al., 1995) or sucrose (Tripathi & Bhatnagar, 1987; Vincent et al., 2021), which are more impermeant to the cell membrane than sorbitol, could be trialled as extracellular space markers. Using either of these compounds at a higher concentration than the 5mM level used in the current study could act as an effective marker for extracellular transport of physiologically relevant small molecules.

Alternatively, utilising different mass spectrometry instrumentation could help elucidate intra- and extracellular pathways. Leading commercially available imaging mass spectrometers are capable of
5 \mu m lateral resolution. However, at this scale, tissue preparation strategies to better preserve tissue morphology become increasingly important. In addition, sensitivity can suffer with the decreased laser irradiation spot size, therefore improvements in detection sensitivity are important. In recent years, the development of MALDI-2 has expanded the capabilities of MALDI and increased its sensitivity for detecting low-abundance molecules. MALDI-2 works by using two lasers: one to desorb the sample and another to ionize it. This dual laser approach has been shown to produce higher ion yields and improved sensitivity compared to traditional MALDI (Soltwisch et al., 2015). This technology has the potential to enable MALDI-IMS with higher sensitivity and high lateral resolution in the low micrometre range (Barré et al., 2019).

SIMS (Benninghoven, 1973) is a technique capable of imaging tissues, single cells, and microbes revealing chemical species with sub-micrometre spatial resolution. While SIMS has several advantages, it also has certain limitations. For instance, the ability to identify secondary ions has been limited due to limitations of the instrumentation, such as the lack of fragmentation capabilities and low mass resolution and accuracy (Smith et al., 2011). This can impact the accuracy of fragment SIL ion detection. Additionally, SIMS also requires specialized training and sample preparation procedures, which may limit its accessibility to researchers.

The Fourier transform-based mass spectrometry technology, SIMS FTICR, instrument provides high mass resolving power and mass accuracy (Smith et al., 2011). However, this technology is relatively new, and therefore, relatively less accessible to researchers, compared to other MS-based technologies. An alternative is ToF-SIMS (Fisher et al., 2016), which can generate chemical maps with an order of magnitude better lateral resolution than the FTICR-SIMS, and the NanoSIMS (Behrens et al., 2012; Levenson et al., 2015) instrument offers sub-100 nm spatial resolution in chemical imaging. Many commercial ToF-SIMS instruments are also capable of depth profiling that allows three-dimensional reconstructions of cell and tissue structure.
6.4. Applying the MALDI-IMS and GC-MS experimental approach to new areas of investigation

In this thesis I have outlined my development, optimisation, and utilisation of a spatially and temporally resolved mass spectrometry approach to visualise the delivery, uptake and metabolism of glucose in normal bovine lenses. In this final section I will discuss how my experimental approach can be potentially utilised in future work to increase our understanding of how the metabolic requirements of different lens regions are met to maintain the overall transparent and refractive properties of the lens and how those requirements are compromised in diabetic and age-related nuclear cataract.

6.4.1. Dissecting glucose uptake and metabolism pathways in the normal lens

In this study I have determined the presence and cellular localisation of GLUT isoforms in the bovine lens. However, confirming their functionality in each lens region is important to further define the delivery of lens nutrients. An inhibitor such as Glutor, which has been mainly utilised to inhibit both GLUT1 and GLUT3 isoforms (Reckzeh et al., 2019; Temre et al., 2022), would be suitable to determine between extracellular and intracellular glucose transport. This would be particularly relevant to uptake in the lens cortex. In the future, utilising a combination of GLUT inhibitors and ouabain treatment to disrupt the microcirculation could be integrated with my spatial multi-MS approach to investigate the role of the microcirculation in the delivery of glucose to the lens nucleus.

Metabolic flux analysis is a crucial component in the study of cellular metabolism, providing insight into the dynamic nature of metabolic pathways and their regulation. This study has utilized GC-MS data to confirm the detection of selected internal standards (SIL) of glucose, sorbitol, glucose-6-phosphate (G6P), and fructose. However, the limited number of SIL metabolites detected, which was primarily based on the observation of SIL fragment ions, is insufficient for a comprehensive analysis of metabolic flux. One of the challenges encountered during this study was the potential overlap between SIL fragment ions and isotopic peaks from endogenous metabolites, which limited the confidence of metabolite assignments. The limitations of the current chromatography, instrument sensitivity, and mass
resolution also contributed to the lack of additional SIL metabolites detected. To advance the understanding of metabolic flux in the lens, future studies may require the use of additional instrument runs and comparison with metabolic standards. The utilization of various organic matrices and on-tissue chemical derivatization reagents to enhance the performance of MALDI-IMS has been discussed recently. These techniques, which have shown promise in improving the analysis of biological samples, can potentially be applied in the lens to enable the detection of a greater number of metabolic intermediates. By implementing these approaches, it may be possible to uncover additional metabolic pathways within the lens, leading to a more comprehensive understanding of its metabolic activity (Zhou et al., 2021).

6.4.2. Studying metabolic changes associated with the development of diabetic cortical cataract

My experimental approach could not only be used to gain insights into the normal functioning of the lens, but also to evaluate the altered metabolic profile in different regions of lenses with diabetic cataract. In diabetes, the elevated level of glucose is metabolised through the polyol pathway in the lens, producing sorbitol and fructose. In addition to the osmotic challenge caused by elevated intracellular sorbitol, elevated lens glucose leads to a decrease in GSH and an increase in ROS (Chan et al., 2008; Lee & Chung, 1999). The induced osmotic and oxidative stress work synergistically to inhibit the ability of fibre cells to regulate their volume. This leads to cell swelling and a loss of the ordered fibre cell architecture that promotes lens transparency in the outer cortex. In addition, proteins are thought to be modified by the formation of advanced glycation end (AGEs) products, which alter the structure and function of crystallins to induce the formation of high molecular weight aggregates, and cataract. In the laboratory, exposure of the lens to hyperglycaemia is typically used as a model of diabetes. My spatially resolved mass spectrometry technique holds great promise for resolving metabolic changes in a diabetic lens model. Our approach could be used to investigate the impact of hyperglycaemia on glucose metabolism in different regions of the lens and to gain a deeper understanding of the metabolic changes that occur in a diabetic lens model.
To test the feasibility of this methodology, I have already performed a set of pilot experiments. Lenses were incubated in AAH containing elevated glucose (50 mM, C₆H₁₂O₆) for 24 h. Initial MALDI-IMS showed evidence of sorbitol and GSH accumulation in the OC region of the lens incubated under hyperglycaemia in comparison to lenses incubated under normal physiological conditions. The signals for sorbitol (See Appendix A, Figure 7.10C) and GSH (See Appendix A, Figure 7.10D) were both elevated in all three regions of the lens under hyperglycaemia conditions compared to lens incubated in normal glucose. The elevation of GSH may be evidence of an attempt to counter the oxidative stress introduced by the high glucose levels. This pilot experiment provides strong evidence for the feasibility of our proposed methodology and lays the foundation for future studies to further explore the metabolic changes in diabetic lenses using spatially resolved mass spectrometry techniques.

6.4.3. Studying metabolic changes associated with the development of age-related nuclear cataract

Age-related nuclear (ARN) cataract is a type of cataract that affects the central core of the lens. It is characterized by protein oxidation, protein aggregation, and light scattering (Lim, Umapathy, et al., 2016; Truscott, 2005b). The precise mechanisms underlying the development of ARN cataract are not fully understood, but oxidative stress is thought to play a major role (Reddy, 1990). The human lens has a robust defence system to protect against oxidative damage that utilises GSH as its principal antioxidant. However, GSH levels decline with age, particularly in the lens nucleus. This decline in GSH levels is thought to be due to several factors, including decreased synthesis, increased consumption, and impaired recycling. The decline in GSH levels in the lens nucleus leads to increased protein oxidation and protein aggregation, changes that ultimately manifest as ARN cataract in the elderly (Truscott, 2000a). Therefore, in addition to studying the metabolic changes associated with diabetic cortical cataract, this method can be used in the future to assess the effect of exposing the lens to oxidative stress on protein and metabolite modifications in different regions of the lens. This mass spectrometric technique holds great potential for spatially resolving metabolite changes in an age-related cataract model. In particular, the method could be used to investigate the effect of oxidative stress on GSH, one of the key metabolites...
involved in lens protection, which is a powerful antioxidant that scavenges reactive oxygen species. An oxidative stress model has previously been developed using \textit{ex vivo} bovine lenses exposed to hyperbaric oxygen (HBO). This model appears to mimic some of the changes associated with lens aging, although an overt cataract is not observed (Lim, Vaghefi, et al., 2016). While MALDI-IMS has been used previously to analyse GSH in HBO-treated lenses (Nye-Wood et al., 2016), this analysis can now be expanded to also study the underlying changes to glucose metabolism that occur with lens aging and cataract formation using the HBO model. This approach could ultimately resolve the question of how the normally robust oxygen radical scavenger systems present in the lens fail with advancing age and initiate the observed protein aggregation specifically in the nucleus of the lens. Such experiments are a first step in developing effective antioxidant based therapies to delay the onset of cataract.

In summary, I have developed a consolidated research pipeline to study spatially and temporally resolved metabolomics in the lens. By using this approach, the uptake, transport, and metabolism of glucose in the lens can be investigated under normoglycemia condition. This method promises to be a powerful tool to study metabolic changes in normal lenses and in models of the major forms of lens cataract. In the future I hope this approach could be extended to study the uptake, transport, and metabolism of novel anticataract therapeutic molecules.
Table 7.1: Putative identifications of small molecules from MALDI IMS of bovine lenses

<table>
<thead>
<tr>
<th>Category</th>
<th>ID</th>
<th>Adduct</th>
<th>Predicted m/z</th>
<th>Observed m/z</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar-related</strong></td>
<td>Glucose/Fructose/Myo-inositol$\dagger$$\ddagger$</td>
<td>[M+Cl]$^-$</td>
<td>215.0322</td>
<td>215.0323</td>
<td>0.5</td>
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<tr>
<td></td>
<td>Sorbitol$\ddagger$</td>
<td>[M+Cl]$^-$</td>
<td>217.0478</td>
<td>217.0482</td>
<td>1.8</td>
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<tr>
<td></td>
<td>3-O-methylglucose</td>
<td>[M+Cl]$^-$</td>
<td>229.0478</td>
<td>229.0479</td>
<td>0.4</td>
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<tr>
<td></td>
<td></td>
<td>[M-H]$^-$</td>
<td>193.0717</td>
<td>193.0709</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>[M-H]$^-$</td>
<td>341.1084</td>
<td>341.1082</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Disaccharide$\dagger$$\ddagger$</td>
<td>[M+Cl]$^-$</td>
<td>377.0856</td>
<td>377.0851</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>SIL molecules</strong></td>
<td>SIL Glucose$\ddagger$</td>
<td>[M+Cl]$^-$</td>
<td>221.0523</td>
<td>221.0528</td>
<td>2.3</td>
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<td></td>
<td></td>
<td>[M-H]$^-$</td>
<td>185.0762</td>
<td>185.0768</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>SIL Sorbitol$\ddagger$</td>
<td>[M+Cl]$^-$</td>
<td>223.068</td>
<td>223.0682</td>
<td>1.1</td>
</tr>
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<td></td>
<td>SIL Glucose-6-Phosphate$\ddagger$</td>
<td>[M-H]$^-$</td>
<td>265.0425</td>
<td>265.0424</td>
<td>0.4</td>
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<td><strong>Redox</strong></td>
<td>Glutaminyl-glutamate</td>
<td>[M-H]$^-$</td>
<td>274.1044</td>
<td>274.1038</td>
<td>2.2</td>
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<td></td>
<td>Ophthalmic acid$\dagger$$\ddagger$</td>
<td>[M-H]$^-$</td>
<td>288.1188</td>
<td>288.1197</td>
<td>5.9</td>
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<td>Glutathione (GSH)$\dagger$$\ddagger$</td>
<td>[M-H]$^-$</td>
<td>306.0765</td>
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<td></td>
<td>S-Formylglutathione</td>
<td>[M-H]$^-$</td>
<td>334.0709</td>
<td>334.072</td>
<td>3.2</td>
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<td>S-Lactoylglutathione</td>
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<td>378.0971</td>
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<td>S-(1,2-Dicarboxyethyl)Glutathione</td>
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<td>422.0869</td>
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<td>CySSG$\ddagger$</td>
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<td></td>
<td>GSSG</td>
<td>[M-H]$^-$</td>
<td>611.1441</td>
<td>611.1453</td>
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<td><strong>Energy</strong></td>
<td>AMP$\dagger$</td>
<td>[M-H]$^-$</td>
<td>346.0558</td>
<td>346.0564</td>
<td>1.7</td>
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<tr>
<td></td>
<td>[M-H]</td>
<td>m/z</td>
<td>RT</td>
<td>%Δ</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
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<td>--------------</td>
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<td></td>
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<tr>
<td><strong>ADP†+</strong></td>
<td>[M-H]-</td>
<td>426.0217</td>
<td>426.0217</td>
<td>0.9</td>
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<td><strong>ATP†+</strong></td>
<td>[M-H]-</td>
<td>505.9883</td>
<td>505.9883</td>
<td>0.2</td>
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<td><strong>Lipids</strong></td>
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</tr>
<tr>
<td>CPA(18:1)†+</td>
<td>[M-H]-</td>
<td>417.2412</td>
<td>417.2402</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>PE(18:1/16:0)†+</td>
<td>[M-H]-</td>
<td>716.5236</td>
<td>716.5229</td>
<td>1.0</td>
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</tr>
<tr>
<td>PC(18:1(11Z)/15:0)†+</td>
<td>[M-H]-</td>
<td>744.5549</td>
<td>744.5576</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>PS(18:1/18:0)†+</td>
<td>[M-H]-</td>
<td>788.5447</td>
<td>788.5456</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Beta-Citryl-L-glutamic acid†+</td>
<td>[M-H]-</td>
<td>320.0623</td>
<td>320.0623</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

† Identity confirmed by On-Tissue MS/MS
‡ Identity confirmed by GC-MS
+ Significantly altered m/z feature in fixed v fresh lens PCA
Figure 7.1: GC-MS identification of the SIL Glucose.

(a) GC-MS elution profile of SIL glucose in outer cortex region of lenses incubated in AAH containing SIL-glucose for 30 min (blue), 20 h (white) and control (i.e. no SIL glucose) lenses (green). (b) Mass spectrum collected at the retention time 19.40 min (red), compared to the reference spectrum for non-stable isotope labelled glucose extracted from NIST20 database (blue). Presence of SIL glucose is confirmed in 30 min sample, while example mass spectrum from 20 h sample is shown for improved spectral quality. (c) Schematic diagram describing main proposed fragment ions from panel (b).
Figure 7.2: GC-MS identification of the SIL sorbitol.

(a) GC-MS elution profile of SIL sorbitol in outer cortex region of lenses incubated in AAH containing SIL-glucose for 30 min (blue), 20 h (white) and control (i.e. no SIL glucose) lenses (green). (b) Mass spectrum collected at the retention time 18.95 min (red), compared to the reference spectrum for non-stable isotope labelled sorbitol extracted from NIST20 database (blue). Presence of SIL sorbitol is confirmed in 30 min sample, while example mass spectrum from 20 h sample is shown for improved spectral quality. (c) Schematic diagram describing main proposed fragment ions from panel (b).
Figure 7.3: GC-MS identification of the SIL glucose-6-phosphate

(a) GC-MS elution profile of SIL glucose-6-phosphate in outer cortex region of lenses incubated in AAH containing SIL-glucose for 30 min (blue), 20 h (white) and control (i.e. no SIL glucose) lenses (green).

(b) Mass spectrum collected at the retention time 25.89 min (red), compared to the reference spectrum for non-stable isotope labelled glucose-6-phosphate extracted from NIST20 database (blue). The ion series shown in the spectrum are dominated by the rearrangement of ions from the TMS modified phosphate group without the carbon backbone involved. Therefore, no 13C mass shift was observed (Harvey, 1973). Presence of SIL glucose-6-phosphate is confirmed in 30 min sample through a combination of retention time, spectrum, and intensity trend, while example mass spectrum from 20 h sample is shown for improved spectral quality. (c) Schematic diagram describing main proposed fragment ions from panel (b).
Figure 7.4: Isotopic distribution analysis for identification of SIL compounds detected by MALDI-IMS.

The highest scoring spectra (Method: SQRTP) (Guo et al., 2021) among SIL glucose-incubated samples were selected and reported. A score than greater than 1.5 is considered as a high quality spectrum match. 
(a) SIL glucose [M+Cl]^- (b) SIL sorbitol [M+Cl]^- (c) SIL fructose-1,6-bisphosphate [M-H]^- (d) SIL glucose-6-phosphate [M-H]- (e) SIL UDP-Glucose [M-H]^+ (f) SIL sedoheptulose-7-phosphate [M-H]^-.
Figure 7.5: GC-MS identification of SIL glucose using reference standard.

(A) GC-MS elution profile of standard solutions of SIL glucose (green) and glucose (white). Two isomeric peaks are present at 19.70 min and 20.20 min. (B) Mass spectrum collected at the retention time 19.70 min (red), compared to the reference spectrum for unlabelled glucose extracted from NIST17 database (blue). The specific indicative ions of glucose and SIL glucose are highlighted (green). (C) The overlaid GC-MS elution profile of SIL glucose incubated lens samples (top) and SIL glucose standard (bottom). (D) The GC-MS spectrum at 19.70 min from SIL glucose incubated lens samples (top) and SIL glucose standard spectrum (bottom).
Figure 7.6: GC-MS identification of the SIL sorbitol using reference standard.

(a) GC-MS elution profile of standard solutions of SIL sorbitol (green) and sorbitol (white). (b) Mass spectrum collected at the retention time 20.20 min (red), compared to the reference spectrum for unlabelled sorbitol extracted from NIST20 database (blue). The specific indicative ions of sorbitol and SIL sorbitol are highlighted (green). (c) The overlaid GC-MS elution profile of the SIL sorbitol peak in SIL glucose-incubated samples (top) and SIL Sorbitol standard (bottom). (d) The GC-MS spectrum at 20.20 min in SIL glucose-incubated samples (top) and SIL sorbitol standard spectrum (bottom). (e) An enlarged view of selected indicative ion 310 of SIL sorbitol in the spectra comparison window.
Figure 7.7: GC-MS identification of SIL glucose 6-phosphate using reference standard.

(A) Overlaid GC-MS elution profiles of SIL glucose 6-phosphate in SIL glucose-incubated samples (top) and glucose 6-phosphate standard (bottom). (B) Mass spectra of SIL glucose 6-phosphate in SIL glucose-incubated bovine lens (red) and glucose 6-phosphate standard (blue). (C) An enlarged view of an ion at m/z 387, indicative of SIL glucose 6-phosphate. (D) An enlarged view of an ion at m/z 471, indicative of SIL glucose 6-phosphate.
Figure 7.8: Sequence alignment of (A) GLUT1 and (B) GLUT3.

Areas in red indicate differences in amino acid sequence.
Table 7.2: Statistical analysis of glucose uptake patterns using GC-MS results.

<table>
<thead>
<tr>
<th>SIL glucose Control</th>
<th>P-value</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>OC</td>
<td>IC</td>
<td>N</td>
</tr>
<tr>
<td>0</td>
<td>1 (ns)</td>
<td>1 (ns)</td>
<td>1 (ns)</td>
</tr>
<tr>
<td>0.5h</td>
<td>0.003 (**)</td>
<td>0.291 (ns)</td>
<td>0.901 (ns)</td>
</tr>
<tr>
<td>1h</td>
<td>0.036 (*)</td>
<td>0.003 (**)</td>
<td>0.035 (*)</td>
</tr>
<tr>
<td>2h</td>
<td>0.028 (*)</td>
<td>0.044 (*)</td>
<td>0.041 (*)</td>
</tr>
<tr>
<td>4h</td>
<td>0.079 (ns)</td>
<td>0.112 (ns)</td>
<td>0.094 (ns)</td>
</tr>
</tbody>
</table>

The statistical analysis revealed a significant difference in the pattern of glucose uptake between SIL-incubated lenses and control lenses cultured in AAH containing unlabelled glucose under similar incubation timescale (*: P ≤ 0.05, **: P ≤ 0.01, and ns: P > 0.05).

Table 7.3: Statistical analysis of SIL glucose signal in the nucleus in normal and ouabain-treated lenses measured by GC-MS.

<table>
<thead>
<tr>
<th>Ouabain-treated</th>
<th>P-value (Region: N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.979</td>
</tr>
<tr>
<td>1</td>
<td>0.802</td>
</tr>
<tr>
<td>2</td>
<td>0.732</td>
</tr>
<tr>
<td>4</td>
<td>0.478</td>
</tr>
</tbody>
</table>

Statistical analysis did not show a significant difference between normal and ouabain-treated lenses, although a slight drop in the amount of glucose in ouabain-treated lenses was observed.
Figure 7.9: MALDI-IMS of hyperglycaemia lens model.

MALDI images of axial sections taken from bovine lenses organ cultured in AAH containing 50 mM (HG) and 5 mM glucose for 24 h showing regional differences in (A) glucose (m/z 217.047) and (B) GSH (m/z 306.075). Response of Sorbitol (C) and GSH (D) in hyperglycaemia condition in three lens regions; N, IC and OC. HG: Hyperglycaemia, NG: Normoglycemia.
Mapping glucose metabolites in the normal bovine lens: Evaluation and optimisation of a matrix-assisted laser desorption/ionisation imaging mass spectrometry method

Ali Zahraei, George Guo, Rebecca D. Perwick, Paul J. Donaldson, Nicholas J. Demarais, Angus C. Grey

Abstract
The spatial resolution of microdissection-based analytical methods to detect ocular lens glucose uptake, transport and metabolism are poor, whereas the multiplexing capability of fluorescence microscopy-based approaches to simultaneously detect multiple glucose metabolites is limited in comparison with mass spectrometry-based methods. To better understand lens glucose transport and metabolism, a more highly spatially resolved technique that maintains the fragile ocular lens tissue is required. In this study, a sample preparation method for matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI IMS) analysis of ocular lens glucose uptake and metabolism has been evaluated and optimised. Matrix choice, tissue preparation and normalisation strategy were determined using negative ion mode MALDI-Fourier transform-ion cyclotron resonance MS of bovine lens tissue and validation performed using gas chromatography-MS. An internal standard was applied concurrently with N-(1-naphthylethyl)enediamine dihydrochloride (NEDC) matrix to limit cracking of the fresh frozen lens tissue sections. MALDI IMS data were collected at a variety of spatial resolutions to detect both endogenous lens metabolites and stable isotopically labelled glucose introduced by ex vivo lens culture. Using this approach, initial steps in important metabolic processes that are linked to diabetic cataract formation were spatially mapped in the bovine lens. In the future, this method can be applied to study the dynamics of glucose uptake, transport and metabolic flux to aid in the study of diabetic lens cataract pathophysiology.

Keywords
glucose, lens, MALDI imaging, metabolites

1 | INTRODUCTION

The ocular lens is a biconvex, transparent cellular tissue suspended in the anterior segment of the eye and functions to focus light onto the retina. It is not only a passive optical element but is also characterised by its own unique cellular structure, region-specific biochemistry and physiological function that combine to produce lens optical
highly spatially resolved method to map glucose uptake, transport and metabolism in the ocular lens would aid in elucidating the pathophysiology of diabetic cataract.

Matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI IMS) is capable of mapping the spatial distribution of multiple biomolecules simultaneously from thin tissue sections, without prior knowledge of the chemical constituents and without labelling.\textsuperscript{15,16} While IMS was first developed to map protein and peptide distributions, more recently, it has been used to map small molecules and metabolites and is particularly powerful in this area when coupled to mass analysis via Fourier transform ion cyclotron resonance (FT-ICR).\textsuperscript{17,18} A range of lens metabolite classes have been mapped using MALDI-FT-ICR IMS.\textsuperscript{21,22,23} Several of the glucose metabolites have been detected in other tissues using MALDI matrices\textsuperscript{24,25} and isotopic l-lactosamine monophosphates and bisphosphates imaged elegantly in the brain by using MS\textsuperscript{5} on a linear ion trap.\textsuperscript{26} However, the tissue preparation and MALDI-matrix conditions for glucose and glucose metabolite detection in the lens have not previously been investigated. Therefore, there is a need to optimise lens tissue preparation for MALDI IMS analysis of this essential lens nutrient. In the present study, we systematically optimised experimental conditions to detect glucose in normal bovine lenses. Matrix choice, normalisation method and tissue fixation were all investigated. The ability of MALDI IMS to detect exogenous lens nutrient uptake in ex vivo cultured normal bovine lenses was demonstrated and validated by complementary gas chromatography–mass spectrometry (GC–MS).

2 | MATERIALS AND METHODS

2.1 | Tissue and reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO). All solvents used were high-performance liquid chromatography (HPLC) grade. Bovine eyes were collected from a local abattoir (Auckland Meat Processors, Ltd) and the lenses (~2 years old) were removed anteriorly. Extracted lenses were used for incubation experiments, fixation or frozen immediately and stored at –80°C until required.

2.2 | Ex vivo lens incubations

Bovine lenses were removed from ocular globes anteriorly and immediately incubated in 12 ml prewarmed isotonic artificial aqueous humour (AAH, 125 mM NaCl, 0.5 mM MgCl\textsubscript{2}, 4.5 mM KC\textsubscript{l}, 10 mM NaHCO\textsubscript{3}, 2 mM CaCl\textsubscript{2}, 20 mM sucrose, 10 mM HEPES, pH 7.2–7.4, 290 ± 5 mMol/L containing 5 mM stable isotopically labelled (1\textsuperscript{3}C\textsubscript{5}) glucose) for 30 min at 37°C.\textsuperscript{27,28} Before freezing, samples were rinsed three times in 20 ml isotonic AAH to remove any SIL glucose on the lens tissue surface.
2.3 | Fixation of bovine lenses

Immediately after dissection, bovine lenses were incubated in a solution of phosphate-buffered saline (PBS) with 2% paraformaldehyde (PFA) and 0.01% glutaraldehyde at 72 h at room temperature. Following fixation, lenses were washed three times for 5 min each with PBS to remove excess fixative, then snap-frozen and stored at −80°C until further use.

2.4 | Tissue sectioning

Frozen lenses were mounted on cold chuck using Cryomatrix™ compound (Thermo Scientific) at the base of the tissue only. Axial sections (thickness 20 μm) were cut using a cryostat (Leica CM3050S, Leica Microsystems GmbH, Wetzlar, Germany) at −20°C and collected on to cooled double-sided carbon tape (PrepSciTech, Kiwaan, Australia) attached to nonconductive glass slides (PINK COLORFROST, LabServ, NZL).20

2.5 | Matrix spot preparation for SIL glucose analysis

The MALDI matrices 9-Aminoacridine (9-AA), 1,5-diaminonaphthalenediamine hydrochloride (1,5-DAN HCl), 2,5-dihydroxybenzoic acid (DHBA) and N-(1-naphthalenyl)ethylenediamine dihydrochloride (NEDC) were dissolved in methanol (MeOH):water (90:10 v/v) to a final concentration of 7 mg/mL. SIL glucose was dissolved in the same solvent (1 mg/mL) and then mixed with the different matrices at a ratio of 1:1 (v/v). A volume of 1 μL of the final samples was pipetted onto a stainless steel MALDI target and dried at room temperature.

2.6 | Matrix and IS application for MALDI IMS

The mounted tissue sections were placed in a desiccator for 1 h and allowed to equilibrate to room temperature immediately prior to matrix application. A TM Sprayer (XTX Technologies, Carborndale, NC) was used to apply internal standard (IS) and matrix solutions to tissue sections. 3-O-methyl-D-glucose (3-OMG, 100 μM) was used as the IS due to its chemical similarity to glucose and because it has a molecular weight (C6H12O6, Mw = 182.109) that is different to both endogenous glucose (C6H12O6, Mw = 180.063) and SIL glucose (C6H12O6, Mw = 186.110). NEDC (7 mg/mL in 90% MeOH or 50% EtOH in H2O) was applied using the spray settings: solvent flow rate 0.15 ml per minute, nozzle speed 200 mm per minute, nozzle temperature 70°C, track spacing 2 mm, dry time 0.5 min, four passes. The IS 3-OMG was applied either separately before matrix application or premixed with NEDC solution using the same spray settings. Following NEDC deposition, samples were stored in a vacuum desiccator until data collection.

2.7 | MALDI IMS

MALDI spot and IMS were carried out using a Bruker Solarix XR 7T FT-ICR mass spectrometer using FTMS Control v2.2.0 and FlexImaging v5.0 (Bruker Daltonics, Billerica, MA). MALDI IMS datasets were acquired in negative ion mode from m/z 100 to 1000 with a resolving power (m/Δm) of 66,000 at m/z 400. A Smartbeam II Nd:YAG laser at 355 nm was applied for MALDI employing 200 laser shots per position at a repetition rate of 1 kHz. The laser beam size was set to medium (150 μm sampling) for all images collected, except for the high spatial resolution images, which were collected using minimum (30 μm sampling). Prior to the spectra acquisition, an external calibration was employed using red phosphorus in MALDI MS negative ion mode. Sections from each lens were collected and analysed in the same image so that intensity levels of detected analytes could be compared directly between samples. Lens sections were sampled in random order to avoid sampling order artefacts. In MALDI IMS datasets were imported into SCILS Lab 2D (version 2019P Pro, SCILS GmbH, Bremen, Germany), whereas MALDI spot data processing was conducted using the DataAnalysis software package (Bruker Daltonics; v5.0). MALDI IMS of metabolites in tissue sections were normalised using either root mean square (RMS) method or to the 3-OMG (M+ = C6H12O6) signal, and MALDI images plotted at the theoretical m/z +/-0.005 Da with weak denoising on. Identification of signals detected in the MALDI IMS datasets was made using database interpretation (HMDB and METÁSPACE) of accurate masses and on-tissue MS/MS where possible.

2.8 | Principal component analysis

Principal component analysis (PCA)12,23 was performed with an in-house written R script and Cardinal package24 used for the purpose of discriminant analysis. inZIML data were generated by SCILS Lab 2D and peaks picked with 20 ppm bin size, smoothed with ‘Gaussian’ method and one-pixel smoothing radius and a threshold of 0.3% of maximum intensity. Sampling locations lying outside the tissue sections were removed manually from each lens section to remove off-tissue regions as a source of variation.

2.9 | Sample preparation for GC–MS analysis

Whole bovine lenses (n = 4) underwent identical octane culture in SIL glucose solution (see ex vivo lens incubations) and were then micro-dissected into three lens regions with tweezers. The capsule, containing the lens epithelium, was peeled from the lens fibre cells and discarded. Then the outer cortex (OC) is separated from the denser inner cortex (IC) and the hard, central nucleus (N). Each sample (OC, IC, N) was placed in a separate falcon tube containing 2 ml of 90% MeOH. Samples were agitated and homogenised in the extraction solvent for 45 min at 4°C. Remaining cell debris was removed via centrifugation at 16,000 g for 20 min at 4°C in an Eppendorf 5402
centrifuged and supernatants containing extracted small molecules kept at −80°C until analysis. Samples were then dried in a Speed-Vac Concentrator and underwent automated trimethylsilyl (TMS) derivatization in preparation for analysis via GC-MS.

2.10 GC-MS analysis

GC-MS was used for identification and semiquantification of sugars. Instrument parameters were based on Villas-Boas et al. An Agilent 7890B gas chromatograph coupled to a 5977A inert mass spectrometer with a split/splitless inlet was used. One microfilter of the sample was injected using an Agilent autosampler into a glass split/splitless 4 mm ID straight inlet liner packed with deactivated glass wool. The inlet was set to 230°C, with a split ratio of 25:1, pressure 99.26 kPa, a column flow of 1.3 ml per minute constant flow mode, giving a calculated average initial linear velocity of 39 cm/s. The column was a fused silica ZB-170 30 m long, 0.25 mm ID, 0.15 μm stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl). Phenomenex). Carrier gas was ultra-high purity grade helium (99.99999%, BOC). The GC oven temperature programming started isothermally at 70°C for 5 min, increased 10°C per minute to 179°C; increased 0.5°C per minute to 180°C; held 2 min; increased 10°C per minute to 220°C, held 1 min; increased 2.5°C per minute to 265°C, held 1 min; increased 10°C per minute to 280°C, held 1 min; increased 1°C per minute to 290°C and held for 0.6 min. The transfer line to the Mass Selective Detector (MSD) was maintained at 250°C, the source at 230°C and quadrapole at 150°C. The detector was turned on 4.5 min into the run. The detector was run in positive-ion, electron-impact ionisation mode, at 70 eV electron energy, with an electron multiplier set with no additional voltage relative to the autotune value. Chloroform blanks were run for every 10–12 samples to monitor instrument carryover. Mass spectra were acquired in scan mode from 40 to 600 amu with a detection threshold of 100 ion counts.

2.11 GC-MS data analysis

GC-MS data were converted into .csv files by the function provided in Automated Mass Spectral Deconvolution and Identification System (AMTD). A sublibrary of the spectral database was generated by searching the QC samples against the NIST17 database. The identification result was then combined with an amino acid and sugar standard reference spectral library. Spectra of SIL molecules of interest were annotated and curated manually with the assistance of the NIST17 database. Peak quantitation was performed within the MassLynx package, an in-house R-based package for metabolomics analysis.

2.12 Scanning electron microscopy

The surface morphology of mounted tissue sections was characterised by a JEOL JCM-6000 scanning electron microscopy (SEM) after matrix application. All the samples were coated with a thin layer of gold (5 nm) using a sputter coater (Q150T Plus, Quorum Technologies) before SEM imaging with a magnification of x1100.

2.13 Intensity profile analysis

ImageJ 1.50i (National Institute of Health, USA) was implemented to investigate the intensity of pixels along with a rectangular selection of the image. The analysis feature demonstrated an average profile plot, where the x-axis displays the horizontal distance along tissue diameter and the y-axis the averaged pixel intensity. We present one example that represents 10 replicate datasets analysed by MALDI IMS.

3 RESULTS

We have experimentally probed the optimal tissue preparation method to map the spatial distribution of the major lens nutrient glucose with MALDI IMS using FT-ICR analysis of standards and tissue sections, SEM for matrix crystal morphology analysis, image intensity profile analysis and validation by GC-MS.

3.1 Optimisation of MALDI matrix choice and spray application

In the absence of blood supply, the lens relies on the uptake of glucose from the ocular humours to provide metabolic energy. Therefore, we first analysed a standard of glucose (SIL glucose), used in later ex vivo lens culture experiments, in several different matrices to determine the optimal matrix and solvent conditions to promote desorption/ionisation via MALDI (Figure 1), predicted and observed m/z for all ions reported in Table S2). Signal quality was determined by monitoring ion intensity, signal-to-noise ratio and the presence of matrix ions. Both [M – H]− and [M + Cl]− ions were monitored because these are the most commonly detected negative ions of primary metabolites for standards and particularly from tissue where chloride is known to be present. First, the typical negative ion mode metabolite matrix 9-AA was trialled. While the signal for the [M – Cl]− ion at m/z 221.052 was significant, the matrix background signals were also abundant, and no [M – H]− ions were detected at m/z 185.076 (Figure 1A). Second, 1,5-DAN HCl, which has also previously been used as a metabolite matrix, was also trialled in negative ion mode (Figure 1B). A low-intensity signal for the [M – H]− ion of SIL glucose was detected at m/z 185.076, whereas the [M + Cl]− ion signal, detected at m/z 221.052 was higher than the [M – H]−, but not abundant in relation to matrix ions. Keeping in mind that ultimately this method would be applied to unwashed tissue sections to map endogenous and exogenous metabolite distributions, a more salt-tolerant matrix was trialled next. SIL glucose was analysed in DHB matrix; however, no ions for SIL glucose were detected in either polarity (data not shown), consistent with previous studies. DHB
was not pursued further because it also lacked the ability to map metabolites of the SIL glucose, such as SIL glucose-6-phosphate, which are generally detected in negative ion mode. Last, we trialled NEDC, an inorganic salt that has previously been used to detect metabolites,41,42 metal ions,43 lipids and GSH in ocular lens tissue.44 A strong response for the [M + Cl]− ion was detected at m/z 221.052 (Figure 1C), with a weak signal for the [M − H]+ ion, and no signal detected in positive ion mode (data not shown). There was also a very limited background signal from the matrix, suggesting that NEDC may be a good candidate to detect glucose and its related molecules in lens tissue.

The ocular lens is a difficult tissue to generate high quality tissue sections from due to the different tissue hydration states of its cortical and nuclear regions. Several tissue preparation methods have been developed to generate lens tissue sections for MALDI IMS analysis, including MeOH soft-lancing,45 tape lancing,40 and sucrose treatment.46 These approaches have utilised fresh-frozen tissue. Metabolite profiles in other tissues have also been obtained from fixed tissue,47,48 which, due to the fixation process, stabilises the metabolites during the sectioning process. Fixed tissues can also produce better macroscopic and microscopic morphology that is more suitable for higher spatial resolution MALDI IMS analysis.49,50 Therefore, tissue sections from both fresh frozen and fixed lens tissue were generated, and matrix spray application parameters were determined. Optical images were captured to assess tissue section morphology and SEM images were collected to determine matrix crystal morphology (Figure 2). With the intention of ultimately focusing on the accurate spatial mapping of particular classes of lens nutrients (i.e., glucose), in addition to matrix application, we also trialled combinations of matrix and IS by spray application to determine the optimal protocol.

When matrix was applied in ethanol ([EtOH] : water 50:50 v/v), tissue section morphology remained largely intact (Figure 2A); however, SEM imaging showed that matrix crystal formation was poor (Figure 2E), suggesting that the number and quality of mass spectral signals would be limited. Spray deposition of matrix compounds in
MeOH-water (90:10 v/v) has been successfully employed for previous MALDI IMS analysis of the ocular lens, and this solvent was used for three different matrix applications. First, the IS was sprayed on bovine lens section prior to matrix application. When this routine was applied to bovine lens sections, large cracks appeared in the tissue (Figure 2B). This resulted in low quality MALDI IMS results due to tissue discontinuities coupled with uneven matrix crystal formation across the cracked tissue sections (Figure 2F). Next, the IS was first combined with a matrix solution before spray deposition using the same parameters as previous applications. While there was some tissue cracking, this was generally limited (Figure 2C), and matrix crystal formation on the tissue surface was relatively uniform (Figure 2G). Matrix crystal uniformity was not due to the presence of IS in the matrix solution because matrix spray alone also showed limited tissue cracking and relatively uniform crystal formation (data not shown). Last, this same spray routine was applied to sections from fixed lens tissue. While the cracking pattern (Figure 2G) and matrix crystal morphology (Figure 2H) was different on fixed tissue, the concurrent application of IS/matrix spray was deemed the optimal protocol to minimize tissue cracking and improve the morphology of matrix crystals. Both fresh frozen and fixed tissues were analysed by MALDI IMS and data quality assessed by considering the number of signals, signal intensity, signal-to-noise ratio and signal spatial distribution.

3.2 | Optimisation of tissue preparation

To assess signal differences between fixed versus fresh frozen tissue, we conducted a PCA analysis (Figure 3). All sampling locations on tissue sections from each treatment group were compared, that is, tissue sections were not separated into anatomically distinct regions before comparison. The two treatments clustered distinctly in PC1, indicating large differences between the treatments based on signal intensity (Figure 3A). A feature plot of PC1 (Figure 3B) generated a list of m/z features that contributed to the variation between PFA fixed and fresh frozen samples (see Table S2), showing the lens metabolites most affected by tissue fixation. MALDI images of a selection of these m/z features are plotted in Figure 3C (left: fixed, right: fresh frozen). The abundant lens antioxidant GSH (Figure 3CJ) showed high signal in the cortex and low/no signal in the nucleus of the lens for both preparation methods. However, the fixed tissue signal for GSH was lower than that of the fresh frozen. Similar results were also observed for the GSH analogue ophthalmic acid (Figure 3C8), where the signal for both preparations is predominately located in the cortex, but the overall intensity is lower in fixed tissue. Another GSH-related molecule t-cysteine-GSH disulphide (CySSG) (Figure 3CII), which has previously been detected in the bovine lens IC, was again detected in the inner lens cortex of fresh frozen lenses, but no signal was registered for the fixed tissue. Finally, a signal assigned ATP was high in the OC of the fresh frozen tissue, but absent from the fixed lens sample (Figure 3CIV). However, not all signals in the fixed tissue preparation showed decreased intensity compared with fresh frozen. The signal for putative lens glucose (Figure 3CV) and sorbitol (Figure 3CIV) were elevated in fixed tissue, particularly in the lens nucleus. While this putative elevation of sugar was interesting, together, these results showed that the relative intensities and distributions of multiple lens small molecules were affected by fixation. With our focus on spatially resolving glucose, its metabolites and other lens metabolites such as antioxidants that are involved in the maintenance of lens transparency and affected by glucose metabolism, this suggested that
fixation prior to MALDI IMS analysis was not suitable. Moreover, because the mechanism of PFA fixation is through its reaction with primary amines to form methylene bridge crosslinks,\(^{32,55}\) glucose is not expected to be fixed through this treatment. Therefore, fresh frozen tissue was utilised for the remainder of the study.

3.3 | Determination of MALDI IMS data normalisation method

When using MALDI IMS to map a specific nutrient or class of metabolites, it is best to utilise a data normalisation approach based on the spray application of a chemically similar standard to account for any ionisation differences that result from different anatomical regions or local tissue environments. While human lens GSH does not appear to suffer significantly from ionisation differences in different lens regions,\(^{44}\) the effect of lens tissue microenvironment on the ionisation of glucose and related molecules is unknown and was therefore tested in our bovine lens samples (Figure 4). Additionally, the method of IS application was tested, because the best method of application, that is, application underneath tissue section, on top of tissue section prior to matrix application or simultaneous IS/matrix application seems to differ depending on the molecule of interest.\(^{34-36}\) The glucose analogue 3-OMG was chosen as the sprayed IS due to its chemical similarity yet different molecular weight to both endogenous glucose and its metabolites and the targeted SIL glucose and its metabolites in the ex vivo cultured lenses. MALDI images were plotted and signal intensity plots along the optical axis, that is, from anterior to posterior pole, were generated following sequential IS/matrix application (Figure 4A, upper) and simultaneous IS/matrix application (Figure 4A, lower). The signal intensity of 3-OMG was nonuniform across the bovine lens sections and indicated ion suppression in the lens cortex relative to the nucleus (Figure 4A, red). Hence, when the distribution of putative glucose (m/z 215.032) was plotted with RMS normalisation, the signal was more abundant in the nucleus relative to the cortex (Figure 4A, green) and reversed when normalised to
3-OMG (Figure 4A, blue). Signal intensity plots of the MALDI images were very similar (Figure 4B, red and green), particularly for the putative endogenous glucose signal normalised to IS (Figure 4B, blue) suggesting that simultaneous spray application of IS/matrix was an appropriate method for lens nutrient mapping by MALDI IMS.

To confirm the identity of m/z 215.032, on-tissue MS/MS could not be performed because CID of this [M + Cl]⁺ ion yielded no structural information due to the loss of the [Cl]⁻ adduct, and the intensity of the [M – H]⁻ ion was too low. Therefore, GC-MS was performed on bovine lenses dissected into the OC, IC and nucleus (N) regions to determine both the relative abundance of the putative identities of m/z 215.032 (e.g., glucose, fructose, myo-inositol) and their abundance in the different lens regions (Figure 4Cii). This GC-MS analysis was then used to confirm the most suitable method of MALDI IMS data normalisation. The analysis showed that, for endogenous compounds, myo-inositol was much more abundant in all lens regions than glucose and fructose. To compare the relative abundance of the two mass spectrometry approaches, MALDI IMS data were digitally dissected into OC, IC and N (Figure 4Cii) and relative quantification of the signal at m/z 215.032 (glucose, fructose or myo-inositol) plotted for each region using both RMS and IS normalisation (Figure 4Ciii). Together, these results confirmed that IS normalisation was appropriate to map glucose-like metabolites and that the abundant signal at m/z 215.032 in the MALDI IMS data should be assigned myo-inositol. Signals for other endogenous metabolites, such as glucose monophosphate(s) (m/z 259.022) and sorbitol (m/z 217.022), were also detected and were localised predominantly to the lens cortex (data not shown).
3.4 Demonstration of method on ex vivo incubated bovine lenses

With the appropriate MALDI IMS, IS/matrix application and normalisation method established, bovine lenses that had been incubated in normoglycaemic AAH containing SIL glucose were analysed (Figure 5). Sections underwent both low spatial resolution analysis (150 µm spot-to-spot sampling, Figure 5B) to capture the whole lens section and high spatial resolution analysis (30 µm spot-to-spot sampling, Figure 5C) to test the sensitivity of the established approach. In addition to the uptake of SIL glucose into the lens, evidence for at least two metabolic pathways (Figure 5A) was spatially detected. SIL glucose-6-phosphate/fructose-6-phosphate ($[M + Cl^-]$ = m/z 265.042), the early metabolic intermediates in a number of metabolic processes such as glycolysis and de novo myo-inositol synthesis, was detected in the MALDI IMS data (Figure 5C). This signal was assigned glucose-6-phosphate/fructose-6-phosphate because existing knowledge of glucose metabolism in the ocular lens suggests that glucose-6-phosphate/fructose-6-phosphate is likely to be more abundant than glucose-1-phosphate under normal conditions. In addition, SIL sorbitol ($[M + Cl^-]$ = m/z 223.068) was detected and is part of the polyol pathway, which in some species plays a pathological role in diabetic lens cataract formation by converting excess glucose to sorbitol to cause osmotic cell damage (Figure 5C,ii). The identity of these molecules was confirmed by both GC-MS analysis of metabolites extracted from microdissected lenses (see Figures S1-S3) and the absence of signal for these metabolites in MALDI IMS datasets of control lenses (see Figure 5A). However, this approach was unable to distinguish between glucose-6-phosphate and fructose-6-phosphate.

In addition, on-tissue MS/MS was unable to confirm the identity of the ion signal assigned glucose-6-phosphate/fructose-6-phosphate due to the lack of structural information gained when performing CID on a $[M + Cl^-]$ ion.

4 DISCUSSION

A tissue preparation method for MALDI IMS analysis of bovine lens metabolites has been optimised, validated by complementary GC-MS analysis of dissected lens regions and used to detect uptake of SIL glucose and its metabolism in cultured bovine lenses. First, matrix choice was optimised using SIL glucose. Next, an appropriate method to control for ionisation differences was tested. Application of IS prior to matrix application is a typical approach to minimise ionisation differences from different tissue regions. As a result of this routine application, large tissue cracks appeared and uneven matrix crystal formation led to poor quality MALDI images, and in this respect, the sequential IS/matrix application method was rejected. The simultaneous IS/matrix spray was deemed the best matrix application protocol to limit tissue cracking and promote matrix crystal formation. Next, tissue fixation was assessed for its efficacy in preserving both tissue morphology and ion signal by MALDI in lens tissue, because some studies have reported the detection of small molecules (e.g., lipids and metabolites) from fixed tissue. In the present study, fixation with PFA had a significant effect on the spatial distributions and intensities of many endogenous metabolite signals. This was not surprising because the lens contains high concentrations of small molecules, such as GSH and ophthalmic acid, which are significant contributors.

**FIGURE 5** Detection of polyol metabolic pathway in cultured bovine lenses. (A) Schematics of common metabolites of glucose in the lens. (B) Optical scan (i) and matrix-assisted laser desorption/ionisation (MALDI) image of stable isotopically labelled (SIL) glucose (ii) in an axial section of a lens incubated for 30 min ex vivo. (C) Higher resolution MALDI images from regions marked by red box in panel B of (i) SIL glucose-6-phosphate (G6P)/fructose-6-phosphate (F6P) (m/z 265.042), (ii) SIL glucose signal (m/z 221.052) and (iii) SIL sorbitol (m/z 223.068). Dashed red box denotes region of lens section where higher resolution images are taken from...
to the lens antioxidant defence system and therefore long-term tissue transparency, and their chemical fixation has been demonstrated. 15

Although the PFA fixation chemical reaction is very complicated, few published studies have considered the fundamental molecular chemical reactions that underly the fixation process. 16-18

Previous work has demonstrated the formation of methylene bridges by the reaction of PFA with amine groups; thus, the targeted components become somewhat confined in tissue samples. In the present study, signal intensity for fixable lens small molecules, such as GSH and cysteic acid, were lower compared with fresh frozen tissue likely due to molecular cross-linking and the inability of the subsequent matrix application and MALDI process to ionize these molecules (see Figure 3C,i-k). Interestingly, due to fixable molecules being cross-linked together, subsequent mass spectrometry analysis can generate peaks from molecules that are less involved in the fixation process. 19

Indeed this seems to be the case with N-glycan imaging of fixed and embedded tissue by MALDI IMS, where protein signal is eliminated due to chemical cross-linking, whereas glycan imaging is possible following PNGase F treatment. 20

Therefore, this may explain the possible enhancement of some signals of sugar-like molecules (myo-inositol and sorbitol, see Figure 3Cv and v6) detected in the fixed lens preparation. A second possibility is that some nonvolatile small molecules are washed out of the lens during the fixation process, which would alter the complement of signals that are detected during the MALDI IMS analysis.

Irrespective of this mechanism, the present study aimed to develop a method to detect lens glucose uptake, transport and metabolism, and because glucose contains no amine groups and is not fixable by formaldehyde-based chemicals, we would not predict chemical tissue fixation to be amenable to glucose detection. Wash-out of the SGL glucose signal by treatment with a chemical fixative would be particularly detrimental to studies of lens glucose uptake because cells at the surface of the lens that have taken up SGL glucose during lens culture would be exposed to the fixative solution first and for the longest time during fixation. Dehydration-based fixation was not trialled because the SGL metabolites of interest are also soluble in EtOH/MeOH and their spatial distributions and relative abundance would, therefore, be compromised if this were used. While chloroform has been used to enhance glucose metabolism detection in brain tissue, 21 any washing of the fragile lens tissue would disrupt the tissue section structure and lead to poor image quality.

In the process of optimizing the data normalization for MALDI IMS, GC–MS was used to identify the endogenous signal at m/z 215.032. In other MALDI IMS studies of the lens and other tissues, m/z 215.032 has been assigned glucose. 22-24 However, this signal could also represent other ketoic metabolites such as fructose and myo-inositol, which are also commonly found in biological tissues. GC–MS analysis showed that there was very little free endogenous glucose present in the lens, and the signal at m/z 215.032 was predominantly myo-inositol. Myo-inositol can be synthesized de novo from glucose and is an important component of inositol phosphates, which are important secondary cell signalling messengers, and phosphatidylinositol lipids, which are a small component of lens phospholipids. 25 In addition, myo-inositol is thought to be an osmoregulator in cultured bovine lens cells. 26 The presence of myo-inositol in the bovine lenses in the current study is consistent with previous studies reporting myo-inositol concentrations in the lens of 7-30 mM kg⁻¹ of the wet lens. 27-29

Using a 30 min incubation, SGL glucose was detected in the outer lens cortex, and its metabolites glucose-6-phosphate/fructose-6-phosphate and sorbitol were also detected. The assignment of m/z 221.052 as SGL glucose is valid for both technical and metabolic reasons. Firstly, MALDI analysis of glucose, myo-inositol and fructose standards suggests that, in NEDC matrix, glucose kinases with greater efficiency than myo-inositol and fructose (Figure S5). Secondly, our analysis showed that glucose is not readily metabolized to myo-inositol in the lens because no SGL myo-inositol was detected in the GC–MS data. However, SGL fructose levels were detected by GC–MS analysis at approximately 30% of SGL glucose levels (data not shown). These results are consistent with previous 14C glucose uptake studies that have shown little accumulation of radioactivity in the inositol fraction, with it remaining as glucose or being converted to sorbitol or fructose. 30 Therefore, while SGL fructose may be present in the incubated lenses and contribute to the signal detected at m/z 221.052 in the MALDI-IMS data, the isomerisation efficiency data suggest that this signal is predominantly SGL glucose.

The detection of SGL glucose-6-phosphate/fructose-6-phosphate was expected because these are the initial metabolites in several metabolic processes, including glycolysis, which is the main energy source for the lens OC cells because they contain both nuclei and mitochondria to facilitate this process. 31 The detection of sorbitol, albeit at low levels, was also expected, because the polyol pathway is active in many animal lenses. While the activity of aldose reductase, the enzyme responsible for converting glucose into sorbitol, is high in the lens, 32,33 the relatively low levels of sorbitol may be a result of two factors. First, that these lenses were only incubated in SGL glucose for 30 min, therefore limiting the time for conversion to take place. Second, these lenses were incubated under normoglycaemic conditions, where the proportion of glucose converted to sorbitol is considerably lower (3%) than under hyperglycaemic conditions (~30%). 34 Interestingly, some cofactors involved in these first steps of glucose metabolism, such as ATP/ADP for the conversion of glucose to glucose-6-phosphate in glycolysis, were also detected using the developed tissue preparation method (see Figures S6 and S7), whereas others, such as NADPH for the conversion of glucose to sorbitol, were not. Previsouly, 9-AAG has been used to detect NADPH in bovine lens tissue, 35 whereas other metabolic intermediates such as pyruvate and lactate have also been detected in other tissue. 36 Therefore, it appears that for a comprehensive analysis of the metabolome by MALDI IMS, it remains necessary to use multiple MALDI matrices.

Nevertheless, it is encouraging that both SGL glucose-6-phosphate/fructose-6-phosphate and SGL sorbitol were detectable in the 30 min incubation, and in the future, longer incubation times will be used, which will increase the amount of glucose taken up into the lens, and the time for glucose metabolism to take place, and hence
the ability to detect it by MALDI IMS. In addition, using a range of
incubation time points, studies of metabolism that were previously
demonstrated by MALDI IMS of the mouse brain may also be possi-
ble in the lens.

5 | CONCLUSIONS

A MALDI IMS method to detect and map glucose and related metabo-
lites in the bovine lens was optimized, validated by GC-MS and used
to monitor glucose uptake and metabolism in normal bovine lenses
under normoglycaemic conditions. This method promises to be a
powerful spatially resolved tool to study the behaviour of glucose in
hyperglycaemic lenses as a model of diabetic lens pathologies, with
the potential to extend this approach to study the uptake, transport
and metabolism of novel antiprismatic therapeutic molecules. With
careful evaluation, this approach could also be considered for appli-
cation in other tissues to enhance glucose metabolism detection and
mitigate lens suppression effects.

ACKNOWLEDGEMENTS

The authors wish to thank the Maurice and Phyllis Paykel Trust for small
equipment funding, and the Health Research Council of New Zealand
and the Marsden Fund of New Zealand. We also acknowledge Erica
Zarate and Sarco Green in the University of Auckland Mass Spectrometry
Centre and Mass Spectrometry Hub for support with GC-MS data
collection and analysis. We greatly thank Prof Jadranka Tracaj-Sejdic
and Dr Albreza Akbarnejad, University of Auckland, Polymer Biointerface
Centre, for SEM imaging and technical assistance.

ORCID

Ali Zohrevand  https://orcid.org/0000-0003-3499-224X
George Guo  https://orcid.org/0000-0003-2217-5525
Paul J. Donaldson  https://orcid.org/0000-0002-4008-1138
Nicholas J. Demianakis  https://orcid.org/0000-0002-0666-9405
Angus C. Gray  https://orcid.org/0000-0002-1540-1080

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

Mapping Glucose Uptake, Transport and Metabolism in the Bovine Lens Cortex

Ali Zahraei1, George Guo1,2, Kyriakos G. Vamava1,3, Nicholas J. Demarais1,3, Paul J. Donaldson1 and Angus C. Grey1,2,4

1Department of Physiology in the School of Medical Sciences, Auckland, New Zealand; 2Mass Spectrometry Hub, Auckland, New Zealand; 3School of Biological Sciences, University of Auckland, New Zealand

Purpose: To spatially correlate the pattern of glucose uptake to glucose transporter distributions in cultured lenses and map glucose metabolism in different lens regions.

Methods: Ex vivo bovine lenses were incubated in artificial aqueous humour containing normoglycaemic stable isotopically-labelled (SIL) glucose (5 mM) for 5 min-20 h. Following incubations, lenses were frozen for subsequent matrix-assisted laser desorption/ionisation (MALDI) imaging mass spectrometry (IMS) analysis using high resolution mass spectrometry. Manually dissected, SIL-inoculated lenses were subjected to gas chromatography-mass spectrometry (GC-MS) to verify the identity of metabolites detected by MALDI-IMS. Normal, unincubated lenses were manually dissected into epithelium flat mounts and fibre cell fractions and then subjected to either gel-based proteomic analysis (Gel-LC/MS) to detect facilitative glucose transporters (GLUTs) by liquid chromatography tandem mass spectrometry (LC-MS/MS). Indirect immunofluorescence and confocal microscopy of axial lens sections from unincubated fixed lenses labelled with primary antibodies specific for GLUT 1 or GLUT 3 were utilised for protein localisation.

Results: SIL glucose uptake at 5 min was concentrated in the equatorial region of the lens. At later timepoints, glucose gradually distributed throughout the epithelium and the cortical lens fibres, and eventually the deeper lens nucleus. SIL glucose metabolites found in glycolysis, the sorbitol pathway, the pentose phosphate pathway, and UDP-glucose formation were mapped to specific lens regions, with distinct regional signal changes up to 20 h of incubation. Spatial proteomic analysis of the lens epithelium detected GLUT1 and GLUT3. GLUT3 was in higher abundance than GLUT1 throughout the epithelium, while GLUT1 was more abundant in lens fibre cells. Immunohistochemical mapping localised GLUT1 to epithelial and cortical fibre cell membranes.

Conclusion: The major uptake site of glucose in the bovine lens has been mapped to the lens equator. SIL glucose is rapidly metabolised in epithelial and fibre cells to many

Abbreviations: Da, Daltons; FT-ICR, Fourier Transform Ion Cyclotron Resonance; GC-MS, gas chromatography-mass spectrometry; Gel-LCMS, gel-based proteomics; GLUT, facilitative glucose transporter; IMS, imaging mass spectrometry; LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry; MALDI, matrix-assisted laser desorption/ionisation; MeOH, Methanol; m/z, mass-to-charge ratio; NEDC, N-(1-naphthyl) ethylenediamine dihydrochloride; PBS, phosphate buffered saline; SIL, stable isotope-labelled.
INTRODUCTION

As an avascular, transparent organ in the anterior eye, the ocular lens plays a crucial role in our sense of sight (Donaldson et al., 2017). The transparent and refractive properties of the lens are established by a unique tissue architecture that minimizes light scattering and a geometry and refractive index gradient (GRIN) that contributes to the correct focusing of light onto the retina (Donaldson et al., 2017). The anterior surface of the lens consists of a single layer of epithelial cells which at the lens equator divide and initiate a process of cell differentiation to form highly elongated secondary lens fibre cells that reside in the outer cortex of the lens. As part of this differentiation process, cell nuclei and other organelles such as mitochondria are degraded in these fibre cells in order to remove these light scattering elements from the light pathway (Bassnett, 2002; Bassnett, 2009). In addition, these differentiating fibre (DF) cells express new proteins, including the crystallins, which contribute to the formation of the GRIN (Ullhorn et al., 2008). When DF cells lose their organelles, they become mature fibre cells in the inner cortex of the lens that in turn surround the primary fibre cells that were initially laid down in uveo and which form the nucleus or core of the lens (McAvoi et al., 1999). This continual process of epithelial cell division, fibre cell differentiation and internalization of existing cells means that the lens grows throughout life and that a gradient of fibre cells of different ages with distinctly different properties and metabolic demands exists in the lens. While it is the structural organisation of the lens that establishes the transparent and refractive properties of the lens, the lens is not a passive optical element (Donaldson et al., 2017), but a biological tissue that requires the input of energy to drive the structural and functional processes that actively maintain its optical properties. The primary source of energy for the lens is glucose, which in the absence of a blood supply must be directly sourced from the surrounding ocular humours, delivered to the different regions of the lens and subsequently metabolised.

In lieu of a blood supply, glucose is taken up from its surrounding humours, where it is present in the aqueous and vitreous humour at approximately 3.2 mM (Davies et al., 1964) and 3.0 mM (Kokavec et al., 2016) in humans, respectively. While the normal concentration of glucose within the lens itself varies between species, it is in the order of 10 mg/100 g tissue (Kuck, 1965). The mechanism of how glucose is delivered and taken up from the surrounding humours has attracted significant research, and our understanding of these mechanisms has developed over time. It was initially hypothesised that glucose was only delivered to and taken up by the epithelial cells located on the lens anterior surface, with the transfer of glucose to the underlying fibre cell mass occurring via passive diffusion via a gap junction-mediated pathway (Goodenough et al., 1980). However, the discovery of glucose transporters in fibre cell membranes throughout the lens suggests that lens fibre cells can directly take up extracellular glucose in all regions of the lens (Merrimian-Smith et al., 1999; Merrimian-Smith et al., 2003; Lim et al., 2017). This regional distribution of glucose transporters is more consistent with the existence of an internal microcirculation system that has been proposed to use circulating fluxes of ions and water to drive the extracellular delivery of nutrients to the lens of the lens faster than can be achieved by passive diffusion alone (Donaldson et al., 2001; Mathias et al., 2007). The existence of this microcirculation system and its role in actively maintaining the lens’s optical properties has been established using a variety of techniques (Gao et al., 2011; Vaghefi et al., 2011; Candia et al., 2012; Vaghefi et al., 2011). Moreover, MRI studies that tracked the extracellular delivery of contrast agents to different lens regions show that solute delivery to the lens nucleus did indeed occur faster than would be expected by passive diffusion alone and that this delivery was abolished by inhibiting the microcirculation system (Vaghefi and Donaldson, 2018). Although these studies did not specifically study glucose delivery, they suggest that small solutes like glucose enter the lens at the anterior and posterior poles via an extracellular pathway that preferentially delivers them to the lens nucleus where they can then be taken up into fibre cells by the transporters known to be expressed in these cells.

Glucose uptake is mediated by two major protein families: the GLUT family that mediates the facilitative diffusion of glucose; and the SGLT family that utilises the energy stored in the Na⁺ electrochemical gradient to actively accumulate intracellular glucose (Wright et al., 2011). GLUT and SGLT isoforms have different affinities for glucose, allowing it to be taken up from extracellular environments with varying glucose levels. The presence and spatial distributions of GLUTs and SGLTs in the lens have been investigated at the mRNA and protein level in a number of species, including mouse, rat (Merrimian-Smith et al., 1999; Merrimian-Smith et al., 2003), bovine, and humans (Lim et al., 2017). Both GLUT1 and GLUT3 have been found in rodent and human lenses, while in the bovine lens, only GLUT1 has been found by Western blotting (Lim et al., 2017). It is clear that GLUTs are essential for lens transparency since the knockout of GLUT1 in a mouse model led to lens cataract formation (Swarup et al., 2018).

Once inside the cell, glucose can be utilised in many metabolic processes to release energy in the form of ATP, which is required to maintain the structural integrity and, therefore, transparency of the lens (Vrensen, 1991; Donaldson et al., 2017). In the lens, it is primarily metabolised by three pathways (Figure 1): glycolysis (Kinoshita, 1953), the pentose phosphate pathway (hexose monophosphate shunt) (Giblin et al., 1981), and the polyol pathway (Dvorin et al., 1973; Kinoshita, 1990). Glycolysis is essential to maintain lens physiological function and tissue
transparency over many decades of life since perturbations to major enzyme-mediated steps in glycolysis result in lens swelling and cataract (Kinoshita, 1955; Hejtmanik et al., 2015). The lens has a similar glycolytic pathway to other tissues, and within the lens, it was traditionally believed that the primary location of glucose metabolism in the lens was the epithelium (Swarup et al., 2018). For example, aerobic glycolysis and metabolism via the citric acid cycle are possible only in the lens epithelium and peripheral fibre cells that contain cell nuclei, mitochondria, and other cellular organelles. However, the aerobic metabolism performed in the lens only accounts for ~20–30% of the total lens ATP production, while only consuming ~3% of the glucose supplied to the lens (Hockwin et al., 1971; Trayhurn and Van Heyningen, 1972). The remaining 70% of lens glucose metabolism is carried out under anaerobic conditions (Bron et al., 1993), which produces lactate that is thought to contribute to a measurable pH gradient from the periphery to the centre of the lens (Bassnett and Duncan, 1986).

The environment in which the lens sits also plays a role in determining metabolic function and lens transparency. The lens sits in a mildly hypoxic environment in the normal eye, yet it can actively control its ion balance, maintain ATP levels, and synthesise proteins. However, exposure of cultured lenses to oxidative stress induced by exposure to hydrogen peroxide (Giblin et al., 1986) or hyperbaric oxygen (Giblin et al., 1988) stimulates the pentose phosphate pathway by increasing hexokinase activity (Hejtmanik et al., 2015). Despite not generating a large amount of ATP, the pentose phosphate pathway is essential because it uses the enzyme glucose-6-phosphate dehydrogenase to synthesise substantial amounts of NADPH (Figure 1) that is used by glutathione reductase to maintain redox balance in the lens by regenerating GSH from oxidised GSSG (Giblin et al., 1981). In addition, NAPDH is used by the third glucose metabolic pathway in the lens, the polyol (sorbitol) pathway. This route was initially described by van Heyningen in 1959, who observed the accumulation of polyols in the lens (Dvornik et al., 1973). In this pathway, sorbitol is formed by aldose reductase using NADPH as a cofactor, which is then converted to fructose by a second enzyme, polyol (sorbitol) dehydrogenase, using nicotinamide adenine dinucleotide (NAD+) as a coenzyme (Figure 1). Under normal physiological conditions, almost one-third of the glucose entering the human lens is metabolised through this sorbitol pathway (Jedrziak et al., 1981). In the human lens, the majority of aldose reductase, and its activity, is present predominantly in the epithelium (Jedrziak et al., 1981). Elevated levels of sugars such as glucose in the blood and AH, as occurs in diabetes (Davies et al., 1984), have been linked to hyperglycaemia-related changes to the lens and cortical cataract formation (Bron et al., 1993). Sorbitol is osmotically active, and therefore an overabundance of sorbitol can draw additional water inside the cell, causing cell swelling and damage, which disrupts the ordered lens cell architecture to cause light scattering. While this mechanism has been demonstrated in species such as dogs (Murata et al.,
2001) and rodents (Kador et al., 2007), aldose reductase activity in the human lens is lower (Jedziniak et al., 1981), and its role in the aetiology of diabetic cataract is less clear.

Not only do they appear to be important differences in the activity of metabolic pathways between species, recent molecular screening also suggests that there are some important differences in the expression of GLUTs and SGLTs between rats, bovine and human lenses. Due to the unpredictable supply of human lenses that can be used in functional experiments, the bovine lens may be a good model to study lens function and cataract formation. Therefore, a previously established normoglycemic model of ex vivo bovine lens incubations that utilises stable isotonically-labelled (3l)cqglucose (Zahraei et al., 2020) has been characterised in the current study. Glucose utilisation and metabolite transport were traced through time by MALDI (Matrix-assisted laser desorption/ionisation) IMS (imaging mass spectrometry), with SIL metabolite validation performed via gas chromatography-mass spectrometry (GC-MS).

In addition, proteomics was used to confirm the presence of GLUTs in the bovine lens and immunohistochemistry to localise GLUT transporters that mediate uptake of glucose from the humour.

MALDI-IMS showed the initial sites of SIL glucose uptake and metabolism occurred predominantly in the epithelial and DF cells located at the lens equator. However, longer incubations of up to 20 h showed that SIL metabolites were able to penetrate to other regions of the lens, including the lens nucleus. Interestingly, a proteomics approach detected both GLUT1 and GLUT5 in normal bovine lenses, and their relative abundance changed as epithelial cells differentiated into fibre cells. Taken together, this work establishes the metabolomic and proteomic profiles of glucose metabolism of the normal lens, which can be used as a baseline for future spatially resolved screening of the metabolic changes that occur in diabetic cataract.

MATERIALS AND METHODS

Tissue and Reagents

Commercially available primary antibodies that were used for immunohistochemistry (IHC) to detect glucose transporters in the bovine lens are detailed in Supplementary Table S1. The secondary antibody goat anti-rabbit IgG-Alexa Fluor® 488 and the nucleic cell marker DAPI were both purchased from ThermoFisher Scientific (Waltham, MA, United States). The cell membrane marker WGA-Alexa Fluor 594 was purchased from Life Technologies (Carlsbad, CA). Phosphate buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) was obtained from Sigma-Aldrich (St Louis, MO). All solvents used were HPLC grade, and other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Ex vivo Lens Incubations

Fresh bovine eyes were collected from a local abattoir (Auckland Meat Processors, Ltd.). Lenses were dissected from ocular globes anteriorly and directly incubated in 12 ml of pre-warmed isotonic artificial aqueous humour (AAH, 120 mM NaCl, 0.5 mM MgSO4, 4.5 mM KCl, 26 mM NaHCO3, 2 mM CaCl2, 1 mM NaH2PO4·H2O, 10 mM HEPES, pH 7.2–7.4, 290 ± 5 mM Osm/L) containing 5 mM stable isotonically-labelled (3l)cqglucose ([U-3l]cqglucose, Ms = 186.110], with un-labelled AAF containing glucose ([13C6]cqglucose, Ms = 180.063) as a control. Lenses were incubated for times that ranged from 5 min to 20 h at 37°C in 5% CO2 using a tissue incubator (Heracell VIOS 160 CO2 incubator, ThermoFisher Scientific, Waltham, MA, United States). Following incubation, samples were rinsed three times in 20 ml isotonic AAH to remove any SIL glucose on the lens tissue surface and then stored at −80°C (Zahraei et al., 2020).

Tissue Preparation for MALDI-IMS

Frozen lenses were mounted on cold chucks using Cryomatrix™ compound (ThermoFisher Scientific, Waltham, MA, United States). A cryostat (Leica CM3050S, Leica Microsystems GmbH, Wetzlar, Germany) was used to cut axial sections with a thickness of 20 μm. Sections were then immediately mounted on cooled double-sided carbon tape (ProSciTech, Kirwan, Australia) attached to non-conductive glass slides (PINK COLORFROST, LabServ, NZ). Collected sections were stored in a vacuum desiccator for at least 1 h and equilibrated to room temperature immediately before the application of the matrix. A N-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) solution (7 mg/mL in 99% EtOH) containing an internal standard (IS) 3-OMG ([M + CD = m/z 229.0473] was applied using a TM-Sprayer (HTX Technologies, Carrboro, NC). Following matrix/IS spraying, slides were stored in a vacuum desiccator until used for data acquisition (Zahraei et al., 2020).

MALDI Imaging Mass Spectrometry

MALDI-IMS of whole bovine lens sections was acquired using a raster step size of 150 μm on Solarix XR 7T FT-ICR instrument using FTMS Control v2.2.0 and flexImaging v5.0 software (Bruker Daltonics, Billerica, MA). Higher spatial sampling using a raster step size of 30 μm was used to assess SIL glucose uptake in specific lens cortical regions. Tissues were analysed in negative-ion mode using adjacent sections and data acquired in the mass-to-charge ratio (m/z) range of m/z 100–1,000 with a resolving power (m/Δm) of 66,000 at m/z 400. The laser scanned across sections in x and y, with 200 laser shots summed for each position at a repetition rate of 1 kHz. While laser power was optimised for each data set, the laser beam dimensions were matched to the raster step size by setting it to "medium" for all overview images collected and "small" for higher spatial sampling data. Before the acquisition, an external m/z calibration was applied using red phosphorus in MALDI MS negative ion mode. Since sections from all timepoints could not fit into the instrument simultaneously, samples were run immediately following each other to minimise sampling variability. Lens sections were also sampled in random order to avoid sampling order artefacts. Data sets from all incubation timepoints were then imported into SCiLS Lab 3D (version 2020 Pro, SCiLS GmbH, Bremen, Germany), and combined into a single data set. MALDI images were plotted at the theoretical
m/z ± 0.005 Da with normalisation to the IS signal without denoising function. Identities were assigned based on accurate mass matching, isotope distribution analysis, and comparison to GC-MS data where possible (see below) (Zahraei et al., 2020). A subset of metabolites MALDI images was plotted as 3D surface plots, and intensity profiles generated to show signal intensity changes across lens sections using Image software (v1.49 m, National Institutes of Health, United States).

**Immunohistochemistry and Confocal Microscopy**

Normal bovine lenses were fixed in 2% paraformaldehyde that contained 0.01% glutaraldehyde for 72 h immediately following removal from the ocular globe. Prior to labelling 16 μm thick axial lens sections with primary antibody, sections were permeabilised in 0.5% Tween 20® for 20 min before incubation in blocking solution (3% BSA, 3% Normal Goat Serum, in x1PBS) for 1 h. Sections were then incubated overnight in GLUT1 or GLUT3 primary antibodies (Supplementary Table S1) diluted 1:200 in blocking solution. After incubation, sections were washed in x1PBS and incubated in a secondary antibody conjugated to Alexa Fluor-488. Sections were then washed and incubated with a mixture of Alexa Fluor WGA-594 and DAPI diluted at 1:100 and 1:1,000, respectively, in PBS for 1 h at room temperature to visualise the lens cell morphology (Lim et al., 2017). Following a series of washes in PBS, sections were mounted in Vectashield (Vector Laboratories, CA, United States) and imaged using a ×60 objective lens on an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) with Fluoview 2.0c software. Digital images (2048 × 2048 pixels) were collected using optimised gain settings that captured the entire dynamic range of the signal at each tissue location. Images were pseudo-coloured using Adobe Photoshop software (v23.1.1, Adobe Inc., San Jose, California).

**Tissue Microdissection for Metabolite and Protein Analysis**

Bovine lenses (either fresh, untreated or cultured with SII glucose as above, n = 6) were micro-dissected with tweezers. The capsule containing the lens epithelium was peeled away from the underlying lens fibre cells. An 8 mm surgical punch was used to collect samples from central, peripheral and equatorial regions of the epithelium, and samples pooled for protein preparation. Samples from peripheral fibres were also collected by using tweezers to peel off cellular material from the periphery of the decapsulated lenses. The denser underlying inner cortical tissue was removed to expose the hard, central nucleus, and tweezers were used to collect fibre cells from this central lens region.

**Protein Preparation and SDS-PAGE**

Tissues from each micro-dissected lens region were homogenised in an ice-cold lysis buffer (5 mM Tris, pH 8.0, 5 mM EDTA, 5 mM EGTA) that contained a protease inhibitor cocktail (Roche, Mannheim, Germany), using a hand-held homogeniser (Ultra-Turrax®, IKA-Werke, Staufen, Germany). Following centrifugation at 15,000 g for 30 min at 4°C, the pellet was separated from the supernatant. Both the pellet and supernatant were loaded and separated on an 10% SDS PAGE gel (Mini-PROTEAN® Bio-Rad Laboratories Inc., Hercules, California, United States), following standard protocols.

**Gel-Based Proteomics (GelC/MS)**

A prominent band at the Mw 53 kDa, corresponding to the molecular weight of GLUTs, was cut from samples of each collected region using a 6 × 2 mm punch. The gel bands were placed in different 1.5 mL Eppendorf tubes and diced into cubes. The gel bands were prepared for LC/MS analysis following standard protocols. Briefly, the bands were washed and de-stained with 50% acetonitrile in ammonium bicarbonate buffer (50 mM). Following de-staining, the bands were dehydrated with acetonitrile, reduced using dithiothreitol and coconitently alkylated with iodoacetamid. Subsequently, the bands were dehydrated again and were finally subjected to enzymatic digestion using 12.5 ng/μl porcine trypsin (Promega Corp., Madison, Wisconsin) in a temperature-controlled microwave at 45°C for 1 h. The digest was acidified to half digestion, and peptides were injected onto a 0.3 × 10 mm trap column packed with Reprosil C18 media (Dr. Maisch HPLC GmbH, Ammerbuch, Germany) and desalted for 5 min at 10 μL/min before being separated on a 0.075 × 200 mm picofrit column (New Objective, Inc., Littleton, Massachusetts, United States) packed in-house with 3 μm Reprosil C18 media. A 30%-50% solvent B gradient was applied at 300 nl/min using a NanoLC 400 UPLC system (Eksigent Technologies, Redwood City, California), where solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The picofrit spray was directed into a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (AB Sciex LLC, Framingham, Massachusetts) scanning from 300 to 2000 m/z for 200 ms, followed by a MS/MS scans on the 35 most abundant multiply-charged peptides (m/z 80–1,600). The mass spectrometer and HPLC system were controlled by the Analyst TF 1.7 software package (AB Sciex LLC, Framingham, Massachusetts, United States). The resulting MSMS data were searched against a database comprising Uniprot bovine entries appended with a set of common contaminant sequences using ProteinPilot version 5.0 (AB Sciex LLC, Framingham, Massachusetts, United States). Search parameters were as follows: Sample Type = Identification; Search Effort = Thorough; Cys Alkylation = Iodoacetamide; Digestion = Trypsin. The peptide summary exported from ProteinPilot was further processed in Excel (v2102, Microsoft Corp., Albuquerque, New Mexico) using a custom macro to remove proteins with Unused Scores below 1.3, to eliminate inferior or redundant peptide spectral matches, and to sum the intensities for all unique peptides from each protein.

**GC-MS Analysis**

Micro-dissected fibre cell regions were placed in separate falcon tubes containing 2 ml of 90% MeOH. Samples were agitated and homogenised in 90% MeOH for 45 min at 4°C. The remaining cell debris was removed via centrifugation at 16,000 g for 20 min at 4°C in an Eppendorf 5420 centrifuge, and supernatants
containing extracted small molecules were kept at -80°C until required for analysis. Samples were then dried in a Speed-vac Concentrator (Thermo SPD121P, ThermoFisher Scientific, Waltham, MA, United States) and underwent automated trimethylsilyl derivatisation in preparation for analysis to identify SIL sugars using an Agilent 7890B gas chromatograph coupled to a 5977A inert mass spectrometer (Zarate et al., 2017). Instrument parameters for GC-MS analysis were based on Villas-Boas et al. (2006) and analysis was performed according to Zahrzei et al. (2020). GC-MS data were converted into .cif files by the function provided in the Automated Mass Spectral Deconvolution and Identification System (AMIDS). A sub-library of the spectral database was generated by searching the QC samples against the NIST17 database. The identification result was then combined with an amino acid and sugar standard reference spectral library. Spectra of SIL molecules of interest were annotated and curated manually with the assistance of the NIST17 database.

RESULTS

We have used a multi-omics approach to characterise the delivery, uptake, and metabolism of glucose in different regions of the bovine lens. Spatial resolution at multiple scales has been preserved using tissue punches and confocal imaging for protein analysis and microdissection and MALDI-IMS for metabolite analysis.

Glucose Delivery

To initially assess the pattern of glucose uptake, ex vivo bovine lenses were incubated in AAH containing a normoglycaemic level of SIL glucose ([13C6] glucose), which due to its shift in mass (+6 amu) can be detected as a m/z signal in the mass spectrum that is distinct from the spectrum of endogenous glucose ([13C6]H12O6) and other isobaric metabolites. Initially, incubations from 5 min to 20 h were used to assess global uptake (Figure 2). A signal detected at m/z 221.0528 in the MALDI-IMS data was assigned as SIL glucose ([M + Cl−] since it was within 2 ppm of the predicted m/z of SIL glucose (Table 1), was not present in control lenses incubated in AAH containing unlabelled glucose (Figure 2A), and was previously not detected in control samples analysed by GC-MS (Zahrzei et al., 2020). Closer inspection of the spatial distribution of timepoints up to 1 h incubation showed initial uptake of SIL glucose occurred at the equator and then spread around the entire periphery of the lens. This signal was more uniformly distributed in the lens outer cortex at 2–8 h, while at 16 and 20 h SIL glucose signal had reduced markedly in the lens anterior region, and a very low signal was also detected in the lens nucleus. Digital dissection of three replicates of each timepoint into cortex and nucleus (Figure 2D) showed signal intensity changes for m/z 221.0528 were most prominent in the cortex, but the signal was also detectable in the nucleus at longer time points even though it was of low intensity relative to the cortex (Figure 2C). When intensity plots were normalised to the maximum signal in each region, the SIL glucose signal was present in the nucleus after 16 h of incubation in SIL glucose (Figure 2D).

Because of the low signal intensity of the SIL glucose signal in the deeper lens regions, we chose to focus on the initial (5–30 min) uptake of glucose in the epithelium and peripheral fibres (Figure 3). After a 5 min incubation, the SIL glucose signal (m/z 221.0528) was most intense around the equatorial region, with a higher signal in the epithelial and outer cortical region just anterior to the equator. Signal intensified after 15 min,
Extending both posteriorly and anteriorly, and continued to spread to the entire lens periphery after 30 min (Figure 3A). This preferential uptake of 35S glucose at different lens surface locations was quantified in the equatorial, anterior, and posterior regions (Figure 3B) and average changes in intensity were plotted as a function of time for three lenses (Figure 3C). This analysis showed 35S glucose uptake from the surrounding media was strongest in the equatorial region of the lens, before being detected in the anterior and posterior regions of the lens (Figure 3C). Together these results suggested a differential affinity or rate of glucose uptake occurred in the equatorial region of the lens that consisted of equatorial epithelial cells and DF cells.

Glucose Uptake
To determine whether the observed pattern of 35S glucose uptake was due to the presence of GLUTs at different quantities in different lens regions, proteomics with relative quantification was performed on membrane protein fractions prepared from the different tissue regions (Figure 4). In the first instance, tissue from epithelial explants (Figure 4A, left) and the cortical and nuclear regions (Figure 4A, right) of the lens were sampled to
determine the presence or absence of different GLUT isoforms in a non-quantitative manner. Cell membrane proteins from each preparation were separated via SDS-PAGE, and the gel band corresponding to the molecular weight of GLUTs (~53 kDa) was cut, trypticised, and peptides separated via reverse-phase HPLC and detected via MS/MS. Interestingly, this approach detected peptides from both GLUT isoforms 1 and 3 in each lens region (see Supplementary Figure S1). Further targeted mass spectrometry could be used in the future to determine relative amounts in the different lens fibre cell regions. Next, to focus on further understanding, the pattern of initial SIL glucose uptake revealed by MALDI-IMS in peripheral lens tissues, tissue punches from central, peripheral, and equatorial epithelial regions were collected and compared to outer cortical fibre cells harvested from the lens equatorial region (Figure 4A), using the above Gel-ICP approach.

GLUT1 was detected in all lens epithelial regions, and in lens fibre cells, with a combined peptide number of 11 across all regions (Figure 4B, upper), confirming the previous detection in the bovine lens by Western blot analysis (Lim et al., 2017). Several other proteins were detected in this band, with almost complete coverage of vimentin (data not shown), an intermediate filament protein that forms part of the cytoskeleton which is known to be expressed in the lens epithelium and DF cells (Sandlands et al., 1991). Consistent with the less spatially resolved sampling above, GLUT3 was also detected in all lens epithelial regions and outer lens fibre cells, with 16 peptides detected across all analysed regions (Figure 4B, lower). When GLUT signals were normalised to the vimentin signal to control for different cell densities that are present in different lens epithelial regions (Wu et al., 2015), the levels of GLUT1 remained relatively constant (Figure 4C). In contrast, the levels of GLUT3 trended down from central to the equatorial epithelium (Figure 4D); however, these differences were deemed not statistically significant by ANOVA. Relative quantitation of GLUT1:GLUT3 showed that GLUT1 was less abundant than GLUT3 in all regions of the epithelium. However, in outer cortical fibre cells, that ratio reversed and GLUT1 was more abundant (Figure 4E). To further confirm the presence of GLUTs in the bovine lens, and determine their subcellular location, immunofluorescence confocal microscopy was utilised.

![Figure 4](image-url)
Axial lens sections were triple labelled with the cell membrane marker WGA (red), DAPI to mark cell nuclei (blue), and antibodies against GLUT1 or GLUT3 (green), and representative images of the epithelium and underlying fibre cells collected from designated regions from the anterior pole to equator (Figure 5A). For GLUT3, using an antibody targeting the C-terminal tail of the protein, no labelling was observed (data not shown). This was surprising since peptides from the C-terminus of GLUT3, the epitope for the majority of trialled GLUT3 antibodies (see Supplementary Table S1), were detected by mass spectrometry (see Figure 4B). However, this is consistent with previous Western blot analysis that was unable to detect GLUT3 in bovine lenses ([Johnson et al., 2017]). In contrast, GLUT1 was detected predominantly in the cell membrane of epithelial cells at the anterior pole, with a low level of labelling in underlying lens fibres (Figures 5B,F). A similar pattern was detected in lens epithelial cells positioned mid-way between the anterior pole and equator (Figures 5C,G). In epithelial cells located in the peripheral epithelial zone, GLUT1 immunolabelling was more diffuse, and some punctate labelling was also detected in the underlying lens fibres (Figures 5D,H). Finally, at the lens equator where lens epithelial cells are differentiating and elongating (i.e., the transitional zone), pronounced cell membrane labelling for GLUT1 was detected, in addition to punctate cytoplasmic labelling (Figures 5E,J). A low level of signal equivalent to anterior pole labelling levels was also detected in lens fibre cells at the posterior pole (data not shown). MALDI-IMS of sections from bovine lenses incubated for 30 min in AAH containing SIL glucose showed uptake of m/z 221.0528, assigned SIL glucose, in regions where GLUT1 immunolabelling was detected (Figures 5J–M). The spatial correlation of GLUT1 labelling and SIL glucose signal suggests that GLUT1 mediates the uptake of glucose in these specific regions of the normal bovine lens cortex. However, a role for GLUT3 cannot be ruled out, and genetic tools and/or pharmacological interventions could be used in the future to determine the relative roles of GLUTs 1 and 3 in glucose uptake in the bovine lens cortex.

**Glucose Metabolism**

Once taken up into the cell, glucose is rapidly metabolised to produce ATP to drive other signalling and metabolic processes and reducing equivalents such as NADH and NADPH. Since lens glucose is predominantly metabolised by glycolysis (to produce ATP), the pentose phosphate shunt (to produce NADPH), and the sorbitol pathway, analysis of the fate of SIL glucose was performed by mapping the distribution of SIL glucose metabolites in lenses incubated for up to 20 h in SIL glucose AAH (Figure 6). For reference, the pattern and time course of SIL glucose...
throughout the lens is shown again in Figure 6A. In addition to this SIL glucose signal, signals for SIL glucose metabolites from each of the three metabolic pathways for glucose were detected by MALDI-IMS (Figures 6B–F). The identities of these signals were validated through a combination of accurate mass matching, isotopic distribution comparison (Supplementary Figure S2) and previous GC-MS analysis of identically incubated lenses (Supplementary Figures S3–S5). The results for each pathway are summarised in Table 1 and presented below.

The glycolytic pathway: The initial step in glycolysis, and a number of other metabolic pathways, is the phosphorylation of glucose. After 5 min a very low signal was observed at m/z 265.0432, representing a SIL hexose-6-phosphate, which is likely glucose-6-phosphate, glucose-1-phosphate, fructose-1-phosphate, or potentially a mixture of these metabolites (Figure 6B). Previous GC-MS analysis supported the assignment of glucose-6-phosphate (G6P) to this signal (Zahraei et al., 2020). The signal at 5 min was localised to the same region of lens epithelium that takes up SIL glucose. In longer incubations, the signal for G6P distributed to the entire epithelium (by 15 min) and then throughout the entire peripheral cortex (at timepoints longer than 2 h). Finally, signal intensity was observed in the entire cortex after several hours of incubation. At 20 h, a low signal level was also detected in the nucleus.

Later steps in the glycolysis pathway result in the formation of fructose-1,6-bisphosphate (F16BP). A signal that matched the predicted m/z for SIL F16BP was detected at m/z 345.0106 (Figure 6C). It was detected at very low levels after 5 min of incubation and was restricted to the outer cortical fibre cells even after several hours of incubation. These relatively low signal levels may indicate either a lower concentration of F16BP, its relatively transient nature in the cell before being metabolised further or that the MALDI conditions used to detect it were not optimal. Previous reports have shown that this metabolite can also be detected by the MALDI matrices 9-AA (Deldicke et al., 2015; Nye-Wood et al., 2016) and 1,5-DAN (Liu et al., 2014; Calvano et al., 2018). Therefore in the future, more detailed mapping of glycolysis pathways in the lens may require the parallel use of multiple matrices optimised for specific metabolites.

The pentose phosphate pathway: In the oxidative phase of the pentose phosphate pathway, glucose-6-phosphate is metabolised
to ribulose-5-phosphate, and in the process, a number of reducing equivalents (NADPH) are produced, which help protect against oxidative stress by reducing oxidised glutathione. In the non-oxidative phase, ribulose-5-phosphate is metabolised in one of two ways to sedoheptulose-7-phosphate (STP) (Lal et al., 1995; Sawyergill et al., 1995). STP is then cyclised back via fructose-6-phosphate to undergo glycolysis, or can be further metabolised to erythrose 4-phosphate to be used in the synthesis of aromatic amino acids. A signal at m/z 295.0510, matching the predicted m/z for SIL STP, was detected in all lenses incubated in SIL glucose (Figure 4D). This signal was generally limited to the most peripheral tissue regions, suggesting localisation to peripheral fibre cells and epithelium, with some stronger regions of signal generally on the lens anterior surface. At longer timepoints, a low signal was detected in the lens cortex, in a region where signals for both SIL glucose and SIL G6P were also detected (see Figures 6A,B).

A signal at m/z 571.0772 was also detected, although only at timepoints longer than 30 min (Figure 6E). This signal matched the predicted m/z for SIL UDP-glucose, a metabolite that is a precursor to glycogen and is also involved in the synthesis of glycosphingolipids. Signal for SIL UDP-glucose was relatively uniform around the entire lens, initially appearing in the peripheral cortex (at 1 h) before increasing in intensity and spreading throughout the entire lens cortex. However, no SIL glycosphingolipids were detected, potentially due to their concentration being below the limit of detection, that their biosynthesis takes longer than the 20 h incubation period used in this study, or that MALDI sampling conditions were not optimised for glycosphingolipid detection.
The polyol pathway: Another major metabolic pathway present in the lens is the polyol (sorbitol) pathway, which converts glucose to sorbitol through the enzyme aldose reductase. Aldose reductase is known to be present in the bovine lens (Ed Corso et al., 1989), and its activity is elevated in hyperglycaemia (Srivastava et al., 2005). Under normal glucose conditions, a signal at m/z 223.0868 that matched the predicted m/z for SIL sorbitol was localised to the anterior lens surface after 30 min of incubation, indicating the formation of SIL sorbitol (Figure 6F). Over time, signal intensity for SIL sorbitol increased and was localised to the cortex at 8 h and longer timepoints. In addition, this signal was also detected in the lens nucleus at 16–20 h.

To more clearly display the SIL metabolite signals detected in the lens nucleus, a subset of the two-dimensional MALDI images presented in Figure 6 were re-plotted as three-dimensional surface plots, where both the colour and height of the signal represents the signal intensity. MALDI images of the six SIL metabolites detected in both 4 and 20 h incubations were plotted, and intensity plots of each signal through the lens equator were generated (Figure 7). While it is clear the MALDI-IMS did not detect SIL metabolite signals in the lens nucleus after only 4 h of incubation, signals for SIL glucose, and SIL sorbitol in particular, were evident in the nucleus after 20 h of incubation. These results tend to suggest that SIL glucose is being delivered to the nucleus, but the levels detected are reduced due to the local metabolism of glucose to sorbitol which appears to be accumulated in the lens.

**DISCUSSION**

In this study, we have used MALDI-IMS to visualise regional differences in the delivery, uptake and metabolism of SIL glucose in the bovine lens and have used proteomic analysis (LC-MS/MS) and immunohistochemistry to validate our findings. Using this multi-pronged approach, we have shown that SIL glucose initially preferentially enters the lens at the equator (Figure 2) in a region where the abundance of GLUT1 relative to GLUT 3 increases (Figure 4). Furthermore, the SIL glucose taken up in these peripheral regions of the lens was available for use in the three main glucose metabolism pathways as indicated by the presence of metabolites of SIL glucose metabolism (Figures 6, 7). At longer time points, SIL glucose and a subset of SIL glucose metabolites were detected in the deeper regions of the lens, albeit at a lower level than what was observed in the outer cortex of the lens (Figures 6, 7), a result that is consistent with the identification of GLUT1 transporters in the nucleus of the lens (Supplementary Figure S1; Lim et al., 2017). These results show that our novel approach can be used to map the delivery, uptake, and metabolism of glucose in the bovine lens and that there are differences in how glucose is metabolised in the different regions of the lens.

The results obtained in this study using MALDI-IMS are consistent with those obtained using Raman spectroscopy (Hu et al., 2015), and NMR approaches (Cheng et al., 1991; Nakamura et al., 2003; Sawada et al., 2005). In these previous studies, glucose uptake and its metabolism to sorbitol were mapped in bovine and rabbit lenses organ cultured under hyperglycaemic conditions. In bovine lenses exposed to 50 mM for up to 4 days, glucose and sorbitol were restricted to the outer cortex, with glucose levels decreasing over time following initial uptake and a corresponding increase in cortical sorbitol levels (Sawada et al., 2005). In rabbit lenses incubated in 35.5 mM glucose for 28 h, glucose was found throughout the lens but was more abundant in the outer cortex, while sorbitol was abundant in the lens nucleus, and lactate was also detected (Cheng et al., 1991). While valuable, these studies suffered from poor spatial resolution, poor time resolution, and a limited number of metabolites were detected. Using our higher resolution MALDI-IMS approach, we have also shown that glucose and sorbitol were also present predominantly in the lens outer cortex, with the accumulation of sorbitol following the depletion of glucose signal as it was metabolised by the polyol pathway, albeit in a normoglycemic model.

Since signals for SIL glucose are not normally detected in the lens (Figure 2A), we have been able to use MALDI-IMS to visualise the delivery of SIL glucose to different regions of the lens. However, although this initial delivery of SIL glucose from the bathing solution to the lens must occur via the extracellular space, MALDI-IMS does not have the resolution to determine whether the SIL glucose signal subsequently detected in a specific region of the lens originates in either the extracellular and/or intracellular compartments. Because of the relative volumes of the two compartments, we believe that the majority of the SIL glucose signal we detect in any given region is predominately intracellular SIL glucose. Supporting this is the rapid appearance of a signal for SIL sorbitol, which is generated from SIL glucose by the cytoplasmic enzyme aldose reductase. Thus, while our MALDI-IMS approach can detect where in the lens SIL glucose accumulates, it cannot resolve how that SIL glucose was delivered to a specific region of the lens. To resolve extracellular delivery of SIL glucose, next generation MALDI instruments or alternative higher spatial resolution IMS approaches such as secondary ion mass spectrometry will be required (Niehaus et al., 2019; Spivey et al., 2019).

Despite the above caveats about the ability of MALDI-IMS to resolve extracellular delivery of SIL glucose to the lens we believe the early time points of SIL glucose in the peripheral epithelium and equatorial region (see Figure 3) can be attributed to direct uptake of SIL glucose from the extracellular space in what appears to be a localised region of enhanced uptake. A similar equatorial region of enhanced uptake of radioactive cysteine was found in monkey lenses (Sweeney et al., 2003). The enhanced SIL glucose signal in this region does not appear to be due to a higher density of cells, which is a known feature of the peripheral epithelial region (Wu et al., 2015), since the proteomic data was normalised to vimentin signal to account for this. These results suggest that in this region of the lens, where epithelial cells differentiate into fibre cells that undergo extensive elongation, increased levels of glucose are required to meet the high energy demands of DNA replication and protein and lipid synthesis. The observed initial preferential uptake of SIL glucose in this equatorial region can be explained by enhanced penetration of SIL glucose into the lens due to the relative lack of tight junctions between epithelial cells in this region which has been observed for the mouse lens (Goodenough
et al., 1980). Alternatively, the observed uptake distribution could be due to an increasing cell membrane area in elongating cells, which has been proposed to explain the enhanced Na+/K+
ATPase channel current observed at the lens equator relative to the central epithelium (Lim et al., 2000).

Interestingly in the present study, both GLUT1 and GLUT3 were detected by our proteomic approach, a result that was consistent with a previous study that analysed samples collected from the bovine lens using laser micro-dissection (Wang et al., 2008). In contrast, antibody-based approaches to detect GLUT3 in the current study by immunofluorescence and previously by Western blot (Lim et al., 2017) failed to detect the protein in the bovine lens. While the precise epitopes of the GLUT3 antibodies used in this study are not known, a large portion of the C-terminal tail of GLUT3, which is the region targeted by most antibodies and two of the antibodies trailed here (see Supplementary Table S1), was detected by LC-MS/MS (see Figure 4B). Possible reasons that the antibody will not bind to a target sequence include inaccessibility, either through protein structure or interaction with other proteins, post-translational modification (PTM) to amino acids in the epitope, such as phosphorylation, or low homology between the sequence used to raise the antibody and the target protein in the tissue. Since unmodified peptides of GLUT3 were detected via LC-MS/MS, PTM is unlikely to explain the inability of the antibody to detect GLUT3. In addition, SDS-PAGE and Western blotting, as used previously, breaks protein-protein interactions yet was unable to detect GLUT3. This suggests that the GLUT3 antibody epitope is not masked by protein binding. Finally, and most likely is that the antibody may not bind with enough affinity to bovine GLUT3 since there may not be enough sequence homology between the murine and bovine sequences (see Supplementary Figure S6). In comparison to GLUT1, which has both homologous and non-homologous epitopes in human and bovine (Supplementary Figure S6A), the GLUT3 sequence appears more varied between species (Supplementary Figure S6B). Nevertheless, while the present study reports GLUT3 is indeed present throughout the bovine lens, GLUT1 appears to be the major isoform and is present in both epithelial and fibre cells. This is in contrast to the rat lens, where GLUT3 was determined to be the major isoform present in fibre cells (Merriman-Smith et al., 1999; Merriman-Smith et al., 2003), yet the current result is consistent with the previous report by Lim et al. (2017). Future experiments that either selectively inhibit these GLUTs, or use compounds that are selectively transported by these isoforms, may be utilised to determine the relative contributions that each isoform makes to the uptake of glucose in the bovine lens epithelium and peripheral fibres. It is also possible that other isoforms of GLUTs or SGLT2 are present in the bovine lens; however, previous Western blotting did not find SGLT1 or SGLT2 in the bovine lens tissue (Lim et al., 2017). Hence, the SGLT transporter family were not targeted for detection in the current study.

Once taken up into cells, we found that SIL glucose was able to be consumed by the three main glycolytic pathways in the lens since SIL glucose metabolites from each pathway could be detected by MALDI-IMS (Figure 6). Unfortunately, a higher number of SIL metabolites could not be detected by either MALDI or GC-MS, possibly because of the transient nature of some metabolites and their rapid metabolism to other metabolic intermediates. The failure to detect other metabolites in the IMS data could be due to the MALDI matrix conditions used, which were optimised for the detection of glucose and its immediate metabolites, and may lack the sensitivity to detect other common endogenous metabolites in glycolysis and the citric acid cycle that could be detected by employing other MALDI matrices (Dekker et al., 2015). In addition, further GC-MS analysis could be performed to target additional SIL metabolites for detection to study metabolic flux in the different lens regions.

While the highest signal intensities for SIL glucose were detected predominantly in the metabolically more active outer cortical regions of the lens, weak signals were also present in the lens nucleus, at longer incubation times (Figures 2, 6, 7). Furthermore, more intense signals for the metabolites SIL G6P and SIL sorbitol, relative to SIL glucose, were also detected in the lens nucleus at these longer incubation times. While the observed SIL glucose uptake pattern may represent uptake via GLUTs in the lens epithelium and outer cortical fibres, followed by passive diffusion via an intracellular pathway mediated by gap junctions (Goodenough et al., 1980), the appearance of SIL glucose and its metabolites in the nucleus within 20 hrs is too rapid to occur via passive diffusion alone. It has been predicted that the time required for molecules such as glucose to move into the centre of a bovine lens via simple diffusion to be more on the order of several days (Mathias et al., 1997). Thus while the current experiments cannot rule out the possibility that the observed accumulation of SIL glucose and its metabolites in the lens nucleus occurs via an intracellular pathway, the time course is more consistent with a delivery via an extracellular route as has been detected for MRI contrast agents (Vaghfe et al., 2011). In these experiments, MRI contrast agents were used as an extracellular tracer molecule to show that solutes could be delivered to the nucleus faster than is predicted via passive diffusion (Vaghfe and Donaldson, 2018) via a pathway that was localised to the lens sutures (Vaghfe et al., 2012). If indeed SIL glucose was also delivered to the lens nucleus via this extracellular sutureal pathway, then the GLUTs, shown to present in the bovine lens nucleus (Figure 4), would then be able to transport it into nuclear lens fibres, where the local metabolism of this SIL glucose would produce the signals for SIL G6P, and SIL sorbitol observed in our MALDI images (Figures 6, 7). Consistent with this notion of local metabolism of glucose, a previous report has confirmed the presence of aldose reductase in the bovine lens nucleus (Liu et al., 1989). However, due to the previously mentioned spatial resolution limitations of the MALDI-IMS technique, it is not possible to confirm that the observed appearance of SIL glucose and its metabolites in the lens nucleus occurs via an extracellular pathway. While higher spatial resolution techniques will be required to directly visualise this movement, experiments designed to inhibit the microcirculation system (Vaghfe and Donaldson, 2018) will show whether this accumulation of SIL glucose and its metabolites is driven by the microcirculation system.
In summary, we have developed and validated an approach to map the delivery, uptake, and metabolism of glucose in the bovine lens. This approach can not only be used in the future to investigate how glucose is delivered to the lens nucleus by incorporating protocols to perturb the microcirculation, but also to determine what effects exposing the lens to hyperglycaemia have on glucose metabolism in the different lens regions. Hence using this combination of spatially resolved MS techniques, we should be able to determine the role played by the lens microcirculation in the delivery, uptake, and metabolism of glucose in the normal and diabetic lens.

CONCLUSION

Glucose is first detected entering the lens in the peripheral epithelial and equatorial regions according to MALDI-IMS. GLUT1 and GLUT3 are present in the bovine lens. GLUT1 is the predominant isoform in lens fibres, GLUT3 is the predominant form in the lens epithelium. Glucose is metabolised primarily in the epithelium and outer cortex, with some evidence of metabolism in the lens nucleus. Higher resolution techniques are required to show extracellular metabolites.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: The European Molecular Biology Laboratory’s European Bioinformatics Institute (EMBL-EBI) Proteomics Identification Database (PRIDE), https://www.ebi.ac.uk/pride/, PXD033022.

AUTHOR CONTRIBUTIONS

AZ performed all incubations, MALDI-IMS studies, and prepared samples for GC-MS and LC-MS/MS. GG performed quantitative analysis of MALDI-IMS, analysed GC-MS data, and provided signal identification validation data. KV prepared samples for LC-MS/MS and collected and analysed proteomics data. ND operated MALDI-FT-ICR equipment and provided technical interpretation and feedback. PD provided biological results interpretation and manuscript editing. AG conceived experiments and supervised work. All authors involved in drafting and editing final manuscript.

FUNDING

The Maurice and Phyllis Paykel Trust provided a grant-in-aid for small equipment (MALDI IMS matrix application device). The Health Research Council of New Zealand (#20/872) provides salary, stipend and consumable support for AZ, GG, and AG. The University of Auckland Mass Spectrometry Hub provides salary support for KV and GG.

ACKNOWLEDGMENTS

The authors wish to thank the Maurice and Phyllis Paykel Trust for small equipment funding, and the Health Research Council of New Zealand. The immunofluorescence imaging data reported in this paper were obtained at the Biomedical Imaging Research Unit (BIRU), operated and funded by Technical Services (Faculty of Medical and Health Sciences, UoA). The BIRU receives significant support from the School of Medical Sciences (Faculty of Medical and Health Sciences, UoA) through academic oversight and directorship. We also thank the University of Auckland Mass Spectrometry Hub for advice and assistance in sample preparation, data collection and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.901407/full#supplementary-material

REFERENCES


Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Appendix C: Conference abstracts

Presented at the Faculty of Medical and Health Sciences (FMHS) HealtheX conference, University of Auckland, Virtual, 15th September 2020, Oral presentation

Mapping Glucose Metabolites in the Normal Bovine Lens: Evaluation and Optimisation of a MALDI Imaging Mass Spectrometry Method

A. Zahraei¹, G. Guo¹, N.J. Demarais², P.J. Donaldson¹, A.C. Grey¹

¹The University of Auckland Faculty of Medical and Health Sciences, Auckland, Auckland, New Zealand
²The University of Auckland Faculty of Science, Auckland, Auckland, New Zealand
E-mail: ac.grey@auckland.ac.nz

Abstract

Background:
Glucose is one of the major nutrients in the lens, which is taken up by the lens from the aqueous humour. Elevated glucose can lead to diabetic cataract, so developing a method to visualise lens glucose uptake would be useful to study the lens pathology. A range of lens metabolites have previously been mapped by matrix assisted laser desorption/ionisation– imaging mass spectrometry (MALDI IMS); however, the tissue preparation and MALDI matrix conditions for glucose and glucose metabolite detection have not previously been investigated.

Objectives:
The aim of this study is to optimise a reproducible tissue preparation methodology to map uptake and metabolism of stable isotopically-labelled (SIL) glucose in cultured normal bovine lenses by MALDI IMS.

Methods:
Matrix choice, tissue preparation, and normalisation strategy were determined using MALDI IMS of bovine lens tissue.

Results:
With the appropriate MALDI IMS, matrix application and normalisation method established, ex vivo bovine lenses that had been incubated in normoglycemic AAH containing SIL glucose were analysed.
Using different time points of incubation, the initial uptake of SIL glucose was detected in the outer lens cortex, while its metabolism and transport into the deeper lens were also detected at longer time points.

Discussion:
A MALDI IMS method to detect and map glucose and related metabolites in the bovine lens was optimised and used to monitor glucose uptake and metabolism in normal bovine lenses under normoglycemic conditions. This method promises to be a powerful spatially-resolved tool to study the behaviour of glucose in a model of diabetic lens pathologies.
Evaluation and optimisation of a MALDI imaging mass spectrometry method to map glucose metabolites in the normal bovine lens

A.Zahraei¹, G. Guo¹², N.J. Demarais²³, P.J. Donaldson¹, A.C. Grey¹²

¹School of Medical Sciences, University of Auckland, New Zealand
²Mass Spectrometry Hub, University of Auckland, New Zealand
³School of Biological Sciences, University of Auckland, New Zealand
E-mail: ac.grey@auckland.ac.nz

Abstract

The ocular lens is a transparent cellular tissue suspended in the anterior segment of the eye, and functions to focus light onto the retina. The lens is not only a passive optical element but is also characterised by its own unique cellular structure, region-specific biochemistry, and physiological function that combines to produce lens optical performance. Glucose is one of the major nutrients in the lens, which is taken up by the lens from the aqueous humour. Elevated glucose can lead to diabetic cataract, so developing a method to visualise lens glucose uptake would be useful to study the lens pathology. A range of lens metabolites have previously been mapped by MALDI IMS; however, the tissue preparation and MALDI matrix conditions for glucose and glucose metabolite detection have not previously been investigated.

Objectives:

The aim of this study is to optimise a reproducible tissue preparation methodology to map uptake and metabolism of stable isotopically-labelled (SIL) glucose in cultured normal bovine lenses by MALDI IMS.

Methods:

Matrix choice, tissue preparation, and normalisation strategy were determined using MALDI IMS of bovine lens tissue. First, the best MALDI matrix for ionisation of SIL glucose was chosen using standard compared in several different MALDI matrices. Next, two tissue preparation approaches, fresh frozen and fixed tissues, were evaluated based on the molecular targets’ detection. Finally, to minimize the
cracking of tissue sections, the efficiency of applying an internal standard/matrix was assessed. These experiments were validated by complementary GC-MS analysis.

Results:
The optimised matrix and internal standard method used application of these simultaneously. With the appropriate MALDI IMS, matrix application and normalisation method established, ex vivo bovine lenses that had been incubated in normoglycaemic AAH containing SIL glucose were analysed. Using different time points of incubation, the initial uptake of SIL glucose was detected in the outer lens cortex, while its metabolism and transport into the deeper lens were also detected at longer time points. We detected two common metabolic glucose pathways in the lens. A MALDI IMS method to detect and map glucose and related metabolites in the bovine lens was optimised and used to monitor glucose uptake and metabolism in normal bovine lenses under normoglycaemic conditions. This method promises to be a powerful spatially-resolved tool to study the behaviour of glucose in a model of diabetic lens pathologies, with the potential to extend this approach to study the uptake, transport, and metabolism of novel anti-cataract therapeutic molecules.
Mapping glucose uptake, transport and metabolism in the bovine lens

A. Zahraei¹, K.G. Varnava¹, G. Guo¹, P.J. Donaldson¹, A.C. Grey¹, N.J. Demarais²

¹The University of Auckland Faculty of Medical and Health Sciences, Auckland, Auckland, New Zealand
²The University of Auckland Faculty of Science, Auckland, Auckland, New Zealand
E-mail: ac.grey@auckland.ac.nz

Abstract

Background:

As a primary nutrient, glucose is required to drive the functional processes that actively maintain lens transparency. In lieu of a blood supply, glucose is taken up from its surrounding humours, and it can be utilised in many metabolic processes.

Objectives:

This study aims to map glucose uptake and metabolism in cultured lenses and correlate the pattern of glucose uptake to glucose transporter distributions and abundance.

Methods:

Bovine lenses were incubated in artificial aqueous humour containing normoglycaemic stable isotopically-labelled (SIL) glucose. Following incubations, lenses were either fixed for immunohistochemical labelling and microscopy analysis or frozen for subsequent MALDI imaging mass spectrometry (IMS). The lens epithelial layer and fibre cell fractions were utilised for either IMS or proteomic analysis. Indirect immunofluorescence and confocal microscopy were utilised for protein localisation.

Results:

SIL glucose uptake was initially concentrated in the peripheral epithelium and lens equatorial region. Glucose is gradually distributed throughout the epithelium and the cortical lens fibres. SIL glucose metabolites from glycolysis, the sorbitol pathway, and the pentose phosphate pathway were also detected.
Spatial proteomic analysis of the lens epithelium detected GLUT1 and GLUT3. Immunohistochemical mapping localised GLUT1 to epithelial and cortical fibre cell membranes.

Discussion:

The major uptake site of glucose in the bovine lens has been mapped to the peripheral epithelium. SIL glucose is rapidly metabolised in epithelial and fibre cells to many metabolites, which are most abundant in the metabolically more active cortical fibre cells compared to central fibres.
Evaluation of glucose uptake and metabolism in the normal bovine lens by MALDI imaging mass spectrometry

A. Zahraei1, K.G. Varnava1,2, G. Guo1,2, N.J. Demarais2,3, P.J. Donaldson1, A.C. Grey1,2

1School of Medical Sciences, University of Auckland, New Zealand
2Mass Spectrometry Hub, University of Auckland, New Zealand
3School of Biological Sciences, University of Auckland, New Zealand
E-mail: ac.grey@auckland.ac.nz

Abstract

As a primary nutrient, glucose is required to drive the functional process to actively maintain lens transparency. In lieu of a blood supply, glucose is taken up from its surrounding humours, and it can be utilised in many metabolic processes. This study aims to map glucose uptake and metabolism in cultured normal bovine lenses and correlate the pattern of glucose uptake to glucose transporter distributions and abundance. Bovine lenses were incubated in artificial aqueous humour containing normoglycemic stable isotopically-labelled (SIL) glucose from 5min to 20 hours. Following incubations, lenses were frozen for subsequent MALDI imaging mass spectrometry (IMS). Along with the IMS experiment, a proteomic analysis was utilised to detect glucose transporters (GLUTs) throughout the lens, including lens epithelium. Spatial proteomic analysis detected GLUT1 and GLUT3 in different ratios in both epithelium and fibre cells. SIL glucose uptake at 5 mins was localised predominantly in the peripheral epithelium and lens equatorial region. Glucose gradually distributed throughout the epithelium and the cortical lens fibres in subsequent timepoints. SIL glucose metabolites were mapped in the glycolysis and pentose phosphate pathways. In addition, UDP-glucose formation was detected. Finally, the conversion of Glucose to sorbitol was detected, initially concentrated on the anterior pole, likely in the lens epithelium. The primary uptake site of glucose in the bovine lens has been mapped to the peripheral epithelium. SIL glucose is rapidly metabolised in epithelial and fibre cells to several metabolites, which are most abundant in the metabolically more active cortical fibre cells in comparison to central fibres. In the future we will apply this approach to map changes in a hyperglycaemia model to mimic diabetic...
cataract. This approach extends our understanding of normal lens function and may inform metabolic changes related to lens pathology.
Mapping glucose uptake and metabolism in the normal bovine lens

A. Zahraei¹, K.G. Varnava¹,², G. Guo¹,², N.J. Demarais²,³, P.J. Donaldson¹, A.C. Grey¹,²

¹School of Medical Sciences, University of Auckland, New Zealand
²Mass Spectrometry Hub, University of Auckland, New Zealand
³School of Biological Sciences, University of Auckland, New Zealand
E-mail: ac.grey@auckland.ac.nz

Introduction:
As a primary nutrient, glucose is required to drive the functional processes that actively maintain lens transparency. In lieu of a blood supply, glucose is taken up from its surrounding humours, and it can be utilised primarily in three metabolic pathways: glycolysis, the pentose phosphate pathway, and the polyol pathway. However, there is a lack of understanding of how glucose uptake and metabolism occur in the lens in physiological and pathological conditions. This study aims to optimise the methodology for the spatially precise mapping of glucose uptake and metabolism in cultured normal bovine lenses and correlate the pattern of glucose uptake to glucose transporter distributions and abundance.

Methods:
Bovine lenses were incubated in artificial aqueous humour containing normoglycaemic stable isotopically-labelled (SIL) glucose from 5 min to 20 hrs. Following these incubations, the lenses were frozen for subsequent MALDI IMS, or micro-dissected and analysed by GC-MS to validate the identification of spatially-mapped metabolites. MALDI IMS of lens sections was acquired using a spatial sampling resolution of 150 um on SolariX XR 7T FT-ICR. Data were acquired in negative mode and range m/z 100 – 1000. Additionally, glucose transporters (GLUTs) were mapped throughout the lens by spatial micro-punching of lens tissue regions and subsequent gel-based proteomic analysis.

Primary data (results):
SIL glucose uptake at 5 min was localised predominantly in the lens equatorial region. Glucose is gradually distributed throughout the epithelium and the cortical lens fibres and eventually to the deeper lens nucleus at subsequent timepoints. Several SIL glucose metabolites were also mapped and indicated
the presence of metabolism via glycolysis and the pentose phosphate pathway. Furthermore, the conversion of glucose to sorbitol was detected, initially concentrated at the anterior lens surface, likely in the lens epithelium. From its primary uptake site of the equatorial region of the lens, glucose was rapidly metabolised in epithelial and fibre cells into its metabolites, which were most abundant in the metabolically more active cortical fibre cells, in comparison to central fibres. Spatial proteomic analysis of the lens epithelium detected the presence and differential expression of GLUT1 and GLUT3. GLUT3 was expressed in higher abundance than GLUT1 throughout the epithelium, while GLUT1 was more abundant in lens fibre cells. The establishment of this methodology will allow us to map pathological changes in a hyperglycaemia model to mimic diabetic cataract. This approach extends our understanding of normal lens function and may inform metabolic changes related to lens pathology.

Mass spectrometry-related innovations (highlights):

This work establishes a consolidated research pipeline to study metabolomics and proteomics in the lens to enable future spatially-resolved screening of metabolic changes in a diabetic lens cataract model.
MAPPING GLUCOSE UPTAKE AND METABOLISM IN THE NORMAL BOVINE LENS

Ali Zahraei1, Kyriakos Varnava1,4, George Guo1,2, Nicholas Demarais1,2, Paul Donaldson1, Gus Grey1,2
1. Department of Physiology in the School of Medical Sciences, University of Auckland
2. Mass Spectrometry Hub, University of Auckland
3. School of Biological Sciences, University of Auckland

INTRODUCTION

Glucose is required to drive the functional processes that actively maintain lens transparency. The lens consists of an epithelial cell monolayer that covers a posterior surface (Figure 1A, yellow), while the bulk of the lens is made up of highly elongated fibre cells (Figure 1A, blue). Throughout life, epithelial cells divide and differentiate into fibre cells in the germinative region. Cell division, differentiation and elongation requires energy to synthesise new proteins and lipid that characterises lens fibre cells. Glucose is taken up from its surrounding humour and can be metabolised via several metabolic pathways (Figure 1B). However, there is a limited understanding of how glucose uptake and metabolism occur in the lens under normal physiological conditions.

RESULTS

Initial glucose uptake in bovine lens

![Initial glucose uptake in bovine lens](image)

GLUTs are expressed in the bovine lens

![GLUTs are expressed in the bovine lens](image)

CONCLUSION

GLUT1 and GLUT3 are expressed in the bovine lens. Specifically, GLUT1 is the predominant isoform in the lens fibres, and GLUT3 is the predominant form in the lens epithelium. Glucose first enters the lens in the peripheral epithelial and equatorial regions as evidenced by MALDI IMS. The posterior lens fibres also contain functional GLUT transporters. Glucose is metabolised primarily in the epithelium and outer cortex, with some evidence of metabolism in the lens nucleus.

METHODS

Freshly dissected bovine lenses were incubated from 5 minutes to 20 hours in artificial aqueous humour containing 5mM (5m) stable isotopically-labelled (SL) glucose. Then, the lenses were either frozen for subsequent MALDI IMS, or micro-dissected and analysed by GC-MS to validate the identity of spatially-mapped metabolites. MALDI IMS of lens sections used a spatial sampling resolution of 30μm and 100μm on a dynamic linear MALDI FT-ICR (Figure 2; method detailed in Zahraei et al., 2020). Data were acquired in positive ion mode in the range m/z 100 – 1000. Glucose transporters (GLUTs) were also detected throughout the lens by spatial micropunching of lens tissue regions and subsequent gel-based proteomic analysis. Finally, immunostaining and fluorescence confocal microscopy was used to localise GLUT1 in different lens regions (Zahraei et al., 2022).

CONCLUSION

GLUT1 and GLUT3 are expressed in the bovine lens. Specifically, GLUT1 is the predominant isoform in the lens fibres, and GLUT3 is the predominant form in the lens epithelium. Glucose first enters the lens in the peripheral epithelial and equatorial regions as evidenced by MALDI IMS. The posterior lens fibres also contain functional GLUT transporters. Glucose is metabolised primarily in the epithelium and outer cortex, with some evidence of metabolism in the lens nucleus.

SIGNIFICANCE

This work establishes a consolidated research pipeline to study metabolomics and proteomics in the lens to enable future spatially-resolved screening of metabolic changes in pathological models such as diabetic cataracts.
LIST OF REFERENCES


doi:10.3390/nu11051186


