The Colours of Diabetes
– Advances and novel applications of molecular optical techniques for studies of the pancreas

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1. ABSTRACT

Diabetes is a rapidly increasing health problem. In a global perspective, approximately 415 million people suffered from diabetes in 2015 and this number is predicted to increase to 640 million by 2040. To tackle this pandemic there is a need for better analytical tools by which we can increase our understanding of the disease. One discipline that has already provided much needed insight to diabetes etiology is optical molecular imaging. Using various forms of light it is possible to create an image of the analysed sample that can provide information about molecular mechanistic aspects of the disease and to follow spatial and temporal dynamics.

The overall aim of this thesis is to improve and adapt existing and novel optical imaging approaches for their specific use in diabetes research. Hereby, we have focused on three techniques: (I) Optical projection tomography (OPT), which can be described as the optical equivalent of x-ray computed tomography (CT), and two vibrational microspectroscopic (VMS) techniques, which records the unique vibrational signatures of molecules building up the sample: (II) Fourier-transform infrared vibrational microspectroscopy (FT-IR) and (III) Raman vibrational microspectroscopy (Raman).

The computational tools and hardware applications presented here generally improve OPT data quality, processing speed, sample size and channel capacity. Jointly, these developments enable OPT as a routine tool in diabetes research, facilitating aspects of e.g. pancreatic β-cell generation, proliferation, reprogramming, destruction and preservation to be studied throughout the pancreatic volume and in large cohorts of experimental animals. Further, a novel application of multivariate analysis of VMS data derived from pancreatic tissues is introduced. This approach enables detection of novel biochemical alterations in the pancreas during diabetes disease progression and can be used to confirm previously reported biochemical alterations, but at an earlier stage. Finally, our studies indicate that Raman imaging is applicable to in vivo studies of grafted islets of Langerhans, allowing for longitudinal studies of pancreatic islet biochemistry.
In summary, presented here are new and improved methods by which optical imaging techniques can be utilised to study 3D-spatial, quantitative and molecular/biochemical alterations of the normal and diseased pancreas.
2. PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers that will be referred to in the text by their corresponding roman numerals (I - IV).


* These authors contributed equally to this work

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3. PAPERS NOT INCLUDED IN THIS THESIS


4. ABBREVIATIONS

2D 2-dimensional
3D 3-dimensional
A-Value Alignment value
ACE Anterior chamber of the eye
BABB Benzyl alcohol benzyl benzoate
BCM $\beta$-cell mass
CCD Charged coupled device
CLAHE Contrast-limited adaptive histogram equalisation
CLSM Confocal laser scanning microscopy
COM Centre of mass
COM-AR Centre of mass - axis of rotation
DFTA Discrete Fourier Transform Alignment
DL Duodenal lobe of the pancreas
EM-OPT Emission - optical projection tomography
FBP Filtered back projection
FT-IR Fourier-transform infrared vibrational microspectroscopy
GDB Glutamic acid decarboxylase
GL Gastric lobe of the pancreas
GLP-1 Glucagonlike peptid-1
GLUT2 Glucose transporter 2
HDR High dynamic range
IA-2 Tyrosine phosphatase-like proteins insulinoma antigen-2
IDDM Insulin-dependent diabetes mellitus
IF-OPT Image-fusion - optical projection tomography
IHC Immunohistochemistry
IR Infrared
LADA Latent autoimmune diabetes of the adult
LSFM Light sheet fluorescence microscopy
MCR-ALS Multivariate curve resolution - alternating least squares
MODY Maturity onset diabetes in the young
MRI Magnetic resonance imaging
MVA Multivariate analysis
NIR Near-infrared
NIR-OPT Near-infrared optical projection tomography
NOD Non obese diabetic mouse
NOD-H2.b Mice congenic to NOD
OCT Optical coherence tomography
OPLS-DA Orthogonal projections to latent structures - discriminant analysis
OPT Optical projection tomography
PET Positron emission tomography
RF Radiofrequency
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>SL</td>
<td>Splenic lobe of the pancreas</td>
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<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
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<td>SPECT</td>
<td>Single-photon emission tomography</td>
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<td>SPIM</td>
<td>Selective plane illumination microscopy</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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<td>T1D</td>
<td>Type 1 diabetes</td>
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<td>T2D</td>
<td>Type 2 diabetes</td>
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<tr>
<td>TM-OPT</td>
<td>Transmission - optical projection tomography</td>
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<tr>
<td>UM</td>
<td>Ultramicroscopy</td>
</tr>
<tr>
<td>VMS</td>
<td>Vibrational microspectroscopy</td>
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<td>WHO</td>
<td>World health organisation</td>
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5. INTRODUCTION
Diabetes is one of the fastest growing health issues in the world. An estimated 415 million people were diagnosed with the disease in 2015 and this number is predicted to increase to 640 million by 2040. The social costs associated with diabetes, such as health service, loss in productivity and disability benefits, is estimated to be over 670 billion USD annually and this figure is predicted to exceed 800 billion USD by 2040.

5.1 The pancreas
An organ that plays a key role in the development of diabetes is the pancreas. The pancreas is an endodermally derived organ with three primary lobes: the splenic lobe (SL), the gastric lobe (GL) and the duodenal lobe (DL). The organ has two distinct functions; (I) an exocrine function, where digestive enzymes are secreted into the intestine to ensure nutrient uptake and (II), an endocrine function, where hormones are secreted into the blood stream to regulate blood homeostasis and food intake. The exocrine part of the pancreas makes up ~90% of the pancreatic mass and is composed of acinar cells, which produce digestive enzymes (proteases, lipases and amylases) that aid in the breakdown of food to facilitate nutrient uptake by the intestines. The much smaller endocrine component (~1–2%) is composed of the islets of Langerhans and is found scattered throughout the organ. The islets of Langerhans are composed of alpha- (α), beta- (β), delta- (δ), epsilon- (ε) and pancreatic polypeptide (PP) or gamma- (γ) cells. These cells secrete the hormones insulin, amylin, glucagon, somatostatin, ghrelin and pancreatic polypeptide respectively. Glucagon and insulin from α- and β- cells are antagonistic hormones. After food intake the blood glucose levels rise resulting in an insulin release from the β-cells, which mediate the uptake and conversion of glucose to glycogen in muscle, liver and fat cells. During periods of low blood sugar, the α-cells release glucagon. This starts the transformation of glycogen to glucose which is released from the liver. Somatostatin acts as a regulator for α- and β- cell secretion and once it is released it stops glucagon and insulin from being secreted. The secretion of pancreatic somatostatin by δ-cells is regulated by many factors such as blood glucose concentration, gut acidity as well as paracrine signalling. The β-cells make
up the majority of the islets (~80 % in mice). In mice, the β - cells are tightly packed and located in the core of the islet with the other endocrine cells forming a surrounding envelope. In humans the endocrine cells are intermingled but the β-cells are still in majority.

5.2 Diabetes mellitus (DM)
The endocrine disease diabetes mellitus is characterised by high blood glucose, either due to the lack of insulin producing β-cells or the body’s inability to respond to insulin stimuli. In 1872, during an archaeological expedition to Luxor, Egypt, a 3500 year old papyrus was found that contained information regarding how to treat polyuria, which is one of the most common symptoms of diabetes. The earliest record of the word diabetes, as a description of the disease, is from 270 BC. The word diabetes comes from the Greek word diabainein that means “a siphon” believed to refer to that people suffering from the disease urinates extensively. In 1670, the word mellitus (sweet) was added by Tomas Willis so that the disease would not be confused with the unrelated hormonal disease diabetes insipidus. Diabetes is commonly divided into two major types: Type 1 (T1D) and Type 2 (T2D). There are however more forms and subtypes of this complex disease including gestational diabetes, pancreatitis induced diabetes, latent autoimmune diabetes of the adult (LADA), cortisone induced diabetes, maturity onset diabetes of the young (MODY) and Alzheimer’s disease (sometimes referred to as type 3 diabetes). In early days, diabetes was hard to treat and the only therapies existing were starvation diets. Fredrick M. Allen and Eliott P. Joslin were two American diabetes specialists that proved that lowering the calorie intake could prolong the life of diabetic patients. However the patients eventually died from malnutrition. It was not until Banting and Best discovered insulin in the 1920s that diabetes became treatable in such a way that the patient could live a relatively normal life. Symptoms of T1D and T2D include excessive urination, abnormal thirst, fatigue and unexplained weight loss. T1D develops fast (weeks to months) and the signs are often obvious. For T2D the symptoms are initially more difficult to detect since the disease develops subtle over years and symptoms is often discovered during routine check-ups.
5.2.1 T1D

T1D, or insulin-dependent diabetes mellitus (IDDM), is also known as juvenile onset diabetes and usually begins early in life, although it can also develop in adulthood. T1D accounts for around 5-10 % of diabetes cases. It is a chronic disease characterised by insulin deficiency. As in most cases of diabetes, the initial symptoms are increase in thirst (polydipsia), appetite (polyphagia) and urination (polyuria). In contrast to T2D, the symptoms can arise rapidly. Eisenbarth proposed a model in 1986 regarding the proneness and steps to acquire T1D. First, the effected individual should carry specific genotypes of the HLA (human leucocyte antigen) in IDDM1 (insulin-dependent diabetes mellitus locus 1) resulting in an increased genetic susceptibility towards developing T1D. Having this genotype in combination with other predisposing genotypes in addition to exposure to certain environmental factors may trigger an immune response. Such environmental factors may include infectious toxins or even food. The immune response triggers the activation of autoreactive lymphocytes that invade the pancreas and insulitis (inflammation of the islets of Langerhans) occurs. The destruction of the β–cells are primarily caused by T-cells (CD4+ and CD8+) . Present during this process is also pancreas specific autoantibodies targeting e.g. insulin, GAD (glutamic acid decarboxylase) and IA-2 (tyrosine phosphatase-like proteins insulinoma antigen-2). When the remaining β–cells cannot produce and secrete sufficient amounts of insulin to clear the blood from the glucose surplus, the person develops hyperglycaemia and is diagnosed with diabetes.

5.2.2 T2D

T2D is the most common type of diabetes ~90 % and is often referred to as adult onset diabetes, even though it has been reported in children below 15 years of age. The characteristics of T2D are insulin resistance, impaired insulin secretion and excessive glucagon secretion. T2D patients frequently suffer from hyperlipidaemia and hypertension, often referred to as metabolic syndromes, that increases the risk of cardiovascular diseases. In settings of insulin resistance, the β–cells try to compensate by increasing insulin production and secretion. Eventually the β–cells will become unable to secrete sufficient amounts of insulin, which results in hyperglycaemia. In healthy individuals, glucagon secretion is blocked by high
blood glucose. In a T2D patient however, the regulation of α-cell secretion is impaired and do not respond to hyperglycaemia, resulting in continuously high blood glucose levels 22,24.

Insufficient physical activity combined with obesity are strongly linked with T2D and it has been shown that lifestyle interventions such as dietary regulations and physical exercise in many cases effectively may prevent disease progression 25. Other treatments for T2D include Metformin (which lowers hepatic gluconeogenesis) Glucagonlike peptid-1 (GLP-1) agonists (which inhibits glucagon release and stimulates insulin secretion) and finally insulin, which many T2D patients eventually have to receive to regulate their blood sugar levels 26.

5.2.3 Rodent models for diabetes
Several animal models (primarily rodents such as mouse (Mus musculus) or rat (Rattus norvegicus) that mimic diabetes disease progression, in several or individual steps, have been created and/or identified. Diabetes in these models can be induced surgically (partial pancreatectomy), transgenically (e.g. by depleting or overexpressing genes involved in insulin signalling), chemically or genetically/spontaneous 27,28. In the most commonly utilized chemically induced diabetes models, the β-cells are destroyed by administrating cytotoxic compounds such as Alloxan or Streptozotocin (STZ). Both are glucose analogues that accumulate in the β–cells through the glucose transporter type 2 (GLUT2). These chemicals have different mechanisms of destroying the β–cell, Alloxan increases the cytosolic calcium concentration, which causes cell destruction, and STZ creates DNA damages and produces high levels of superoxide radicals leading to cell necrosis 29,30. Examples of genetic/spontaneous models are the bio breeding rat that develops T1D 31, Zucker Diabetic Fatty rat that is used for T2D and obesity research 32 and the db/db mouse that lacks the leptin receptor resulting in obesity and T2D 33. Two of the perhaps most frequently used mouse models in diabetes research are the Non Obese Diabetic (NOD) mouse model for T1D and the ob/ob mouse model for aspects on T2D.
5.2.4 The NOD mouse

The NOD mouse is frequently used in diabetes research as a model for autoimmune diabetes. In NOD, the disease develops spontaneously and the first signs of autoimmune T-cell infiltration into the pancreas can be detected around 3 - 4 weeks after birth, with full-blown insulitis at around 8-9 weeks and overt diabetes manifesting around 12-14 weeks of age \(^{34,35}\). However, there may be significant variations even within a litter. One of the strongest reasons for T1D susceptibility in the NOD mice is the Idd1 locus that encodes for the major histocompatibility complex class (MHC) II molecule I-Ag7. The human homologue, HLA-DQ8 has been shown to be able to replace the IA-g7 in transgenic mice with diabetes \(^{36}\). Susceptibility can also be linked to the sex of the mice with a higher incident of diabetes in females (60–80 %) compared to males (10 %) \(^{37}\). Apart from the Idd1, multiple other loci contributing to the development of T1D have been identified, where some have potential homologues in the human genome \(^{38}\). Furthermore, development of diabetes in the NOD mice is highly influenced by the general health status of the mice, e.g. animals affected by infections will not develop diabetes at the same rate \(^{39-41}\).

5.2.5 The ob/ob mouse

The leptin deficient \(ob/ob\) mouse \(^{42}\) has become a widely studied model for initial aspects of metabolic disturbances leading to type 2 diabetes, including insulin resistance and obesity \(^{43}\). \(Ob/ob\) mice are grossly overweight and are hyperglycaemic, hyperinsulinemic, have high blood pressure and high heart rate \(^{44}\). Their phenotype resembles symptoms commonly associated with human T2D. At birth, there are no visual differences between homozygote and heterozygote littermates, but at 2 weeks they develop hyperinsulinemia and exhibit significant weight gain. After weaning the mice suffer from overt hyperglycaemia and blood glucose continues to increase up to 4 months. At this point the mice have high food intake and grow rapidly \(^{45}\). The heterozygote littermates are often used as experimental controls \(^{46-48}\). \(Ob/ob\) mouse islets are hyperplasic and the \(\beta\)-cells are hypertrophic. The above features are most likely a way to compensate for the increased demand for insulin. If \(ob/ob\) mice are fasted over night the blood glucose lowers to nearly normal levels. Furthermore it is possible to revert most of the \(ob/ob\)
mice phenotypes by exogenic administration of leptin \(^{44}\). The \(\beta\)-cells in \(ob/ob\) mice also accumulate lipids leading to islet hyperlipidaemia which is one cause of \(\beta\)-cell malfunction \(^{49}\).

### 5.3 Imaging the pancreas in diabetes

The possibility to generate a precise and true visualisation of an organ, tissue or cell is of key importance to provide basic as well as detailed understanding of biological processes and to produce mechanistic insight into disease development. The anatomy of the pancreas with the comparably smaller endocrine portion spread throughout the much larger exocrine parenchyma, as well as its irregular shape and loose texture (rodents) makes it exceedingly difficult to produce an overview of the organ with sufficient resolution to detect and measure the islets of Langerhans. Moreover, the pancreas is positioned deep in the abdomen, close to other big organs and major blood vessels, which further complicates \textit{in vivo} analyses. Pancreatic \(\beta\)-cell mass (BCM) may strongly influence diabetes disease progression and the possibility to monitor islet distribution and BCM is a key element of many diabetes-related research undertakings. Commonly, the tedious and time-consuming task of intervallic sampling is used to measure BCM. Sections from different areas of the pancreas are gridded and mathematical formulas are used to estimate the BCM based on the cross-sectional area of the \(\beta\)-cells \(^{46,50,51}\). Recent improvements that reduce the amount of sections required increased the speed and also lowered the cost of the method \(^{52}\). However, it should be noted that stereological sampling techniques always provide a result that is an extrapolation of 2D data and that the spatial information is incomplete. Therefore, new and/or improved imaging techniques capable of visualizing and quantifying BCM, as well as other ongoing cell types, during diabetes disease progression is needed. This field has seen a remarkable development in the past decade and presented below are a selection of techniques that are in use or have potential for imaging in diabetes.

#### 5.3.1 Nuclear imaging techniques

Nuclear imaging techniques use radioactive tracers (PET, SPECT) or nuclear magnetic spin (MRI) to visualize the object to be imaged. These techniques are generally considered to be non-invasive. These techniques are standard and are used
in hospitals every day. Although nuclear imaging is not used as a routine screening for diabetes, recent studies on obesity and diabetes, fat monitoring as well as coronary artery calcification suggest that nuclear imaging techniques could aid in diabetes detection.

5.3.1.1 Magnetic resonance imaging (MRI)

MRI is a standard tool in hospitals to non-invasively visualize anatomical structures. The technique uses a strong static magnetic field to polarise hydrogen protons (typically from water) inside the object, aligning them with the magnetic field. This alignment will cause a wobbling of the hydrogen nucleus at a certain frequency, known as a Larmor frequency, which is proportional to the strength of the magnetic field. By changing this radiofrequency (RF) in pulses via a secondary magnetic field orthogonal to the static field, the wobble can be changed. Between the pulses of the RF, the field returns to its relaxant state and emits another frequency, which is recorded and converted into a tomographic image. By moving the object/patient along the magnetic static field, tomographic images are acquired that can be combined to form a larger 3D visualisation. However, the resolution of the images acquired is dependent on the strength of the magnets used to create the field. Higher resolution images require stronger magnets and high-resolution MRI machines are expensive, posing a problem for researchers aiming at using MRI for mouse models.

Contrast agents need to be used in order to use MRI to monitor β-cells. A contrast agent should possess the following criteria; it should be non-toxic, β-cell specific and give a strong and stable contrast so that it may be differentiated from other tissues. Given this, MRI has been implemented in diabetes research using animal models by applying nanoparticles coupled to a small molecule known to accumulate in β-cells. Balla et al. could achieve in vivo single cell detection of islets of Langerhans in mice. However, the detection was performed in a research MRI machine that possessed greater field strength than that of clinical machines, meaning that in vivo visualisation was not possible. In 2015, Gaglia et al. showed that with the use of MRI it is possible to detect early pancreatic inflammation in T1D patients using dextran-coated nanoparticles that are phagocytosed by macrophages.
5.3.1.2 Positron emission tomography (PET) and single-photon emission computed tomography (SPECT)

These imaging techniques require that radioactive tracer molecules are injected into the animal/patient. During the decay of the radioactive tracer gamma rays are emitted, which are collected by detectors placed around the object of interest. The information collected is used to trace the origins of the gamma rays in space and over time. With this information at hand, it is possible to create an image based on the radiotracers position. Two techniques that use gamma detection are positron emission tomography (PET) and single-photon emission computed tomography (SPECT). The difference between these two techniques lies in the tracer used. In SPECT imaging, a single gamma ray is emitted during the decay, which is collected by the detectors. In PET imaging, a positron is produced. The positron will react almost immediately with an electron and this reaction emits two gamma rays that are collected by the detectors \(^{57,58}\). In settings of diabetes, the SPECT and PET techniques have mainly been used for studies of peripheral tissues affected, e.g. to detect increased risks for cardiac disease \(^{59,60}\) or to study changes to blood flow in the brain or in glucose metabolism \(^{61}\). However, using PET with radioligands specific to the vesicular monoamine transporter 2 (VMAT2), expressed by \(\beta\)-cells, demonstrates the possibility to monitor changes in BCM over time \(^{62,63}\). Recently, Brom et al. demonstrated that radionuclide labelling of Exendin-3 (a GLP1 analog) could be used for SPECT based monitoring of BCM in vivo \(^{64}\). These, and other studies suggest that PET and SPECT holds great potential as tools for direct studies of pancreatic BCM, with the potential translation to clinical settings.

5.3.5 Optical imaging techniques

There are many types of optical imaging modalities. One thing they have in common is that they utilize light to create a visualisation of the object or the process investigated. The light used is often in the visual spectra. However, for optical imaging of biological tissues, the lower part of the near-infrared region (700 – 900 nm) is more favourable (Paper II). Listed below are a few established and novel optical imaging techniques already implemented or in development for diabetes research.
5.3.6 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) has become an imaging workhorse. Regular fluorescent microscopes are plagued by out of focus light that comes from areas that are in another focal plane to the object studied. To increase image quality in CLSM, the out of focus light is excluded by adding a pinhole. Commonly a specific laser is required for each desired wavelength for multichannel imaging. However, recent developments of white lasers with tuneable filters increase the detection capabilities for CLSM, allowing for multiple channels to be imaged using one laser. The technological development in the field of microscopy is always ongoing. For example, the 2014 Nobel Prize in chemistry was awarded to Betzin, Moerner and Hell for their invention of the super-resolution fluorescence microscope, a technique that is also referred to as nanoscopy. This technique increased the spatial resolution of fluorescent microscopes from ~250 nm down to <20 nm. With the use of a clearing agent matching the tissues refractive index (a measurement of the propagation of light through an object) penetration depths up to 100 μm has been achieved. In studies of the developing and adult pancreas, CLSM has been widely used by its own or in combination with other techniques to detect islet infiltration. However, the high resolution comes at a cost; the technique suffers from its inability to visualise large specimens and typically the penetration depth is less than 500 μm. Hence, the technique is not suitable for imaging of the intact pancreas, even in small experimental animals such as mice. Multiphoton microscopy may increase the penetration depth by using light of longer wavelengths less prone to photon attenuation from tissue. The main difference between multiphoton and regular CLSM is the excitation method, in regular CLSM the excitation light is usually of a shorter wavelength than the emission light, whereas in multi- and two-photon microscopy the excitation light is of longer wavelengths. Furthermore in these techniques excitation only takes place in the focal plane. A very interesting CLSM approach for diabetes research takes advantage of the eye as a natural body window. By transplanting islets into the anterior chamber of the eye (ACE), longitudinal high-resolution imaging studies of the islet of Langerhans has been made possible. These studies have been performed on mice as well as primates where the transplanted islets have been shown both to mimic the
phenotype of endogenous islets and to revert symptoms of diabetes in their host animal. Coppieters et al. presented a CLSM-based method for analysing the interaction between T- and β-cells during T1D in mice in vivo. Hereby, pancreata were extravitalised and imaged using a two-photon microscope to detect transgenically expressed markers.

5.3.7 Light sheet fluorescence microscopy (LSFM)

In light sheet fluorescence microscopy (LSFM), also known as selective plane illumination microscopy (SPIM) or ultramicroscopy (UM) the light is passed through the sample orthogonal to the lens. By only illuminating a small transversal section of the sample in the focal planes, the out of focus light is eliminated. The sample is moved through the light sheet (alternatively the light sheet is moved through the sample) to acquire images which are later put together to create a 3D image stack. LSFM has the capacity to visualise relatively large specimens at single-cell resolution, and the technique has recently been used to visualise the developmental anatomy of the pancreas. Although not suitable to image BCM distribution in pancreata from larger cohorts of animals, ongoing studies in our own laboratory suggest that LSFM may become a powerful complement to OPT by enabling investigation of areas of interest in studies of whole pancreatic specimens at higher resolution (Fig. 1).

Figure 1. LSFM allows for high resolution imaging of the islet of Langerhans in intact mouse pancreas. Image depicts hyperplastic islets of an adult ob/ob mouse duodenal lobe labelled for insulin. Volume rendering is done in the Drishti software. Scale bar: 250 µm. Picture by C. Nord and S. Parween.
5.3.8 **Optical coherence tomography (OCT)**

Optical coherence tomography (OCT) is a technique that is often described as ultrasound that uses light instead of sound waves. Light is directed through the tissue and the resulting light reflection, or backscatter, is used to create an image of the tissue analysed. The backscatter properties are different depending on tissue composition, making it possible to visualize structures without any prior labelling.\(^{76}\) However, OCT is not able to detect fluorescence, which in some cases may be a drawback. Further, absorption and light scattering of the tissue limit the imaging depth but structures up to 2 mm are feasible to image with a lateral resolution of \(>15\) \(\mu m\).\(^{77}\) Taking advantage of the fact that the backscattering of the islets of Langerhans is different from that of the surrounding exocrine parenchyma, in combination with recent developments that extends the focus of OCT, enables imaging as well as \textit{in vivo} quantitive assessments of murine islets of Langerhans.\(^{78,79}\) In 2015 Berclaz et al. provided a technique that combines OCT with the ACE model (described in section 7.4.5) to allow for label free, longitudinal, \textit{in vivo} assessments of islet function, vascularisation as well as islets of Langerhans T-cell infiltration.\(^{80}\)

5.3.9 **Optical projection tomography (OPT)**

OPT has since its introduction in 2002\(^ {81}\) proven to be a useful tool in 3D studies of specimen in the mm - cm range. As such it has in many respects filled a previous imaging gap by enabling assessments of specimens too large for CLSM. OPT has provided important information in studies of diverse model systems and organs, particularly in the field of developmental biology.\(^ {82-86}\)

OPT has two principal working modes: transmission and emission. In transmission mode (TM-OPT), the light is passed through the specimen and the projection view captures the absorption of the specimen. This mode can be used for \textit{in situ} hybridisation studies or for the detection of gene expression using \(\beta\)-galactosidase activity or other type of non-fluorescent dyes. In emission mode (EM-OPT), the specimen is labelled with fluorophores (usually conjugated to antibodies), which are excited by incoming light of a certain wavelength. The emitted light, at a different wavelength, is captured by the imaging chip of a charged couple device (CCD).
From here onwards, EM-OPT is referred to if not otherwise indicated. What distinguishes OPT from most other optical imaging modalities, is that the signal intensity in a pixel does not correspond to a certain coordinate in 3D space. Instead it corresponds to the sum of the intensities along/through a straight line of the sample (Fig. 2 D).

The OPT scanning procedure is conducted as follows: a specimen which has been previously embedded in low melting point agarose, cut into a quasi-cylindrical shape to reduce light diffraction and made transparent in a clearing solution is attached in the imaging chamber. The clearing solution is required to reduce the diffraction of the light from the tissue (In an ongoing study, our laboratory is working to adapt a range of alternative clearing agents for pancreatic imaging. Hereby, a clearing solution may be selected to meet the specific imaging demands, e.g. with regards to compatibility with transgenic reports, tissue expansion/shrinkage opacity etc.). Once mounted in the imaging chamber, the sample is rotated around its own axis, usually in steps of 0.45 or 0.9 degrees and an image is acquired at every step, resulting in a full documentation of the rotated specimen. The acquired projection images may then undergo post-acquisition enhancements (see section 7.1.2 and 7.3) or directly be reconstructed into tomographic sections using a filtered back-projection algorithm (FBP) (Fig. 3 B and C). The tomographic sections corresponding to the sample can then be visualized using imaging software (Fig. 3 D). There are multiple imaging softwares that can handle tomographic sections and do various measurements, such as volumetric quantification based on intensities.

Figure 2. The basic OPT scanning procedure. (A) The sample is mounted in an agarose block to immobilise the tissue and to facilitate handling during optical clearing. It is then attached to a stepper motor (B). (C) Lenses focus the light onto a CCD chip and a projection view (D) is captured every step during the 360° rotation. Image adapted from 81.
Figure 3. Illustration of the OPT process. (A) The sum of the intensities along a straight line through the sample is recorded into one pixel of a projection view. A thicker sample results in a higher intensity pixel. (B) Comparison of FBP with 8 projection views and (C) 800 projection views. (D) Tomographic images are stacked to recreate the sample in 3D. Red line (A) indicates the area from where the tomographic images (C and D) are derived.

Optimisation and adaption of OPT has brought the sample size from embryonic scale to intact or semi-intact organs (such as kidney, liver or pancreas) from adult research animals. As made possible by computational and IHC improvements, OPT’s capacity to visualise specifically labelled structures throughout the volume of adult organs presents new opportunities. In diabetes research, this possibility provided, a means by which the entire BCM distribution could be imaged and quantified in an intact mouse pancreas at high resolution (Fig. 4) 87.

Figure 4. Optical projection tomography allows for spatial and quantitative assessments of the rodent pancreas. The image depicts an adult mouse SL. The islets are reconstructed based on the signal from insulin antibody labelling (red) and the anatomy of the pancreas (grey) is based on tissue autofluorescence. Adapted from 87.
However, BCM quantification carried out with OPT was initially associated with a degree of manual editing of the tomographic sections, mainly including the removal of optical artefacts. Labelling β–cells immunohistochemically with an antibody recognising insulin most often results in differences in contrast between islets due to the amount of insulin present in the islets. During the reconstruction of the projection views an intensity range is set in order to account for this variation, with the main goal not to lose any data either due to saturation or by cutting out intensities. To equalise the intensity of the islets and to reduce user subjectivity as much as possible, contrast limited adaptive histogram equalization (CLAHE) was introduced and applied to the projection views, resulting in projection views with a more homogenous contrast and less intensity variability, facilitating the reconstruction setup. 88. As such, the OPT technique has facilitated a range of studies regarding aspects of normal β-cell distribution as well as in studies of β-cell destruction, proliferation and regeneration. Examples of studies in which OPT has played a pivotal role include the assessment of autoimmune destruction in the NOD model, showing that small islets are more sensitive to the autoimmune attack 35. The technology further played a role in demonstrating that the mouse pancreas harbours significantly more islets than previously reported and that there is a significant difference in β-cell mass as well as islet distribution between the lobes of the pancreas 88. OPT has further enabled the assessments of the impact from selective gene deletions on total pancreatic β-cell mass. E.g. a selective disruption of the T-cell factor 7-like 2 gene found to be associated with T2D through genome wide association studies was found by OPT to reduce the BCM with 30 % 89. OPT could also in a study presented by Van de Casteele et al. contribute to demonstrate that the bHLH transcription factor Neurogenin 3 is required for β-cell neogenesis and proliferation in murine pancreata subject to pancreatic duct ligation 90. At present a number of studies are being performed in our laboratory in which OPT plays a key role in addressing aspects of BCM dynamics in different diabetes disease models.

5.4 Biochemical profiling of the pancreas
Currently there exists no technique that provides accurate, unbiased, high throughput analysis of pancreatic tissue biochemical composition at the cellular level, especially if the spatial context is to be preserved. Most analyses are instead
conducted on circulating biomarkers or peripheral tissues. Pancreatic biopsies for routine diagnostics have been investigated but are considered unfeasible due to the high risk of post-operative complications. Imaging techniques such as PET, SPECT and MRI (discussed above) have relatively low spatial resolution and their dependency on the measurement of a contrast agent interacting with its target, reduce their potential to broadly study complex biochemical alterations that may occur in the pancreas during disease progression. Proteomics and metabolomics may provide abundant biochemical information. However, random screens using these techniques may prove difficult and prior knowledge is generally required to generate useful information. A powerful technique for biochemical profiling is vibrational microspectroscopy.

5.4.1 Vibrational microspectroscopy (VMS)

All molecules have repetitive periodic motions, known as vibrations. The frequencies of these vibrations are based on a number of factors such as the atoms involved, their symmetry and bond strengths. As a consequence, each compound has a unique set of vibrations with defined frequencies. In vibrational microspectroscopy (VMS) additional energy is added in the form of photons to these molecular vibrations and the resulting changes are recorded. There are three primary VMS techniques: (I) Near-infrared spectroscopy, (II) Fourier-transform infrared (FT-IR) microspectroscopy and (III) Raman microspectroscopy. Both FT-IR and Raman are non-invasive techniques and do not require external agents for detection. Instead a compound is detectable if exhibits molecular dipole moment changes (IR active), or changes in molecular polarisation (Raman active). Given this, not all compounds can be detected by VMS as not all vibrations are IR or Raman active such as noble and diatomic gases. Although it for some scientific questions may be disadvantageous not to be able to detect gases, it is for most studies assessing biomedical tissues highly beneficial. This is because, if diatomic gases were VMS active, air would pose a major problem for the analysis.

In FT-IR microspectroscopy, the absorption of polychromatic light by the sample is measured and plotted in the form of a spectrum. The location of bands /peaks in the spectrum carries information about the nature of the vibration(s), such as identity, whereas the intensity provides information about the abundance of
the vibration such as concentration. In Raman microspectroscopy, the energy is added in the form of monochromatic light and the so-called inelastic scattering of photons is tracked upon interaction with the sample. This inelastic scattering (Raman scatter effect) can be divided into two types: (I) stoke lines from photons that have lost energy through inelastic scattering and (II) anti-stoke lines from photons that have gained energy. The stoke and anti-stoke lines contain similar information about molecular composition and structure as FT-IR spectra. The amount of Raman scatter is very small compared to the Rayleigh scatter, one for every million photons. The Rayleigh (elastic scattering) are photons of the same wavelength as the added light. These have not interacted with the sample, and therefore contain little or no valuable information, and are discarded by filtering.

Different VMS techniques are complimentary. FT-IR microspectroscopy is fast and sensitive to polar bonds, but requires a high degree of sample preparation including the removal of water as the strong absorption of infrared light by water often disturbs the analysis. Water is often less of a problem for Raman microspectroscopy and sample preparation is much easier or not even required at all. However, Raman microspectroscopy is commonly slower than FT-IR microspectroscopy and autofluorescence problems frequently occur that mask the already weak signals. Another difference is the spatial resolution. For standard FT-IR microspectroscopy the diffraction is limited to the 5 - 20 \(\mu\)m range \(^{97}\) while the limiting factor for Raman microspectroscopy is the laser spot size which can be focused to sub-\(\mu\)m ranges.

FT-IR and Raman have previously been used to detect phenotypic markers of diseases \(^{98-100}\). In diabetes related research studies, VMS studies have mainly been conducted on peripheral tissues such as in monitoring of chemical alterations in the rat soleus muscle \(^{101}\) or changes in blood plasma proteins during T1D \(^{102}\). However, few studies have directly targeted the pancreas itself, and those that exist have mainly been performed \textit{ex vivo} by targeting a specific compound, e.g. beta amyloid deposits in the pancreas of T2D patients \(^{103}\). The reason for this primarily lies in problems related to biological diversity, the complexity of the acquired spectral data and (such as overlapping of non-diagnostic-bands (bands
masking each other)) and shifting of bands (a slight chemical variation shift of the vibrations so that the band is detected elsewhere in the spectra).

Raman and FT-IR hold the potential to acquire full spectral profiles of the pancreas that accounts for all chemical compounds observed simultaneously. Since all components may be monitored, the possibilities to detect new markers or changes related to the disease progression would increase in comparison to that if only one or a few specific, known components were targeted. However, the above mentioned problems need to be circumvented in order to utilize VMS techniques for complete biochemical profiling. One way to resolve issues of this kind is to use the multivariate analysis or multivariate image analysis.

5.4.2 Multivariate analysis
A multivariate image is an image that contains both spatial (x, y and z) and spectral dimensions. In image analyses, the goal is to extract information from the image useful for classification, characterisation and/or detection of features/defects/errors in the imaged object. This information is usually presented as numerical values based on image characteristics such as intensity or as in VMS spectral bands. Multivariate analysis (MVA) can simultaneously analyse the possible relationships between existing variables, allowing for analyses of extreme complexity to be performed. One type of MVA is orthogonal projections to latent structures-discriminant analysis (OPLS-DA) which is a dimensional reduction analysis based on the orthogonal projections to latent structures (O-PLS). This means that it reduces the number of variables by combining them into new principal components (PC). In short OPLS-DA tries to find the variation between pre-defined classes in the dataset. In previous studies of biological samples, OPLS-DA analyses of VMS data was able to differentiate between cell types in a mouse liver as well as detecting previously unknown chemical differences in cell types in the aspen wood. OPLS-DA has however previously not been implemented in studies of the diabetic pancreas.
6. AIMS OF THIS THESIS

- To develop strategies to generally improve structural features in data acquired by OPT, for specific use in pancreas imaging and diabetes research.

- To increase the sample size limit and multichannel capacity for OPT through hardware configuration improvements.

- To establish and to validate multivariate image analysis of vibrational microspectroscopic data as a means for broad biochemical profiling of the pancreas.
7. RESULTS AND DISCUSSION

7.1 Paper I: Image processing assisted algorithms for optical projection tomography

Since the introduction of OPT in 2002\(^1\), a number of bespoke and one commercial scanner have been constructed\(^8\). OPT scanners require meticulous calibration as well as quasi-perfect alignment of the imaged sample. If this is overlooked, the acquired data will have low or insufficient quality for further analysis. To facilitate pre- and post-acquisition processes in OPT imaging, as well as to reduce processing times, a set of computational tools were developed to aid the operator and to reduce user subjectivity.

7.1.1 Centre of mass-axis of rotation (COM-AR)

The way the sample is mounted in the OPT machine is elegant and will be discussed in Paper II section 7.2.3. The sample is rotated using a stepper motor before each projection view is acquired. Thereafter, the acquired projection views need to be aligned with their corresponding opposing view (180°). Therefore, the importance of sample positioning cannot be underestimated. If the sample is tumbling elliptically and/or is not rotating around its own axis, the resulting tomographic reconstructions will be blurry. The further away the samples centre of mass is from the axis of rotation the worse the artefacts will become. The tumbling motion will also be a limiting factor for the magnification used; less tumble enables higher magnification. It might be relatively easy to spot the centre of a sample that is rather homogenous in shape such as a liver lobe or a kidney, but for a more heterogeneously shaped structure, such as the pancreas, the centre of mass can be difficult to establish.

In an attempt to aid the user with positioning of the sample in OPT imaging, a previous presented method that uses the four opposing sample projections\(^9\). With this approach, the user decides where on the sample the region of interest (ROI) is rather than assuming that the whole sample is the ROI. This gives the operator the option to focus on a specific part of the specimen. However, it also means that the whole sample might not be optimally positioned on the rotational axis. In most OPT
assessments the entire object is the ROI. Therefore an approach that can accurately and objectively determine the centre of the entire specimen is of utmost importance. Other methods dealing with centre of rotation are either complex, focused on small specimen or strictly TM-OPT \textsuperscript{110-112}. Therefore we designed a protocol that determines the centre of mass by using two 90° projections, referred to as centre of mass-axis of rotation (COM-AR). This technique relies on the samples autofluorescence in combination with a specific signal derived from IHC labelling. The first step is to distinguish the structure from the background in the projections (Fig. 5).

<table>
<thead>
<tr>
<th>Input</th>
<th>COM calculation</th>
<th>COM superimposed</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="" /></td>
<td><img src="image2.png" alt="" /></td>
<td><img src="image3.png" alt="" /></td>
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**Figure 5. The COM-AR facilitates the positioning of the sample on the axis or rotation.** (A) The input projection view acquired in the GFP channel gives an outline of the sample based on its autofluorescence. (B) Binary image that is created for the COM calculation, black dashed line is the calculated COM of that view. (C) Specific signal projection, in this case derived from β–cells labelled with antibodies against insulin, COM superimposed as a white dashed line.

Segmentation/thresholding based on the tissue autofluorescence is carried out with the help of the expectation maximisation method (a method to estimate parameters using in this case the highest and lowest intensity). The projection is made into a binary image to facilitate estimation of the objects shape (Fig. 5 B). The centre of mass (COM) for the sample may then be calculated for each of the two projection views (black dashed line in Fig. 5 B). To facilitate the alignment in the scanner, a
vertical line corresponding to the COM is superimposed on the signal channel (white dashed line in Fig. 5 C) in which normally more specific features are present. These features may then be used as additional reference points. Relying on a computer-based script to centre a sample has both benefits and drawbacks. It facilitates the use of the machine and speeds up the pre-acquisition process, making the OPT scanning procedure more user-friendly and standardised. However, if the autofluorescence from the specimen is insufficient, the algorithm cannot calculate the COM correctly.

7.1.2 Discrete Fourier transform alignment (DFTA)

Virtually all machines suffer from some sort of inconsistency and errors. In OPT, a small tilt between the camera and the centre of the stepper motor axis will result in a misalignment of the projection views. This misalignment can be measured by overlaying mirrored (0°-180°) projection views. Ideally they should produce a perfect match. Typically, in the commercial Bioptonics 3001 scanner as well as in the original OPT setup, there will almost always be a shift between the camera and the axis of the stepper motor, no matter how careful the COM alignment was performed. If this shift is not corrected for it will result in blurry and/or distorted tomographic sections. To correct for the shift, the projection views are moved horizontally until they overlap. This may be performed in reconstruction software such as NRecon (NRecon, Bruker, Belgium). The resultant correction value is referred to as the post-acquisition alignment value (or A-value). For most samples, the A-value changes throughout the samples vertical orientation. Therefore the operator must reconstruct the sample in multiple segments, each with their own A-value, adding to the longevity of the process. Previous reports demonstrate that post-acquisition processing can improve the reconstruction results with regards to increased resolution and artefact reduction. However, these methods involve hardware adaptations and/or are highly time consuming.

With the aim to facilitate procedure of the A-value correction we developed an approach in which the sample was digitally divided into blocks (in this case 8 pixels high), and the A-value of each block was calculated (Fig. 6). In order to simplify the calculation of the shift in between the projection views as well as to improve the capabilities to handle large amount of data Discrete Fourier
transformation (DFT) was used. There are different ways to represent data, e.g. the “spatial domain” could be built up by intensities and coordinates whereas frequencies and amplitudes build up the “frequency domain”. DFT converts the data from the “spatial domain” to the “frequency domain” since shifts as the ones calculated for the A-value are easier to detect in the “frequency domain”.

Figure 6. DFTA simplifies reconstruction by unifying the A-value throughout the specimen. The A-value is measured in 8 pixels high blocks and plotted in a graph. The slope of the curve is used to rotate the projections to form one A-value for the entire specimen. Image adapted from 116.

By this approach, the optimal A-value for each block could be put into the reconstruction software resulting in a faster and more accurate reconstruction procedure. This procedure however still required that the sample had to be reconstructed in segments. By calculating the angle of the slope and adding a pivot point corresponding to where the A-value is zero, or close to zero, it was possible to rotate the projections around the pivot point to form a unified A-value. Hereby, the reconstruction could be carried out without dividing the sample into segments, which further increased processing speed and most importantly the quality of the data.
Figure 7. COM-AR in combination with DFTA significantly improves the reconstructed data. (A) Standard OPT protocols often produces blurriness of peripheral object. (B) OPT data processed with COM-AR together with DFTA shows improved quality. Scale bar in B corresponds to 2 mm in A – B.

In conclusion, the implementation of COM-AR and DFTA generally contributes to increased data quality in OPT based assessments of specimens of irregular shapes (with associated difficulties in axis alignment and A-value tuning). COM-AR facilitates optimal sample positioning in the OPT machine, which ensures that the collected projection views are of high quality, whereas DFTA aids in the reconstruction process by unifying the A-value. When applied to OPT data of the BCM distribution in the rodent pancreas, these tools have significantly improved the quality of the reconstructed islets of Langerhans, especially those located in the periphery of the gland (Fig. 7).
7.2 Paper II: Near infrared optical projection tomography for assessments of β-cell mass distribution in diabetes research

Despite the established capacity for whole pancreas OPT-based imaging developed in the laboratory, limitations of the technique prevented certain research assessments. In particular, larger models, such as the rat pancreas, are exceedingly difficult to image and the possibility to produce multichannel images of sufficient quality is inadequate. To address these shortcomings and to produce a standardized sequential protocol incorporating the diverse tools and procedures developed, we performed a study aiming to take advantage of the near infra-red (NIR) spectrum in OPT imaging (see Fig. 8). Below, key aspects of this protocol will be discussed.

Figure 8. Optimised OPT protocol allows for quantitative assessments of specimen on the cm scale. Flowchart depicting the processes involved for OPT imaging. Sections discussed in this thesis are: Near Infrared-OPT (7.2.2), Sample holder (7.2.3), COM-AR (7.1.1), A-value tuning (DFTA, 7.1.2), Tomographic reconstruction (5.3.9).

7.2.1 The near infrared window
The near infrared (NIR) region of the light spectrum (600 nm – 1200 nm) is often referred to as the “optical window” in imaging. In this range light absorption from tissues such as fat, blood (haemoglobin) or fur is significantly lower than in
wavelengths below 600 nm\(^{117}\). The reduction of endogenous tissue light absorption is highly beneficial since its interference with the desired signal often impose a problem for many imaging tasks. One quality aspect of an image is its signal to noise ratio (SNR). A higher ratio indicates that the images suffer from fewer disturbances from unwanted signal (noise). Further, longer wavelengths are capable of penetrating deeper into tissue before they are diffracted, meaning that larger tissues can be studied. There is however a limit of how long wavelengths that can be used since eventually noise contamination from water will appear, as in the case for FT-IR (discussed in 5.4.1)

**7.2.2 Near-infrared OPT (NIR-OPT)**

The excitation power of most standard research light sources (usually mercury arc) is fairly low at the higher wavelengths (around 680 nm and above). In Emission-OPT, this manifests as prolonged exposure times. For a commonly used excitation wavelength (594 nm) used in OPT for quantification of BCM of mouse pancreata the exposure time is regularly around 1s / projection view in the Bioptonics scanners and around 2s for the original OPT setup\(^{35,68,81,87,88}\). Acquiring images at 680 nm increases the exposure time up to five times in the original OPT setup. For the commercial Bioptonics 3001 scanner, it is difficult to use wavelengths above 647 nm since the sensitivity of the camera is too low at higher wavelengths, filter changes are not easily carried out and the software prohibits exposure times longer than 10s. Longer exposure times may further increase so called photo bleaching, i.e. fluorophores that are exposed to exiting light will fade over time.

By replacing the original mercury-vapour light source with a metal halide lamp, we could significantly increase the excitation power and thus the excitation possibilities in higher wavelengths. A prerequisite to capture images in the NIR range is the quantum efficiency (measurement of light sensitivity) of the detector in this part of the spectrum. To improve signal detection in the NIR-region, a CCD -detector with a quantum efficiency of >90 % even at 800 nm was fitted to the original OPT setup\(^{81}\). In addition, custom built filter sets allowing for optimal excitation and channel separation of the fluorophores, as well as a large mirror with higher light reflectance to maximise the excitation possibilities was fitted, altogether enabling OPT imaging
in the NIR spectrum (NIR-OPT). Additional optimisation of the setup has been conducted since the publication of the study. Most importantly, fitting the instrument with a LED-light source, which is even more powerful in the NIR range, further decreased the exposure times for the longer wavelengths. In addition, the LED-light source has no start up time, very little heat production and it can be directly switched on and off in short intervals without risking damage or reduction of its lifespan. To further facilitate imaging in low/weak light conditions gain is applied in the NIR-OPT setup. Gain can be applied digitally or analogously. Digital gain is applied after the analog to digital conversion of the image. It amplifies the entire image signal by multiplying the values building up the image, resulting in that all the information is increased, including the noise. In NIR-OPT imaging analog gain is used. This means that the amplification is applied before the digital conversion so that the full imaging depth can be used. As a result the sensitivity can be increased but not at the expense of higher background, as in the case of digital gain.

7.2.3 Prevention of unwanted specimen movements in the vertical plane

Unwanted movement during the scan in any direction will highly impact the final result. In the original configuration of the OPT scanner (as well as in the commercial 3001 scanner), the sample commonly moved along the vertical axis (z-axis) due to how the sample was placed in the scanner. The sample in these configurations was attached to the stepper motor by gluing the agarose embedded sample to a mount. In principal a metal rod is attached to the scanners stepper motor, either via its own magnet or a magnet on the machine. However, the most commonly used clearing solution for OPT (Benzyl alcohol: benzyl benzoate (BABB) 1:2 ratio), dissolves a range of glues tested for this purpose. This resulted in that the sample in many cases gradually dropped along the z-axis or even fell off, rendering the entire scan useless. To overcome this issue, a mount was devised with a “crown” fitted to one end of a metal cylinder allowing for a very rigid fixation of the sample by using needles to secure the upper part of the agarose (Fig. 9).
This technique completely eliminated the use of glue and produces scans without measurable shifts in the z-axis direction. Further, by programming a short pause in the stepper motors rotation between each scan, movements caused in the agarose by the rotations of the stepper motor could be eliminated.

7.2.4 Benefits of NIR-OPT imaging for pancreas assessments

In virtually all biomedical research fields, the possibility to visualise multiple structures in an individual specimen is often key to address the research question at hand. For studies of the adult pancreas, previously described setups, including the commercial Bioptonics 3001 scanner, allowed essentially only for the quantification of one channel due to poor SNRs in the available wavelengths. In diabetes research, e.g. in the study of autoimmune processes or in grafting experiments (where the relation of the graft and the vascular network is of interest when evaluating potential grafting sites) increasing multichannel capacity would carry considerable advantages.

Adapting OPT imaging to the NIR-range extended the available spectrum for labelling from 647 nm to 800 nm as the upper limit. This extension allows for at least two additional quantifiable channels with high SNR suitable for quantification of structures such as islets of Langerhans and interacting/neighbouring cell types (see Fig. 10 and 11).
**Figure 10. NIR wavelengths reduce endogenous tissue autofluorescence.** Adult mouse duodenal lobe (DL) whole mount stained with a primary antibody against insulin and with fluorophore conjugated secondary antibodies (A) 488 nm, (B) 594 nm, (C) 680 nm and (D) 750 nm. Scale bar: 1 mm in A – D.

**Figure 11. NIR-OPT extends the spectrum allowing imaging of additional features in additional channels.** Adult NOD mouse DL depicting the insulin producing β–cells (blue), larger blood vessels expressing α–smooth muscle actin (red), infiltrating CD3+ T-cells (green) and anatomy/outline (grey/black). The anatomy/outline of the organ based on autofluorescence. Scale bar: 1 mm.

The additional channels with high SNR also reduces the need for operators trained in manual intervention of images and with a specific subject knowledge and understanding of how the sample is presented in tomographic images related to the end result in 3D-space.

In all, by the adaptation of OPT to the NIR spectrum, the upper size limit of OPT, previously considered to be around the size of an adult mouse pancreatic lobe (~1
cm length) could be significantly increased. By NIR-OPT, adult rat pancreatic lobes, 6x larger (v/v) than the mouse equivalent could be successfully reconstructed and quantified. This opens up for studies of diabetes models that have previously been inaccessible by OPT at least if intact specimens were to be analysed. For example, in a recent study, performed in collaboration with the Norwegian University of Science and Technology, pancreata from 24 adult Goto-Kakizaki rats were analysed with NIR-OPT. The collected data contains quantitative and spatial information regarding the BCM distribution throughout the pancreatic volume, down to the level of the individual islets. Hereby, it was demonstrated that a certain type of bariatric surgery, sleeve gastrectomy, is superior to duodenojejunostomy in preserving BCM in this T2D model. Several other rat models are currently under investigation using the NIR-OPT technology. Another utility for NIR-OPT has been in the evaluation of non-invasive imaging approaches. For example NIR-OPT in combination with SPECT imaging was recently used to cross validate the efficiency of a radiotracer (111In-exendin-3) for β-cell assessments in normal and diabetic rats. Using OPT as a “gold standard”, this study showed similar patterns for the radiotracer (imaged by SPECT) and the IHC labelling for insulin (imaged by NIR-OPT), indicating that this radiotracer holds promise for BCM imaging.
7.3 Paper III: Improving signal detection in emission optical projection tomography via single source multi exposure image fusion

Some specimens are particularly difficult to visualise using OPT. This is due to detectors having a certain dynamic working range. If the intensities of the sample differs too much and/or exceed the allowed range for the detector, the operator has to compromise between; (I): capturing low intensity features and saturating high intensity features, (II): lose low intensity features and capture high intensity features or (III): lose some of the low intensity features and saturate some of the high intensity features (Fig. 12). The data lost in all of the above scenarios cannot be retrieved after image acquisition. The CLAHE method (discussed in section 5.3.9 of the introduction) may homogenize the signal heterogeneity in a sample but once pixels are saturated or the intensity is not high enough there is no data to equalise.

Figure 12. Heterogeneity in the intensities across a sample complicates the acquisition process. Adult C57BL/6 left lateral liver lobe labelled for smooth muscle α-actin expressed by larger blood vessels. (A) Short exposure setting where strong signal is optimally captured results in that low intensities are lost. (B) Intermediate exposure setting where most of the sample is captured optimally and both high and low intensities areas are lost, either due to saturation or too low intensity. (C) Long exposure setting where low intensities are captured optimally whereas the high intensity areas are saturated. Arrows corresponds to areas where information is lost due to either saturation or low intensity. Arrowheads corresponds to areas that are captured optimally.

Hence, a method making it possible to analyse a specimen that contains an extremely high variability in intensity is desired to deal with particularly challenging samples which span from low to high contrast. Examples of such scenarios include
antibody based staining of blood vessels in organs such as the pancreas and the liver. The latter organ is frequently used in experimental models of islet transplantation in T1D\textsuperscript{120}. Multichannel imaging with NIR-OPT would enable assessments of the islets distribution within the liver in relation to the vascular system.

One possibility to tackle the problem of highly variable contrast ratios is to use so-called image fusion. There are many types of image fusions and perhaps the most recognised method is the high dynamic range (HDR) method, found as a feature in most modern smartphone cameras. HDR works by retrieving the cameras response function (how incoming light is mapped to image intensity values as well as its sensitivity at different wavelengths) for a number of exposures resulting in that the intensity values exceeds the range of a standard image format, hence “high dynamic range”. In 2012, Fei et al. presented a TM-OPT HDR method for embryonic scale samples\textsuperscript{121}. This method combines 9 images with different exposure times to create a single HDR image. Though it would be possible to use this method on larger samples and with EM-OPT, it would potentially induce photo-bleaching and the size of the data sets would increase significantly. In addition, HDR violates the dynamic range of normal image formats so that special non-standard file formats or additional conversions are needed before viewing is possible. The complexity of the HDR method, including the need to retrieve the cameras response function, are additional drawbacks.

With this in mind, a data driven fusion approach with the aim of generating the best possible contrast was devised. This method, which we refer to as image fusion–OPT (IF-OPT), combines the best of two (or three) worlds by capturing both low and high intensity objects/structures. A set of 3 projection views typically corresponding to the 3 images in Figure 13, (A) low-, (B) intermediate- and (C) high intensity are used for the fusion. Treating the 3 images as dimensions (making up a 3D space) a principal component analysis is used to decide how to optimally fuse the information from the three exposures in order to maximise the contrast in the final image.
Figure 13. IF-OPT facilitates the reconstruction of samples with intensities of high variabilities. Adult C57BL/6 left lateral liver lobe labelled for smooth muscle α-actin expressed by larger blood vessels. Arrowheads correspond to problem areas that are rendered reconstructable by IF-OPT.

When IF-OPT was performed on biological specimens (liver, kidney) as well as a digital phantom, there was a visual improvement of contrast in the image. In a blinded evaluation researchers ranked IF-OPT against existing enhancement methods performed on a liver specimen. In over 90% of the cases, IF-OPT was chosen as the method that visualised most of the structures in the sample. Even with a high-end camera, with a high dynamic range such as the one fitted in our NIR-OPT system, the IF-OPT method significantly improved the structural display.

In comparison to the HDR method, IF-OPT does not overstep the dynamic range of normal imaging formats and can therefore be used directly. E.g. in this case the projection views can be reconstructed without any form of conversion. Furthermore, the use of only 3 exposures helps to reduce potential photo bleaching, scanning time and data storage. Finally, it should be noted that research questions regarding expression levels should exclude IF-OPT and CLAHE based image enhancements, since these methods alters/equalises the intensity across the specimen, which is not the case for HDR based fusion.
7.4 Paper IV (manuscript): Multivariate image analysis facilitates label-free, biochemical profiling of the diabetic pancreas

The ability to detect chemical alterations occurring in an organ or tissue is a key component in understanding disease etiology. Despite the epidemic proportions of diabetes, limited tools are available to biochemically characterize the pancreas, i.e. the key organ affected by the disease, especially if the spatial context is to be preserved. The ability to analyze biochemical changes directly in pancreatic tissue would greatly support attempts to understand mechanistic aspects of the disease and aid in prognostic and/or diagnostic assessments. As discussed in section 5.4.1, VMS techniques have been used to study peripheral tissues in settings of diabetes. However due to the complexity of the acquired spectral data (discussed in section 5.4.1) studies of the pancreatic tissue itself are scarce. By the development of multivariate image analyses techniques many of these hurdles have been overcome.

This study aimed to establish a technique by which complete biochemical profiles of the pancreas may be derived independent of any contrast agents. This was attempted by combining two complementary approaches for vibrational microspectroscopy (FT-IR and Raman (see section 5.4.1)) with MVA (see section 5.4.2).

7.4.1 Label free detection and classification of cell types in pancreatic tissue

To assess the detection potential of OPLS-DA (see section 5.4.2) from VMS data, tissue from C57Bl/6 mouse pancreata was subjected to FT-IR and Raman imaging. A full vibrational spectrum was collected from non-labelled tissue sections. Consecutive sections, IHC labelled for insulin was used to determine where the insulin producing β-cells were located relative to the assignment of the chemical profiles derived from the VMS spectra. Hereby both endocrine and exocrine regions could be identified. Note that consecutive sections with labelled structures are needed initially to create chemical composition reference libraries of the tissues.

By selecting 10 pixels in the spectral image from areas representing each cell type (known from the IHC), two classes based on their chemical composition were created (endocrine and exocrine). By matching the chemical composition of the
remaining unassigned pixels to one of the two classes, a false colour map (FCM) that reflected the location of the cell types, as determined by IHC, could be created (Fig. 14).

Figure 14. OPLS-DA of VMS data can differentiate between exocrine and endocrine tissues of the mouse pancreata. β-cells IHC labelled for insulin (A) acts as a guide when selecting pixels during class assignment in the spectral image (B). Triangles indicate pixels where the spectra was selected (blue = exocrine cells and red = endocrine cells). FCM derived based on OPLS-DA classification of the tissues chemical composition (C). Scale bar: 25 µm in A - C.

An increased number of classes (cell types) posed no difficulty to the OPLS-DA analysis (manuscript IV, Fig. S2). In order to evaluate the diagnostic potential of the method, a transgenic mouse strain, RIP-HAT expressing human islet amyloid polypeptide (hIAPP) forming toxic amyloid deposits, similar to human T2D, was analysed. Hereby, we could identify bands previously assigned to amyloid deposits.

7.4.2 VMS in biochemical profiling of diabetes disease models
To test whether OPLS-DA of VMS data of pancreatic tissue could be used to detect biochemical changes linked to diabetes disease progression, two frequently used models for diabetes were assessed, the NOD model for T1D and the leptin deficient ob/ob model for T2D/insulin resistance and obesity (see sections 5.2.4 and 5.2.5). A summary of the results is given below.

7.4.3 Biochemical profiling of the NOD mouse model
For NOD mice profiling three different stages were selected based on previous knowledge of disease progression \cite{35}. These were 1 week (pre-insulitis), 3 weeks (around the onset of insulitis) and 9 weeks (full blown insulitis). Included as control was the congenic NOD.H2-b strain.
Most significantly, OPLS-DA of NOD VMS data acquired with FT-IR and Raman showed that 3 week old NOD mice have a unique endocrine and exocrine chemical composition compared to all other stages irrespective of genotype (Fig. 15). In VMS, an increase in intensity of a band corresponds to an increase in the amount of a chemical compound and vice versa. When analysing the spectral data of 3 week old NOD mice compared to earlier and later stages and stage matched controls (peak assignment), changes in peak intensities could be observed in bands assigned to proteins (decreased), DNA (increased) and collagen (increased) (manuscript IV, supplementary table 2). The specific nature and relevance for disease progression is yet to be determined.

Our data further indicate that it is possible to distinguish the age between the groups based on the chemical composition regardless of the genotype (manuscript IV, Fig. 3). It should be noted that these plots are in 3D, and that further separation occur in the z-axis.

Figure 15. Raman based OPLS-DA enables biochemical classification of mice from different age groups and strains. Squares corresponds to NOD mice and triangles corresponds to NOD.H2-b control mice. The shading depicts the age, light = 1 week, medium = 3 week and dark = 9 week.

7.4.4 Biochemical profiling of the ob/ob mouse model

OPLS-DA was carried out on ob/ob mice at two stages, 3 weeks (lean) and 9 weeks (obese) with their controls (+/−). As in the NOD analysis these mice could be separated into groups of age, genotype and disease stage based on their chemical compositions. When analysing the peaks of the spectra, previously unreported as
well as reported changes such as higher proportion of lipids, collagen and β-sheet structures as well as lower proportions of carbohydrates and nucleic acids per area could be detected. Notably some changes could be detected at earlier stages than with other existing technology. For example, the accumulation of IAPP has been shown by radioimmunoassay first at 16 weeks\textsuperscript{122}. With our approach accumulation of β-sheet proteins could be observed well before week 9.

### 7.4.5 VMS (Raman) \textit{in vivo}

Raman microspectroscopy has successfully been applied as a tool in disease diagnostics and recently it is presented as an intra operative tool for cancer detection\textsuperscript{123,124}. Prompted by our results on pancreatic tissues \textit{ex vivo}, we attempted to acquire the chemical composition of islets of Langerhans \textit{in vivo}. To accomplish this, we performed Raman microspectroscopy on islets transplanted to the anterior chamber of the eye (ACE) using the eye as a natural body window\textsuperscript{125} (section 5.3.6). Islets of Langerhans from \textit{ob/ob} and C57BL/6 mice were syngeneically transplanted onto the iris of the host mice. Applying Raman microspectroscopy with virtually the same settings as for the chemical profiling of the NOD and \textit{ob/ob} mice on section, spectra could be collected from both the iris as well as from the grafted islets of Langerhans. When analysing the spectral data there was little to no detectable difference in chemical composition of the iris tissue between the \textit{ob/ob} and C57BL/6 mice (see figure 16 B comparing green (C57BL/6) and black (\textit{ob/ob}) lines. However, the composition in the islets of Langerhans was found to have differences that showed similarities with changes found in the dry tissue sections from the chemical profiling of the \textit{ob/ob} mice, such as increase in β-sheet structures (see greyed box around 1600 \text{1/cm} in Fig 16 B). Furthermore a detectable peak previously assigned to insulin could be detected (greyed box around 680 \text{1/cm}). In further pursuit of the diagnostic capabilities of Raman microspectroscopy a set of whole organ (mouse pancreata) as well as human pancreatic biopsies were analysed. This to assess whether the pancreatic capsule enclosing the organ would pose a problem for future \textit{in vivo} studies. Intact pancreata from NOD and NOD.H2-b mice were analysed through the tissue capsule and the resulting spectral profiles allowed for identification of the animals strain (NOD or NOD.H2-b). The tissue capsule did
not pose any difficulties when the same procedure was applied on human pancreatic biopsies. To exclude the possibility that the spectrum was derived from the capsule it was overlaid with a spectrum acquired from an area inside of the biopsy. Little to no difference between the spectra was detected (Supplementary Figure 6 in the manuscript).

Figure 16. In vivo Raman analysis of grafted islets of Langerhans may be used for longitudinal studies of the biochemical changes related to diabetes progression. (A) Bright field image of islets of Langerhans grafted to the iris of an \textit{ob/ob} mouse. (B) Raman spectra representing the iris from a C57BL/6 (green), a \textit{ob/ob} (black) and a grafted islet of Langerhans from a C57BL/6 (blue) and from a \textit{ob/ob} (red).

In conclusion using MVA on VMS data could provide novel insights related to biochemical changes during disease progression. These findings may be used as guides/hints for other targeted methods such as proteomics and metabolomics etc. Furthermore, combining the presented approach with the ACE model where the grafted islets are proposed to mimic the endogenous islet situated in the pancreas of the animal would allow for longitudinal assessments of grafting efficiency, drug treatments as well as dietary related changes at a molecular level.
8. SUMMARY AND CONCLUSIONS

Figure 17. Schematic illustration summarising the key findings and developments presented in this thesis. Applying the suggested improvements to OPT facilitates and improves data collection and quality. MVA of VMS provide detailed information about the chemical composition of the material analysed which may provide new insights to previously known or unknown disease processes. Abbreviations: Multivariate analysis (MVA), Vibrational microspectroscopy (VMS), Centre of mass – axis of rotation (COM-AR), Discrete Fourier transformation analysis (DFTA) Image fusion - optical projection tomography and Near infrared – optical projection tomography.

Presented in this thesis is a combination of computational tools and hardware developments that improve the quality of the acquired OPT data. By facilitating pre- and post-acquisition procedures they further contribute to reduce user subjectivity. Jointly, these developments have played a central role in studies addressing aspects of normal pancreatic anatomy, β-cell preservation (including
analysis of the effect of Ba)\textsuperscript{118}, β-cell destruction (of STZ treated mice, manuscript in preparation), regeneration and proliferation\textsuperscript{90}. They have further contributed to important information in studies of other organs, diseases and model systems\textsuperscript{126-128}. Hopefully this will aid in transferring the OPT from being a highly specialised technique, available to a small portion of the research community, to a more readily used optical technique such as the CLSM. The developed approach for MVA of VMS is still in its infancy but shows great potential as a tool in diabetes research/diagnostics, on its own or in conjunction with other techniques.
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10. REFERENCES


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