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Imaging the Pancreatic Beta Cell

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1. Introduction

The development of advanced technologies for pancreatic beta-cell imaging intended for medical practice, preclinical testing and experimental diabetes research has become a field of intense study over the last decade. Insulin deficiency and hyperglycemia in type 1 diabetes (T1D) occurs as a result of selective T-cell-mediated autoimmune destruction of pancreatic beta-cells. During the first stage of T1D, known as insulinitis, the islets of Langerhans are subjected to a massive invasion of a mixed population of leukocytes, followed by a selective destruction of pancreatic beta-cells. During the second stage of T1D, hyperglycemia develops as a result of impaired insulin production by damaged or disabled beta-cells (Mathis & Gaglia, 2010). In patients with T1D immune infiltration, the destruction of beta-cells and reduction in beta-cell mass (BCM) precede clinical manifestation of the disease. Furthermore, dynamic changes in BCM correlate with the time course of diabetes progression and the efficiency of anti-diabetic treatment. In clinical practice, the evaluation of islet inflammation is currently based on monitoring serum titers of antibodies directed against certain beta-cell antigens (Eisenbarth et al., 2002). The assessment of BCM is limited primarily to functional tests of insulin secretion in response to secretagogues (Robertson, 2007), the results of which reflect the specific mechanisms of beta-cell function. These diagnoses may however be affected by anti-diabetic treatments as well as other factors. Histological analyses provide the most accurate BCM determination in humans and are achieved by an examination of pancreas specimens obtained from patients undergoing pancreatic surgery. Due to the heterogeneous distribution of pancreatic islets within the pancreas, this procedure can provide BCM estimations within a specific pancreatic area rather than the whole organ (Meier et al., 2009). The analysis of BCM in whole pancreas samples, obtained during autopsy, is impractical. Hence, the development of non-invasive diagnostic techniques that enable detection of pancreatic islet inflammation in humans at risk for T1D and to estimate BCM would become powerful tools for both the early diagnosis and effective treatment of T1D.

Existing imaging technologies differ in terms of the type of energy that is used to generate visual information (e.g. positrons, photons, X-rays, sound and radiofrequency waves), the depth of penetration, spatial and temporal resolution, sensitivity, as well as the category of information that can be obtained using the technique (e.g., anatomical, physiological, cellular, or molecular). Extensive efforts are ongoing to create a non-invasive clinical imaging modality for beta-cell imaging based on the magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) platforms. Limited employment of beta-cell imaging in clinical practice has

fostered great interest in using animal models of T1D at all stages of diabetes research. Adaptation of the modalities that are extensively used in clinical practice, such as MRI, PET and SPECT/CT for imaging animal models, allows the creation of experimental systems with high spacial resolution. Methods of optical imaging for the visualization of the pancreas from the molecular to the cellular level can be employed to test new therapeutic strategies as well as to develop new contrast agents for non-invasive T1D monitoring. These methodological approaches may eventually be applied to human T1D care in the early diagnosis of diabetes and the monitoring of disease progression and effectiveness of drug treatment. Since several recent reviews and book chapters focusing on nuclear imaging techniques using tracers for the visualization of specific tissue binding sites, such as PET and SPECT, and multimodality platforms PET/CT and SPECT/CT, as well as the application of these techniques for beta-cell imaging, have been recently published (Ahlgren & Gotthardt, 2010; Gotthardt, 2010; Hutton & Beekman, 2010; Ichise & Harris, 2010; Townsend, 2010), we will in this review focus on optical imaging techniques and MRI. In this chapter we will overview recent advances in beta-cell imaging using these imaging technologies with an emphasis on optical projection tomography (OPT), an advanced 3D imaging technology, developed to bridge the gap between MRI and microscopy techniques, employing optical sectioning approaches.

2. Magnetic resonance imaging

MRI has become one of the most practical, non-invasive imaging technologies over the last decades. MRI utilizes the principle of nuclear magnetic resonance and detects molecules containing nuclei with magnetic dipole properties or unpaired nuclear spins, such as hydrogen nuclei in water and other organic compounds (like lipids). In a strong static magnetic field produced within the MR scanner, hydrogen nuclei become aligned (either parallel or anti-parallel) to the magnetic field (these orientations correspond to the low- and high-energy states of the nuclei, accordingly). A temporary radiofrequency (RF) pulse, generated by a transmitter RF coil, changes the orientation of nuclear spins in the magnetic field, and is accompanied by absorbing energy. Following this pulse, nuclei return to their equilibrium (relaxation) state, emitting energy at resonance, or Larmor frequency, which can be detected by the receiver RF coil as an MRI signal (Rinck, 2001). The decaying signal is characterized by two relaxation time constants: T1 (the longitudinal relaxation time) and T2 (the transverse relaxation time), which depend on the physical and chemical properties of the tissue, the molecular environment of the nuclei and the strength of the static magnetic field. In different micro-environments, nuclear spins show different relaxation parameters that are detectable as different MRI signals at each point in the image. Differences in the relaxation behavior of tissues exposed to a magnetic field of a certain strength and RF pulses of a specific sequence determine image contrast and can be used for the monitoring of pathological processes in both organs and tissues (Budinger & Lauterbur, 1984). Detailed descriptions of MRI physics, engineering principles, instrumentation and contrast agent mechanisms have been presented in a number of publications (Gadian, 1995; Rinck, 2001; Artemov, 2003; Cassidy & Radda, 2005; Medarova & Moore, 2009).

In clinical practice, MRI has become a safe procedure because neither ionizing radiation nor a radiopharmaceutical agent is used for the scanning procedure. In comparison with other non-invasive imaging technologies, MRI possesses such advantages as high spatial resolution, unlimited penetration depth and high contrast for soft tissues which allows the

collection of both anatomical and functional data. By applying contrast agents that can specifically label various cells types based on antigen expression levels, MRI can be used as a molecular imaging technique. Its key disadvantage is low sensitivity to molecular probes as compared with nuclear imaging methods such as PET and SPECT. This aspect has become a key challenge for the use of MRI in beta-cell imaging of both endogenous and transplanted islets. The introduction of a modified protocol for islet transplantation and immunosuppression, widely known as the Edmonton protocol, was followed by the successful replication of this procedure in many clinical centers around the world. Hereby islet transplantation from deceased donors has become a therapeutical option for patients with T1D, whose condition cannot be adequately managed (Shapiro et al., 2000). By utilizing this protocol, excellent metabolic control and independence from insulin injections could be achieved by 80% of the patients 1 year and ~20% 5 years after islet transplantation illustrating its promise for T1D treatment (Ryan et al., 2005). However, the long-term outcome of transplantation in many cases is influenced by substantial graft loss and damage of transplanted islets resulting from numerous adverse factors such as immune attack, glucose-induced deterioration, immunosuppression therapy etc., making the timely, non-invasive monitoring of islet grafts extremely important. To non-invasively resolve either native pancreatic islets of 100 to 400 micrometers in size and scattered throughout the pancreas, constituting approximately 1-2% of the total pancreatic volume under normal physiological conditions and considerably less in T1D, or islets transplanted into the liver, contrast agents with high beta-cell specificity are required.

2.1 MRI contrast agents for imaging pancreatic islets

Contrast agents used for MRI can be classified as positive, which primarily cause a reduction in T1 relaxation time and bright contrast, or negative, which cause a shortening in transverse T2 relaxation time and dark contrast. T1 contrast agents are usually compounds of small molecular weight, containing a paramagnetic metal, such as gadolinium or manganese, reduce T1 time, increasing the intensity of the signal in T1-weighted images. T2 contrast agents, e.g. superparamagnetic iron oxide (SPIO) nanoparticles, are usually iron-oxide nanocrystals covered by a polymer shell which cause a shortening of the T2 relaxation time, decreasing the signal intensity in MR images. T2 contrast agents are considered more sensitive than T1 contrast agents. (Rinck, 2001).

Imaging of beta-cells in native or transplanted islets is challenging because the magnetic properties of islets do not sufficiently differ from those of the surrounding tissues to be resolved non-invasively using existing contrast agents. However, *ex vivo* labeling of islets destined for transplantation with SPIO nanoparticles allows the MRI tracking of islet grafts within recipient organs. The potential for MRI to allow the direct visualization of the location and distribution of islet grafts labeled with the SPIO contrast agent ferucarbotran (approved for clinical application) and transplanted into the livers of streptozotocin (STZ)-treated and control recipient rats was first demonstrated by Jirak and co-authors (2004). The efficiency of islet transplantation was also evaluated by the restoration of normoglycemia in STZ-treated rats within 1 week of the procedure. *Ex vivo* co-labeling of human pancreatic islets with modified magnetic nanoparticles carrying the near-infrared fluorescent dye Cy 5.5 allowed both the MRI monitoring of prelabeled islets, transplanted either under the kidney capsules or into the livers of healthy and STZ-treated nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice and the cross-evaluation of the islet grafts using optical imaging (Evgenov et al., 2006). Additionally, accumulation of nanoparticles in the endosomal structures of labeled islets did not affect glucose-induced insulin secretion *in vitro*.

By employing a transfection technique combining the electroporation and *ex vivo* labeling of rat and porcine islets with the SPIO contrast agent Feridex, complexed with poly-L-lysine, Tai and associates (2006) showed a good correlation between signal loss and increasing numbers of islets transplanted under the rat kidney capsule, imaged using a standard clinical 1.5 T scanner. Labeling of mouse and xenotransplanted human islets with T1 contrast agent-GdHPDO3A (Gd-gadolinium, HPDO3A-10-(2-hydroxypropyl)-1,4,7,10 tetraazacyclododecane-1,4,7-triacetic acid) was sufficient for direct MRI imaging with high spatial resolution and contrast of subrenal mouse islet grafts and intrahepatic human islet xenografts, respectively (Biancone et al., 2007).

To prevent the rejection of transplanted pancreatic islets by the immune system of a recipient, various immunoisolation techniques have been tested. One of the most widely used immunoisolation approaches employs the encapsulation of pancreatic islets within an immunoprotective alginate coating, which creates a semipermeable membrane around the transplanted islets and prevents antibody penetration into the capsule, while still allowing passage of glucose, insulin, and other metabolites (Lim & Sun, 1980, Sun et al., 1996). Chemically modified microcapsules can serve as multimodal contrast agents for the non-invasive monitoring of transplanted islets. Barnett et al. (2007) used MRI-detectable magnetocapsules, prepared with alginate containing Feridex, for the encapsulation of cadaveric human islets prior to transplantation into the livers of swine. Engrafted magnetocapsules were visualized by MRI as hypointense signal voids both immediately and 3 weeks after injection into the portal vein. There were functional *in vivo* and induced sustained human C-peptide levels. Incorporation of perfluorocarbons, hydrocarbons with most hydrogen atoms replaced by fluorine-19 (^{19}F), including perfluoropolyether (PEPE), in alginate capsules not only increases islet cell oxygenation and glucose-stimulated insulin secretion but also enables ^{19}F -MR imaging. Due to the absence of MRI detectable fluorine in the body, ^{19}F -labeling ensures high specificity of the signal and a lack of background signal (Barnett et al., 2011). Employment of a modified capsule coating could provide immunoprotection for xenografted islet cells, increasing the effectiveness of transplantation. It could also be used as a multimodal contrast agent for the monitoring of all steps of islet transplantation. To obtain better discrimination of labeled cells from the image background, Srinivas and co-authors (2007) applied *ex vivo* labeling of T cells using a PEPE agent and performed *in vivo* monitoring of T-cell migration to the pancreas after cells were transferred into non-obese diabetic (NOD) mice using spin density-weighted ^{19}F -MRI. Because of the negligible background, ^{19}F -MRI insures selective visualization only of labeled cells. Simultaneous acquisition of conventional ^1H images in the same session allows for a combination of images of labeled cells and images of anatomical structures.

In a pilot study, Toso et al. (2008) showed the feasibility and safety of MRI for the non-invasive monitoring of islet grafts prelabeled with SPIOs and transplanted into the livers of patients with T1D. Saudek and colleagues (2010) further developed this approach by implementing and optimizing new MR sequences using a scanner with a higher magnetic field strength (3T). Pancreatic islets labeled with ferucarbotran and transplanted into the livers of T1D patients with pre-transplant negative C-peptide levels were detected as hypointense areas on T2-, T2*-, and T2/T1-weighted images. These patients were followed for 24 weeks (Fig.1 (I)). Hence, MR imaging could play an important role not only in the monitoring and quantifying of transplanted islet engraftment and survival, but at all stages of islet transplantation, including screening of potential transplant recipients, for guiding the transplantation procedure and for monitoring post-transplantation complications in combination with other techniques for clinical imaging. (Low et al., 2010).

2.2 MRI based functional BCM monitoring

Imaging of endogenous pancreatic beta-cells in a living organism is more challenging, than the detection of *ex-vivo* labeled islet grafts, and requires highly specific beta-cell contrast agents to be applied. Prospective contrast agents for *in vivo* beta-cell imaging should meet the following requirements: 1) the agent should be sufficiently selective to beta-cells and not be taken up by adjacent cells and tissues, 2) it should provide a stable signal of high intensity, reflecting functional state and viability of beta-cells, 3) the agent should not have side toxic effects and should be retained by beta-cells during the entire period of time, required for scanning (Ahlgren & Gotthardt, 2010). It would obviously be of great practical value if a contrast agent for MRI could enable imaging of a parameter directly related to beta-cell function. A promising approach in this direction is based on the specific glucose-stimulated uptake of manganese ions by beta-cells. The paramagnetic manganese (Mn^{2+}) ion, a T1 relaxation-promoting contrast agent, has been tested by Antkowiak and co-authors (2009) for the non-invasive assessment of beta-cell function using MRI in control and STZ-treated C57BL/6J mice. Manganese mimics calcium by entering pancreatic beta-cells through voltage-gated Ca^{2+} channels after glucose challenge. Accumulated manganese in beta-cells represents integrated calcium signaling over time and can be detected as a brightening on T1-weighted images. Quantification of the normalized signal intensity showed a 51% increase in control mice after glucose infusion compared to the 9–20% increase in STZ-treated animals. These findings exhibit the potential of Mn^{2+} -enhanced MRI as a technique for the non-invasive monitoring of native beta-cell function, with sufficient sensitivity for the detection of functional BCM under normal physiological conditions and during progressively developing diabetes.

2.3 MR Imaging of microvascular changes and auto-immune inflammation in T1D

Endocrine cells in the pancreas are surrounded by a dense, glomerular-like capillary network and receive about 10% of total blood flow to the pancreas (Bonner-Weir & Orci, 1982, Svensson et al., 2003). The islet vasculature plays an important role in the regulation of endocrine cell function and glucose homeostasis is determined by both beta-cell secretory function and the microcirculation in the endocrine pancreas (Ballian & Brunicardi 2007, Kostromina et al., 2010). The development of T1D is commonly associated with progressive changes in the pancreatic islet microcirculatory system, including early augmented microvascular permeability, which allows transendothelial migration and the homing of T-cells, followed by a destruction of pancreatic beta-cells (Savinov et al., 2003, Savinov & Strongin, 2009). Microvascular alterations precede other symptoms of diabetes, including insulinitis and hyperglycemia, as observed in various T1D animal models (Colantuoni et al., 1988, Enghofer et al., 1997) and patients with diabetes (Haller, 1997). Non-invasive detection of islet microvascular abnormalities as an early biomarker of pancreatic islet damage is considered a promising approach for the early diagnosis and monitoring of T1D.

Microvascular alterations associated with the onset and progression of insulinitis were detected using MRI in a murine model of induced T1D (i.e., BDC2.5 T-cell receptor transgenic mice) after intravenous injection of long-circulating magnetofluorescent nanoparticles. It was demonstrated that accumulation of extravasated nanoparticles resulted from increased microvascular permeability and invading phagocytic macrophages, allowing probe uptake correlating with the aggressiveness of insulinitis in these transgenic animals (Denis et al., 2004). Using the same, high-resolution, MRI approach, non-invasive real-time

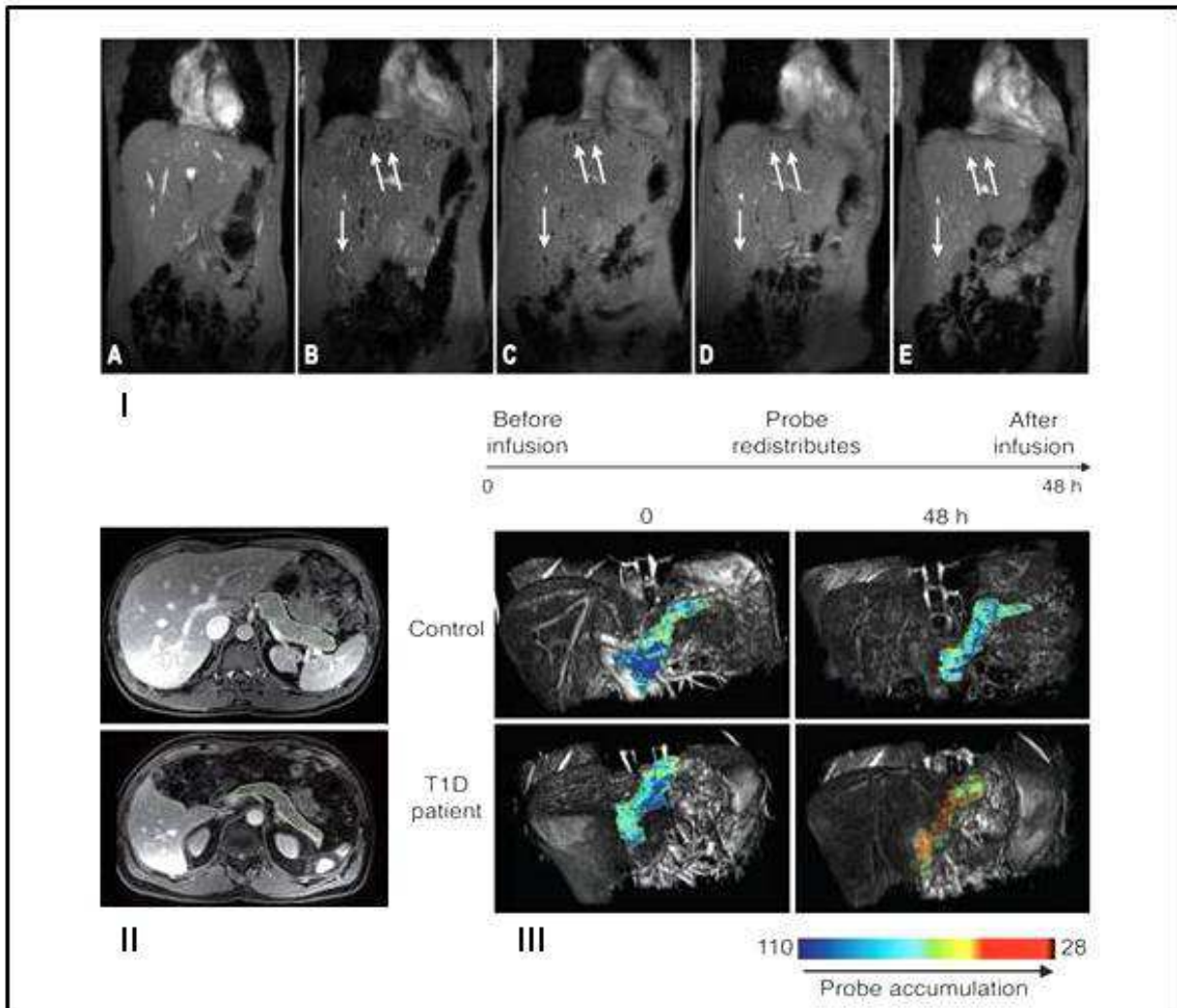


Fig. 1. I. Magnetic resonance imaging in a T1D patient on the day before transplantation (A), 1 day (B), 1 week (C), 4 weeks (D), and 24 weeks after transplantation (E). Typical areas of signal loss attributed to the detected islets are indicated by arrows (Saudek et al., 2010).

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II. Pancreas volume index (PVI) of recently diagnosed diabetes patients is less than that of controls. Representative single MR-VIBE (volumetric interpolated breath-hold examination) slices at the level of the body/tail of the pancreas are shown with the pancreas outlined in green. The control subject (upper panel) and the individual with recent onset T1D (lower panel) have total estimated pancreas volumes of 119 ml and 50 ml respectively.

III. Insulinitis may be visualized by MRI. Shown are T2-pseudocolor reconstructions of the pancreas overlaid on 3D-VIBE images, measured before and 48 hours after MNP infusion. T2 values in the pancreas are similar between the patient and control subjects before infusion but different after infusion. (II & III adapted from Gaglia et al., 2011). © Copyright 2011 by American Society for Clinical Investigation. Reproduced with permission of American Society for Clinical Investigation.

quantification of microcirculatory changes, resulting from pancreatic inflammation during the progression of diabetes and after its reversal using anti-CD3 monoclonal antibody therapy in NOD mice, was performed (Turvey et al., 2005). Semi-quantitative assessment and direct visualization of microvascular changes in the pancreata of STZ-treated mice were performed using MRI after injection of a long-circulating contrast agent PGC-Gd-DTPA-F (a protected graft copolymer linked to gadolinium diethylenetriaminepentaacetic acid residues labeled with fluorescein isothiocyanate) (Medarova et al., 2007). A significantly greater accumulation of PGC-Gd-DTPA-F in the pancreata of diabetic animals was revealed compared with control mice. *Ex vivo* histological analysis confirmed not only extensive distribution of PGC-Gd-DTPA-F within pancreatic vasculature, but also a substantial leakage of the probe into the islet interstitium in diabetic animals whereas the fluorescent signal in control mice was determined predominantly in the vascular compartment.

Based on the results of experimental findings in animal models, MR imaging of magnetic nanoparticles has recently been successfully applied in a pilot clinical study to non-invasively visualize islet inflammation in recent-onset diabetic patients (Gaglia et al., 2011, Fig.1 (II&III)). It was presumed that magnetic nanoparticle retention in the pancreas emerged as a result of vascular leakage and uptake by macrophages, which were evaluated using local T2 changes, and was dependant on the aggressiveness of insulinitis. This approach permitted the non-invasive detection of inflammatory events in the pancreas of patients at an early stage of diabetes and could be useful for monitoring the efficiency of drug therapy.

3. Optical imaging

Optical imaging is based on the detection of photon transmission through biological tissues. The propagation of light through tissue is affected by two simultaneous processes: absorption and scattering. Both effects depend on the wavelength and depth of penetration (Ntziachristos, 2010). A significant part of our current knowledge about the pathogenesis of diabetes is based on analyses performed with conventional bright-field or epifluorescence microscopy, including the microscopic assessment of multiple histological sections, which has been widely used for analysis of experimental animal models of T1D for decades. Traditional microscopic examination of fixed tissue sections (normally 10-20 μm) ensures high quality of images because only a small part of photons are scattered.

3.1 Microscopy and morphometric analyses

Histological microscopic analysis is commonly used for the evaluation of progression and severity of insulinitis in the pancreases of diabetic animals. The insulinitis score is usually expressed in conventional units and reflects the degree of islet infiltration by mononuclear cells. In many experimental studies, this technique has been employed for the evaluation of the dynamics of islet infiltration as well as for the assessment of progressive BCM loss in relation to insulinitis in animal models for T1D (Wicker et al., 1987; Signore et al., 1994). The ratio of islet area to the entire pancreatic area is considered a very informative indicator of pancreatic islet development and reflects the dynamics of BCM reduction with T1D progression (Li et al., 2000). For the quantification of this parameter, multiple sections or series of overlapping histological images can be acquired using a microscope equipped with a motorized stage (Zito et al., 2004). After the computerized "stitching" of multiple single planes, every composite image represents a histological section through the whole pancreas, containing a large number of islets. A similar quantitative approach is used for whole slide

scanning, a recent technology that combines ultra-resolution digital scanning of glass slides with archiving, viewing, and image analysis of whole slide scans. After scanning the whole slide, all islets in the pancreatic section can be identified using either semi-automated or automated computer-assisted image analysis. This technology is widely used for the monitoring of pancreatic morphology in T1D animal models (Soltani et al., 2007).

Epifluorescence microscopy of the entire pancreas was performed by Kilimnik et al. (2009) for the assessment of pancreatic islet distribution *in situ* using transgenic mice that express green fluorescent protein (GFP) specifically in beta-cells under the control of the mouse insulin I promoter (MIP, Hara et al., 2003). After dissection, fixation and treatment with sucrose and glycerol, the pancreas was placed on a glass slide and examined at different depths. Integrated two-dimensional images obtained by forming a montage of single planes acquired along the Z-axis were processed and used for morphometric analyses. However, conventional epifluorescence microscopy has a limited ability to resolve the three-dimensional (3D) tissue architecture that provides both stereological data and 3D visualization.

3.2 Confocal laser scanning microscopy

In contrast to epifluorescence microscopy, confocal laser-scanning microscopy (CLSM), collects fluorescent photons from a single, illuminated, focused spot, excluding fluorescent signals from objects that are outside the focal plane. Single optical sections through fluorescent objects are generated after point-by-point scanning across the specimen. Parallel optical sections obtained by moving the focal plane along the Z-axis can be combined, assembling a 3D stack. The application of confocal microscopy for 3D analysis has become an important advance because it does not require sectioning and avoids the spatial distortion of biological structures, which is an inevitable result of tissue embedding, processing, and sectioning. A detailed comparative analysis of human pancreatic islets and islets isolated from non-human primates and mice of different strains, including their architectures and compositions using CLSM, have been performed by Brissova et al. (2005). Entire isolated islets were subjected to optical sectioning along the Z-axis at 1- μm intervals, after which optical sections were reconstructed into a 3D stack and analyzed using image analysis software. It was found that human islets were not only heterogeneous in terms of cell composition but also had a pattern of alpha, beta, and gamma cell distribution within the islets that differed from that of murine islets. Experimental studies performed by Cabrera et al. (2006) with the assistance of CLSM showed that human islets contained fewer beta-cells and more alpha cells than mouse islets, amounting to 60% and 30% of the total number of endocrine cells, respectively. Furthermore, all types of endocrine cells were found in close proximity to islet microvessels, but without any particular alignment along the microvasculature. A unique architecture and cellular composition of human islets is associated with a distinct physiology, as evaluated by oscillations in membrane potentials and intracellular Ca^{2+} responses to high glucose concentrations. A recent review on islet architecture and comparative anatomy of islets in different species of animals from an evolutionary point of view has been presented by Heller (2010).

3.3 Intravital microscopy

Intravital microscopy has become a powerful technique for studying the dynamics of biological processes and visualizing the cellular and sub-cellular events in the native environments of living animals. Due to a limited depth of penetration, application of the methods of *in vivo* microscopy to the imaging of most internal tissues, including the

pancreas, requires special surgical tissue preparation methods. There are three main categories of tissue preparation which are based on the type of surgical method used: chronic-transparent windows (abdominal window, body window, dorsal skinfold chamber, etc.), exteriorized tissue preparation, and *in situ* preparations (Jain et al., 2005). Chronic abdominal window preparation, developed for direct intravital microscopy of the pancreas, is used mainly for studies of the growth of pancreatic tumors and the effects of anti-cancer therapies (Tsuzuki et al., 2001). A body window was designed for longitudinal *in vivo* imaging of islets, isolated from transgenic mice expressing proinsulin II tagged with a fluorescent reporter protein Timer, which changes colour from green to red in the first 24 h after synthesis, and transplanted under the kidney capsule (Bertera et al., 2003). By using this technique fluorescently labeled beta-cells and T cells can be simultaneously investigated through the window device placed and sutured over the site of islet transplantation after underlying skin and body wall are removed.

The dorsal skinfold chamber is a valid model for the intravital microscopic study of pancreatic islet grafts, in which the striated skin muscle serves as a bed for the transplants (Menger et al., 1992). After contrast enhancement is achieved by intravenous injection of fluorescently labeled dextran (FITC-dextran, TRITC-dextran), the microcirculatory bed of transplanted islets can be identified as a distinctive, glomerular-like vascular plexus and used for the quantitative analysis of all microvascular segments in individual islet grafts using traditional techniques (Menger et al., 1994). Intravital microscopy of the exteriorized pancreas, which is located deep in the area between the spleen, stomach and duodenum, is tightly connected with the surrounding tissues and possesses essential opacity, is extremely challenging and is not widely practiced compared with transparent and easily accessible organs, like the mesentery and a few skeletal muscles (Intaglietta & Messmer, 1983; Ley et al., 1986; Hudlicka, 1998; Segal, 2005). Exposure of the pancreas requires extreme care because this tissue is quite fragile and easily develops hemorrhages. Furthermore, to identify and locate islets that could be used for imaging, an additional contrast enhancement of the islets should be used. Pancreatic islets can be revealed after intravenous injection of dithizone (diphenylthiocarbazone, DTZ), a chelating agent that combines with zinc in islets to form a visible complex (Bunnag et al., 1963) that possesses fluorescent properties in the red spectrum (Denis et al., 2004, Martinic & Herrath, 2008). However, the fluorescence of DTZ complexes in the living pancreas fades rather quickly, so this type of staining cannot be used for long-lasting *in vivo* experiments. Generation of transgenic mice with pancreatic beta-cells genetically tagged with GFP, or with its yellow or cyan derivatives that can be detected *in vivo*, has become a valuable tool for the real-time identification of pancreatic islets containing fluorescently labeled beta-cells (Hara et al., 2003). To visualize fluorescent protein-labeled beta-cells in the intact pancreas, Hara et al. (2006) developed an imaging technique that combines reflection and confocal microscopy. Visualization of the intact pancreas from MIP-GFP and red fluorescent protein (RFP) transgenic mice showed that the distribution of fluorescently labeled beta-cells within the pancreas was not even, as the majority of islets were located around large blood vessels, and some clusters of beta-cells were found around the pancreatic and common bile ducts. By crossing neurogenin-3-GFP mice with MIP-RFP mice the authors generated rainbow mice in which beta-cells and beta-cell progenitors were labeled with different fluorescent proteins and used for the study of pancreatic development at the molecular level.

Intravital microscopy of the pancreatic microcirculation has benefited greatly from genetically tagged fluorescence labeling of pancreatic beta-cells. The relationship between

the pattern of blood flow through the pancreatic islets and islet cell composition and its influence on islet cell secretion has been a topic of many scientific discussions. Different orders of endocrine cell perfusion within an islet can have significant physiological consequences, because hormones secreted by one cell type can affect the secretory function of another cell type. Different hypotheses about the organization of blood flow in islets have been attributed to three models that describe the pattern of pancreatic islet blood flow: mantle-to-core, core-to-mantle and polar flow (Menger et al., 1994, Brunicardi et al., 1996, 1997). Most experimental data have been obtained using scanning electron microscopy-corrosion cast studies or intravital microscopy using fluorescent markers such as FITC-labeled red blood cells, FITC-dextran or fluorescent microspheres. To further investigate the relationship between pancreatic islet microcirculation and islet cell organization, dynamic optical imaging employing high-speed CLSM of the pancreases of IP-GFP mice has been used (Nyman et al., 2008, Fig.2). To assess 3D flow patterns in the islets, a time series of z stacks with an imaging rate of 70–72 frames per second after bolus injection of rhodamine dextran was acquired. Average fluorescence intensities were evaluated over time for selected regions of interest at different locations within the islet microvasculature using the 3D data. The analysis revealed two major directional blood flow patterns in the mouse islets: 1) the inner-to-outer type of perfusion, where the core of beta-cells is upstream of the non-beta-cell mantle, which was found in the majority of islets and 2) the top-to-bottom blood flow pattern, where the direction of blood flow is not dependant on cell type, which was found in 35% of islets. Due to the limited depth of penetration inherent in confocal microscopy, these results represent flow patterns obtained for islets located near the surface of the dorsal pancreas, revealing the predominance of the inner-to-outer type of islet perfusion, in which the products of beta-cell secretion likely have a regulatory effect on the secretory activity of non-beta-cells and ensuring the interaction of different types of endocrine cells in the regulation of blood glucose levels.

An intravital setup for islet monitoring, similar to those described above, relies on a different technology. Optical coherence tomography (OCT) is an interferometric technique that amplifies photons backscattered by the tissue. As such, it could be described as the optical equivalent of ultrasound imaging. Villiger et al., (2009) showed, by implementing an extended focus scheme (extended focus optical coherence microscopy, xfOCM, Leitgeb et al., 2006), that islets backscatter light stronger than the surrounding pancreatic tissue, allowing for the visualization of islets in the exteriorized pancreas of mice. Hence, although to some extent limited by its penetration depth (~300µm), this technique enables imaging of endogenous islets without the use of contrast agents.

3.4 TPLSM and MPLSM imaging

More recent advances in optical imaging platforms may further facilitate diabetes research and the development of new therapies for T1D, imaging of intracellular beta-cell metabolism and molecular probe generation. Examples of such approaches include the application of two- and multi-photon laser confocal scanning that enables 3D and longitudinal assessments of fluorescently labeled molecular structures in living pancreatic islets in both *in vitro* and intravital systems. Imaging of living cells and tissues with multi-photon microscopy provides important advantages over visualization performed using conventional confocal microscopy, including substantially increased depth of penetration and reduced phototoxicity in 3D imaging (Dunn & Young, 2006; Benninger et al., 2008).

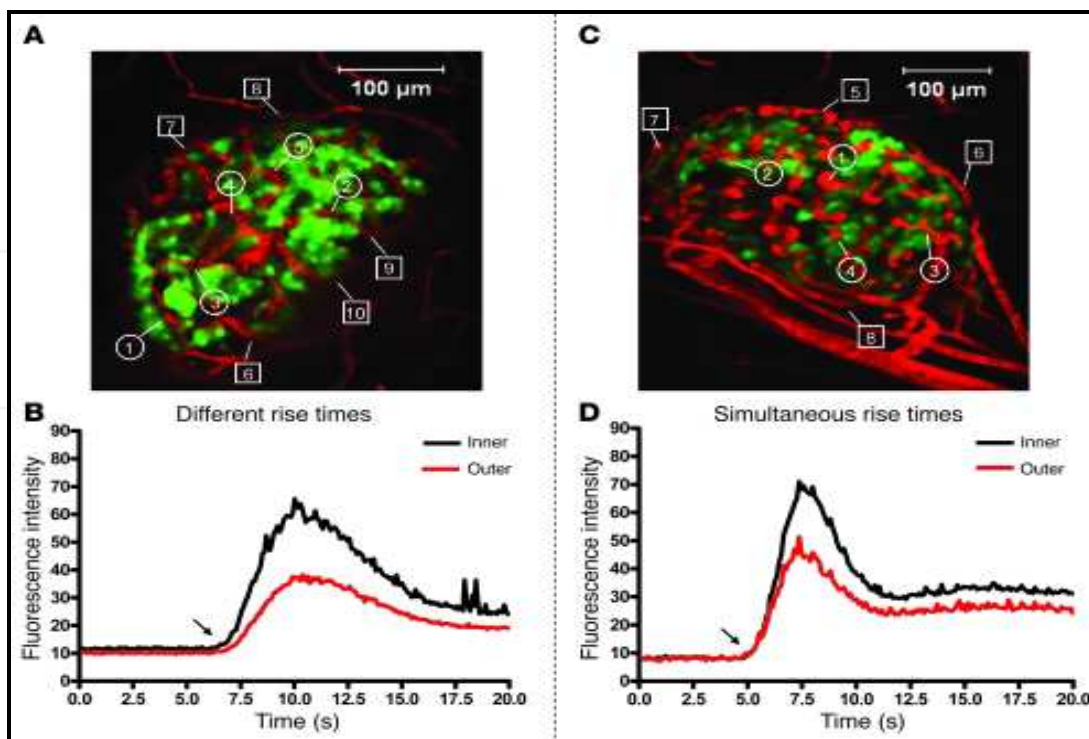


Fig. 2. Intravital laser-scanning confocal microscopic imaging of MIP-GFP mouse pancreases after injection of rhodamine dextran tracer into the vasculature. Temporal resolution analysis demonstrates different islet blood flow patterns. (A and C) Two different islets (single-plane images), shown with inner (circles) and outer (squares) vessel regions selected and marked for fluorescence intensity measurement after a bolus of rhodamine dextran. Scale bars: 100 μm . (B and D) Average inner vessel fluorescent intensity time courses (throughout the z plane) compared with average outer vessel fluorescence intensity time course for the islets in A and C, respectively. Arrows denote rise times. Average rise time for the inner vessels was either (B) before that of outer vessels, as demonstrated by increased fluorescence intensity shown first by inner vessels, or (D) no different from outer vessels, which demonstrates that different islets had different perfusion patterns (Nyman et al., 2008). © Copyright 2008 by American Society for Clinical Investigation. Reproduced with permission of American Society for Clinical Investigation.

Conventional fluorescence microscopy is based on the absorption of a single photon by a fluorescent molecule (fluorophore), which results in a transition of the molecule into an excited state with a higher energy level. Relaxation to the initial state leads to emission of a single photon of a wavelength longer than the excitation wavelength. High excitation intensity, which can be provided by ultra-short infrared pulsed lasers with twice the wavelength (or half the frequency) of the standard excitation beam within a shorter period of time than the time of fluorescence decay of the fluorescent molecule, can lead to the simultaneous absorption of two photons each with half the energy required for single-photon excitation. Focusing the excitation beam into a small volume is achieved using lenses with high numerical aperture. Due to this very precise focusing, the only location where the absorption takes place is in this focal volume, but not at other depths either below or above the focal plane through which the excitation light passes. However, fluorophores do not experience a two-photon effect, ensuring one of the most important two-photon LSM

(TPLSM) advantages - reduced photobleaching-which occurs only in the focal area but not within the surrounding tissue. The high excitation wavelength allows for deep tissue penetration. While in conventional confocal microscopy the scattering of excitation light induces fluorescence outside the focal plane, in TPLSM the scattered photons do not contribute to the fluorescence signal. Since fluorescence is derived only from the focal plane, the fluorescence signal can be achieved without a pinhole but with an external detector placed immediately after the objective, thus increasing the efficiency of signal collection. TPLSM therefore displays several important advantages over conventional confocal microscopy by allowing confocal images to be obtained with increased imaging depth and high light-gathering efficiency but with less photobleaching and specimen damage outside the focal volume. Hence, TPLSM makes it possible to undertake a number of studies related to beta-cell imaging at the cellular and sub-cellular level.

Coppieters and co-workers (2010) developed an approach based on intravital two-photon microscopy of the pancreas to study the dynamic interactions between individual T cells and pancreatic beta-cells in real time. An acute transfer model for autoimmune diabetes was employed by injection of fluorescently labeled splenocytes harboring diabetogenic T-cell receptor-transgenic CD8 T cells into reporter mice with beta-cells expressing both GFP and antigen. The authors were able to detect the transferred T cells around the islets, which were accessible for intravital examination by two-photon microscopy, and to track both T-cell motility and interactions with beta-cells.

Based on TPLSM, a platform for the non-invasive and longitudinal *in vivo* imaging of functional pancreatic islets transplanted into the anterior chamber of the eye at a single-cell resolution, has been developed. Non-invasive TPLSM monitoring of the islets isolated from mice expressing GFP under control of the rat insulin-1 promoter (RIP-GFP) and transplanted into the iris through the cornea was performed together with the visualization of the islet vascular network after injection of Texas Red-conjugated dextran (Speier et al., 2008a). Originally, the anterior chamber of the eye was chosen as a transplantation site because of its immune-privileged properties (Niederhorn, 2002). Being a natural body window, the anterior chamber of the eye can be easily accessed with LSM and TPLSM through the cornea, providing sub-cellular resolution in real time and the possibility for longitudinal monitoring of islet cytoarchitecture, vascularization and innervations as well as beta-cell metabolism, after islet engraftment on the iris (Speier et al., 2008b). Hence, this approach enables studies of beta-cell signal transduction, gene expression and mechanisms involved in exocytosis, to be conducted with fluorescent probes in a natural physiological environment with vascular and nervous supply re-established after transplantation (Leibiger et al., 2010).

3.5 Optical projection tomography

Optical projection tomography (OPT) Sharpe et al., (2002), is a relatively recent technique that was developed as a tool for 3D visualization of gene and protein expression patterns in biomedical specimen at the mm-cm scale. The technology in many ways bridges the gap between microscopy techniques utilizing optical sectioning such as CLSM (with a penetration depth limited to a few hundred micrometers) and non-optical techniques like MRI (commonly used for imaging at the level of the entire organism). The 3D images generated by OPT are volumetric data sets similar to those generated by other tomographic techniques and therefore the same visualization techniques may be used. Hence, OPT could

be described essentially as computed tomography (CT), which instead of using x-rays employs light in the visible part of the spectrum (Sharpe, 2003). The basic OPT scanner setup includes a rotary stage driven by a stepper motor for positioning and holding the specimen, imaging lenses for focusing and magnifying the image, two types of illumination: a white light diffuser for the transmission imaging and an arrangement for imaging in the fluorescence mode and a charged coupled device (CCD) camera to capture the raw projection images. For OPT imaging, the specimen is embedded in an agarose gel and subjected to optical clearing. It is then attached onto a rotary stage of the scanner and immersed in an imaging chamber, filled with an organic solvent, most frequently, Murray's Clear (also referred to as BABB), prepared as a mixture of benzyl alcohol and benzyl benzoate. Placement of the sample in an index-matching medium reduces scattering of light and heterogeneity of refractive index within different parts of the specimen during scanning. While the specimen is rotated through 360°, light transmitted through the specimen is focused on the CCD chip by lenses and images are captured at each step-rotated position. For the reconstruction of raw projection data into a 3D voxel stack, a filtered back-projection algorithm is used (Sharpe, 2004). The technique has already contributed to addressing a broad range of biological questions in diverse systems such as human, mice, chicken, fly, zebrafish and plants (see e.g. Sarma et al., 2005; Kulandavelu et al., 2006; Fisher et al., 2008; McGurk et al., 2007; Bryson-Richardson & Currie 2004; and Lee et al 2006). However, due to inherent properties of pancreatic (and other) tissue (including strong endogenous autofluorescence and light scattering effects) in combination with difficulties in obtaining sufficient reagent penetration (primarily for antibodies), the technique was initially limited to studies of the embryonic pancreas. In this respect OPT has been a powerful resource for spatial and quantitative phenotypical analyses of pancreas development and in assessments of tissue interactions during pancreas organogenesis (Hecksher-Sørensen et al., 2004; Asayesh et al, 2006; Sand et al., 2011). By combined improvements in protocols for sample preparation, tomographic imaging and computational processing we could adapt the technique to also allow for imaging of the intact adult mouse pancreas (Alanentalo et al., 2007). By nature of the tomographic process, this enabled a practical approach to generate quantitative data (number and volume), spatial coordinates (x, y, z) and 3D reconstructions of individual molecularly labelled objects (e.g. insulin labelled islets of Langerhans) throughout the volume of the pancreas with close to cell level (~15µm) resolution. We further described how such global assessments of the pancreatic constitution (or disease state) could be used to guide confocal resolution regional assessments based on the expression of molecular markers, interacting cell types, spatial coordinates, morphology and volume of individual objects (e.g. an islet) throughout the volume of the gland Alanentalo et al., (2008). Hence, OPT imaging allows for detailed, spatial, quantitative and statistical analyses of the pancreatic BCM distribution down to the level of the individual islets with high molecular specificity and without the need for extrapolation of 2D data. Studies of dynamic events, such as the autoimmune induced destruction of beta-cells during T1D progression, highlights the added value of the OPT technique in such assessments. By allowing for the generation of full islet size distribution profiles, as opposed to average values such as mean islet volume, volume weighted mean, total islet volume and total islet count, important information regarding these processes may be revealed. As an example we recently addressed, by OPT, quantitative and spatial aspects of the autoimmune induced β -cell destruction during T1D progression in the NOD mouse. Although limited to "frozen moments in time" (OPT is due to the requirement for chemical

processing of the tissue limited to ex-vivo assessments), we could hereby provide evidence for a preferential depletion of “smaller” islets during the initial phase of the autoimmune attack, a compensatory growth potential of the larger insulin positive islets during the later stages of the disease as well as new information on the insulinitis process itself including its apparently random distribution at onset, local variations during its further development, and the formation of structures resembling tertiary lymphoid organs at later phases of insulinitis progression (Alanentalo et al., 2010) (see Fig. 3). The possibility to extract this type of cross-relatable spatial and quantitative data suggests that OPT imaging may facilitate numerous diabetes related research areas encompassing those involving evaluations of strategies for therapy and restoration of BCM, interpretations of how metabolically induced phenotypes manifest as diabetic disorders, the screening for rare events or cell niches in genetically engineered mice, the effect of targeted gene ablation on BCM etc.

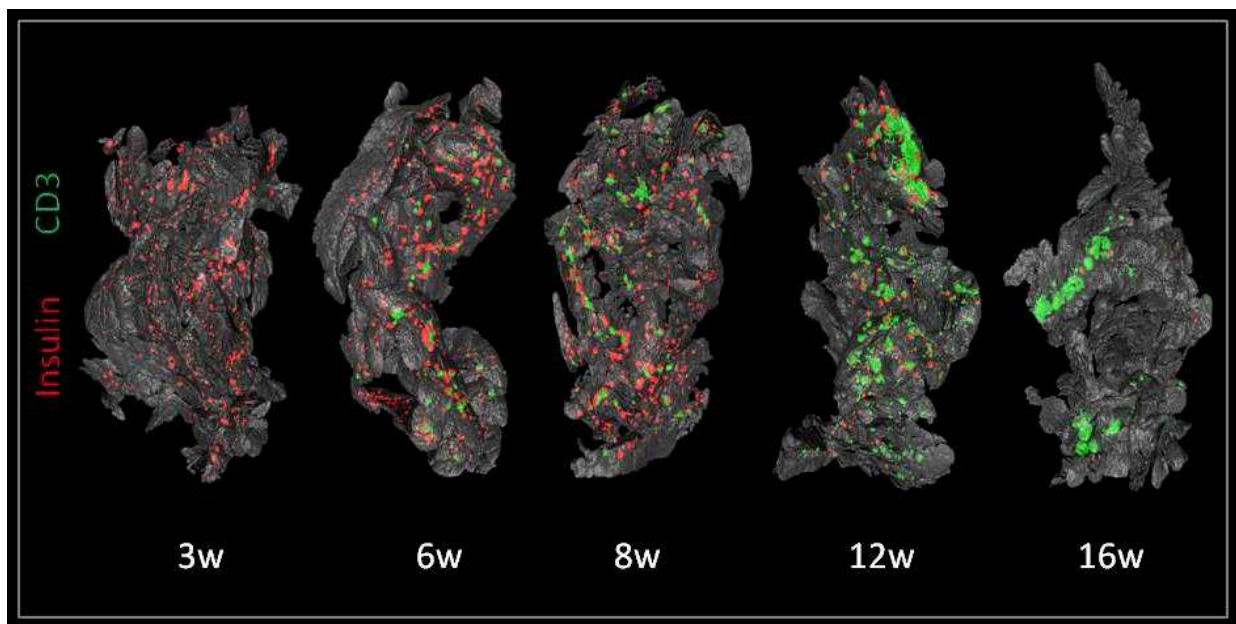


Fig. 3. Spatial assessment of the progression of autoimmune insulinitis in the NOD mouse. Isosurface rendered OPT images of representative pancreata (duodenal) from NOD mice at 3, 6, 8, 12, and 16 weeks. Ins⁺ islets (red) are reconstructed based on the signal from insulin-specific antibodies and infiltrating T-cells (green) based on the signal from CD3-specific antibodies. A high-quality color representation of this figure with scale bars included is available in the online issue (Alanentalo et al., 2010) © Copyright 2010 by American Diabetes Association. Reproduced with permission of American Diabetes Association in the format other book via Copyright Clearance Center.

As with any tomographic imaging technology, OPT is associated with a number of technological hurdles. These most commonly relate to artifacts introduced by the tomographic reconstruction process itself or such that are of optical nature. Several recent reports have described improved reconstruction algorithms and/or other computational tools to further enhance OPT imaging (Birk et al., 2011; Walls et al., 2005, 2007.). As OPT will find its way into more research laboratories, studying different aspects the pancreas and/or T1D disease aetiology, improvements of this kind that more specifically address challenges associated with OPT imaging of organs on the pancreatic scale could be expected. OPT has

an important advantage in that it can produce 3D images of both non-fluorescent and fluorescent specimen, i.e. it can operate in both transmission and emission mode (Sharpe, 2010). This holds the benefit of permitting visualization of pancreatic structures or cell types labeled with standard laboratory markers. Hence, in contrast to other imaging modalities, allowing for *in vivo* or *in vitro* imaging of the intact pancreas, OPT imaging may be performed with a huge selection of available or custom designed antibody markers pertinent to diabetes research. By the possibility to operate in emission mode, and thereby the option for the detection of markers at different fluorescent wavelengths, OPT thus appears well suited for “whole pancreas” monitoring of cellular ratios or cellular interactions under various genetic or pathologic conditions. Preliminary studies performed in our laboratory suggests that this potential may be further expanded by taking advantage of the increased channel separation and signal to noise ratio obtained by using wavelengths in the near infrared spectrum (U. Ahlgren, unpublished observation). An obvious drawback for OPT imaging, at least for studies of the adult rodent pancreas, is the non-existent capacity to perform longitudinal analyses. This is to some extent compensated for by the relative ease by which large number of specimen, e.g. at different stages of disease progression, may be investigated. Although current protocols for tissue processing and antibody labelling are relatively lengthy (~ 10-12 days), they require limited “hands on” and large series of pancreata may be run in parallel (Alanentalo et al., 2010). Further developments in automation of tomographic reconstruction and image processing may further increase the speed and accuracy by which post-scan processing of OPT data may be performed. It should be noted however that the large amount of digital data produced by OPT imaging impose specific challenges for storage capacity and for various aspects of image processing and analyses. A typical pancreas scan (projected and reconstructed images) on a commercial instrument produces in the range of 7-8 GB of data. Although the OPT technique does not allow for the visualization of diabetogenic processes in live mice, there is a potential application for the technique in attempts to develop new strategies for non-invasive beta-cell scoring by other imaging modalities. Preliminary data obtained in our laboratory suggests that by the possibility to detected multiple fluorescent markers, as is further facilitated by the adaptation of OPT to near infrared imaging, OPT could play an important role in the development of new contrast agents for non-invasive assessments of pancreatic cell types by nuclear imaging techniques such as MRI. In this respect, the uptake specificity and readout of essentially any contrast agent being developed for non-invasive imaging (of e.g. beta-cells), that may be also fluorescently labelled, should be possible to cross evaluate, *ex vivo*, by antibody staining (e.g. for insulin) imaged in another fluorescent channel. Finally, it should be emphasized that the potential use for OPT imaging in T1D research is not limited to assessments of the pancreas. By the adaptation of OPT to imaging of intact mouse organs (and larger tissue specimen) of adult mice (Alanentalo et al., 2007), it may well find important applications in experimental research on islet engraftment and in studies of vascular complications of diabetes.

4. Concluding remarks

A correlation between changes in BCM and the development of T1D is generally accepted. The development and refinement of imaging technologies that enables assessments of BCM

dynamics and its distribution is therefore highly needed in both pre-clinical and clinical settings. To this end, a range of imaging platforms have been developed, with each one associated with strengths and weaknesses for parameters such as tissue penetration depth, spatial resolution, contrast agent specificity/availability etc. Together, these imaging modalities represent technologies for assessments of diabetogenic processes from the cellular and sub-cellular level, to the level of the whole organ or entire organism. Given the title of this chapter, it should be emphasized that the potential of these techniques for experimental diabetes research or in clinical applications goes far beyond the study of the beta-cells. Obviously, they have important applications for the study of other cells and tissues affected by- or mediating the disease, for example in the diagnostics of leukocyte infiltration. In this chapter, we have attempted an overview of the current status and future potential of MRI and a number of optical imaging modalities, including CLSM, M/TPLSM and OPT. Of these, the optical techniques have restricted possibilities for imaging of larger specimen due to their limited tissue penetration depth. However, by their generally speaking high spatial resolution, the abundant selection of available biomarkers and the possibility for simultaneous detection of different targets in the investigated tissue they are well suited for studying mechanistic aspects of T1D in experimental animal research. In contrast, MRI, PET and SPECT at present represent the technologies of choice for assessments of human subjects due to their “unlimited” penetration depth, non-invasive characteristics and the possibility to perform longitudinal studies. Although SPECT and PET are not covered by this chapter, it should again be emphasized that these techniques similarly to MRI hold great potential for the monitoring of BCM in clinical settings. Hereby, it should be noted that whereas MRI has the highest spatial resolution, with the potential of monitoring individual islets, it has the lowest sensitivity while the opposite is true for PET. Still, a number of technological hurdles, some of which are shared for all three modalities, for their routine use in clinical practice needs to be resolved. The perhaps major issue in this respect is the identification of suitable beta-cell ligands/ contrast agents. As discussed in section 3.5 of this chapter the development and/or identification of such agents may be facilitated by the use of *ex-vivo* optical techniques for whole organ imaging such as OPT. Given the dramatic technological and methodological advances that been presented during the past decade, it seems likely that many of the problems that currently impede beta cell and pancreas imaging, of spatial and quantitative nature, are to be resolved in an over-viewable future. Although, no technique can be expected to cover all the needs for imaging in diabetes research a palette of complementary techniques exist today that provides solutions for a vast variety of imaging tasks. Another important challenge for the future is the development of imaging strategies that also enables extraction of specific functional aspects of the native beta-cells. As development tends to progress in the direction of combinatorial/multimodal approaches, it seems likely that techniques for imaging-based multivariate analyses will be an important part of tomorrows toolbox for diabetes research and diabetes care.

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6. Abbreviations

3D - three-dimensional

BCM - beta-cell mass

CLSM - Confocal laser scanning microscopy

DTZ - Dithizone

LSM - Laser scanning microscopy

MPLSM - Multi photon laser scanning microscopy

MNP - Magnetic nanoparticles

MRI - Magnetic resonance imaging

MR - Magnetic resonance

OCT - Optical coherence tomography

OPT - Optical projection tomography

PET - Positron emission tomography

SPECT - Single photon emission computed tomography

SPIO - Superparamagnetic iron oxide

STZ - Streptozotocin

T1D - Type 1 diabetes

TPLSM - Two photon laser scanning microscopy

xfOCM - extended focus optical coherence microscopy

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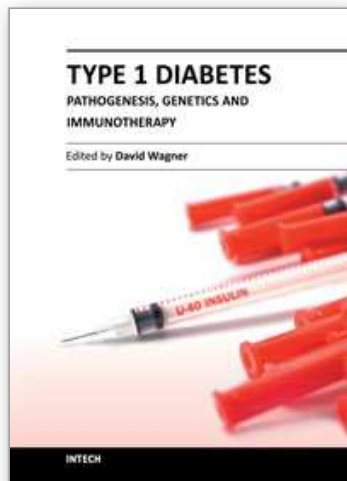
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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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