Published in final edited form as:
Acta Physiol (Oxf). 2012 February ; 204(2): 178–185. doi:10.1111/j.1748-1716.2011.02301.x.

Non-invasive *in vivo* imaging of pancreatic β-cell function and survival – a perspective

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Abstract

A major problem in medical research is to translate *in vitro* observations into the living organism. In this perspective, we discuss ongoing efforts to non-invasively image pancreatic islets/β-cells by techniques, such as magnetic resonance imaging and positron emission tomography, and present an experimental platform, which allows *in vivo* imaging of pancreatic β-cell mass and function longitudinally and at the single-cell level. Following transplantation of pancreatic islets into the anterior chamber of the eye of mice and rats, these islets are studied by functional microscopic imaging. This imaging platform can be utilized to address fundamental aspects of pancreatic islet cell biology *in vivo* in health and disease. These include the dynamics of pancreatic islet vascularization, islet cell innervation, signal-transduction, change in functional β-cell mass and immune responses. Moreover, we discuss the feasibility of studying human islet cell physiology and pathology *in vivo* as well as the potential of using the anterior chamber of the eye as a site for therapeutic transplantation in type 1 diabetes mellitus.

Keywords

diabetes mellitus; fluorescence microscopy; *in vivo* optical imaging; islets of Langerhans; pancreatic β-cell mass

The incidence of both forms of diabetes, i.e. type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), is increasing, T2DM reaching epidemic proportions. Genome-wide association studies revealed that more than 30 candidate genes are involved in the pathogenesis of T2DM, most of them potentially linked with pancreatic islet/β -cell function. This shifts the paradigm of diabetes being primarily due to insulin resistance of the

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Conflict of interest
P-OB is co-founder of BioCrine AB, IBL is consultant to BioCrine AB.

classical insulin target tissues liver, muscle and fat towards a more central role of the β-cell in the pathogenesis of this disorder. Hence, it pinpoints the importance of an intact integrated signal-transduction for a proper function and survival of the β-cell. There is a growing body of evidence that the β-cell relies on multiple signals provided by the architecture of the islet of Langerhans for an appropriate function. To understand the dynamics of β-cell function and survival under normal conditions and the development of β-cell dysfunction in diabetes, these processes must be studied in the context of the intact islet of Langerhans *in vivo* where the capillary network is intact and the innervation is adequate. Here we present an experimental platform, which allows non-invasive imaging of pancreatic β-cell function and survival longitudinally with single-cell resolution.

The physiology of islets has been extensively investigated *in vitro*

There is a wealth of information about the physiology of rodent islets *in vitro*. A variety of techniques have been applied to isolated islets to record membrane electrical activity, changes in cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, mitochondrial membrane potential and hormone release. The main signal-transduction cascades have been dissected in detail and the different steps involved in the stimulus-secretion coupling have been elucidated for rodent β-cells. Glucose is effectively taken-up by the β-cell glucose transporters. It is metabolized in glycolysis and Krebs-cycle, which results in an increased ratio of ATP to ADP in the cytoplasm. This closes ATP-sensitive potassium channels (K_{ATP} channels) and leads to cell membrane depolarization, opening of voltage-gated Ca^{2+} channels, increase in $[Ca^{2+}]_i$ and finally, insulin secretion. As a consequence of this chain of events, insulin secretion is tightly coupled to changes in the extracellular glucose concentration. Besides this K_{ATP}-channel-dependent 'triggering' pathway, glucose in addition acts through a so-called K_{ATP}-channel-independent 'amplifying' pathway to achieve maximal insulin exocytosis.

Methods for *in vivo* imaging of pancreatic islet function are scarce

In contrast to isolated islets *in vitro*, glucose reaches endocrine islet cells in the native pancreas through the microvasculature and this arrangement, for sure has an impact on islet cell function. In addition, native islets are innervated and surrounded by a dense basement membrane. Imaging islets in the living organism is challenging and unfortunately, non- or minimal-invasive technologies to monitor islet cell function and survival longitudinally, specifically in terms of molecular signal-transduction processes, have not been developed.

To study β-cell function in a relatively intact environment, pancreatic slices containing islets were used for electrophysiological recordings (Speier & Rupnik 2003). In this set-up, islets retain their structural integrity and are not subjected to enzymatic digestion, but are isolated from the organism. Islets have been imaged after transplantation under the kidney capsule, where it has been possible to visualize the vascularization process (Bertera *et al.* 2003, Nyqvist *et al.* 2005). However, the instability and inaccessibility of this transplantation site makes this approach difficult, in particular for functional and longitudinal microscopic studies.

Other emerging efforts to image β-cells *in vivo* include magnetic resonance imaging (MRI) and position emission tomography (PET), (reviewed in Paty *et al.* 2004, Souza *et al.* 2006, Lin *et al.* 2008, Moore 2009). Although MRI has been successfully applied to monitor the mass change of transplanted β-cells/islets in diabetic rats (Koblas *et al.* 2005), these methods are limited because they poorly discriminate between exocrine and endocrine tissues, thus failing to accurately measure β-cell mass. This is likely because of the low abundance of islets and the lack of contrast from the surrounding tissue in the pancreas. An additional major challenge is identifying markers that can be combined with imaging reporters that are

specific to endocrine cells of the pancreas. While it is expected that these methods will eventually improve quantifying islet mass *in vivo*, they may not be useful to visualize subtle changes in apoptosis, vascularization or innervation. Even with the use of high-contrast materials or tissue-specific luminescence, these techniques are limited by high background signal from surrounding tissues, lack spatial resolution (<100 μm) and do not allow functional monitoring of islets.

Optical imaging techniques, including confocal microscopy and optical coherence tomography, have been recently applied to study the biology of islets in the pancreas or in transplanted islets (reviewed in (Lin *et al.* 2008, Virostko & Powers 2009). To perform these studies, islets and their associated structures, such as blood vessels and nerves, need to be labelled with probes that emit light or that affect light scattering. Using an early marker of apoptosis (annexin V) coupled to a fluorophore emitting near-infrared radiation, β-cell death can be detected in diabetic mice *in vivo* (Medarova *et al.* 2005). To image with single-cell resolution, however, the pancreas needs to be externalized. This approach has been used to investigate blood flow in pancreatic islets (Nyman *et al.* 2008) and has been proposed to investigate immune responses to islets in the pancreas (Martinic & von Herrath 2008). Longitudinal studies, however, are unlikely to be possible because the pancreas cannot be externalized repeatedly.

An alternative approach is to apply fluorometric imaging techniques that have been used for *in vitro* islet cell characterization, such as epifluorescence microscopy, confocal laser scanning microscopy (CLSM) and two-photon laser scanning microscopy (TPLSM), to islets grafted in a site that is readily accessible for imaging, sufficiently stable for longitudinal and functional studies, and in which islets are vascularized and innervated. This site is the anterior chamber of the eye (Speier *et al.* 2008a,b).

The anterior chamber of the eye as a natural ‘body-window’ for *in vivo* imaging

The anterior chamber of the eye can be used as a natural body-window to non-invasively image pancreatic β-cell function and survival longitudinally in the context of the intact islet under *in vivo* conditions (Speier *et al.* 2008a, b). The cornea is transparent and the grafts are easily vascularized and innervated because of the rich blood and nerve supply of the iris that forms the bed of the anterior chamber of the eye. The iris contains nerve fibres that have been shown to invade the intra-ocular grafts (Taylor *et al.* 1978). Therefore, the autonomic input to the grafted tissue can be modulated non-invasively.

The anterior chamber of the eye is a classical transplantation site used since the early 1870s (van Dooremaal 1873), when van Dooremaal made the observation that tumour cells injected into the anterior chamber of the eye formed progressively growing tumours. More than 60 years later, Sir Peter Medawar discovered that skin grafts survived for prolonged intervals in the eye. Since then, the anterior chamber of the eye has been widely used as a model system to study not only immune deviation but also growth and differentiation of a variety of tissues, including ovaries (Falck 1959), peripheral and central nervous tissues (Bickford-Wimer *et al.* 1987) and also pancreatic tissue grafts (Adeghate & Donath 1990).

Utilizing the anterior chamber of the eye as an imaging platform will move *in vitro* islet studies into real-time *in vivo* islet physiology. Fluorescent tools and probes that have been used to image structure and function in isolated islets *in vitro* can now be applied to intra-ocular islet grafts. It is easy to envisage applications of this platform for studying the mechanisms of vascularization and innervation after transplantation, inflammatory

responses, as well as physiological responses to changes in plasma glucose concentration or pharmacological intervention (Fig. 1).

Pancreatic islets following transplantation into the anterior chamber of the eye are functional

After transplantation of mouse islets into the anterior chamber of the eye, these islets engraft on the iris and are readily observed and imaged through the cornea. Immunohistochemical staining of engrafted islets shows that the proportion of insulin containing β-cells and glucagon-containing α-cells does not change after transplantation (Speier *et al.* 2008a,b). Moreover, comparing the histology of islets transplanted into the eye with islets in the pancreas reveals no changes in islet composition (Ilegems *et al.* 2010). In fact, electron micrographs show that fine structures such as fenestrae within the capillary endothelial cell layer are maintained. Engrafted islets are functional and contribute to glucose homeostasis of the recipient mouse. After transplantation of syngeneic islets (approx. 300 islet equivalents) into the anterior chamber of the eye of streptozotocin-induced diabetic C57BL/6 mice, all animals achieve normoglycaemia within 2 weeks (9.2 ± 2.2 days). Removal of the islet graft-bearing eye resulted in prompt return to hyperglycaemia (Speier *et al.* 2008a). Thus, engrafted islets are responsible for glycaemic control in the recipient mouse.

The anterior chamber of the eye: a transplantation site to study dynamics of vascularization

Both revascularization and reinnervation of islets in the process of engraftment are of vital importance for islet cell function and survival (Korsgren *et al.* 1993, Adeghate 2002, Andersson *et al.* 2004, Brissova & Powers 2008, Richards *et al.* 2010). The vascularization of transplanted islets in the anterior chamber of the eye can be investigated by using angiographic imaging. Syngeneic mouse islets that express green fluorescent protein in their pancreatic β-cells [rat insulin promoter (RIP)-GFP mice (Speier *et al.* 2008a)] are transplanted into the eyes of C57Bl/6 mice. Because islets can be readily observed and retrieved, it is feasible to image repeatedly the same group of islets. To visualize blood vessels, the fluorescent dye rhodamine dextran is injected into the tail vein. Blood vessels can be seen contacting grafted islets <1 week after transplantation. The vascularization pattern becomes more complex with time and 10 days after transplantation smaller vessels branching from larger vessels in grafted islets can be seen. Tie2-GFP mice, that express GFP in their endothelial cells can be used as recipients to explore the vascularization process non-invasively. Longitudinal experiments demonstrate that the vascularization process reaches a plateau after 4 weeks, showing a vasculature density of the islets equivalent to that found in the pancreas.

Hence, this platform will enable researchers to image angiogenesis at high resolution in real-time in a mammalian model. This platform will also allow studies of the microcirculation within the pancreatic islet under normal and diabetic conditions and thereby reveal possible islet vascular complications associated with the disorder.

The anterior chamber of the eye: a transplantation site to study dynamics and nature of innervation

An important task in islet cell biology as well as pathology is to understand the dynamics, nature and origin of innervation of the various endocrine cells. When transplanted into the anterior chamber of the eye, tissues become innervated by the autonomic nerve supply of the iris (Rodriguez-Diaz *et al.* 2009). The use of mice expressing GFP specifically in neurones

[e.g. B6; 129S-Mapt$^{tm1(EGFP)Klt}$ or ChAT^{BAC}-eGFP (Tallini *et al.* 2006)] as recipients of the grafts allows for non-invasive visualization of the innervation process and thereby the possibility to follow elongating nerve axons. With retrograde tracing from the eye, one will be able to investigate which brain areas project to the various endocrine cells.

By using the *in vivo* imaging platform, it is thus possible to follow in real-time innervation and nature of innervation of the various islet cells subsequent to transplantation. Not only can one investigate how this process is affected in diabetes but also how important this innervation is for overall signal-transduction patterns and thereby the function and survival of the various endocrine cells.

The anterior chamber of the eye: a transplantation site to study signal-transduction in pancreatic β-cells *in vivo*

To fully understand the molecular mechanisms that lead to β-cell dysfunction in diabetes and to develop strategies strategies aiming at preventing β-cell dysfunction, signal-transduction pathways in β-cells have to be investigated under natural conditions in the islet *in vivo*. Hence, a potential strategy to achieve this is based on online monitoring/imaging of key events in β-cell signal-transduction in pancreatic islets that are transplanted into the anterior chamber of the eye to allow non-invasive *in vivo* monitoring using biosensors that reflect (1) glucose metabolism/ATP production, (2) $[Ca^{2+}]_i$, (3) exocytosis, (4) stimulus-induced insulin gene transcription, (5) total β-cell mass, (6) apoptosis and (7) regeneration. Biosensors to be used in these experiments are genetically engineered proteins that carry fluorescent probes, which allow imaging of cellular signalling events by measuring with spatio-temporal resolution fluorescence resonance energy transfer (FRET) between suitably labelled interacting partners as well as the fluorescence intensities of signalling components *per se*. Various genetically engineered Ca^{2+}-biosensors are available and have been successfully used *in vitro* and *in vivo* (reviewed in (Kotlikoff 2007). Biosensors reflecting insulin gene expression (insulin promoter-driven GFP) and apoptosis (C-DEVD-Y) have been extensively tested in *in vitro* studies (Leibiger *et al.* 1998, 2001, Kohler *et al.* 2003, Uhles *et al.* 2007). Biosensors can be specifically expressed in β-cells by employing the insulin promoter. A strategy will be to use virus-based expression vectors for transduction of islets *in vitro* prior to their transplantation into the eye.

Similarly, expressing these biosensors under the control of the glucagon or somatostatin promoter will allow real-time imaging of signal-transduction in pancreatic α- and δ-cells respectively.

The anterior chamber of the eye: a transplantation site to study changes in pancreatic β-cell mass

To investigate the feasibility of non-invasive imaging of β-cell death, we transplant islets from RIP-GFP mice (expressing GFP in their β-cells under control of the RIP) into the anterior chamber of the eye and, after complete engraftment and vascularization, we monitor cell death following intravenously administered annexin V (which binds to the surface of early apoptotic cells) conjugated to allophycocyanin (APC). Transplanted RIP-GFP islets imaged in mice with regular blood glucose levels display normal morphology and absence of annexin V-APC labelling. β-cell death in mice transplanted with RIP-GFP islets is induced by intravenous administration of alloxan. After 24 h, substantial loss of GFP fluorescence and structural changes in the reflection of the islet grafts are observed, indicating loss of β-cells. Administration of annexin V-APC at this time point results in strong labelling of islet grafts. High magnification imaging reveals that most annexin V-APC labelling is found in graft regions devoid of GFP fluorescence. These data illustrate

that β-cell death can be imaged non-invasively and longitudinally in real-time under *in vivo* conditions in islets engrafted in the eye (Speier *et al.* 2008a). These experiments also demonstrate that engrafted islets in the anterior chamber of the eye can be used as visible representatives of their natural counterparts in the pancreas. This allows monitoring longitudinally, the gain in β-cell mass in the ob/ob mouse as well as the loss of β-cells in response to autoimmune destruction in the T1DM BB-rat.

The anterior chamber of the eye: a transplantation site to study immune reactions at the cellular level

Development of T1DM and its treatment by islet transplantation covers two fundamental aspects in immunology research, namely autoimmune destruction of cells (T1DM) and allograft rejection following transplantation (islet transplantation in T1DM). The here-presented experimental platform allows for the first time monitoring, in real-time, the respective immune responses *in vivo* at the cellular level (Abdulreda *et al.* 2010). In a model of allograft transplantation, DBA/2 mouse islets are transplanted into diabetic C57BL/6 recipients without immune-suppression, which leads to intra-ocular graft loss. When C57BL/6(B6.129P2-Cxcr6tm1Litt/J) mice expressing GFP in T-lymphocytes are used as recipients, T-lymphocytes can be tracked to show that increasing cellular infiltration correlates with loss of islet morphology and function. Collectively, intra-ocular islet allografts are rejected via a T-cell-mediated destructive process resulting in hyperglycaemia. These results show that real-time, repetitive live imaging of immune cell infiltration and islet morphology is feasible in the anterior chamber of the eye. The ability of monitoring individual islets non-invasively in the same animal over time is an invaluable advantage of this model compared with conventional transplantation sites. Note-worthy is that these data clearly illustrate that the immune privilege, which is transiently provided by this transplantation site is finally broken following vascularization of the graft.

The anterior chamber of the eye: a transplantation site to study human pancreatic islet cell physiology as well as cell pathology

It is clear from our own as well as other studies (Brissova *et al.* 2005, Cabrera *et al.* 2006) that the human pancreatic islet is different from rodent islets in terms of both structure and function. Therefore, it is of utmost importance to clarify signal-transduction processes in human islets under normal conditions and why these do not function properly in diabetes. To be able to integrate the complex processes involved in the living organism, a systems biology approach is needed. In this context, the application of the *in vivo* imaging platform will be mandatory. An interesting challenge for the success of these experiments is to develop a humanized mouse model (Caicedo *et al.* 2009, Rodriguez-Diaz *et al.* 2009). For this purpose human islets are transplanted into immunodeficient nude mice, whose endogenous β-cells have been chemically destroyed, and subjected to our imaging platform. Similar to rodent islets, the dynamics of human islet vascularization can be monitored longitudinally. Moreover, it will be possible to study the function of human islet cells in an *in vivo* setting, which will then allow relating human islet cell function to glucose homeostasis in the organism.

The anterior chamber of the eye: a novel clinical transplantation site for the treatment of T1DM

The observation that pancreatic islets engraft in the anterior chamber of the eye and are able to maintain glucose homeostasis in mice that are rendered diabetic by chemically destroying

their own β-cells raises the fundamental question as to whether the anterior chamber of the eye represents a novel clinical transplantation site for the treatment of patients with T1DM.

The eye is an interesting novel potential site for clinical transplantation because of the immune privilege properties, the simple procedure, the easy non-invasive monitoring of the graft, the possibility of delivering local immunosuppression and the potential for systemic tolerization through anterior chamber-associated immune deviation as discussed in Stein-Streilein & Streilein (2002). Notably, if needed, immunosuppressive drugs may reach efficacy at lower levels by local application into the eye compared with systemic application. Contrary to the intrahepatic portal system, which is currently the site of choice for therapeutic islet transplantation, vascularization in the eye is rapid and effective and there is no inflammation after blood contact, thus limiting massive islet loss associated with the high vulnerability of islets in the immediate post-transplantation period.

To test the anterior chamber of the eye as a suitable transplantation site in non-human primates, we performed a pilot study where we transplanted two times 18 000 islet equivalents into the eye of a streptozotocin-diabetic baboon in combination with anti-CD154 monotherapy (Perez *et al.* 2011). The ophthalmological examination showed no inflammation, no immune response and no sympathetic ophthalmia. Over the monitored period of time, i.e. 357 days, blood glucose homeostasis improved, insulin C-peptide levels (evidence for insulin production) in the aqueous humor and in the circulation increased, and the HbA1c values (measure of long-term glucose regulation) decreased. Intra-ocular islet grafts thus manifestly contributed to glucose homeostasis and improved glycaemic control in this diabetic baboon.

Conclusions

A major problem in medical research in health and disease of today is to translate *in vitro* observations into the *in vivo* situation. While efforts are made to improve the specificity and resolution for imaging β-cell mass by PET and MRI, these techniques will not have the possibility provided by high-resolution optical imaging, i.e. to study β-cell biology and pathology at the single-cell level. The herein-described imaging platform allows non-invasive, longitudinal *in vivo* imaging of cell function and survival at single-cell resolution in a multicellular environment. Because the pancreatic islet is a micro-organ consisting of several endocrine and non-endocrine cell types where the function of each cell type is affected by cell–cell interactions, autocrine, endocrine and paracrine feedback loops as well as humoral and neural factors, this imaging platform in combination with the existing vast variety of transgenic (knock-out, knock-in, etc.) animal models will allow studies of islet cell biology in health and disease in a broad spectrum of disciplines of life sciences. Although no obvious differences in islet architecture, vascularization and innervation between engrafted islets in the anterior chamber of the eye and endogenous islets in the pancreas have been observed, other signalling input provided by, for example, the exocrine pancreas are missing. Thus, future work will have to reveal additional strengths and limitations of the presented imaging platform.

Acknowledgments

This study was supported by grants DK-58508, DK-075487 (to A.C.), and DK-084321 (to A.C.) from the US National Institutes of Health, by funds from Karolinska Institutet and by grants from the Swedish Research Council, the Novo Nordisk Foundation, the Swedish Diabetes Association, the Berth von Kantzows Foundation, the Knut and Alice Wallenberg Foundation, VIBRANT (FP7-228933-2), Skandia Insurance Company, Ltd., Strategic Research Program in Diabetes at Karolinska Institutet, the World Class University program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (CR31-2008-000-10105-0), and the Family Erling-Persson Foundation.

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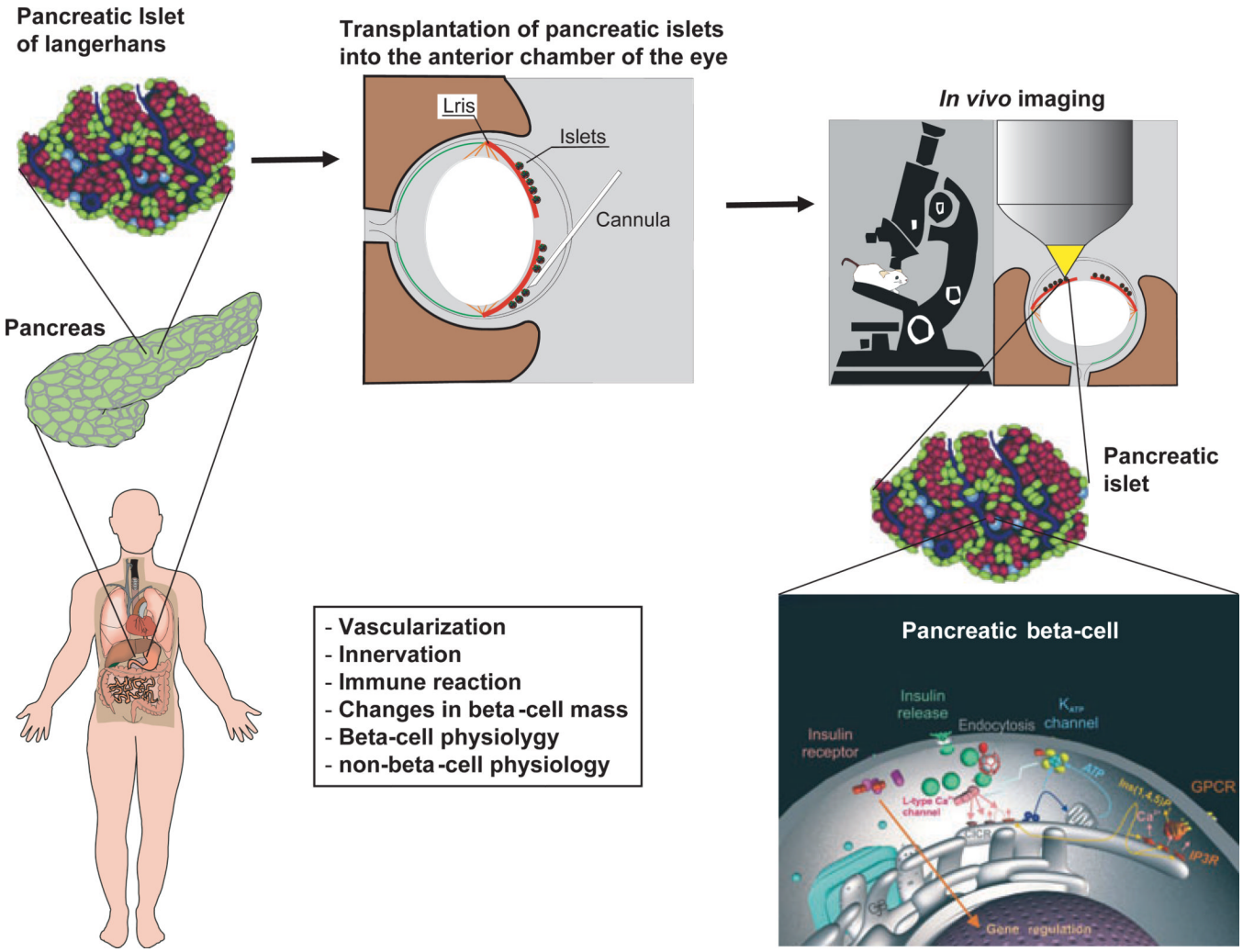

Figure 1.

Schematic illustration of the *in vivo* imaging platform to study pancreatic islet/β-cell physiology and pathology. Following their isolation, pancreatic islets of Langerhans are transplanted into the anterior chamber of the eye, where they engraft on the iris, become vascularized and innervated. As the cornea represents a natural body-window, this transplantation site allows non-invasive, repetitive *in vivo* imaging of the islet with high resolution, i.e. at the single-cell level, and to study various aspects of islet/β-cell function and survival under normal as well as diabetic conditions.