

Chronicle of an Islet Death Foretold

**Innovative Methodological Approaches to
Understand the Fate of Pancreatic Islets in
Transplantation**

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Cover illustration

Artistic representation of an encapsulated islet, highlighting extracellular matrix distribution, both fibrillar – a remanent of intra-islet blood capillaries – and as nodal accumulation. The encapsulated islet is surrounded by an exaggerated infiltration of immune cells.

Image created using AI, based on images acquired during this thesis.

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Abstract

This thesis focuses on understanding the fate of pancreatic islets in transplantation, in the context of treating Type 1 Diabetes (T1D). It investigates factors that affect the survival and function of isolated pancreatic islets, such as hypoxia, anoikis (cell death due to detachment from the extracellular matrix), and immune stress. It also explores encapsulation strategies to protect transplanted islets. Methodological developments include 3D microscopy for the morphological evaluation of whole islets, and the use of standardized Digital Image Analysis (DIA) to assess islet graft biocompatibility.

Background

Type 1 Diabetes is an autoimmune disease characterized by the destruction of pancreatic beta cells, leading to insulin deficiency. Standard care involves insulin therapy, but its limitations have prompted the exploration of alternatives like pancreatic islet transplantation, a procedure aiming to restore endogenous insulin production. However, islet transplantation faces challenges like limited donor availability, and islet loss due to hypoxia, anoikis, and immune rejection.

Objectives

1. Develop a comprehensive 3D imaging protocol for the evaluation of the extracellular matrix (ECM) distribution in pancreatic islets under hypoxic conditions.
2. Investigate hypoxia-induced ECM alterations in whole isolated islets.
3. Standardize and automate digital image analysis for objective evaluation of transplanted islet biocompatibility.

4. Evaluate the biocompatibility of bioencapsulated islets by means of immune reaction assessment.

Methods

Light Sheet Fluorescence Microscopy (LSFM) was used to acquire 3D images of whole, isolated islets, allowing mapping of ECM proteins. This involved tissue clearing, multiplex immunofluorescence, and digital image analysis.

A semi-automated DIA protocol was developed, to quantify immune cell infiltration around encapsulated islets transplanted subcutaneously. The documented validation process helps reduce bias, and the methodological approach provides a standardized method to evaluate biocompatibility.

Results and discussion

The first hypothesis was that hypoxia – a significant stressor affecting islets post-transplantation – would lead to distinct morphological changes in the ECM and cellular organization of pancreatic islets, impacting their survival and function.

Under hypoxia, ECM proteins (Collagen IV, Fibronectin, and Laminin) were not uniformly distributed but instead exhibited a central clustering pattern within the islet. This clustering correlates with areas of deformed and scattered nuclei, suggestive of central necrosis.

Furthermore, E-Cadherin, a marker of cell-cell adhesion, was found to be significantly diminished or absent in the central regions of hypoxic islets, further supporting the notion of cellular detachment and death.

The morphological evidence of ECM distribution in suffering islets supports the use of strategies such as bio-encapsulation to enhance isolated islets survival and function. Indeed, encapsulation can serve as a scaffold to put bioactive materials,

such as ECM proteins and cues, in direct contact with the peripheral, ECM-deprived cells.

Bioencapsulation of islets using various bioactive materials and cells (RGD-alginate, decellularized human pancreatic ECM, mesenchymal stem cells, and a zwitterionic anti-fouling coating) enhances islet survival and function in vitro. Thus, the second hypothesis was that enhanced islet survival, coupled with immune-modulating stimuli of the biocapsules, would also reduce immune cell infiltration, and improve the biocompatibility of islet grafts.

The DIA method effectively quantified the immune cell infiltrates (lymphocytes and macrophages) around different types of encapsulated islets. Capsules containing bioactive materials showed significant increased immune cell infiltration compared to standard alginate capsules.

These results suggest that, even if the studied bioencapsulation strategy has shown promising results in vitro, it fails to modulate the immune response, and even worsen immunological outcomes in vivo. This highlights the need for careful optimization of bioencapsulation materials to balance immune protection with islet survival. In this sense, standardized evaluation methods, such as the one proposed in this thesis, are crucial for enhancing the success of islet transplantation.

Conclusion

This thesis advances the understanding of pancreatic islet transplantation by highlighting the effects of hypoxia on islets' microstructure, supporting the use of bioencapsulation to enhance islet survival, and by proposing a robust methodology for evaluating graft-triggered immune reaction.

I. Introduction

This introductory chapter will focus on pancreatic islets, outlining their role in the aetiology and potential treatment of Type 1 Diabetes (T1D). Beginning with the identification and aetiology of T1D, the chapter will cover the clinical presentation and current standard of care. It will then describe pancreatic islets, including their structure, function, and the current clinical application of islet transplantation. The factors affecting the survival and function of isolated islets grafts – such as hypoxia, anoikis, and immune stress – will be discussed. The chapter will conclude with an overview of bioencapsulation strategies designed to protect and enhance the viability of transplanted islets.

I.A Type 1 diabetes: from disease identification to current treatment strategies

I.A.1 Disease identification

The term diabetes mellitus comprises a set of prevalent endocrine disorders, characterized by sustained hyperglycaemia¹. Within these condition, Type 1 Diabetes, refers to the complete insulin deficiency, a syndrome caused by the absence of endogenous insulin secretion due to beta cell depletion^{1,2}. Beta cells are a subset of cells of the pancreatic islet of Langerhans, specializing in insulin synthesis and secretion to glycaemic stimuli¹.

T1D is a prevalent disease, affecting more than 8 million people worldwide³. The condition entails increased morbidity and mortality, notably through chronic micro-vessel damage impacting heart, kidneys, eyes and nerves¹.

I.A.2 Disease mechanisms

T1D is an autoimmune disease resulting from multifactorial causes, involving a complex interplay between genetic and environmental factors⁴. Genetic predisposition, such as specific human leukocyte antigen (HLA) haplotypes, contributes significantly to disease susceptibility^{2,5,6}. Additionally, environmental triggers, such as viral infections or dietary factors, are believed to initiate or accelerate the autoimmune response that results in beta cell mass depletion^{1,6,7}. The immune response involves cellular and humoral components. Cellular immunity is evidenced by insulinitis produced by the islet's invasion by certain subsets of autoreactive T lymphocytes^{6,8-10}. The humoral response involves the production of autoantibodies, regulated by B and T lymphocytes. These include autoantibodies against insulin, glutamic acid decarboxylase (GAD), IA-2 antigens, and zinc transporter ZnT8 (it is worth noticing that the exact mechanism of cell destruction is not yet completely understood)^{8,9}.

I.A.3 Clinical presentation

T1D usual presentation is hyperglycaemia during childhood. Typical symptomatology includes polyuria, polydipsia, and weight loss⁴. Polyuria results from elevated serum glucose surpassing 180 mg/dL (10 mmol/L), leading to increased urinary glucose excretion, causing osmotic diuresis, and potentially presenting symptoms like nocturia, bedwetting, or daytime incontinence in a previously continent child. Polyuria leads to hypovolemia, dehydration and can alter the body's electrolyte balance. Both hypovolemia and hyperglycaemia can lead to polydipsia. Finally, weight loss is the result of both hypovolemia, increased catabolism, and the anorexia caused by ketosis. An important

complication of T1D is diabetic ketoacidosis. This is an acute, severe, metabolic manifestation, an exacerbation of dehydration, electrolyte loss, hyperglycaemia and ketosis.

T1D has a plethora of manifestations during the course of the disease, that escape the scope of this thesis. It is important to notice that the condition is chronic, and no widespread cure is currently clinically available¹¹.

I.A.4 Standard of care of T1D: insulin therapy

A century ago, the prognosis for individuals with T1D was exceedingly poor, with life expectancy measured in months. In 1921, Frederick Banting, Charles Best, James Collip, and John Macleod successfully isolated and purified insulin from canine pancreases^{12,13}. This marked the inception of insulin therapy, a pivotal moment in the history of diabetes management. Its application in human patients led to remarkable improvements in glycaemic control. Figure 1 illustrate this change, and Figure 2 provides an overview of the history of islets and insulin¹⁴⁻¹⁹.

Although insulin therapy alleviates the debilitating symptoms of diabetes, allowing patients to lead healthier and longer lives, managing the symptoms of T1D necessitates meticulous attention. Indeed, blood glucose artificial control requires careful monitoring, adaptation of insulin administration, and dietary management^{4,20}. These constraints impose a considerable burden on individuals, potentially leading to increased stress and disruption of daily activities²¹.

Since the discovery of insulin, diabetes management has witnessed significant progress. Advances in insulin formulations, such as long-acting and rapid-acting insulins, insulin analogues, and insulin delivery methods like insulin pumps and

continuous glucose monitors (CGMs), have improved glycaemic control and patient quality of life^{20,22}.

The current standard of care for managing T1D centres around the administration of exogenous insulin^{4,20}. The objectives of such a treatment are (a) maintain glucose levels at individual's target, while limiting episodes of hypoglycaemia, (b) to manage cardiovascular risk factors effectively, and (c) having the less impact possible in the individual's daily life²⁰. Insulin treatment significantly enhances the prognosis of T1D, but it does not address the underlying autoimmune process responsible for the disease. As a result, individuals with T1D require lifelong insulin therapy for optimal disease management. Considering the limitations of insulin therapy, a substantial effort is made to explore alternative treatment options that target the root cause of type 1 diabetes²³. One such approach is pancreatic islet transplantation, which aims to replace the endogenous beta-cell population and potentially offer a cure for the disease^{23,24}.



Figure 1. The first patient to receive insulin was Leonard Thompson (14 years old) in January 1922, at Toronto General Hospital. After this achievement, treatment evolved quickly. Insulin offered an effective treatment for T1D patients, otherwise condemned to short and painful lives.

Adapted from Flier et al.²⁵.

Figure 2: Evolution of Type 1 Diabetes (T1D) Treatment

1. Pancreatic Islet Discovery (1869):

- German anatomist Paul Langerhans discovers pancreatic islets, who describes these clusters of cells as "small, circumscribed structures" in the pancreas.

2. Islet Function Elaboration (1902-1920):

- English physiologist Sir Edward Schafer coins the term "islets of Langerhans."
- Oskar Minkowski and Joseph von Mering demonstrate that removing the pancreas causes diabetes in dogs, linking pancreas function to diabetes.

3. Insulin Discovery (1921-1922):

- Extraction of insulin from the pancreas.
- First successful insulin administration to a human patient.
- Insulin's life-saving impact on diabetes patients becomes widely recognized.

4. Insulin Refinement and Mass Production (1922-1940):

- Insulin purification techniques improve, enhancing its therapeutic efficacy.
- Eli Lilly and Company mass-produces insulin, ensuring wider availability.
- Introduction of long-acting insulin, such as NPH insulin, provides extended glycaemic control.

5. Introduction of Synthetic Human Insulin (1980s):

- Genetic engineering techniques enable the production of synthetic human insulin using bacteria like *E. coli*.
- Synthetic insulin reduces allergic reactions and ensures a more reliable supply.

6. Whole Pancreas Transplantation (1966-present):

- First performed in 1966.
- It remains the gold standard for T1D patients with renal failure, offering insulin independence and improved survival.

7. Islet Transplantation (1980s-present):

- Clinical application of islet transplantation began in the 1980s, notably with the first successful autologous islet transplantation after total pancreatectomy for chronic pancreatitis in 1980.
- The first case of transient insulin independence after islet allotransplantation (using donor islets) in a patient with type 1 diabetes was reported by David Scharp in 1990.
- These advances laid the groundwork for modern islet transplantation protocols, such as the Edmonton Protocol introduced in 2000, which significantly improved outcomes for people with T1D.

8. Development of Insulin Analogues (1990s-2000s):

- Introduction of rapid-acting analogues (e.g., lispro, aspart) for quicker post-meal glucose control.
- Long-acting analogues (e.g., glargine, detemir) with extended duration to cover basal insulin needs.

9. Continuous Glucose Monitoring and Insulin Pumps (2000s-present):

- Integration of CGM technology provides real-time glucose level monitoring.
- Insulin pumps become more sophisticated, offering precise insulin delivery based on CGM data.

10. Artificial Pancreas and Closed-Loop Systems (2010s-present):

- Development of closed-loop systems combining insulin pumps and CGM for automated insulin delivery.
- Advancements toward an artificial pancreas for more personalized and automated diabetes management.

I.B Pancreatic islets

The pancreas, located in the upper abdomen, is an amphicrine gland, i.e., has both endocrine and exocrine functions. It is predominantly exocrine (comprising 99% of its structure): the pancreatic acini secrete pancreatic juice into the duodenum via the pancreatic duct. This juice contains bicarbonate (which neutralizes gastric acid entering the duodenum from the stomach), and digestive enzymes that facilitate the breakdown of carbohydrates, proteins, and fats in ingested food.

The endocrine pancreas represents only 1% of the organ. Pancreatic islets, also known as islets of Langerhans, are micro-organs within the pancreas, which main role involves regulating blood glucose levels through the secretion of hormones such as insulin, glucagon, somatostatin, and pancreatic polypeptide.

I.B.1 Glucose homeostasis

Beta cell depletion impairs insulin production and disrupts glycaemic homeostasis. Insulin secretion in response to elevated blood glucose involves a tightly regulated sequence of cellular events, which can be expanded as follows:

1. Insulin synthesis and granule formation²⁶⁻²⁸

Beta cells synthesize insulin through a multi-step process:

- The *INS* gene produces preproinsulin mRNA, translated into a 110-amino-acid precursor.
- In the endoplasmic reticulum, preproinsulin loses its signal peptide to form proinsulin, which folds into three disulfide-bonded domains.

- Proinsulin is cleaved in secretory granules into mature insulin (51 amino acids) and C-peptide, co-stored with amylin in dense-core vesicles.

2. Glucose sensing and metabolism^{27,29}

- **Glucose uptake:** glucose enters β -cells primarily via GLUT1/3 transporters in humans (GLUT2 is predominant in rodents).
- **Glycolysis:** intracellular glucose is phosphorylated by glucokinase (rate-limiting step), metabolized to pyruvate, and oxidized in mitochondria to generate ATP.

3. Electrophysiological triggering pathway^{27,30}

- **ATP/ADP ratio:** elevated ATP closes ATP-sensitive K^+ (K_{ATP}) channels, reducing K^+ efflux.
- **Membrane depolarization:** resting potential (-70 mV) shifts to $\sim$ -50 mV, opening voltage-gated Ca^{2+} channels (VDCCs).
- **Calcium influx:** Ca^{2+} entry ($\approx$ 10 μ M intracellular) activates SNARE proteins (e.g., syntaxin, SNAP-25), triggering granule fusion.

4. Biphasic insulin secretion^{29,31}

- **First phase (0–10 min):** rapid exocytosis of 50–100 *readily releasable pool* (RRP) granules near the plasma membrane.
- **Second phase (10+ min):** sustained release from the *reserve pool* (RP), replenished by microtubule-driven granule mobilization.

5. Amplifying pathways²⁹

Glucose metabolites enhance secretion independently of Ca^{2+} :

- **NADPH and acyl-CoAs** potentiate exocytosis machinery (e.g., PKC, PKA).
- **Pyruvate kinase** generates ATP microdomains near K_{ATP} channels, sustaining depolarization.

6. Co-secretion of regulatory factors^{32,33}

- **Amylin:** slows gastric emptying and suppresses glucagon.
- **C-peptide:** protects vascular endothelium and reduces oxidative stress.

Insulin acts on various target tissues, including muscle, liver, and adipose tissue. Insulin's hypoglycaemic effect is achieved by facilitating glucose uptake into cells, promoting glycogen synthesis, and inhibiting glucose production in the liver⁴.

Together with insulin, other hormones contribute to glucose homeostasis. During feeding, elevated glycaemia elicits coordinated secretion of insulin and somatostatin while suppressing glucagon, with concurrent release of amylin and pancreatic polypeptide³⁴. Amylin, co-secreted with insulin from β -cells, slows gastric emptying, suppresses postprandial glucagon, reduces hepatic glucose output, and promotes satiety³⁵. Pancreatic polypeptide, secreted by PP cells, modulates digestive functions by inhibiting exocrine secretion, slowing gastric emptying, and inducing hypothalamic satiety signals³⁶. In fasting, this pattern reverses – glucagon rises as insulin, somatostatin, amylin, and polypeptide release decline. Gut-derived incretins (GLP-1, GIP) amplify glucose-stimulated insulin exocytosis via cAMP-PKA/Epac pathways, and epsilon-cell-derived ghrelin inhibits insulin and stimulates somatostatin secretion for counter-regulation³⁷⁻³⁹. The redundant interplay among these hormones ensures precise glycaemic control.

I.B.2 Pancreatic islet microanatomy

Pancreatic islets are composed of a variety of cells, including different endocrine cell types surrounded by extracellular matrix (ECM) and irrigated by a vascular network⁴⁰. The following sections will approach, separately, the cell populations, the ECM, the vascularization and innervation of in situ islets.

I.B.2.a Cell populations

Endocrine cell populations

The endocrine cellular components in humans are, in increasing abundance, the following⁴⁰:

- **Epsilon cells** constitute less than 1% of the total islet cell population^{41,42}. These rare cells produce the hormone ghrelin³⁷.
- **Pancreatic Polypeptide (PP) cells**, also known as gamma cells or F cells, represent approximately 1-5% of the islet cell population⁴³. Located predominantly at the periphery of the islets, these cells secrete pancreatic polypeptide upon food ingestion³⁶.
- **Delta cells** count for 3-10% of the islet's cells^{43,44}. They produce somatostatin.
- **Alpha cells** constitute approximately 15-20% of islet cells, this proportion being significantly lower in rodent islets⁴⁴. These cells produce and release glucagon^{32,34}.
- **Beta cells** are the most abundant cell type within the islet, constituting approximately 40-70% of the total islet cell, though this percentage is lower than the 70-80% typically found in rodent islets^{44,45}. These cells produce, store, and secrete insulin, as well as amylin³⁵.

Human pancreatic islets display a complex and heterogeneous architecture, with β , α , and δ cells intermingled throughout the islet rather than organized in distinct layers as seen in rodents (a β -cell core surrounded by α and δ cells in the periphery) – Figure 3^{45,46}.

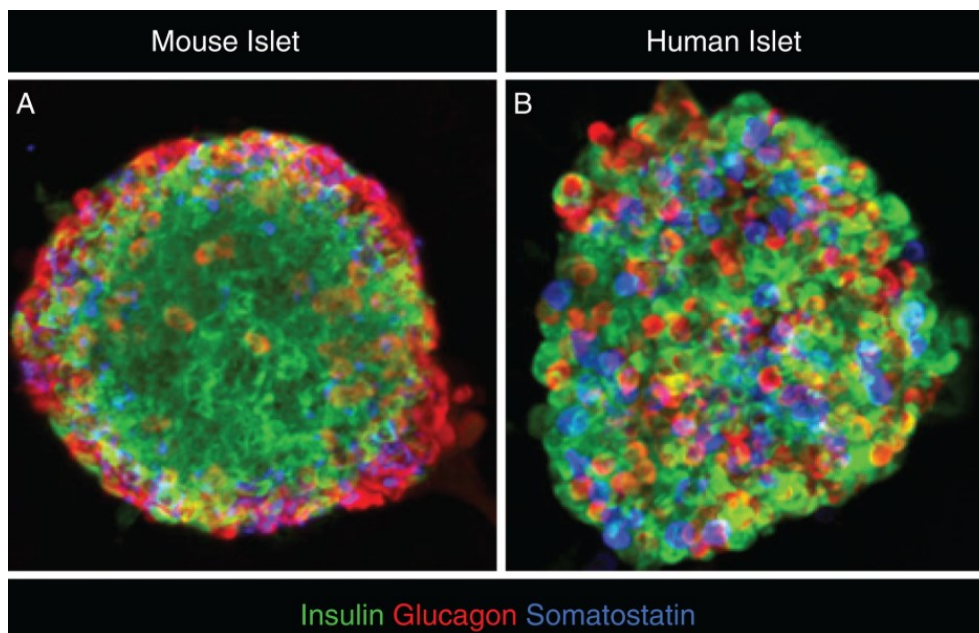


Figure 3. *Comparative confocal laser scanning microscopy images, of immunofluorescence for insulin (green), glucagon (red), and somatostatin (blue) in mouse (A) versus human (B) pancreatic islets. Note the peripheral localization of alpha and delta cells in the mouse islet. Comparison taken from Aamodt et al⁴⁶, adapted from Brissova et al⁴⁷.*

While endocrine cells form the functional core of pancreatic islets, a variety of specialized non-endocrine cells also play essential roles in islet physiology, immune regulation, and metabolic homeostasis.

Vascular Compartment

- **Islet Endothelial Cells (IECs)** form a highly specialized microvasculature characterized by fenestrated capillaries ten times denser than in exocrine tissue^{48,49}. These cells express unique markers such as nephrin and α -1 antitrypsin, distinguishing them from endothelial cells in other tissues⁵⁰. Their fenestrae, regulated by vascular endothelial growth factor (VEGF) from beta cells, facilitate rapid bidirectional transport of insulin and metabolites⁴⁸. IECs also secrete basement membrane components like collagen IV and laminin^{49,51}. During development, IECs guide islet morphogenesis through angiocrine signals⁵⁰.
- **Pericytes** envelop ~40% of islet capillaries, identified by NG2 and PDGFR- β expression⁵². Their cytoplasmic extensions span multiple endothelial cells, modulating capillary diameter and blood flow via contractile activity⁵². Pericytes also stabilize endothelial junctions and secrete trophic factors, with their depletion exacerbating beta cell dysfunction in hyperglycaemic conditions^{49,52}.

Immune Cells

- Resident **macrophages** are primarily situated in perivascular niches, and account for around 2% of the total islet cell population⁵³. These cells express markers such as CD14, CD163, and CSF1R^{54,55}. They extend filopodia into the lumens of blood vessels to sample circulating antigens and are capable of presenting insulin peptides via MHC-II⁵⁶. Additionally, resident macrophages secrete IL-1 β and TNF- α to

stimulate β -cell proliferation and produce MMP9, which is involved in extracellular matrix remodelling⁵⁴.

- **Dendritic Cells (DCs)** populate mouse islets at densities of 8–10 per islet, residing near vasculature with minimal migration⁵⁴. Human islet DCs remain less characterized.

Stromal and Support Cells

- **Islet Stellate Cells (ISCs)** have been identified in rat and human islets^{57,58}. They share similarities with pancreatic stellate cells (PSCs) of the exocrine pancreas, including the expression of markers such as α -smooth muscle actin (α -SMA), nestin, and glial fibrillary acidic protein (GFAP)^{58,59}. In healthy islets, ISCs contribute to the structural integrity and paracrine support of endocrine cells, potentially playing a supportive role in islet viability and function⁵⁹.
- **Fibroblasts** are a diverse population of mesenchymal-derived cells that play a key role in forming and maintaining the extracellular matrix and connective tissue within the islet⁶⁰. In normal islets, fibroblasts help maintain tissue homeostasis by producing ECM molecules such as collagen, fibronectin, and proteoglycans, which provide structural support for islet cells and blood vessels⁶⁰. They also secrete survival and angiogenic factors, including VEGF, FGF, and IL-6, which can promote islet cell viability, enhance revascularization, and support β -cell function⁶⁰⁻⁶².

Neural Components

Schwann Cells have been described to form a reticular network along islet margins, ensheathing autonomic nerve terminals, in mice⁶³. Ultrastructural studies show their membranous processes intercalate with β -cells, potentially modulating neuro-insular signalling⁶³.

I.B.2.b Cell–cell interactions

In addition to their ECM anchoring, pancreatic islets possess a network of adhesion molecules that mediate cell–cell interactions, ensuring coordinated hormone secretion and tissue homeostasis. Key molecules include:

- **E-Cadherin:** is the principal cadherin in islets, mediating strong homophilic adhesion between β -cells and other endocrine cells^{64,65}. It supports islet cell clustering, maintains islet architecture, and synchronizes insulin secretion by enabling calcium signalling coordination⁶⁵. Loss of E-cadherin disrupts islet morphology, impairs glucose-stimulated insulin secretion, and reduces β -cell survival^{64,66}.
- **N-Cadherin,** which contributes to adherens junctions and facilitates secretory granule transport in α - and β -cells^{67,68}.
- **Neural Cell Adhesion Molecule (N-CAM)** is critical for cell-type segregation, maintaining α -cell peripheral localization (in the rat), and preserving islet ultrastructure^{69,70}.
- **Cell Adhesion Molecule 1 (CADM1):** a member of the immunoglobulin superfamily, CADM1 mediates homophilic interactions and facilitates nerve–islet cell communication. In humans, it

is expressed across all major islet cell types, while in mice, it is α -cell-predominant^{71,72}.

- **Connexins**⁷³
 - **Connexin 36 (Cx36)**: forms β -cell gap junctions, enabling electrical coupling and synchronized insulin secretion.
 - **Connexin 43 (Cx43)**: expressed in islet endothelial cells, supporting vascular-endocrine crosstalk.
- **α - and β -Catenins**: intracellular adaptors that link cadherins to the actin cytoskeleton, regulating polarity and survival^{74,75}.

I.B.2.c Extracellular matrix

In addition to the cellular components, the pancreatic islet is surrounded by an extracellular matrix. The ECM is a complex network of proteins and molecules that provides structural support and influences cellular behaviour^{40,76}. The ECM within the islet consists of various components, including collagens, fibronectin, laminin, proteoglycans⁷⁶. These components play crucial roles in cell adhesion, signalling, and modulating the local microenvironment within the islet⁷⁴. The following sections will describe the ECM's structure, how it is secreted and remodelled, the differences in ECM across species and developmental stages, to finally deep dive into the main ECM molecules' structure and function.

Structural organization of in situ islets ECM

In native pancreatic tissue, islets exist within a complex three-dimensional microenvironment composed ECM⁷⁷. In most species, islets are typically

surrounded by an incomplete capsule consisting of a single layer of fibroblasts and collagen fibres, forming what is known as the peripheral, peri-insular or peri-islet basement membrane (BM) (Figure 4)^{60,77}. This specialized form of ECM serves as the physical interface between the endocrine islet tissue and the surrounding exocrine pancreas.

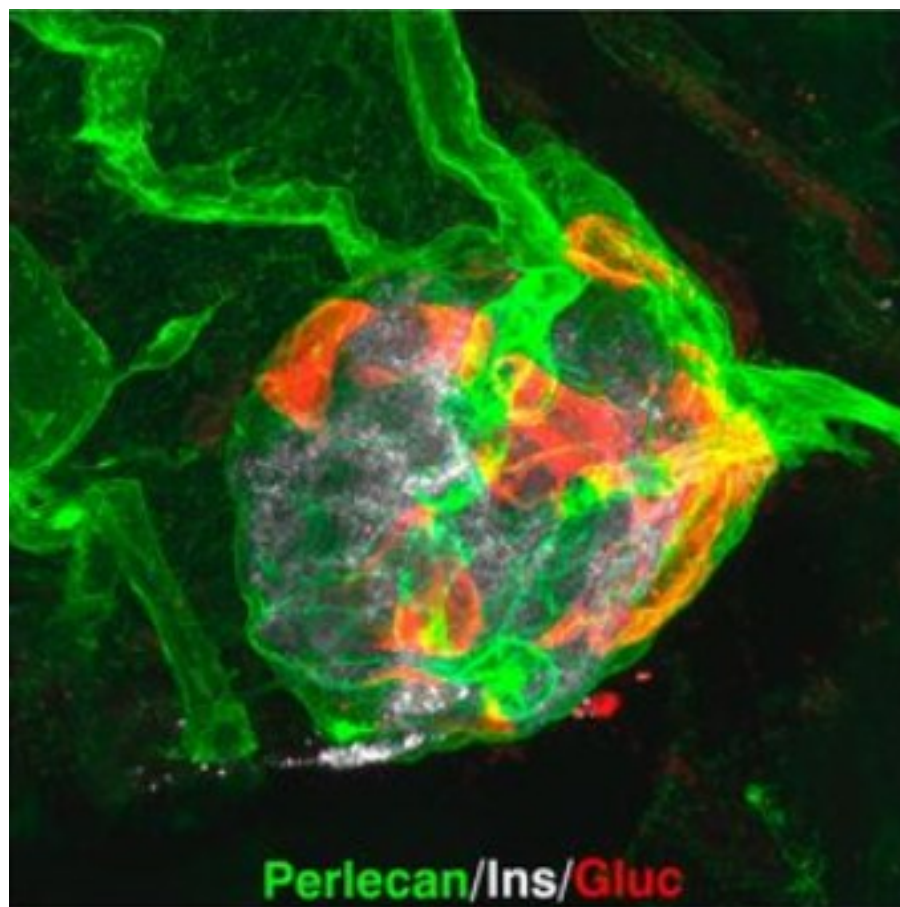


Figure 4. Confocal microscopy - 3D projection of a whole-mount healthy mouse pancreas, highlighting the peri-islet capsule through IF staining: insulin (white), glucagon (red), and perlecan (green) – a proteoglycan essential for interconnecting collagen IV and laminins. The islet is encased within this peri-islet basement membrane. Taken from Korpos et al⁷⁸.

The ECM is not merely restricted to the periphery of islets but extends throughout their interior via the intra-islet vasculature, in what is known as the perivascular basement membrane⁶⁰. In human islets, the double-layered perivascular membrane can be subdivided as vascular (in contact with the endothelium) and endocrine (in contact with endocrine cells) (Figure 5)^{79,80}. Studies of mouse islets indicate that islet endocrine cells lack their own BM and instead interact directly with the vascular endothelial BM (Figure 6)⁷⁸. This arrangement provides a polarity to β -cells within islets, with one face oriented toward the vasculature, facilitating efficient insulin secretion directly into the bloodstream (Figure 7)⁴⁴.

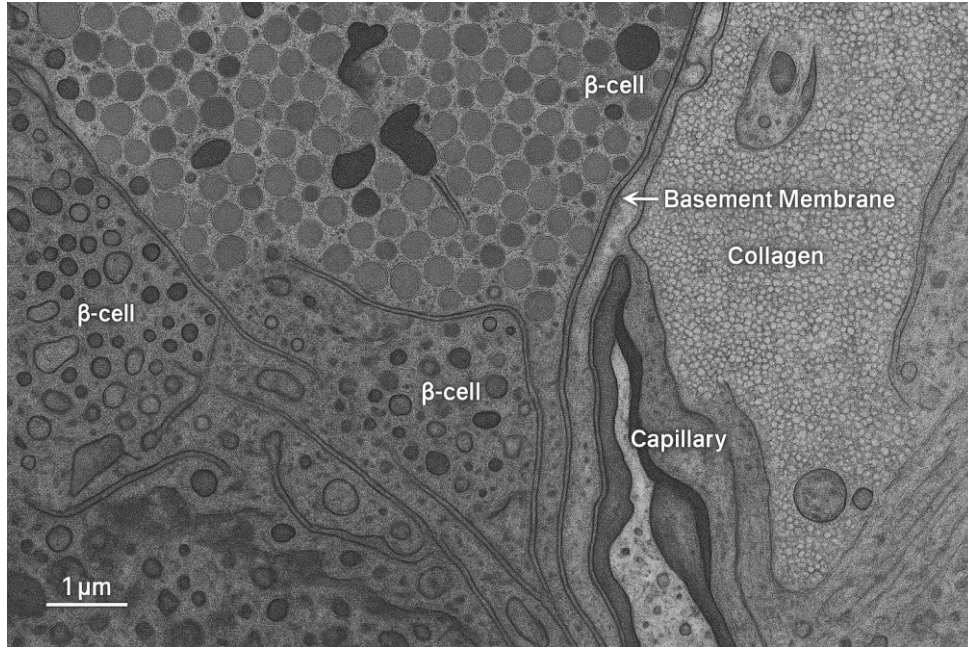


Figure 5. Transmission electron microscopy of human islet in situ (intact pancreas): note the two-layered vascular BM, and the well-arranged collagen fibres. Taken from Cross et al; image enhanced using AI^{79} .

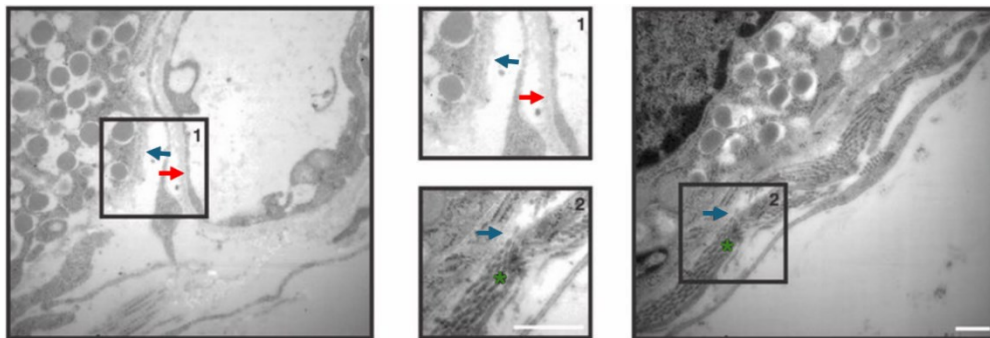


Figure 6. An electron micrograph of a mouse pancreatic islet in situ shows the peri-islet basement membrane (blue arrow) surrounding β -cells and the endothelial BM of blood vessels (red arrow; inset 1). Fibrillar collagens are visible beneath the peri-islet BM (arrow; inset 2). Scale bar = 1 μ m. Taken from Korpos et al.⁷⁸

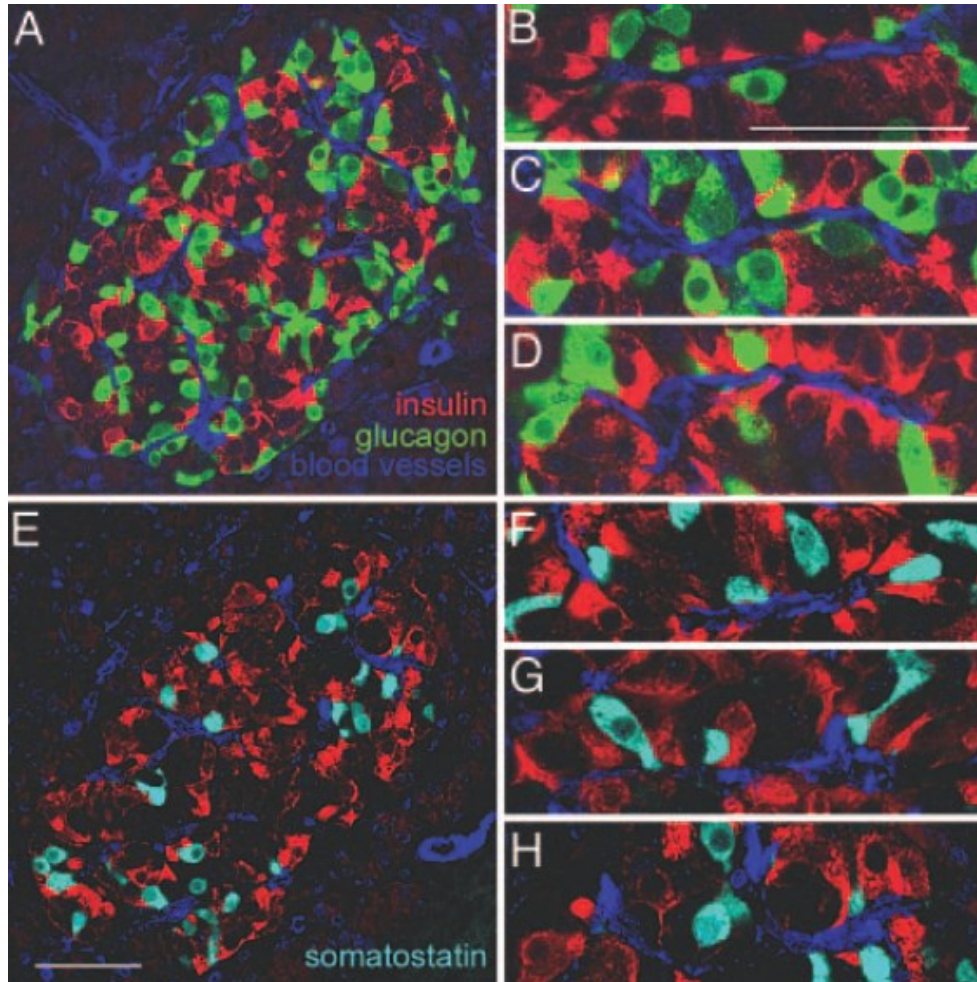


Figure 7. Endocrine cells within human islets were found to be in close, yet seemingly random, contact with vascular cells. The majority of cells producing insulin (red), glucagon (green), and somatostatin (cyan) were situated near vascular cells that tested positive for both smooth muscle cell actin (indicative of vascular smooth muscle cells, blue) and CD34 (a marker for endothelial cells, blue). It is also noteworthy that numerous blood vessels appeared sectioned along their longitudinal axis, which provided the opportunity to observe extended segments (as shown in panels B–D and F–H, which are magnified sections of panels A and E, respectively). Confocal microscopy of IF stained pancreas sections. Scale bar = 50 μm . Taken from Cabrera et al⁴⁴.

ECM Secretion and remodelling

In the pancreas, the ECM surrounding and within islets undergoes continuous remodelling through the balanced processes of protein secretion and degradation^{40,81}. Several cell types contribute to ECM production in native islets. The peripheral islet capsule is primarily secreted by fibroblasts, while the perivascular ECM is produced by pericytes and endothelial cells⁸¹. In addition to ECM production, various cell types (e.g., stellate cells, macrophages, pericytes, beta cells) within and around islets express matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that regulate ECM turnover⁸².

Differences in ECM among species and developmental stages

The composition, thickness, and continuity of ECM on the islet periphery vary considerably across species^{60,83}. Examples include canine islets, which generally possess more substantial matrix coverage than rat and human islets, and porcine islets, having discontinuous periinsular BM⁶⁰. Further differences will be addressed in the description of each ECM protein.

Additionally, the distribution of matrix proteins differs between developing and mature islet tissue. For instance, during murine ontogeny, the islet ECM undergoes structural and compositional maturation that parallels the functional specialization of endocrine cells⁸⁴. Laminin isoform switching represents a key mechanism in this process – while laminin-111 ($\alpha1\beta1\gamma1$) predominates in the developing mouse pancreas, this foetal isoform becomes replaced by laminin-511 ($\alpha5\beta1\gamma1$) in adult islets^{84,85}. Additionally, early pancreatic progenitor cells emerging from ductal structures display a distinct matrix signature rich in

vitronectin (VN), fibronectin (FN), and collagen IV (Coll-IV)^{46,60}. As islets coalesce into their characteristic mantle-core architecture during late gestation – a structural organization specific to rodents – the matrix profile shifts toward Coll-IV and laminin networks with concomitant reduction of FN and VN^{46,86}. This developmental pattern mirrors conserved mechanisms observed across epithelial basement membrane systems, where provisional matrices containing VN and FN support migratory processes during organogenesis before being replaced by Coll-IV/laminin in mature tissues^{60,84}.

Meanwhile, human islets develop within a unique double basement membrane architecture featuring distinct laminin isoforms in the vascular ($\alpha 4/\alpha 5$ -containing) and endocrine ($\alpha 5$ -exclusive) compartments⁸⁴. While laminin-511 persists as a key component in adult human islets, the developmental transition from foetal $\alpha 1$ -containing isoforms appears less pronounced compared to mice⁸⁴. Human pancreatic progenitors emerging from ductal networks similarly utilize provisional FN/VN-rich matrices, but maintain Coll-VI as a predominant collagen species throughout maturation^{60,84}. Of note, mature human islets retain fibronectin, mainly in their peripheral extracellular matrix, although FN levels are reduced compared to developmental stages^{60,82}.

In the isolated islet, although ECM composition has been described and quantified (but only to a certain extent), its distribution (3D architecture) remains to be elucidated. The current understanding is that the peripheral BM is lost during isolation, but little is known of the remaining intra-islet ECM, with reported changes in ECM content depending on the time of culture^{79,83,87}.

Specific matrix elements and their functions

This section describes the main matrix components, together with their described functions in situ. ECM molecules effects on the isolated islet will be discussed separately in the following section.

Laminins

Laminin-511 ($\alpha 5\beta 1\gamma 1$) constitutes the dominant isoform in mature human islets, forming the structural backbone of the peri-islet basement membrane^{80,84,88,89}. Unlike murine islets where laminin-111 predominates during development, human islets retain laminin-511 as a persistent architectural component into adulthood⁸⁴. Laminin-332 ($\alpha 3\beta 3\gamma 2$) is also constitutively expressed, though at lower baseline levels, and shows stress-inducible regulation under inflammatory conditions⁹⁰.

Spatial Architecture (illustrated in Figure 8)

Human islets exhibit a unique double BM organization around intra-islet vasculature:

- Vascular BM: contains laminin-411/421 ($\alpha 4\beta 1\gamma 1/\alpha 4\beta 2\gamma 1$) alongside laminin-511^{80,84}
- Endocrine BM: exclusively composed of laminin-511, forming a continuous peri-islet sheath that invaginates with penetrating vessels⁸⁴

This dual-layer system creates distinct microdomains - the inner vascular BM supports endothelial interactions, while the outer endocrine BM interfaces directly with β -cells through polarized Lutheran glycoprotein

receptors⁸⁰. Laminin-511 shows pan-islet distribution but concentrates at the islet periphery, mirroring collagen VI networks⁸⁵.

Functional Mechanisms

Laminin-511 orchestrates critical β -cell functions through:

1. Receptor-mediated signalling^{82,84,91}:
 - Lutheran glycoprotein binding enhances glucose-stimulated insulin secretion (GSIS) via PI3K-dependent pathways
 - $\alpha 3\beta 1$ integrin interactions promote β -cell proliferation and insulin transcription, potentially through FGFR5 co-receptor activation
2. Stress adaptation^{82,90-92}:
 - IL-1 β upregulates laminin-332 expression, correlating with improved β -cell survival under inflammatory stress
 - Laminin-511 preserves β -cell mass during isolation by inhibiting anoikis through Bcl-2/Bax ratio modulation

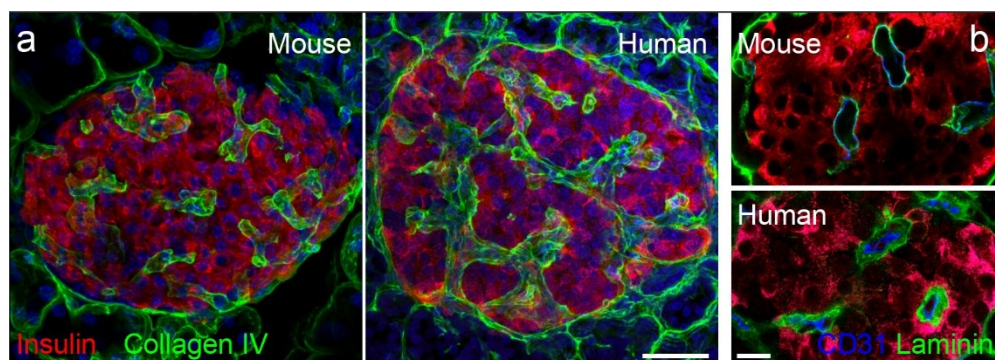


Figure 8. a) Maximum intensity projections from confocal microscopy of mouse and human islets, immunolabeled for insulin (red) and collagen IV (green). b) Confocal micrographs of islet regions in mouse and human samples, triple-stained for insulin (red), laminin (green), and CD31 (blue). Scale bars: (a) 40 μ m; (b) 10 μ m. Taken from Almaça et al⁹³.

Collagens

While collagen IV and VI are the most prominent collagens in human pancreatic islets, other types-including I, III, V, and XVIII-are also present and contribute to islet structure and function. The distribution and significance of each collagen type varies among species and within islet compartments^{82,88,89,94,95}.

Collagen IV

Collagen IV (Col-IV) forms the structural foundation of pancreatic islet basement membranes, with distinct isoform expression patterns across species. In human islets, Col-IV $\alpha 1/\alpha 2$ heterotrimers dominate both vascular and peri-islet BMs, maintaining continuity across the peri-islet sheath and intra-islet capillaries^{79,86,88}. Murine islets similarly rely on Col-IV $\alpha 1/\alpha 2$ during development, but exhibit dynamic isoform transitions: $\alpha 1/\alpha 2$ persists in vascular BMs, while peri-islet BMs shift toward laminin-dominated networks post-maturation^{78,96}.

Spatial organization (illustrated in Figure 7)

In human islets, Col-IV localizes to two compartments:

- Vascular BM: envelops intra-islet capillaries as part of a double-BM structure, co-localizing with laminin-411/421^{79,86}
- Peri-islet BM: Forms a discontinuous scaffold intertwined with collagen VI at the islet periphery, providing mechanical resilience during isolation⁷⁹

In murine islets, Col-IV is described both in the peri-islet BM layer and coating the intra islet capillaries^{93,96}.

Functional mechanisms: enhancement of adhesion, motility, and insulin secretion via integrin signalling^{97,98,99}.

Collagen VI is the most abundant collagen in the human pancreas, found predominantly at the islet-exocrine interface and peri-islet regions⁸⁹. It is also present in peri-islet and intra-islet locations in both human and porcine islets^{95,100}. In native islets, it stabilizes the peri-islet basement membrane¹⁰¹.

Collagens I, III, V and XVIII are less studied. They localise mainly in the periphery of islets and in the exocrine tissue of the pancreas. Their roles is believed to be mainly structural (Col-III and Col-V)^{88,89,95,102}.

Fibronectins and RGD-containing components

Fibronectin (FN) is a large, multifunctional glycoprotein. A key feature of fibronectin is the presence of the RGD (arginine-glycine-aspartic acid) motif within its structure. This short amino acid sequence is essential for integrin-mediated adhesion, as well as for downstream signalling that promotes cell survival and function^{88,89}.

Baseline presence: mature human islets retain fibronectin, mainly in their peripheral extracellular matrix. However, FN levels are reduced compared to developmental stages, where provisional FN/vitronectin-rich matrices dominate progenitor niches⁶⁰.

Functional role: FN enhances β -cell viability through⁸⁹:

- Upregulation of Bcl-2 anti-apoptotic proteins
- Stimulation of glucose-stimulated insulin secretion via $\alpha 5\beta 1$ integrin binding
- Promotion of insulin mRNA synthesis, though without altering glucose sensitivity

Glycosaminoglycans (GAGs) and Proteoglycans

The major GAGs in islets include heparan sulphate (HS) and hyaluronan, while key proteoglycans include heparan sulphate proteoglycans (HSPGs) such as syndecans and perlecan (Figure 9)^{78,88,89,103}.

HS is highly expressed in both the peri-islet basement membrane and intracellularly within β -cells in human and mouse islets^{89,103}. Together with perlecan and syndecans (HSPGs), they maintain islet architecture, support β -cell survival, and enhance insulin secretion¹⁰⁴.

Hyaluronic acid, a non-sulfated GAG, is unique among GAGs as it is not attached to a protein core and is synthesized at the plasma membrane rather than in the Golgi apparatus. In normal islets, hyaluronan is involved in cell adhesion, migration, and tissue organization. Of note, in vitro, excess hyaluronan impairs β -cell insulin secretion^{89,104}.

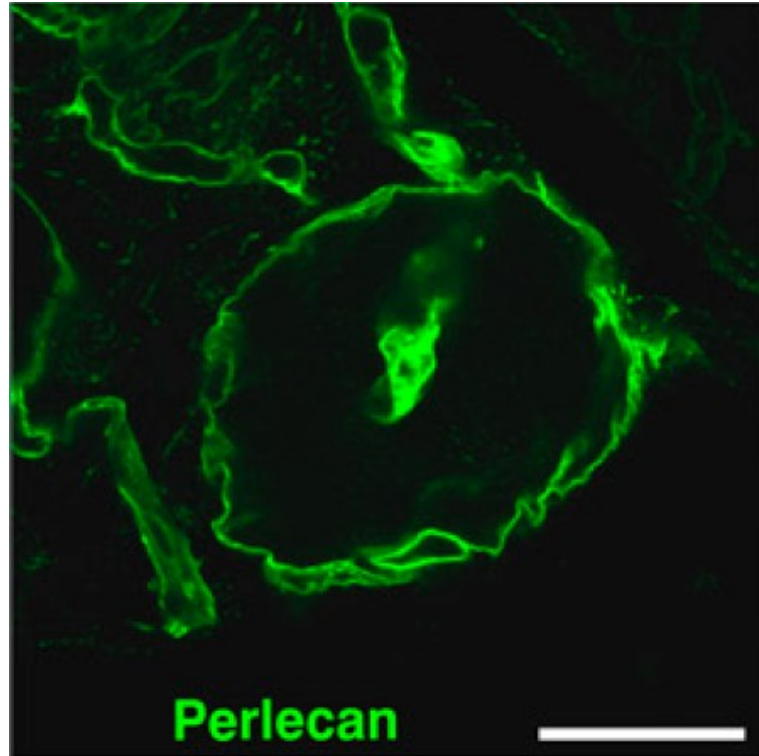


Figure 9. Confocal microscopy, optical section of a whole-mount of mouse pancreas, illustrating the peri-islet BM and endothelial BMs. IF for Perlecan, a HSPG. Scale bar = 100 μ m. Taken from Korpos et al⁷⁸.

Nidogen/Entactin

Nidogen, also known as entactin, is a multifunctional glycoprotein. It serves as a crucial structural linker, connecting major BM proteins such as laminins and collagen IV, thereby ensuring the integrity and function of the islet ECM. Nidogen is found within the peri-islet basement membrane and the

vascular BM that surrounds intra-islet capillaries in both human and rodent islets^{88,89}.

Others

Additional extracellular matrix components identified in pancreatic islets include fibrillin, thrombospondin, vitronectin, and tenascin. However, detailed information about these proteins is not provided here due to the limited research available on them.

ECM effects on isolated islets

Many of the effects attributed to ECM molecules come from studies using these molecules to support *isolated* islets (co-culture, supplementation, co-transplantation, co-encapsulation, etc.). Some key effects attributed to ECM molecules in this context (regardless of species) are summarized in Table 1.

ECM Component	In vitro effects	In vivo effects
Laminins	- Culturing islets on $\alpha 5$-laminins (e.g., laminin-511/521) maintains normoxia, prevents central necrosis, and preserves insulin secretion for ≥ 7 days¹⁰⁵ - Laminin-332 enhances β-cell proliferation and protects against cytokine-induced apoptosis¹⁰⁶ - Specific laminin sequences (e.g., RGD, PDSGR) in alginate capsules improve GSIS and viability⁹²	- Transplantation of islets cultured on laminin-511-coated surfaces normalizes blood glucose in diabetic mice within 3 days¹⁰⁵
Collagen IV	- PEG hydrogels containing collagen IV increase GSIS by 2-fold over 32 days and synergize with laminin for 6-fold enhancement¹⁰⁷ - High concentrations (>100 $\mu\text{g/ml}$) suppress GSIS in alginate capsules⁹²	- Collagen IV-modified scaffolds improved islet survival and function and reduce time to euglycemia in a mouse model¹⁰⁸
Collagen VI	- At 0,1-10 $\mu\text{g/ml}$, improves viability (87% survival vs. controls) and oxygen consumption but does not enhance GSIS⁹⁴	-

Collagen I	- Type I oligomeric collagen macroencapsulated islets showed a density-dependent improvement in viability, cytoarchitecture, and insulin secretion¹⁰⁹	- STZ-induced diabetic mice treated with type I oligomeric collagen macroencapsulated islets exhibited rapid (within 24 h) diabetes reversal and maintenance of normoglycemia for 14 (immunocompromised), 90 (syngeneic), and 40 days (allogeneic)¹⁰⁹
Fibronectin	- FN-mimetic peptide PR_b increases ECM fibronectin deposition, improving survival and liposome delivery to islets¹¹⁰ -Fibronectin culture enhanced islet viability and increased insulin content¹¹¹	- Fibronectin-pretreated islets improved glycaemic control (lower plasma glucose, higher insulin) within 2 weeks and enhanced glucose tolerance at 9 weeks post-transplantation in a syngeneic rat model¹¹¹
Nidogen/Entactin	- Co-assembly with laminin/collagen IV reconstructs peri-islet basement membranes, improving viability.¹¹² - Nidogen-1 reduces hypoxia-induced apoptosis in pseudo-islets by 50% and enhances insulin secretion.¹¹³	-

Cell-ECM interactions in islets

Integrin-mediated cell adhesion

Pancreatic islet cells engage with the extracellular matrix mainly through integrin receptors, heterodimeric transmembrane proteins composed of α and β subunits that mediate bidirectional mechanochemical signalling^{89,114}. In human islets, integrin expression is both complex and developmentally regulated. Mature human β -cells prominently express **α v β 5 integrin**, a heterodimer identified as a specific surface marker for human β -cells that persists even in type 1 diabetic islets devoid of insulin^{89,114}. Other critical integrins include **α 3 β 1** (collagen/laminin-binding), **α 5 β 1** (fibronectin-binding), and **β 1-containing heterodimers**, which collectively regulate survival, insulin secretion, and morphogenesis^{106,115,116}.

Key functional roles attributed to integrins include, regardless of species:

- **β 1 Integrin**: essential for postnatal β -cell expansion and glucose-stimulated insulin secretion. β 1 knockout models exhibit impaired insulin secretion, reduced β -cell mass, and disrupted FAK/ERK and Akt signalling^{115,116}. β 1 also mediates adhesion to collagen I/IV and laminin-511, with glycosylation of its extracellular domain enhancing ligand-binding capacity in 3D islet structures¹¹⁷.
- **α 5 β 1 Integrin**: binds fibronectin via RGD motifs and synergy sites, promoting β -cell proliferation, insulin mRNA synthesis, and anti-apoptotic Bcl-2 expression^{82,118}. This interaction stabilizes focal adhesions and enhances mechanical resilience during isolation.

- **$\alpha 3\beta 1$ Integrin:** critical for β -cell survival on collagen IV, activating FAK/ERK pathways while suppressing caspase-3 cleavage. Its blockade reduces PDX1 expression, impairing β -cell differentiation¹⁰⁶.
- **$\alpha v\beta 5$ Integrin:** uniquely marks human β -cells and facilitates adhesion to provisional matrices (e.g., vitronectin) during developmental islet morphogenesis^{106,114}.

Other Cell-ECM interactions

Although less studied than integrins, islet cells rely on diverse non-integrin receptors to interact with their ECM – summarized in Table 2.

Non-integrin receptor	Binds to:
Discoidin Domain Receptors (DDR1/2) ¹¹⁹	Collagens I–VI
CD44 ¹²⁰	Hyaluronan
Lutheran glycoprotein ^{46,80}	Laminin-511 in the peri-islet basement membrane
Dystroglycan ⁴⁶	Laminin-111

I.B.1.c Vascularization of healthy islets (in situ vascularization)

Vascular architecture and blood supply

Pancreatic islets possess a unique vascular structure that ensures optimal oxygenation in their native environment. The islets have a dense glomerular-like angioarchitecture, with tortuous and fenestrated capillaries, that is dependent on vascular endothelial growth factor (VEGF) (Figure 10 and 11)^{46,121}.

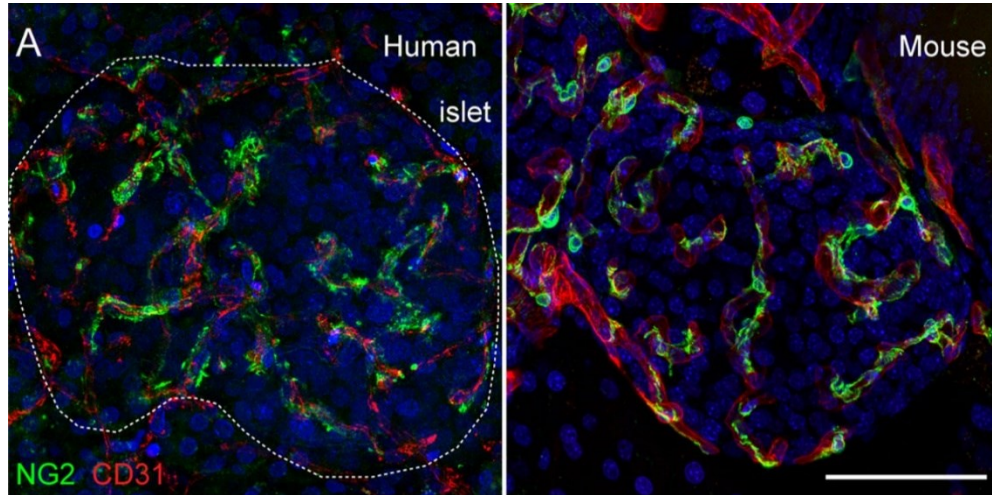


Figure 10. Confocal microscopy images presented as maximal projections compare pancreatic sections from a 16-year-old human (left – delimited by dotted lines) and a 2-month-old mouse (right). These sections were immunolabeled for pericytes (detected via NG2 marker, green) and endothelial cells (CD31, red). Scale bar = 50 μ m. Taken from Gonçalves et al.¹²¹

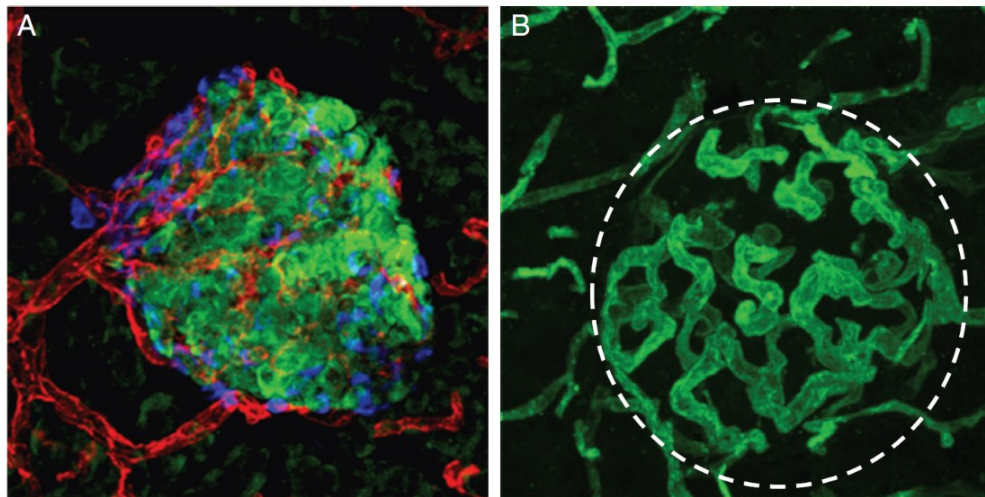


Figure 11. Confocal microscopy - IF of a mouse islet in situ. (A) insulin (green), glucagon (blue) and endothelial cells (CD31+, red). (B) note the intricate and tortuous intra-islet vascularization (CD31+, green). Taken from Aamodt et al.⁴⁶.

Although pancreatic islets constitute only 1-2% of the total pancreatic volume, they receive 5-10% of the pancreatic blood flow^{122,123}. This disproportionate blood supply indicates that the amount of oxygen per islet volume transported by haemoglobin significantly exceeds that of other pancreatic parenchyma. The extensive vascularization appears to serve multiple purposes beyond oxygen delivery, including facilitating β -cell replication and enabling rapid disposal of secreted hormones¹²².

Islets' blood flow is regulated in response to changes in blood glucose levels, to modulate insulin and glucagon secretion⁴⁶. When glycaemia is elevated, as in the post-meal state, islets receive increased perfusion to facilitate glucose uptake by cells, thus enhancing insulin release (see innervation).

Oxygen Tension in Native Islets

Measurements of oxygen tension in large superficial rat islet pancreatic islets using Clark microelectrodes have reported values of approximately 31-37 mmHg, which is higher than in many other visceral organs¹²⁴. This relatively high oxygen tension likely reflects the substantial oxygen requirements for adequate glucose sensing and metabolism in pancreatic β -cells¹²².

However, despite the rich vascular network, oxygen levels are not uniform across all islets in the pancreas. The hypoxia marker pimonidazole, which accumulates in tissues with oxygen tension below 10 mmHg, reveals significant heterogeneity in islet oxygenation. Remarkably, approximately 20-25% of all native islets in healthy rats show low oxygenation ($pO_2 < 10$ mmHg). This

suggests the existence of a subpopulation of dormant low-oxygenated islets that may constitute a functional reserve of endocrine cells¹²².

This variability in islet oxygenation appears to correlate with heterogeneity in blood perfusion. A study using microspheres demonstrated that islet blood perfusion is highly variable, with some islets receiving substantially more blood flow than others. Interestingly, this heterogeneity in blood perfusion seems to be relatively constant over time, as islets containing microspheres after initial injection tend to also contain microspheres after repeated injections¹²².

I.B.1.d Innervation

The autonomic nervous system densely innervates the pancreatic islet with both sympathetic and parasympathetic fibres. In human islets, it is sympathetic axons that primarily innervate smooth muscle cells associated with blood vessels, thus regulating local islet blood flow, helping to fine-tune hormonal secretion. Innervation varies amongst species; for example, in mice endocrine cells are directly in contact with both types of autonomic fibres⁴⁶.

I.B.2 Current clinical application of islet transplantation

Islet transplantation is a specialized procedure that involves the grafting of pancreatic islets from a donor pancreas into individuals with T1D. The primary objective is to restore normal insulin production and function in recipients, ultimately aiming to achieve exogenous insulin independence and improve glycaemic control. The Edmonton Protocol, the world's largest program of its kind, has brought islet allotransplantation to the clinical setting^{23,24,125}. Due to short term procedural risks, and the risks of chronic immunosuppression, islet

transplantation is currently reserved to only severe cases of T1D, notably patients suffering from frequent unawareness hypoglycaemia^{23,24,125}. Furthermore, due to islet lost upon transplantation (around 50%), and further diminution of the beta cell mass in the chronic setting, currently 1-to-3, and up to 4 donors are needed per recipient¹²⁶.

I.B.3 Islet transplantation procedure

The islet transplantation procedure involves several sequential steps²³:

1. Donor pancreas procurement: the organ is harvested from a deceased donor. Donor characteristics, including age, body mass index, and absence of diabetes in the donor, may affect islet yield.
2. Islet isolation: the pancreas is flushed and cooled with preservation solution and then surgically removed and transported to the isolation center. Islets are highly susceptible to oxygenation – thus, harvesting necessitates reduced times of warm and cold ischemia. The pancreas is distended with collagenase, and the islets are separated from the exocrine tissue using a density gradient.
3. Purification, using a series of washes and centrifugation steps to remove any remaining exocrine tissue and debris.
4. Culture: purified islets are cultured in a specialized medium to improve their viability and function before transplantation.
5. Infusion: the islets are infused into the recipient's liver through a minimally invasive procedure known as percutaneous transhepatic portal embolization. The islets then engraft within the liver and begin to produce and release insulin into the bloodstream. The liver is the only

current clinically available implantation site, although others are used in research.

6. Post-Transplant monitoring: to evaluate the function and success of the islet transplantation, including screening for rejection and assessment of glycaemia and insulin requirements.

I.B.4 Islet (graft) functional mass

Because not all transplanted islets may be fully functional, as well as important islet loss upon grafting, the number of islets to be transplanted relies on their overall capacity, as a graft, to survive and to secrete enough insulin to regulate glycaemia.

The current approach to quantify the functional mass of islets is the islet equivalent or IEQ¹²⁷. This standard is defined as the tissue volume of a perfectly spherical islet with a diameter of 150 μm . This standardized unit allows to assess and compare the efficacy and functionality of islet preparations for transplantation or experimental purposes, across different studies and laboratories.

A patient usually requires more than 10.000 IEQ per kilogram of bodyweight, usually in several injections¹²⁵. However, these criteria only take into account the number of islets, normalized by size¹²⁷. They do not consider the state of individual islets.

I.B.5 Benefits and limitations of islet transplantation

Benefits of islet cell transplantation include enhanced glycaemic control through the restoration of endogenous insulin production, potentially leading to decreased reliance on exogenous insulin, and in some cases, even achieving insulin independence. Additionally, there is a decreased likelihood of experiencing severe hypoglycaemia. Current evidence suggests long-term safety with minimal risks apart from those associated with the transplant procedure itself. Moreover, recipients often experience an improvement in their quality of life by alleviating the burdens associated with managing diabetes and its complications¹²⁸.

Limitations include the constrained availability of donor organs, which poses a considerable challenge in extending the treatment to a larger patient population. Despite progress in immunosuppressive therapies, the risk of rejection of transplanted islet cells persists, necessitating continued monitoring and management. Furthermore, the requirement for immunosuppression carries potential side effects and long-term implications. Additionally, procedure's cost and accessibility are barriers to its widespread adoption. Also, long-term outcomes can vary among recipients, adding further complexity to its effectiveness¹²⁸.

While islet transplantation has shown promising outcomes, some challenges still need to be addressed. Beside the limited availability of donor organs, the major limitation of islet transplantation is the poor long-term graft survival and function, with patients requiring 2-to-3 individual grafts. Indeed, a major islet loss is observed after transplantation, due to three main conditioners: hypoxia, anoikis, and immune damage.

I.C Conditioners of islets' survival, function, and loss after isolation

The main stressors responsible for pancreatic islet loss in the context of transplantation are hypoxia, anoikis and the immune reaction. They function as an interdependent continuum, where intensity varies by context (e.g., islet quality, implantation site) and affects individual islet cells heterogeneously. Furthermore, these stressors share molecular pathways that may amplify cellular stress, ultimately driving islet dysfunction and death.

I.C.1. Hypoxia

Islet isolation and in vitro culture

Islet isolation disrupts their dedicated vasculature, initiating a first wave of hypoxia. Surgical excision and vascular cross-clamping rapidly reduce intra-pancreatic partial oxygen pressure (pO₂) to below 5 mmHg within 30 minutes^{129,130}. Cold preservation slows but does not halt oxygen consumption, and enzymatic digestion further severs vascular connections, rendering islets dependent on passive oxygen diffusion^{129,131}. After isolation, islets are cultured in vitro, where oxygen is supplied from the surrounding medium, creating a radial oxygen gradient¹²³. While the periphery of the islet may experience normoxic conditions (21% O₂, ~160 mmHg), the core of larger islets (>250 μm) often drops below 10 mmHg, resulting in central hypoxia and necrosis¹²³. High seeding densities and increased medium depth exacerbate this effect by limiting oxygen diffusion¹³².

Hypoxia in transplantation

Upon intraportal transplantation (the current standard in the clinical practice), oxygen tension plummets immediately upon implantation, dropping to 5–15 mmHg due to the portal blood environment and thrombus formation from Instant Blood Mediated Inflammatory Reaction (IBMIR), which creates diffusion barriers and leaves distal islet regions oxygen-starved. By day 3 post-transplantation, pO_2 worsens to <10 mmHg, hitting its lowest point at ~5 mmHg during Days 4–7, exacerbated by the oxygen consumed by infiltrating macrophages. Partial revascularization can marginally improve pO_2 to 5–10 mmHg in by day 14, but fibrosis restricts diffusion. By 1 month, chronic hypoxia stabilizes at 6–8 mmHg, driving progressive graft failure estimated at 50% annual decline in viability^{123,133-139}.

Transplantation Sites

A critical factor of graft oxygenation is the site of transplantation. Currently, intra-portal infusion is the most used approach in the clinic. There is active investigation on alternative sites; some are exemplified in Table 3, together with their oxygen levels.

Site	pO ₂ (mmHg)	Measurement Method	Transplant Model	Timing
Liver ¹³⁶	~5	Clark microelectrodes	Rat → diabetic rat, syngeneic	9-12 weeks post transplantation
Subcutaneous ¹⁴⁰	~4	Optical sensors	No transplant	-
Omental pouch ¹⁴¹	30–40	Clark microelectrodes	Mouse/human islets → Mouse models	1 month post transplantation
Kidney capsule ¹³⁶	~6	Clark microelectrodes	Rat → diabetic rat, syngeneic	9-12 weeks post transplantation
Kidney capsule ¹⁴⁰	~40	Optical sensors	No transplant	-
Spleen ¹³⁶	~7	Clark microelectrodes	Rat → diabetic rat, syngeneic	9-12 weeks post transplantation

I.C.1.a Effect of hypoxia on islets

Depending on intracellular O₂ partial pressure (i.e., the level of hypoxia), the islet's cells can either (a) adapt and survive, (b) suffer necrosis, due to disruption of ATP production, leading to failure of critical cellular processes, with irreversible damage and cell lysis; or (c) engage apoptosis pathways^{123,142-144}. Key mechanisms involved in hypoxia-induced apoptosis in islets are^{123,133,143-148}:

1. Activation of signalling pathways, notably hypoxia-inducible factors (HIFs) and reactive oxygen species (ROS).
2. Dysregulation of proliferative pathways: ROS can notably induce an upregulation of the mitogen-activated protein kinase kinase (MAPK) pathway, and a downregulation of extracellular signal-regulated kinases (ERKs) pathway, blocking proliferation and inducing apoptosis.
3. Bcl-2 family proteins: under moderate hypoxic conditions, the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family is altered. Pro-apoptotic proteins, such as Bax and Bak, are activated, leading to mitochondrial outer membrane permeabilization (MOMP) and the release of Cytochrome C.
4. Mitochondrial pathway: the release of Cytochrome C from the mitochondria initiates a cascade of events within the cell. Cytochrome C binds to Apaf-1 (apoptotic protease-activating factor 1), forming the apoptosome complex. This complex activates caspase-9, which in turn activates downstream effector caspases, such as caspase-3, resulting in the cleavage of vital cellular substrates and ultimately leading to cell death.
5. DNA damage and p53 activation: the DNA damage (due to ROS) response activates the tumour suppressor protein p53, which can either

promote cell cycle arrest and DNA repair or initiate apoptosis. Under hypoxic conditions, the balance may shift towards the activation of p53-mediated apoptosis.

It is important to note that the balance between apoptosis and adaptive mechanisms in response to moderate hypoxia is complex and can depend on various factors such as the duration and extent of oxygen deprivation, cellular heterogeneity within the islets, and individual cell variations.

I.C.1.b In vitro model and detection of hypoxia in isolated islets

When studying hypoxia in islet transplantation, there is some consensus regarding the O₂ partial pressure, with many groups using, in vitro, 1% or 7.6 mm Hg^{123,142,143,145,149,150}.

Carbonic anhydrase 9 (CA9) is an hypoxia marker that is upregulated in response to low oxygen levels in tissues^{145,151}. It is a member of the carbonic anhydrase enzyme family, which catalyses the reversible conversion of carbon dioxide to bicarbonate and protons. CA9 is involved in regulating pH and acid-base balance within cells and is commonly expressed in hypoxic environments, including solid tumours and ischemic tissues.

The upregulation of CA9 is primarily mediated by the Hypoxia-Inducible Factor 1 alpha (HIF-1 α) pathway¹⁵¹. HIF-1 α is a transcription factor that plays a central role in the cellular response to hypoxia. Under hypoxic conditions, HIF-1 α translocates to the nucleus, where it forms a heterodimer with the constitutively expressed HIF-1 β subunit. This HIF-1 complex binds to specific hypoxia response elements (HREs) in the promoter regions of target genes, including CA9, initiating their transcription. HIF-1 α has a short half-life, making it difficult

to detect and measure. CA9, on the other hand, is a more stable protein, thus allowing easier detection.

I.C.2. Cell death induced by islet detachment from ECM – anoikis

During the isolation and transplantation process, islets are subjected to mechanical and enzymatic stress, disrupting their natural ECM connections. The loss of cell adhesion to the ECM and cell-to-cell contacts, which normally occur within the native pancreatic tissue, can trigger a cellular response known as anoikis^{152,153}.

Anoikis is a type of programmed cell death that occurs when cells detach from the extracellular matrix or surrounding tissue^{152,153}. It is a mechanism that helps to maintain tissue homeostasis by eliminating cells that have become detached from their normal environment. Anoikis is controlled by the integrin signalling pathways, and the absence of signals coming from the extracellular compartment or even an inadequate signal (wrong ECM composition) enhances integrin disengagement and integrin-dependent signalling^{46,152,154}. The subsequent ERK signalling inhibition decreases anti-apoptotic protein levels and activates pro-apoptotic proteins⁴⁶. Both intrinsic and extrinsic types of apoptosis can be initiated, and once one islet cell has triggered anoikis, it has the ability to transmit the death signal to neighbouring cells via the production of cytokines and other paracrine factors, resulting in the impairment of the whole islet survival and function^{152,155-157}.

A compensatory mechanism, bypassing the integrin pathways (by activation default of the focal adhesion kinase (FAK) and downstream targets, notably the MAPK/ERKs pathways), induce the upregulation of the alpha and beta-catenins, resulting in the cell hyperexpression of E-Cadherin^{46,158}. This can be

understood as a reinforcement of cell-to-cell adhesions in the absence of ECM anchoring.

While ECM loss induces anoikis, it has been extensively reported that the addition of ECM proteins (collagen, laminin, and fibronectin) can enhance islet survival and function: matrices can counteract anoikis by mimicking the natural extracellular matrix and providing a favourable environment for cell survival^{46,87,153,159-164}.

I.C.3. Immune stress

Upon transplantation, a series of reactions occur, that affect survival and function of islets. The first is the instant blood-mediated inflammatory reaction (IBMIR), characterized by the activation of the innate immune system and the coagulation cascade in response to the exposure of transplanted islets to blood^{126,148,160,165-168}. Complement activation, platelet aggregation, and clot formation can occur, leading to ischemia and damage to the transplanted islets.

Concomitantly, islets trigger an alloimmune response, due to differences in leukocyte antigens (HLAs or donor species leukocyte antigens in case of xenotransplants) between the donor islets and the recipient^{24,166,167}. T cells, a key component of the adaptive immune system, recognize the transplanted islets as foreign and initiate an immune response, leading to the destruction of transplanted islets^{24,167}. This reaction is summarized in Figure 12 (left panels). The immune infiltrates release various pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interferon-gamma (IFN- γ)^{166,169}. These cytokines can directly damage the transplanted islets, impair their function, and induce apoptosis. They can also activate other immune cells, perpetuating the inflammatory response and

contributing to islet destruction. As stated before, anoikis and hypoxia also induce cytokine secretion, which further boosts immune reaction, creating a vicious circle of inflammation and cell death¹¹.

To mitigate the immune reaction from the recipient against transplanted islets, immunosuppressive medications are often administered ¹⁶⁷. Immunosuppressive drugs, such as calcineurin inhibitors, and antiproliferative agents, inhibit immune cell activation and reduce inflammation, preventing rejection of the transplanted islets. The use of corticosteroids is controversial, due to risks of islet toxicity. Additionally, immune modulation strategies aim to selectively target immune cells involved in the alloimmune response, promoting tolerance, and reducing the need for long-term immunosuppression. Figure 12 provides an overview of current therapies.

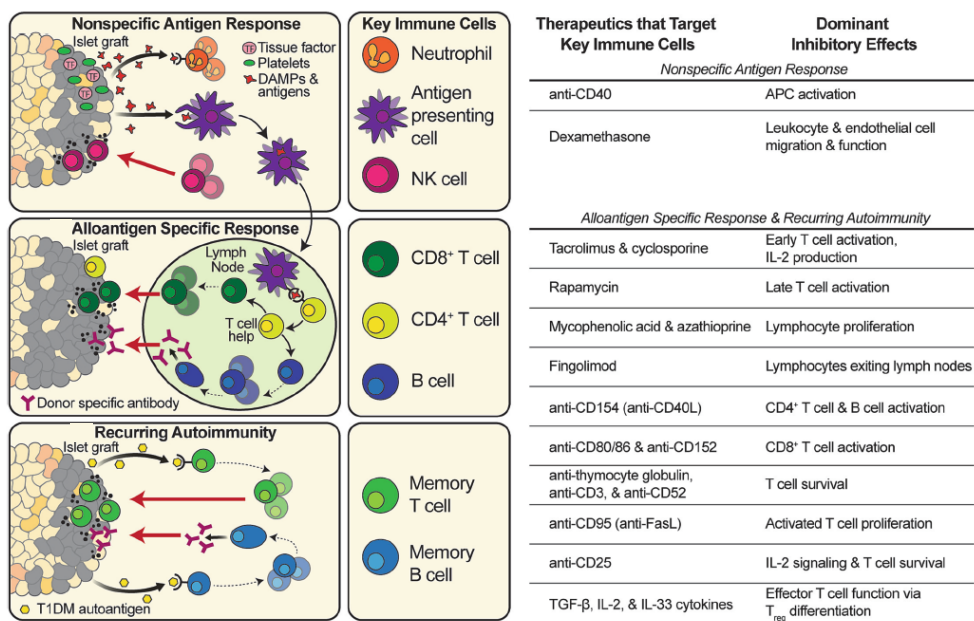


Figure 12: Main cellular contributors to the immunological rejection of transplanted allogeneic cells and common therapeutic targets. Taken from Lansberry et al ¹⁶⁷.

I.D Bioencapsulation as a strategy to support and protect transplanted islets

Hypoxia, anoikis and the immune reaction hinder islet survival and function. Reducing the impact of these factors should rescue islets and ensure better graft outcomes.

Encapsulation is defined as the wrapping of the islets within a material which can selectively allow the passage of nutrients and hormones while preventing immune cells from reaching the islets¹⁷⁰⁻¹⁷⁶. The capsule can also serve as a scaffold to support bioactive materials that can modulate aspects such as oxygenation, ECM contact and immune modulation. The main interactions between the islet, the capsule and the host are summarized in Figure 13.

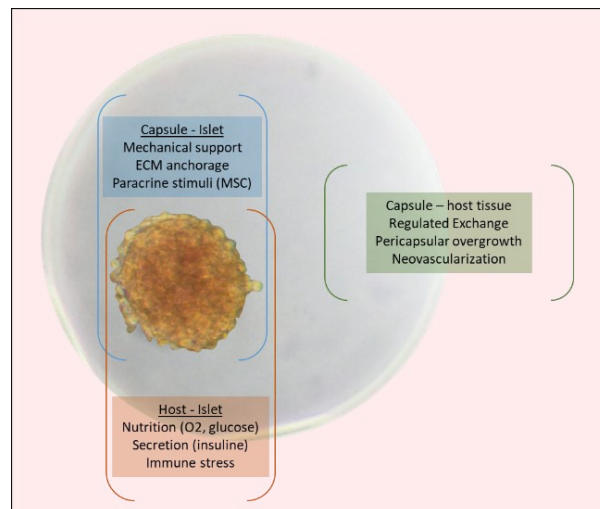


Figure 13. Conceptually, islet encapsulation creates three distinct interactions: the islet and the capsule (e.g., the capsule can serve as a scaffold to support islet's contact with bioactive moieties); between the islet and the host, which determines nutrition, hormone clearing and small-molecule immune reaction (cytokines); and between the capsule and the host, determining the PFO, but that can also react locally to stimulate, e.g., neovascularization.

I.D.1 Encapsulation

Encapsulation can be done at different scales, including nanoencapsulation (very small-scale coatings), microencapsulation (small-scale coatings), and macroencapsulation (larger-scale devices)¹⁷⁰⁻¹⁷⁴.

Nanoencapsulation involves enclosing individual islets in biomaterial compositions at a very small scale, typically with a thickness of less than 100 nanometres. Nanoencapsulation aims to provide a close interaction between the islets and their surrounding microenvironment, facilitating better nutrient transport.

Microencapsulation concerns the encapsulation of islets in small capsules or beads made of one or several biomaterials. These capsules can range in size from micrometres to millimetres.

Macroencapsulation involves encapsulating a larger number of islets in devices or structures that are bigger in size compared to microcapsules. These macro-devices aim to overcome limitations such as clumping of capsules and difficulty in recovery after transplantation.

The main advantages of encapsulation are¹⁷⁰⁻¹⁷⁶:

1. Immune protection, by directly blocking the contact with immune cells. This limits direct immune lysis and rejection.
2. Semipermeability: allow the diffusion of nutrients, insulin, and oxygen, while limiting the entry of immune cells and antibodies, thus preserving islet functionality while protecting them from immune-mediated damage.
3. Capsules serve as a scaffold, which can contain different biomaterials. By playing with different formulations, several groups have developed

supportive microenvironments for the islets, optimizing their function and insulin secretion.

4. Encapsulation techniques can be scalable, to support clinical demand.

An important aspect of encapsulation techniques is their influence in hypoxia. Transplanted islets depend on local vascularization at the site of implantation. Although capsules allow oxygen diffusion (to different extents), they lengthen the physical distance between cells inside the capsule and blood vessels outside it¹⁷⁷. Yet, the type of materials used can enhance neo-vascularization (see next section).

Amongst the limitations and challenges of encapsulation, it is worth mentioning¹⁷⁰⁻¹⁷⁶:

1. Pericapsular Fibrotic Overgrowth (PFO), which can impede nutrient and insulin exchange, leading to a decline in islet function over time. Capsules, even empty, behave as foreign objects, thus triggering PFO. Key determinants of this reaction are capsules' size and physical characteristics such as rugosity.
2. Capsules do not provide complete immune-isolation, as immune cells can still interact with the encapsulated islets through the semi-permeable matrix, via secreted inflammatory factors such as cytokines, that lead to immune stress.
3. Capsule stability, i.e., the durability of the encapsulation barrier over time, especially in dynamic physiological environments.
4. Size constraints: depending on the encapsulation method used, there may be limitations in the number of islets that can be encapsulated within a given volume, affecting the overall efficacy of the treatment.

Many of the advantages and limitations of encapsulation depend on the materials and techniques used. Thus, composition and capsule quality will be the main determinants of biocompatibility, and with this, the capacity of the capsule to actually protect the islet from cellular immune reaction, while limiting small molecules immune stress, and ensuring nutrition and key pro-survival and pro-function moiety contact.

I.D.2 Biomaterials

Several materials have been studied in this context, ranging from inert to biologically active compounds, as well as the incorporation of cells (like mesenchymal stem cells) and drugs. Jeyagaran et al. have recently reviewed the current trends; Table 4, adapted from this publication, offers an oversight on the challenges and strategies to enhance islet transplantation¹⁶⁹. It is out of the scope of this thesis to systematically review encapsulation techniques and biomaterials; the following section will focus on the specific biomaterials and techniques used in this thesis in the context of the BIOCAPAN project.

Challenges	Strategies	Methods
Acute hypoxia	Hypoxia preconditioning	Islets pre-exposed to intermittent hypoxia
		Islets preconditioned in carbon monoxide gas
	Oxygen-releasing molecules	Perfluorocarbons (PFCs)
		Calcium peroxide (CPO)
		Haemoglobin (Hb)
	External oxygen supply	β Air device (oxygen refueling technique)
		Oxysite (encapsulation of CPO within polydimethylsiloxane)
Chronic hypoxia	Pre-vascularization	Subcutaneously transplanted islets followed by 50 % oxygen inhalation treatment
		Pre-implantation an agarose rod containing cyclic oligopeptide SEK-1005
		Pre-implantation of FGF-releasing collagen-based sheet
		'Device-less' transplant technique
	Re-vascularization	Delivery systems for growth factors VEGF or FGF
		Co-transplantation of bone-marrow or adipose mesenchymal stem cells (MSCs) with encapsulated islets to improve angiogenesis
		Coating 1,12-dodecanedioic acid (DDA) on a chitosan pouch
		Co-transplantation with accessory cells such as endothelial progenitor cells
Foreign body response	Immune isolation	Polyacrylonitrile and polyvinyl chloride copolymers (PAN/PVC)
		Polyurethane and polyvinyl pyrrolidone copolymers (PU/PVP)
		Polytetrafluoroethylene (PTFE) membranes
		Triazole thiomorpholine dioxide (TMTD)-modified alginate encapsulation
		Co-incorporation of islets and Tregs in PLGA scaffolds.
		Silicon

Foreign body response	Immune isolation	Aluminium/aluminium oxide (Al/Al ₂ O ₃)
		Electrical charges modification
	Implant topography modification	Avoid sharp edge
		Surface roughness
		Modification of the size of spherical implants in a certain range
	Anti-fouling materials	Zwitterionically modified alginates
	Coating techniques	Coating 1,12-dodecanedioic acid (DDA) on a chitosan pouch
		Coating PEG/alginate around the islets
		Rapamycin-PEG coated alginate microsphere
	Delivery system for immunomodulatory cells and immunomodulators	PLGA scaffolds with controlled release of TGF- β 1
		Co-incorporation of islets and Tregs in PLGA scaffolds
		Islets encapsulated within PEG containing a crosslinked haemoglobin
		Treatment of immunomodulatory agents after islet transplantation (e.g. cyclosporine, dexamethasone)
		New immunosuppressive treatment (CTLA4-Ig combined with anti-CD154 mAb treatment)
		on nonobese diabetic mice transplanted with alginate-encapsulated islets
		Co-encapsulation of CXCL12 and islets in alginate capsule to suppress T-cells function
		Co-encapsulation of tacrolimus-loaded PLGA microspheres and islets in a hydrogel
		Co-encapsulation of TLR2-modulating pectin polymers and alginate in a microcapsule
		Co-transplantation of Fas ligand with streptavidin (SA-FasL) microgels and islets
	Robotic drug delivery technique	Dynamic Soft Reservoir (DSR) that utilized mechanical oscillation to mitigate fibrotic capsule formation

1.D.3 The BIOCAPAN approach

BIOCAPAN was a multicentric project financed by the European Horizon 2020 program (grant agreement ID 646272). Its participants were the Commissariat à l'Energie Atomique et aux Energies alternatives (France), Université catholique de Louvain (Belgium), Charité-Universitätsmedizin Berlin (Germany), NovaMatrix (Norway), NanoImmunotech (Spain), Université Joseph Fourier (France), Etablissement Français du Sang (France), European Research Services GmbH (Germany), and Wake Forest University Health Sciences (USA).

BIOCAPAN's primary objective was to design a GMP-grade bioactive microcapsule, enhancing the long-term functionality and survival of pancreatic islets by preventing pericapsular fibrotic overgrowth, ensuring in situ oxygenation, innovatively reconstructing the extracellular matrix microenvironment, and modulating the immune system. The project also aimed to develop and apply a standardized, reproducible, GMP-grade microfluidic microencapsulation platform¹⁷⁸.

The selection of components for the BIOCAPAN project (RGD-Alginate, ultrapure human pancreatic extracellular matrix, human mesenchymal stem cells, and chitosan) followed a rigorous multi-criteria approach integrating previous scientific evidence, partner expertise, scalability and regulatory readiness. The choice was based on demonstrated efficacy in in vitro validation studies. A clear example of this is the use of NovaMatrix's GMP-grade production (ISO 9001:2015/13485:2016 certified) and ultrapure alginate manufacturing experience. The scalability issue was also approached from a capsule manufacturing perspective, with CEA developing a microfluidic approach that can be easily reproduced, thus having the capacity to rapidly encapsulate high

numbers of islets, such as those needed for a standard transplantation (3.000 IEq per kg body weight, thus 210.000 IEq for a standard adult).

I.D.3.i Alginate

Alginate is a naturally occurring biopolymer derived from brown algae (Phaeophyceae) and is composed of linear polysaccharides¹⁷⁹. There are two main types of alginates: guluronic acid-rich (G-alginate) and mannuronic acid-rich (M-alginate)¹⁸⁰. Amongst its characteristics, it is important to highlight its biocompatibility, ease of gelation, and semipermeable properties^{179,180}. It has been widely used in islet encapsulation, demonstrating its capacity to protect from the immune response while allowing proper nutrient and insulin exchange¹⁷⁹⁻¹⁸².

In the islet encapsulation process, purified islets are mixed with an alginate solution, and the choice of G-alginate or M-alginate can influence the properties of the resulting hydrogel^{179,181}. For instance, G-alginate tends to form more rigid hydrogels, whereas M-alginate leads to softer hydrogels.

The gelation process, known as ionotropic gelation, involves the use of divalent cations, such as calcium (Ca²⁺) or barium (Ba²⁺), to cross-link the negatively charged alginate molecules. When the alginate solution containing islets is exposed to a solution of Ca²⁺ or Ba²⁺ ions, it induces the formation of a stable hydrogel matrix surrounding the islets^{179,181}. This process results in the creation of alginate microcapsules, which provide a protective environment that shields the islets from direct contact with the host's immune system.

I.D.3.ii RGD

RGD, which stands for Arginine-Glycine-Aspartate, is a short amino acid sequence that plays a crucial role in cell adhesion and cell-matrix interactions⁹². It is a recognized cell-adhesive motif, commonly found in extracellular matrix proteins such as fibronectin and vitronectin¹⁵⁵. When incorporated into the encapsulation material, RGD facilitates cell-matrix interactions, allowing the islet cells to attach and anchor to the surrounding hydrogel^{92,155,183}. By providing a supportive environment for the encapsulated islets to adhere and spread, RGD can improve cell viability and function. Furthermore, the incorporation of RGD into the encapsulation material has shown promise in mitigating pericapsular fibrotic overgrowth¹⁸³.

I.D.3.iii Extracellular matrix

The incorporation of ECM in the encapsulation system aims to create a more biologically relevant microenvironment, both biochemically and biomechanically, facilitating cell-matrix interactions – thus contributing to islet survival and secretion^{92,164,184,185}.

ECM possesses immunomodulatory properties that can help modulate the immune response at the transplantation site¹⁵⁵. It may promote a more tolerogenic microenvironment, potentially reducing the risk of immune rejection against the encapsulated islets.

In addition, ECM can support the formation of new blood vessels (angiogenesis) in the vicinity of capsules^{155,185}.

ECM can be derived from various sources; BIOCAPAN used an innovative approach, incorporating ultrapure, soluble ECM derived from decellularized human pancreas. This was based on in vitro results showing that this biomaterial

enhanced mechanical stability as well as the function of encapsulated pancreatic islets¹⁶⁴.

I.D.3.iv Mesenchymal stem cells

In the field of islet transplantation, mesenchymal stem cells (MSC) have been associated with pro-angiogenic, anti-inflammatory and immunomodulatory effects¹⁸⁶. Particularly in the context of islet encapsulation, MSC have been shown to (a) enhance viability and functionality in encapsulated islets^{187,188}, (b) improve graft oxygenation and re-vascularization (neo-angiogenesis)^{188,189}, and (c) to reduced fibrosis around transplanted islets¹⁹⁰.

I.D.3.v Antifouling

Antifouling strategies aim to minimize the non-specific adhesion of biomolecules and cells to the encapsulation material, thus reducing pericapsular fibrotic overgrowth^{191,192}. In the context of islet encapsulation, zwitterionic materials (that contain equal numbers of positively and negatively charged groups) effectively resist protein and cell adhesion¹⁹³. The balanced charge distribution creates a repulsive force against proteins and cells, preventing their attachment to the encapsulation surface. One such a zwitterion is chitosan, a biocompatible polysaccharide derived from chitin¹⁹⁴. The cationic nature of chitosan interacts with the negatively charged cell membranes and proteins, hindering their adhesion to the encapsulation surface.

In summary, BIOCAPAN evaluated the in vivo biocompatibility of islets encapsulated with size-adapted capsules, supplemented with biomaterials and cells that had proven their efficacy in supporting islet survival and function in vitro.

The immune reaction in this type of models is very important and necessitates sensitive methodology to be able to differentiate and categorize dense cellular infiltrates. Thus, there is a need to develop and validate a digital image analysis technique, that reduces bias by limiting user input, and by analysing large amounts of data, both from the number of images and by their size (whole slide).

II. Objectives

This thesis is divided into two main parts, aiming at (1) understanding factors affecting islets survival after grafting and (2) providing tools for objective evaluation of islets morphology or biocompatibility, before and after transplantation.

Before transplantation, islet isolation disrupts native vascularization and ECM, leading to hypoxia and anoikis. Furthermore, after grafting, islets are still submitted to hypoxia until reconnection to the host vascularization. Despite the shared molecular pathways between hypoxia and anoikis (ERK, MAPK) and the key role of ECM in survival and function of isolated and transplanted islets, the impact of hypoxia on ECM organization has seldom been studied.

The first part of this work was thus dedicated to evaluating the effects of hypoxia on islets ECM distribution:

Objective 1: Development of a 3D Imaging Protocol

- Establishment of a comprehensive and validated methodology for clearing, whole mount immunofluorescence staining, 3D imaging, software reconstruction and analysis of whole islets;
- Development of qualitative and quantitative analysis of immunolabeled molecules in isolated human islets;

Objective 2: Investigation of Hypoxia-Induced ECM Alterations

- Analysis the distribution of ECM proteins (Collagen IV, Fibronectin, Laminin) and E-Cadherin in isolated human islets;
- Examination of the morphological changes in these molecules under hypoxic conditions.

To counter the effects of hypoxia and anoikis, islets can be encapsulated using biomaterials and cells, thus providing direct contact with key moieties, and profiting from their immunomodulatory effects.

The second part of this thesis explores the evaluation of the immune response triggered by transplanted bio-encapsulated islets in a preclinical model:

Objective 3: Methodological standardization and automation

- Validation of an automated digital image analysis (DIA) quantification method applied to whole-slide immunohistochemistry for more objective and standardized evaluations.

Objective 4: Evaluation of biocompatibility of encapsulated islets implantation

- Description and quantification of the immune reaction against transplanted capsules;
- Comparison of the effectiveness of bio-encapsulation against simple encapsulation.

III. Methodologies used in this project for the evaluation of morphology and biocompatibility of pancreatic islets

III.A 2D: whole slide imaging

Whole slide imaging (WSI) is a digital pathology technique that involves scanning entire slides, thus permitting to create high-resolution digital images of entire samples (vs snapshots of a specific area). This technology allows for the comprehensive visualization and analysis of tissue at a microscopic level¹⁹⁵⁻¹⁹⁷.

WSI involves a series of steps starting with sample preparation, where tissue samples undergo standard histological or immunohistochemical procedures and staining. These prepared slides are then loaded into a slide scanner for high-resolution image capture, with the scanner software subsequently stitching together multiple images to create a seamless digital representation. The resulting digital slides are stored in a proprietary format optimized for viewing and analysis, allowing pathologists and researchers to examine them using specialized software¹⁹⁶.

WSI offers numerous advantages^{195,196}. Firstly, it enhances efficiency by digitizing slides, which enables remote access and saves time and resources. Secondly, digital slides can be viewed at various magnifications, enhancing diagnostic accuracy. Additionally, digital slides are easily stored, retrieved, and shared electronically, promoting collaboration and data exchange.

On the other hand, some inherent disadvantages to this technology need to be mentioned^{195,196}. The implementation of WSI systems can be costly due to the substantial investment required in scanners, software, and infrastructure, both for its procurement as for maintenance. Additionally, users require specific training in the use of both hardware and software. Finally, this WSI demands

sufficient quality assurance and validation, to maintaining accuracy and consistent image quality.

III.B 3D microscopy

III.B.1 Light Sheet Fluorescence Microscopy

There are several 3D microscopy techniques that have been applied to pancreatic islets, notably confocal microscopy, two photon microscopy, micro and nano tomography, and light sheet fluorescence microscopy.

Light Sheet Fluorescence Microscopy (LSFM), also known as selective plane illumination microscopy (SPIM), is an advanced imaging technique used to visualize biological samples with high spatial resolution in a non-destructive manner¹⁹⁸⁻²⁰¹. LSFM is particularly well-suited for 3D imaging of large, intact specimens, such as tissues, organs, embryos, and small organisms as well as for 3D cell cultures like spheroids and organoids.

The key principle behind LSFM involves illuminating the sample with a thin sheet of laser light, perpendicular to the detection axis. This creates an optical section of the sample, where only the focal plane is illuminated. The fluorescence emitted from the illuminated plane is captured by a camera, allowing for high-speed imaging of (only) the selected plane. Adjacent focal planes are then imaged in order to cover the entire sample and are subsequently reconstructed as a 3D image¹⁹⁸⁻²⁰¹. Figure 14 summarizes the procedure.

Advantages of LSFM include reduced photobleaching and phototoxicity, achieved by utilizing a thin sheet of light for selective sample illumination, thereby minimizing photodamage compared to widefield or confocal microscopy. LSFM enables high-speed, high penetration depth, and 3D imaging¹⁹⁸⁻²⁰¹.

However, LSFM has some disadvantages. It requires specific sample preparation techniques to ensure optimal imaging (tissue clearing methods are described further on). It is a pricy technology and requires complex equipment. Furthermore, handling and processing the large volumes of data generated by LSFM imaging can be computationally intensive, often requiring specialized software tools¹⁹⁸⁻²⁰¹.

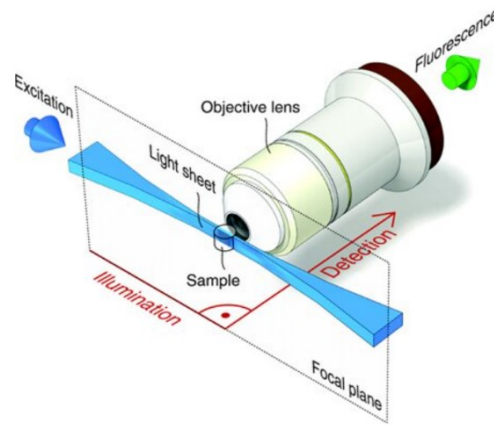


Figure 14. In a LSF microscope, notably the Zeiss μ 1, the sample “hangs” in the center of a virtual chamber, with excitation lasers and an objective at its sides. The object can move in the x , y , and z axis, as well as spin around the vertically axis. The laser beams are extremely slim, which limits object excitation to a single plane in space: it is this thin layer of fluorescent tissue that is detected by the objective lens. Image taken from Stegmaier et al²⁰².

III.B.2 Tissue clearing

The principle of tissue clearing is to reduce light scattering to enable deep-tissue imaging. This is achieved primarily through refractive index matching between the tissue and the imaging solution. The refractive index of a substance denotes its ability to alter the speed of light propagation within a medium compared to vacuum conditions^{203,204}. Biological samples exhibit a diverse refractive index range, spanning from 1.33 (water) to 1.66 (bones), with intermediate values for proteins and lipids typically falling between 1.4 and 1.6²⁰⁵.

The physical principles of LSFM require the studied sample to be transparent: by homogenising the sample's components refraction indexes, light scattering is reduced, and thus the sample's opacity. This homogenization is achieved by tissue clearing technologies, which remove compounds with outlier refractive indexes (like lipids) and bleach pigments^{203,204}.

There are many tissue-clearing methods; Vietes-Prados and Renier have recently reviewed them²⁰³. They can be broadly categorized into three main families based on their approach to achieving refractive index homogenization.

1. Aqueous methods like CUBIC rely on hydration to disaggregate lipids into micelles, in conjunction with hyperosmotic solutions²⁰⁶
2. Hydrogel-based techniques, (e.g., CLARITY), uses protein crosslinking enhancement to help stabilize tissue structures, thus permitting the use of strong detergents and accelerated sample permeabilization²⁰⁷
3. Organic solvent-based methodologies, like 3DISCO use a combination of dehydration and immersion in solvents with a high refractive index²⁰⁸

III.C Digital Image Analysis (DIA)

III.C.1 Visual evaluation versus computer-assisted analysis

Visual evaluation of stained tissue sections is currently the standard practice in clinical routine²⁰⁹. Although visual evaluation according to specific criteria is very fast, it is subjected to intra- and inter-observer variations²¹⁰. A more robust, but highly time consuming, approach is point-counting, requiring a high number of images to ensure data quality^{210,211}.

The human eye's detection limit is characterized by its ability to distinguish distance between two separated points (spatial resolution) and to detect significant differences in intensities (abundance). In general, the human eye can perceive intensity changes of high amplitude (for instance, detecting the difference between 100 points and 200 points). However, detecting smaller incremental changes (for instance, distinguishing between 100 points and 101 points) becomes more challenging. This phenomenon is attributed to the logarithmic nature of human perception, which is why it is more challenging to discern subtle differences when quantities are already relatively high. This inherent limitation can introduce subjectivity and inconsistency when relying solely on visual assessment, highlighting the importance of digital image analysis for accurate and objective quantification of such subtle changes.

III.C.2 Digital Image analysis methodological principles

DIA involves the computer-based processing and quantification of images obtained from biological samples. The core objective of DIA is to transform pictures into numbers. Computational algorithms convert various aspects of an

image, such as pixel intensities and shapes, into numerical data that can be analysed, compared, and statistically interpreted^{209,210,212}.

A typical DIA protocol involves a systematic workflow that can be summarized as follows^{210,212,213}:

1. **Preprocessing:** raw images often contain noise and artifacts. Preprocessing steps like background subtraction, noise reduction, and contrast enhancement are applied to improve image quality and compensate for staining variations across batches of images.
2. **Segmentation:** this step isolates objects of interest from the background, or in other words, it separates the image in a set of meaningful regions or segments. Depending on the application, objects can be cells, tissues, particles, or other structures.
3. **Classification:** segmented objects can be refined by further classifying them according to specific features like size or staining intensity.
4. **Feature extraction:** its purpose is data reduction by measuring (extracting) certain attributes or properties associated with the classified segments (i.e., objects) that distinguish them from other objects. Relevant attributes or features can be size, shape, intensity, or spatial relationships.
5. **Quantification:** extracted features are quantified, assigning numerical values to the attributes.

Digital image analysis presents several advantages^{210,212,213}:

1. **Objective evaluation:** eliminates subjectivity in data interpretation and limits intra- and inter-observer variability. Evidently, DIA is not flawless and depends on algorithms that are, in fine, defined by humans.

However, it proposes a clear overview on the process: algorithms and images can be shared and subject to peer scrutiny.

2. **Continuous quantitative measurements**, vs visual scoring that use discrete measurements.
3. **Standardization**: protocols allow for consistent measurements, thus ensuring that evaluations are reproducible and comparable across different experiments and research groups.
4. **High Throughput**: with (semi-)automated analysis, large numbers of images can be processed quickly without compromising accuracy. This enables the treatment of significant amounts of data, enhancing the statistical robustness of the results.

IV. Results

IV.A.1 3D evaluation of ECM of isolated hypoxic islets (Paper A)

This first paper approaches the two first objectives of this thesis: a methodological one, namely the development of a 3D imaging protocol applied to whole isolated islets; and the quantitative and qualitative evaluation of the distribution of ECM proteins (Collagen IV, Fibronectin, Laminin) and E-Cadherin within the islet, thus permitting the examination of the expression changes in these molecules under hypoxic conditions.

Islet transplantation implies the loss of most of the islet's ECM during isolation, and low-oxygen conditions following implantation. Both the diminution of islet's ECM content and hypoxia are detrimental to islets. Using Light Sheet Fluorescence Microscopy, this paper will explore how hypoxia affects ECM distribution over time. For this, isolated islets are cultured in either standard or hypoxic conditions (to mimic the period ensuing transplantation). The resulting micro-anatomical changes are compared from a three-dimensional perspective, allowing both quantification and the evaluation of spatial relationships.

3D evaluation of the extracellular matrix of hypoxic pancreatic islets using light sheet fluorescence microscopy

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ABSTRACT

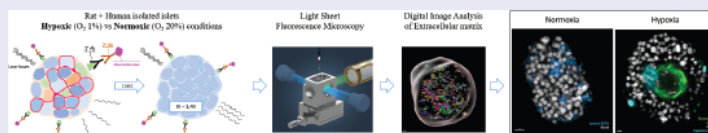
Pancreatic islet transplantation is a promising treatment for type 1 diabetes, but the survival and function of transplanted islets are hindered by the loss of extracellular matrix (ECM) during islet isolation and by low oxygenation upon implantation. This study aimed to evaluate the impact of hypoxia on ECM using a cutting-edge imaging approach based on tissue clearing and 3D microscopy. Human and rat islets were cultured under normoxic (O₂ 21%) or hypoxic (O₂ 1%) conditions. Immunofluorescence staining targeting insulin, glucagon, CA9 (a hypoxia marker), ECM proteins (collagen 4, fibronectin, laminin), and E-cadherin (intercellular adhesion protein) was performed on fixed whole islets. The cleared islets were imaged using Light Sheet Fluorescence Microscopy (LSFM) and digitally analyzed. The volumetric analysis of target proteins did not show significant differences in abundance between the experimental groups. However, 3D projections revealed distinct morphological features that differentiated normoxic and hypoxic islets. Under normoxic conditions, ECM could be found throughout the islets. Hypoxic islets exhibited areas of scattered nuclei and central clusters of ECM proteins, indicating central necrosis. E-cadherin was absent in these areas. Our results, demonstrating a diminution of islets' functional mass in hypoxia, align with the functional decline observed in transplanted islets experiencing low oxygenation after grafting. This study provides a methodology combining tissue clearing, multiplex immunofluorescence, Light Sheet Fluorescence Microscopy, and digital image analysis to investigate pancreatic islet morphology. This 3D approach allowed us to highlight ECM organizational changes during hypoxia from a morphological perspective.

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Introduction

Type 1 Diabetes Mellitus (T1D) is a disease that affects more than 8 million people worldwide and is a leading cause of morbidity in developed countries¹. T1D has a complex etiology and is characterized by a lack of endogenous production of insulin^{2,3}. It is normally treated with the daily administration of insulin via injections or pumps⁴.

A promising alternative is the transplantation of pancreatic islets of Langerhans^{5,6}. Several studies have demonstrated the capacity of grafted islets to regulate blood glucose physiologically^{5,7}. However, this procedure is limited to a handful of centers worldwide and is clinically applied under very specific criteria, due to still suboptimal graft survival, necessitating 2-to-3 donors per recipient.

To be transplanted, islets are isolated from human cadaveric pancreas. Isolation and transplantation induce a series of processes that compromise islets' survival and function. Among them, cell-ECM detachment and hypoxia play a predominant role^{8,9}.

During isolation, most of the islets' native extracellular matrix (ECM) is lost due to enzymatic digestion, triggering a process of cellular apoptosis named anoikis^{10,11}. The mechanisms enrolled in anoikis have been largely reported, demonstrating that the scarcity of ECM triggers (a) compensatory mechanisms, e.g., the overexpression of E-Cadherin, reinforcing cell-to-cell adhesion, and (b) a series of molecular pathways resulting in apoptosis¹²⁻¹⁵.

Islets' ECM is composed of many proteins, of which Collagen 4, Fibronectin, and Laminin have been identified as forming the basal membrane (BM), a capsule that surrounds islets and that is lost during isolation, with Collagen 4 and

Laminin also coating the islet's inner microvasculature^{10,14,16-18}. Conversely, ECM supplementation after isolation reduces cytokine stress and fibrosis and overall enhances graft survival and long-term insulin production^{10,13,16,19-24}.

Another issue to overcome for optimal islet transplantation is oxygenation. Isolation destroys the islets' dedicated blood supply²⁵. Upon implantation, islets suffer from hypoxic conditions until a new microvasculature is developed around them^{26,27}. Islets' oxygenation, relying on O₂ diffusion, varies between the implantation sites but has been consistently reported as being under the islets' physiological needs²⁷. Hypoxia has been shown to decrease insulin secretion and to induce several forms of cell death, from necrosis to apoptosis^{28,29}.

Interestingly, hypoxia and cell-ECM detachment trigger similar molecular mechanisms: in a dialog of activation and inhibition of regulators such as the MAPK cascade, p38, JNK, and ERK, both processes lead to the induction of cytokine secretion, which in turn intensifies death mechanisms, as well as the dysregulation of the Bcl-2 family leading to apoptosis^{9,13,16,30,31}. Thus, ECM attachment and oxygenation are both determinants of islet survival and function.

Little is known about the modifications of islets' ECM after isolation, especially under hypoxic conditions^{9,32}. We hypothesize that hypoxia hinders remodeling, as this process necessitates active secretion of proteins. Therefore, we have developed a protocol of 3D imaging to evaluate the influence of hypoxia on isolated islets' ECM remodeling.

A 3D approach allows for both protein quantification and qualitative evaluation. 3D imaging improves visualization of the islet architecture, permitting a better analysis of the distribution of cells and extracellular matrix^{33,34}. Recent

developments in light sheet microscopy and tissue clearing have made possible the efficient imaging of whole organs or organ subunits, like islets^{35,36}. Light sheet fluorescence microscopy (LSFM) is a technique that uses a single plane (sheet of light) illumination, allowing fast, high-resolution, multichannel imaging of thick samples³⁷. To reach 3D imaging without signal loss in depth, tissues need to be transparent, even for small, dense structures like pancreatic islets ($\varnothing$ 50-250 μm). This is achieved using clearing techniques that homogenize the refractive index of the different structures of the organ, thus reducing diffraction and light absorption while preserving shape and immunofluorescence³⁵.

Our study is a proof of concept, demonstrating the feasibility of a LSFM-based approach to isolated islet evaluation, and highlights the importance of an efficient 3D approach. We explored the influence of hypoxia on ECM remodeling, providing morphological evidence of ECM changes in islets in the context of transplantation. To the best of our knowledge, we are the first to validate such a process applied to isolated islets and to address the effects of hypoxia on the ECM from a 3D perspective³⁸.

Materials and methods

Islet procurement

Rat islets. All protocols were approved by the Ethical Committee [2020/UCL/MD/016]. Male Wistar rats were put under inhalatory anesthesia and euthanized by exsanguination after laparotomy. 10 ml of Collagenase P solution (1 U/ml - Roche, #11213873001) was injected into the pancreas through the bile duct, under direct observation, to ensure homogeneous

distribution throughout the organ. The pancreas was then dissected free and stored in ice-cold Krebs awaiting processing.

Digestion was done by putting the pancreas at 37 °C in a water bath for 15 minutes. Digestion was controlled by direct observation and was stopped by adding cold Krebs, followed by 3-4 washes. Islets were then handpicked under a dissection microscope.

Human islets were procured at Tebubio. Two batches were used in our study: 58 years old, BMI 29, HbA1c 5.0%, and 32 years old, BMI 26.9, HbA1c 4.9%. After reception, they were cultured for 48 hours, as instructed by the provider, to allow for recovery.

Experimental workflow

After isolation, islets were divided into three groups of approximately 400 islets and cultured in RPMI (Invitrogen, #11875093) supplemented with fetal bovine serum 10% (Lonza, #26140079), penicillin-streptomycin 100 U/ml (Lonza, #09-757F), glutamine 2 mM (Lonza, #BE17-605E), and 10 mM or 5 mM glucose for rat and human islets, respectively. Islets of the first group were harvested after overnight culture in normoxic (O₂ 21%, CO₂ 5%) conditions (Normoxia Day 1 (ND1)). Islets from the two other groups were cultured for a total of 5 days either in normoxic or hypoxic (O₂ 1%, CO₂ 5%, AirLiquide, mix #23209469-10) conditions (Normoxia Day 5 (ND5) and Hypoxia Day 5 (HD5)). Hypoxia was reached by placing the culture dishes in a hypoxic chamber (StemCell, #27310).

Viability

An islet sample was taken from culture dishes, centrifuged, and incubated in 200 μ l of 1% fluorescein (Thermo Fisher, #119241000) and 1% propidium iodide (AcrosOrganics, #440301000) in PBS for 5 minutes, then rinsed for 10 minutes in PBS. Islets were placed in culture dishes (Greiner, Cellview, #627871) and imaged using an epifluorescence microscope (Zeiss AxioObserver.z1). Images were processed with QuPath software (v0.3.0) using an ad hoc protocol to measure the stained areas in the green (viable cells) and red (dead cells) channels³⁹. Viability was calculated as the ratio between the green area over the area of the combined channels.

Functional assessment

Glucose-stimulated insulin secretion (GSIS) tests were performed with batches of 3-4 islets. Hand-picked islets (similar size) were incubated at 37°C for 1 hour, in 1 mL Krebs buffer containing glucose either 1mM or 15mM. At the end of the incubation, the culture supernatant was collected for measurement of insulin (homemade assay⁴⁰) and glucagon (Merck Millipore, Burlington, MA), and islets were collected and sonicated in 10 mmol/l Tris, 0.2 mol/l NaCl and 10 mmol/l EDTA for measurement of their DNA, insulin, and glucagon content. All secretion experiments were carried out in duplicate.

Fixation and storage

After culture, islets were washed three times with PBS, fixed using 4% formaldehyde supplemented with 0.1% Triton X-100 (Sigma, #T9284) for 3 h, followed by three washes of 5 minutes using PBS. They were then stored at 4°C

in a solution of PBS, 0.01% formaldehyde, and 0.005% sodium azide until further processing (maximum 1 month).

Antibodies validation

All antibodies used are listed in Extended Data - Table 1 – Antibodies.

Absence of cross-reactivity of secondary antibodies

Fixed islets were embedded in 1% agarose and then included in paraffin. Sections of 5 µm were cut using a microtome.

2D sections of islets were incubated with each primary antibody individually, which was then revealed either with the adequate fluorochrome-conjugated secondary antibody or with a mix of all secondary antibodies. Hoechst was used for nuclear staining.

Antibody penetration

Islets were treated as described in the 3D IF section, up to finalizing the primary antibody step. Islets were then fixed using formaldehyde 4% for 1 hour at RT, included, and cut. Then, sections were either (a) incubated with anti-mouse and anti-rabbit secondary antibodies for 1 hour at RT, together with Hoechst for nuclear staining, or (b) followed standard IF staining (primary and secondary antibodies, as described before).

Representative images of the validation tests can be found in the Extended Data – Figure 1.

3D immunofluorescence

Unless otherwise stated, all steps were performed in 24-well plates using volumes of 1 ml under agitation at room temperature. Rinsing was done by washing three times for 5 minutes.

Fixed islets were washed with 0.05% Triton-X100 in PBS, followed by a permeabilization step in 0.1% Triton-X100 in PBS for 1h. Unspecific binding block was done with a 3 hours incubation in 5% BSA (Carl Roth, #3854.3), 0.1% Triton-X100, and 0.01% sodium azide in PBS. Primary and secondary antibodies were incubated in a volume of 500 µl at 37°C under agitation in PBS containing 1% BSA, 0.1% Triton-X100, and 0.01% sodium azide. Antibodies (primaries and secondaries) were incubated for 3 or 7 days each, respectively for human and rat islets, with three washing steps of 20 minutes in between. Finally, islets were washed and fixed for 1 hour in formaldehyde 4%. Antibodies are listed in Extended Data – Table 1.

Clearing and embedding

Islets were cleared by placing them in 1 ml of CUBIC1 solution (Extended Data – Table 2) for 24 hours³⁶. After washing with PBS, islets were handpicked to eliminate as much supernatant as possible, and placed in a well with 1 ml of 1% agarose low melting point (Sigma, #39346-81-1) and kept at 56 °C. Islets were gently aspirated using a capillary (ø 1 mm) and left at room temperature for 2 hours to solidify. The agarose rods were then de-molded and incubated for 4 hours at 37°C in a solution of CUBIC2 (Extended Data – Table 2) diluted 1:1 in water. Finally, samples were incubated overnight under agitation at 37°C in

100% CUBIC 2 (refractive index = 1.4929 at room temperature) containing 1 µg/ml Hoechst 3342 (Invitrogen® #H3570) for nuclear staining.

Light sheet fluorescence microscopy (LSFM)

The agarose rods were aspirated into a capillary (ø 1 mm), which was placed on an adequate sample holder and introduced in the chamber of the LSFM (Zeiss Lightsheet.z1) filled with CUBIC2. For imaging, the tip of the agarose rod containing the islets was pushed out of the capillary. Acquisition parameters were kept constant for all samples.

Acquisition parameters are summarized in Extended Data - Table 3 - Confocal vs LSFM.

For rat islets, acquisitions were performed with 5x/0.1 illumination and 5x/0.16 detection objectives, using dual side illumination with pivot scan.

To be able to image ECM details, human islets were imaged using 10x/0.2 illumination objectives and a 20x/1.0 detection objective.

Laser scanning confocal microscopy

After LSFM imaging, an agarose rod containing islets was placed in a dish with a coverslip bottom and imaged with a Zeiss LSM800 microscope using a 20x/0.8 Plan-Apochromat objective.

Image processing

The two image sets generated using dual-side acquisition were fused into one image, using a discrete Fourier transformation with Zeiss Zen Black Edition (version 9.2.8.54).

Image analysis

Images were converted using Bitplane's Imaris Batch Converter and imported into Imaris Arena. Imaris (v9.8) was used to produce orthogonal and 3D projections to evaluate islets macroscopically and to perform quantifications. Each islet was treated individually.

For quantitative analyses, each islet was first delineated in 3D using a surface wizard, applying a threshold to the sum of the pixel values of all channels smoothed with a Gaussian blur. When needed, artifacts were manually discarded, and islets were separated if they coexisted in the same image.

Target protein expression was quantified using an ad-hoc protocol provided in the Extended data – DIA protocols. Briefly, it consisted of a pre-processing step using a Gaussian filter and a detection based on a threshold using either absolute intensity or background subtraction (depending on the protein). No post-processing filters were applied. Results were expressed as the percentage of stained volume over the islet volume.

Statistics

All calculations were computed using IBM SPSS (v25). Datasets were evaluated using Q-Q plots and the Shapiro-Wilk test; if necessary, transformations were

performed to ensure normality. Results are presented as mean +/- SD of non-transformed data.

Correlations were evaluated using Pearson's Correlation Coefficient and means comparisons were done in a two-by-two independent means fashion. ANOVA was used to evaluate the influence of condition on islet volume.

Results

Study Design

This study is divided into two parts: (a) the technical validation of the methodology (immunofluorescence, clearing, LSFM, and digital image analysis), using rat and human islets, and (b) the evaluation of the effect of hypoxia on target proteins abundance and distribution in human islets. Figure 1 summarizes the methodology workflow.

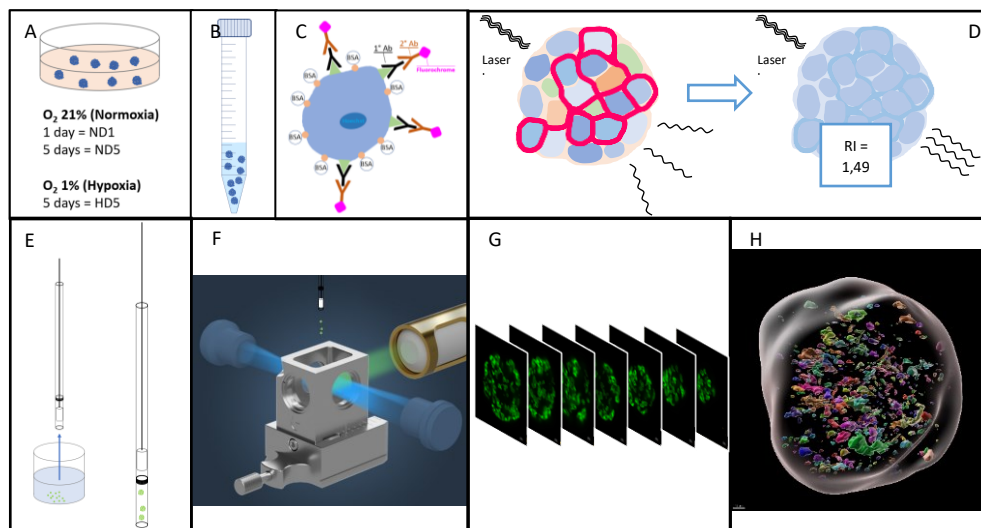


Figure 1. *Workflow of the study. A) Islet culture in different oxygenation conditions. B) Fixation and storage. C) Immunofluorescence: permeabilization, unspecific site blockage, primary and secondary antibodies incubation (3 and 7 days each, for human and rat islets, respectively). D) Using the CUBIC approach, refractive indexes homogenization. E) Embedding of fluorescent and transparent islets in agarose and aspiration in a capillary. F) Positioning of the islets, in an agarose rod, inside the imaging chamber of the LSFM (adapted from Zeiss® *z.1* user manual). G) Imaging of sequential virtual sections. H) Image processing: 3D region of interest (grey) containing volumes stained for Fibronectin.*

For both parts, we have compared three experimental groups: islets cultured in normal conditions for one day (ND1, Normoxia Day 1), and islets subjected to five days of either normal or hypoxic culture conditions (ND5, Normoxia Day 5, and HD5, Hypoxia Day 5).

In the technical part, we validated hypoxic conditions, primary and secondary antibodies, and we correlated the digital image analysis (DIA) quantification of insulin and glucagon to gold standard measurements using radioimmunoassay (RIA).

We then applied our validated methodology to morphologically evaluate and quantify Collagen 4, Fibronectin, Laminin, and E-Cadherin in individual islets.

Light Sheet Fluorescence Microscopy provides high-quality 3D images of islets.

Among the available 3D imaging technologies, we compared confocal laser scanning microscopy and LSFM acquisitions of the same immunostained and cleared human islet ($\varnothing$ 180 μ m). On both microscopes, z-stacks were acquired sequentially for each of the four fluorescence channels with a 20x detection

objective (specifications are summarized in Extended Data – Table 3). The whole, full-depth islet was imaged. The comparison is illustrated in Figure 2.

Sample transparency was verified with LSFM and confocal microscopy: sharp staining with preserved fluorescence intensity was observed throughout the whole sample (in XY and in Z).

We identified several advantages of the LSFM over confocal acquisitions. First, imaging was much faster with LSFM (3.5 min per islet vs. 31 min with confocal), mainly owing to the detection system (camera vs. detector). Secondly, as illustrated in Figure 2, although optimal step size was applied on both systems (according to Nyquist criteria), axial resolution was superior with LSFM. Indeed, 3D projections of confocal images displayed a flattened appearance, while similar islet diameters were measured laterally and axially on LSFM images without any image processing. Thirdly, confocal images were edgier but more speckled, especially for Hoechst-stained nuclei. This complicates further segmentation in the DIA process. The resolution of LSFM images allowed for visualization of subcellular structures such as nuclei, cell membranes, and insulin granules.

Taken together, our observations validate the clearing methodology and highlight LSFM as the most efficient whole islet imaging technology.

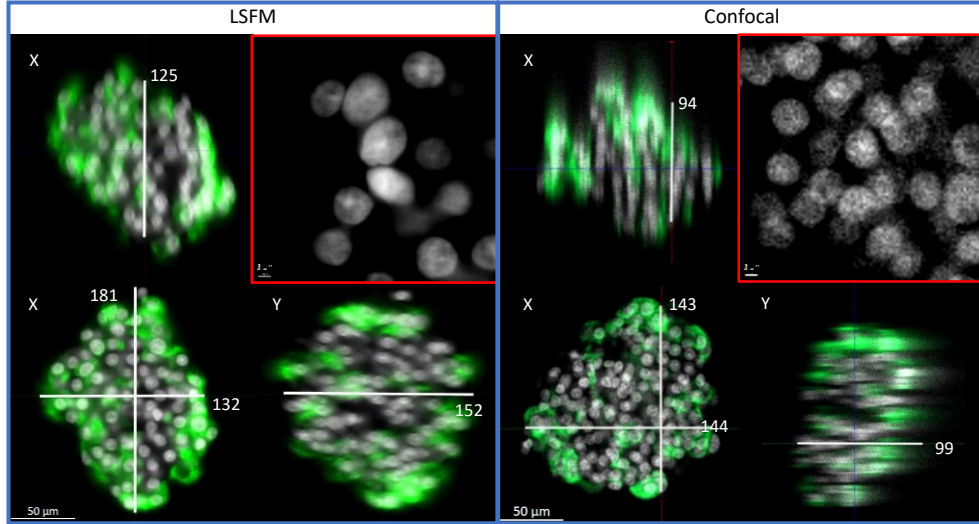


Figure 2. Comparison of imaging quality using LSFM and Confocal microscopies. The same human islet was imaged with both microscopes, with IF for insulin (green) and nuclei (Hoechst, white). The position varies as it is impossible to acquire systematically from the same angle with two different microscopes. Bottom left of each data set: 2D cross-section in the middle of the islet (X/Y view). Top-left and bottom right: X/Z and Y/Z orthogonal projections. Top right (red square): magnification of Hoechst-labelled nuclei in X/Y. Image resolution was superior in confocal microscopy, with overall crispier staining. However, this was detrimental to proper nuclear segmentation in DLA, requiring additional pre-processing steps. Orthogonal projections highlight the flattening of the sample in z and spherical aberrations when using confocal microscopy.

Hypoxia hinders islets' survival and function – validation of the hypoxic experimental model

To validate the effect of hypo-oxygenation, rat and human islets were cultured in hypoxic conditions using a hypoxia chamber with O_2 at 1% for 5 days (HD5)

and compared to islets cultured in normoxic conditions for 1 or 5 days (ND1, ND5). Immunofluorescence for Carbonic Anhydrase 9 (CA9) was used to identify hypoxia (Figure 3). CA9 is part of the hypoxia cascade and a more stable downstream target of HIF-1^{29,41}. As summarized in the graph of Figure 3, CA9 was present predominantly in the hypoxia group, although sporadic CA9-positive cells were identified in the ND1 and ND5. There were no statistically significant differences between the normoxia groups, while a significant difference was observed between HD5 and ND1 ($p=0.006$) and ND5 ($p=0.007$).

After 5 days in culture, viability was, respectively for normal and hypoxic conditions, 88% and 62% in human islets ($p=0.04$), and 79% and 29% in rat islets ($p=0.001$). Overall, this shows that hypoxia diminishes islet survival.

Rat islets were then incubated with 1 mM (G1) or 15 mM (G15) of glucose to assess their functionality. Insulin secretion rose from 1.9 (G1) to 7.5 (G15) ng/hour/islet for ND1 and from 2.6 (G1) to 3.6 (G15) ng/hour/islet for ND5 and dropped to 0.2 (G1) to 0.3 (G15) ng/hour/islet for HD5, $n=12$ per condition, $p<0.01$. A graphic illustrating these differences can be found in the Extended Data – Figure 2. These results show that glucose-stimulated insulin secretion diminishes at ND5 and is virtually stopped at HD5, showing the detrimental effect of hypoxia on glucose-stimulated insulin secretion.

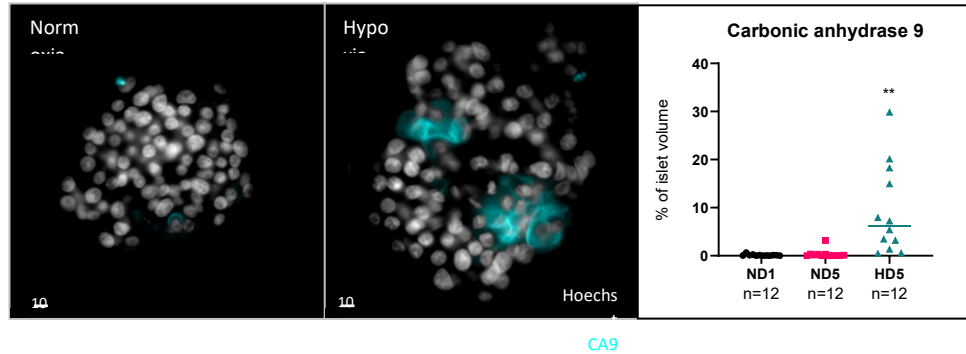


Figure 3. Left and central panels: representative 2D cross-sections in the middle of the human islets, with IF for CA9 and Hoechst. CA9 is a trans-membrane protein, used here as a marker of hypoxia. Its presence after 5 days in normal culture conditions was sporadic (left), while under hypoxic conditions (group HD5, right), it could be found in all islets, expressed in large areas. Note the alteration in nuclear shape in areas around hypoxia. Right panel: quantification of CA9, expressed as percentage of total islet volume, showing significant presence of CA9 after 5 days in hypoxia.

Insulin and glucagon quantification: digital image analysis (DIA) produces similar results to radioimmunoassay (RIA)

To validate immunostaining quantification on 3D images by DIA, we compared the impact of hypoxia on insulin and glucagon contents measured by DIA and by RIA. Measurements were performed on different rat islets from the same batch. DIA quantification generated volumetric values normalized to islet volume, while protein weight was measured by RIA and normalized to DNA content.

Figure 4 illustrates that both techniques showed consistency between DIA and RIA measurements, notably the maintenance of insulin content under hypoxia, and an increase or decrease of glucagon abundance, respectively in ND5 and HD5 conditions ($p < 0,01$).

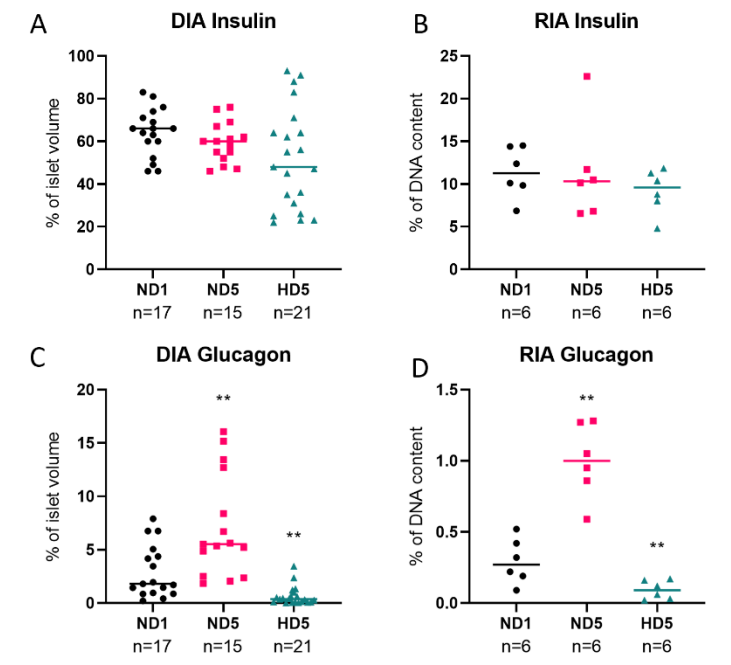


Figure 4. Comparison of the quantification of rat islets' insulin content with two different techniques. A) and C) percentage of volume occupied by insulin or glucagon inside the islet, determined by Digital Image Analysis. B) and D) weight of these proteins measured by radioimmunoassay, expressed as a percentage of DNA content.

Both DIA and RIA depict similar data distribution. N represents the number of islets.

3D quantitative evaluation of islets and ECM components

Human islets were used to address changes in the ECM during hypoxia. To ensure that islet size did not bias our analysis, we confirmed using ANOVA that culture conditions did not influence islet size ($p=0.277$). Furthermore, the influence of size on specific cues was evaluated. They will be mentioned only if they are significant.

The effects of hypoxia on Collagen 4, Fibronectin, Laminin, and E-Cadherin were evaluated on human islets. Insulin and glucagon staining allowed islet identification, thus avoiding the bias of analyzing non-endocrine islet cells.

Quantifications were performed using ad-hoc protocols created with Imaris® software, as illustrated in Figure 5. From a volumetric point of view, the mean amounts of studied proteins were not significantly different among the different conditions (Figure 6).

Taken together, these results show that hypoxia does not affect Collagen 4, Fibronectin, Laminin, and E-Cadherin overall content.

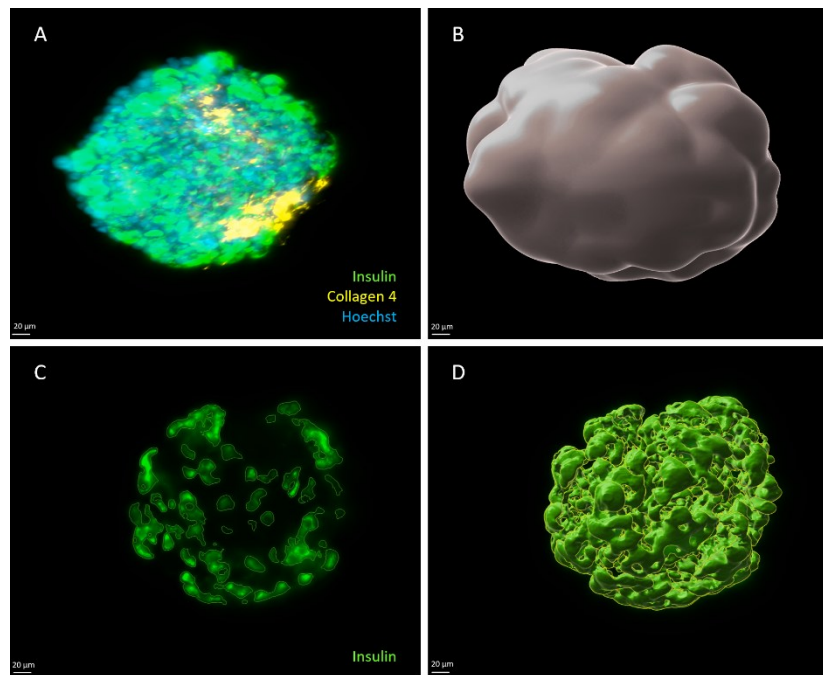


Figure 5. *Digital Image Analysis process. A) 3D projection of an islet before processing; B) 3D projection of a Region of Interest, used to calculate total islet volume. C) 2D projection showing target protein (insulin) identified using a standardized intensity threshold; D) 3D projection of the target protein stained volume.*

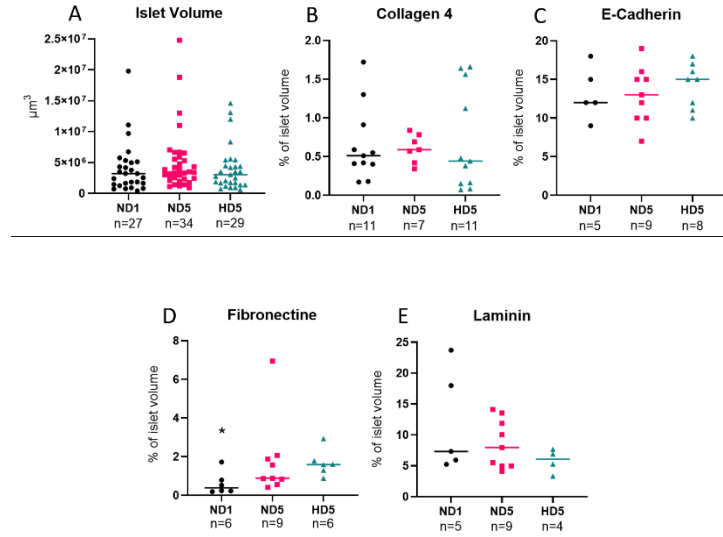


Figure 6. Summary of quantifications of protein content in human islets using DLA. Each dot in the graph represents an islet, and horizontal lines represent the mean value of the set. Statistical significance is shown with a *. A) Islet volume did not differ significantly amongst Groups. B to E) Target protein abundance expressed as a percentage of total islet volume. Means do not differ significantly, especially after 5 days of culture ($p > 0.05$).

3D qualitative morphological analysis of islet ECM components

Volumetric quantifications fail to grasp the complexity of islet ECM. To better understand the distribution of the studied proteins, we analyzed images qualitatively, using a systematic approach to evaluate islets morphologically.

All islets were deprived of their basal membrane: we could not identify ECM proteins surrounding islets. We identified ECM inside the islet, although its distribution varied among groups. These morphological differences in the distribution of target proteins are summarized in Fig. 7.

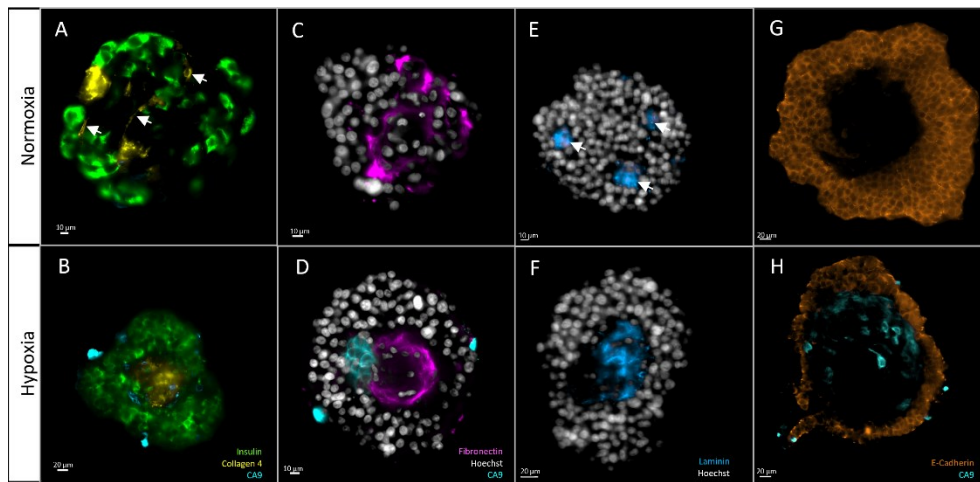


Figure 7. Morphological analysis of target proteins in human islets, after 5 days of either Normoxia (top row) or Hypoxia (bottom row). 2D cross sections in the middle of the islets. The same display parameters are identical for each protein. A) Collagen 4 can be found in structures resembling blood vessels in group ND5 (arrows). B) Central scar showing condensed Collagen 4 stain; note hypoxic cells (CA9-positive) around this area. C) Fibronectin is found throughout the islet in normoxic conditions. D) In hypoxia, it accumulates in the center of the islet, in the fashion of scarring tissue. This last picture is also a clear example of what we name architectural loss: ECM condensation, and scarce nuclei, which also present altered morphology, like irregular shape and small size. Note the presence of hypoxic cells (CA9) around the fibrotic core. E) Laminin forms small, scattered nodes in normoxic conditions. F) In hypoxia, it is found in central scars. G) E-Cadherin, a membrane marker, is found in the outer layers of islets while avoiding central areas. H) These holes in the signal are more evident in hypoxic conditions, where the central area can be populated by hypoxic cells (CA9-positive).

Collagen 4 was found around structures resembling blood vessels in the ND1 and ND5 groups, being predominant in the latter. In HD5, vessel-shaped structures could not be identified. Also, Collagen 4 was found in scarring areas in all groups, although these were more prominent in HD5, where they occupied large areas in the center of the islets.

Fibronectin was present throughout the islet. Outside the areas of scarring, it had a characteristic intercellular distribution. This mesh-like structure was more evident at ND5. In HD5, however, fibronectin tended to accumulate in central scars, surrounded by hypoxic areas.

Laminin, like the other ECM proteins, tended to accumulate in scarring areas. These areas were evident in HD5, presenting the central scarring already described. However, in ND5 these areas were smaller and scattered throughout the islet. We named these laminin-stained areas nodes. We found that the mean number of nodes was significantly lower in HD5, compared to ND1 and ND5 (respectively 1.5, SD 0.5, n=4, vs. 6.4, SD 0.6, n=5, and 7, SD 1.8, n=9 $p<0.05$) (Extended Data – Figure 3). This observation suggests that hypoxic conditions induce extensive central scarring, while in normoxia, the scarring is present but circumscribed to smaller but more numerous, scattered areas. Of note, node number and islet volume were correlated ($PCC = 0.864$, $p<0.01$).

Taken together, the morphological analysis shows that ECM proteins (Collagen 4, Fibronectin, and Laminin) tended to accumulate in central areas of scarring tissue, suggesting central necrosis, which was more evident after 5 days of culture, especially in the hypoxia group. These areas were characterized by (1) nuclei with hyperintense signal, scattered and with aberrant shape, and (2)

accumulation of ECM proteins presenting high density and a disorganized morphology (Fig. 7 B, D, F, and G).

E-Cadherin presented a typical membrane distribution. In the ND1 group, staining was homogeneous, with only some sporadic areas of higher intensity. The islets from the ND5 group presented some areas of more intense staining, spatially related to the few zones stained for CA9. However, islets at HD5 presented many areas of high signal intensity, with an overall mean intensity significantly higher than other groups (ND1= 8598, SD 1318, n=5, ND5= 6463, SD 657, n=9, vs HD5= 14805, SD 1762, n=8, arbitrary units, $p<0.01$) (Extended Data – Fig. 3).

ND5 and HD5 were characterized by the presence of central holes, which correlated with islet size (PCC = 0.887, $p<0.01$). When hole size was expressed as a percentage of total volume, we identified a tendency for bigger holes in HD5, although not statistically significant (2.5%, SD 2.5, n=5, 3.1%, SD 1.3, n=9, and 8.6%, SD 4, n=8, for ND1, ND5, and HD5, respectively) (Extended Data – Fig. 3).

Taken together, our results show the feasibility of volumetric quantification of Collagen 4, Fibronectin, Laminin, and E-Cadherin. In addition, the 3D approach for morphological assessment provides deep insights into protein distribution, exposing differences among experimental conditions that cannot be grasped with mere total protein quantification or 2D analysis.

Discussion

This study is a proof of concept, demonstrating the applicability of a methodology for 3D imaging and analysis. Our protocol permitted the assessment of specific proteins in isolated pancreatic islets, both quantitatively and qualitatively. We have applied this methodology to evaluate the impact of hypoxia on E-Cadherin and three proteins of the extracellular matrix, namely Collagen 4, Fibronectin, and Laminin. To the best of our knowledge, we are the first to propose a complete protocol using a 3D approach for IF, clearing, LSFM, and DIA applied to rat and human, isolated pancreatic islets.

Our results support the validation of the immunofluorescence (IF) protocol, both for the absence of antibody cross-reaction and for homogeneous antibody penetration. To ensure diffusion, several factors needed to be optimized, notably pre-permeabilization, antibody concentration, buffer composition, and incubation time and temperature³⁶. Here, we have successfully applied it to both human and rat islets.

During validation, our results showed that when compared to a standard measurement method, i.e., radioimmunoassay, the DIA approach can similarly highlight differences in insulin and glucagon expression. Although meant as a validation step, our results open the door to a significant improvement in protein analysis. LSFM can indeed provide information on individual islets instead of the pulled measurements obtained by RIA or other proteomics quantification methods. It also provides information on protein distribution within each islet.

Following ECM loss during isolation, islets have been shown to undergo ECM remodeling^{11,32}. Nevertheless, the mechanisms underlying this phenomenon in

the context of hypoxia remain largely unexplored^{9,32}. We propose here a technical approach to explore the complexity of the variations of ECM in hypoxic islets via qualitative and quantitative analyses.

The overall content of Collagen 4, Fibronectin, and Laminin, assessed by volumetric quantification, was not significantly affected by hypoxia. This can be understood from the point of view of energy consumption: any change in ECM after isolation involves an active process. In hypoxic conditions, the ability to secrete insulin is significantly reduced, as demonstrated by the glucose-stimulated insulin secretion (GSIS) test. Consequently, the remodeling of extracellular proteins may also be compromised, since this process relies on the secretion of enzymes. Furthermore, we have shown that through morphological analysis, only possible in a 3D setting, we could highlight subtle differences that could illustrate the influence of hypoxia on ECM. In other words, even if protein content is comparable, hypoxia induces a loss of architecture, resulting in a localized, central concentration of ECM proteins, creating scars inside the islet. We hypothesized that these areas are virtually lost and that the functional volume of the islet is thus reduced. In addition, the presence of hypoxic cells around necrotic cores could imply the presence of transitioning areas, where the rescue of dying cells could be a therapeutic target.

We observed that the E-Cadherin signal was absent in the central areas of the islet. As these central areas are occupied by an accumulation of ECM in other IF combinations, we hypothesized that holes correspond to areas of central scarring. Reports have shown an enhanced expression of E-Cadherin where ECM is absent and vice versa, suggesting compensatory mechanisms¹².

These advances can support the development of new strategies to improve islet transplantation. For example, as many groups have proposed the use of ECM proteins and moieties to enhance survival and function^{10,13,16,19-24}, it would be interesting to explore their impact on the 3D structure of islets. Moreover, our technique could be applied to examine islets *ex vivo* to assess the impact of support techniques such as encapsulation or the use of biomaterials, and to evaluate islet morphology at different stages after transplantation.

Nevertheless, the described methodology has its limitations. LSFM remains an onerous technology, although it has become more common over the years³⁷. Also, our LSFM has only four excitation channels, limiting the number of concomitant IF stains. Furthermore, the selection of the antibodies can be challenging, as they need to come from different species and be sufficiently specific to avoid cross-reactions, limiting the possible combinations of stains.

Another limitation is the length of the protocol. For human islets, it takes ten days from fixation to DIA results. However, this length is determined mainly by antibody incubation times; in our experience, this could still be reduced as the protocols evolve and are optimized. Furthermore, standardization could include the automatization of DIA, reducing analysis time and enhancing data quality⁴².

Albeit these surmountable limitations, we have developed a robust, validated methodology that could prove a major tool in a rapidly evolving field^{5,43}. As nowadays most quantification tools available cannot grasp morphology or spatial distribution, we believe that our 3D approach can play an important role in bettering the comprehension of isolated islets' anatomy. Furthermore, our contributions to the understanding of ECM morphology could have a direct impact on improving therapeutic approaches.

Conclusions

In this study, we have applied an incipient yet innovative technology to the study of pancreatic islets. With our 3D evaluation of ECM proteins and E-cadherin, we shed new light on the morphology of islets and demonstrated changes developed during hypoxia. Our findings fill a theoretical gap in the understanding of the remodeling of ECM after islet isolation, from a morphological perspective.

Data Availability

Images, including individual metadata and performed analyses, are available and free for use at <https://doi.org/10.14428/DVN/GVARMX>

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Ethics Declarations

The authors declare no competing interests.

Founding

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IV.A.2 Supplementary data

Extended Data – Table 1

List of antibodies used for 3D immunofluorescence

Target	Host	Conjugation	Clone	Supplier	Reference	RRID	Original concentration	Dilution
Primary antibodies								
Insulin	rabbit	-	C27C9	Cell Signaling	3014	AB_2126503	59 µg/ml	1:500
Glucagon	mouse	-	K79bB10	Sigma-Aldrich	G2654	AB_259852	8.1 mg/ml	1:2000
Collagen 4	mouse	-	M3F7	ThermoFisher	1282-MSM3-P0	N/A	200 µg/ml	1:100
Fibronectin	rabbit	-	polyclonal	abcam	Ab23751	AB_447656	1 mg/ml	1:200
Laminin	rabbit	-	polyclonal	abcam	Ab11575	AB_298179	0.7 mg/ml	1:100
E-Cadherin	rabbit	-	24E10	Cell Signaling	3195S	AB_2291471	54 µg/ml	1:200
Carbonic anhydrase 9	goat	-	polyclonal	R&D Systems	AF2188	AB_416562	0.2 mg/ml	1:500
Secondary antibodies								
Rabbit IgG (for rat islets)	Chicken	AlexaFluor 488		ThermoFisher	A-21441	AB_2535859	2 mg/ml	1:500
Rabbit IgG (for human islets)	Donkey	AlexaFluor 488		ThermoFisher	A-32790	AB_2762833	2 mg/ml	1:500
Goat IgG	Donkey	AlexaFluor 568		ThermoFisher	A-11057	AB_2534104	2 mg/ml	1:500
Mouse IgG	Donkey	AlexaFluor 647		ThermoFisher	A-31571	AB_162542	2 mg/ml	1:500 (for Collagen 4) 1:2000 (for Glucagon)

	Primary antibodies	Matched secondary antibodies
Multiplex IF #1_human islets	Insulin	Donkey anti-Rabbit_AlexaFluor 488
	Collagen 4	Donkey anti-Mouse_AlexaFluor 647
	Carbonic anhydrase 9	Donkey anti-Goat_AlexaFluor 568
	+ Hoechst3342	
Multiplex IF #2_human islets	Fibronectin	Donkey anti-Rabbit_AlexaFluor 488
	Glucagon	Donkey anti-Mouse_AlexaFluor 647
	Carbonic anhydrase 9	Donkey anti-Goat_AlexaFluor 568
	+ Hoechst3342	
Multiplex IF #3_human islets	Laminin	Donkey anti-Rabbit_AlexaFluor 488
	Glucagon	Donkey anti-Mouse_AlexaFluor 647
	Carbonic anhydrase 9	Donkey anti-Goat_AlexaFluor 568
	+ Hoechst3342	
Multiplex IF #4_human islets	E-Cadherin	Donkey anti-Rabbit_AlexaFluor 488
	Glucagon	Donkey anti-Mouse_AlexaFluor 647
	Carbonic anhydrase 9	Donkey anti-Goat_AlexaFluor 568
	+ Hoechst3342	
Multiplex IF #5_rat islets	Insulin	Donkey anti-Rabbit_AlexaFluor 488
	Glucagon	Donkey anti-Mouse_AlexaFluor 647
	Carbonic anhydrase 9	Donkey anti-Goat_AlexaFluor 568
	+ Hoechst3342	

Extended Data – Table 2

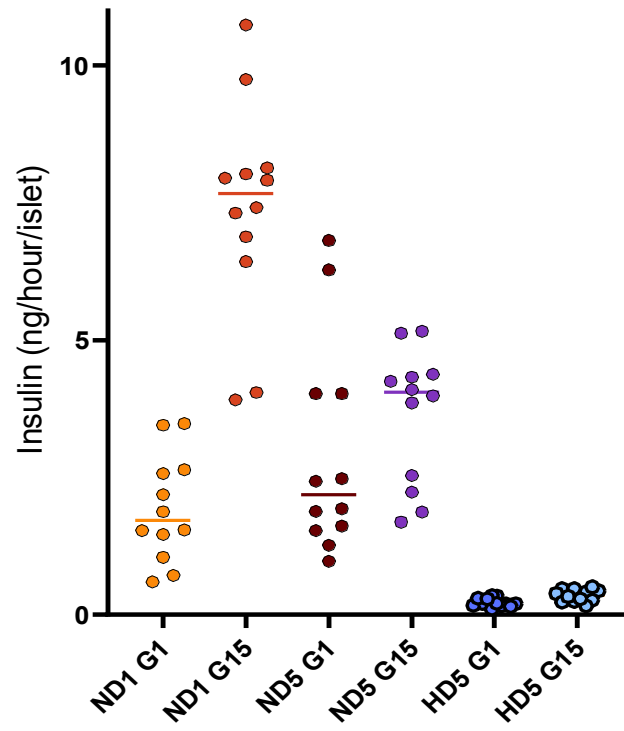
Recipes of house-made CUBIC solutions

	Component	Supplier	Reference	Quantity
CUBIC 1	N, N, N', N'-tetrakis (2-hydroxypropyl) ethylenediamine	Sigma (Merck)	122262	25%
	Urea	Sigma (Merck)	U5378	25%
	Triton X-100	Sigma (Merck)	T9284	15%
	Distilled H ₂ O			35%
CUBIC 2	Sucrose	MP	821713	50%
	Urea	Sigma (Merck)	U5378	25%
	2,2',2''-nitrilotriethanol	Sigma (Merck)	90279	10%
	Distilled H ₂ O			15%

Extended Data – Table 3

Acquisition data - Confocal vs LSF microscopies

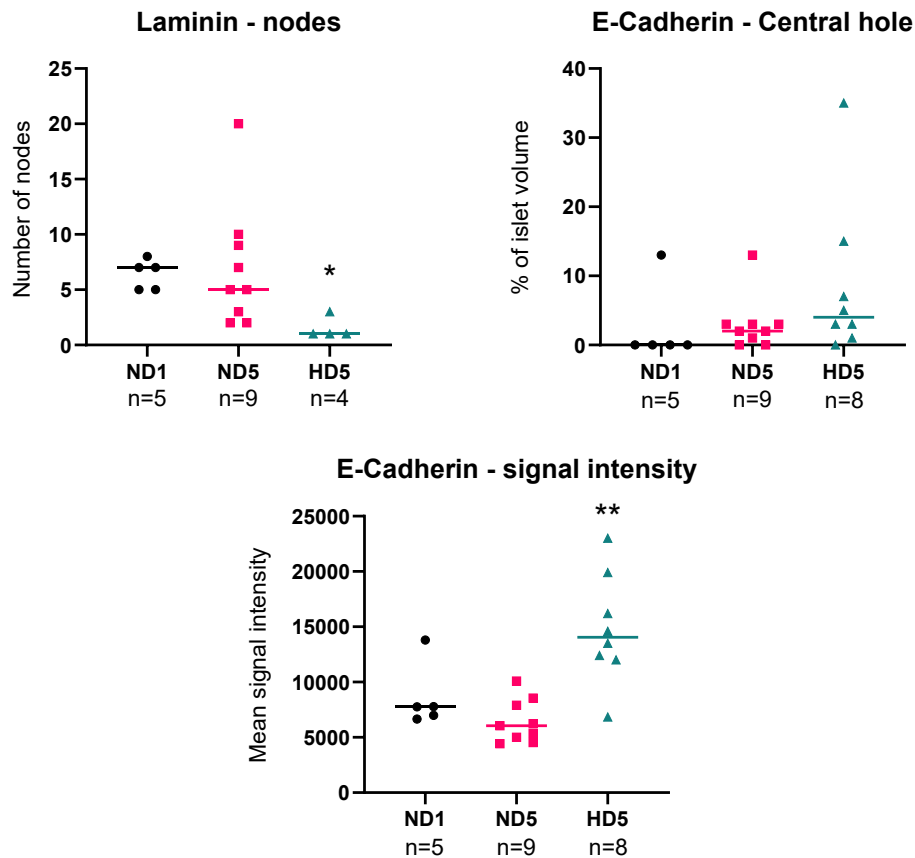
	Confocal	LSFM
Objective	20x/0.8 Plan-Apochromat	20x/1.0 Clr Plan Neofluar
Laser lines	405, 488, 561, 640 nm	405, 488, 561, 638 nm
Detection wavelenghts	400-529, 495-585, 559-627, 645-700 nm	420-470, 505-545, 575-615, LP660 nm
Detector	GaAsP-PMT	PCO.Edge sCMOS camera
Pixel depth	8 bits	16 bits
Frame size	512x512	1920x1920
Z step	0,46 µm	0,41 µm
Slices	296	560
Total thickness	135,7 µm	226,4 µm
Time	31 min	3,5 min



Extended data – Figure 1. Comparison of glucose stimulated insulin secretion measurements using radioimmunoassay.

Under normoxic conditions, insulin secretion diminishes after five days in cultures. It is virtually stopped at HD5, showing the detrimental effect of hypoxia on secretion.

n = 12 per group

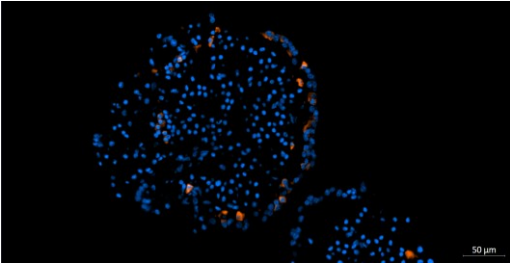
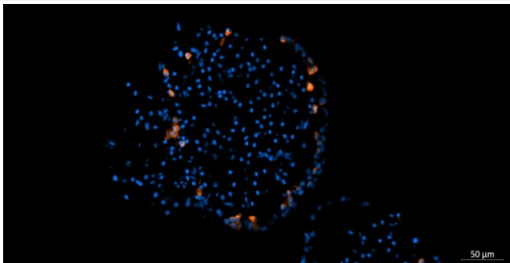
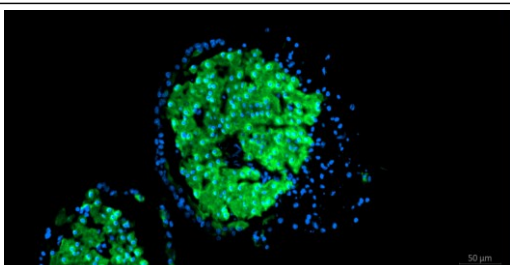
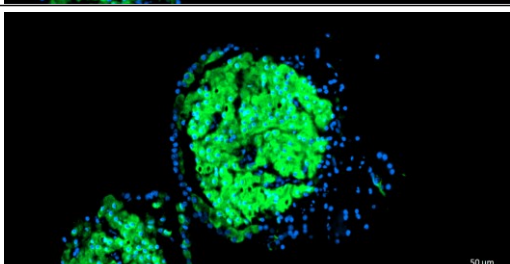


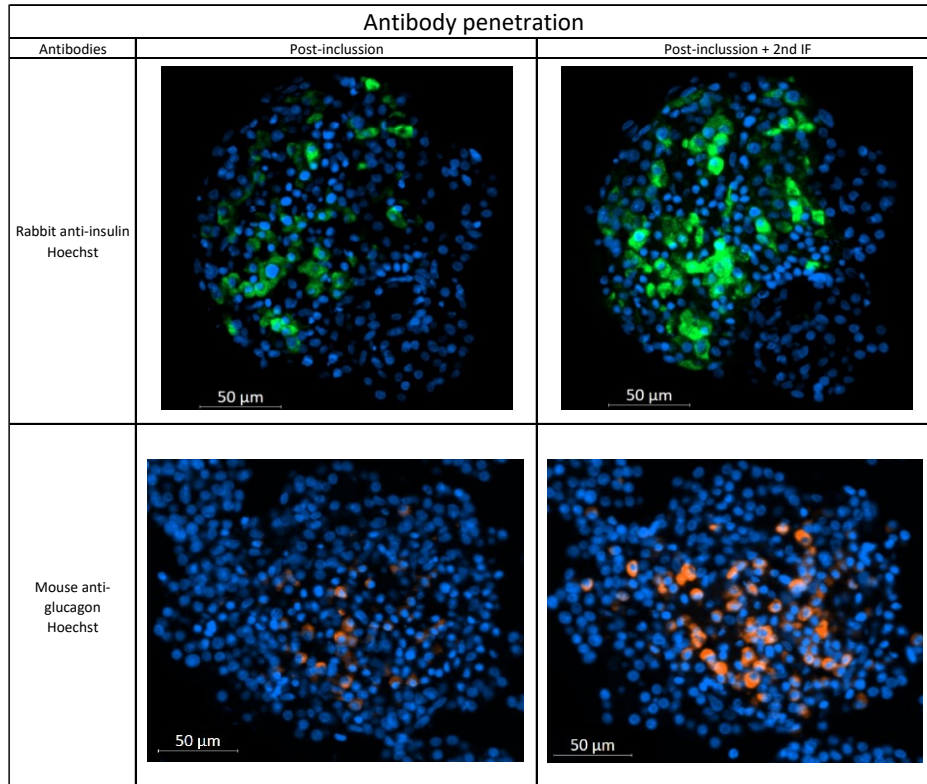
Extended information – Figure 2. Comparison of specific cues of protein distribution.

A) The number of nodular accumulations of laminin was significantly lower in hypoxia, where this protein was found in a big, central scaring area ($p < 0.05$). In normoxic conditions, nodes were smaller and scattered throughout the islet.

B) E-Cadherin presented a peripheral distribution, avoiding central areas that we denominate “holes”. These holes were mainly absent in ND1, small in ND5 and occupied extensive areas in HD5.

C) E-Cadherin IF mean signal intensity was significantly higher in HD5 ($p < 0.01$). This complements our morphological observations, where peaks of signal were detected in the vicinity of CA9-positive cells.

Cross-reactivity of secondary antibodies		
1° antibodies	2° antibodies	Representative image
Glucagon	Specific	
Glucagon	Mix	
Insulin	Specific	
Insulin	Mix	
CA9	Mix	



Extended data – Figure 3. Validation of antibodies. Taken together, these evaluations show that in the absence of primary antibody, the secondary antibodies do not recognize neither unspecific epitopes, nor cross react with any primary antibodies from other species. In addition, antibodies reach the center of the islets, implying that quantifications reflect the entire protein volume.

IV.A.3 Commentary

This study provides a methodology combining tissue clearing, multiplex immunofluorescence, Light Sheet Fluorescence Microscopy, and digital image analysis to investigate pancreatic islets morphology. This 3D approach allowed us to highlight ECM organizational changes during hypoxia.

Findings show that under hypoxic conditions, ECM proteins (Collagen IV, Fibronectin, Laminin) and E-Cadherin did not exhibit significant differences in which regards to quantity. However, distinct traits in protein and nuclear morphology were identified. Hypoxic islets exhibited areas of scattered nuclei (pointing to cell death) and central clusters of ECM proteins. E-cadherin was absent in these areas. Our results, demonstrating a diminution of islets' functional mass in hypoxia, are in line with the functional decline observed in transplanted islets experiencing low oxygenation after grafting.

In hypoxic islets, ECM was concentrated in the center of the islet in an unorganized manner. Around it, what could be identified as areas of suffering tissue, with abundant E-Cadherin and CA9. These peripheral areas of surviving islet cells could benefit from the close contact with ECM moieties.

These results support the use of bioencapsulation, which can, in addition to the mechanical shielding of islet, serve as a scaffold to nestle bioactive materials and cells – thus keeping the periphery of the islet in close contact with pro-survival factors and moieties. However, adding components to a capsule can alter its biocompatibility. This aspect will be developed in the following Paper.

IV.B In vivo evaluation of bioactive-encapsulated islet grafts (Paper B)

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BRIEF COMMUNICATION

Xenotransplantation WILEY

Semi-automated digital quantification of cellular infiltrates for in vivo evaluation of transplanted islets of Langerhans encapsulated with bioactive materials

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Abstract

Background: In the field of xenotransplantation, digital image analysis (DIA) is an asset to quantify heterogeneous cell infiltrates around transplanted encapsulated islets.

Materials and Methods: RGD-alginate was used to produce empty capsules or to encapsulate neonatal porcine islets (NPI) with different combinations of human pancreatic extracellular matrix (hpECM), porcine mesenchymal stem cells (pMSC) and a chitosan anti-fouling coating. Capsules were transplanted subcutaneously in rats for one month and then processed for immunohistochemistry. Immunostainings for macrophages (CD68) and lymphocytes (CD3) were quantified by DIA in two concentric regions of interest (ROI) around the capsules. DIA replicability and reproducibility were assessed by two blind operators. Repeatability was evaluated by processing the same biopsies at different time points. DIA was also compared with quantification by point counting (PC).

Results: Methodology validation: different sizes of ROIs were highly correlated. Intraclass correlation coefficients confirmed replicability and reproducibility. Repeatability showed a very strong correlation with CD3 stains and moderate/strong for CD68 stains. Group comparisons for CD68 IHC at each time point proved internal consistency. Point counting and DIA were strongly correlated with both CD3 and CD68. Capsule biocompatibility: Macrophage infiltration was higher around capsules containing biomaterials than around empty and RGD-alginate-NPI capsules. Lymphocytic infiltration was comparable among groups containing cells and higher than in empty capsules.

Conclusion: We validated a semi-automated quantification methodology to assess cellular infiltrates and successfully applied it to investigate graft biocompatibility, showing that neonatal porcine islets encapsulated in alginate alone triggered less infiltration than capsules containing islets and bioactive materials.

KEYWORDS

digital image analysis, encapsulation, islet transplantation

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1. Introduction

Encapsulated islet xenotransplantation has been proposed as a therapeutic option for Type 1 diabetes¹⁻⁶. Encapsulation protects the graft from the immune response, thus contributing to viability and functioning of islets.^{4,5} Although less immunogenic, encapsulated cells can still lead to surrounding immune infiltrates, which needs an evaluation generally dependent on cell counting.⁵⁻⁷ However, manual identification of cells encounters important limitations, such as morphological criteria (giant cells), operator dependence and errors, and being very time-consuming.

These problems could be overcome by using Digital Image Analysis (DIA) quantification, a methodology relying on algorithms to standardize measurements of specific features on a biopsy image. The relevance of this technology has been discussed elsewhere.⁸

Here, we propose a standardized, whole-slide quantification methodology to quantitatively assess immune cell infiltrates to better evaluate encapsulated islet transplants and apply this to subcutaneous grafts.

We explored bioactive encapsulation, using RGD-alginate and decellularized human pancreatic extracellular matrix (hpECM) that offer specific moieties capable of enhancing islet survival and function⁹; mesenchymal stem cells (MSC) that have been proven beneficial when co-encapsulated with islets⁷; and an antifouling coating (ZW) to prevent cell adhesion and therefore hinder fibrosis¹⁰.

2. Materials and Methods

Islet were isolated as described elsewhere¹¹ and cultured for eight days before encapsulation to allow maturation. Biomaterials¹² and MCSs¹³ production is described in the Supporting Information.

2.1 Capsule production and composition

Microcapsules (ϕ 500 μm) were produced using an in-house microfluidic device as described elsewhere.¹⁴ Capsules shared shape, size, and quality parameters.

Specific capsule composition of the experimental groups can be found in the Supporting Information. For the in vivo evaluation of biomaterials, where three conditions were compared, RGD-alginate was used to produce (1) empty RGD-alginate capsules (control), to encapsulate (2) neonatal porcine islets (NPI) only (basic), or (3) NPI and hpECM, pMSC and ZW (bioactive).

2.2 In vivo evaluation

All procedures were approved by the local ethics committee [2016/UCL/MD/02]. 300g male Wistar rats were anesthetized and two thousand capsules injected subcutaneously through an 18G needle.

After one month, implants were dissected free, fixed in formaldehyde 4% and embedded in paraffin. Following evidence from pilot studies showing a heterogeneous graft size and reaction (Figure 1), each biopsy was cut into three parts and included in separate paraffin blocks.

5 μm -thick sections underwent haematoxylin-eosin staining or immunohistochemistry (IHC). The antibodies used were: for lymphocytes, rabbit anti-CD3 (Abcam, ab828) followed by anti-rabbit Envision-HRP secondary antibodies (Dako, K4003); for macrophages, mouse anti-CD68 (Abcam, ab31630) followed by donkey anti-mouse-HRP (Jackson ImmunoResearch, 715-035-151). Immunostainings were revealed with DAB and nuclei counterstained with haematoxylin. The slides were then digitalized at x20 magnification using a slide scanner (Leica® SCN400).

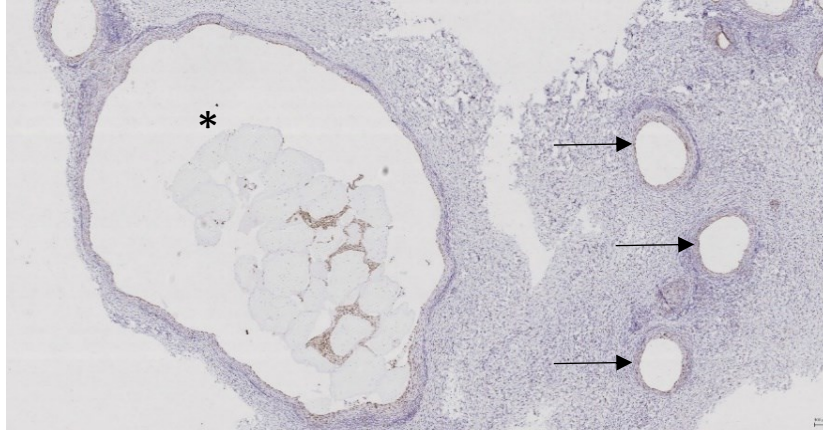


Figure 1 - Illustration of isolated (arrows) and agglomerated (*) capsules. Scale bar: 100 μm .

2.3 Image analysis: Immunohistochemistry quantification

Each *biopsy* was divided into three *blocks*, from each of which two *images* were produced for analysis. For methodological questions, comparisons were done using blocks or images. Pooled biopsy results were used to address biological questions.

Scanned images were analysed using Visiopharm® software with house-made algorithms designed with Author®. Briefly, after grafted tissue (i.e. the capsules) automatic detection, two regions-of-interest (ROIs) were automatically drawn by a first concentric 30 μm dilation from the external border of the capsule (ROI 1), followed by an additional 100 μm dilation (ROI 2). For both steps, corrections were applied when needed (debris, folds, absence of tissue). To detect the immunostained cells, an optimized colorimetric filter was used, and a detection threshold was empirically determined (Figure 2). Finally, the percentage of stained area was calculated.

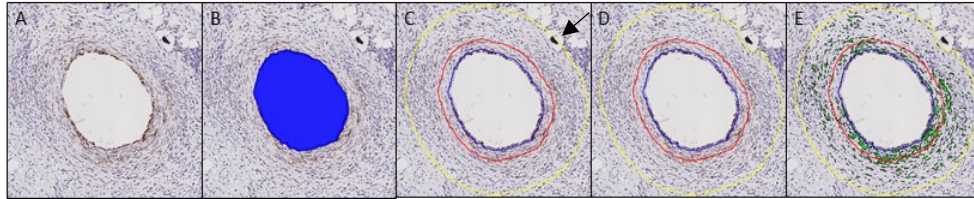


Figure 2 - Illustration of the automated digital analysis. (A) original image, (B) capsule detection, (C) drawing of ROIs (blue=capsule, red=ROI 1, yellow=ROI 2), (D) manual correction (debris, marked with an arrow), (E) detection of DAB-stained cells (green).

2.4 Methodology validation

Three variables were investigated to validate the methodology: replicability, reproducibility, and repeatability. All analyses were performed based on ROI 1.

2.4.1 Replicability: inter-observer tissue detection variability

Two independent operators (one naïve and one experienced) analysed a set of sections stained for CD3 and CD68, blindly processing the images (specifically the manual exclusion of non-exploitable areas) but maintaining the same detection threshold.

2.4.2 Reproducibility: inter-observer DIA variability

Another set of sections stained for CD68 was analysed by two independent operators (one naïve and one experienced), blindly processing the images and defining independent detection thresholds.

2.4.3 Repeatability: immunostaining variability

The same operator analysed different sections of the same biopsy set, independently immunostained (by the same person) for CD68 and CD3 at different time points (4 for CD68; 2 for CD3).

2.4.4 Comparison with point counting

Images were generated using ImageJ®. Four hundred μm^2 ROIs were drawn at x20 magnification, over selected scanned biopsies, taking the capsular border as one of the limits, and cropped. Using the Grid Tool, one hundred crosses were placed on top of each image and manually counted. A cross was counted as positive if any part of it was in touch with the DAB-stained area. The same images were processed using Visiopharm®, measuring the overall percentage of staining.

2.5 Statistics

All calculations were computed using IBM SPSS®.

A cubic root transformation was performed to normalize data.

Analyses were done per image, per block (mean value of two images of the same slide) or biopsy (mean values of two or three blocks). For inter-observer correlations, image-to-image comparisons were performed, using intraclass correlation coefficient (ICC). Block-to-block correlations were done using Pearson's. A linear mixed model was used to answer biological questions.

Data are presented as a percentage of IHC positive area in ROI 1 (mean $\pm$ standard deviation) unless otherwise stated.

P values < 0.05 were considered significant.

3. Results

A summarized workflow of this study is shown in Figure 3.

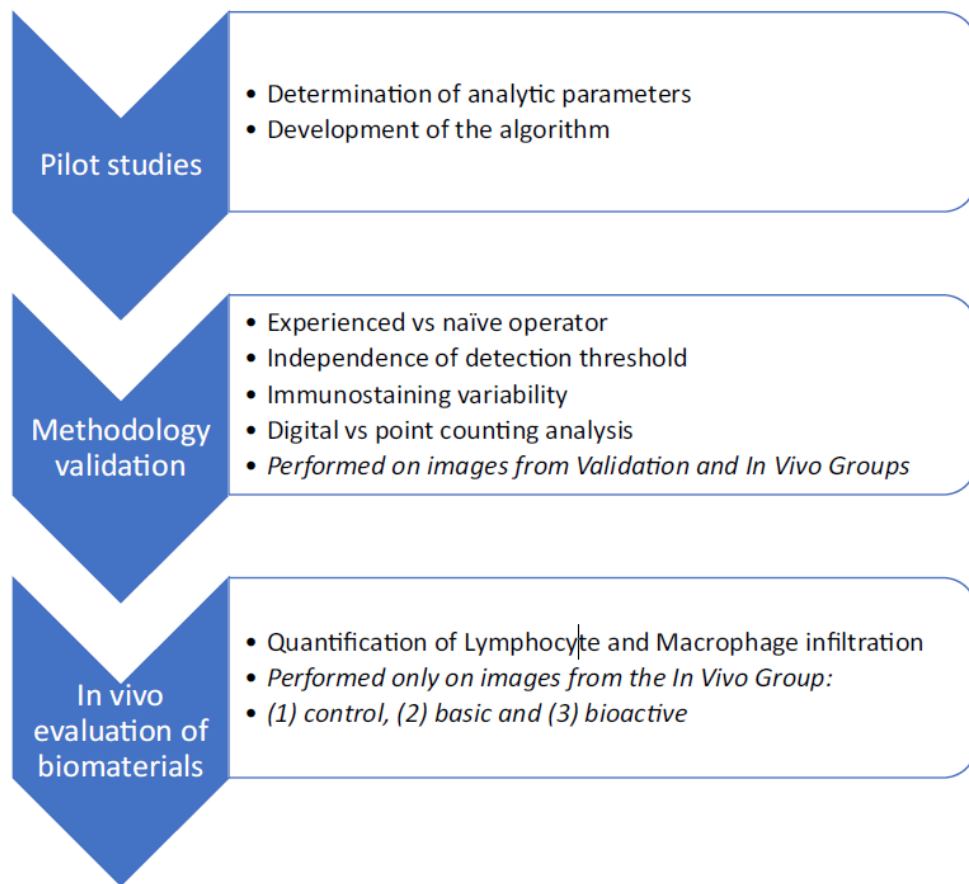


Figure 3 – *Workflow followed for methodology validation and immune infiltrates quantification.*

3.1 Methodology validation

3.1.1 Replicability

To assess the inter-observer tissue detection/correction variability, lymphocyte and macrophage infiltration index was quantified on 31 (CD3) and 46 (CD68) whole slide images.

Lymphocyte infiltration was 4.42% (± 1.89) and 4.70% (± 2.07) for operators one and two respectively. ICC was 0.989 for average measure, with CI95 between 0.946 and 0.996 ($p < 0.001$).

Macrophage infiltration was 17.07% (± 12.94) and 19.00% (± 13.94) for operators one and two respectively. ICC was 0.982 for average measure, with CI95 between 0.941 and 0.993 ($p < 0.001$).

An experienced operator (the developer of the DIA quantification methodology) and a naïve operator obtained similar, highly correlated results suggesting good automatic capsule detection with minor impact of manual corrections.

3.1.2 Reproducibility

To evaluate the inter-observer variability in the complete DIA workflow, macrophage infiltration was quantified on whole slide images of 118 sections immunostained for CD68. The infiltration was 10.83% (SD 6.41) and 12.20% (SD 6.40) for operators one and two respectively. ICC was 0.934 for average measure, with CI95 between 0.852 and 0.965 ($p < 0.001$). Higher absolute values obtained by operator two (naïve) reflect a less stringent staining detection threshold.

These results show that a DIA workflow requiring minimal corrections produced comparable and highly correlated results, even when independent detection thresholds are used.

3.1.3 Repeatability

The experimental immunostaining variability was then investigated. Non-consecutive sections (time points 1, 2 and 3) and consecutive sections (time points 3 and 4) of the same biopsies were immunostained, scanned and analysed at the different time points (1 to 4). Values for each time point can be found in the Supporting Information.

For lymphocytes, CD3 IHC was performed only for consecutive sections (time points 3 and 4). Pearson's correlation between blocks was 0.875 (n=12 per time point, $p<0.001$).

For CD68 (macrophages), data from time points correlated as follows: 1 to 2, ICC 0.608, $p<0.01$; 1 to 3, ICC 0.476, $p<0.01$; 1 to 4, ICC 0.503, $p<0.01$; 2 to 3, ICC 0.611, $p<0.01$; 2 to 4, ICC 0.394, $p<0.05$; and 3 to 4, ICC 0.431, $p<0.05$; n=29 per time point.

Groups stained for CD68 were compared at each time point using a mixed model (see Supporting Information); a statistical difference was evidenced for each paired comparison. These results expose extrinsic variability, resulting in different values at each time point. However, correlations between time points were very strong for CD3 and moderate/strong for CD68. Furthermore, the statistical difference amongst quantified macrophage infiltration around experimental groups evidences the internal consistency of DIA.

3.1.4 Point counting

From each section of selected biopsies with IHC for CD3 and CD68, ten images were quantified by point counting (PC) and automated % stained area obtained by DIA. Values of PC were summed per biopsy and compared to DIA values using Pearson's correlation with a list-wise approach. Results are summarized in Figure 4.

Although mean values obtained by DIA and PC differ, both methodologies were highly correlated.

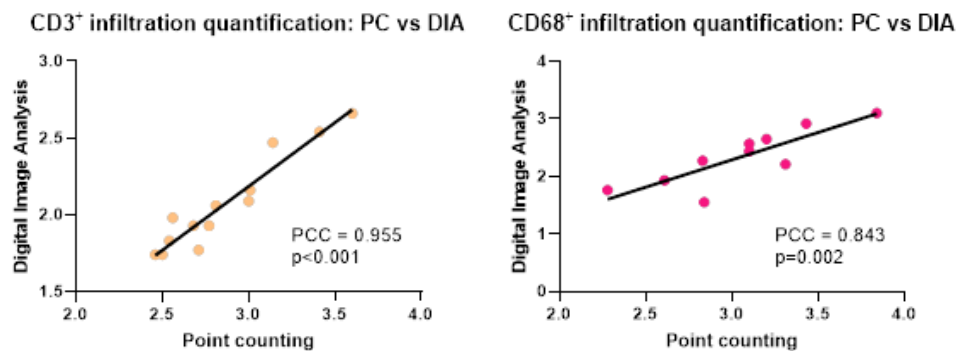


Figure 4 – correlations between point counting and digital image analysis with Pearson's Correlation Coefficient (PCC). Values correspond to transformed data.

3.2 Evaluation of regions of interest

To explore the influence of the thickness of the ROI, calculations were done on ROI 1, ROI 2 and their sum (ROI 1+2).

ROI 1, close to the capsule, represents an area usually infiltrated by immune cells. ROI 2 intended to include tissue of the injection site. The sizes of these ROIs were designed to consider the heterogeneity of infiltrate distribution in the tissue. ROIs correlations are shown in Figure 5.

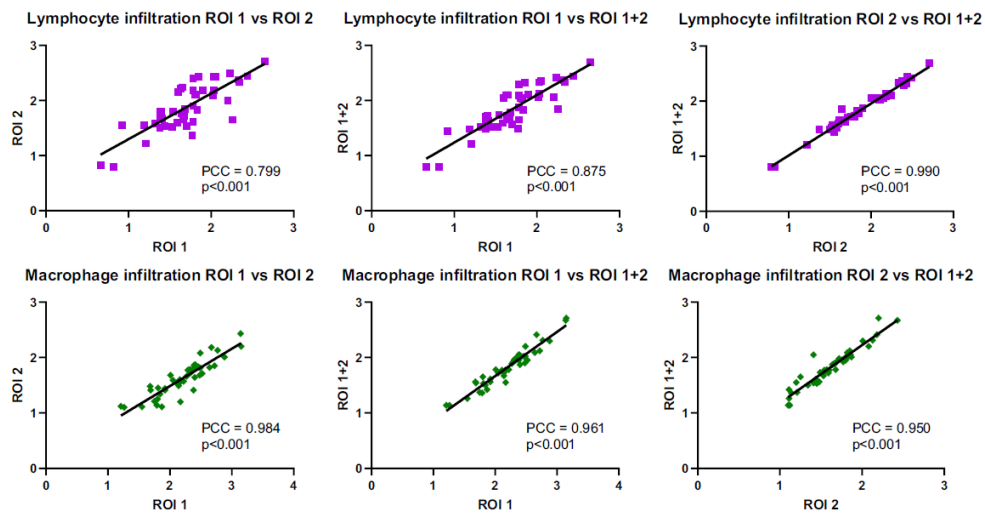


Figure 5 – Correlations and Pearson's Correlation Coefficient (PCC) amongst infiltration measurements for CD3 (lymphocytes, TOP) and CD68 (macrophages, bottom) in the ROI1 (proximal ROI), the ROI2 (distal ROI), and the two ROIs taken together. Values correspond to transformed data.

Moreover, measurements reflected the distribution observed by direct microscopy, where macrophages tend to be found near the capsules (ROI 1) and lymphocytes in the far periphery (ROI 2). Images illustrating this distribution can be found in the Supporting Information.

High correlation amongst ROIs supports the use of ROI dilatation between 30 and 100 μm from the graft.

3.3 Biomaterials evaluation

To illustrate the application of this DIA methodology, infiltrates were quantified in the three in vivo Groups previously described: (1) control, (2) basic and (3) bioactive capsules. Comparisons were done using a linear mixed model to exploit the different values obtained for each individual (i.e. each n corresponds to four to six data points). Infiltration was quantified in all ROIs; results for ROIs 1+2 are presented in Figure 6.

Taken together, these results show that (1) encapsulated NPI trigger a lymphocytic infiltration; (2) this lymphocytic infiltration is not influenced by the supplementation of hpECM, pMSC and ZW; (3) biomaterials trigger an important macrophage infiltration.

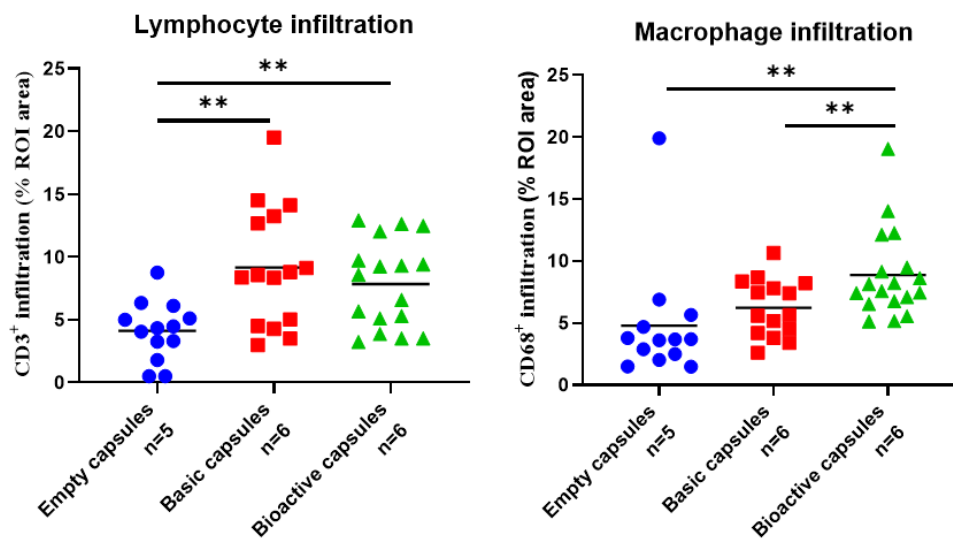


Figure 6 - Lymphocyte infiltration was higher for both types of capsules containing cells. For macrophages, islets encapsulated in alginate alone performed better than those co-encapsulated with biomaterials, with no statistical difference with empty capsules. **, $p < 0.010$. Values correspond to untransformed data.

4. Discussion

The rat subcutaneous tissue has been widely evaluated for experimental transplantation of islets. Porcine islets could be an alternative to humans islets to palliate donors' scarcity.¹⁵

The immune reaction triggered by grafted encapsulated islets is characterized by immune cell infiltration in the vicinity of the capsule that can lead to graft rejection. To evaluate biocompatibility on experimental biopsies, a DIA protocol was developed, allowing measurement of immune infiltrates. To improve quantification accuracy, each biopsy was cut into three pieces that were processed separately and analysis was performed on entire tissue sections (rather than on selected fields of view). Quantitative DIA in IHC permitted operator independent assessments, using contiguous scales, while relying on objective and standardized criteria for systematic analysis.

DIA has been used for quantification of lymphocytes and macrophages in various contexts.^{16,17} In the field of islet transplantation, DIA has been applied on immunofluorescence quantification, although reports lack methodology validation.^{6,18} In this study, we present experiments proving an IHC semi-automated quantification methodology that is replicable, reproducible, and transposable to two different immunostainings.

The validation procedure permitted exposure of IHC and biological variabilities. When different sections of the same biopsy piece were submitted to the exact same protocol at different time points, the values obtained differed, reflecting irregularities in the immunostaining outcomes, a problem already approached in the literature.¹⁹⁻²¹ Another source of irregularities is biological variability, which can be addressed by augmenting the number of analysed slides, which is easily achievable by using DIA. Although extrinsic variability is present, we could

evidence correlations amongst the different time points and the internal coherence of the methodology was proved.

DIA quantification strongly correlated to a widely used method (PC). Values obtained from the latter were higher, as has been described in the literature.^{16,22} In addition, the semi-automatization of image analysis allows an important gain in time, which compensates for the costs of DIA software.⁷⁻⁹

Using DIA to assess infiltrates has many advantages. Computer-assisted analysis can integrate enormous amounts of data in a comprehensible and efficient fashion. It provides consistent results since objective parameters are clearly defined. In our approach, the user was only asked to identify the non-exploitable tissue, which requires very low expertise and virtually no learning curve.

Nonetheless, some limitations need to be noted. DIA relies on digitalized images of IHC-treated biopsies. Steps occurring before analysis can be influenced by uncontrollable parameters such as human handling, antibodies and solutions' age and quality, and scanning parameters like light intensity. It is therefore important to have quality controls of IHC protocols to ensure repeatable results. In this sense, DIA can work retroactively to ensure quality adjustment of IHC protocols.

To show the applicability of DIA, we assessed the biocompatibility of three formulations of capsules grafted subcutaneously in rats. As expected, control (empty) capsules trigger immune infiltration, in line with what has already been described in the literature.⁴ The presence of xenogeneic cells inside the basic and bioactive capsules triggers a florid infiltration of lymphocytes, evidencing cellular recognition independent of capsule composition. On the other hand, macrophage infiltration was higher around bioactive capsules, speaking of non-cellular pathways triggered by biomaterials.

Building on our results, more markers could be investigated using the same DIA approach to allow a more extensive evaluation of biocompatibility, such as characterizing sub-populations of the infiltrating cells (for example, macrophage M1/M2 polarization), and evolution of the reaction over time^{23,24} or blood vessel distribution.

We offer a new standardized DIA technique to quantify immune infiltration in grafted capsules irrespective of tissue heterogeneity. By this means, we identified the most important source of error as being the immunostaining. We also successfully investigated the biocompatibility of different biomaterials, showing that NPI triggered a milder immune infiltrate when encapsulated in RGD-alginate alone than when co-encapsulated with mesenchymal stem cells, human extracellular matrix and a zwitterionic anti-fouling coating.

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IV.B.2 Supplementary data

Materials and methods specifications/Documents files

Islet isolation, biomaterials and mesenchymal stem cells

Neonatal pig islet (NPI) isolation was approved by the local ethics committee and carried out as described elsewhere¹. Briefly, pancreata were harvested from exsanguinated piglets, cut into 1- to 3-mm³ pieces in cold Hank's balanced salt solution (HBSS; Life Technologies) supplemented with 0.25% bovine serum albumin (BSA; Roche, Basel, Switzerland), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Westburg), 10 mM glucose, 1% penicillin, 1% streptomycin, and 0.1% amphotericin B (Westburg). Enzymatic digestion was done using 10 mg/pancreas collagenase V (Sigma-Aldrich, Darmstadt, Germany). Digested tissue was then filtered on a 500- μ m mesh, washed three times in cold HBSS, and cultured in HAM-F10 (Life Technologies) supplemented with 0.5% BSA, 50 μ M IBMX, 10 mM glucose, 2 mM glutamine, 10 mM nicotinamide (Sigma-Aldrich), 1% penicillin, and 1% streptomycin.

Capsules were produced with a mixture of alginate (PRONOVA SLM100) at 2.5% w/w and RGD-coupled alginate (NOVATACH™ MVG GRGDSP - GRGDSP-coupled high G high MW alginate) at 0.5 mg/ml.

Adipose-tissue-derived porcine mesenchymal stem cells were provided by Dr Sandrine Lablanche (Université Grenoble-Alpes, France).² For encapsulation, they were used at 150 per islet equivalent.

Ultra-pure extracellular matrix from human pancreata was provided by Dr Giuseppe Orlando (Wake Forest University, USA). Decellularization and

solubilization are described elsewhere.^{3,4} It was used at a concentration of 0.1 mg/ml

For the antifouling treatment, a chitosan derivate was used, whose zwitterionic characteristics help change the electrostatic charge of the capsule surface. A ZW chitosan bearing 26 +/- 5% phosphorylcholine groups was kindly provided by Prof. K. Glinel (UCLouvain, Belgium).

Capsules composition

Group	Type	n (rats)
Validation	Alginate RGD + NPI + pMSC	4
	Alginate RGD + NPI + pMSC + ZW	2
In vivo	Alginate RGD (empty capsules)	5
	Alginate RGD + NPI	6
	Alginate RGD + NPI + pMSC + ECM + ZW	6

Group	Type	n (rats)	Used in:
Validation	Alginate RGD + NPI + pMSC	4	Methodology validation
	Alginate RGD + NPI + pMSC + ZW	2	Methodology validation
In vivo	Alginate RGD (empty capsules)	5	Methodology validation and biological evaluation
	Alginate RGD + NPI	6	Methodology validation and biological evaluation
	Alginate RGD + NPI + pMSC + ECM + ZW	6	Methodology validation and biological evaluation

Results

Repeatability evaluation

Mean values for each time point for IHC stainings

IHC	Type of capsules	n	Time point 1		Time point 2		Time point 3		Time point 4	
		(blocks)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CD68	Control (empty)	11	9.22	8.41	6.83	4.18	4.93	3.22	3.59	4.69
	Bioactive	18	15.92	5.60	9.39	3.41	8.65	3.53	9.41	5.15
CD3	Control (empty)	6					4.74	3.39	5.70	3.95
	Bioactive	6					12.06	5.98	9.20	6.80

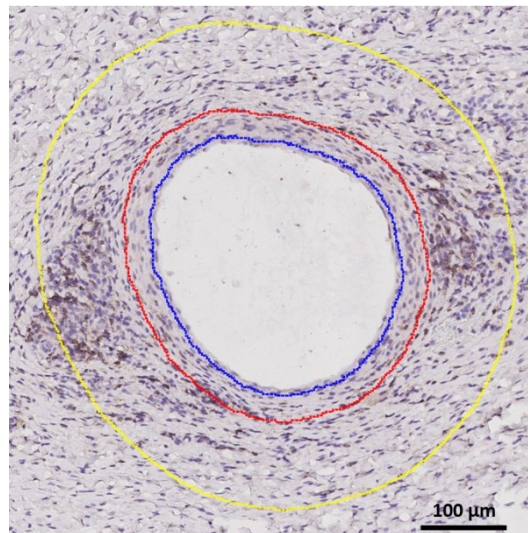
Comparison, at each time point, between groups with IHC for CD68 using a mixed model.

Time point	Control (empty) capsules			Bioactive capsules			Sig.
	n	Mean	SD	n	Mean	SD	
1	11	9,2	8,4	18	15,9	5,6	0,002
2	11	6,8	4,2	18	9,4	3,4	0,041
3	11	4,9	3,2	18	8,7	3,5	0,008
4	11	3,6	4,7	18	9,4	5,2	0,001

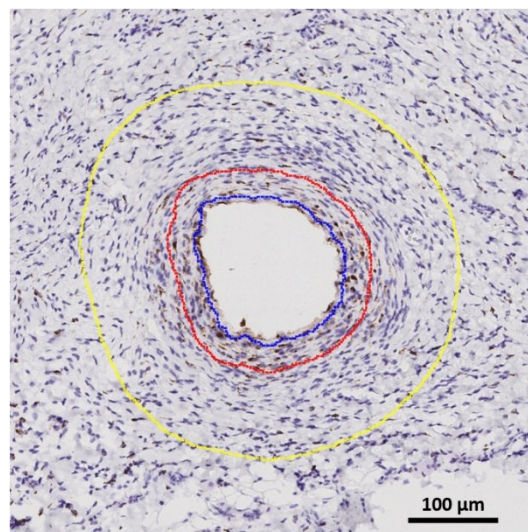
Distribution of infiltration of lymphocytes (a) and macrophages (b)

Blue= capsule delimitation, Red= ROI 1, Yellow= ROI 2

a) CD3 distribution (mostly on ROI2)



b) CD68 distribution (mainly on ROI 1)



Supplementary data References

1. Mourad NI, Perota A, Xhema D et al. Transgenic Expression of Glucagon-Like Peptide-1 (GLP-1) and Activated Muscarinic Receptor (M3R) Significantly Improves Pig Islet Secretory Function. *Cell Transplant* 2017;26:901-911.
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IV.B.2 Commentary

The article presents a study on the semi-automated digital quantification of cellular infiltrates for evaluating transplanted islets encapsulated with bioactive materials. It validated and applied a DIA method for standardized whole-slide immunohistochemistry evaluations, allowing to quantify cell infiltrates around transplanted encapsulated islets, aiming to improve the assessment of graft outcomes in xenotransplantation.

By utilizing digital image analysis to quantify heterogeneous cell infiltrates, the study offers a more precise and objective method for assessing the biocompatibility of encapsulated islet transplants. The validation of a semi-automated DIA quantification demonstrates the reproducibility and reliability, and enhances the credibility and applicability of the study's findings.

In addition, this paper explores the use of bioactive encapsulation materials such as RGD-alginate, decellularized human pancreatic extracellular matrix, mesenchymal stem cells, and an anti-fouling coating. Quantification of the immune reaction against transplanted capsules revealed the presence of immune infiltrates around all capsule types, with lymphocytes (CD3-positive) more prevalent in cell-containing capsules and increased macrophages (CD68-positive) in biomaterial-containing capsules. The methodology showed correlations among different time points and internal coherence, highlighting the advantages of using digital image analysis in assessing infiltrates.

Although biomaterials have the potential to improve islet survival and function, the specific biomaterial combination studied in this paper triggered a notably heightened cellular response in contrast to islets solely encapsulated in alginate.

This suggests that the materials and cellular elements in bioencapsulation have a higher immunogenicity compared to standard encapsulation, potentially compromising clinical success.

Building on these results, the study demonstrates that, although not exempt of limitation, semi-automated DIA quantification is a robust tool to evaluate encapsulated islets xenografts, and emphasizes the importance of addressing biological variability.

V. Discussion

In his *Chronicle of a Death Foretold*, Gabriel García Márquez reveals the fate of his protagonist on the very first page, then meticulously reconstructs the events from multiple perspectives to uncover the truth and understand why Santiago Nasar was destined to die. This thesis adopts a similar investigative approach. The facts – i.e., the significant loss of transplanted islets – are well established, and the physiological and molecular mechanisms underlying cell death have been detailed in the literature. Yet, there remains a pressing need to characterize isolated islets and transplants from a morphological standpoint, continuing the detective work necessary to unravel the causes behind the suboptimal outcomes of islet grafts. My humble contribution to this field has been to adapt advanced technologies and validate methodologies, particularly in microscopy and image analysis, to provide fresh perspectives on this enduring problem – adding another chapter to the chronicle of an islet death foretold.

The methodologies used, and the biological implication of the results will be discussed in two main sections. The first examines the technical aspects, beginning with an overview of the general impact and perspectives of the approaches used, followed by a focused evaluation of each method individually. The second section addresses biological considerations, with particular attention given to strategies to enhance the success of islet transplantation.

Technical discussion

This thesis explored methodological approaches in the context of islet transplantation, from evaluating islets themselves after isolation, to the quantification of the reaction triggered by their grafting.

From a methodological perspective, this thesis achieved the following objectives:

- For islet evaluation, the establishment of a comprehensive and validated methodology for clearing, whole mount immunofluorescence staining, 3D imaging, software reconstruction and analysis of whole islets, as well as the development of qualitative and quantitative analysis of immunolabeled molecules in isolated human islets (Objective 1);
- For graft evaluation, the validation of a semi-automated DIA quantification method applied to whole-slide immunohistochemistry (Objective 3).

General considerations

DIA transforms subjective visual assessments into objective, quantifiable data using standardized algorithms, greatly improving efficiency and scalability in both research and clinical settings – an essential goal for initiatives like BIOCAPAN, especially in research and clinical settings with high sample volumes²⁰⁹⁻²¹².

DIA is anchored in the positivistic paradigm, which values empirical observation, objective measurement, and quantitative analysis as the basis for scientific inquiry. However, it can sometimes oversimplify the inherent

complexity of biological phenomena. Qualitative analysis can enhance research by uncovering patterns, anomalies, and contextual subtleties that quantitative data alone may miss – thereby deepening understanding and generating new hypotheses for future quantitative exploration²¹⁴.

The integration of artificial intelligence (AI) and machine learning (ML) further enhances image analysis (both qualitative and quantitative)²⁰⁹. For example, by enabling automated, nuanced interpretation of IHC and IF data – such as distinguishing α and β cells in live islets with high precision, or segmenting β cells for 3D mapping and subcellular analysis²¹⁵⁻²¹⁷. AI ensures consistency, reduces inter-observer variability, and recognizes subtle data patterns, but requires thorough validation, can struggle with generalizability, and often lacks transparency in its decision-making (most AI software function as a “black box”)²¹⁸⁻²²⁰.

Methodological validation is essential for reliable IHC/IF, as these techniques are still susceptible to analytical variability, both of the technique itself, as well as in the quantification. Validation involves selecting appropriate antibodies, identifying sources of variability (e.g., staining intensity), and establishing standardized protocols and quality controls. Automated stainers and control samples can further improve consistency. While validation increases reliability and regulatory compliance, it is resource-intensive and requires ongoing oversight as protocols evolve^{221,222}.

Key Points for Paper A (3D imaging of isolated islets)

Main achievements:

- Developed and optimized 3D imaging protocol for isolated rat and human islets, including whole-mount immunostaining, clearing, LSM microscopy, and digital image analysis.
- Overcame technical hurdles: antibody penetration, limited fluorophore combinations, secondary antibody cross-reactivity.
- Created open-source, high-resolution 3D image datasets, enabling detailed reconstructions and future studies.
- Showed how hypoxia changes ECM morphology (not quantity), highlighting central necrosis and transitional zones.

Potential applications:

- ECM mapping could be completed by targeting other molecules (example, other collagens, nidogen), as well as integrin expression; for example, $\alpha3\beta1$ (collagen/laminin-binding) or $\alpha5\beta1$ (fibronectin-binding), to evaluate if anchoring dynamics change in isolated islets.
- Characterizing post-isolation morphological changes in intra-islet capillaries and their associated vascular ECM, as β -cell polarization is structurally dependent on interactions with the peri-vascular basement membrane^{44,93}. Correlate residual vascular locations with isolated islets' ECM morphological cues, such as the laminin nodes described in the Paper.
- Evaluating encapsulated islets, by assessing aspects such as capsule uniformity, and the behaviour of biomaterials and cells inside the capsule

(longitudinal changes in MSCs or supplemented ECM). Assess biomaterial effects on the peripheral, healthy regions of islets, and on transitional zones (areas surrounding the necrotic core of islets); i.e., if biomaterials have the capacity to affect the suffering areas, even if they are not in direct contact with it.

- Tracking angiogenesis within vascularized scaffolds, assessing metrics like vessel density, guiding design improvements in bioengineered bioartificial pancreases.

Key challenges on isolated islet's ECM evaluation are:

- The dynamic nature of ECM, which changes with development, and disease processes.
- Variability across species and experimental conditions.
- Technical challenges in preserving and visualizing ECM structures: enzymatic degradation is inherently variable and difficult to standardize, as isolation procedures typically rely on visual criteria to determine when digestion is complete.

Proposed future directions:

- Incorporating conjugated primary antibodies, to reduce steps and protocol duration.
- Complementary technologies (correlative imaging or laser capture microdissection coupled with quantitative proteomics) to enhance results robustness by differentiating biological vs. technical signals.
- Enhanced qualitative analysis, notably by combining DIA with AI.

Key Points for Paper B (2D DIA of graft biocompatibility)

Main achievements:

- Used semi-automated whole-slide DIA on IHC-stained tissue to quantify immune cell infiltration to assess biocompatibility of encapsulated islet grafts.
- Improved robustness via increased sampling, validation, and automation.
- Demonstrated capacity to analyse large quantities of images

Identified limitations:

- 2D methods lack spatial depth, may misinterpret structures.
- Destructive sectioning thus limiting sampling.
- Single-marker detection restricts insight.
- While cellular components (i.e., immune infiltrates) are quantified, the method fails to incorporate the intricate architecture of ECM fibres, hindering a comprehensive view of fibrosis.

Proposed future directions:

- Combine DIA with multiplex IHC/IF to enable the simultaneous analysis of multiple biomarkers, to explore aspects such as macrophage subpopulations (M1/M2) within the same tissue section.
- Expand biocompatibility analysis to include non-cellular fibrotic components. Since islets actively remodel their native ECM, investigate their capacity to remodel peri-transplant fibrosis⁶¹.

- Assess vascular density and function. For example, a study in subcutaneous canine implants demonstrated a strong positive correlation between foreign-body capsule thickness around implanted subcutaneous devices (a similar scenario as with capsules) and vascular density, suggesting that hypoxic conditions within thicker fibrotic capsules drive compensatory angiogenesis²²³.
- Explore 3D imaging (LSFM, nano-CT) to better map biocompatibility: the en bloc extraction of implants with surrounding inflammatory tissue in Paper B's model creates an opportunity for 3D imaging techniques, to comprehensively map fibrosis, inflammatory cell distribution (like infiltration routes and hotspots around the islets graft), and proto-vascularization across the entire implant²²⁴.

Biological considerations

In type 1 diabetes, the medical approach has focused on understanding the causes (to allow for prevention), diagnostic and therapeutical criteria (to treat the right patient at the right time), with glycemia normalization as its cornerstone, determining T1D's morbidity and mortality⁴. From the discovery of insulin, the control of glycemia using insulin has guided treatment^{4,12,20}. In the absence of endogenous production of the hormone, injections of exogenous insulin permit the regulation of blood glucose levels, with varying degrees of success^{4,11,20}.

An alternative to insulin treatment is pancreatic islet transplantation, which takes on the role of endogenous regulator of glycemia. Enormous progress has been made in this approach, both by academia and the private sector. However, even if islet transplantation is applied in the clinic, it is not yet routine practice^{23,24,125}.

There are three main factors conditioning the survival and function of isolated and grafted islets: hypoxia, anoikis and immunity^{133,147 152,153 11,165-168}.

The molecular and physiological aspects of these processes have been extensively explored; however, understanding the morphological changes occurring within islets has remained limited. This thesis advances the field by employing 3D imaging techniques to investigate the spatial distribution of key extracellular matrix molecules – Collagen IV, Fibronectin, Laminin – and E-Cadherin in isolated human islets. These analyses have clarified the organization and distribution of these molecules, as well as their alterations under hypoxic conditions, thereby directly fulfilling the objectives outlined in Objective 2.

These results support the notion that isolated islets exhibit a layered morphology (peripheral-to-core structural organization), serving as a spatial biomarker of cellular health:

- Peripheral zones with intact ECM architecture and consistent E-Cadherin junctions can correlate with viable, functional islet cells
- Transitional areas (elevated CA9/E-Cadherin signal) may represent structurally compromised but salvageable cells
- Central regions exhibiting ECM fragmentation and disrupted cell-adhesion patterns map to hypoxic, necrotic zones

This morphology, that still need to be correlated with viability, can establish architecture as a predictor for survival outcomes, where specific structural features (e.g., ECM distribution, cell-cell contact integrity) directly reflect – and potentially regulate – functional resilience. Furthermore, this structural understanding can guide the development of bioengineering strategies such as bioencapsulation, which put peripheral areas of islets in direct contact with pro-functional and pro-survival biomaterials.

While many alternative biomaterials offer unique benefits, each comes with significant drawbacks-ranging from mechanical instability and immunogenicity to scalability and regulatory hurdles. In a sense, BIOCAPAN was a choice amongst others: it is thus critical that the in vivo evaluation of the project's capsule was based on robust data. DIA ensured this, and allowed for precise quantification of immune infiltrates around grafted bio-encapsulated islets, helping to demonstrate that the reaction triggered by bioactive capsules surpassed that of simple, alginate capsules, implying that the biomaterials and cells used in this bioencapsulation approach are more immunogenic than standard encapsulation (Objective 4).

These findings underscore the need for continued innovation. Promising directions for future research include further investigation of biomaterials, developing strategies to enhance oxygenation, and evaluating alternative sources of insulin-producing cells.

Alternative biomaterials

A variety of biomaterials have been explored for islet encapsulation. As a substitute to alginates, a well-studied alternative is agarose, a seaweed-derived polysaccharide that forms hydrogels capable of preserving islet viability and providing a degree of immunoprotection. However, agarose capsules tend to be mechanically weaker than alginate ones, increasing the risk of rupture during implantation, and they have shown inconsistent results in preventing immune reactions over time^{179,225,226}.

Collagen-based systems, especially those using oligomeric collagen, offer a biophysical environment that closely mimics natural pancreatic tissue and can support islet structure and function. Yet, conventional collagen gels are prone

to rapid contraction, which can compress islets and impair their function, and the process of purifying high-quality collagen is labour-intensive, making it less suitable for large-scale use.^{109,179}.

Synthetic polymers like polyethylene glycol (PEG) and poly(lactic-co-glycolic acid) (PLGA) have also been considered. PEG is attractive for its hydrophilicity and customizable chemistry, allowing for thin, conformal coatings that do not hinder nutrient diffusion. However, PEG lacks natural cell-adhesion motifs and often requires additional functionalization, while its degradation products may cause inflammation^{227,228}. PLGA allows for precise control of degradation rates and can be used for drug delivery, but its acidic byproducts can damage islets and attract immune cells, leading to fibrosis^{229,230}.

Strategies to mitigate post-transplant hypoxia

Incorporating oxygen carriers into capsules, or co-transplanted with islets, can provide temporary oxygen supplementation while graft vascularization develops. These compounds – capable of reversible oxygen binding – address the critical limitation of oxygen's low aqueous solubility (blood carries $\sim 30\times$ more oxygen than water). Major classes include Haemoglobin-based carriers (HBOCs), Perfluorocarbons (PFCs), and natural carriers, such as haemoglobin, hemerythrin, myoglobin²³¹⁻²³⁴.

The BIOCAPAN project initially explored the use of a haemoglobin-based oxygen carrier, but abandoned it due to manufacturing scalability barriers, highlighting the translational gap between experimental designs and clinically viable solutions.

From a vascular perspective, two main strategies can enhance islet transplantation: direct islet revascularization and pre-vascularization of the transplantation site.

A promising approach involves direct revascularization of grafts prior to transplantation, using engineered endothelial cells. A recent study demonstrates that human reprogrammed vascular endothelial cells (R-VECs) can functionally revascularize pancreatic islets both in vitro and in vivo. When co-transplanted subcutaneously with human islets into diabetic mice, R-VECs established anastomoses with the host microcirculation, enabled durable engraftment (with grafts remaining functional for over 20 weeks), and consistently reversed hyperglycaemia²³⁵.

Optimizing transplantation sites presents another alternative to improve graft oxygenation. Current sites, particularly the liver, pose significant challenges – including hypoxic stress, immune exposure, and procedural risks – that contribute to substantial graft loss. Pre-vascularization emerges as a transformative strategy to address these limitations by engineering *de novo* vascular networks in accessible, extrahepatic sites (e.g., subcutaneous or omental spaces) prior to transplantation. For instance, growth factor-releasing scaffolds (e.g., bFGF-loaded agarose rods) implanted 1–2 weeks before transplantation improved glycaemic control by reducing ischemic stress and accelerating graft integration²³⁶. Similarly, catheter-induced pre-vascularized pockets – generated by temporarily placing a vascular access catheter for 14 days – leveraged foreign body reaction-mediated angiogenesis to create a vascularized niche. This "device-less" approach achieved diabetes reversal in mice while requiring significantly fewer islets than conventional transplantation into non-pre-vascularized sites²³⁷. These pre-vascularization approaches highlight the

potential of site optimization to minimize islet requirements and improve clinical feasibility.

Alternative sources of insulin-producing cells

Even if encapsulation can limit stressors and provide better islet transplantation outcomes, there is still a critical limitation: donor scarcity. While approaches like BIOCAPAN aim to enhance transplanted islet survival and function to mitigate these issues, alternative strategies seek to circumvent donor shortages entirely. These include xenotransplantation (e.g., porcine islets), stem cell-derived insulin-producing cells, and bio-engineered pseudo-islets.

Xenotransplantation using porcine islets is supported by the ethical and logistical advantages of pigs as a scalable donor source. Non-porcine xenotransplantation candidates face significant limitations compared to pigs. Non-human primates (e.g., baboons) were initially explored for their evolutionary proximity but were abandoned due to ethical concerns, zoonotic risks, and poor scalability. Rodents (rats/mice) are used preclinically to study immune rejection but lack therapeutic relevance due to insulin structural differences (3 amino acids vs. 1 in pigs). Fish islets (e.g., tilapia) exhibit temperature-sensitive insulin incompatible with human physiology and provoke immune incompatibility. Pigs remain the sole viable xenotransplant source due to insulin similarity, scalability, and clinical precedent²³⁸⁻²⁴⁰.

Limitations of xenotransplantation, other than limited graft survival due to already discussed causes, include the exacerbated immune reaction triggered by inter-species mismatch, pig-carried infections (notably porcine endogenous retroviruses - PERVs), and different dynamics in insulin secretion between

human and porcine islets^{238,240,241}. To palliate this, genetic engineering has significantly advanced porcine islet xenotransplantation, showing enhanced secretion profiles, reduced rejection and prolonged graft survival²⁴²⁻²⁴⁴.

An alternative to *isolated* islet transplantation is the use of Stem Cell-Derived islets (SCIs), which offer a scalable and immune-modulable. Induced pluripotent stem cell (iPSC)-derived islet organoids now achieve 70–80% insulin-positive cells through stepwise differentiation protocols mimicking pancreatic development. These stem-cell-derived pseudo-islets (that will be described later) exhibit glucose-stimulated insulin secretion and calcium flux dynamics comparable to islets²⁴⁵⁻²⁴⁷.

In vivo results include the Vertex Pharmaceuticals' VX-880 trial, which demonstrated 100% insulin independence in type 1 diabetes patients at 1 year using allogeneic iPSC-derived islets and immunosuppression, and ViaCyte's encapsulated pseudo-islets (VC-02), showing sustained C-peptide production but limited clinical efficacy due to poor engraftment^{248,249}.

The main limitations in the use of SCIs and iPCS are their tumorigenic risks, genetic and epigenetic instability, immune rejection (described even with autologous iPSCs), differentiation efficiency and functional maturity (as glucose responsiveness and vascularization are still suboptimal), and scalability and manufacturing challenges²⁵⁰.

Pseudo-islets are engineered cell aggregates designed to replicate the cellular organization and functional properties of native pancreatic islets, while offering the advantage of uniform size. They can be generated from various sources, including reaggregated dissociated primary islet cells from human, rodent, or

porcine origin. Beyond primary cells, pseudo-islets may also be assembled from stem cell–derived cells or immortalized β -cell lines. Additionally, chimeric approaches that combine stem cell–derived cells with dissociated primary islet cells can enhance their maturation and functional capacity²⁵¹⁻²⁵⁵.

One of the most significant advantages of pseudo-islets is their improved insulin secretion capability. Studies have shown that glucose-stimulated insulin secretion is substantially higher in pseudo-islets compared to human islets²⁵⁶. This enhanced function is maintained over extended culture periods, with pseudo-islets showing stable insulin and glucagon content from 6 to 14 days in laboratory settings²⁵¹.

However, the development of pseudo-islets faces several challenges, including viability and functional maturity, clinical-grade scalability, together with limitations due to the cell source used, as discussed previously for SCIs and iPSCs²⁵⁷.

Future outlook

A durable, scalable, and clinically viable islet transplantation solution will only be realized through the deliberate integration of advanced biomaterials, alternative cell sources, optimized implantation sites, and cutting-edge imaging and analysis tools. This thesis underscores that progress in any single area is insufficient; only a holistic, systems-level approach can address the multifactorial challenges of islet graft survival, function, and accessibility for patients with type 1 diabetes.

VI. Conclusions

The research presented here represents a significant advancement in the understanding of pancreatic islet morphology and has the potential for improving islet transplantation outcomes. By developing and refining a methodology that encompasses 3D immunofluorescence, clearing, Light Sheet Fluorescence Microscopy, and digital image analysis, I have described crucial insights into the effects of hypoxia on the extracellular matrix of isolated islets. My findings have shed light on the structural changes of ECM after islet isolation, providing morphological support to the concept of anoikis. Furthermore, I have filled a critical theoretical gap by describing ECM changes in hypoxia-exposed islets, offering valuable insights into post-isolation ECM behaviour. In doing so, I have established criteria for defining healthy islet tissue, redefining the concept of functional mass, which could enable more precise measurements of islet grafts.

Healthy areas in the periphery of islets, present even under hypoxic conditions, back the use of biomaterials that can support ECM attachment, enhance oxygenation, and modulate the immune response to rescue and improve islet transplantation outcomes. Encapsulation, serving as a scaffold for these biomaterials, has shown promise in enhancing islet survival and function in vitro. To study the effect of bioencapsulation in vivo, I have developed and validated a technique for automated quantification of immune infiltrates, enabling precise differentiation between various implant types. Surprisingly, the results indicate that the use of biomaterials, while beneficial for islet survival and function, may have unintended consequences when combined with encapsulation, emphasizing the need for a comprehensive, standardized biocompatibility assessment.

This thesis underscores several key perspectives for future research. 3D microscopy and digital image analysis will remain essential tools for evaluating complex structures like pancreatic islets and understanding phenomena such as biocompatibility. Protocol validation will continue to be paramount in ensuring robust and replicable results, while the incorporation of qualitative 3D analysis will provide deeper insights into our quantitative findings.

Efficiency will be a central focus for methodological progress, with adaptations to materials and protocol optimization. The potential for automation, guided by artificial intelligence, will play a pivotal role in processing large datasets and ensuring unbiased analysis, ultimately advancing our understanding of pancreatic islets, and improving the success of islet transplantation therapies.

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