Fluorescence lifetime metabolic mapping of hypoxia-induced damage in pancreatic pseudo-islets

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Abstract
Pancreatic islet isolation from donor pancreases is an essential step for the transplantation of insulin-secreting β-cells as a therapy to treat type 1 diabetes mellitus. This process however damages islet basement membranes, which can lead to islet dysfunction or death. Posttransplantation, islets are further stressed by a hypoxic environment and immune reactions that cause poor engraftment and graft failure. The current standards to assess islet quality before transplantation are destructive procedures, performed on a small islet population that does not reflect the heterogeneity of large isolated islet batches. In this study, we incorporated...
Fluorescence lifetime imaging microscopy (FLIM) is a powerful optical method that can probe changes in metabolic state in vitro and in vivo in real time, and can serve as diagnostic tools of pathological tissues in situ [12–14]. The time-dimensional characteristic of FLIM combined with multiphoton (MP) microscopy enables the visualization of endogenous nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), and the discrimination between free and protein-bound forms based on their respective lifetimes [15]. Free NADH has a short fluorescence lifetime $\tau_1$ (300–800 ps), while $\tau_2$ of the protein-bound NADH is longer (1000–6500 ps). Similarly, free FAD has a longer fluorescence lifetime $\tau_2$ (2300–2900 ps), and the lifetime of bound FAD $\tau_1$ is shorter (300–455 ps). Based on the respective contribution of $\tau_1 (\alpha_1)$ and the optical oxidative ratio (FAD/FAD + NADH), the metabolic equilibrium between glycolysis and oxidative phosphorylation can be evaluated in living cells. FLIM has been used to differentiate metabolic processes including proliferation, differentiation, metabolic switching in tumors, and apoptosis in various cell types [16–19]. The metabolic machineries of pancreatic $\beta$-cells are designed to sense blood glucose fluctuations and respond accordingly by the secretion of insulin [20]. $\beta$-cells uptake glucose via their transporters of the GLUT family, which is further processed by glycolysis and oxidative phosphorylation, producing NADH and FAD [21]. The increase in ATP leads to the closing of K+ATP channels and is followed by Ca2+ influx, necessary for the exocytosis of insulin granules.

Here, we demonstrate that FLIM can be utilized to monitor the dynamic metabolic changes in $\beta$-cells upon glucose stimulation. By combining the detection of (pre-) hypoxic cellular response and the identification of a profile for glucose-stimulated islets, we introduce FLIM as a tool for the screening of single pancreatic pseudo-islets. Identification and exclusion of damaged and glucose nonresponsive isolated islets prior to transplantation has the potential to improve the clinical outcomes for type 1 diabetic patients receiving an islets transplant.
2 | EXPERIMENTAL SECTION

2.1 | Cell culture and pseudo-islet assembly

The EndoC-βH3 (Univercell Biosolutions, Toulouse, France) cell line was cultured as previously described [22]. We modified the protocol by seeding 2000 cells that aggregated into pseudo-islets using nonadherent 96 well U-bottom plates (Thermo Fisher Scientific, Waltham, Massachusetts). Pseudo-islets were cultured under normoxic conditions (37°C, 5% CO₂, 20% O₂) for a total period of 5 days. Hypoxic conditions were defined as 37°C, 5% CO₂ and 1% O₂. Before being subjected to hypoxia, cells were seeded and cultured for 48 hours under normoxic conditions to allow the assembly of cells into pseudo-islets. Pseudo-islets were in hypoxia for periods ranging from 3 to 48 hours. To track the pseudo-islet size over time, brightfield images were taken every 24 hours using a brightfield microscope (Zeiss, Jena, Germany). Diameters were analyzed using the software ImageJ V1.52p.

2.2 | GSIS assay

Prior to any GSIS assay, pseudo-islets were incubated for 24 hours in starvation medium (Optiβ2, Univercell Biosolutions).

2.2.1 | Standard GSIS

Pseudo-islets were grouped by six per well and washed twice with Krebs buffer (Univercell Biosolutions), supplemented with 1% BSA (Krebs-BSA) (Thermo Fisher Scientific). Pseudo-islets were synchronized for 1 hour in Krebs-BSA and washed twice afterwards. Pseudo-islets were subsequently incubated for 1 hour with Krebs-BSA, Krebs-BSA supplemented with 20 mM glucose (Gibco, Waltham, Massachusetts), and Krebs-BSA for a second time. After each incubation, supernatants were collected and stored at −20°C until further detection of insulin with an ELISA assay (Ultrasensitive Insulin ELISA, Mercodia, Uppsala, Sweden).

2.2.2 | On-FLIM GSIS procedure

Pseudo-islets were loaded into a microfluidic chip as previously described [22]. A low-pressure four-port switching valve (IDEX Health & Science, Oak Harbor, Washington) was used to allow the switch between Krebs-BSA and Krebs-BSA supplemented with 20 mM glucose. Pseudo-islets were incubated for a total of 135 minutes with Krebs-BSA (corresponding to the synchronization phase and 1 hour under Krebs-BSA) before switching to 20 mM glucose for 327 minutes. For nonstimulated GSIS (control), the pseudo-islets were subjected to a total of 459 minutes in Krebs-BSA. The synchronization phase in Krebs-BSA was not measured. Each islet was monitored at the same focus level by acquiring FLIM images every 27 minutes continuously for the duration of the experiments.

2.3 | Immunohistological analyses

Pseudo-islets were washed with PBS (Gibco), embedded in Histogel (Thermo Fisher Scientific), fixed with 4% PFA, and subjected to paraffin-embedding using a Shandon Citadel 1000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Then, 3-μm-thick sections were prepared using the microtome RM2145 (Leica, Wetzlar, Germany). Sections were deparaffinized using xylene and rehydrated by graded ethanol (100%-50%). Then, 1% Triton-X permeabilization and antigen retrieval were performed as described before utilising the following antibodies: anti-insulin: guinea-pig IgG, 1:200 dilution, A0564 (DAKO, Santa Clara, California); anti-vascular endothelial growth factor (VEGF): Rabbit IgG, 1:400 dilution, RB-9031-P (Thermo Fisher Scientific); anti-HIF-1α: rabbit IgG, 1:500 dilution, ab51608 (Abcam, Cambridge, UK) and anti-caspase-3: rabbit IgG, 1:100 dilution, ab13847 (Abcam) [23]. To visualize nuclei, sections were incubated with 4,6-diamidino-2-phenylindol (DAPI) solution with a concentration of 2 μg/mL (Sigma-Aldrich, St. Louis, Missouri) for 10 minutes. Mounting was performed with Molecular Probes Prolong Gold Anti Fade solution (Invitrogen, Carlsbad, California). Immunofluorescence images were obtained using a Zeiss LSM 880 (Zeiss) and analyzed using Zeiss Zen Blue software and ImageJ V1.52p. Staining intensities (VEGF and caspase-3) were evaluated via the mean gray value per pixel within a region of interest (ROI) defined as DAPI+ areas. HIF-1α-stained images were quantified by three independent unbiased observers. Cells were counted as HIF-1α+ when nuclei were exhibiting a double staining with DAPI and HIF-1α. The ratios of HIF-1α+ cells were calculated by dividing the number of HIF-1α+ cells by the number of total DAPI+ cells per pseudo-islets.

2.4 | Multiphoton imaging and FLIM data acquisition

TCSPC-based fluorescence decay measurements were performed with a Zeiss LSM 880 (Zeiss) coupled with a
Ti:Sapphire femtosecond laser (MaiTai HP Spectra Physics, Santa Clara, California) and a two-channel NDD BIG2.0 GaAsP PMT detector (Becker & Hickl GmbH, Berlin, Germany). NADH and FAD autofluorescence was induced with a two-photon excitation at a wavelength of 700 nm and 5% laser power through a ×63/1.4 NA C-plan apochromat objective (Zeiss). Emission light was filtered in the range of 450 to 490 nm for NADH, and 500 to 550 nm for FAD. Total image acquisition time was 161 seconds at a resolution of 512 × 512 pixels (524.8 μs/pixel). Instrument response function was recorded at 900 nm from crystalline urea (Sigma-Aldrich). Normoxic and hypoxic pseudo-islets were transferred either into μ-slides Angiogenesis (ibidi GmbH, Gräfelfing, Germany), or into a microfluidic chip for FLIM measurements as previously described [22]. All FLIM measurements were performed at 37°C using a microscope stage top incubation system (ibidi heating system, ibidi GmbH).

### 2.5 FLIM data analysis

SPICImage (Becker & Hickl GmbH) was used to perform biexponential decay fittings with a 30% threshold of maximum photon count to remove the background. The quality of fit was decided based on a mean χ² value smaller than 1.1 per image. ASCII images for α, τ and χ² were exported for further analysis. Concentric ROI segmentation based on the outline of the islet was performed on each FLIM image using MATLAB R2020a (The MathWorks Inc., Waltham, Massachusetts). The ROIs were equally spaced at 10 μm in order to calculate mean values for cells at similar depths (MATLAB code available upon request).

#### 2.5.1 Optical oxidative ratio calculation

The optical oxidative ratio was defined as the total photon counts of FAD divided by the sum of FAD and NADH photon counts [24]. The calculation of the optical oxidative ratio was performed after the image processing by SPICImage and MATLAB software, which includes suppressing the background.

#### 2.5.2 Lumen ratio calculation

The lumen size was evaluated using an additional threshold of 25% of the total photon count images after background removal by the SPCImage software (MATLAB code available upon request).

### 2.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California). Results are shown throughout the entire manuscript as mean ± standard deviation. Outliers were removed using Grubb’s test with a confidence interval of 0.05. Normality was assessed using Shapiro-Wilk tests. Normal distribution was assumed for low n-number samples (n < 8). All n-numbers, applied tests, and corresponding significances for each result are listed in the figure legends.

### 3 RESULTS AND DISCUSSION

#### 3.1 Human pancreatic β-cells engineered to form glucose-responsive pseudo-islets in vitro

To mimic the insulin-secretory function of endocrine pancreas, we modified a previously described protocol using human pancreatic β-cells, aggregated at 2000 cells per pseudo-islet [22]. After 5 days of culture, pseudo-islets had properly aggregated into clinically relevant spheres with a mean diameter of 101 ± 5.4 μm (Figure 1A), which were similar in size to previous studies demonstrating a relationship between islet size and functionality in vitro and in vivo in terms of survival rate after transplantation [25, 26]. Smaller rat islets (<125 μm) were superior than larger islets (>150 μm) in terms of insulin release in vitro and restoring glycemic control in a diabetic rat model [26].

The main function of pancreatic β-cells is to secrete insulin in response to glucose stimulation. Pseudo-islets were stimulated with 20 mM glucose and showed a significant increase in insulin secretion upon stimulation (0 mM: 0.63 ± 0.1 mU/L insulin/pseudo-islet vs 1.66 ± 0.6 mU/L insulin/pseudo-islets at 20 mM) (Figure 1B). After stimulation, pseudo-islets returned to their basal insulin secretion level. Immunofluorescence staining showed a homogeneous distribution of insulin throughout the pseudo-islets (Figure 1C). Altogether, these data show the dynamic response of human pseudo-islets to glucose, and their potential to mimic the functional unit of the islets of Langerhans that produce insulin.

#### 3.2 FLIM enables the noninvasive monitoring of hypoxia-induced cell death in vitro

Hypoxia leads to β-cell dysfunction, which is a major factor contributing to the overall poor efficiency of the Langerhans islet isolation and transplantation procedures
Severe and prolonged hypoxia (<1% oxygen) induces nonreversible cellular changes resulting in programmed cellular death. To evaluate the impact of severe hypoxia on our pseudo-islet in vitro system, key hypoxic expression markers were evaluated over time, including vascular endothelial growth factor (VEGF), HIF-1α and cleaved caspase-3 (Figure 2).

Adult pancreatic islets continuously secrete VEGF to maintain blood vessel density and proper fenestration [28]. In response to decreased oxygen tension, pancreatic β-cells secrete VEGF as part of an adaptive response to hypoxia [29]. Here, we showed a significant increase in VEGF after 1 hour under hypoxia (Figure 2A).

Our results showed that HIF-1α, which is induced by a decrease in oxygen in the cytoplasm, significantly increased after 6 hours under hypoxia (Figure 2B). The HIF-1α protein, which is stabilized by hypoxic conditions, has a large number of target genes, including HIF-1 and VEGF [30]. Here, we observed a potentially coordinated and significant increase of HIF-1α and VEGF after 1 hour under hypoxic conditions. After 6 hours under hypoxic conditions, HIF-1α expression increased further and reached a plateau, while VEGF expression slowly decreased.

HIF-1α exerts both pro- and anti-apoptotic effects, depending on the severity of hypoxia [31]. Under severe hypoxic conditions, HIF-1α can trigger hypoxia-induced apoptosis, which can be measured by the expression of cleaved caspase-3 [32]. Here, cleaved caspase-3 was significantly upregulated from 12 to 24 hours under hypoxia (Figure 2C). These results suggest that pseudo-islets under hypoxic conditions initiate an adaptive response from 1 to 4 hours, seen by a rapid increase in VEGF. HIF-1α may exert a pro-apoptotic effect via HIF-1α between 4 and 6 hours that leads to the activation of programmed cell death via cleaved caspase-3 between 6 and 12 hours under hypoxia. Identification of dead cells (i.e. after completion of apoptosis) is possible using MP images of the endogenous NADH. The appearance of a hypoxic core, which was depicted by the loss of NADH autofluorescence intensity, can be observed in pseudo-islets at 12 hours under hypoxia (Figure 2D).

FLIM can identify metabolic changes in living cell cultures and in vivo [33]. Here, we were interested in whether FLIM has the fidelity to identify metabolic changes arising from the early adaptive response to hypoxia before the activation of cleaved caspase-3 and following nonreversible cellular changes. Therefore, FLIM images were acquired from the endogenous NADH and FAD autofluorescence of pseudo-islets under normoxia and hypoxia for 3, 6 and 12 hours. Each metabolic profile was characterized by the FLIM parameters \( \tau_1, \tau_2 \) and \( \alpha_1 \) from the respective coenzymes NADH and FAD, as well as the optical oxidative ratio (FAD/FAD + NADH) (Figure 2E-J). NADH \( \tau_1 \) represents the fluorescence lifetime of free NADH, whose major contribution arises from cytosolic NADH in opposition to bound NADH found in the oxidative phosphorylation chain and characterized by \( \tau_2 \) [34]. NADH and FAD lifetimes are highly sensitive to changes within their microenvironment, such as pH, solvent polarity or viscosity [33]. We showed that hypoxia induced an increase in NADH \( \tau_1 \) at 3 and 6 hours, while NADH \( \tau_2 \) was most affected after 3 hours (Figure 2E,F). Hypoxia is known to trigger a switch from aerobic to anaerobic glycolysis, which is a protective strategy against the production of reactive oxygen species (ROS) [35]. It is also the means with which NAD$^+$ is recovered.
FIGURE 2  Hypoxia-induced cellular response in human pseudo-islets is detectable with fluorescence lifetime imaging microscopy (FLIM). Immunofluorescence staining of, A, vascular endothelial growth factor (VEGF), B, hypoxia-inducible factor 1α (HIF-1α) and, C, cleaved capase-3 in pseudo-islets under normoxic and hypoxic conditions and the corresponding quantification (right). n ≥ 3. D, Multiphoton (MP) imaging and quantification of the lumen size of hypoxic pseudo-islets over a time period of 12 hours. n ≥ 10. E-J, Time lapse FLIM analysis of hypoxic pseudo-islets showing, E, nicotinamide adenine dinucleotide (NADH) τ1, F, NADH τ2, G, flavin adenine dinucleotide (FAD) τ1, H, FAD τ2, I, NADH α1 and, J, the optical oxidative ratio based on endogenous fluorescence of FAD/FAD + NADH. n ≥ 10. Scale bars equal 50 μm. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. *P < .05; **P < .01 and ****P < .0001
from NADH, which is required to produce ATP. This process produces a large amount of lactic acid. The following acidification of the cytosol may be responsible for the significant change in NADH $\tau_1$. The mitochondria produce ROS via the complexes I and III of the electron chain transporters, whose production levels increase when oxygen levels drop in a range of 5% to 0.5% [35]. Interestingly, oxidative stress arising from ROS has been shown to increase NADH $\tau_2$, which is observed here after 3 hours under hypoxia (Figure 2F) [36]. The recovery of NADH $\tau_2$ at 6 and 12 hours under hypoxia may reflect the activation of antioxidant pathways [37].

Under hypoxia, FAD $\tau_1$ and $\tau_2$ significantly decreased after 6 and 12 hours (Figure 2G,H). The majority of the endogenous fluorescence from FAD arises from the mitochondria when FAD is in a complex with lipoamide dehydrogenases and the electron transfer flavoproteins, whose contributions represent ~50% and ~25% of the total intensity, respectively [38]. The contribution from the electron change transporter is considered negligible, and the remaining 25% of the FAD intensity is not associated with metabolism. Therefore, change in FAD $\tau_1$ and $\tau_2$ are strongly associated with the mitochondrial microenvironment.

NADH $\alpha_1$, which is the contribution of the free NADH over the total amount of photons collected, significantly decreased in pseudo-islets after 12 hours under hypoxia when compared with the normoxic controls (Figure 2I). This describes a redistribution from free to bound NADH forms to facilitate more efficient ATP production in the mitochondria. The nucleus increases its energy demands, as it prepares the cells for apoptosis [39]. Decrease in NADH $\alpha_1$ has been reported in vivo in murine keratinocytes undergoing apoptosis [19]. These data are corroborated with a significant increase in optical oxidative ratio at 12 hours under hypoxia, implying that the relative amount of mitochondrial FAD (free and bound) increased when compared to NADH (free and bound) (Figure 2J).

We showed that FLIM can detect metabolic changes that are induced by the hypoxic environment in a nondestructive manner in pancreatic $\beta$-cells. Moreover, our data reveals that the NADH lifetimes $\tau_1$ and $\tau_2$ can be used as indicators of the early adaptive hypoxia-induced cellular response, occurring before the appearance of the major peak in HIF-1$\alpha$ or cleaved caspase-3.

### 3.3 Spatial distribution of FLIM outputs allow the detection of early adaptive hypoxia-induced cellular response in pseudo-islets

During the isolation process, pancreatic islets are severed from their vasculature and rely on diffusive properties from the surrounding media for their oxygen and nutrient supply. The diffusion gradient is depending partially on the islet size, which is highly heterogeneous in the native pancreas [40]. In addition, each islet has a different degree of vascularization and composition of $\alpha$-, $\beta$-, $\delta$-, $\gamma$- and PP-cells [41]. FLIM can be used to assess the metabolic state of single cells, which can reveal the heterogeneity within single islets or pseudo-islets. We hypothesize that FLIM can discriminate subregions within one pseudo-islet that differentially react to hypoxic conditions, either by an adaptive response to hypoxia or by initiating a programmed cell death. Therefore, we segmented FLIM acquisitions to create ROIs based on their spatial distribution (see Section 2) within the pseudo-islets as illustrated in Figure 3a. For each ROI representing a 10 $\mu$m increment in depth, NADH and FAD lifetimes $\tau_1$ and $\tau_2$, NADH $\alpha_1$, and optical oxidative ratio were assessed (Figure 3B-G).

Under normoxic conditions, the first ROI (0-10 $\mu$m) is metabolically different than all other ROIs: a significantly higher optical oxidative ratio and lower NADH $\alpha_1$ indicate that cells within the first ROI rely on higher oxidative phosphorylation rates. This is likely due to the direct contact between nutrients/oxygen and $\beta$-cells.

Under hypoxia, FLIM parameters NADH $\tau_1$, FAD $\tau_2$, NADH $\alpha_1$ and optical oxidative ratio were significantly affected by the spatial distribution of the corresponding ROIs. Hypoxia severely impacted FLIM parameters in the central region of the pseudo-islets (30-80 $\mu$m), starting from 3 hours under hypoxia, characterized by a steady increase of NADH $\tau_1$, decrease in FAD $\tau_2$, decrease in NADH $\alpha_1$, and increase in optical oxidative ratio (Figure 3B,E-G). In the pseudo-islet core, hypoxia is most likely <1% oxygen due to the oxygen consumption of the outer cells. Under such conditions, cellular mechanisms concentrate on antioxidant-producing pathways that come at the expense of glycolytic ATP production [37]. After a prolonged time under those severe conditions, the apoptotic cascade is activated, which may be responsible for the further increase in oxidative phosphorylation [42].

In contrast, in the outermost ROI (0-30 $\mu$m), a significant decrease in NADH $\alpha_1$ was observed after 12 hours under hypoxia, while no changes were noted in either NADH $\tau_1$ or optical oxidative ratio. FAD $\tau_1$ and $\tau_2$ decreased after 6 hours only in the first ROI (0-10 $\mu$m). This suggests that the cells in the first ROI may have initiated an adaptive response to hypoxia and are able to maintain their glycolytic rate for a longer period.

NADH $\tau_2$ and FAD $\tau_1$ were not affected by the spatial distribution within the pseudo-islets (Figure 3C,D). While NADH $\tau_2$ oscillated during hypoxia, FAD $\tau_1$ was only affected in the first ROI (0-10 $\mu$m). Interestingly, both
FIGURE 3  Segmentation of fluorescence lifetime imaging microscopy (FLIM) images reveal the heterogeneity of hypoxic pseudo-islets in vitro. A, Schematic illustration of the segmentation of FLIM images creating 10 μm-deep incremented regions of interest (ROIs). B-G, FLIM analysis of hypoxic pseudo-islets over time, and segmented per ROI, showing, B, nicotinamide adenine dinucleotide (NADH) $\tau_1$, C, NADH $\tau_2$, D, flavin adenine dinucleotide (FAD) $\tau_1$, E, FAD $\tau_2$, F, NADH $\alpha_1$ and, G, the optical oxidative ratio based on endogenous fluorescence of FAD/FAD + NADH. n $\geq$ 10. Scale bar equals 50 μm. Two-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons test. §§§: significant difference between ROIs $P < .0001$. *: Significant difference between groups within one ROI. *$P < .05$; **$P < .01$, ***$P < .001$ and ****$P < .0001$
NADH $\tau_2$ and FAD $\tau_1$ are protein-bound lifetimes within the mitochondria, which may reduce their sensitivity to microenvironmental changes. The segmentation of FLIM outputs allowed the discrimination of surviving and apoptotic cells within a single pseudo-islet. As expected, the innermost ROIs are

![FLIM analysis](image)

**FIGURE 4** In situ fluorescence lifetime imaging microscopy (FLIM) probes glucose-responsiveness of normoxic pseudo-islets in vitro. FLIM analysis over time of nonstimulated pseudo-islets and glucose stimulation with 20 mM shows, A,B, nicotinamide adenine dinucleotide (NADH) $\tau_1$, C,D, NADH $\tau_2$, E,F, NADH $\alpha_1$ and, G,H, the optical oxidative ratio based on endogenous fluorescence of flavin adenine dinucleotide (FAD)/FAD + NADH. I, Representative images of NADH $\alpha_1$ over time during glucose stimulation. Corresponding time points are shown with red arrows in F. n = 4. Scale bar equals 50 μm.
strongly affected by hypoxia, as indicated by a change in NADH lifetimes, NADH $\alpha_1$, and oxidative ratio. These changes will likely lead to the nonreversible initiation of apoptosis [19, 42]. The $\beta$-cells located in the outer ROIs were characterized by a lower NADH $\alpha_2$ and stable values for NADH $\tau_1$, FAD $\tau_2$ and optical oxidative ratio. These metabolic changes suggest that the $\beta$-cells were affected by hypoxia by redistributing free to bound NADH. However, the stable values of NADH and FAD lifetimes suggest that these $\beta$-cells have reached a metabolic equilibrium under hypoxia via an adaptive response.

### 3.4 FLIM can noninvasively identify metabolic oscillations of glucose-responsive pseudo-islets

The major prerequisite of transplanted pancreatic islets is the regulation of glucose homeostasis by sensing glucose and secreting appropriate amounts of insulin [1]. Hypoxia decreases aerobic glycolysis, thus reducing the $\beta$-cell capacity to secrete insulin upon glucose stimulation [43]. Depending on the severity of the hypoxia-induced cell damage, the downstream effects of HIF-1$\alpha$ activation in $\beta$-cells can be transient and reversible. Therefore, assessing the metabolic changes due to glucose stimulation is essential to identify functional islets. Here, we characterized the glucose-response of functional pseudo-islets using FLIM. Normoxic pseudo-islets were stimulated with 20 mM glucose as previously described [22]. FLIM images were acquired during the glucose stimulation of single pseudo-islets over time and compared to nonstimulated pseudo-islets (Figure 4).

In the first 60 minutes of analysis without glucose, NADH $\tau_1$ steadily increased overtime, while NADH $\alpha_1$ decreased, which reflects the known metabolic changes due to glucose starvation (Figure 4A,E) [44]. Prolonged starvation resulted in NADH $\tau_1$, FAD $\tau_1$ and FAD $\tau_2$ oscillations, steady decrease in NADH $\tau_2$, and an increase in NADH $\alpha_1$ and optical oxidative ratio (Figures 4A,C,E, G and S1). The absence of glucose diminishes the glycolytic flux and forces mitochondrial adaptation. Carbon sources can be provided, for instance by glutamine, which can fuel the TCA cycle and maintain ATP levels [45]. Additional pathways can be initiated to provide energy, such as the $\beta$-oxidation of fatty acids or the pentose phosphate pathway [37]. The pentose phosphate pathway produces nicotinamide adenine dinucleotide phosphate (NADPH), which may be responsible for the increase of free NADH after 60 minutes, as NADPH endogenous fluorescence is included in the NADH signals [46].

Stimulation of $\beta$-cells with glucose impacted NADH and FAD lifetimes, oscillations of NADH $\alpha_1$ and led to a drop of the oxidative ratio (Figures 4B,D,F,H,I and S1). Glycolytic flux abruptly increases as glucose is transported into the cells, which was seen in our data by the immediate changes in NADH and FAD lifetimes and spiking of NADH $\alpha_1$. Similarly, aerobic glycolysis produces large amounts of NADH, correlating with the decrease in oxidative ratio.

Hypoxia-induced cellular changes can be reversible depending on their severity. For instance, 6 hours under hypoxia altered the mitochondrial lifetimes of NADH and FAD, while NADH $\alpha_1$ and optical oxidative ratio were stable, suggesting a moderate impact of hypoxia. To investigate the glucose-response of pseudo-islets cultured under hypoxia for 6 hours, FLIM metabolic profiles were assessed during stimulation with 20 mM glucose (Figure 5). We detected large fluctuations in NADH $\tau_1$ and NADH $\alpha_1$, which did not resemble the metabolic response of stimulated normoxic pseudo-islets (Figure 5A,C). Nevertheless, the value of NADH $\tau_2$ before glucose stimulation under hypoxia was similar to normoxia (Figure 5B,E). Upon stimulation, NADH $\tau_2$ under hypoxia spiked and plateaued at higher values compared with normoxia (Figure 5B). The optical oxidative ratio of hypoxic pseudo-islets, which was overall higher than in normoxia, dropped upon glucose stimulation similarly to normoxic pseudo-islets (Figure 5D). The profile of FAD lifetimes was similar to normoxia as well (Figure S2). Stimulation of pancreatic $\beta$-cells with glucose and following insulin secretion requires both glycolysis and oxidative phosphorylation [47]. After 6 hours under hypoxia, pseudo-islets were still glucose-responsive, as indicated by NADH $\tau_2$, FAD $\tau_1$, FAD $\tau_2$ and the optical oxidative ratio. The stimulation lead to an increase in the total NADH compared to FAD, which most likely is produced by an increase in glycolysis. However, the high value of NADH $\tau_2$ indicated an important change within the mitochondrial microenvironment. This change may have arisen from the increasing levels of ROS due to hypoxia and the loss of the metabolic rescue mechanisms of a functional cell, which further increased as the TCA cycle and oxidative phosphorylation are enforced during insulin secretion. Prolonged hypoxia is known to lead to $\beta$-cell dysfunction, characterized, in part, by a loss of glucose-response [43]. Here, we showed that pseudo-islets after 6 hours under hypoxia still respond to glucose based on FLIM, which was validated by a standard GSIS assay (Figure S3). However, important changes in the metabolic response of the coenzymes NADH and FAD were detected suggesting cellular
stress. Our data demonstrates how FLIM can detect metabolic impairment in hypoxic pseudo-islets in comparison with standard methods such as GSIS assays.

4 | CONCLUSION

Developing noninvasive tools to assess the quality of isolated pancreatic islets prior implantation has the potential to improve the outcomes of Langerhans islet transplantation to treat type 1 diabetes. In this study, we used an in vitro system mimicking the insulin-producing and glucose-responsive characteristics of the islets of Langerhans and combined it with FLIM to assess pseudo-islet quality and their glucose-response under standard and hypoxic conditions. FLIM identified and discriminated between minor and severe hypoxia-induced cellular damages in β-cells. FLIM further allowed the marker-free and noninvasive detection of a metabolic response due to glucose stimulation in functional living pseudo-islets, which can be used to assess the severity of hypoxia-induced damages during insulin secretion.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

Aline Zbinden, Daniel A. Carvajal Berrio, Max Urbanczyk, Shannon L. Layland and Katja Schenke-Layland: Designed the experiments and wrote the manuscript. Aline Zbinden, Daniel A. Carvajal Berrio, Max Urbanczyk, Mariella Bosch, Sandro Fliri, Chuan-en Lu and Abiramy Jeyagaran: Performed experiments, collected and analyzed data. Peter Loskill and Garry P. Duffy: Gave conceptual advice.

**DATA AVAILABILITY STATEMENT**

The main data supporting the findings of this study are available within the article and its supplementary information. The raw data generated in this study are available from the corresponding author upon reasonable request.

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