Islet Assessment for Transplantation

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Abstract

Purpose of review—There is a critical need for meaningful viability and potency assays that characterize islet preparations for release prior to clinical islet cell transplantation (ICT). Development, testing, and validation of such assays have been the subject of intense investigation for the past decade. These efforts are reviewed, highlighting the most recent results while focusing on the most promising assays.

Recent Findings—Assays based on membrane integrity do not reflect true viability when applied to either intact islets or dispersed islet cells. Assays requiring disaggregation of intact islets into individual cells for assessment introduce additional problems of cell damage and loss. Assays evaluating mitochondrial function, specifically mitochondrial membrane potential, bioenergetic status, and cellular oxygen consumption rate (OCR), especially when conducted with intact islets, appear most promising in evaluating their quality prior to ICT. Prospective, quantitative assays based on measurements of OCR with intact islets have been developed, validated and their results correlated with transplant outcomes in the diabetic nude mouse bioassay.

Conclusion—More sensitive and reliable islet viability and potency tests have been recently developed and tested. Those evaluating mitochondrial function are most promising, correlate with transplant outcomes in mice, and are currently being evaluated in the clinical setting.

Keywords

Oxygen consumption rate; viability; potency; release criteria; marginal mass; purity

Introduction

Islet cell transplantation (ICT) is emerging as a promising approach for the treatment of selected patients with type 1 diabetes mellitus [1–8]. ICT is currently in a phase III multicenter clinical trial [9] to determine if it will become the standard of care. There is an urgent need for reliable assays that characterize the islet product for release prior to transplantation. Development of such assays is mandated by the Federal Drug

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Administration (FDA) and has been the subject of investigation for the past decade. To provide a framework for understanding the current state of the art, this article first reviews the numerous approaches that have been proposed and tested, than focuses on the most recent results and promising assays for clinical ICT.

Current specifications for lot release prior to clinical islet transplantation

The FDA mandates that for any cellular and tissue-based product, the manufacturer must be able to demonstrate that it can be safely and reproducibly manufactured [10]. This is generally done by characterizing the product and establishing specifications for product release. Lot release specifications for islet products include demonstration of **safety** (i.e., sterility, mycoplasma, pyrogenicity/endotoxin, and freedom from adventitious agents) and assessments of several key product characteristics that include, but are not limited to, **identity, purity, viability, and potency**. The current specifications for release of islet products within the United States are summarized in Table 1. These specifications function to exclude preparations that are contaminated, highly impure, grossly damaged, or do not contain significant numbers of islets. It is currently accepted that these specifications provide reasonable estimates of islet safety, identity, and purity, but do not provide meaningful measures of viability or potency of the preparation [12–15]. Therefore the establishment and validation of useful islet viability and potency tests is urgently needed. The sections that follow focus on existing and emerging islet viability and potency tests, including those that are based on measurements of oxygen consumption rate (OCR), which appear to be the most promising.

Limitations of the tests currently used for islet lot release prior to clinical transplantation

Many of the methods currently used to assess islet preparations were developed nearly 20 years ago [11]. The advantages and limitations of tests currently used for islet product release, which were recently discussed in detail [16], are summarized in Table 2A.

Sampling from an islet suspension

An important issue in characterizing islet preparations, relevant to all assays, is sampling. Obtaining a sample from a suspension that is representative of the whole preparation is critical [11,16]. Nevertheless, maintaining a homogeneous islet suspension while sampling is challenging, as islets settle rapidly. Differences in size and density of aggregates can lead to significant differences in settling velocity and exacerbate this problem. The extent of the systematic error introduced during this type of sampling is unknown. To minimize random error during sampling, multiple replicates should be collected. However, the additional time and analysis required for collection of multiple samples, the concern about introducing contamination and removing islets that otherwise could be transplanted to benefit the recipient all pose limitations. Currently, only duplicate samples of 100 µL (derived from a 100-mL islet suspension) are collected and counted, an amount that may not represent the entirety of the preparation.

Measurements of the amount of islet tissue

Quantification of the total amount of islets in an islet preparation is critical because it ultimately determines the islet dose that is transplanted. The method most widely used currently is manual, visual counting of islet equivalents (IE) under a light microscope following dithizone (DTZ) staining to determine the total volume of islet tissue and its purity. This method has advantages and limitations (*See* Table 2A) that are described in detail elsewhere [11,16]. Methods for estimating the total number of cells or volume of

tissue in a preparation include measurements of intracellular deoxyribonucleic acid (DNA), cellular nuclei counts, large particle flow cytometry [17], and packed tissue volume. These methods do not provide islet- or β-cell specific information, so they require an independent estimate of the purity (fractional volume of islet tissue or β-cells). Such estimates can be obtained using a variety of methods [16], including morphological analysis with electron and/or light microscopy [18*], immunohistochemistry with laser scanning confocal microscopy, or laser scanning cytometry [19]. Recent studies indicate that conventional DTZ staining overestimates purity by 20–30% as compared to measurements with electron and light microscopy [20] and total number of IE by as much as 90% [18*] as compared to recently-developed, more accurate methods that combine nuclei counting with light microscopy.

Measurements of viability

The current viability assay used for clinical islet product release is based on assessing membrane integrity with fluorescein diacetate/propidium iodide (FDA/PI) (*See* Table 1). Characteristics and limitations of this assay are outlined in Table 2A and detailed elsewhere [11,16]. A major limitation of this assay is that it does not reflect true viability because it may not account for cells undergoing early apoptosis or dying by other modes of cell death, during which cells have not yet developed damage to their cell membrane. Furthermore, it does not correlate with the diabetic nude mouse bioassay (NMB) or clinical ICT outcomes.

Measurement of islet function (potency)

The β-cells within the islets have a specific, dedicated function, the dynamic release of insulin in response to a glucose stimulus. Therefore, one would expect that assessment of islet function should be straightforward, particularly if the insulin secretion rate of a preparation can be easily measured. Measurements of basal and glucose-stimulated insulin secretion (GSIS) could theoretically be used to provide a meaningful measure of the amount of viable and functional IE (or β-cells) in a preparation if one assumes that insulin secretion from an islet population is relatively constant when normalized on a per viable IE or per viable β-cell basis. Unfortunately, GSIS does not correlate with clinical transplant outcomes [14–15,21]. There are several likely reasons for this persistent finding. Stresses associated with pancreas preservation, islet isolation, and islet purification may lead to extensive degranulation and/or insulin leakage (from dead or dying islet cells). Conceivably, islets that do not secrete insulin at expected rates, but are nonetheless viable, may recover when transplanted into the recipient. In other words, low GSIS may not necessarily imply irreversibly impaired secretory function and, thus, GSIS does not correlate with clinical outcomes. Furthermore, insulin leakage from dead or damaged cells may be difficult to account for (because this contribution to the total insulin cannot be reliably estimated), may interfere with proper calculation of insulin secretion rate and stimulation index, and may complicate the interpretation of the results of the GSIS assay. Insulin secretion is also particularly sensitive to the local partial pressure of oxygen (pO_2) and assay procedures usually do not account for that [22].

The mouse bioassay as an *in vivo* islet potency test and a surrogate islet potency validation tool

According to the FDA [10], a suitable potency assay is one that demonstrates that the clinical product possesses the specific ability to provide the desired clinical effect. The diabetes reversal (DR) resulting from islets engrafted under the kidney capsule of immunodeficient nude mice correlates with clinical transplant outcomes and is currently accepted as the gold standard for testing islet potency [14–15,23–24]. However, the time (days to weeks) required for this assay to produce interpretable results renders it

retrospective. Nonetheless, correlation of real-time, *in vitro* tests with transplant outcomes in the NMB can establish other such tests as acceptable surrogate potency tests. Several recently-proposed islet potency tests are therefore being judged based on their ability to predict DR in the NMB [19,25–36*,**]. Even though the NMB is the premiere method available to researchers for the assessment of islet potency, it suffers from numerous limitations (*See* Table 2B). These limitations include the length of time required to obtain a meaningful outcome, the complexity of the surgical procedure, the difficulties in maintaining diabetic mice and timing diabetes induction with the unpredictable availability of human islets, the negative impact of impurities on outcome [15,37–39], the transplant site (kidney capsule), which may be more prone to the presence of impurities and/or dead tissue than the clinical transplant site (the liver), and the inability to account for immune rejection or the effects of immunosuppressive drugs that are present in the clinical setting. There have been recent attempts [40–41] to provide other *in vivo* islet potency tests that are alternatives to the diabetic NMB. These alternatives can potentially overcome some, but not all of the limitations of the NMB.

Desired characteristics of islet potency tests

The assays under consideration for use as potency tests for islet characterization prior to clinical transplantation should be reliable, cost-effective, operator-independent, reproducible, and transferable to other labs, work with relatively small (yet representative) islet numbers (100–500 IE), not require islet handpicking (which may bias the results), and should be able to provide real-time results (i.e., completed within hours). Given the heterogeneity of islet preparations and the intrinsic difficulties in characterizing them, assays that possess all of the desired characteristics may be very difficult to develop. This difficulty is reflected in the fact that, despite the intense effort dedicated to develop, implement, and validate a number of assays over the past decade, consensus behind any single assay has not yet been reached. Key viability and potency assays under consideration for the assessment of clinical islet preparations are described next.

Islet viability and potency tests under consideration

Table 2C summarizes some of the more recently explored assays used in islet quality assessment, highlighting their key strengths and identified weaknesses. Despite the landscape of flavors available to researchers, many of these assays are most valuable when used in the study of individual cells rather than cell aggregates.

Islets are three-dimensional, multi-cellular aggregates composed of several different cell types, including the β-, α-, δ- and PP-cells. Most assays used to assess cellular viability, apoptosis, or mitochondrial health, have been designed for suspensions or cultures of individual cells, not aggregates. Consequently, the development of techniques to study the quality of an islet preparation provides unique challenges. Because the diameter of an islet equivalent is 150 μm, it is necessary to consider mass transport limitations, particularly if an assay relies on the availability of molecular oxygen. The relatively large size of the islet makes fluorescence microscopy difficult, subjecting any such analysis to background signal and operator bias that is simply unique to the study of intact multi-cellular clusters. To circumvent some of the islet shape and size limitations, techniques have been developed to break apart the islets. Digestion with serine proteases and mechanical agitation may be used to dissociate islets into suspensions of their constituent cells, but these techniques result in significant damage to the cells and possibly death by anoikis [43–44], leading to the loss of as much as 50% of the original cell populations [45–47]. To minimize the problems associated with islet disaggregation, gentler formulations have been created and used [19]. Yet, it is unclear whether the negative effects of dissociating individual islet cells from one

another can be fully minimized. Furthermore, islet preparations have varying amounts of impurities, which complicates the use of any technique designed with the expectation that the studied tissue is comprised entirely of islets. Differentiating the non-endocrine tissue from the islets poses additional difficulties.

Cell Membrane integrity tests

These assays interrogate the integrity of the cellular plasma membrane and rely on differential staining using newer combinations of both cell membrane permeable and impermeable dyes [12,16], but have been unable to fully obviate the problems encountered with the current viability stains used prior to product release (i.e., FDA/PI). In fact, some of these proposed stains introduce new issues, such as islet toxicity [16]. 7-aminoactinomycin D (7-AAD, a membrane impermeable dye) has been used on disaggregated cells in combination with flow cytometry (FACS) to enable quantification of the fraction of cells that are viable by membrane integrity, but the method nonetheless requires the undesirable dissociation of the intact islets [16]. An alternative approach relies on sequential staining of membrane compromised cells within intact islets using 7-AAD. After initially staining with 7-AAD, the nuclei of the entire preparation are released from intact islets using a detergent and subsequently counted by hemacytometer or FACS [48–49]. The initial count (of non-viable cells) is divided by the second count (of total nuclei) to present a ratio equivalent to fractional viability (FV). This technique bypasses the limitations associated with islet disaggregation of multi-cellular spheroids, such as islets; however, as a membrane integrity test, it only accounts for dead cells with compromised cell membranes [16,49].

Other cell death and mitochondrial assays

Several assays attempt to characterize the degree of apoptosis within islet preparations [29*, 31**]. These assays may depend on the timing of the measurement as it relates to the onset of apoptosis. The magnitude and timing of the responses may also vary between cell types and the unique nature, intensity, and duration of encountered stresses [16]. Importantly, these cell death markers may not be reliable indicators of irreversible damage. Even though mechanistic information regarding the cell death process can be obtained, individual assays may not capture all dying cells and still suffer from limitations that are related to islet size and its three-dimensional structure. A recent report describes a method to study several apoptosis and cell death-related markers (including VADFMK, Annexin V, and Fura Red) simultaneously using FACS and shows that this sort of multi-parametric analysis may more reliably characterize the quality of an islet sample [29*]. Another paper [31**] describes an elegant approach to combine fluorescence imaging of mitochondrial membrane potential (MMP) and Ca^{2+} leakage with measurements of insulin secretion, determined by enzyme-linked immunosorbent assay (ELISA). The system involved perfusing a microfluidic chip containing intact islets. The future of islet quality assessment may continue to leverage these types of multimodal techniques in the attempt to map a quality "fingerprint" of islet preparations prior to their consideration for transplantation.

Assays have also been developed to probe the state of mitochondrial health, which span a range of relevant indicators, through assessing the ability of a cell to reduce tetrazolium salts [16,33], to replenish ATP [27*,28,30*,42**], or to maintain MMP [16,19,31**]. Tetrazolium assays like MTT have fallen slightly out of favor because many variables or conditions, not limited to mitochondrial activity, can affect the ability of a preparation to reduce tetrazolium salts [16]. In contrast, tests that measure the relative abundance of high energy phosphates (or the ADP/ATP ratio) have reportedly shown promise in predicting ICT outcome in mice [27*–28]. However, the ADP/ATP ratio must be interpreted with caution, because the concentrations of these metabolites fluctuate rapidly with changing conditions. Furthermore, as recently pointed out [42**], the ADP/ATP ratio does not reflect the true

viability of an islet preparation and unlike the ATP/DNA ratio fails to account for non-viable cells containing no ADP or ATP. Additionally, even though ATP and ADP measurements are simple, inexpensive, and quick to obtain, islet ADP measurements based on luminescence may be unreliable as they have frequently provided negative concentration estimates [42**]. MMP dyes are used as surrogate measures of mitochondrial health, in that they preferentially accumulate in healthy and polarized mitochondria. Both laser scanning cytometry [19] and the microfluidic system described earlier [31**] have been used to correlate MMP with the quality of preparations composed of dissociated and intact islets, respectively.

Oxygen Consumption Rate (OCR)

Measurements based on OCR, which is related to mitochondrial function, have been extensively used to assess the viability and health of cells in a variety of fields [50–55], including islets [26,32–34**,56,57**,58] and β-cell lines in tissue engineered constructs [59–61]. Several groups have recently focused their efforts on characterizing islet viability and potency using OCR measurements and in some cases correlating these measurements with outcomes in the NMB [26,32,34**,57**]. Reports on islet respiratory activity include measurements based on scanning electrochemical microscopy [27*] and oxygen sensitive phosphorescence lifetime or fluorescence intensity in a variety of configurations [26,32–34**,56,57**,58]. The instrumentation and methodologies employed along with the strengths and limitations of each approach are outlined in Table 3. The approach for indirectly measuring OCR using fluorescence intensity in a multi-well plate oxygen biosensor system (OBS) has the distinct advantage of being high-throughput and convenient but in its current form suffers from several major limitations that prohibit its reliable use [16,26]. Recent efforts to bypass some of the inherent limitations of the OBS [62] may enable more reliable and effective use of this method in islet potency assessment.

Recently published data obtained with the most basic approach, using optical pO_2 sensors in stirred microchambers [33], demonstrate that transplanted OCR (OCR_{TX}, a measure of the amount of viable tissue) and OCR/DNA (a measure of viability) are sufficient when used in combination to predict outcomes in diabetic mice transplanted with rat [56–57**], porcine [33], and human [26,32,34**,58] islets. These studies suggest that information on the functional capacity of the islets or β-cells is not necessary for predicting transplantation outcomes in mice. In fact, the most recently reported study with rat islets transplanted in immunosuppressed diabetic mice [57**] clearly demonstrated this relationship between OCR_{TX} and OCR/DNA of the transplanted islets and diabetes reversal in mice. When the results of these transplantations were plotted such that the ordinate was OCR_{TX} and the abscissa was OCR/DNA of the transplanted islet sample, the data segregated into three regions: (1) an upper and right-most portion, where diabetes was reversed in all animals, (2) a lower left, where diabetes was not reversed in any animals, and (3) a narrow band in the middle in which both outcomes were represented. In this study, sensitivity and specificity analyses on OCR_{TX} and OCR/DNA exhibited values of 93% and 94%, respectively, in predicting diabetes reversal. Importantly, the marginal mass for DR was not fixed [57**] but rather depended on OCR/DNA, and increased from 2,800 to over 100,000 IE per kilogram recipient body weight (KgBW) as OCR/DNA decreased. These findings are consistent with reports that neither OCR_{TX} nor OCR/DNA, when used individually, correlated with transplant outcomes in mice [15,63].

Correlation of transplantation outcomes with rat islets was substantially better than that obtained with human islet preparations [32]. There are several likely explanations for this finding, which include: (a) the absence of non-islet tissue in rat preparations, (b) the large fraction of nonviable tissue at low OCR/DNA, and (c) the large number of human islets, in

contrast with the small number of rat islets, required to reverse diabetes in mice. The predicted probabilities of DR with rat islet transplants were sharply defined with a large domain at 100% cure, whereas the analogous plot for human islet transplants [32] had angled contours of roughly constant slope with virtually no domain of 100% cure, although such a domain might have been attainable if there had been preparations of higher OCR/DNA. The absence of data in the high OCR/DNA range was a limitation of the study with human islets [32].

Data obtained with a porcine-to-non-human primate (xenogeneic) model suggest that sustained insulin independence is dependent on both OCR_{TX} per KgBW and OCR/DNA (unpublished observations). Interestingly, initial data obtained with pure and impure clinical autologous and single-donor, allogeneic islet transplants suggest that in these cases (especially islet auto-transplants), the OCR dose normalized per KgBW alone may sufficiently correlate with clinical outcomes (unpublished observations).

Of particular interest are attempts to extract information on islet potency based on glucose-stimulated OCR, which may be more representative of β-cells and their functional capacity [26–27*,34**,56,58]. This index has been represented either as a ratio of the measured OCR in the presence of high glucose divided by the OCR in low glucose (OCR_{hglc}/OCR_{lglc}) or simply the difference in measured OCR in the presence of high and low glucose (ΔOCR_{glc}) [34**,56,58]. Publications detailing these procedures report reasonable correlations with the NMB and suggest that there may be an advantage in using these indices for clinical islet potency assessment. It remains to be seen if the challenges associated with widespread implementation and inherent limitations of these complicated methodologies [16] can be overcome and whether the promising results attained with research models will translate into the clinical setting. Work currently under way with clinical auto- and allo- and pre-clinical xeno- transplant models is expected to provide further insight into these issues and help identify and establish islet potency tests that are truly predictive of transplant outcomes.

Conclusion

The islet product release criteria that screen preparations before clinical allogeneic ICT are currently unable to predict post-transplant success from failure. More sensitive and reliable islet viability and potency tests have been recently developed and tested. Those assessing mitochondrial function, particularly those that measure the OCR of an islet preparation, appear to be the most promising and correlate with transplant outcomes in the NMB. These tests are currently being evaluated in the clinical setting and preliminary results are encouraging. Assays that characterize cell composition and molecular profiles may be useful in further defining the islet product and may provide useful information on islet immunogenicity and pro-inflammatory potential. The recent clinical success in reversing diabetes with single-donor, allogeneic transplants, will further enhance our ability to define potency tests and islet characteristics that are predictive of transplant outcome.

Abbreviations

ICT	Islet cell transplantation
OCR	Oxygen consumption rate
FDA	Federal Drug Administration
EU	Endotoxin unit
IE	Islet equivalent(s)

DTZ	Dithizone
FDA/PI	Fluorescein diacetate/propidium iodide
NMB	Nude mouse bioassay
DNA	Deoxyribonucleic acid
GSIS	Glucose-stimulated insulin secretion
pO_2	Partial oxygen partial pressure
DR	Diabetes reversal
SLM	Standard light microscopy
FM	Fluorescence microscopy
C	Calcein AM
EH	Ethidium homodimer
EB	Ethidium bromide
SYTO®	Green membrane permeable fluorescent dye
AO	Acridine orange
FACS	Fluorescent-activated cell sorting (or flow cytometry)
7-AAD	7-aminoactinomycin D
VADFMK	Membrane permeable caspase ligand (inhibitor)
PS	Phosphatidylserine
MTT	Tetrazolium salt, 3-(4;5-dimethylthiazol-2-yl)-2;5-diphenyl tetrazolium bromide
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
MMP	Mitochondrial membrane potential
FV	Fractional viability
ELISA	Enzyme-linked immunosorbent assay
ΔOCR_{glc}	Defined as the measured increment in OCR when stimulated by glucose
OCR_{TX}	Transplanted OCR, which represents viable islet dose
OCR/DNA	Measure of OCR normalized to DNA represents the FV of cellular/ islet preparation
OCR_{GS}	Glucose-stimulated OCR
OCR_{hglc}/OCR_{lglc}	Defined as the Stimulation Index a ratio of OCR measured at high glucose concentrations (16.7 or 33.3 mM) to OCR measured at high glucose concentrations (2.8 or 5.6 mM)
$\Delta OCR_{glc}/DNA$	Defined as the OCR Index a ratio of the estimated ΔOCR_{glc} normalized to DNA
OBS	BD Biosciences Oxygen Biosensor System®
KgBW	Kilogram body weight (of transplant recipient)

Acknowledgments

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TABLE 1

Product release criteria for clinical islet preparation

TYPE OF TEST	PRODUCT TEST	SPECIFICATION	TYPE OF SAMPLE
Safety	Endotoxin	< 5 EU/kg	Supernatant of islet suspension in transplant media
	Gram stain	No organisms detected within limits of assay	
Identity	Islet count (IE/kg)	5,000–20,000 (1st transplant)	Islets in transplant media
		3,000–20,000 (Re-transplants)	
	Purity	≥ 30%	
Viability	Dye exclusion (FDA/PI)	≥ 70%	Islets after overnight culture and in transplant media
Potency	Glucose stimulated insulin release (ELISA)	Stimulation Index >1	Islets after overnight culture

EU = Endotoxin unit

IE = Islet equivalent, defined as a volume of islet tissue equal to that of a sphere having a 150 µm diameter [11]

DTZ = Dithizone

FDA/PI = Fluorescein diacetate/propidium iodide

TABLE 2

TABLE 2A

Strengths and limitations of assays currently used prior to islet product release for clinical transplantation

ASSAY	STRENGTHS	LIMITATIONS
Islet count (IE)	Relatively easy to perform Counts Experienced islet isolation centers have standardized procedures	Visual assessment of 3D islet in 2D planes contributes to error Sample may not be representative of whole preparation Presence of contaminant tissue (e.g., exocrine cells, ganglia, etc) may complicate counts
Purity (DTZ)	Stain differentiates between exocrine and islet tissue Relative ease of use Rapid assessment	Visual assessment of 3D islet in 2D planes contributes to error Provides no information regarding viability of preparation
Cell membrane integrity (FDA/PI)	Relative ease of use Can be performed prospectively Fractional viability can be estimated by dye exclusion	Visual assessment of 3D islet in 2D planes contributes to error Impossible to identify irreversibly damaged cells whose plasma membranes have not yet been permeabilized FDA may be additionally cleaved by lipases or esterases from non-endocrine tissue, over-estimating the true islet viability Visual counting is operator dependent Background fluorescence (with certain combinations or high concentrations of dyes) can obscure approximations Counterstain may not provide enough contrast Dyes rely on diffusion to penetrate into islet core Lack of correlation with mitochondrial function assays, NMB and clinical outcomes Does not discriminate endocrine (islet) from exocrine (contaminant) tissue
Glucose-stimulated insulin secretion (GSIS)	May provide information regarding potency of islet preparation	Unable to predict true islet potency or transplant outcome Islets may not be as responsive to glucose stimulus *in vitro* but may still reverse diabetes *in vivo* Difficult to account for degranulation of β cells following glucose stimulus or "leaky" cells with damaged plasma membranes

TABLE 2B

Strengths and limitations of the diabetic nude mouse bioassay

ASSAY	STRENGTHS	LIMITATIONS
Nude mouse bioassay (NMB)	Most reliable *in vivo* assessment of islet potency Results correlate with clinical outcome	Assay can only be used retrospectively (days to weeks for outcomes) Impure preparations may yield false negative transplant outcomes The severity and duration of the diabetic state of the mouse affects the predictive outcome of the assay Islets are transplanted into the kidney capsule, not into the hepatic portal system (thereby not fully representing the current clinical protocol) Mice are susceptible to developing other conditions (e.g., infection) that can also affect outcome Does not account for immunologic rejection or the effect of immunosuppressive agents on islets The assay carries several practical challenges (e.g., induction of diabetes needs to be timed with islet isolation)

TABLE 2C

Advantages and disadvantages of assays being under consideration for clinical islet quality assessment

ASSAY	REFERENCES	ADVANTAGES	DISADVANTAGES
Membrane integrity tests			
SLM/FM C/EH SYTO®/EB AO/PI	[12,15,16]	Similar advantages as FDA/PI (See Table 2A) Some stains may exhibit greater sensitivity in detecting islet cell membrane damage	Similar disadvantages as FDA/PI (See Table 2A) Certain dyes are chemically unstable or form crystals which can manifest as visual artifacts Some dyes can exhibit islet toxicity (fragmentation, e.g., C)
FACS 7-AAD† Topro3†	[16,19,29]	Minimizes diffusion limitations More quantitative Minimizes operator dependence Allows possibility of β-cell specificity Membrane integrity can be approximated using the 7-AAD sequential staining procedure	Requires dissociation (except sequential staining with 7-AAD) of islet aggregates, resulting in irreversible cell damage and loss (i.e., anoikis) Relative subjectivity in gating cellular subpopulations in FACS Requires expensive equipment and training
Other cell death and mitochondrial assays			
Caspase activation (VADFMK†)	[25,29]	Detects early apoptotic events Rapid measurement	Provides "snapshot" of early apoptotic events, but may not detect late apoptotic or necrotic cells May not account for caspase independent mechanisms of cell death May require dissociation of islets
PS Externalization (Annexin V†) **DNA Fragmentation** (TUNEL†) **Ca^{2+} Leakage**(Fura Red†)	[25,29,31]	May detect both apoptosis and necrosis	Difficult to use prospectively, because the assay may require histological staining and subsequent analysis (i.e., Annexin V) May require dissociation of islets
Reduction potential **Tetrazolium salts**	[16,33]	Detects reducing capacity of islets Relative ease of use Inexpensive Useful in comparing effects of single variables on the oxidative state of a preparation Can be performed on intact islets	Reduction of salts involves complex reactions and may reflect local pO$_2$ changes or differences in the compositions of cell culture media Accumulation of insoluble byproduct of reduction reaction (in MTT assay) is toxic to assayed cell preparation
Bioenergetic status ADP/ATP ATP/DNA ATP/protein ATP/IE	[27–28,30,42]	Relative ease of use Inexpensive Low islet requirement (~100 IE) ATP and ADP play a particularly critical role in insulin secretion (ie, islet function) Can be performed on intact islets	ATP concentrations fluctuate rapidly (short half-life) and are sensitive to transient changes in local conditions (i.e., glucose levels, pO$_2$, pH) Islets are difficult to assay because of differences between environmental conditions experienced by cells located in the core versus the periphery ADP/ATP measurements do not account for non-viable cells ADP measurements by luminescence assay may be unreliable
Mitochondrial membrane potential JC-1 TMRE† Rh123	[16,19,31]	Detects loss of mitochondrial polarization, which occurs during early apoptosis and during necrosis	Difficult to quantify absolute changes in MMP May require dissociation of islets

IE = Islet equivalent, defined as a volume of islet tissue equal to that of a sphere having a 150 µm diameter [11]

DTZ = Dithizone

FDA/PI = Fluorescein diacetate/propidium iodide

NMB = Nude mouse bioassay

Abbreviations and Legend

SLM = Standard light microscopy

FM = Fluorescence microscopy

C = Calcein AM, green membrane permeable fluorescent dye

EH/EB= Ethidium homodimer or ethidium bromide, red-orange membrane impermeable fluorescent dye

FDA = Fluorescein diacetate, membrane permeable dye that fluoresces green after cleavage by non-specific esterases

PI = Propidium iodide, red membrane impermeable fluorescent dye

SYTO® = Green membrane permeable fluorescent dye

AO = Acridine orange, green membrane-permeable fluorescent dye

IE = Islet equivalent, defined as a spherical aggregate of pancreatic endocrine cells of 150 µm diameter

FACS = Fluorescent-activated cell sorting (or flow cytometry)

7-AAD = 7-aminoactinomycin D, membrane-impermeable fluorescent dye

VADFMK = Membrane permeable caspase ligand (inhibitor)

PS = Phosphatidylserine

MTT = Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

DNA = Deoxyribonucleic acid, measured using commercial fluorimetric assay

ATP = Adenosine triphosphate

ADP = Adenosine diphosphate

MMP = Mitochondrial membrane potential

[†]Assay has been used on islets in conjunction with FACS, which requires the dispersal of islet clusters. Dissociating islets typically involves harsh enzymatic digestion with serine proteases that results in the disruption of cell-matrix interactions, cellular damage and death (e,g., anoikis). It is important to note that FACS analysis in itself is associated with inherent limitations, including the relative subjectivity of gating cell sub-populations, the large sample required for analysis (~1000s IE), high cost of equipment, extensive training and complex methodology that is susceptible to error

TABLE 3

Current methodologies used in the measurement of oxygen consumption rate

SYSTEM	METHOD	REFERENCE	MEASURED QUANTITIES	ASSAYED TISSUE	ADVANTAGES	DISADVANTAGES
Perifusion bioreactor	Phosphorescence lifetime	[56]	ΔOCR_{glc}	Rat islets	Real-time assessment of transient dynamics (e.g., glucose responsiveness, Ca^{2+} blockade, protein synthesis inhibition) May provide β-cell specific information	Complex system with limited to use in research
		[34]	ΔOCR_{glc} OCR_{TX}	Human islets		
Stirred microchamber	Fluorescence quenching	[33]	OCR/DNA OCR/cell	βTC3 cells Rat islets Porcine islets	Quantitative, rapid and prospective assessment of an islet preparation Operator independent	May not differentiate between OCR attributed to islets or other cells in a preparation
		[32]	OCR_{TX} OCR/DNA	Human islets		
		[57]	OCR_{TX} OCR/DNA	Rat islets		
Static culture	Fluorescence microplate reader	[58]	OCR_{GS} OCR_{hglc}/OCR_{lglc}	NHP islets Human islets	Simple, inexpensive and rapid assessment	May not provide accurate estimates of OCR Limited experience with its use in this application Complex theoretical estimation of pO_2
		[26]	OCR ΔOCR_{glc}/DNA	Human islets		

OCR = Oxygen consumption rate

ΔOCRglc = Defined as the measured increment in OCR when stimulated by glucose (3 – 20 mM)

OCRTX = Transplanted OCR, which represents viable islet dose

DNA = Deoxyribonucleic acid, measured using commercial fluorimetric assay

OCR/DNA = Measure of OCR normalized to DNA, represents the fractional viability of a cellular/islet preparation

OCRGS = Glucose-stimulated OCR

OCRhglc/OCRlglc = Defined by the authors of the paper[37] as the Stimulation Index, a ratio of OCR measured at high glucose concentrations (16.7 or 33.3 mM) to OCR measured at basal concentrations (2.8 or 5.6 mM)

ΔOCRglc/DNA = Defined as the OCR Index, a ratio of the estimated ΔOCRglc normalized to DNA