Viability Analysis of Cryopreserved Rat Pancreatic Islets Using Laser Scanning Confocal Microscopy

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We have developed a digital image analysis technique to assay the viability of frozen–thawed pancreatic islets by using laser scanning confocal microscopy (LSCM) in conjunction with double fluorescent staining [acridine orange/propidium iodide (AO/PI)]. Freshly isolated rat pancreatic islets were cultured for 18–24 h and then brought to a 2 M concentration of dimethyl sulfoxide (Me₂SO) by serial addition at decreasing temperatures. Ice was nucleated in the islet suspension at a defined temperature (−10°C), followed by a controlled period for equilibration and then cooling in a programmable bulk freezer at rates of 0.3, 1, 3, 10, and 30°C/min to −70°C. Samples were then stored in liquid nitrogen. Subsequent to rapid thawing and serial dilution with sucrose solution to remove Me₂SO, AO/PI-stained individual islets were prepared for imaging on the LSCM. A series of optical sections through individual stained islets were obtained and processed to obtain high-contrast images at two different wavelengths; 488 nm and 514 nm for viable and damaged tissue, respectively. Image analysis algorithms consisted of template masking, generation of histograms of the pixel intensity profile, and gray level thresholding to obtain binary images. The total percentages of both types of tissue in the islet were computed by summing the two populations in each serial section. The spatial distributions of viable and damaged tissue were calculated from the three-dimensional (3-D) data base for both cultured (control) and cryopreserved islets. The 3-D spatial distributions of damaged and viable tissue in the islets were computed by determining the normalized distance of each viable/damaged voxel from the centroid of the islet volume to a mathematically estimated 3-D superquadric surface used to estimate the outer boundary of the islet. Further, each isolated damaged cell was identified and its volume determined. These studies indicate that cryopreserved islets exhibit shape distortion and a decrease in the numerical density of cells in comparison to unfrozen controls. Maximal survival was observed at the slower cooling rates. Accordingly, damage was found to occur throughout the 3-D islet volume in distinct spatial distributions for islets frozen at the slower and the faster cooling rates. Further, it was found that the volume of the majority of damaged cells identified was consistent with that of cells ranging in diameter from 5 to 9 μm.

INTRODUCTION

Widespread successful transplantation of pancreatic islets as a curative treatment of diabetes mellitus is predicated on the ability to store freshly harvested donor tissue for extended times with minimal diminution in viability and function. Studies have indicated that a critical mass of viable β cells is necessary for a successful transplantation (39). These studies have potentiated the use of cryopreserved islets to meet the requirement of a large number of islets needed to induce normoglycemia in diabetic patients (29, 38). Thus, quantitative determination of viability and the mass of β cells present in cryopreserved and thawed tissue is essential for optimal design of a preservation protocol and success of the subsequent transplant.

Many biochemical and physiological measurements have been used as indices of viability to determine the maximal recovery of islets subjected to freeze–thaw regimens (2, 8, 10, 20, 21, 23, 25, 27, 34, 40). Most of these studies have successfully demonstrated the viability of cryopreserved islets in terms of their capacity to secrete insulin. However, there is a considerable interest in quantifying the three-
dimensional (3-D) spatial distribution of the damaged cells and their individual and aggregate volumes in response to specific cryopreservation protocols. This information would provide an indication of the type and percentage of damaged cells and would be useful as an indirect measure of the mechanism of injury owing to cryopreservation. This paper presents an optical viability analysis technique implemented to investigate the effect of cooling rate on survival of cryopreserved islets.

Confocal microscopy has already been applied successfully to the 3-D imaging of isolated pancreatic islets (7, 18, 19). Accordingly, laser scanning confocal microscopy (LSCM) of frozen–thawed and unprocessed (cultured) islets stained with AO (acridine orange) (17) and PI (propidium iodide) (14, 15, 35) was used in conjunction with image processing to determine the ratio of viable and damaged cells in individual islets and the 3-D spatial distribution of the viable and damaged tissue.

**MATERIALS AND METHODS**

**Isolation of Rat Pancreatic Islets**

Individual islets were isolated by enzymatic digestion of the excised pancreas from Fischer 344 rats weighing ~250 g. Three to four milliliters of a solution consisting of 3 mg/ml Collagenase P (Boehringer-Mannheim, Cat. No. 1249-002) in Hank’s balanced salt solution (HBSS, Sigma H-8264) at 0°C was injected slowly into the pancreas via a bile duct cannula (36). The perfused pancreas was excised and then digested at 37°C for 19 min. Islets were separated from exocrine tissue on a dextran (Sigma D-3759, MW = 87,000) discontinuous density gradient. The isolated islets were suspended in culture medium containing RPMI 1640 medium (Sigma R-5632, Hepes buffered), 10% fetal calf serum, 2% antibiotics (penicillin, streptomycin, and fungizone, GIBCO Cat. No. 15245-012) and cultured at 37°C in a humidified environment of 95% O₂ and 5% CO₂ for a minimum of 18 h prior to experimentation.

**Cryopreservation Procedures**

For cryopreservation experiments ~100–150 hand-picked islets were suspended in 0.2 ml of culture medium and placed in 5-ml sterilin tubes. A CPA was introduced incrementally, starting with 0.1 ml 2 M dimethyl sulfoxide (Me₂SO, Sigma D-5879) at 22°C, followed by serial additions of 0.1 ml of 2 M and 0.4 ml of 3 M Me₂SO at 22°C and 0°C, respectively (16, 24, 31). Ice was nucleated in the suspending solution at −10°C, and the specimen was held for a 5-min equilibration period at this temperature before transferring to a Kryo-10 digitally programmable and controlled freezer for cooling. Five different cooling rates were employed: 0.3, 1, 3, 10, and 30°C/min to reduce the temperature to −70°C. The islets were then transferred directly to a liquid nitrogen dewar and stored at −196°C.

Rapid thawing from −196°C was effected by continuous shaking of the tube in a 37°C water bath until nearly all visible ice was melted and then immersion into an ice bath (16). Me₂SO removal was achieved by addition of 1.6 ml of 1.5 M sucrose at 0°C, followed by serial dilution with 0.5 ml of RPMI 1640 medium. Subsequent additions of RPMI 1640 medium were carried out at 22°C with 0.5-, 1-, and 2-ml volumes. Finally, the islets were centrifuged at 100g and resuspended in culture medium. Frozen–thawed islets were incubated for a few hours at 37°C to allow for recuperation from the stress incurred during the cryopreservation procedure. The islets were then stained with AO/PI (for approximately 45 min) and imaged using a Zeiss LSM-10 confocal microscope. Ten to twenty serial sections were obtained through each islet (fluorescent-stained islets before and after cryopreservation) at increments varying between 3 and 7 μm (Fig. 1).

**Quantitative Analysis Using Digital Image Processing**

The first step involved the processing of each 2-D (512 × 512) image in the sequence of N sections (for both control and frozen–
Fig. 1. Series of 14 optical sections through an islet thawed after freezing at 10°C/min. (a) Viable cells imaged at 488 nm; (b) damaged cells imaged at 514 nm.
thawed data sets including images obtained at 488 nm, viable tissue, and 514 nm, damaged tissue). The images were initially binarized using algorithms described in detail elsewhere by Merchant et al. (19).

The total number of pixels at an intensity of 255 (indicating the local presence of the fluorescent stain) was recorded for each cross section of the viable and damaged data sets for both unprocessed and cryopreserved islets. The sum of the total pixels for N sections was computed, and the ratio of the sum of the viable tissue to that of the nonviable was determined.

A further analysis procedure was performed to determine the spatial distribution of the viable and damaged tissue in both the control and cryopreserved islets. In order to expedite future 3-D computations, the spatial resolution of the 512 × 512 images was reduced to 128 × 128 by applying simple decimation algorithms (22). The 3-D spatial distribution of tissue was determined by identifying each voxel (viable and damaged) and computing its relative location in the islet. The spatial location of tissue within the islet was measured by estimating a 3-D surface for the islet and computing the normalized distance of each voxel from the surface. Superquadric primitives without deformation (3) were employed to compute the 3-D surface. Since our aim was to approximate a smooth surface to define the shape of islets, parametric deformations were not implemented.

Initially, the centroid of the islet volume was defined as that point where all the mass of the islet can be concentrated without changing the first moment of the islet about any axis (18). The islet surface was estimated by formulating a least square minimization of the superquadric cost function with the processed 3-D data points as input (5). The inside–outside/cost function, \( F(x, y, z) \), of the superquadric surface was defined by the equation

\[
F(x, y, z) = \left( \left( \frac{x}{a_1} \right)^{2\epsilon_1} + \left( \frac{y}{a_2} \right)^{2\epsilon_2} + \left( \frac{z}{a_3} \right)^{2\epsilon_3} \right)^{\frac{1}{\epsilon_2}},
\]

where \( x, y, \) and \( z \) are the position coordinates in 3-D; \( a_1, a_2, a_3, \epsilon_1, \epsilon_2, \) define the superquadric size; and \( \epsilon_1 \) and \( \epsilon_2 \) are the shape parameters.

The input 3-D points were initially translated and rotated to the center of the world coordinate system (denoted by the subscript \( W \)) and the superquadric cost function in the general position is as follows (5):

\[
F(x_w, y_w, z_w) = F(x_w, y_w, z_w; \ a_1, a_2, a_3, \epsilon_1, \epsilon_2, \phi, \theta, \psi, c_1, c_2, c_3),
\]

where \( a_1, a_2, a_3, \epsilon_1, \epsilon_2, \phi, \theta, \) and \( \psi \) represent orientation; and \( c_1, c_2, \) and \( c_3 \) define the position in space of the islet centroid. To recover a 3-D surface it is necessary to vary the above 11 parameters to define a set of values such that most of the outermost 3-D input data points will lie on or close to the surface. The orientation parameters \( \phi, \theta, \) and \( \psi \) were neglected in accordance with the rationale of Solina and Bajcsy (30) for the analysis of blob-like objects. Hence, only the size and the shape parameters were...
varied, and the cost function was minimized using the Levenberg-Marquardt method (28). Further, since multiple sets of parameter values can produce identical shapes, typically certain severe constraints are essential to obtain an unique solution. However, since the recovered 3-D surface was used only to represent space occupancy/shape, such ambiguities did not impose a problem (30). The initial estimates for the size parameters were obtained from the input data points, whereas the shape parameters were initially set to 1. The final parameter values for the 3-D surface model were determined based on the criterion that the computed surface would enclose >90% of the 3-D input data points. Figure 2 presents a graph of an estimated superquadric surface illustrating the image tissue voxels enclosed within and/or lying on the 3-D surface along with the outlying tissue voxels.

After the surface model was identified the distance of each viable/damaged image voxel from the centroid of the 3-D islet volume was obtained (18). The distance was then normalized with respect to the length of a vector containing the voxel and extending from the centroid to its intersection with the estimated superquadric 3-D surface. This calculation was implemented in a spherical coordinate system as shown in Fig. 3. As illustrated, ρ defines the distance of voxel P from the origin O which is fixed at the centroid, ρc, the length of the vector originating at O, passing through the voxel P, and terminating at the point of intersection with the superquadric surface, S. The coordinates of voxel S are then (ρc, θ, φ), i.e., voxels P and S have similar θ and φ values and different ρ values. Thus, ρc is easily obtained using the equation

where the parameters a₁, a₂, a₃, e₁, and e₂ are estimated via the nonlinear least square minimization of the superquadric cost function.

After ρc was obtained, the normalized distance of voxel P from the centroid was computed as ρ/ρc. Thus, all the voxels inside the estimated 3-D surface had a normalized distance value less than 1, and surface voxels a value of 1. Each tissue voxel was then assigned to a regional group as a function of its computed normalized distance from the centroid. Thereby, 10 serial annular shells were obtained, each having a normalized shell width of 0.1. Thus, the spatial distribution of viable and damaged tissue was computed in the form of a histogram, i.e., the number of voxels were determined for each shell depending upon the normalized distance from the centroid.

The final step of the analysis involved image processing operations on only the 3-D im-

\[
\rho_c = \frac{1.0}{\sqrt{\left(\left(\frac{\sin \phi \cos \theta}{a_1}\right)^{2e_2} + \left(\frac{\sin \phi \sin \theta}{a_2}\right)^{2e_2} + \left(\frac{\cos \phi}{a_3}\right)^{2e_1}\right)^{\frac{1}{e_1}}}}.
\]
ages of damaged cells after cryopreservation and has been described in detail elsewhere by Merchant et al. (18). The 3-D volume of damaged cells and/or clusters of damaged cells in cryopreserved pancreatic islets was implemented by isolating each PI-stained region (red) and then computing its volume (18). Since the volume of a typical islet cell varies from \( \sim 100 \) to \( 300 \ \mu m^3 \), only indicated cell volumes greater than \( \sim 50 \ \mu m^3 \) were isolated; small regions were assumed to be noise and neglected in the analysis. This would exclude the possibility of actually eliminating a nuclear volume because only the brightly fluorescing cells are captured and it is unlikely that a cell nucleus would appear isolated in the image. As an example, considering an 3-D image of \( 512 \times 512 \times 20 \) spatial resolution, captured using an 40× objective with a magnification factor of 20, and a \( z \)-interval of \( 3 \ \mu m \); the volume of an individual voxel is \( 0.6337 \ \mu m^3 \) (calibrated for the Zeiss LSM 10). Hence a volume of <\( 50 \ \mu m^3 \) corresponds to <78 voxels, which is only a small fraction of the size of imaged cells (\( \sim 160-475 \) voxels) and can be excluded as noise.

**RESULTS**

The algorithms described above were used to determine the 3-D distribution of the freeze–thaw-induced tissue damage and the percentage survival for islets frozen at five different cooling rates: 0.3, 1, 3, 10, and \( 30 ^\circ C/\text{min} \). Five experiments were performed for each cooling rate, and five islets were imaged and analyzed for each cryopreservation experiment. Further, five unprocessed (cultured) islets were imaged and analyzed as controls for each of the 25 cryopreservation experiments performed. For each of the 25 experiments performed, the islets were harvested from two Fisher 344 rats, a random sample of 5 incubated islets was imaged as controls before cryopreservation, and a random sample of 5 islets was imaged after the freeze–thaw procedures. Hence, the islets for each of the different cooling trials were obtained from different animals, but the islets from each trial were compared to their own controls. Further, a study was performed to compare the spatial distribution of viable tissue in cultured islets stained with AO to that stained with fluorescein diacetate (FDA). Cultured islets were stained with FDA (5 mg/ml in acetone) for 15 min and scanned using the 514-nm argon laser. Similarly, the spatial distribution of deliberately damaged tissue was evaluated via supravital PI staining of islets that were frozen by immersion in liquid nitrogen without cryoprotection to intentionally induce injury. The frozen islets were thawed and stained with PI for 45 min prior to confocal imaging.

**Effect of Cooling Rate on Survival**

The percentage survival in islets thawed after freezing at various cooling rates and in unprocessed control islets is presented in Table 1. The percent survival computed as the ratio of the total number of viable tissue voxels to the sum of the viable and damaged tissue voxels for each cooling rate implements the average of 5 experiments (total 25 islets). Similarly, the control data are averages of 5 experiments each consisting of 5 islets. The control islets consistently exhibit \( \sim 79\% \) viable cells. Further, for each cooling rate values for the normalized survival were computed as the ratio of the percent survival after cryopreservation to the percent survival of unprocessed/control tissue. Following the cryopreservation procedures the percentage of the damaged cells varied as a function of different cooling rates employed. The best results were obtained at the slow cooling rates of 0.3 and \( 1 ^\circ C/\text{min} \) yielding a normalized survival value of \( \sim 0.94 \), and the lowest survival was observed at the faster cooling rate of \( 30 ^\circ C/\text{min} \) with a normalized value of 0.54. The variance values recorded are considerably high; however, this is expected because a relatively small random sample is used to represent a large population; furthermore, the survival of the cryopreserved islets is also dependent on various other factors such as the size of the islet (qualitative analysis of the numerous islets processed indi-
TABLE 1

<table>
<thead>
<tr>
<th>No. of islets</th>
<th>% Survival</th>
<th>Cooling rate (°C/min)</th>
<th>No. of islets</th>
<th>% Survival</th>
<th>Normalized survival w.r.t. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>(µ ± σ)</td>
<td></td>
<td>(n)</td>
<td>(µ ± σ)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>84 ± 10</td>
<td>0.3</td>
<td>25</td>
<td>78 ± 22</td>
<td>0.93</td>
</tr>
<tr>
<td>25</td>
<td>71 ± 15</td>
<td>1.0</td>
<td>25</td>
<td>70 ± 28</td>
<td>0.98</td>
</tr>
<tr>
<td>25</td>
<td>80 ± 15</td>
<td>3.0</td>
<td>25</td>
<td>62 ± 19</td>
<td>0.78</td>
</tr>
<tr>
<td>25</td>
<td>82 ± 14</td>
<td>10.0</td>
<td>25</td>
<td>51 ± 28</td>
<td>0.62</td>
</tr>
<tr>
<td>25</td>
<td>79 ± 17</td>
<td>30.0</td>
<td>25</td>
<td>43 ± 20</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 1 shows the effect of cooling rate on the survival of cryopreserved rat pancreatic islets. It was observed that the larger islets were more susceptible to freeze−thaw-induced damage than the smaller islets, and variations of other parameters of the freezing protocol, that may have a substantial influence on the survival.

**Statistical Analysis**

The viability data from all the different experiments performed was evaluated using the Student’s t test to determine the significance of the results obtained. Two analysis tests were performed for each cooling rate using hypothesis test for (A) ≥ 90% normalized survival and (B) > 50% normalized survival. For the hypothesis test of ≥90% survival, using a 95% confidence interval the probabilities were 0.96 and 0.88 for the slow cooling rates of 0.3 and 1°C/min, whereas the probabilities for the faster cooling rates of 3, 10, and 30°C/min were 0.1, 0.11, and 0.015, respectively. The probability was >0.6 at each of the five cooling rates for the hypothesis test of achieving a survival >50%. Hence, for the data presented the slower cooling rates were optimal for cryopreservation of rat pancreatic islets.

Further, a paired t test was performed to compare the various cooling rates using a 95% confidence interval (i.e., α = 0.05). As seen in Table 2 there is little or no difference between the slower cooling rates, whereas a significant difference is observed between the slower and faster cooling rates. The normalized survival obtained by employing cooling rates of 0.3 and 1°C/min is similar (P = 0.456), whereas that between 0.3 and 30°C/min is significantly different (P = 0.035).

**Evaluation of the Numerical Density of Imaged Tissue along the Optical Axis**

The numerical density of imaged tissue along the z axis was evaluated to determine the effect of the absorption and/or scattering of light on the scanned data while optically sectioning deeper into thick samples. A plot of the number of pixels counted in x−y planes along the z axis for two islets of different sizes is presented in Fig. 4. In the ideal case, the profile of imaged pixels would be symmetric about the center plane of the islet. As seen in Fig. 4, the numerical density of the imaged tissue decreases as the thickness of the islet increases. This effect is more striking in the thicker specimens (islet 1; ~120 µm in size).

**TABLE 2**

<table>
<thead>
<tr>
<th>Cooling rates (°C/min)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 vs 1.0</td>
<td>0.459</td>
</tr>
<tr>
<td>0.3 vs 3.0</td>
<td>0.081</td>
</tr>
<tr>
<td>0.3 vs 10.0</td>
<td>0.024</td>
</tr>
<tr>
<td>0.3 vs 30.0</td>
<td>0.035</td>
</tr>
<tr>
<td>1.0 vs 3.0</td>
<td>0.033</td>
</tr>
<tr>
<td>1.0 vs 10.0</td>
<td>0.047</td>
</tr>
<tr>
<td>1.0 vs 30.0</td>
<td>0.004</td>
</tr>
<tr>
<td>3.0 vs 10.0</td>
<td>0.213</td>
</tr>
<tr>
<td>3.0 vs 30.0</td>
<td>0.012</td>
</tr>
<tr>
<td>10.0 vs 30.0</td>
<td>0.137</td>
</tr>
</tbody>
</table>
ble tissue at the periphery, indicating a diffuse islet boundary. This is expected because the superquadric surface is estimated without considering parametric deformation, and a smooth surface is applied without obtaining an exact fit. Some of the cells are likely to have been dislodged from the surface during the isolation and handling procedures.

Distribution of Viable Tissue in Islets Stained with FDA

In order to provide a comprehensive description of the tissue imaged via the supravital stains (AO/PI) employed, data \((n = 9)\) from another stain (FDA) are presented in Fig. 6. The frequency of occurrence of viable tissue as a function of position in the islet is normalized with respect to the theoretical reference volume. A comparative measurement was performed to evaluate the effects of the specificity of staining on the total imaged volume. The total imaged volume is significantly lower than the theoretical volume in both cases (Figs. 5 and 6). However, the percentage of imaged tissue using FDA was relatively higher than that obtained using AO (data not shown); this may occur due to the differential staining characteristics of the two stains. The tissue stained by FDA represents the cytoplasmic components of the cell, whereas the AO-stained tissue consists of the cell nucleus and some of its surrounding regions, accounting for the reduced numerical density of the imaged tissue in the case of AO when compared with FDA.

Distribution of Deliberately Damaged Tissue in Islets Stained with PI

Islets were deliberately damaged by immersion into liquid nitrogen without cryoprotection and stained with AO/PI to obtain high densities of damaged cells with no indication of viable tissue. The images of nonviable tissue stained by PI are characterized by isolated cells that are separated from their surroundings due to preferential localization of the stain within damaged cells. The measurement of spatial distribution of nonviable tissue as...
Fig. 5. Spatial distribution of tissue in control islets. The normalized distance is plotted vs the frequency of occurrence of viable or damaged tissue divided by the theoretical reference volume.

Presented in Fig. 7 indicates a relatively lower percentage of imaged cells when compared with the viable tissue stained using either FDA or AO. The void volume is very high owing to a combination of various factors including the inherent limitation of the imaging process.

Fig. 6. Spatial distribution of tissue in control islets stained with FDA (n = 9). The normalized distance is plotted vs the frequency of occurrence of viable tissue divided by the theoretical reference volume.
Fig. 7. Spatial distribution of tissue in deliberately damaged islets stained with PI ($n = 4$). The normalized distance is plotted vs the frequency of occurrence of damaged tissue divided by the theoretical reference volume.

as explained earlier, and the preferential staining properties of PI.

**Spatial Distribution of Scanned Viable/ Damaged Tissue**

The frequency of occurrence of viable tissue divided by the total imaged viable tissue was plotted versus the normalized distance from the centroid for the AO and FDA stains to provide an estimate of the relative extent and distribution of viable tissue present within cultured islets (Fig. 8). As illustrated the numerical density of the viable tissue for both the AO and FDA stains is highest at a distances of $\sim 0.7-0.8$ from the centroid and low in the central regions at distances of $0.1-0.2$ from the centroid. Further, it is seen that the spatial distribution for both AO/FDA stained tissue is similar, indicating an homogenous distribution of the imaged tissue.

Similarly, the frequency of occurrence of damaged tissue divided by the total imaged damaged voxels was plotted versus the normalized distance from the centroid for the deliberately damaged islets stained with PI and for the cultured islets stained with PI (Fig. 9). This analysis suggests that the numerical density of the damaged tissue is highest at a distance of $0.7-0.8$ from the centroid, as in the case of the viable tissue. Further, in the case of the deliberately damaged tissue, the numerical density of cells is relatively lower at the periphery, indicating the presence of an irregular (ragged) outer edge.

**Distribution of Damaged Tissue in Cryopreserved Islets**

The data from cryopreserved islets was analyzed in different formats to evaluate the distribution and the density of damaged tissue in localized shells defined by progressively increasing the distance from the centroid within the 3-D islet volume. The results from the cryopreservation experiments are presented in Figs. 10 and 11.

The normalized distance is plotted vs the frequency of occurrence of damaged tissue divided by the sum of the viable and damaged
tissue for each protocol, thereby providing an estimate of the relative extent and distribution of damage present within the 3-D islet volume at the various cooling rates (Fig. 10). There is a marked increase in the density of the damaged tissue for more rapid cooling rates (0.3 and 1 vs 3, 10, and 30°C/min). This observation is consistent with the results of the survival data presented earlier (Table 1).

In order to assess the 3-D spatial distribution of the damaged tissue at various cooling rates, the normalized distance was plotted vs the frequency of occurrence of damaged tissue divided by the sum of only the damaged tissue voxels enclosed by the 3-D islet volume (Fig. 11). It is evident from these results that the spatial distribution of damaged tissue can be distinguished for the islets cryopreserved at the slow and the fast cooling rates. The slower cooling rates show increased damage toward the periphery and relatively less damage in the interior when compared to the faster cooling rates, with the damage gradually increasing from the center toward the periphery. However, at faster cooling rates the damage is biased toward the core and lower at the peripheral regions. A probable cause for these effects may be the greater susceptibility of islets frozen at the faster cooling rates to intracellular ice formation. The slower cooling rates allow greater time for water efflux from the cells, reducing the risk of intracellular ice formation. However, the islets frozen at slower cooling rates are exposed to higher osmotic gradients for longer periods, which may result in higher degrees of injury to the peripheral regions which are exposed to significant osmotic stresses.

**Estimation of Cell Volume in Damaged Tissue**

Individual cells representing damaged tissue were identified and isolated using the region labeling procedure (13, 18). Figure 12
Fig. 9. Distribution of damaged tissue in control islets ($n = 19$) and deliberately damaged islets stained with PI ($n = 4$). The normalized distance is plotted vs the frequency of occurrence of damaged tissue divided by the sum of all viable and damaged tissue voxels for control islets. The normalized distance is plotted vs the frequency of occurrence of damaged tissue divided by the total imaged damaged tissue voxels for islets with excessive damage.

Fig. 10. Distribution of damaged tissue at various cooling rates as a fraction of the total measured voxels.
Fig. 11. Distribution patterns of damaged tissue observed at various cooling rates.

presents a plot of the volume ($\mu m^3$) versus the frequency of damaged cells normalized to the total number of isolated regions measured. It is evident that the volume of the majority of the islet cells after cryopreservation is in the range of 100–300 $\mu m^3$. This can be compared to the average volume of a mammalian cell which is computed for cells ranging in diameter from 5 to 9 $\mu m$ to lie in the range of approximately 65–380 $\mu m^3$, indicating that the majority of the damaged cells have been identified individually. Thus, it is likely that the larger volumes represent clusters of cells. These measurements confirm the ability to au-

Fig. 12. Damaged cell volume measurements for an islet. These measurements closely represent the results observed in all islets processed.
automatically identify individual cells in an islet when the packing density is sparse.

**DISCUSSION**

The cryopreservation of islets has been intensely investigated and numerous studies have shown islet survival after freeze–thaw procedures using a variety of cooling rates (1). The cryopreservation protocol implemented in our study was designed after Rajotte et al. (24) and Taylor and Benton (31) with minor modifications. The islets were equilibrated with 2 M Me₂SO for 10 min to allow for partial osmotic equilibration (11, 37). However, this study does not include nor compare the optimum cryopreservation protocol with the numerous other freeze–thaw protocols implemented earlier by other investigators. Furthermore, since our laboratory is not equipped for assessing the islet viability via the more conventional approach of insulin secretion in response to glucose, we are unable to include data on this functional assay.

From results of the viability analysis we see that the maximal survival was obtained at the slowest cooling rates of 0.3 and 1°C/min we tested. These results are consistent with the high survival rates observed at similar cooling rates by several other investigators (21, 23, 24, 31) using independent means of assessment. The 3-D spatial distribution of tissue within the islet is presented via supravital staining using different stains, namely, AO/PI and FDA, and the imaged data is analyzed with respect to a mathematically estimated 3-D surface. These results show a homogenous distribution of the stains within the islet (Fig. 8); however, the percentage of the imaged tissue is considerably diminished when compared with the theoretical maximum. This low percentage of imaged tissue is manifested owing to four major factors, as follows.

(i) The 3-D surface was calculated by using superquadric primitives without deformation. Since our aim was to approximate a smooth surface to define the shape of islets, parametric deformations were not implemented. This approach was taken because, although some unprocessed and most cryopreserved islets may exhibit irregular edges, in the case of the cryopreserved islets the cell density is significantly reduced, and the edges are more irregular. It is assumed that increased geometric irregularity of the outer boundary of cryopreserved islets is a manifestation of processing injury. Therefore, mathematical surfaces of geometric parameters similar to those of unprocessed islets were fit to provide consistency of analysis. The more ragged edge of cryopreserved islets should be anticipated as a decreased density of fluorescing voxels in the outer volume shells. Further, the final parameter values for the 3-D surface model were determined based on the criterion that the computed surface would enclose >90% of the 3-D input data points (Fig. 2). Thus, this approach of not estimating an exact fit partially accounts for the higher percentage of theoretical volume when compared to the imaged volume.

(ii) An inherent limitation of confocal imaging is that a gradual loss of resolution and contrast is seen when optically sectioning deeper into thick samples. This phenomenon is evident due to absorption and refraction of light inside the tissue, which leads to a distortion of excited and emitted light resulting in low contrast in deeper focal planes (6, 7, 12). Further, the imaging process is highly dependent on the intensity of the staining, and optical absorption effects cause difficulty in obtaining uniform images throughout the entire depth of the islets. Thus, optical sections obtained at regions deep inside the tissue are imaged such that only a fraction of the islet is captured.

(iii) The fluorescent probes (AO/PI) used for the viability assay stain viable and nonviable cells differentially. When used as a supravital stain, AO accumulates in acidic organelles such as lysosomes and insulin-containing secretory granules. The viable cells fluoresce green with pale green nuclei and orange lysosomes. The images obtained show brightly stained cells and surrounding regions. PI enters nonviable cells causing them to flu-
oresce brightly red. The images show isolated cells occurring throughout the 3-D volume. Thus, the scanned images may reflect only the brightly stained (green) acidic regions within and surrounding the viable cells, and/or only the brightly stained (red) nonviable cells, and not the entire 3-D volume of an islet, thus accounting for a low density of the imaged volume.

(iv) Approximately 20% of the islet volume is composed of blood vessels and the intercellular matrix, which are imaged as void spaces. Since the theoretical volume is calculated by assuming that the outer mathematical surface is completely filled with cells the actual presence of these void spaces reduces the percentage of total volume which could be measured experimentally.

The analysis of the spatial distribution of damage presented here indicates that for slow cooling rates, the injury is greater at the periphery. The pattern of damage is concentrated more toward the central regions as the cooling rate increases. Further, in the case of the faster cooling rates an overall higher degree of damage is observed extending through the islet. These results are in agreement with the morphological and microscopic observation of other workers (20, 27). Since the tissue frozen at faster cooling rates is susceptible to intracellular ice formation, this is a probable cause of the extensive damage observed in the islets frozen at the faster cooling rates.

To the present time there have been numerous studies performed to determine optimal techniques of processing islets for low-temperature storage (1, 4), including investigations to examine each of the component steps of a cryopreservation protocol such as the cooling and warming rates (31, 33), CPA equilibration times and concentrations (11, 19, 26, 32), ice nucleation and intracellular ice formation (9), and thermal equilibration periods. Most of these studies have successfully demonstrated viability of the processed islets using independent methods of assessment to determine the functional capacity of the cryopreserved tissue to secrete insulin. These studies have made a tremendous impact resulting in the successful application of islet cryopreservation to human clinical transplantation. However, these studies have not addressed the issue of examining the physical occurrence of damage within the processed tissue, which contributes to a complete understanding of the fundamental phenomena underlying the mechanism of freeze–thaw-induced injury.

We present the scope of laser scanning confocal microscopy in conjunction with digital image processing as a potential tool to evaluate the 3-D manifestation of injury within cryopreserved tissue and discuss one of the major limitations of confocal imaging encountered when imaging thick tissue samples (>100 μm in size). This technique can be easily extended to other applications, biologically oriented or otherwise, to determine the spatial distribution of 3-D data. The technique and algorithms described can be extrapolated to applications in cryopreservation with confocal imaging of other tissue types.

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