RESEARCH PAPER

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Functional assessment of automatically sorted pancreatic islets using large particle flow cytometry

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The size composition of human islet preparations has been attributed to functional potency, islet survival and transplantation outcomes. In the early post-transplantation phase islets are supplied with oxygen by diffusion only and are at risk of critical hypoxia. The high rate of early islet graft dysfunction is in part attributed to this condition. It has been presumed that islets with smaller diameter, and therefore smaller diffusion distance, are superior to large islets regarding early survival rate and graft function. In this study we aimed to evaluate Complex Object Parametric Analysis and Sorting (COPAS) as a device for automated sorting of human islets. The use of COPAS was validated for accuracy and sensitivity using polystyrene beads of known diameters. Based on time of flight relative to particle isolated islets were then automatically sorted and analyzed for viability and function using handpicked islets as control. Our results suggest that COPAS enables the automated and accurate sorting of islets with no negative impact on their integrity and viability. Thus, COPAS is an adequate tool for size-specific analysis of pancreatic islets and may be considered as part of a platform for automated high-throughput screening of pancreatic islets.

Introduction

Pancreatic islet transplantation has evolved into a relevant treatment option for patients with type 1 diabetes. During the isolation procedure islets are deprived of their naïve endothelial glomerular-like network[1,2] and microenvironment and then transplanted "naked" into the portal system of the liver. Engraftment and revascularization of the islets is a slow process and a relevant blood-flow is re-established not earlier than 10–14 days after transplantation.[3,4] Until then, islets are supplied with oxygen by diffusion only, and therefore, are highly susceptible to hypoxic damage. Moreover the intraportal environment shows naturally a rather low oxygen tension in the range of 5 mmHg, which is only a fraction of the oxygen tension within the native pancreas.[1,2,4] Recent studies demonstrated that islets with smaller diameter are superior to larger islets during the early engraftment period, presumably due to the better gas exchange (O_2, CO_2) as a consequence of the shorter diffusion distance. Accordingly, it has been reported that smaller islets display greater O_2 consumption and insulin secretion rates than larger islets.[4-7]

The COPAS (Complex Object Parametric Analysis and Sorting) technology is based on traditional single cell flowcytometry, but it is designed for the analysis and sorting of objects with diameter from 20–700 μm. Current applications include beads, small seeds, Drosophila embryos, other like sized model organisms and large cells/cellular clusters. The continuous flow system can analyze objects using five parameters: size, optical density and spectrums of fluorescence. Objects are aligned in the centre of a sheath fluid, which produces a stable laminar flow and carries the object to the flow cell. Here, they pass axially and individually through the focus of an Argon 488 nm laser beam. The resulting signals are then measured and recorded by forward scatter and fluorescence detectors. The relative size of each object is measured by an axial light-loss detector, which calculates the time the light blockage signal remains above a pre-set threshold level: this parameter corresponds to the time of flight (TOF). TOF is related to an object axial length and was the essential parameter used for this study. Sorting and dispensing decisions are based on user-selected ranges of TOF. In the case an object meets the pre-defined criteria, the airflow is briefly paused and the object is sorted to the collection dish.

In the present study we aimed to validate the potential of the COPAS instrument for automated size-based sorting of islets, a crucial pre-requisite for accurate study of their size-dependent characteristics.

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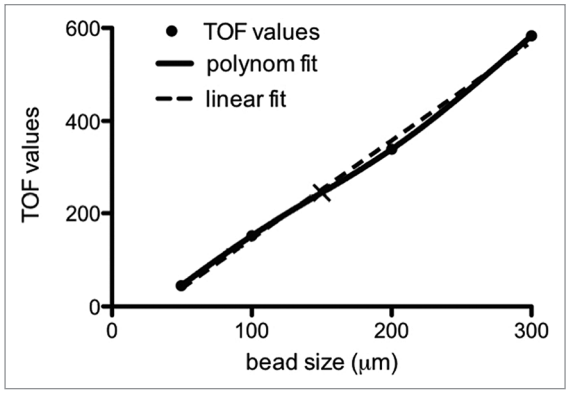

Figure 1. Mathematical correlation between polystyrene particle size and time of flight (TOF). The cut off point of 244 for a particle with diameter of 150 μm is indicated by the slanted black line.

Results

Calibration of the COPAS. Initial validation of sensitivity and accuracy of the COPAS was performed using polystyrene particles whose diameter range mimicked that of a typical mixture of isolated islets. Particles were run on the COPAS and TOF values were collected. Correlation of the data obtained with given particle sizes allowed for polynomial function analysis and calculation of a standard curve[8] (**Fig. 1**). Based on this standard curve, it was determined that the cut off TOF value for sorting islets smaller and larger than 150 μm was 244. The sorting gates were set accordingly for islet sorting (**Fig. 2A and B**).

Accuracy of islet sorting. Since COPAS cannot discriminate between aggregates of exocrine cells and islets, the purity of the islet preparation prior to sorting is a critical factor. Accordingly, COPAS sorting was carried out on three independent islet isolation whose purity was >85% pure, as estimated by microscopy analysis after dithizone staining. The outcome of the automated islet sorting was also validated by visual inspection of each islet group using a microscope equipped with an eyepiece grid. Ninety five percent of the small islets sorted with COPAS had a diameter smaller than 150 μm, whereas in the group of larger islets 98% had a diameter bigger than 150 μm (**Fig. 2C and D**). The corresponding 5 and 2% erroneously sorted islets were found in the next neighboring size category.

Islet viability. The membrane integrity of handpicked and COPAS sorted islets was determined by FDA/PI staining and fluorescent microscopy (**Fig. 3A–D**). The viability of

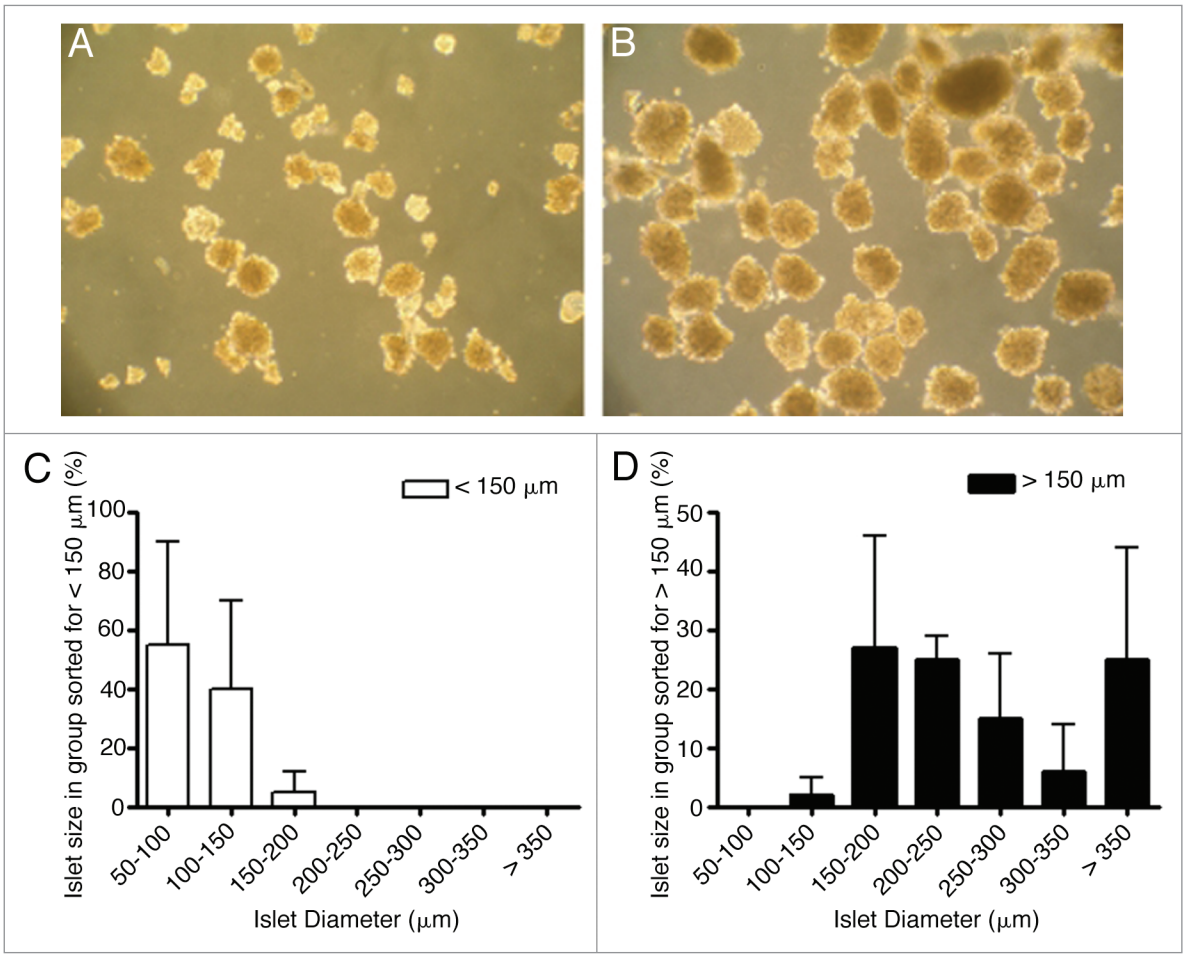

Figure 2. (A and B) Representative bright field pictures (20x magnification) of COPAS sorted islets with diameter <150 μm (A) and >150 μm (B). (C and D) Size distribution of sorted islets with diameters <150 μm (C) and >150 μm (D) as assessed by visual inspection. (n = 3).

manually and automated sorted islets did not significantly differ (<150 μm, handpicked: 92 ± 1.5%, COPAS: 88 ± 7.2%; >150 μm, handpicked: 91 ± 1.3%, COPAS: 87 ± 6.3%) (**Fig. 3E**), suggesting that the COPAS procedure was not harmful. This viability assay, however, has limitations as cells at early stages of apoptosis, i.e., prior to damage of their plasma membrane, may escape detection.[20-22]

Glucose-stimulated insulin secretion. The functional integrity of handpicked and COPAS sorted islets was further assessed by comparing their insulin secretion. Manually and automatically sorted small islets exposed to 3.3 mM glucose for 1 h secreted 3.31 ± 0.82 ng insulin/μg DNA and 2.19 ± 0.35 ng insulin/μg DNA, respectively. Upon stimulation with 16.7 mM glucose for 1 h insulin secretion increased to 5.52 ± 1.26 ng insulin/μg DNA for handpicked and 4.47 ± 0.83 ng insulin/μg DNA. Handpicked and COPAS sorted large human islets secreted at rest 5.41 ± 0.55 ng insulin/μg DNA and 4.57 ± 0.47 ng insulin/μg DNA, respectively. After stimulation with 16.7 mM glucose their insulin secretion increased to 10.52 ± 2 ng insulin/μg DNA and 9.6 ± 0.4 ng insulin/μg DNA (**Fig. 4A**). Stimulation indices showed no significant difference between islet groups based either on their size or method of sorting (**Fig. 4B**). Hence, the sorting method did not appear to alter insulin secretion.

Discussion

Islet size has an influence on islet transplantation outcome and survival. Recent publications show that mouse smaller islets perform better than larger islets when exposed to hypoxia followed syngeneic transplantation under the kidney capsule.[4,5,12] The smaller islet diameter implies a shorter diffusion distance and thereby a more effective supply of oxygen and nutrients. Evidence that the COPAS is not detrimental for islet function and viability opens the opportunity of applying this technology for the sorting of thousands of islets of homogeneous size within a few hours—a task that is unfeasible by manual sorting. COPAS-mediated islet sorting could therefore pave the way for high throughput screening of islets from large mammals, such as pigs, and thereby advance the field of islet research and drug development for diabetes therapy.

Materials and Methods

Islet isolation. Human islets (n = 3) were isolated from donor organs with consent obtained for research by next of kin and authorization through Eurotransplant. Islets were isolated following the modified Ricordi method using collagenase NB1 and neutral protease (Serva). Purification was performed with continuous-density Biocoll gradient (Biochrom AB) centrifugation using a COBE 2991 cell processor. Islets were cultured for 24 h in M1A media (Mediatech) supplemented with 32.5 mM L-glutathione (Sigma) prior to experimentation. The purity of the preparations was assessed by microscopy after dithizone staining of the islets.

Islet sorting based upon size by "handpicking" or large particle flowcytometry (COPAS). For handpicking experiments

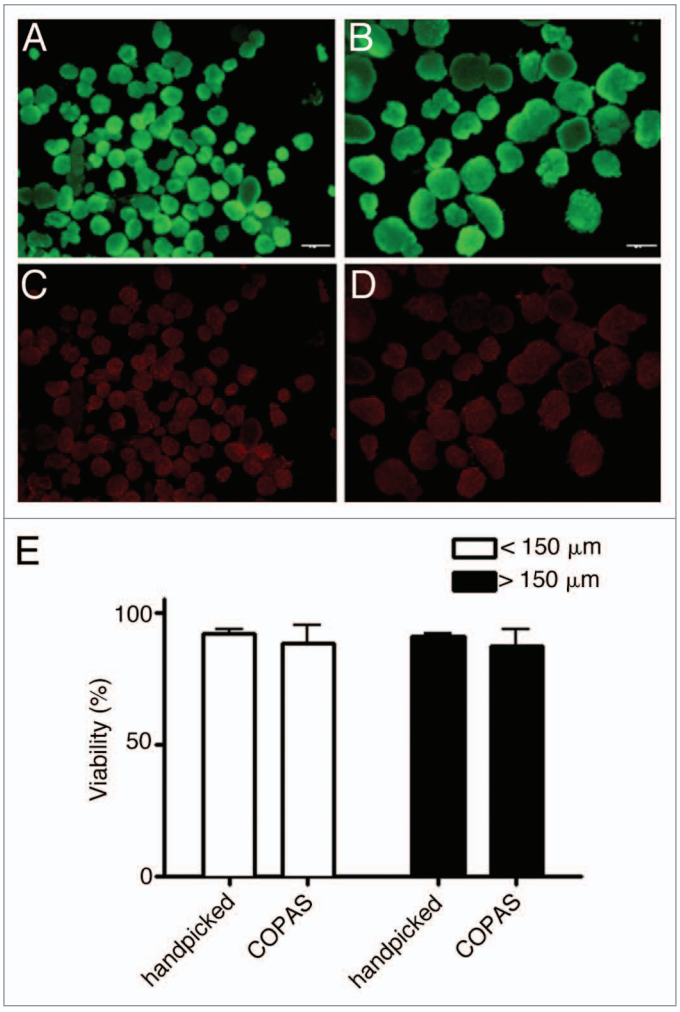

Figure 3. (A–D) Representative fluorescence microscopy pictures (40x magnification) of COPAS sorted islets <150 μm (A and C) or >150 μm (B and D) stained with FDA (A and B) and PI (C and D). (E) Viability of handpicked or COPAS sorted islets. (n = 3).

the islet size was visually determined using an eyepiece grid integrated into an inverted microscope. The islets were manually separated into two groups with diameter smaller and larger than 150 μm. Small and large islets were separate according to sorting gates pre-determined with polystyrene particles of diameters of 49.8, 100, 200 and 300 μm (Union Biometrica). For automated sorting the islets were loaded on the COPAS at a dilution that would result in the detection of 10–30 events/second. In our experience this minimizes the likelihood of two particles being separated simultaneously, thus allowing for the reliable size sorting of 35,000–100,000 islets within 1 h. Following the sorting procedure, the islets were cultured over night prior to further experimentation.

Viability staining. Islet aliquots were transferred to phosphate-buffered saline (PBS) and fluorescein diacetate (FDA) and propidium iodide (PI) were added to a final concentration of 0.5 and 75 μM, respectively. Cell viability was estimated using fluorescence microscopy by calculating the percentage of viable (FDA-positive, green) versus non-viable (PI-positive, red) cells

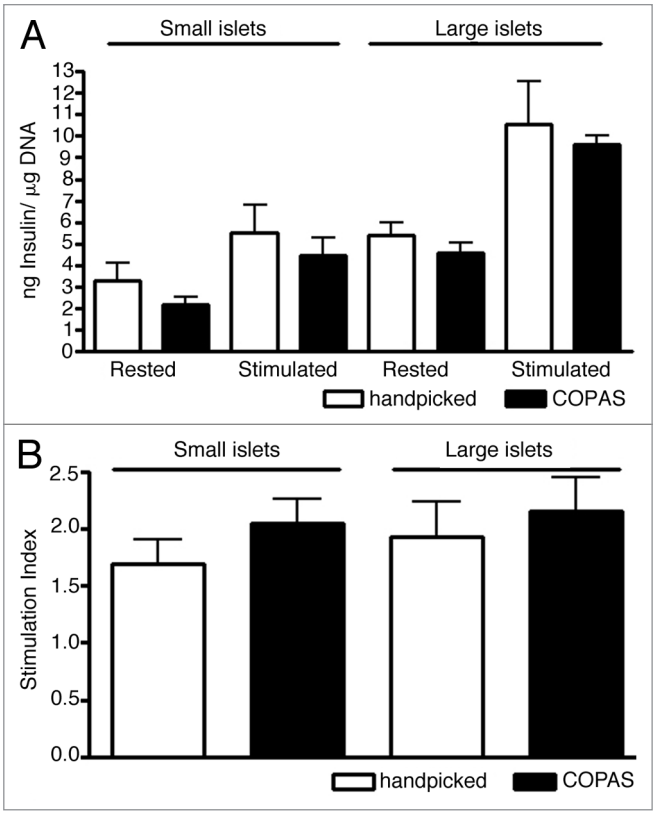

Figure 4. (A and B) Insulin secretion (A) and stimulation index (B) of islets <150 µm or >150 µm after manual or automated sorting.

within each islet. A minimum of 80 islets was analyzed for each sample.

Glucose stimulated insulin release. Islet samples were transferred to Krebs Ringer Buffer (KRB, 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_2 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$) and equilibrated in 3.3 mM glucose for a minimum of 30 min. The islets were then transferred into fresh medium containing either 3.3 mM (resting condition) or 16.7 mM (stimulation condition) glucose for 1 h in a gently shaking water bath at 37°C. Insulin secreted in the supernatant was measured by radioimmunoassay (RIA, Millipore) and normalized to DNA content, as determined after purification with the DNeasy Kit (Qiagen). The stimulation index was calculated as quotient of insulin secreted upon stimulation versus insulin secreted in resting conditions.

Statistical analysis. Results and figures are expressed as mean ± standard error of the mean (SEM).

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