An Overview of COPAS ™ Large Particle Flow Cytometry for the Analysis and Sorting of Mouse, Rat, Pig and Human Pancreatic Islets

Introduction:

Flow cytometry is a useful technique for the analysis and sorting of the individual cells that make up the functional pancreatic islet. Techniques using standard single cell flow cytometers require users to disrupt the islets in order to create suspensions of single cells that are small enough to be run through their flow cell. Functional studies and sorting of intact islets are now possible using large particle flow cytometry. This may provide researchers with additional information regarding cell function in the context of a whole islet as well as exploring other preparative and culture-growth conditions. In this document we describe tests using the COPAS PLUS flow cytometer from Union Biometrica, Inc. for the in-flow analysis and sorting/dispensing of intact mouse, rat, pig, and human islets. The 1000 micron flow cell of this instrument can accommodate objects up to approximately 600 microns in diameter, which is large enough for the analysis of intact islets. Post-analysis of intact islets run through a flow cytometer has shown that viability and functionality are unaffected (References 1 and 2).

In cooperation with Islet Transplantation Centers (University of Wisconsin, Madison; Rudbeck University, Uppsala; UPRS University, Lille; Hospital San Raffaele, Milan; ISMETT, Palermo; University Hospital, Geneva; OCDEM, Oxford), Union Biometrica has provided the COPAS PLUS to open new possibilities on islet research. Several new tests on a diversity of islets have been developed. This opportunity allows researchers and clinicians to optimize the current protocols used in both whole islet research and whole islet transplantation.

The first reports by independent researchers utilizing a Union Biometrica COPAS PLUS for analysis and sorting of islets has been previewed in a series of platform presentations (functional studies) and posters (islet quality control using Newport Green) presented by Fernandez & Hanson of the University of Wisconsin (Madison) at the IPITA 2005 Meeting (Geneva, Switzerland, May 5-7, 2005) and the American Transplantation Congress 2005 Meeting (Seattle, WA, USA, May 21-25, 2005), (Reference 3).

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Large Particle Flow Cytometry

Technology:

COPAS instruments analyze large particles (20-1,500 micron diameter) in a continuously flowing stream at a rate of 10-50 objects/second. Using object size (TOF), optical density (EXT) and intensity of fluorescent markers (FLU) as analytical criteria, particles can be selected and dispensed into Petri-dishes or multi-well plates for further analysis. A gentle pneumatic sorting mechanism located downstream of the flow cell does not harm or change the sensitive objects, making it suitable for fragile and sensitive cells and cell clusters. Samples can be run either live or fixed, and analyzed for detection of various stains, dyes, or fluorescent proteins. Multiple fluorescence excitation and emission wavelengths are available for interrogation of the samples. In the following proof-of-principle investigations an instrument with a three laser configuration (HeCd UV laser, argon-ion laser 488/514 nm and a 670 nm diode laser) was used.

Overview of Application examples:

On the following pages are brief reports on eight different projects which are intended as a quick overview of some of the feasibility experiments that have used COPAS. In some cases only an initial proof-of-principle experiment has been completed so far; in others the methodology is well developed and even published. For more details and to discuss your specific research project, please contact our applications scientists at appsupport@unionbio.com.

I. QC of islets preparations using Newport Green. (**Transplantation** 2005; 80: 729–737)
II. Propidium iodide (PI) and Annexin-V staining of human islets.
III. Heparin coating of islets. (**Diabetes** 56:2008-2015, 2007)
IV. Endothelial cell coating of human islets.
V. Staining of islets with calcein and ethidium homodimer.
VI. TMRE staining and analysis of whole islets.
VII. IBMIR antibody reaction test (**Transplantation** 2008; 85:8 1193-1199)
VIII. Sorting of mouse islets from a raw digest.

I. QC of islet preparations using Newport Green

Validation of Large Particle Flow Cytometry for the Analysis and Sorting of Intact Pancreatic Islets (**Transplantation** 2005; 80: 729–737)

Luis A. Fernandez, Eric W. Hatch, Barbara Armann, Jon S. Odoric, Debra A. Hullett, Hans W. Sollinger, and Matthew S. Hanson

Background:

Accurate quantification of total islet yield is an essential step prior to transplantation and for research. The standard method of manually determining an islet equivalent (IEQ) count is subjective and prone to error. We evaluated Complex Object Parametric Analyzer and Sorter (COPAS) large particle flow cytometry for the determination of islet equivalent counts and purities of islet preparations.

Methods:

Initial validation of the sensitivity and accuracy of the COPAS flow cytometer was performed by analysis and sorting of uniform polystyrene microspheres with sizes similar to islets. Human and Rhesus monkey islets were stained with the zinc-specific fluorescent dye Newport Green to discriminate islet from non-islet tissue. Islet sizes were extrapolated from standard curves obtained using microspheres from which individual islet volumes were calculated. IEQ counts on six islet preparations were performed by the standard manual method and compared with results obtained by automated COPAS flow cytometry.

Results:

The COPAS flow cytometer was highly accurate in the detection and measurement of both polystyrene microspheres and islets. IEQ counts determined by COPAS flow cytometry were consistent with manual counts although subject to error when assessing preparations with significant numbers of islets embedded within acinar tissue. Size-specific islet sorting with retention of morphology and dithizone staining was also shown using the COPAS cytometer.

Conclusions:

COPAS large particle flow cytometry provides a novel automated approach for quantification of intact islets and determination of islet equivalent yield. In addition, the ability to analyze and sort islets based upon user defined criterion opens unique avenues for experimentation.

II. Analysis of islet viability and apoptosis using propidium iodide and Annexin-V staining.

Objective:

Much published research does not address the variable viability between islets, although it is well known that islets quickly deteriorate with time and with exposure to Ficoll. The goal of this experiment is to demonstrate that PI staining can be used with the COPAS PLUS to assay islet viability / activity levels. During the process of enzymatic digestion and the culturing samples of islets can be tested quickly using PI for the amount of dead cells on the exterior of the islets. Detection of PI staining in the interior part of the islets can be done using the Profiler technique.

Results:

In previous experiments we have tested PI staining on cultured human islets in order to analyze and sort them based on the fluorescence intensity (see Image 1 and 1). Microscopic investigation showed that the PI uptake took place only in the exterior cells on the islets. Following these results we made a comparison between excitation of PI by UV and/or 488 nm. In addition, we have used Annexin-V for an indication of apoptosis of the islets and analyzed double stained islets using PI (FLU3) and Annexin-V FITC (FLU2).

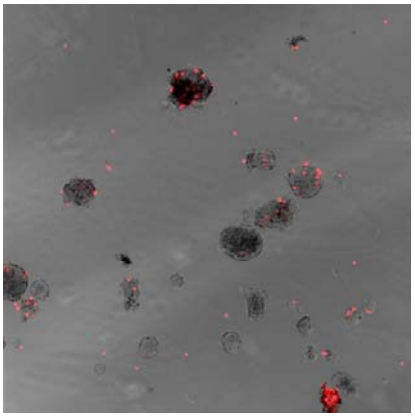

Image 1:
Human islets stained with PI before sorting.

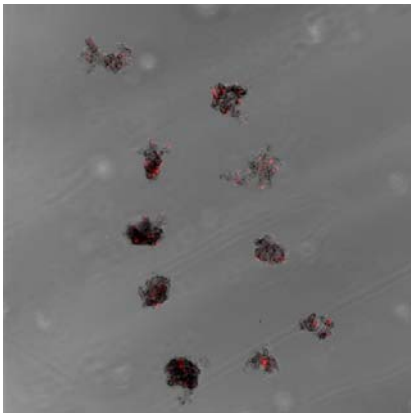

Image 2:
PI positive small fragments sorted from the sample.

Conclusions:

We have tested PI staining using three different laser configurations. The lowest shift for the PI positive stained islets was expected using the 488nm laser only, a higher shift using the 488nm and reduced power UV laser, and the highest shift using the 488nm and normal power UV laser. The shift in the mean channel of fluorescence can be correlated to the size of the islets. This permits quantification of the percentage of dead cells in the islet preparation.

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III. Heparin coating to reduce immune-rejection of human, pig and mouse islets.

Islet Surface Heparinization Prevents the Instant Blood-Mediated Inflammatory Reaction in Islet Transplantation *(**Diabetes** 56:2008-2015, 2007)*

Sanja Cabric, Javier Sanchez, Torbjörn Lundgren, Aksel Foss, Marie Felldin, Ragnar Källen, Kaija Salmela, Annika Tibell, Gunnar Tufveson, Rolf Larsson, Olle Korsgren, and Bo Nilsson

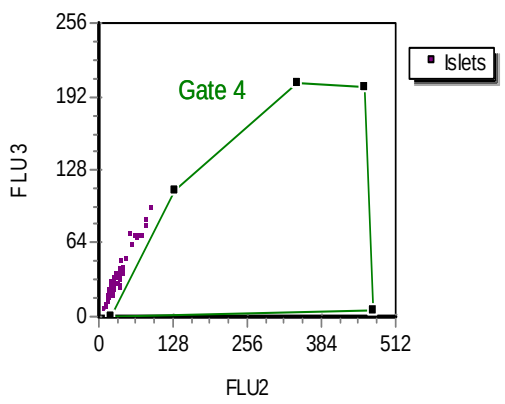
Objective:

Heparin coating is one method of reducing immune-rejection of transplanted islets. The goal of this experiment is to demonstrate that the COPAS PLUS can be used to assay heparin coverage of the islets as the heparin protocol is being further developed. The importance of this method needs to be proven in animal models. The coating procedure has been tested using a frequency resonance procedure. Quantitative data and inhibition tests are in progress using the COPAS PLUS. A test has been performed to prove that the procedure work on mouse islets as well. The results allow the use of heparin coated islets in a mouse model to further investigate the immune-rejection of the transplanted islets.

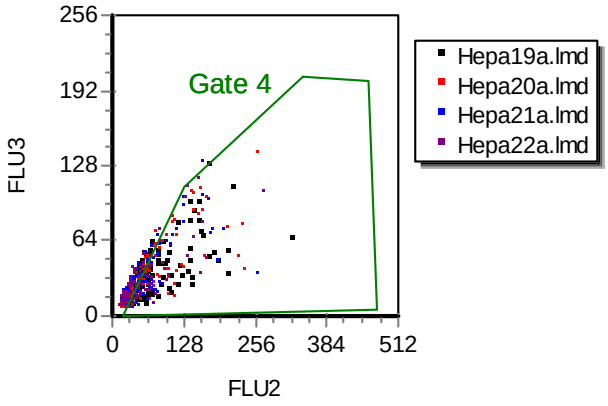
Results:

Purified islets are treated using a series of sequential steps. The first step is to add biotin, the second to add avidin, and the third to add heparin. The final staining step is with anti-thrombin linked to Alexa-488. At every step part of the sample was analyzed to measure the background fluorescence.

The background fluorescence of the heparin coated islets was also measured using anti-thrombin only. In this experiment we have tested 4 different BIOTIN bindings: SNLL, TFP (19a), PEO and SNL on human islets. Initial tests using pig and human islets demonstrated the possibility to use the Alexa-488 stain.
The plots below show overlays of all tested BIOTIN types, comparing unstained and stained islets.



Plot 4: Auto-fluorescence of unstained islets



Plot 5: Heparin coated islets stained with anti-thrombin-Alexa-488

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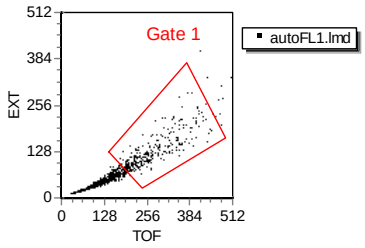
This experiment shows that the TFP biotin exhibits the largest change between autofluorescence and Alexa-488 staining. The highest percentage of positive islets is the TFP biotin as well (94%).

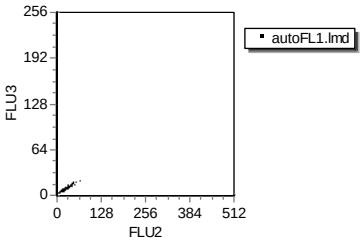
Heparin coating test on mouse islets:

Following the results of the pig and human islets we have optimized the staining procedure and tested it both on human and mouse islets. The mouse islets test was done to confirm that the procedure works well so mouse islets can be used for transplant experiments.

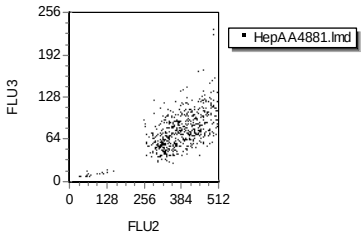
The plots below demonstrate the large difference between stained and unstained islets, thus indicating the positive result of the coating procedure.



Size and Density of mouse islet sample. Region 1 is used for selecting islets and display in the fluorescence plot



Auto-fluorescence of the uncoated mouse islets



Alexa-488 fluorescence of the islets

Conclusions:

Tests on human islets have demonstrated the feasibility of the heparin coating procedure. Follow up experiments were done using different coating intensities to allow quantification of the process. The results show the utility of heparin coated islets in a mouse model to further study the overcoming immune-rejection of the transplanted islets.

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IV. Endothelial cell coating of human islets.

Objective:

Similar to heparin coating, researchers want to do functional tests utilizing endothelial cells as a protective coating on islets. These tests have been designed to demonstrate that COPAS PLUS can also be used to assay the coverage of endothelial cells. The purpose of the test was to evaluate the coverage of the islets by using stained endothelial cells. To assess the process of endothelial cell coating of islets, images were made to determine the extent of endothelial cell coverage on the islets. Coated islets are cultured for one week to allow cell divisions of the endothelial cells (generally two divisions).

Results:

Islets are coated using endothelial cells stained with CellTracker. Each sample was tested and analyzed using a region for the islets based on size (TOF) and density (EXT). Fluorescent positive events were sorted and evaluated by confocal imaging.

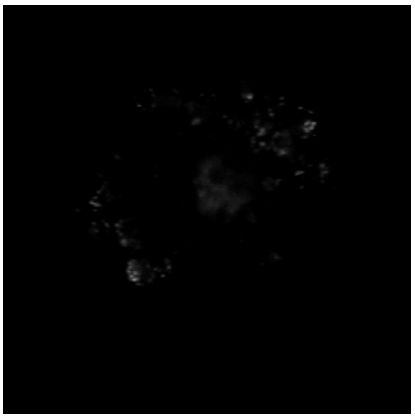

Image 3:
Uncoated islets

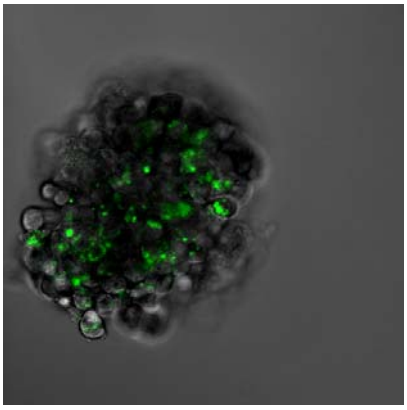

Image 4:
Image of a partially coated islet

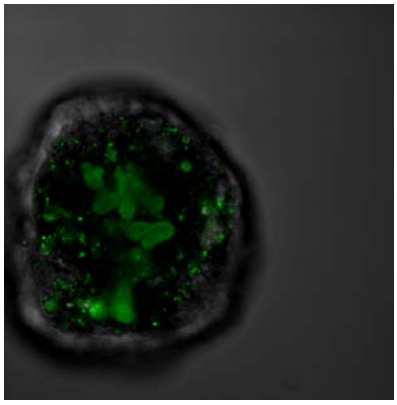

Image 5:
Image of a fully coated islet

Conclusions:

The autofluorescence level of islets is relatively high. Using the logarithmic scaling feature of the COPAS PLUS software, we have adapted the setting to create a gate for measuring positive events. The whole population shows an increase of fluorescence. This indicates that all islets have a certain level of coating by the stained endothelial cells. Clumps of stained endothelial cells were sorted among positive islets and showed bright fluorescence. Some islets have (bright) clumps of endothelial cells attached.

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V. Staining of islets with calcein and ethidium homodimer

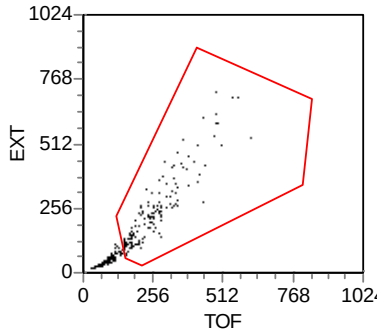
Objective:

This assay is used to measure cell viability. It is a two-color fluorescence assay that simultaneously determines:

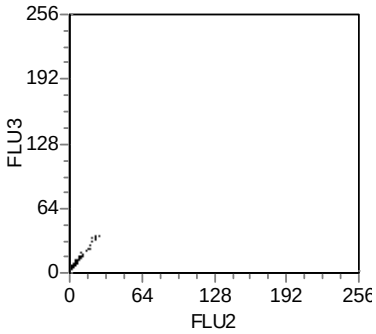
· Live cell number—Live cells have intracellular esterase that convert non-fluorescent, cell-permeable calcein acetoxymethyl (Calcein AM) to the intensely fluorescent calcein. Cleaved calcein is retained within cells.

· Dead cell number—Dead cells have damaged membranes; the ethidium homodimer-1 (EthD-1) enters damaged cells and is fluorescent when bound to nucleic acids. EthD-1 produces a bright red fluorescence in damaged or dead cells. Islet preparations were stained using calcein and ethidium homodimer. The calcein and ethidium staining were done separately and together.

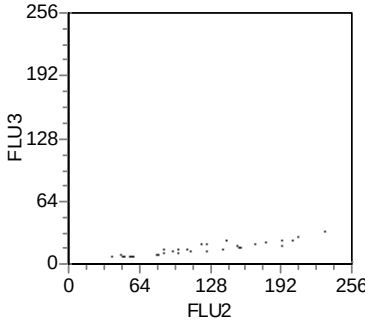
Results:



Dot plot showing size (TOF) and optical density (EXT)



FLU3 represents the red emission Ethidium Homodimer



FLU2 represents the Green emission Calcein

Strong positive events for both dyes were sorted individually. The green emission of calcein is very strong and appears to be present over the total islet. This has to be confirmed by confocal microscopy. The ethidium positive events sorted had some positive cells on the outside of the islet structure.

Conclusion:

Calcein staining of islets is very strong and easily detectable. Combinations of calcein and ethidium homodimer staining can be used to verify culture conditions. This preparation was about 80% pure and had contained few dead islets.

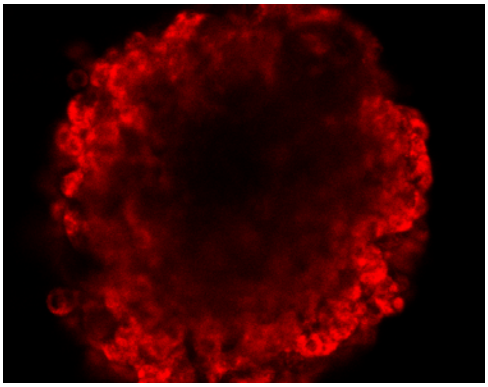
VI. TMRE staining and analysis of whole islets.

Objective:

TMRE has been proven in single cell flow cytometry to be a good dye for testing the mitochondrial potential and activation of cells. We have tested the staining protocol suitable for single cells on whole islets. Settings were created to sort stained islets. In order to verify the penetration of the dye into the islet we used a confocal microscope.

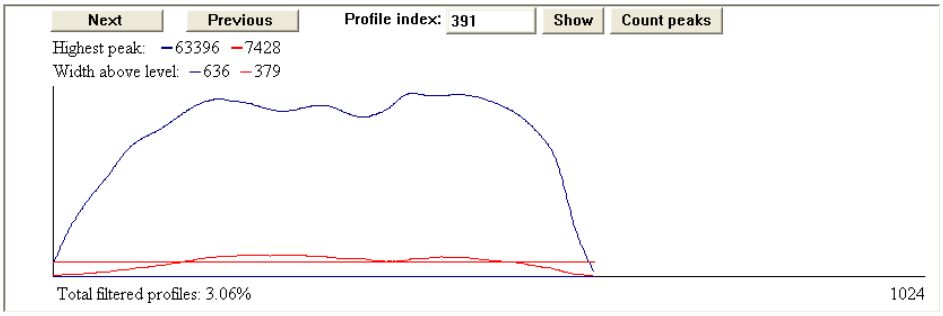
Results:

TMRE stained islets were analyzed and sorted using the COPAS. Sorted islets wee examined by confocal microscopy.



TMRE stained islets. Confocal microscopic control demonstrated that the staining was through the whole islet.

The Profiler II was used to generate profiles of the stained islets.



Profiling image of an islet stained with TMRE. The blue line represents the EXT (optical density) profile of the islet. The red line represents the TMRE staining intensity of the islet. The straight horizontal red line was set for a minimum peak intensity and used as sorting parameter.

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VII. IBMIR antibody reaction test

Acute antibody-mediated complement activation mediates lysis of pancreatic islets cells and may cause tissue loss in clinical islet transplantation *(Transplantation 2008; 85:8 1193-1199)*

Jenny Tjernberg, Kristina N. Ekdahl, John D. Lambris, Olle Korsgren, Bo Nilsson

Background:

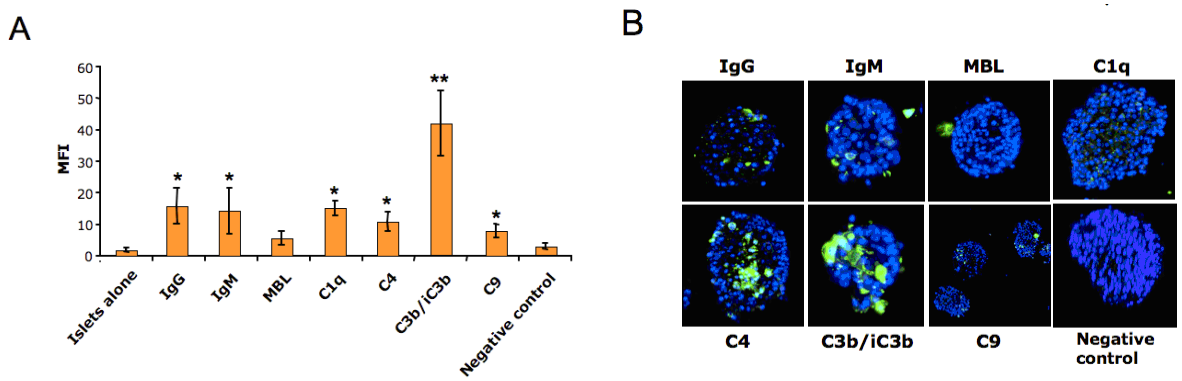
Clinical islet transplantation is associated with loss of transplanted islets necessitating tissue from more than one donor to obtain insulin independence. The instant blood-mediated inflammatory reaction (IBMIR) is one explanation to the tissue loss. Complement activation is an important cytotoxic component of the IBMIR and in the study we have investigated this component in detail.

Results:

Isolated human islets were analyzed by large particle flow cytometry and confocal microscopy after incubation in human AB0-compatible hirudin-plasma. After incubation, the islets bound IgG and IgM, Clq, C4, C3 and C9. The binding of C3b/iC3b was evident already after 5 min. The binding of C3b/iC3b and the generation of C3a and sC5b-9 were inhibited by the complement inhibitor compstatin. Lysis as reflected by propidium iodide and release of C-peptide was also inhibited by compstatin. There were significant correlations between IgM/IgG vs C3b/iC3b and between sC5b-9 and C-peptide.

Conclusion:

The conclusion is that complement is activated by natural IgG and IgM antibodies already after 5 min. The complement activation leads to lysis of cells of the pancreatic islets.



Human islets (n=5) incubated in AB0-compatible hirudin-treated plasma (n=5) for 30 min. The islets were stained for C1q, C3b/iC3b, C4, C9, IgG, IgM, and MBL. Controls consisting of islets incubated without (islets alone) and with mouse isotype IgG1 (negative control) were included. The islets were analyzed by (A) COPAS and (B) confocal microscopy. All values were compared with values obtained from the negative control are given as mean ± SEM (*= P<0.05, **= P<0.01).

VIII. Sorting of Mouse islets from a raw digest.

Objective:

Initial tests to sort the raw digest of mouse islets have been tried. Optimization of the sample preparation using PBS and stirring of the sample were needed. In this experiment we have used freshly prepared TSQ and optimized PBS. The thresholds for the analysis were set high to avoid contamination from very small particles. Contamination by the smaller objects can be avoided by collecting in a 50 micron cell strainer which is placed in the collection device.

Results:

Using the UV laser only (325 nm) we excited TSQ and collected emission in PMT 1 (violet or FLU1), PMT2 (Green or FLU2) and PMT3 (Red or FLU3). Using electronic compensation made the islets more distinct from the unstained population. A secondary selection was based on size (TOF) and optical density (EXT). The sorted islets were checked visually. The sample analysis rate was 50-100 per second.

We have been using the Enrich Sort mode to collect all events in the selected region (lower purity, optimal yield). This sort mode will collect all other events present in the same drop as the TSQ stained islet that will be sorted.

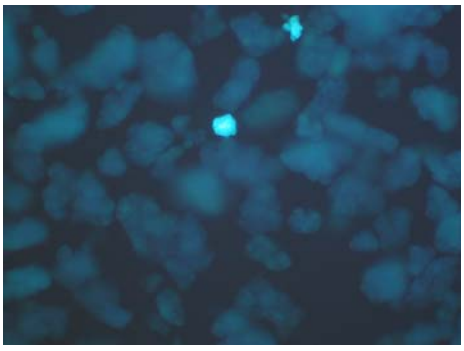


Image of the TSQ stained islets in the raw digest

mage of sorted mouse islets from a raw digest.

Sort calculations:

Time:

After initial testing using the first sample we have sorted for longer times using a second sample. The raw digest, stained with TSQ, was diluted into 100 mL samples with optimized PBS. The flow rate for sample fluid is +/- 5 ml per minute.

The total time for the sorting, including wash time, was 25 minutes.

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Recovery and Yield:

Based on the initial recovery using the Enrich mode we have tested Enhanced sort mode on the second sample. This resulted in a recovery of 20% (1 out of 5 possible events was sorted). Using Enrich mode the recovery was 100%. The total purity of the second sample was 63%, so the effective yield was also 63%.

Total islet count:

From the second preparation (1 pancreas) we expected approximately 200 islets to be available to sort. Using the yield of 63% this should have resulted in 126 islets. From the second pancreas we have collected 112 islets.

Conclusions:

Sorting islets from a raw digest of mouse pancreas has become possible using TSQ staining. The use of compensation clearly indicates the TSQ positive population. Using the Enrich sort mode we can collect approximately 56% of the expected islets that can be recovered by an experienced person by hand. The total time needed for one mouse pancreas is 25 minutes. No operator interference is needed during this time. Collecting the islets in a 50 micron cell strainer allows cleaning of the collected islets easily. Comparative tests using Newport Green (L. Fernandez and M. Hanson, Wisconsin U.S.A.) and TSQ (D. Gray, Oxford, UK) have proven that the islets do not lose their functionality.

References:

1) Gray DW, Gohde W, Carter N, Heiden T, Morris PJ. Separation of pancreatic islets by fluorescence-activated sorting. *Diabetes.* 1989 Jan;38 Suppl 1:133-5.

2) Jindal RM, McShane P, Gray DW, Morris PJ. Isolation and purification of pancreatic islets by fluorescence activated islet sorter. *Transplant Proc.* 1994 Apr;26(2):653.

3) Fernandez, Luis A.; Hatch, Eric W.; Armann, Barbara; Odorico, Jon S.; Hullett, Debra A.; Sollinger, Hans W.; Hanson, Matthew S. Validation of Large Particle Flow Cytometry for the Analysis and Sorting of Intact Pancreatic Islets. *Transplantation*. 80(6):729-737, September 27, 2005.

4) Cabric et al. Islet Surface Heparinization Prevents the Instant Blood-Mediated Inflammatory Reaction in Islet Transplantation. *Diabetes* 56:2008-2015, 2007

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