Automated quantification of pancreatic \( \beta \)-cell mass

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Golson ML, Bush WS, Brissova M. Automated quantification of pancreatic \( \beta \)-cell mass. Am J Physiol Endocrinol Metab 306: E1460–E1467, 2014. First published April 22, 2014; doi:10.1152/ajpendo.00591.2013.—\( \beta \)-Cell mass is a parameter commonly measured in studies of islet biology and diabetes. However, the rigorous quantification of pancreatic \( \beta \)-cell mass using conventional histological methods is a time-consuming process. Rapidly evolving virtual slide technology with high-resolution slide scanners and newly developed image analysis tools has the potential to transform \( \beta \)-cell mass measurement. To test the effectiveness and accuracy of this new approach, we assessed pancreata from normal C57Bl/6J mice and from mouse models of \( \beta \)-cell ablation (streptozotocin-treated mice) and \( \beta \)-cell hyperplasia (leptin-deficient mice), using a standardized systematic sampling of pancreatic specimens. Our data indicate that automated analysis of virtual pancreatic slides is highly reliable and yields results consistent with those obtained by conventional morphometric analysis. This new methodology will allow investigators to dramatically reduce the time required for \( \beta \)-cell mass measurement by automating high-resolution image capture and analysis of entire pancreatic sections.


tended due to variation in image intensity between blank and tissue images (17). Image analysis for quantification of \( \beta \)-cell mass was initially performed by point counting morphometry, pioneered by Susan Bonner-Weir (9, 15, 24, 27); more recently, however, investigators have been using several low-throughput software platforms such as MetaMorph (Molecular Devices, Sunnyvale, CA), ImageJ (National Institutes of Health, Bethesda, MD), and Volocity (PerkinElmer, Santa Clara, CA) that measure \( \beta \)-cell area relative to the area of exocrine or total pancreatic tissue (2, 9, 11, 16, 20–22).

The advent of virtual slide technology together with the development of new analytical tools for virtual slides holds great promise to transform histological analysis of pancreas, which historically has been challenging because of a disproportionately small size of endocrine vs. exocrine compartment and nonuniform distribution of islets throughout the gland. Here, we took advantage of high-resolution slide scanning to assess its suitability for \( \beta \)-cell mass measurement. This approach used a web-based digital image information management interface, allowing remote virtual slide viewing, conferencing, archiving, and image analysis. To determine whether the slide scanning system can accurately measure \( \beta \)-cell mass, we trained an image analysis algorithm within its software suite and then used trained macros to examine pancreata from normal C57Bl/6J mice and from mouse models of \( \beta \)-cell ablation [streptozotocin (STZ)-treated mice] and \( \beta \)-cell hyperplasia (leptin-deficient \( \text{Lep}^{ob/ob} \) mice).

The automated analysis of histological sections sampled systematically throughout the pancreas yielded results that were highly consistent with the outcomes of the conventional morphometric approach. Both automated and conventional analyses showed that \( \beta \)-cell mass in diabetic STZ-treated mice was reduced to 10% of the control, whereas nondiabetic \( \text{Lep}^{ob/ob} \) mice had \( \beta \)-cell mass increased nearly threefold, which was similar to previously reported data (1, 23, 28). Our data indicate that the automated analysis of virtual slides is appropriate for quantitative assessment of \( \beta \)-cell mass. With all the above taken together, we present a new methodology that will allow investigators to dramatically reduce the time required for \( \beta \)-cell mass measurement by automating high-resolution image acquisition and analysis of entire pancreatic sections. This new approach can be adopted for the morphometric assessment of other pancreatic cell types and applied to the analysis of human pancreas specimens.

MATERIALS AND METHODS

Mice and STZ administration. Wild-type and \( \text{Lep}^{ob/ob} \) mice (6, 7, 12) were on a C57Bl/6J background (Jackson Laboratories, Bar Harbor, ME). For STZ treatment, STZ (Sigma, St. Louis, MO) was freshly resuspended in cold 100 mM citrate buffer (pH 4.5) at 52.5 mg/ml and injected at 175 mg/kg body wt. Diabetic mice were euthanized 8 days after STZ injection (fed blood glucose 527 ± 20
mg/dl). All analyses were performed on 10-wk-old mice. Some slides used for the training of the image analysis algorithm were obtained from B6D2 mice at postnatal day 8 (P8). Animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

Tissue fixation, immunohistochemistry, and imaging. After dissection, pancreata were weighed, laid flat in cassettes, fixed for 4 h in 4% paraformaldehyde, dehydrated, and embedded in paraffin (Fig. 1 A). Longitudinal pancreatic sections were cut at a 5-μm thickness, collected at 250-μm intervals, and plated on glass slides. This resulted in the collection of sections from six to eight depths per pancreas. Sections were immunolabeled with guinea pig anti-insulin (Dako, Carpinteria, CA; 1:1,000) and HRP-conjugated donkey anti-guinea pig antibodies (Jackson ImmunoResearch, West Grove, PA; 1:400). Insulin immunolabeling was visualized with a DAB kit (Vector Laboratories, Burlingame, CA), and sections were counterstained with Eosin-Y (Fisher Healthcare, Houston, TX). A schematic of pancreas processing is shown in Fig. 1 A. All images were acquired on a ScanScope CS slide scanner (Aperio, Vista, CA) at ×20 magnification (resolution of 0.5 μm/pixel) and uploaded into a Spectrum digital slide interface (Aperio).

Fig. 1. Workflow for automated β-cell mass analysis. A: schematic of pancreas processing and sectioning. B: schematic of image analysis. Building a macro for accurate image segmentation involves repeated rounds of tissue selection, training, and testing. Steps that should be omitted when tuning a macro are depicted in gray; optional steps are shown in green; required steps are shown in beige.
Building and testing of image analysis macros. A workflow schematic of image analysis is depicted in Fig. 1B. The Aperio software suite consists of a web-based interface (Spectrum v. 11.1.0, recently renamed to eSlide Manager), in which images are viewed and batch processing occurs, and a local workstation interface (ImageScope v. 11.2.0.780) where macro training takes place. Aperio image analysis has several algorithms, including the Genetic Imagery Exploitation toolset, with histology pattern recognition capability (Genie, Los Alamos National Laboratory, Los Alamos, NM), which we used to analyze brightfield images of pancreatic sections. To train the algorithm, images were chosen to reflect differences in sectioning and staining quality. Images of interest were copied within Spectrum and placed within a Genie project. We developed two image analysis macros. Macro 1 was set to perform analysis at ×20 magnification.

This high magnification requires more computational time but can better select individual β-cells and irregularly shaped islets such as those seen in postnatal pancreas and STZ-treated mice. Macro 2 analyzed images at ×10 magnification, which accelerated analysis fourfold. Within the Genie project, three “Classes” of objects were defined, i.e., Glass, Eosin, and DAB (representing insulin labeling). Images within the Genie project were opened with ImageScope and regions of interest assigned to each Class.

It is important to select a broad range of features for each Class to ensure that Genie correctly identifies objects of interest in the tissue section. A subset of these required features is depicted in Fig. 2. To fully encompass the variety in immunolabeling/counterstaining intensity and tissue morphological features necessary for Genie training, on average seven slides were used to generate each image analysis macro. For Glass, we selected clean areas as well as areas with some bubbles and dust. Areas including exocrine tissue, large blood vessels, connective tissue, and occasional pancreatic-associated lymph nodes were classified as Eosin. For Eosin and DAB Classes, we selected areas with and without section folds (including areas in and out of focus) and areas that varied in immunolabeling/counterstaining intensity. When assigning areas with section folds to Classes, selecting folds with surrounding unfolded tissue resulted in more accurate object classification. In addition, regions assigned to the DAB Class had to be selected well within islet boundaries to avoid a haloting effect around islets. Despite high DAB background within connective tissue in some samples, the careful selection of connective tissue regions allowed the macros to distinguish between the connective tissue and islets. We also selected Eosin and DAB areas adjacent to each other to teach the algorithm the spatial relationship between Eosin and DAB Classes. After regions of interest were selected, Spectrum was used to create a Genie training set montage (referred to as “Montage” for short; Fig. 2).

The Montage was then opened in ImageScope, and a new Training Macro was created and saved by using the “Analysis” menu and selecting the “Genie Training v1” algorithm. We adjusted the number of iterations in training to 500 before saving the Training Macro. Next, the Training Macro was run within Spectrum on the newly created Montage. Once the training job was completed, the Montage was reopened in ImageScope and viewed with image annotations and analysis color markup to evaluate the sensitivity and specificity of the Training Macro for the defined Classes of objects such as Glass, Eosin, and DAB (Fig. 3). Low sensitivity indicated that the Training Macro was not detecting the tissue of interest although it was assigned correctly in the Montage. The Training Macro had a low specificity for a given Class if it misclassified regions of interest in a slide. We aimed for a >97% training success rate to achieve a highly accurate image segmentation. We continued with the macro training until no further improvement was observed in either sensitivity or specificity. If >97% sensitivity and specificity were not reached, we examined what area of the slide was classified erroneously. More examples of the misclassified area were then added to the Montage, and macro training was repeated.

Once the Training Macro reached the optimal sensitivity and specificity in our Genie Training Set Montage, we created a Classifier Macro in ImageScope by using the “Analysis” menu and selecting “Genie Classifier v1”. In the image analysis window under “Classifier,” we selected the optimized Training Macro and saved it as the Classifier Macro. The performance of the Classifier Macro was then assessed on small regions of images that were not part of the Genie Training Set Montage. If the Classifier Macro aberrantly assigned tissue types, we added regions with poorly classified tissue to the Genie Training Set Montage and repeated the process above (see Fig. 1B).

Analysis of digital slides with the Classifier Macro can be carried out on a local workstation, but we used server-based batch processing to increase analysis efficiency. The batch processing allowed us to select and analyze slides on a specimen basis using a Classifier Macro optimized for each specimen type (islet phenotype). After batch processing, data for each specimen (mouse) were directly exported into an Excel spreadsheet for further statistical analyses and calculation of β-cell mass.

Any given macro will not be universal for every investigator. Differences in tissue processing and staining between laboratories can change the intensity and staining quality, and as a result of that, the macro needs to be retrained to assure high specificity and selectivity in tissue classification. Depending on the magnification used for
Since the training takes place on the server, the investigator is involved only in reviewing the macro after each training session and in determining whether the training requires adding more tissue features or is final. We predict that the training time will greatly decrease in the foreseeable future with ongoing improvements in server hardware (e.g., increasing number of processing cores and speed) and operating systems.

Analysis of β-cell mass. Images of whole pancreatic sections acquired on a ScanScope CS slide scanner at ×20 magnification were analyzed by both conventional morphometry and a macro-based automated approach. Six to eight slides were analyzed per mouse by each method.

Conventional morphometry was performed with MetaMorph v. 7.7. Because MetaMorph software does not have the capacity to analyze images of an entire pancreatic section captured at ×20 magnification, all images were physically cropped into smaller regions prior to analysis. Islets were manually traced using the MetaMorph region tool, and then islet and exocrine tissue areas were analyzed as described previously (11). Analysis of β-cell mass using this conventional approach required ~10 h per mouse.

Automated analysis of β-cell mass ran in the server environment without any investigator supervision. Image analysis at ×10 magnification (defined in Classifier Macro) took between 10 and 25 min and increased to 20–60 min per slide at ×20 magnification depending on section size. The output of the Genie Classifier Macros was then used to calculate β-cell mass. Since the glass portion of the slide is included in image analysis, the Genie Classifier Macros return DAB%, Eosin%, Glass%, and total analysis area for each slide.

To calculate DAB% in a pancreatic tissue section the following equation was used:

\[
tissue \text{ DAB}\% = \left(\frac{\text{slide DAB}\%}{\text{slide DAB}\% + \text{slide Eosin}\%}\right) \times 100
\]

(1)

Since each slide had a different amount of total tissue, the following equations were used to calculate the total Tissue, DAB, and Eosin area per slide:

\[
\text{slide tissue area} = \text{scanned slide area} \times \left(\frac{\text{slide DAB}\%}{\text{slide DAB}\% + \text{slide Eosin}\%}\right) \times 100
\]

(2)

\[
\text{slide DAB area} = \text{slide tissue area} \times (\text{tissue DAB}\% / 100)
\]

(3)

\[
\text{slide Eosin area} = \text{slide tissue area} - \text{slide DAB area}
\]

(4)

To calculate DAB% per pancreas the following equation was used:

\[
\text{cumulative pancreas DAB}\% = \frac{\sum_{i=1}^{n} \text{slide DAB area}_i}{\sum_{i=1}^{n} \text{slide DAB area}_i + \sum_{i=1}^{n} \text{slide Eosin area}_i} \times 100
\]

(5)

where \( n \) = number of slides/pancreas/mouse.

β-Cell mass was calculated by the following equation:

\[
\text{β-cell mass} = \text{pancreas weight} \times (\text{cumulative pancreas DAB}\% / 100)
\]

(6)

Statistical analysis. Mouse weights, pancreas weights, and β-cell mass were analyzed using Student’s t-test with two tails and one-way
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ANOVA using Prism v. 6 (GraphPad Software, La Jolla, CA). Two-way repeated-measures ANOVA was performed using the *anova* function of STATA 12.1 (StataCorp, College Station, TX) with slide as the repeated measure. A standard two-way ANOVA was used to compare β-cell mass measured by automated vs. conventional method across three mouse strains. Post hoc t-tests were performed to examine pairwise differences among mouse strains. P values < 0.05 were considered significant.

RESULTS

New-generation slide scanning system allows rapid, high-resolution image acquisition of full pancreatic sections. To determine whether an automated slide scanning and analysis system can accurately assess β-cell mass, we used three mouse models with different islet phenotypes: C57Bl6/J controls and STZ-treated and leptin-deficient (*Leplob/ob*) mice. Body weights (15.1 ± 0.7 g, control; 11.8 ± 1.3 g, STZ-treated; and 31.9 ± 1.8 g, *Leplob/ob* mice) and wet pancreas weights (280 ± 21 mg, control; 182 ± 12 mg, STZ-treated; and 270 ± 12 mg, *Leplob/ob* mice) of all three cohorts were consistent with prior reports (1, 6, 7, 12). Pancreatic sections were immunolabeled and imaged at ×20 magnification using the workflow outlined in Fig. 1A. The mean and median sizes of sections were 113.6 and 113.7 mm², respectively, and the mean and median scan times per section were 73.49 and 75.2 s, respectively.

Classifier Macros automate image analysis of pancreatic sections. High-resolution images of pancreatic sections were used to design Classifier Macros for automated analysis of β-cell mass (Fig. 1B). Detailed instructions for building Classifier Macros are outlined in MATERIALS AND METHODS.

We reasoned that using pancreatic sections with islet features encompassing both normal islets and islets from STZ-treated mice would allow us to develop a generic pattern recognition macro sufficiently versatile for all three islet phenotypes. With this goal in mind, we selected P8 tissues for Genie algorithm training, since islets at this stage are small and β-cells are often not adjacent to each other, similar to those in STZ-treated animals. To ensure high resolution for single β-cell detection, this Macro was trained at ×20 magnification using a Genie Training Set Montage that contained 32 different slide/tissue features. The final Classifier Macro (Macro 1) correctly recognized all three classes of objects, Glass, Eosin, and DAB (Fig. 3, A–D'), and was able to detect individual β-cells (Fig. 3B'). We noted that, in some pancreatic sections from the STZ-treated group, small areas between β-cells were occasionally misclassified as Eosin, and glass in between eosin-stained tissue was occasionally misclassified as Eosin. However, this small variation in Macro 1 specificity was <10% and did not affect β-cell mass quantification in the STZ-treated cohort.

Initially, we thought that Macro 1 would be sufficiently sensitive and specific for all three types of tissues, but in *Leplob/ob* pancreas it was unable to detect the interior of some islets. This may have been due to larger islet size or the lower intensity of DAB staining in *Leplob/ob* islets that could be associated with decreased insulin content per β-cell (8). Because of this lower islet recognition by Macro 1 in *Leplob/ob* pancreas and memory limits imposed by the size of our Training Montage, we created Macro 2 (Fig. 3, E–F'). To improve the detection of *Leplob/ob* islets, we incorporated more slide/tissue features into the Genie Training Set Montage (total of 42 features; some examples shown in Fig. 2). Some of these features included lightly stained islets that are common to *Leplob/ob* pancreas (Fig. 2F). However, the larger Montage required decreasing the magnification for Macro training from ×20 to ×10 to balance image processing capacity and analysis speed. Although Macro 2 accurately classified normal and *Leplob/ob* islets (DAB), it was not suitable for detection of individual β-cells in STZ-treated animals due to the lower magnification selected for training. We also noted that the Training Macro frequently displayed a “halo” effect, where some pixels around islets in the adjacent exocrine tissue were classified as DAB (data not shown). This effect was minimized in the analysis by selecting DAB+ areas well within islet borders and adding this feature to the Genie Training Set Montage: see Fig. 2, I and J, for examples of interior outlining of islets that results in minimal haloing in Fig. 3, E–F'.

Because of the vast differences in islet morphological features between STZ-treated and *Leplob/ob* mice, we subsequently used Macro 1 for analysis of β-cell ablation and Macro 2 to measure β-cell hyperplasia. This approach ensured high fidelity of islet detection and analysis accuracy. In contrast, Macros 1 and 2 could be used interchangeably for analysis of β-cell mass in C57Bl6/J controls with <10% variation in the analysis results (data not shown).

Automated image analysis accurately measures β-cell mass. Once the design and testing of Macros 1 and 2 were successfully completed, we applied them to the measurement of tissue DAB% (Eq. 1) in control, STZ-treated, and *Leplob/ob* mice. Each pancreas was subjected to a systematic section sampling at 250-μm intervals throughout the tissue block (Fig. 1A). β-Cell mass was then calculated using Eq. 6.

We first examined the distribution of islets throughout the pancreas by plotting tissue DAB% in relationship to section depth within the pancreas. Our data indicate that tissue DAB% varied considerably with section depth in all three groups of mice (Fig. 4, A–C; note different scale on y-axis in panels A–C). A similar observation was reported by Chintinne at al. (5), who analyzed β-cell mass in adult rat pancreas. The average coefficient of variation was 45% in controls (range 20–77%), 46% in STZ-treated mice (range 20–85%), and 32% in *Leplob/ob* mice (range 20–41%). One outlier in the *Leplob/ob* group had tissue DAB% similar to that of controls (Fig. 4C). Despite this variation, tissue DAB% was significantly different among control, STZ-treated, and *Leplob/ob* mice at every tissue depth examined (Fig. 4D). The exception was the last portion of some tissue blocks (slide 8), where sections were available from only two to three animals per group (Fig. 4D).

Next, we validated our automated macro-based approach by comparing the automated and conventional morphometric analyses for the entire set of scanned images from each mouse. To test whether our macros produced significantly different pancreas DAB% measurements across all mice, we performed a two-way repeated-measures ANOVA, considering each slide/tissue section to be a repeated measure within each mouse (Fig. 5A). We modeled a *between* effect to compare our automated analysis vs. the conventional method. If our macros produced significantly different pancreas DAB% results across this diverse set of samples, we would see a statistically significant effect of the analysis choice; this effect was not significant (*P* = 0.6044). On the basis of these results, we found no
significant difference between our automated analysis and the conventional approach (Fig. 5A).

We also considered the β-cell mass computed across all mice and performed a two-way ANOVA. The effect of the analysis choice was not statistically significant (P = 0.1863), but, as expected, differences in β-cell mass across the three mouse strains were highly significant (P < 0.0001). We then performed pairwise post hoc t-tests between mouse strains for each method (Fig. 5B). Each pairwise comparison was statistically significant. Our data showed that, independently of the analysis method, diabetic STZ-treated mice had β-cell mass reduced by 90% compared with controls, whereas β-cell mass was increased approximately threefold in the leptin-deficient mice, which is consistent with previously published reports (1, 23, 28).

DISCUSSION

Defining how pancreatic β-cell mass relates to function requires accurate measurement of β-cell mass. This task has been challenging due to low abundance of islets and their heterogeneous distribution throughout the pancreas. Here, we show that these challenges can be overcome with a rapidly evolving virtual slide technology that provides automated high-resolution image acquisition and analysis of pancreatic sections.

Histological analysis of β-cell mass relies on the capability to image entire pancreatic sections. New-generation slide scanning systems, such as the one used in the current study, allow automation of this process by capturing images of entire tissue sections at high magnification (×20–40) with unprecedented speed and quality. For example, the imaging time required to generate our dataset from all three animal cohorts was only 2.5 h. Completing this task with conventional instrumentation would likely take several days if not weeks. In contrast to older scanners, new slide scanning at high magnification dramatically increased image resolution, allowing detection of individual β-cells. This is especially important for the monitoring...
of β-cell mass loss, which is frequently associated with significantly perturbed islet architecture.

In addition to new-generation slide scanners, virtual slide technology offers web-based digital slide management and, more importantly, new tools for automation of digital slide analysis. This automation is based on specialized algorithms for image segmentation and is further supported by batch processing of digital slides. Our data from three different strains of mice demonstrate that automated analysis of β-cell mass yields results that are highly consistent with those obtained by conventional morphometry and with previously published data (1, 23, 28).

To automate β-cell mass measurement, we used the pattern recognition algorithm because it is able to classify image components based on a number of features including morphology, color, and texture. This is a great advantage compared with a simple color deconvolution approach, since the pattern recognition algorithm can be trained to develop macros that can easily distinguish the staining in β-cells from the background in non-β-cell compartments such as red blood cells, connective tissue, or acinar tissue. We used DAB labeling for β-cell mass measurement, which is frequently associated with significant perturbations in islet architecture.

In conclusion, having a fast, reliable, and automated method for histological measurement of β-cell mass will greatly benefit analysis of islet/pancreas phenotypes and validation of new approaches and biomarkers for imaging of β-cell mass in vivo. Moreover, we envision that this methodology for automation of β-cell morphometry can be applied to the analysis of other islet endocrine cell types and human pancreatic specimens.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.L.G. and M.B. conception and design of research; M.L.G. and M.B. performance of research; M.L.G. and M.B. analysis and interpretation of data; M.L.G., W.S.B., and M.B. writing, editing, and critical revision of manuscript.

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