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Accuracy Evaluation of Langerhans Islet Volume Estimation from Microscopic Images

Master thesis

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DECLARATION

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Hanna Hlushak

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Master's Thesis title:

Accuracy evaluation of Langerhans islet volume estimation from microscopic images

Abstract:

For successful islet transplantation it is important to input sufficient quantity of islets, hence it is required to know the volume of the transplanted islets. Several methods for estimating the volume from 2D microscopy images are used (the Ricordi table, spherical and ellipsoid models). The aim of this work is to evaluate the accuracy of these methods. With the help of Optical Tomography microscope we made 401 projections of individual islets and created 3D models by image reconstruction. We calculated the individual islet volumes by the isotropic Fakir method and compared them with results from the evaluated 2D methods for 65 real islets and 35 synthetically generated islets. Ellipse fitting method for islet volume estimation performed by plugins of Fiji software was shown to be the most suitable among the evaluated 2D methods.

Key words:

Langerhans islets, volume estimation, OPT microscopy, 2D and 3D methods, synthetic islets.

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1. Introduction

Pancreatic islets (Langerhans islets) are clusters of thousands of hormone producing cells. They are located in the pancreas, one of the most important functions they provide is production of insulin, hormone which regulates metabolic processes in the body and regulates glucose level in blood. Moreover, Langerhans islets take 1-2% of the whole pancreas organ (Olsson and Carlsson 2011).

People with type 1 Diabetes mellitus lack β cells, responsible for producing of insulin, hence they are in need of exogenous sources of insulin (ex. injections). Constant high glucose level results in secondary complications, the most common are such as renal function, vision, cardio-vascular diseases, necrotic tissues. These patients are dependent on daily insulin injections, must constantly have their blood glucose level monitored during the day. The risk of hypoglycemia or hyperglycemia leads to physical and mental distress. All things considered, the quality of live is much affected by the disease.

For years, many of scientific studies and clinical trials were devoted to curing the type 1 DM and improving the life quality of diabetic patients offering different approaches (Fiorina et al. 2008). One of the most promising approaches is pancreatic islets transplantation, which nowadays is an outstanding alternative to the whole pancreatic organ transplant. (Fiorina et al. 2008) Its general idea is removing the donor's pancreas, after which it undergoes the process of digestion and harvesting. Further, purification of Langerhans islets is performed. The islets are then injected into the portal vein in the liver, where they after some time start functioning and recover the endocrine function (Shapiro et al. 2006)

From the technical point of view there are many parameters related to the transplanting procedure to be qualitatively defined and measured: size, distribution, volume, islet equivalent(IEQ), viability (Ricordi C. 1990) (Lehmann et al. 2007) (Buchwald et al. 2009). Volumetric measurements of the islets must be performed before the transplantation, since total volume of islets is one of the main parameters to determine the successful outcome of the procedure, it is important to put enough islets to produce sufficient amount of insulin. For the convenience of use, a concept of an islet equivalent (IEQ) was proposed and accepted as a normalized islet volume. (Ricordi C. 1990). Currently, several methods of volume approximation has been developed for the mentioned applications and are commonly used,

in the work we use those to evaluate the volumes of islets: 1.sheroid model, 2. ellipsoid model(Švihlík et al. 2014), 3. Ricordi table (Ricordi C. 1990).

These methods are proven to be useful algorithms of volumetric evaluation and are based on approximations of shapes from 2D images of pancreatic islets. Ricordi table is the method which gives approximate estimation of volume, based on relation of a certain range of islet diameter to its volumes. The method of sphere is based on diameter determining from the area of segmented islet (measured by pixels), then, volume is estimated by the diameter of sphere. Whereas in the second method ellipsoidal shape of an islet is considered, hence ellipse is fitted to each islet, major and minor semi axes are measured (Dvořák et al. 2016). For this purpose we used software Fiji (Appendix 1) and its plugins available on demand by contacting Professor Jan Kybic (Appendix 1), which automatically evaluate volumes deriving from 2D projections and measuring of islets diameters. Firstly, the islets must be sampled and 2D microscopy images of the islets taken, then the images are binarized and volumes are estimated by Fiji plugins (Švihlík et al. 2014).

In this study we concentrate on measuring of volumes of the human pancreatic islets by introducing volume evaluating method of the islets based on existing isotropic Fakir method (Kubínová and Janáček 1998) and using it as a reference to currently used methods to estimate sufficiency of evaluating volumes of Langerhans islets by these methods. The 3D method is based on using Fakir probes, which are virtual randomly oriented parallel lines. For evaluating of volume of an object, it's needed to measure the total length of intersections between the object and a probe (Kubinova et al. 2005) (Appendix 1).

By contrast with the 2D methods of ellipse and sphere, Fakir method is based on deriving volumes from 3D models, obtained by processing 2D multiple slices, projections of an islet. Optical projection tomography microscope was applied to pancreatic islets in situ before (Alanentalo et al. 2007), but we applied OPT to isolated human pancreatic islets by previously casting them. We used OPT microscopy technology for it allowed us to make a large number of projections of an islet from many angles. Then, using such software as ''NRecon'', designed for basic reconstruction of 3D models from obtained with OPT microscope, ''Data Viewer'', which is used for visualization of 3D reconstructed data in the forms of orthogonal projections, ''VolViewer'' for creating a visual 3D model of a Langerhans islet with functions of 'volume rendering' and maximal intensity projections (MIP) and possibility of turning,

zoom, multiple channels view, virtual slices, 3D measurements and more, we will observe a shape of islets. (Sources for downloading at Appendix 1).

Measuring islets volume with the help of Fakir method, we expect to obtain the high accuracy evaluations with relatively real volumes of the micro-organs and obtain values of volumes obtained by 2D spherical, ellipsoid and Ricordi table methods. We believe, the majority of pancreatic human islets possess rather flat and various irregular, than spherical shapes, hence the current methods based on 2D approximation might not estimate volumes of islets accurately enough. 3D Fakir method can help visualize islets and using it as a reference for 2D methods, estimate the difference and error of the methods.

The main idea of this research is to compare and improve volume evaluating methods of islets and to prove our suggestion the current methods mentioned above might give not give precise volume estimation of pancreatic micro-organ. Using more accurate tool for volume representation, based on OPT picture sequence 3D technology our goal is to estimate the difference between methods volumes. OPT microscopy allows to obtain the volumetric measures utmost approximate to real volumes, which is nearly impossible to obtain with current methods grounded on 2D model calculations, so it can be used as a reference for comparison of the methods.

The work's goals and hypotheses

The goals, which define the purpose of our work can be summarized as following:

1. Estimation of volumes by Fakir method, based on the reconstruction of 3D data from the OPT microscopy technology.
2. Measurement of the volume of islets by the currently used methods based on 2D data (Ricordi table, spherical, ellipsoid method);
3. Perform the comparison of Fakir and currently used methods and estimate an error of the evaluated methods based on 2D images with respect to Fakir estimation.

Hypotheses:

Since current methods do not show a precise approximation, our preliminary observations suggested that especially larger islets have rather flat then round shapes, therefore we assume that the volumes of islets is overestimated by currently used methods.

2. State-Of-The-Art

This chapter will provide a review of literature related to our study, current methods and technologies that are nowadays used for volumetric evaluation of Langerhans islets. We will give an overview to current methods of islet volume approximation which we use in our work, will shortly explain background literature needed to give a support to our work.

A key point for a successes in pancreatic islet transplantation is to transplant an appropriate quantity of islets. Hence the volume of the transplant is a significantly important parameter for outcome of the procedure (Ricordi C. 1990) . For the convenience of use, a concept of an islet equivalent (IEQ) was proposed and accepted as a normalized islet volume (Table 2.1) (Ricordi C. 1990). For instance, to fulfil pre-transplant criteria with respect to a total pellet volume of final preparation is <7 ml of tissue, and the necessary amount of islet equivalents per one kilogram of body weight is >5000 (Buchwald et al. 2009). Thus the right evaluation of amount and volumes of islets is of a great importance for satisfactory arrangement of preparation for the procedure (Shapiro et al. 2006).

2.1. Ricordi Method

Ricordi method was established as a gold standard for laboratories around the world to be a tool for islet quantification before transplantation, which for this purpose uses a microscope with a grid 0-400μm allowing to estimate the diameter of an islet, hence to derive volume out of the distribution of the size of diameters with the help of Ricordi table (Ricordi C. 1990). The diameters are counted manually with the help of a ruler, to give an illustration, Figure 2-1 represents a graft of islets, manually measured by medical experts.

The Ricordi procedure consists in considering islets as circles meaning spheroid shape and evaluating the radius, out of diameters from applied Ricordi table to a grid, hence converting radiuses trough mathematical equations to volumes (IEQ). The majority of islets do not possess a directly spherical shape, for this reason their approximate size is evaluated by an average diameter. The accepted Ricordi algorithm categorizes islets to a classes of diameters, applying 50μm diameter range step and relative conversion factors enable conversion of an each islet diameter value to a volume (Ricordi C. 1990)

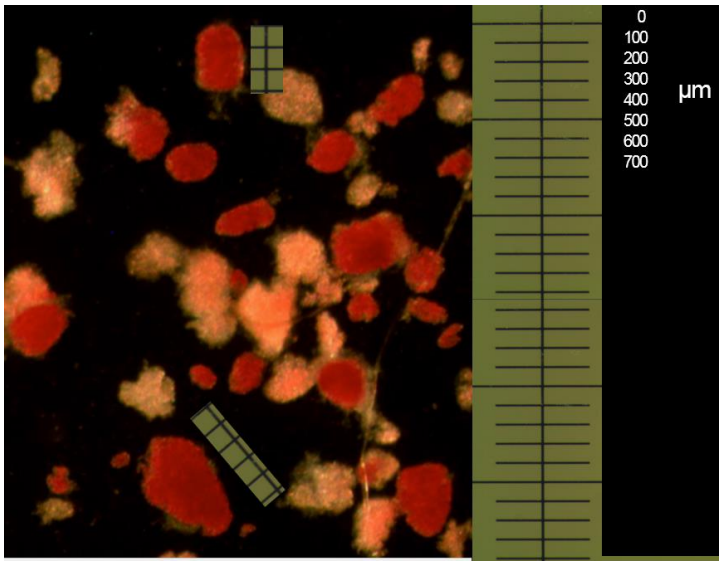


Figure 2.1: Islets, isolated from a cadaverous pancreas with elements of an exocrine tissue, on a standard microscope with 10x objective magnification, ruler of 0-700µm.

Normally, islets less than 50 µm in diameter are not taken in count. According to Ricordi algorithm conversion factors to turn each number of the classified islets N into volumes it is needed to use Table 2.1. With the help of the conversion number it becomes possible to turn islet number into IEQ value. Another key thing to remember is that Ricordi table diameter range ultimate value is 400µm, hence any islet with the diameter equal or larger than this value will have the volume of 27,979,808μ^3, which will not be true for all of the islets exceeding the ultimate diameter in the table.

Table 2.1: Represents the mean volume with respect to the certain diameter range and the conversion factor of islet of 150µm diameter(Ricordi C. 1990)

islet diameter range(µm)	mean volume (μm^3)	IEQ
50-100	294,525	n/6
100-150	1,145,373	n/1.5
150-200	2,977,968	n·1.7
200-250	6,185,010	n·3.5
250-300	11,159,198	n·6.3
300-350	18,293,231	n·10.4
350-400	27,979,808	n·15.8

As illustrated in the Table 2.1, islets of a diameter below 50 μm are not taken in count and the calculated islets diameters are divided into ranges. After that IEQs can be evaluated by following equation:

$$\mathrm{IEQ_{tot}} = \sum_{\mathrm{k}} N_k \lambda_k, \quad \mathrm{k} = 1,2,3,\ldots,\mathrm{K}, \tag{1}$$

Where N is a number of islet in the certain diameter range, K is a number of ranges, λ_k indicates the conversion number (Table 1.1) and can be calculated by (3):

$$\lambda_{\mathrm{k}} = \frac{\mathrm{V_m}}{\mathrm{V_{150}}} = \frac{\mathrm{V_k^{min}} + \mathrm{V_k^{max}}}{2 \cdot \mathrm{V_{150}}} = \frac{(\mathrm{d_k^{min}})^3 + (\mathrm{d_k^{max}})^3}{2 \cdot 150^3}, \quad \mathrm{k} = 1,2,3,\ldots,\mathrm{K}, \tag{2}$$

where $\mathrm{d_k^{min}}$ and $\mathrm{d_k^{max}}$ are the minimal and maximal diameter of a range, which derive $\mathrm{V_k^{min}}$ and $\mathrm{V_k^{max}}$ indicate minimal and maximal volumes respectively, $\mathrm{V_{150}}$ is a volume of an ideal normalized islets with 150 μm diameter and $\mathrm{V_m}$ is defined as a mean volume(Švihlík et al. 2014).

Taking in count, that from the equation of spherical volume of an individual islet:

$$V = \frac{4}{3} \cdot r^3 \pi, \tag{3}$$

Where *r* makes radius of a sphere, follows the volume of a 150 μm diameter islet, which constitutes 1,767,146 μm^3. Hence it is possible to count an IEQ value for each isle(Ramachandran, Huang, and Stehno-Bittel 2014):

$$IEQ = \frac{V}{1{,}767{,}146}. \tag{4}$$

2.2. Method of Spheres

The first approach of islets volumes approximation proposed by Ricordi is widely used nowadays, it approximates the parameters from 2D images of islets. The standard procedure bases on a using a model of an islet, considered to be perfectly spherical with the diameter of 150 μm, where its tissue volume is related to one IEQ via mathematical formulation(Ramachandran, Huang, and Stehno-Bittel 2014)

The spherical volume approximation method is a variation of Ricordi's method, which excludes the rough rounding of volumes limited by the table diameter ranges. Firstly the diameter of the islet is calculated (1), where an islet is considered as a circle:

$$d = 2\sqrt{p^2 \frac{A}{\pi}}[\mu m], \tag{5}$$

where p is a pixel size, if A is an area of a circle in pixels(Švihlík et al. 2014) (Dvořák et al. 2016). Then the volume of an islet is derived from a diameter of an islet and is considered a volume of sphere.

$$V = \frac{d^3}{6} \cdot \pi. \tag{6}$$

2.3. Method of Ellipsoids (Prolate Spheroid Model)

Preliminary studies has shown that pancreatic islets are not perfect spheres, the majority of islets in the natural state are more of an irregular and ellipsoidal (egg-shaped) prolate spheroid appearances than of spherical shapes (Ramachandran, Huang, and Stehno-Bittel 2014)(Buchwald et al. 2009) (Lehmann et al. 2007) (Niclauss et al. 2008).

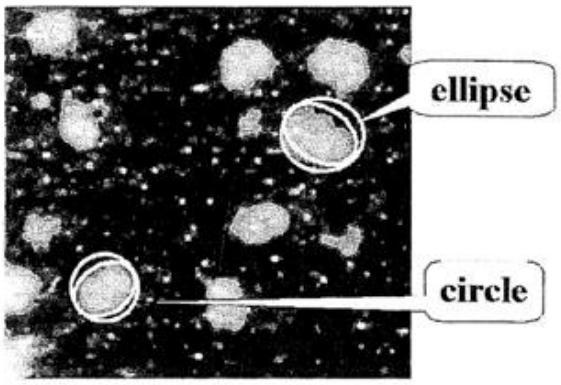


Figure 2.2: More appropriate shape model for an islet than a perfect circle is an ellipse (Girman 2003)

In the second approach, pancreatic islets in 2D plane images are assumed to be ellipses, rather than perfect circles, hence it is possible to evaluate the volume by rotating islet around its main axis and the longest length of an islet is a major axis of the ellipse (Niclauss et al. 2008)

Further, volumes are calculated respectively ellipsoidal (prolate spheroid) shape derived from the form of ellipse with the volume:

$$V = \frac{4}{3} \cdot a \cdot a \cdot b \cdot \pi, \tag{7}$$

where *a* is a major half axis and *b* is a minor one (Girman 2003)(Švihlík et al. 2014). Every islet we consider to be ellipse with one-half major axis *a*, and one-half minor axis *b* then the area of an ellipse is given by $A_e = \pi ab$. Instead of diameter in the case of circle, we use Ricordi's histogram of average length of ellipse's axes(Švihlík et al. 2014)

2.4. Fiji Software and Plugin for Analyzing 2D Images

Fiji is a software for biological image analysis, it is based on public domain software ImageJ, where everyone can edit, modify, create plugins, distribute files and so on. The plugins of ImageJ can be spread between users with the help of integrated system, where Fiji makes the implementation of new algorithms into plugins of ImageJ easier. That is to say, Fiji is a bridge between biology research and computer science (Schindelin et al. 2012).

Fiji concentrates on updating the architecture of ImageJ and allows developing of new solutions for biological image analysis. It introduces a database, various software libraries that allow to use the algorithms in purpose of creating practical tools for a certain biological image analysis issue. In Fiji, the basic codes can be used through many scripting languages, the algorithms are available to be exploited by any user, repeating improvements and updates reach the common base as soon as possible due to fast communication between developers and users (Schindelin et al. 2012)

Plugin for FIJI: Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py was designed by Dr. Jan Švihlík for analyzing of the segmented binarized images of pancreatic islets in Jython language. The basic work principle of the plugin, is that the shape of ellipse and a circle is fitted on each islet on the image of sampled islets and the estimated parameters, like diameter of a circle, length of major and minor axis in ellipse, help to measure the volume of the spheres and ellipsoids and calculate volume values. (Švihlík et al. 2014)

2.5. Fakir Method for 3D Volumetric Measurements

At the present time OPT (optical tomography) technology allows imaging, estimating, and comparison of structures and many other processes concerning a three-dimensional object, it has been used in such applications as measuring volume spaces and maximizing efficiency of output processes.

The OPT technique is able to evaluate geometrical parameters of structures in small objects, like islets, when microscope is taking projections through an object. OPT microscopy, obtains multiple image of sections of a 3D specimen.

Fakir method allows to create a set of virtual probes of freely oriented positions in space with a help of certain software and use it within the sliced images (Kubínová et al. 1999). Fakir isotropic methods is one of stereological methods of spatial volume estimation, which uses the probes. The main principle of this method consists in counting the intersections between the probes and an object, using spatial grids of points, lines and orientations it is possible to evaluate volume in 3D, as well as length and surface area (Kubinova et al. 2005)

2.5.1. Fakir Probes

Fakir probe is a set of parallel virtual lines going through structures of the observed object (Figure 2.3). To proceed the volumetric evaluation measurement of lengths of intercepts between the probe and structure of an object needs to be performed. An object (in our study the object is a Langerhans islet) is pierced with the fakir lines(Kubinova et al. 2005)

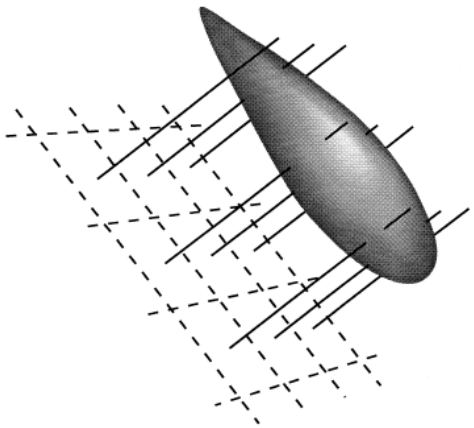


Figure 2.3: Fakir probe coming through a 3D model (Kubínová and Janáček 1998)

As illustrated in Figure 2.4,the fakir probes, partially shifted relatively each other, are mutually perpendicular and create a cubic spatial grid, this gives more efficiency because of negative covariance of projections of the surface comparing to a grid with not shifted probes(Kubinova et al. 2005) (Janacek 1999) , Figure 2.4:

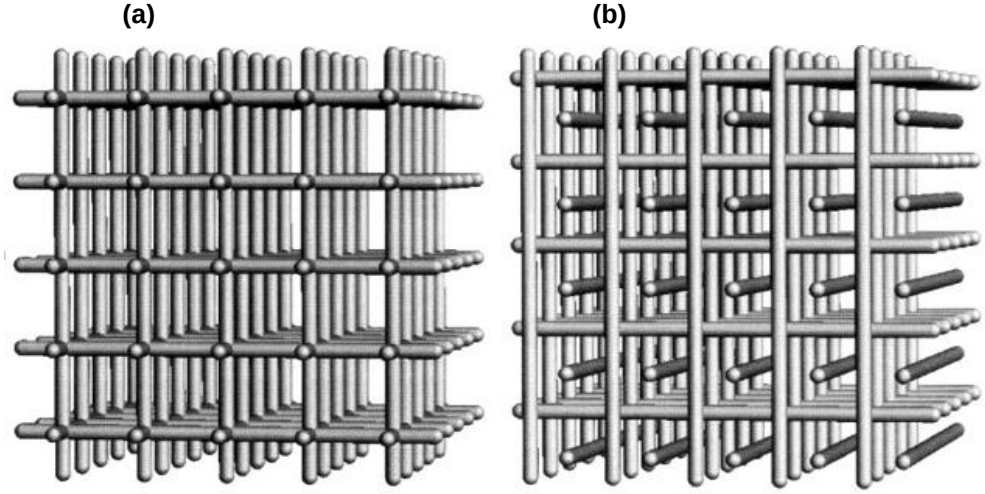


Figure 2.4: (a) Spatial grid of Sandau, (b) Fakir orthogonal probes(Kubínová et al. 1999)

Then, when the grid is settled randomly and is isotropic, for volume measurement of an object by the fakir probes, can be given the formula representing the volume as a sum of lengths of an object-probe intercepts:

$$est\ V = \frac{1}{3} \cdot u^2 \cdot (L_1 + L_2 + L_3) \tag{8}$$

L_m is the length of intercepts, *m=1,2,3* count of probes, *u* is the grid constant(interval between the grid's parallel lines) (Kubinova et al. 2005).

2.5.2. Fakir Software

The method of Fakir probes is implemented in a computer software Fakir (Appendix 1), which consists of several parts, where the one is made for generating of isotropic lines(probes) and another counts length of interceptions between the lines and structure of the observed objects (3.3.5), hence, there is no need to randomize the direction of section stacks as the program creates orthogonal triplet of fakir probes(OTSFP) (Kubínová and Janáček 1998).

This software generates an isotropic set of virtual fakir probes and so it is not necessary to randomize the direction of the stack of sections. The probes with the grid constant and rotation, by rotational vectors around three axis with relative angles are created in the program. The probes are labeled with different colors, depending on the current position of

the line probe, whether it is inside of the section or out of the plane (Figure 3.10). And the volume of the image is focused through by the probes and its intersections with surface in three planes, where program not only counts the intersections of each of the orthogonal triplets with matter, but also it measures length of those line intercepts, hence evaluating of volume of an object can be performed (Kubínová and Janáček 1998).

2.5.3. OPT Microscopy, Contributing to 3D Technology

3D imaging techniques develop rapidly nowadays, allowing to observe an object in details on a picture in 3 dimensional mode, which finds its use in medical, research, educational and other fields. One of the new moving forward techniques is optical projection tomography, combines tomographical sectioning and optical microscopy, which is applied to obtain measurements of objects, using 2D projections. OPT computes 3D volumes by the passing elementary particles (photons) through a measured object.

Optical projection tomography (OPT) microscopy permits 3D reconstruction and vitalization of small objects, whose aim is to obtain reconstructed 3D models cognate to the object's real shape (Sharpe 2003) Among such techniques for 3D imaging as CT, who implements measurement of big objects and confocal microscopy for a very small objects, OPT finds a place in the middle, where the specimen's size ranges from about 0.5mm -15mm (Swoger and Sharpe 2002). An important key to remember, OPT microscopy scanner principle bases on maximizing depth of focus through a specimen and its rotating, by contrast, in confocal microscopy another strategy of reducing depth of focus is used to specify an exact depth in the tissue, to that end, OPT obtains images with the overlook of the inside of a specimen (Sharpe 2003).

2.5.4. OPT Milano

Optical projection tomography microscope 'OPT Milano' was made in collaboration with Polytechnic University of Milan by Andrea Bassi. The microscope can be used for imaging of a very small objects and further reconstruction of 3D models out of the taken projections by OPT Milano, like Langerhans islets and its benefit is more qualitative telecentric optics and also high sensibility of camera, providing high resolution images of projections. It has one disadvantage related to optics, as it does not have the possibility of zooming, so it is needed to change the objectives when bigger size object is being taken projections from. But

in this study we were reconstructing only samples of the small size, Langerhans micro-organs, and no switching of the objective was needed (See more detailed information in Appendix 2).

2.6. Software for 2D to 3D Transformation

The set of images acquired from OPT microscope is needed to be processed, further reconstructed and viewed, which can be done with the help of computer software for images reconstruction, to create a cross section slices of the sample. One of the software used for reconstructions of 3D images from the obtained projections is NRecon made by SkyscanNV, which makes a set of corrections, alignments, reconstructions and more.

2.6.1. NRecon

In the SkyScan's volumetric reconstruction software NRecon projections, which are obtained through rotating of a sample at a certain angle are used to create a cross section images of an observed object, it performs 3D data reconstruction from the projections. The program is divided in two parts, where the first is NRecon, which is a user friendly interface and the other is NRecon Server, the tool and of the program (Skyscan NV 2011).

The main steps of the process of reconstruction are loading the set of data and beginning at the Start page, its functions are: 'preview', where parameters are set and modified, 'fine tuning', which lunches multiple previews for the ease of the adjustments in the preview mode, 'start' for starting the reconstruction and checking the space on the disk and 'add to the batch', which stocks the orders to perform the reconstruction later. The projections can be viewed due to the navigation mode of the display (Skyscan NV 2011).

The parameters are to be managed at Settings page, meanwhile the program remains in the navigation mode to review the adjustments, at the Output page the preview of having reconstructed slice is performed, and the parameters of the final image can be adjusted and viewed at the previewing mode, whereas Advanced page gives an additional possibility to modify the parameters and back at the starting page full reconstruction is being performed or directed to a batch manager (Skyscan NV 2011)

After the reconstruction is done and the 3D reconstructed images are obtained, it can be visualized by Data Viewer software in shapes of orthogonal projections.

2.6.2. Data Viewer

Data Viewer by SkyScan enables the visualization of the reconstructed set of images, hence helps to review the slices, which are shown as a slice sequence movie or as three orthogonal sections, inside of the reconstructed space the images are centered. Hence, the program allows viewing the intersection of three orthogonal sections and turning it all together or separately each slice with the help of mouse control, in the viewing mode. In other words, Data Viewer is a tool for the users and researchers, which is used for the visualization of the data output representing the previously collected data from the sample observation (Skyscan n.d.).

Such extra parameters as smoothing, saving the data in different planes, computing distances and intensities are available in Data Viewer. In order of the user convenience, conversion between the image formats JPEG, BMP, TIFFs is performed along with several adjustments like color, naming, size changing, mixing and rearrangement of the images. All the mentioned functions and options help managing and perceiving the data sets, that were collected (Skyscan n.d.).

2.6.3. VolViewer

The process of reconstruction of the obtained images from OPT microscope is followed by viewing it in volumetric and sectional planes, it can be done with a software VolViewer (Bangham laboratory). The program has several main properties and functions for creating a 3D view of the specimen, to describe VolViewer better: it allows real time volume rendering with the algorithm of 3D slicing, as well as real time per channel brightness and contrast and thresholding operators, it has an option of independent adjustment of volume vision and intensity of up to three data channels, the 3D reconstructed specimen can be viewed in any position and orientation and have 3D parameters measured, filtered and segmented, the surface area can be computed and smoothed. The program makes the function of real-time cutting possible and the specimen can be clipped at any place and position, the local illumination measured by gradient computation. After the object is viewed, the movie or animation of its rotation can be exported for further use. The important features of VolViewer also include stereo rendering and inscription of the interface to other programs as for instance is Mathlab (Appendix 1, Software).

3. Methods

The experiments were performed to evaluate the volumes of islets for 2D and 3D data. In this chapter all the steps about how the procedure was done and for what reason the exact step was chosen will be described in details in order to give an understanding of the carried out procedures concerning volume estimation.

3.1. Overview of the Experiments

For the performing of the islets volumes estimation it was needed to arrange the preparation of imaged material. Hence the islets underwent the procedure of isolation from cadaver (by medical experts), further we proceeded with purification and staining of the micro-organs which will allow for them to be visible enough for the measurements.

The first experiment was aimed on the estimation of Langerhans islets volumes from the OPT microscope outcome. It took place at the laboratory Czech Academy of Science, at the Institute of Physiology Biomathematics Department where the Optical Projection Tomography is situated. The OPT microscope was set on making 401 projections of each of the 100 islets, with the help of two Stages and Tomos software, which align the islet respectively its axis and a camera. Further procedure includes the processing of images obtained and reconstructing them, further by the Fakir probes software the volumes of the reconstructed data measures the volume of the islet (3.3).

The second experiment is about evaluating volumes of the pancreatic islets outgoing from 2D images. This procedure consists of capturing the images by the camera of inverted or stereo microscope, the next step is segmentation of the islets and, using FIGI software implemented plugin of 2M_Binary_140430.py for segmentation and with the help of a plugin Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py, proceed with the volume estimation.

3.2. Islet Preparation and 2D Images Acquisition

To be capable of observing and obtaining 2D images of the Langerhans islets, the micro-organs must be isolated from pancreas. The process of separating of pancreatic islets starts with removing the pancreas from the donor (cadaver). The further process of islet isolation proceeds with digestion in the chemical solution (collagenase), centrifugation and purifying, which can be done only by medical experts.

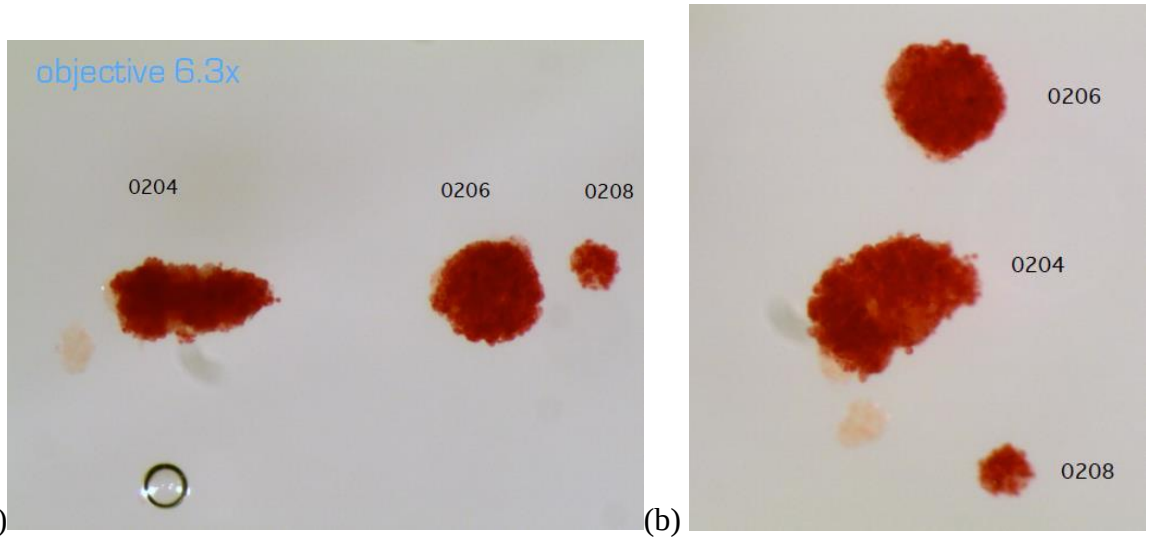
In the laboratory of IKEM, Prague, the isolation of human cadaverous pancreatic islets was

carried out. 100 human islets were isolated from 6 donors (cadavers) by medical experts, during the period of about 6 months. The isolation was carried out randomly, depending on a donor material supply. The time between pancreas isolating and islets imaging was about 10-24 hours. From each donor 5 - 27 islets were sampled.

After the procedure of islet isolation, the pancreatic islets undergo staining to differentiate them from surrounding tissue, when they still contain some endocrine tissue and possess white color. With the help of Dithizone solution the islets obtain red color, which visualizes and utmostly defines isles from other remaining tissues (see Appendix 1, used materials).

In the solution of 10 - 20 islets in 100 µL culture media just removed from incubator 50 µL of DTZ was added, gently swirled and after 30 seconds at a room temperature we could observe the color change. The 4µL HBSS/Albumin is added for preventing islets of being stuck on the plastic surface of the dish (Ricordi C. 1990).

The islets were distributed on the grid in the semblance of red dots. For the imaging of the islets we picked several islets (1 to 3 islets) transferring those to a separate dish with 4 mL HBSS/Albumin and placing it in the middle of the stereo or inverted microscope, equipped with camera. The images were acquired after adjustments of focus and various magnification. We captured from 1-3 islets on each image for further convenience of processing data. The illustration of the islets see Figure 3.1:



(a) (b)

Figure 3.1: Human pancreatic islets number 0204, 0206, 0208 (where 2 is a donor number) on the stereo microscope SZ60, IKEM laboratory.

3.2.1. Islet Casting

After the acquiring 2D data to proceed with 3D imaging at OPT microscope each islet has to be casted. OPT imaging requires the specimen to be casted in gel or to be placed in liquid for the purpose of making the projections and convenient access view at the specimen. However, the casted specimen must be also put in the fluid with the same refraction index as the specimen, to reduce the light refraction throughout the islet, so the light beams pass through in straight lines.

To caste our islets within Agarose gel for the further ability to use it at OPT microscope, the islet was suspended in about 3 cm of gel. For this purpose we used an ordinary syringe of 0.5 cm diameter with inclined sharp cut off the bottom outlet. 500 μL of Agarose melted and kept at 37ºC temperature was poured in the syringe by the pipette with as well cut wider tip, moving plunger downwards to fill it from the cut off part as the plunger moves the opposite direction. Using another pipette with smaller volume of the tip, 1- 3 islets were picked from the graft of the separated islets and slowly inserted into still liquid gel. All this actions have to be performed as fast as possible until the gel hardens, also avoiding bubbles inside the gel is of a big importance, for the reason of the clearness field of view is needed for OPT qualitative projections, in the opposite case the sample can be not be used and must be wasted. The syringes were then kept in cold environment in order to jellify again.

3.3. Fakir Volume Estimation After OPT Imaging

The experiment was performed to evaluate the accuracy of currently used methods which derive volumes using 2D images of the islets. It uses 3D models of the islets reconstructed from 2D projections of OPT Milano microscope.

After the syringes are ready and the islets are casted, they need to be taken projections of on the OPT microscope as soon as possible since while the pancreatic islets are dying they absorb the liquid from the surrounding gel, hence as a result enlargement in size occurs and the volume of the islet changes, consequently outcomes of measurements are not precise.

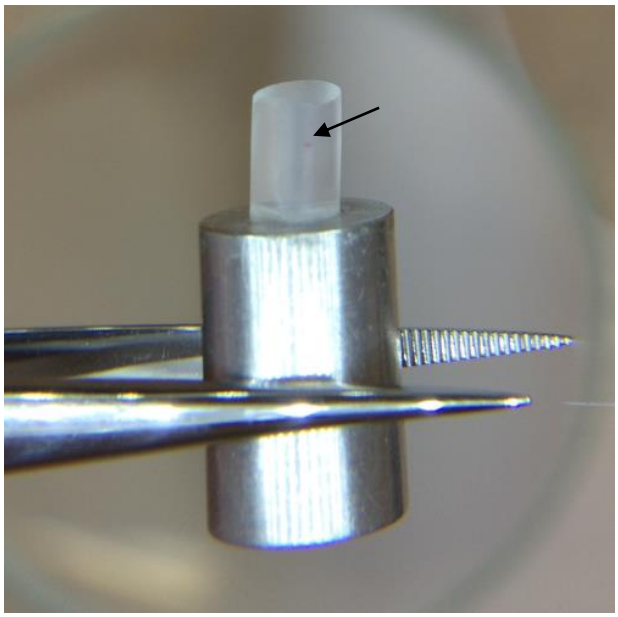
3.3.1. Adjusting of an Islet on OPT System

The islets in solidified gel were taken from the syringe. While the content of one syringe was getting prepared for the OPT imaging, the rest were kept in the fridge as the cool environment prevents the process of volume change of the islets. The cast of one syringe was taken out and

cut accordingly to appropriate size for adjusting it on the aluminum base of the holder of OPT microscope.

On the Figure 3.2 an example of the islet, which was casted in agarose is shown. The casted specimen was attached to an aluminum plate with the help of glue and left to harden and attach to the base better for about 20 minutes, importantly, the casted islet must be attached to it in order not to fall into liquid the specimen put into during the projection acquiring. The glue mass must be sufficient enough to attach the agarose cast, but not preventing the field of view through the islet, as the overdose might cause the glue and agarose gel amalgamating and distorting of the images, thus it is a necessary condition for the high quality images output. The clearer is the gel, without any dust or particles contain, the better are the acquired projections.

The rotating holder is shown on the Figure 3.3, where the quartz cell is filled with matching refractive index fluid and the casted islet is dipped into the liquid.



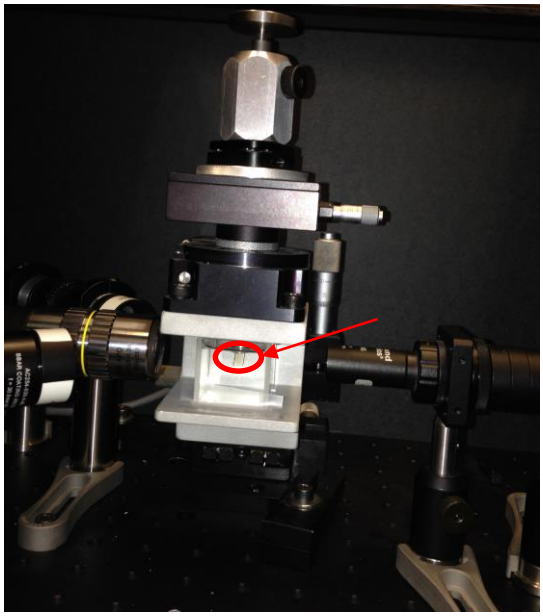


Figure 3.2: Agarose casted islet (islet is shown with the arrow) on the aluminum base of OPT microscope holder.

Figure 3.3: The casted islet on the aluminum plate is attached to the rotational holder and dipped into liquid container.

3.3.2. Islet Centering

The alignment of the sample around the rotation axis according to the instructions for the proper acquisition of projections was performed with the help of Tomos software, which enables visualization of an islet, allowing to rotate it in a life mode and make the sequence of projections. Figure 3.4 shows the operational computer, which was used to manage OPT system, and the Tomos interface with the possibility of live imaging adjustments during acquisition:

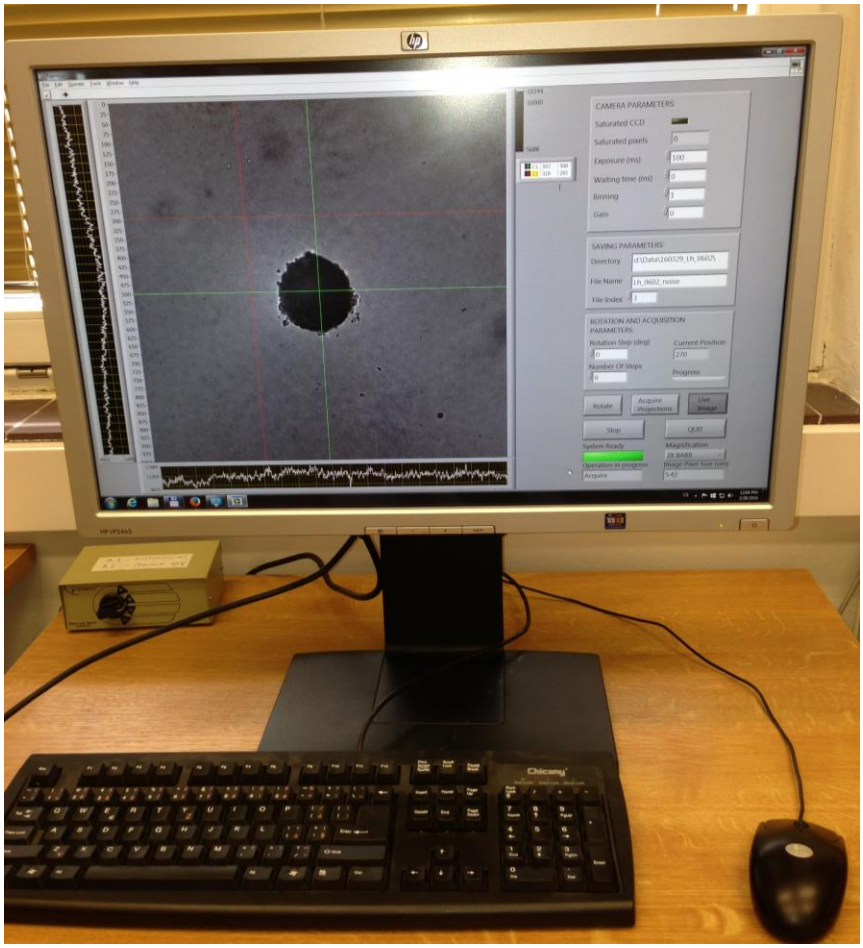


Figure 3.4: Tomos software interface for visualizing and adjusting the islet in life mode before acquiring projections.

According to the instruction from 'Documentary guide' of OPT Milano several steps were made for aligning the islet around the rotational axis:

- One of the axis of the 2 axis Stage arranged parallel and another perpendicularly to the optical axis with the help of "Rotate" command;, which will rotate the islet according to the value of "Rotation step(deg)" which was set on 0.9°;

- "Living image command' chosen to see the islet I real time;
- 3-axis Stage xyz was used for the islet centering and focusing in the camera view field;
- marked the starting position of the islet center with a yellow line on the life image;
- rotating the sample at 180° and obtaining the final position of the sample center;
- moved the sample using 2-axis Stage, translating the axis perpendicular to the optical axis until the center was shifted to the middle in between the final and starting (yellow line) positions;
- Repeat the steps until the centers of the sample are nearly the same in the final and starting position;
- Again rotated by 90°;
- Repeated the procedure several times till the islet rotated around its axis for all 180°.

3.3.3. Acquisition of 401 Projections

Further the rotation of the islet is performed on the holder and the rotation is possible on 360°, with the help of 2-axis Stage the sample was centered on the axis of rotation and 3-axis Stage using translation centers the islet on the camera and moved it to the field of its view. OPT microscope (Appendix 2) uses visible light to illuminate the specimen, while rotation of the islet on 0.9° step, for each turning the set of projection was obtained in the amount of 401 images, which takes up to 1 minute. What takes the longest time is a preparation and centering of the islet, rather than obtaining the projections.

The process of the projection acquisition resembles with the process of CT imaging, where OPT uses visible light instead of x-rays, as the islet in the cast is almost transparent, visible light can easily go through and the projection of a slice can be obtained. In OPT Milano laser diodes LEDs were used as source of excitation, with available wavelengths 405nm, 470nm and 625nm.

3.3.4. OPT Milano Data Reconstruction

The process of reconstruction of the islet projections was performed: after the projections were obtained, they were directed to a chosen folder of the other computer, which has a fast time of processing and possesses a big hard drive, as it needs to have a lot of memory. While normally most images use 8 bits, for the purpose of further editing is better to use 16 bits, as it possesses more details, wider gray scale and the islet tissue can be visualized better.

During the reconstruction it was needed to perform correction of background, "shift" correction and reconstruct 3D data from the obtained images, using NRecon software. Table 1.2 is shown the description of the detailed steps which was needed to use to go all the way through from the data obtained from OPT to reconstructed images, with further 3D visualization of a Langerhans islet.

Table 3.1: OPT Milano data reconstruction steps

1. Background correction. Using Fiji Macro Background correction OPT: a. Select the 'First projection' of dataset; b. Select 'Background image'; c. Select the 'Noise' image.
2. Check the background correction in Zoner Photostudio Pro (folder 'Corrected').
3. 'Shift' correction: a. In Total Commander inside the folder 'Corrected' create a new 'Shift' folder; b. Copy the first and the last image from the data set to this folder; c. Run Fiji: File- Import –Image Sequence- Images from folder 'Shift'; d. Compare the two images at the highest zoom and find the shift(in pixels); e. Apply the shift on the data set: Import- Image Sequence- 401 images of the data set- Plugins- OPT Stack Sample Fall Correction; f. Create new folder 'Shift_correction' and copy log file into it ; g. Copy the name of the log file to Fiji- Save as Image Sequence.
4. Rewrite the tiffs from Fiji in Zoner software (NRecom cannot read *.tiff* from Fiji).
5. Open the data set in NRecon, check misalignment compensation.
6. Make preview, Set ROI(region of interest), create a new folder 'Rec_LH_xx), restrict reconstructed area, press "Start"
7. Start DataViewer, check the reconstruction and orthogonal slices.

The background correction was necessary to perform for making the background more uniform, so the islet can be defined better on the image, during the correction background signal is subtracted from measured signal and performs correction of inhomogeneity in illumination.

The ‘Shift correction’ was made to correct possible shift of the islet in the cast of the gel while obtaining projections, starting image relatively the last. It shows the shift occurred in the particular islet, it can be visible by eye while zooming the image, normally about few pixels. Fiji plugin ‘OPT Stack Sample Fall Correction’ was applied to align the islet projections on the same level, so the first and the last images are not shifted each respectively other.

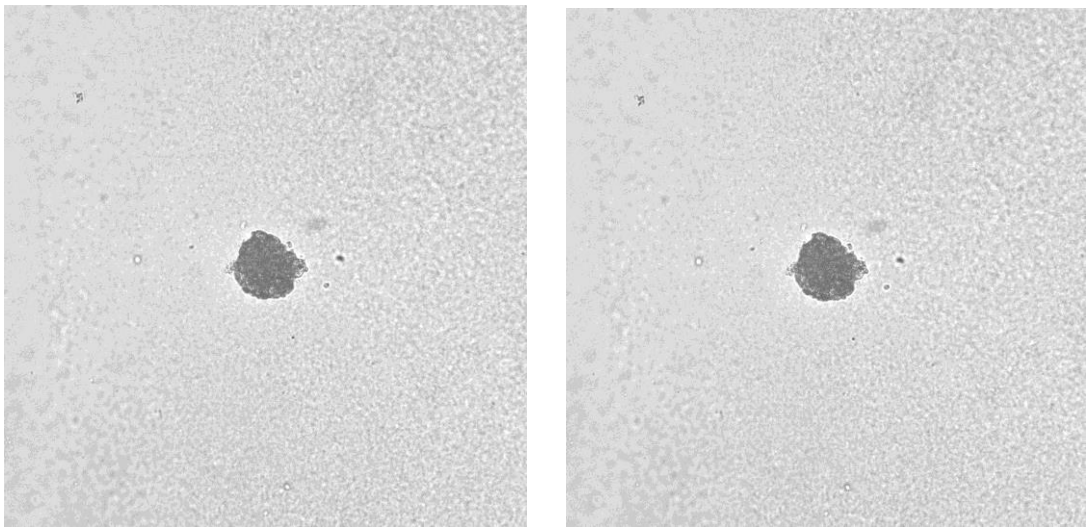


Figure 3.5: Langerhans islets projections named Lh_0601_0_0000 and Lh_0601_0_0400, where 0000 is the first projection and 0400 is the last one. The difference can be slightly visible, about 2 pixels shift occurred during the rotation and acquiring projections. The shift can occur due to different factors, like changing of the gel density, absorbing of liquid from the gel by the islet, so the correction gets rid of the small defect and the reconstruction of 3D volume of the islet is proceeded with more accuracy.

After the reconstruction, the projections of an islet taken at OPT microscope are put together to form a stack of images and the islet can be visualized as orthogonal projections, which allows to go through the slices of the islet and which can be viewed in DataViewer.

DataViewer software shows the orthogonal slices of the pancreatic islet, where it can be shown in 2D (Figure 3.6) and in 3D modes. In 3D mode view the islet can be viewed from three positions: X, Y, Z (blue, green and red lined on the Figure 3.7). All 401 of the reconstructed images create the scene of the whole islet and it can be viewed through, the islet color is defined by grey scale from 0 to 255 level.

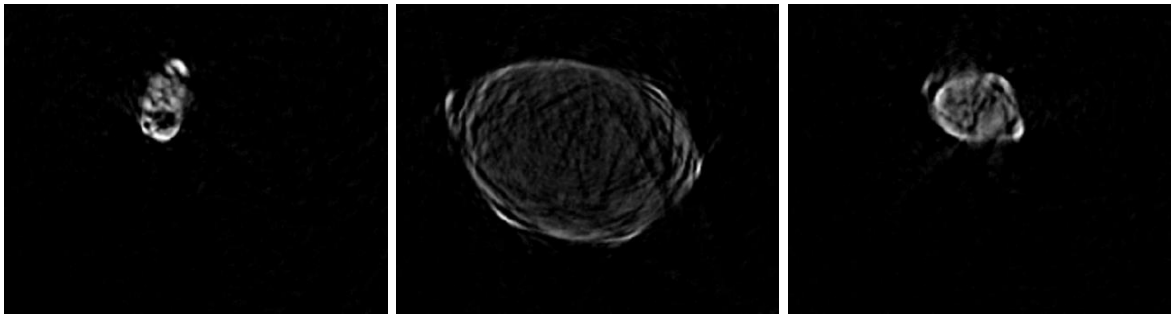


Figure 3.6: Lh_0601_0_rec islet projections in DataViewer, 2D mode, going through the islet along Z axis.

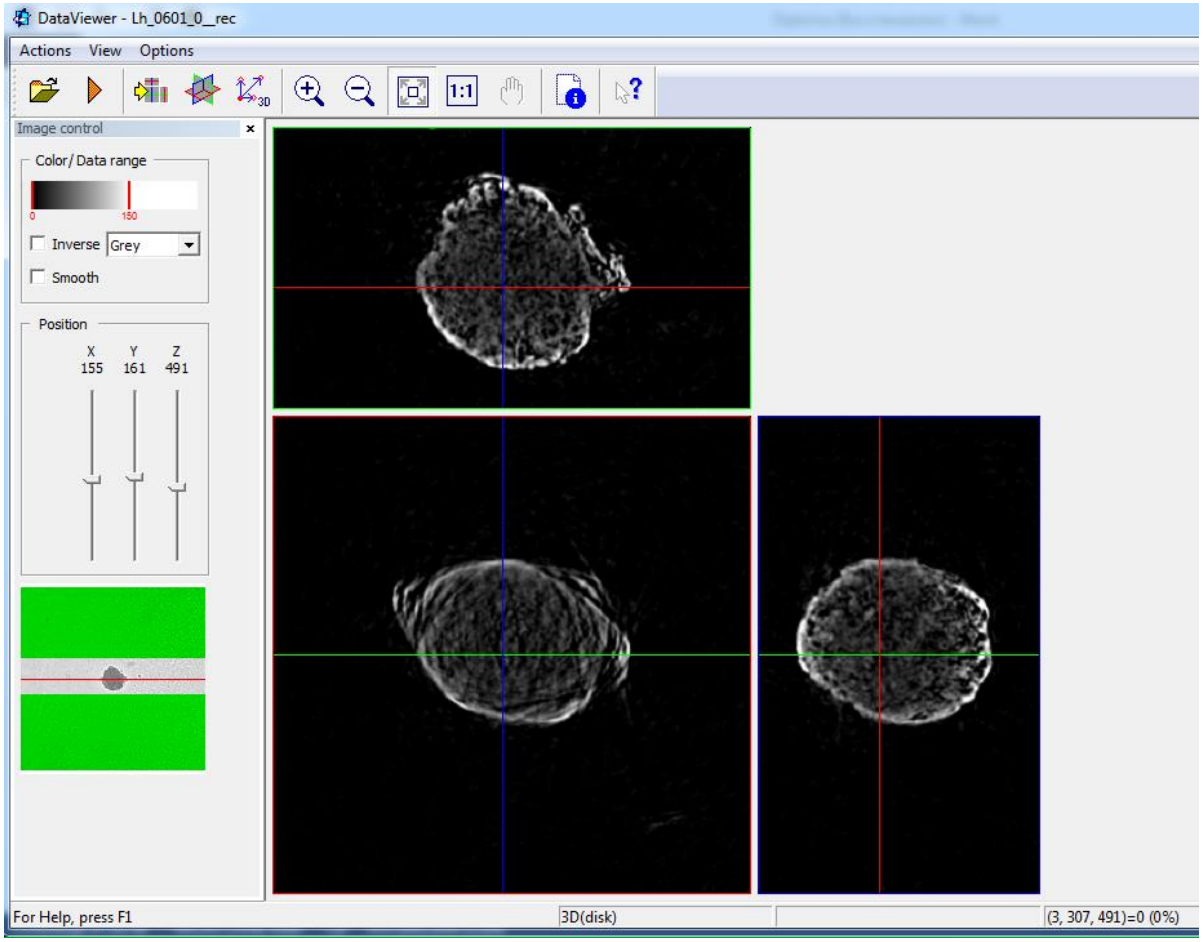


Figure 3.7: 3D reconstruction of Lh_06011_1_rec islet projections in DataViewer software.

3.3.5. Volume Estimation by Fakir Probes Method (Fakir Software)

After the process of reconstruction was carried out and we got all the reconstructed images with the corrected background and shift correction and were checked in DataViewer, we proceeded with volume estimation of the islets with the Fakir tool.

Based on the principle of the Fakir probes (2.4.1), Fakir software is able to evaluate the volume of an islet it using the reconstructed 3D data.

The linear probes are formed in the triple orthogonal grid. The volume of the islet is estimated by the computing the total length of the grid intercepts between the grid and the islet, the more detailed formulation of the total lengths evaluation was given earlier in the State of the Art part of the work (Kubínová et al. 1999). To give the illustration, the grids are shown on the Figure 3.8:

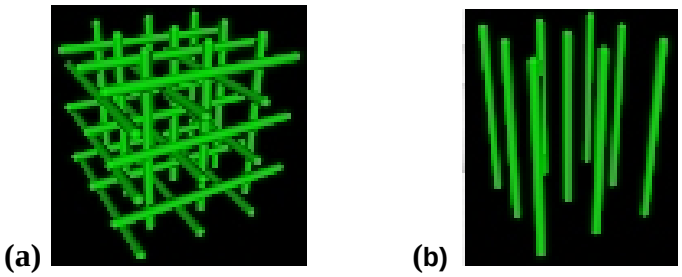


Figure 3.8: The threefold grid (a) made of the parallel line, Fakir probes shown as (b) http://www2.biomed.cas.cz/~janacek/fakir/3dtools.htm

Choosing of the calibration is an important thing to define, in our case the calibration was chosen 1.04785, which serves as a reference for the volume estimation, reference was measured from a glass slide, the step number which was varying (20, 30, 50, 100), defines the density of the spatial grids, depending on the islet size (Figure 3.9).

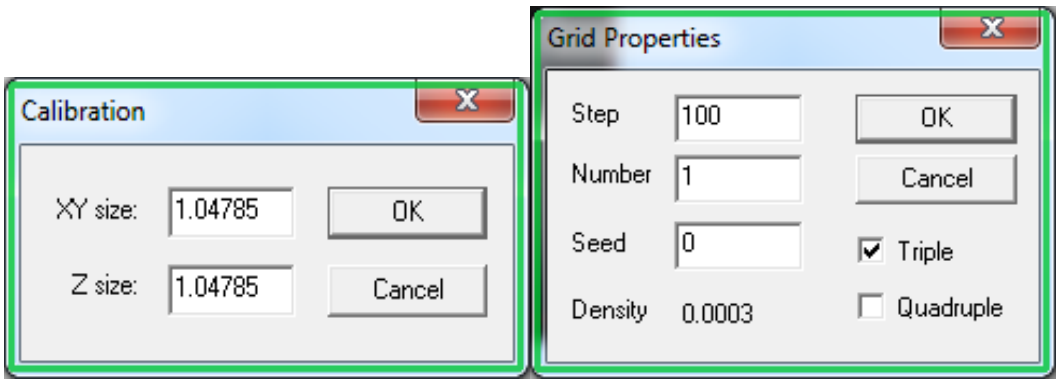


Figure 3.9: The 'Calibration' and 'Grid properties' options in 'Fakir'.

Three windows, which represent different planes are responsible for three grids. On the Figure 1-17 the estimation of islet Lh_0601 volume is illustrated. On the picture, the red lines are different axis, depending on a plane. The green squares are the Fakir probes which go

throughout the object. While operating a particular window, the program shows which grid it is and the number of probes used.

When the probe intercepts the islet surface it changes it color and it means it is situated inside, after it goes out it changes the color back, the program measures the total length of this interceptions to estimate the volume and the amount of the interceptions estimates the area. Probes go through the islet while zooming, going through all the 401 slices of the islet. The process of volume estimation in 1 islet takes about 10-15 minutes.

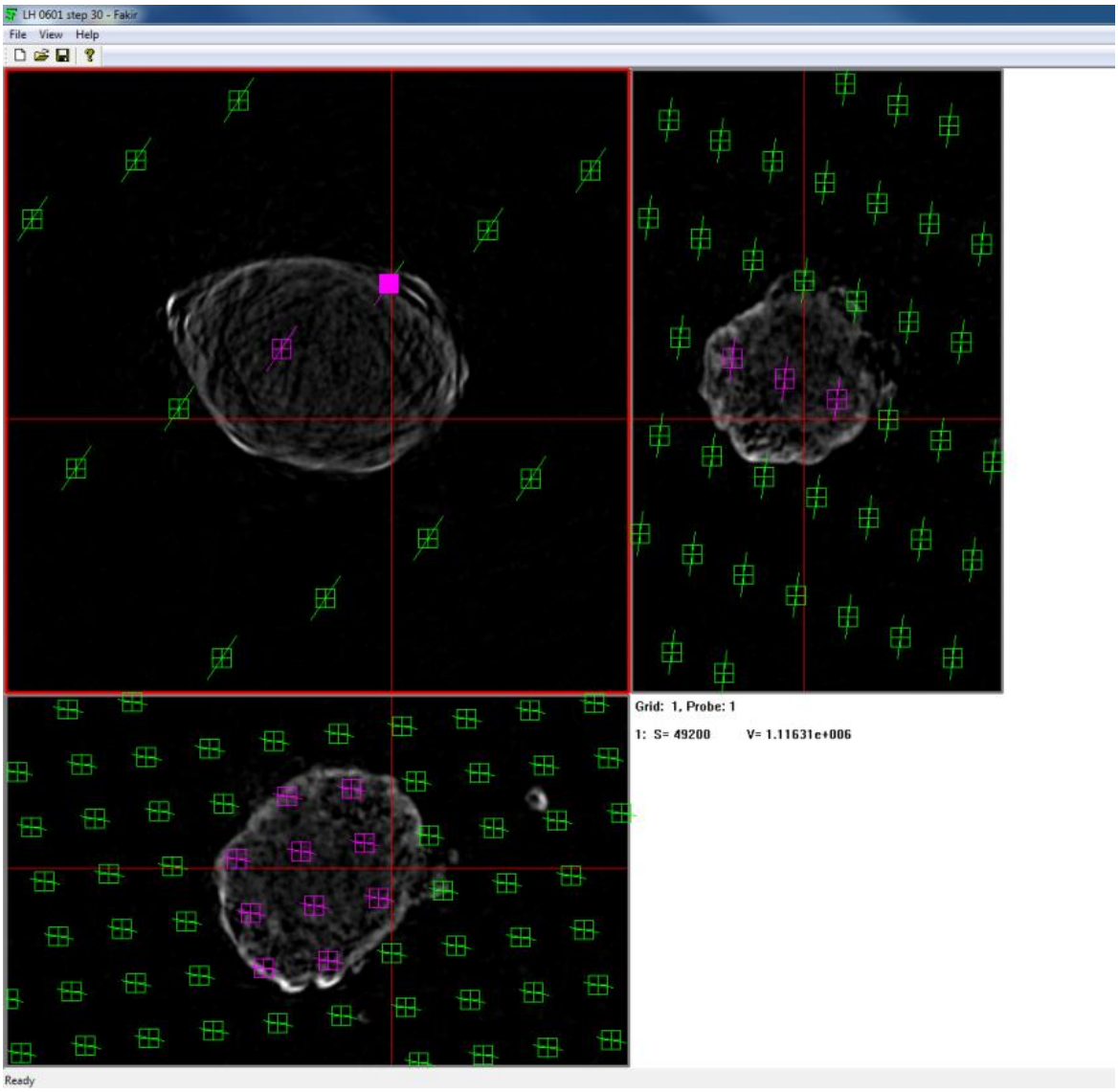


Figure 3.10: Process of volume estimation of Lh_0601 islet with Fakir probes.

After all probes of the three planes are measured the values can be exported in a text note and the measurements are saved in file with *.grd* extension, to be able to review the process again it is possible to Open the saved *.grd* file in the Fakir. For visualization of the reconstructed data, it was opened in the VolViewer software, which allowed to see the islets

in 3D view, to perform rotation along various axis and planes, so each islet could be observed from different sides. On Figure 3.11 the islet Lh_0601 is being rotated in VolViewer:

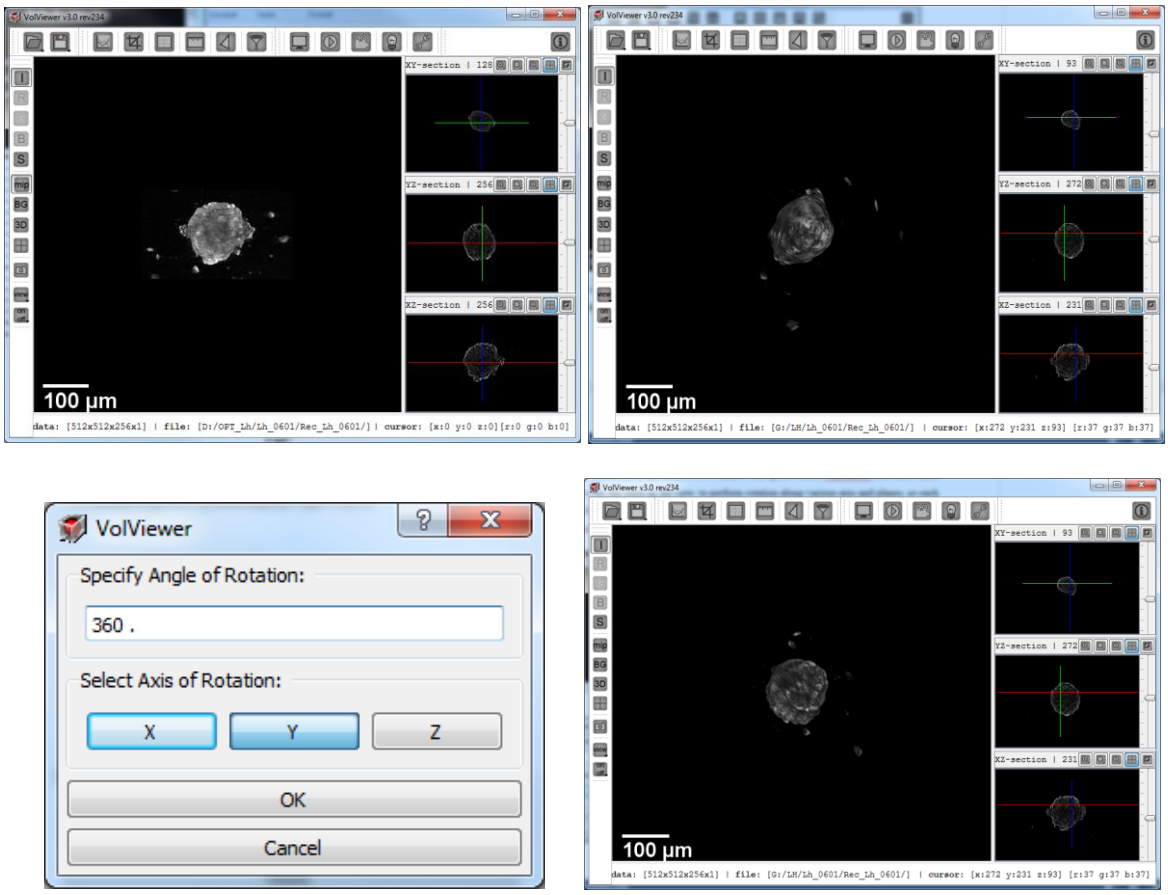


Figure 3.11: Islet Lh_0601 in VolViewer software being observed in 3D view. It was rotated at 360° along XY axis.

The volumes of 72 islets were estimated in Fakir and saved for the ease of use in Excel table. So each islet was named Lh_XY, Lh meaning islets of Langerhans, X was responding to a donor number and Y was a number of an islets as they were sequentially processed. Because of the lack of donors 72 human islets were evaluated, out of which 2D microscopic images of 7 islets were lost in the process or were inappropriate for the qualitative evaluation. To fulfill the experiments of the 100 islets, we used 35 synthetically created islet shape models, with known 3D volumes (code for generating the islets in MATLAB available on CD).

3.4. Volume Estimation for 2D Microscopic Images

This experiment was performed to evaluate volumes of the 100 islets by methods of circular and ellipsoidal approximation, and table of Ricordi, which are currently widely used. The purpose is comparison of the results with the Fakir method to see the sufficiency of the currently used methods.

For the experiment we used 2D images of the Dithisone stained islets captured by camera of stereomicroscope. Each image contained 1-3 islets, each islet on 2D image got its number after it was casted in the syringe, which also had 1-3 islets inside and processed on OPT Milano microscope to make sure each islet on 2D image responds to the same number of a 3D reconstructed islet.

To estimate the volume of the islets from 2D images we used plugins implemented in Fiji software (Švihlík et al. 2014), where the islets are considered to have a circular or ellipsoidal shape, therefore volume is derived according to this approximation as it given more detailed explanation and illustrated at (2.2) (Girman 2003). The other 2D method is a Ricordi method, which uses a table for volume converting from islet diameter (2.3.1-2.3.3). We performed also manual approximation of volume estimation (sphere, ellipse fitting) based on pixel counting (3.4.2).

The plugin of Fiji Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py estimates only binarized (black and white colored) images. So the process of images binarization has to be performed before operating the Islet_Analyzer. The other plugin 2M_Binary_140430.py does the transformation of islet color.

3.4.1. Estimating Islets Volume by Fiji (Method of Spheres and Ellipsoids)

The following steps had to be accomplished to estimate the volumes of 100 islets out of its 2D images:

1. Obtaining the 2D after the islets were isolated and stained. The islets on the images were given numbers according to their OPT imaging numbers. For this purpose, we used stereo-microscope and inverted microscope (Appendix 1). Both types are routinely used for islet quantification in various islet laboratories. The inverted microscope was preferably used in the dark filed mode in order to better distinguish islets from non-islet tissue in the Dithisone staining, for stereo microscope we used light background. The magnification of

objective was marked on each image, for the further estimation of islets volume in proper scale. To give the example of the 2D pictures, Figure 3.12:

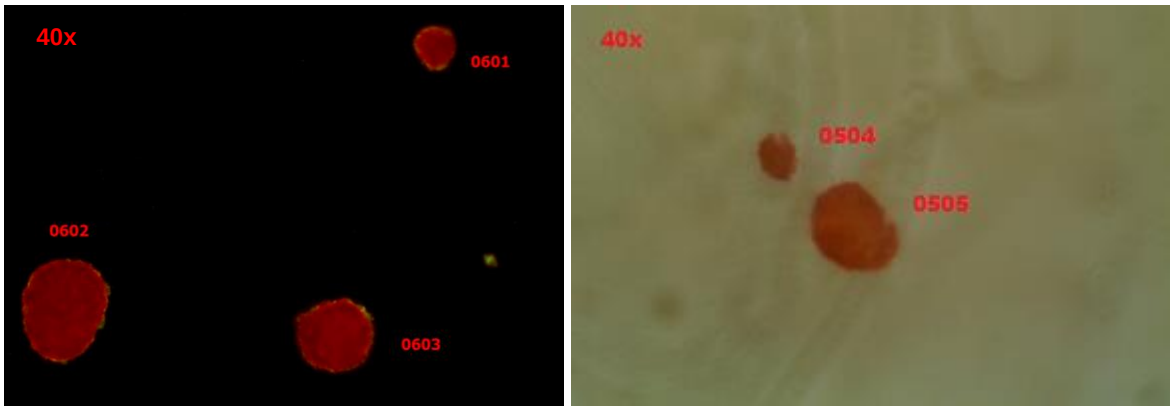


Figure 3.12: Islets of 6 and 5 donors are on the plate, captured by the camera of Inverted microscope CKX41, Olympus at magnification of 40x.

2. Segmentation and binarization of the images using 2M_Binary_140430.py, where the image undergoes thresholding, where background has the value 1 and the islet is 0,:

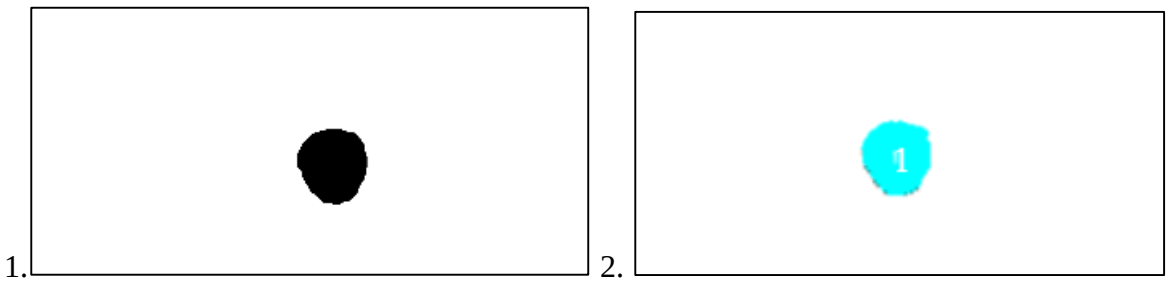


Figure 3.13: 1. Segmented binarized image of Lh_0601. 2. Islet is given a number and the volume is estimated.

Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py

The shapes of circles or ellipses are adjusted to each islet and the approximate volume of the islet is estimated by the sphere and ellipsoid models(Švihlík et al. 2014).

Once the binarized images are opened in the program, the plugin recognizes the islet, gives a number to each islet on the image, fits the circle or ellipse to it and proceeds with analyzing of the islet. The important detail to add operating the plugin is a scaling factor, which is different for microscopes and magnifications, majority of islets were measured by 40x objective of inverted microscope CKX41, where the scaling factor was 1.16 µm/pixel. Islets 0101-0103 were magnified by SZ60 20x with 2.4 µm/pxl, 0104 and 0105 with SZ60 stereo

microscope 20x with 1.3 pixel size and donor 2 islets with 63x magnification on the same stereo microscope with 0.8 pixel size.

Table 3.2: Pixel size (µm/pxl) depending on magnification of the used microscope.

	CKX41	SZ60
20x	2.34	2.4
40x	1.16	1.3
63x	-	0.8

The outcome is a set of values, which are given in multiple Excel documents for each islet, the files are concatenated into one Excel file for ease of use, as we estimated each islet one by one, and for comparison we used such values for each of the 100 islets: diameter of islets from in 3D, 2D and manual counting [µm], volume of spheres [µm^3], [nL], volume of ellipsoids [µm^3], [nL], volume derived from manual estimation, total IEQ from circle, total IEQ number. The time of volume estimation by the plugin is about 30 seconds.

3.4.2. Manual Estimation of Islet Volumes

For more sources of comparison we used manual counting of volume values. Manual estimation was based on pixel counting of islet biggest diameter and perpendicular to it minor diameter, then the mean value of diameter was derived and applied in volume formulation of spherical and ellipsoid method. Pixels were converted to µm multiplying it by pixel size. The formula for volume of spheres ($V= (d_{mean}^3 \cdot \pi)/6$) and ellipsoids ($V= (d_{min}^2 \cdot d_{max}) \cdot \pi/6$) was multiplied by pixel number depending on magnification (as was explained for Fiji plugin).

Figure illustrates the islet being measured manually in Fiji using pixel measures and in 'Paint', the diagonals were counted as a hypotenuse and the diameter obtained its number:

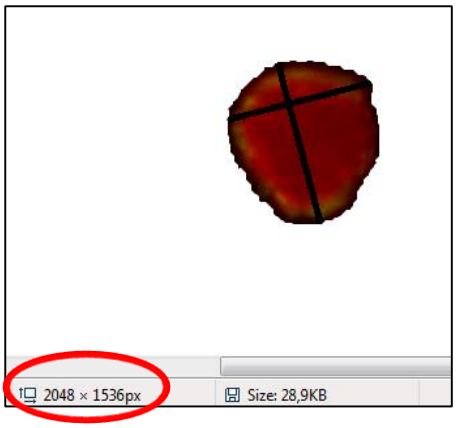


Figure 3.14: Islet Lh_0601 is estimated by pixel counting of diameters (major and minor axis) and volume of islet is derived.

The segmented islets were estimated and the volume was derived, which allowed us to check Fiji plugins and approximate its values manually.

3.4.3. Using Generated Images of Synthetic 3D Islets

In the experiments were estimated 72 islets from 6 donors. Due to different factors like condition of isolated islets, when the islets were not suitable for imaging we were able to qualitatively estimate volumes 72 islets by Fakir method and due to the loss of 7 islets during the experiments itself, we got 65 ‘live’ islets volumes by 2D and 3D methods.

For a better comparison of the methods and estimation of Fiji plugin for volume analyzing, we used images of 35 synthetically created islet-like shape models. 3D volumes of the synthetic islets were known. The islets were generated as 2D images (Figure 3.15) in MATLAB by randomly chosen numbers of radiuses, 3 half axes. We set the minimal and maximal limit of the axes x, y and z. The resolution of the generated images had to be higher than axes numbers. The volumes were measured as a volume of ellipsoid, by multiplying the axes ($V= 4/3 \cdot \pi \cdot x \cdot y \cdot z$).

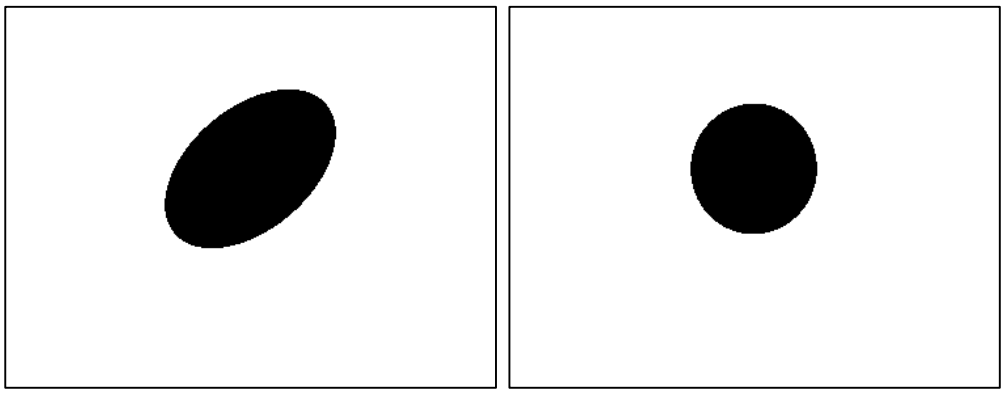


Figure 3.15: Synthetically generated 2D images of islets, with known 3D volume.

To analyze volumes by Fiji (sphere and ellipsoid) we applied the plugin Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py.

3.4.4. Ricordi Table Volume Estimation

One of the methods for volume estimation, which is widely used nowadays is Ricordi method. The method bases on using a table, which directly transfers diameters of islets to volumes and IEQ values (chapter 2.2).

We estimated our islets by Ricordi table, where manually estimated diameter of an islet refers to volume of a sphere of mean diameter in the range (50-100 … 350-400 μm) (Table 2-1). In the case of volume estimation by Ricordi table, islets smaller in diameter than 50 μm are ignored and islets in diameter exceeding 400μm are considered as islets of about 27nL. Some laboratories extend the table to bigger volumes, so the limitation will be higher than 27 nL, in our laboratory we did not extend the table.

In this work we used this method and estimated 65 islets by the table of diameter to volume conversion (Table 2.1), the values were compared with the reference Fakir method.

4. Results

In this chapter outcome of the experiments is presented and the difference and resemblance of the both methods is explained. In the form of graphics and tables, an illustration of the data obtained by processing images of Langerhans islets is given. The main idea of this chapter is to represent the output and the sufficiency of the methods of volumetric estimation of islets. Fakir 3D method and circular and ellipsoidal methods with the help of Fiji software, manual counting and Ricordi table were compared, mainly volumes of the pancreatic islets were taken in count. Because the sufficient volume of pancreatic islets it is one of the main criteria in the successful pancreatic islet transplant, in this chapter the results are aimed on the estimation of adequacy of the used methods for volume approximation.

4.1. Pancreatic Islets Volume Data Obtaining and Processing

We obtained volumes of 65 real islets by 3D method of Fakir and 2D methods like method of sphere and ellipse fitting in Fiji, sphere and ellipse manually estimated and Ricordi table method.

Firstly, we acquired 2D images of the sampled islets on inverted and stereo microscopes (Appendix 2), which were obtained from cadaverous pancreases. Further, the islets were casted and taken 401 projections of on OPT microscope. The projections were reconstructed and volumes of 3D reconstructed islet shapes were estimated by Fakir probes method.

On the other hand, the 2D images were cut out from the background, binarized and processed in Fiji software (by plugins **2.3.3)** for estimating volumes of islets by spherical and ellipsoid methods. Certain pixel size was chosen for each microscope magnification.

The islets were also manually measured in Fiji by pixels and by computing the average diameter of each islet the volumes of sphere and ellipse were measured. Another method for volume approximation of pancreatic islets we used was Ricordi table (Ricordi C. 1990), where measured islet diameters corresponded to certain volumes according to the table.

After obtaining, the data we comprised it into tables and analyzed it through graphical expression. The main comparison was made between 3D and 2D values to show the accuracy of approximation of volumes of pancreatic islets by the current 2D methods. All the tables with the comprised data of real and synthetic islets are available on CD.

As was mentioned before, we estimated 72 islets from 6 donors, and 7 of them were not suitable for undergoing experiments, for this reason this islets were not imaged and not included in comparison. From the data we also excluded 1 islets: Lh_0101 and analyzed it separately as we believe it is composed of two islets and the value of the volume is overestimated, hence it cannot be considered suitable for the sufficient analysis of the 2D methods.

We used 35 synthetically generated islets for fulfilling the amount of 100 and receiving more sources for data analysis, the synthetic islets had known 3D volume value (estimated by randomly chosen x, y, and z semi-axes) and 2D images.

4.2. Comparison of 3D and 2D Methods

The main difference in the methods are the different approaches to volume estimation, so the outcome of the same islets differs in volumes while applying different methods. Fakir 3D method was based on OPT microscopic projections, reconstructing the images and using Fakir software as a tool for estimation of volumes. In 2D methods an approximation of the volume was made by taking the biggest diameter of a sphere fitted on an islet or a fitting an ellipse with the minor and major axis. The 2D methods were proceed with Fiji software, where the program automatically performed binarizing, fitting of ellipse or circle and volume estimation.

Pancreatic islets do not possess spherical shape, they are irregular in nature, so approximation and sphere fitting sometimes results in not sufficient estimating of the volume of the islet by 2D approximation methods. The Figure 4.1 gives an illustration how this approximation (for example circle fitting on islets of irregular shapes) can influence estimation of volume:

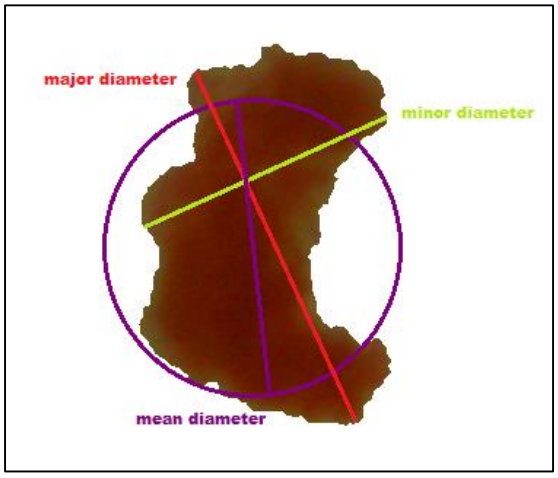


Figure 4.1: Islet Lh_0402, manual approximation of diameter for volume estimation of the islet.

Taking in consideration that an islet can possess flat 3D shape, volume derived from the sphere would influence the volume outcome and result in a deviation from a real volume of the islet. Figure 4.2 is showing reconstructed islet Lh_0402 in and visualized in VolViewer.

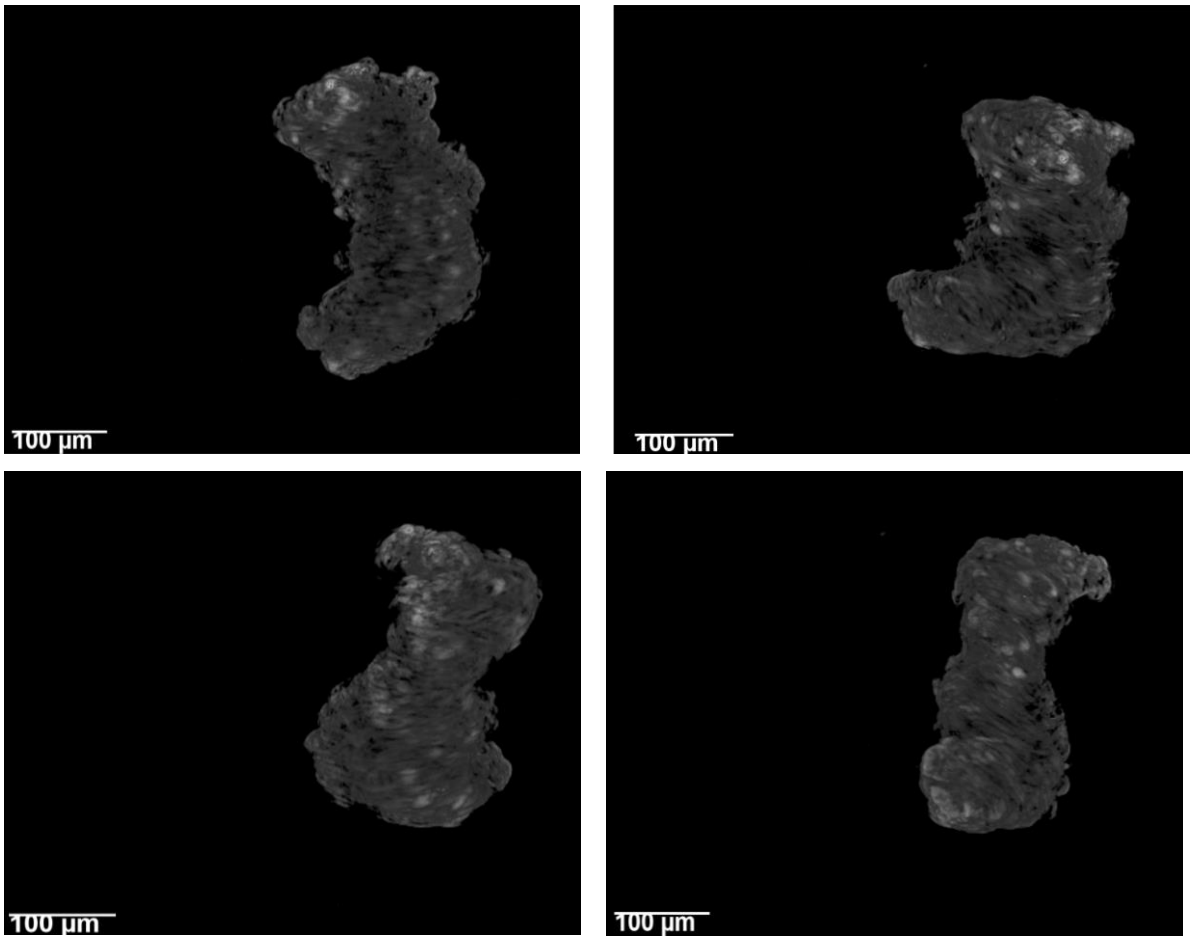


Figure 4.2: Lh_0402 reconstructed and visualized in VolViewer, 3D view from 4 different planes.

For estimation of the islet volumes by 3D Fakir method 401 slices were used. By 2D method islets were evaluated by one image, where the ellipse or circle was fitted.

Fiji results were given by a table of values, which included values according to Ricordi method (quantity of islets in the particular range and IEQ), islet diameters (from circle, ellipse), ellipse axes, area, volumes (from sphere and ellipse fitting). In this work we concentrated on analyzing the values of diameters and volumes from method of spheres and ellipsoids for making comparison with Fakir 3D method, by plotting the values and estimating relative and absolute errors of the methods (All the raw data from Fakir and Fiji is available on CD).

Table 4.1: Fiji values for Lh_0601, estimated from 2D binarized images with 1.16 µm/pxl size.

Lh_0601.png	0	Diameter threshold [µm]													
READ PIXEL SIZE FROM inicamera.txt [µm]:	1.16														
HISTOGRAM ACCORDING TO RICORDI:															
Islet circular diameter [µm]	0	50	100	150	200	250	300	350	400	450	500	550	600	650	700
Frequency	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
IEQ per range	0	0	0.648	0	0	0	0	0	0	0	0	0	0	0	0
IEQ per range - diameter: avg of ellipse axes	0	0	0.648	0	0	0	0	0	0	0	0	0	0	0	0
Index of islets	1														
(2a) MAJOR axis of ellipse length [µm]	146.25														
(2b) MINOR axis of ellipse length [µm]	133.641														
(d) AVERAGE length of axes of ellipses [µm]	139.946														
ANGLE of ellipses [°]	94.5786														
(P1) AREA of object from pixels [µm^2]	15350.6														
(P) AREA of object from ellipses [µm^2]	15350.6														
(V) VOLUME of SPHERES [µm^3]	1430711														
(V) VOLUME of ELLIPSOIDS [µm^3]	1367645														
Index of islets	1														
Diameters of islets computed from circle [µm]	139.803														
IEQ - ratio between SPHERE volume and sphere volume with d = 150 µm	0.80962														
IEQ - ratio between ELLIPSE volume and sphere volume with d = 150 µm	0.77393														
Index of islets	1														
Total IEQ from CIRCLE	0.648														
Total IEQ from ELLIPSE	0.77393														
Sum of areas computed from PIXELS [µm^2]	15350.6														
Sum of areas computed from ELLIPSES [µm^2]	15350.6														
Sum of volumes computed from SPHERES [nL]	1.43071														
Sum of volumes computed from ELLIPSOIDS [nL]	1.36765														

As the result of Fakir estimation we received .grd (allows to run it in Fakir again and see the path of probes coming into and outside of the islet) and.txt extension files with the values of volume and deviation included, also area value (Figure 4.3):

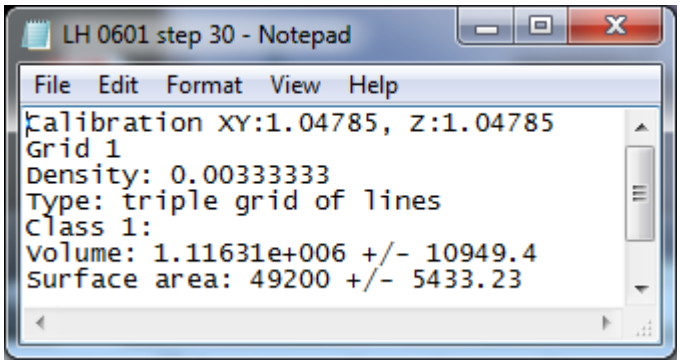


Figure 4.3**: Results of the Lh_0601 saved information of the volume and area estimation with error shown in the text file.**

4.3. Islet Volume Data Analysis

We analyzed the data of 65 real and 35 synthetically generated islets, we used MATLAB and Excel graphics, histograms and boxplots to illustrate dependency, difference, errors of 2D methods with respect to Fakir method.

4.3.1. Real islets

The real islet volumes we estimated by Fakir, spherical and ellipsoid methods (in Fiji and manually), Ricordi table methods. We performed comparison the methods by illustrating distributions of volumes in different methods, estimation of absolute and relative errors.

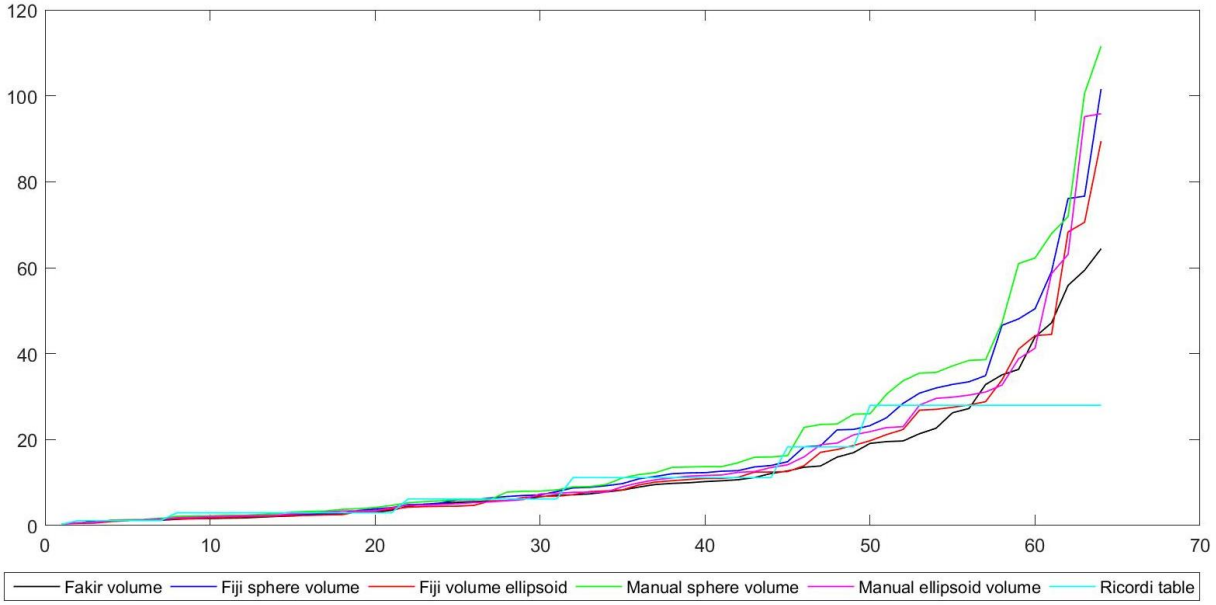


Figure 4.4: Represents sorted volume distribution for all methods independently, where y axis represents sizes of islets and x axis is a position of the islet from smaller volume to bigger. The graphic allows us to see how the values of volumes of our sampled islets obtained by different methods are distributed in (nL), we can see the relation of volumes distribution of all methods.

For illustrative purposes, sampled islets cumulative relative error measured for each method is presented on the Figures 4.5 - 4.7:

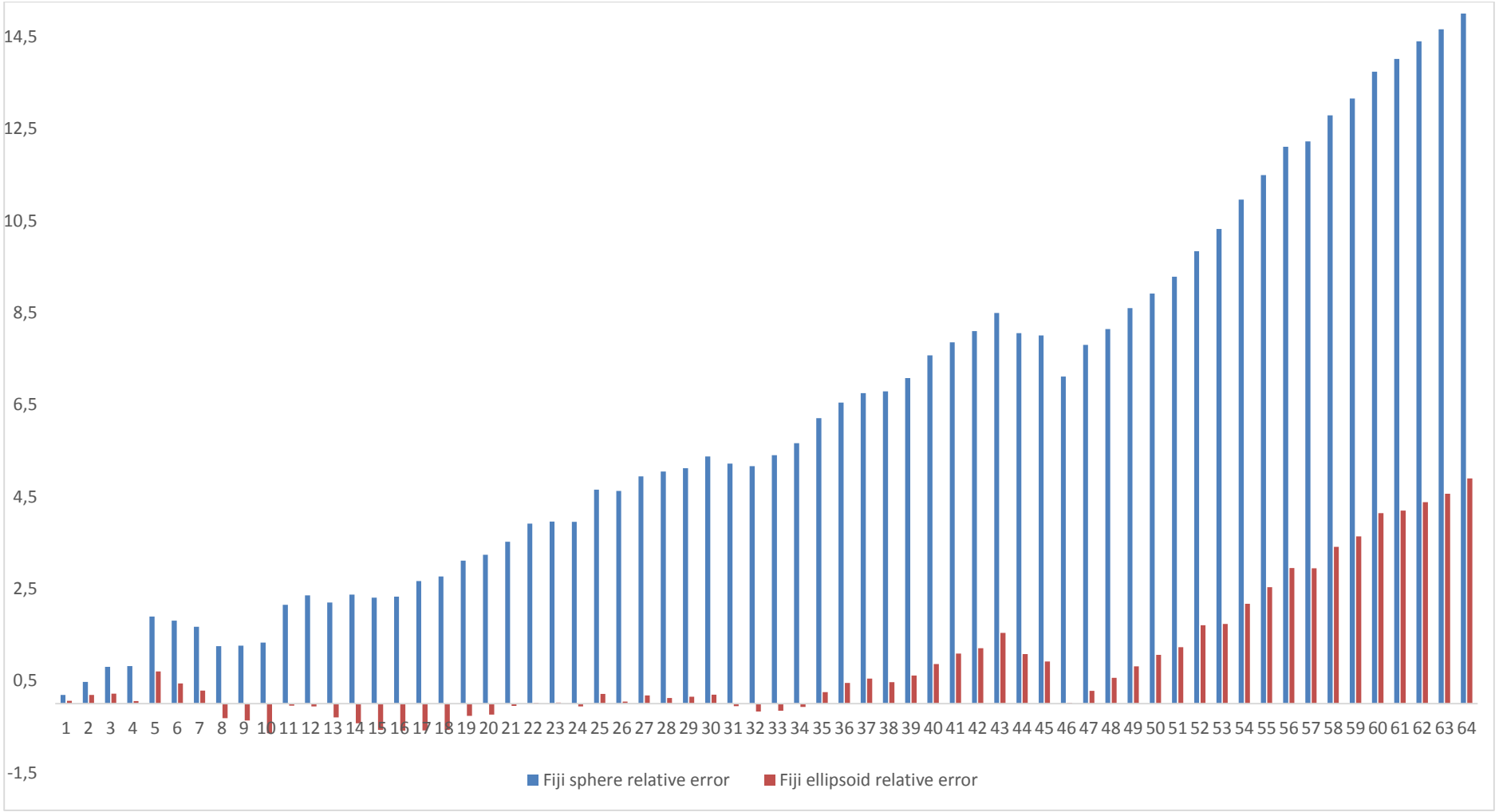


Figure 4.5: Cumulative histogram for 62 islets analyzed together, where x axis is the number of an islet and y axis represents cumulative relative errors of islet volume estimation for method of sphere and ellipsoid in Fiji.

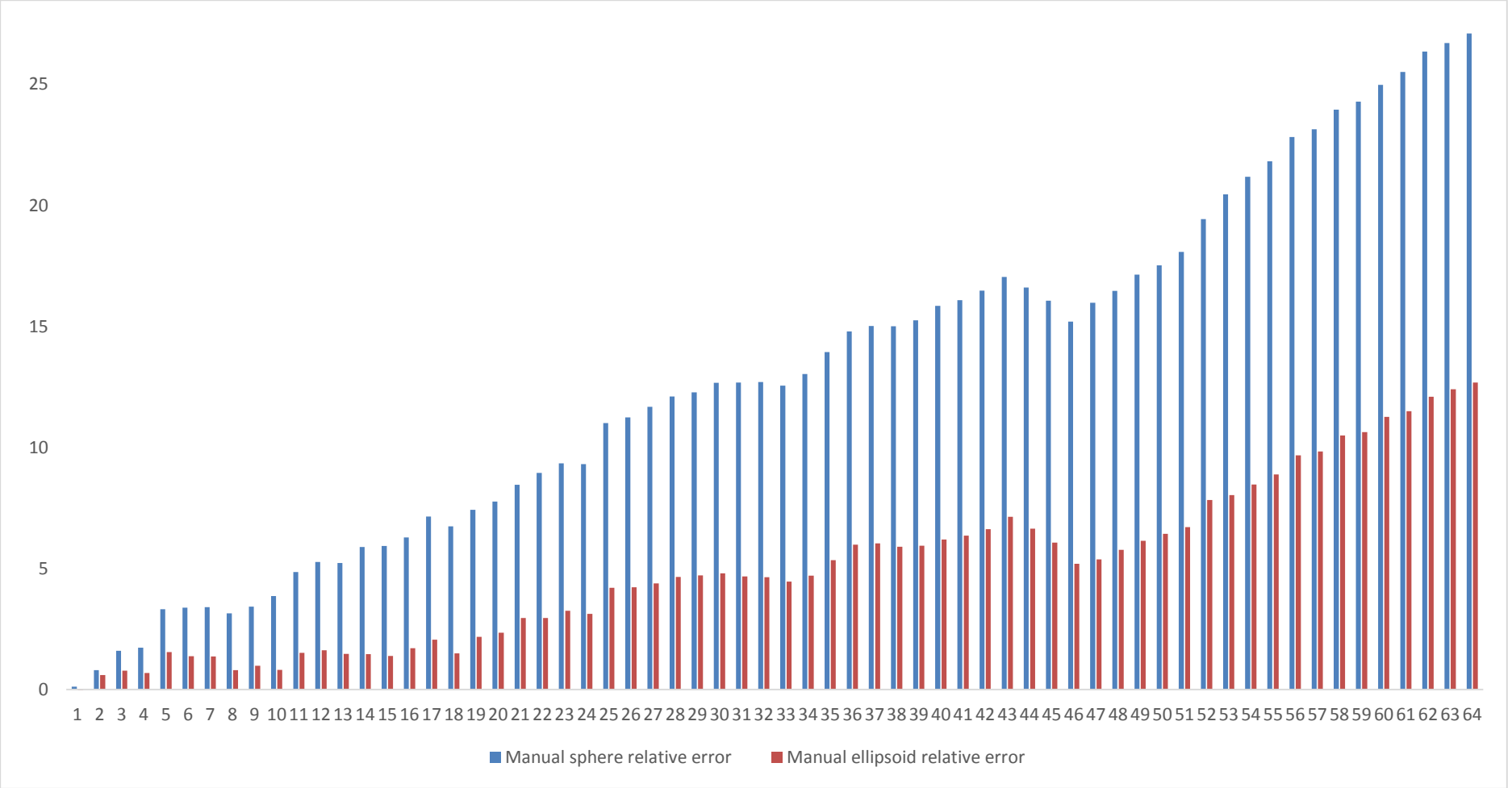


Figure 4.6: : Cumulative histogram for 62 islets analyzed together, where x axis is the number of an islet and y axis represents cumulative relative errors of islet volume estimation for manual method of sphere and ellipsoid.

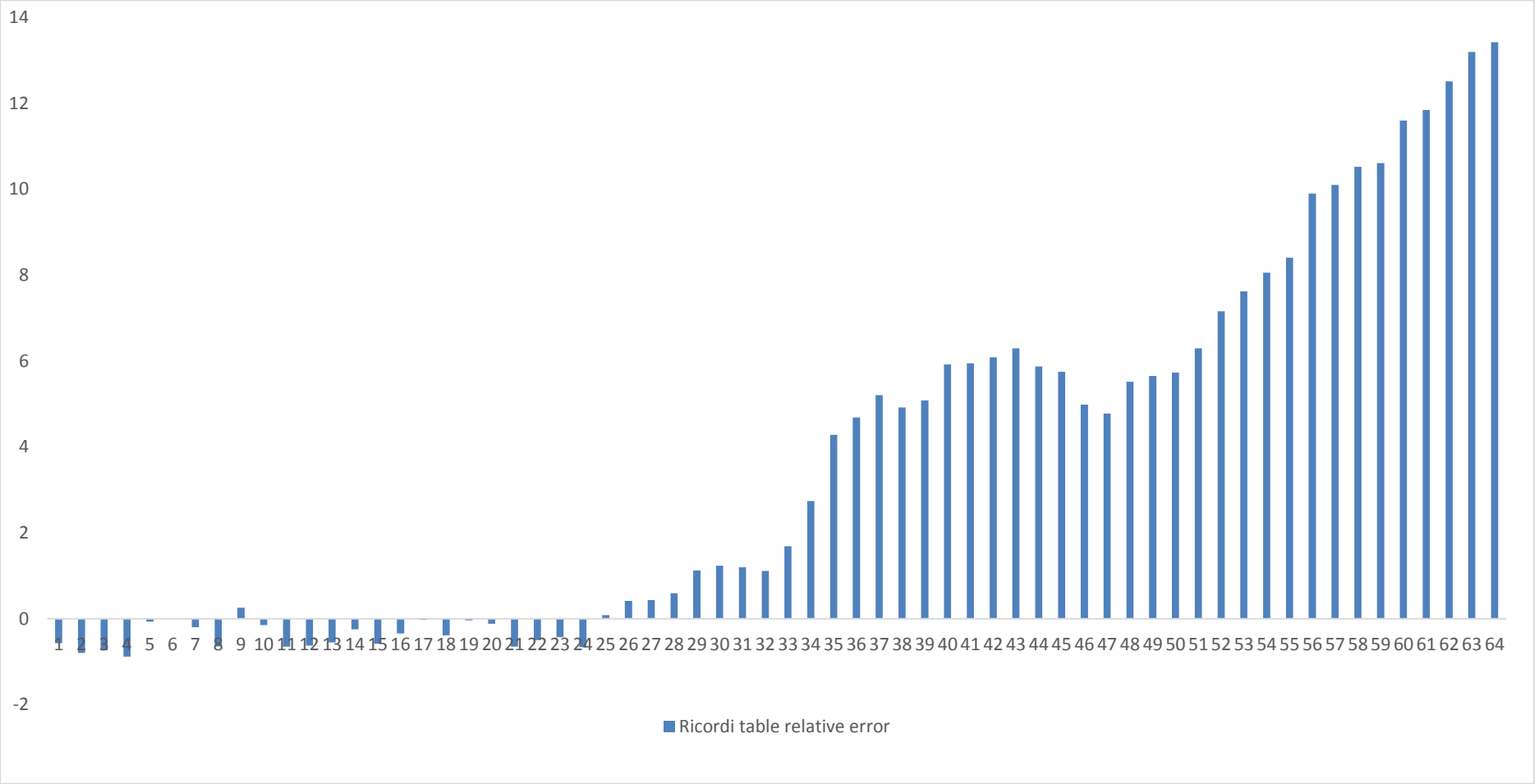
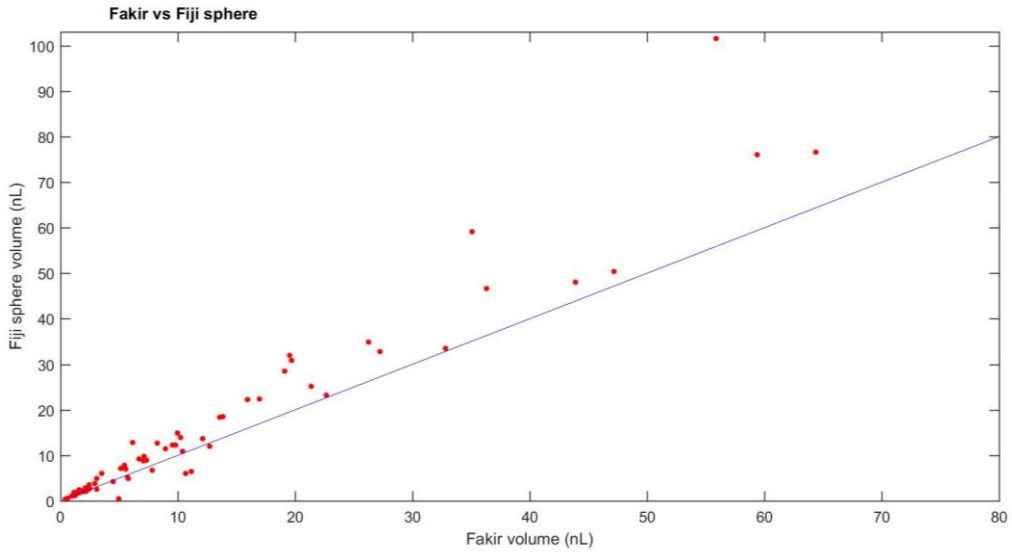
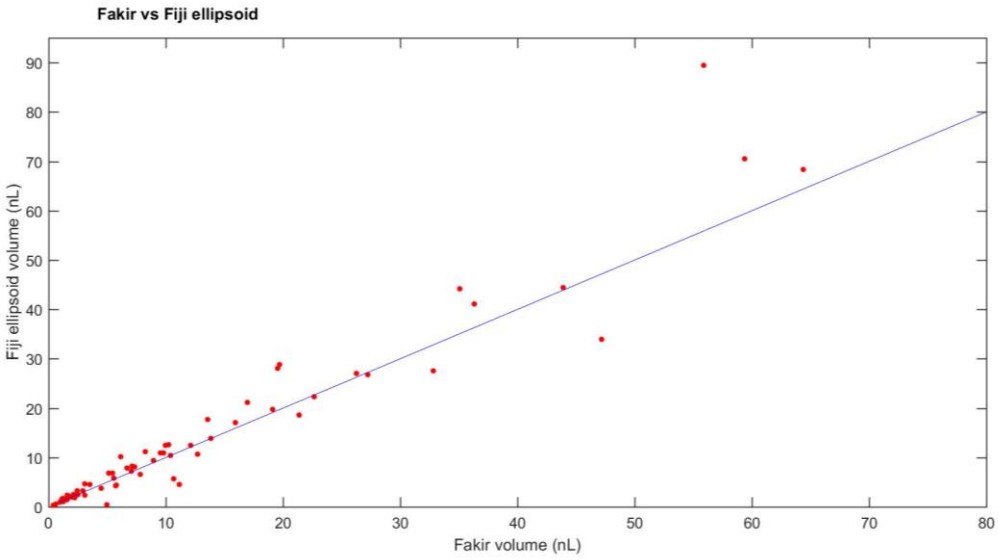


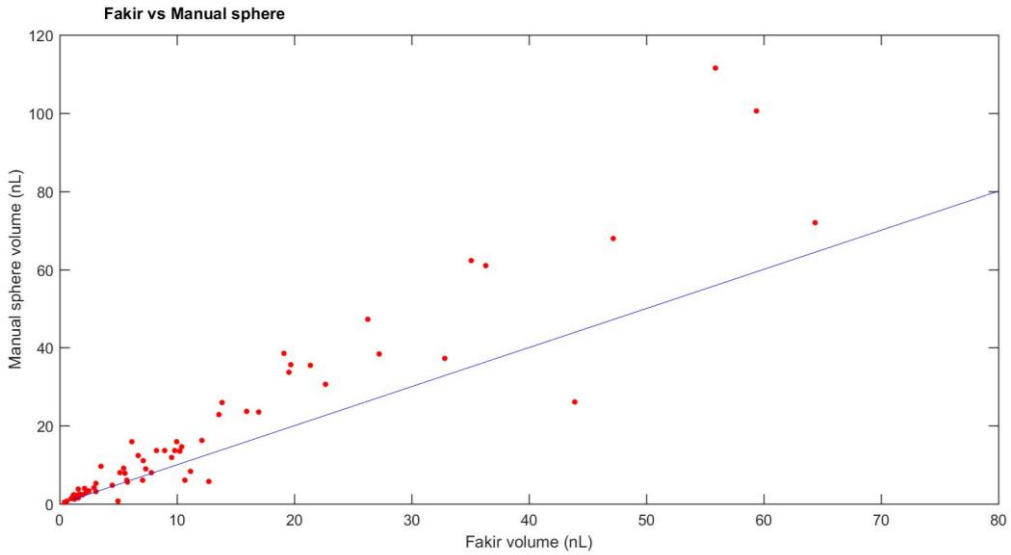
Figure 4.7: Cumulative histogram for 62 islets analyzed together, where x axis is the number of an islet and y axis represents cumulative relative errors of islet volume estimation by method of Ricordi.

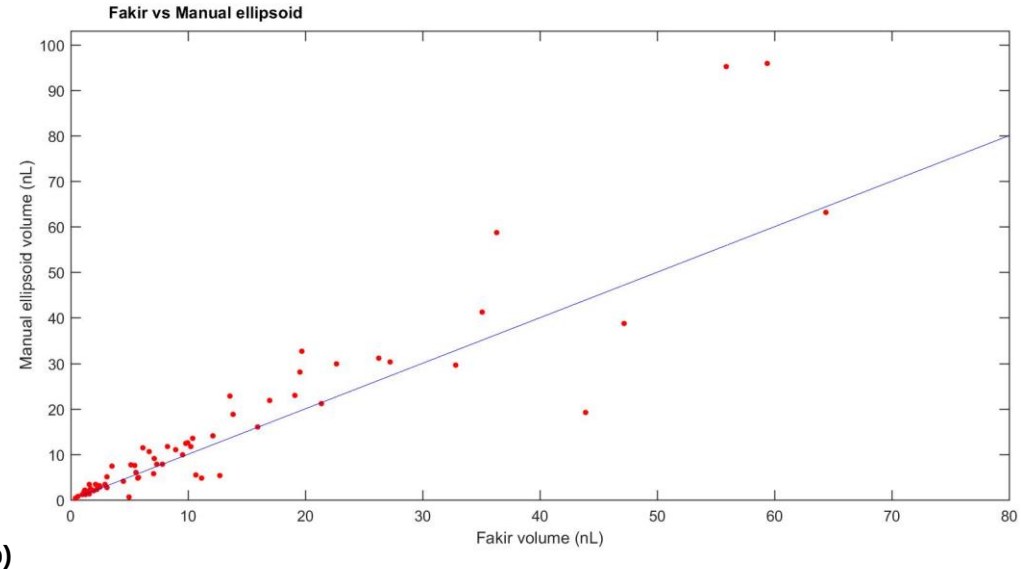
Results presented on Figure 4.8 show a scatter plot of islet volumes measured by different methods. We can see how the volumes are distributed and how each method approximates the volumes in relation to Fakir method. The data is shown in range from 0 -100 nL, where the islets with volumes exceeding this range are not illustrated and are analyzed separately. From Figure 4.8 we can see overestimations or underestimations of volumes comparably to Fakir and volume values scatter change with larger volume of an islet.



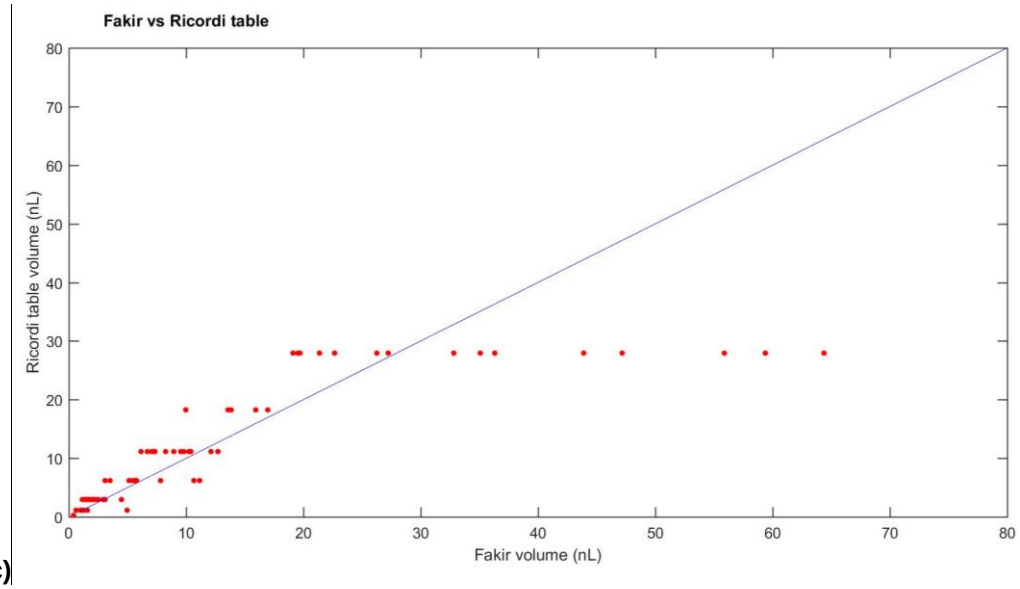


(a)





(b)



(c)

Figure 4.8: Correspondence of 2D methods volumes to Fakir 3D volumes (nL), (a) for Fiji, (b) manual approximation, (c) Ricordi method.

Absolute error allows us to estimate the difference between volumes between Fakir, Fiji (sphere and ellipsoid), manual (sphere and ellipsoid) and Ricordi table. Boxplot of the values represents the absolute error, shows the minimum, median and maximum of the difference between Fakir and the other methods. Volumes of islets, which have an absolute error exceeding the majority of the population, are illustrated as read pluses outside the box. The absolute error was derived from the equation: $\Delta V = V - V_F$, where V is a value of volume of estimated method and V_F is a reference volume measured by Fakir method, it represent the deviation of 2D methods from the values of reference 3D method:

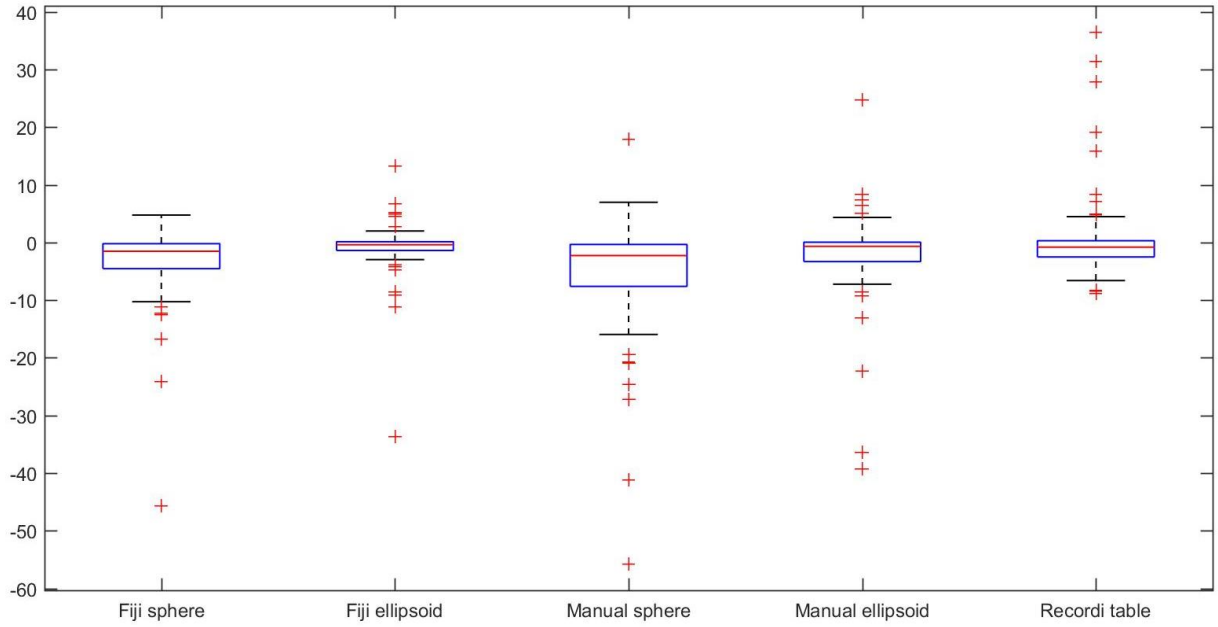


Figure 4.9: Estimated absolute error, the difference between methods in relation to reference Fakir method, data presented in nL.

The relative error represents deviation between volumes in different methods regarding Fakir in range from -1.5 to 1, it is a measure compared to reference value. The relative error was estimated by equation: $\delta_{V_F} = \frac{\Delta V}{V_F} = \frac{V - V_F}{V_F}$, where is a relative error, ΔV is a value of an absolute error:

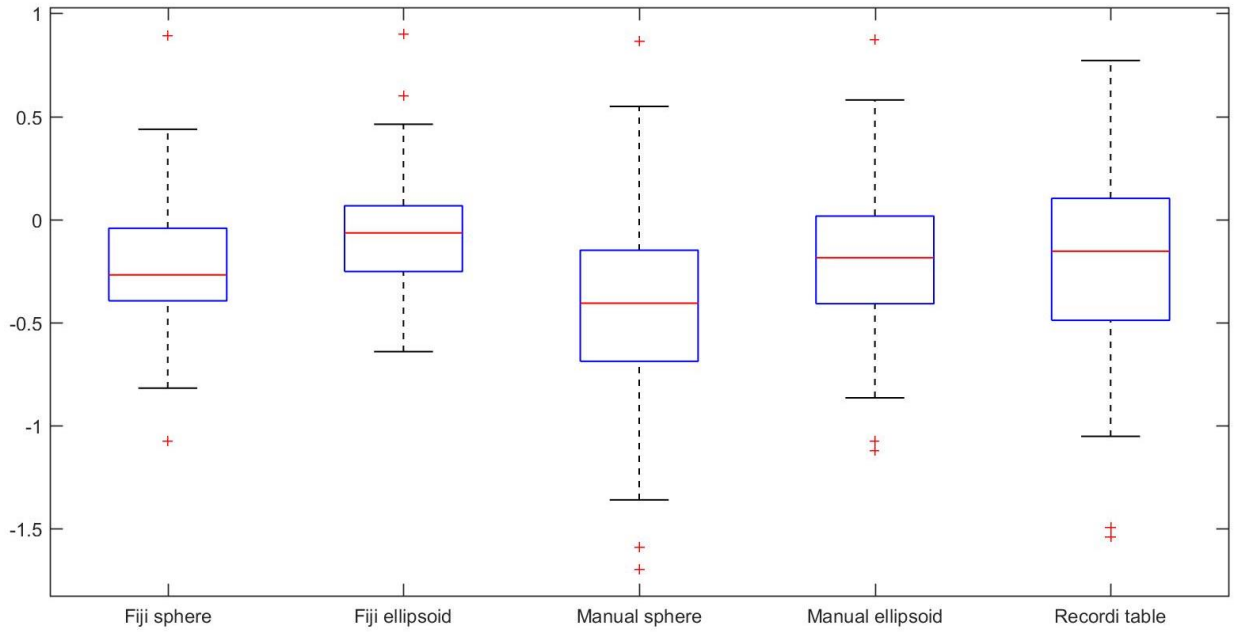


Figure 4.10: Estimated relative error, deviation of 2D methods to reference Fakir method results.

Figure 4.11 shows percentage errors of volume estimation in these big islets, which we additionally estimated to see for which reason the overestimation occurred.

Table 4.2: Volumes of islets in Lh_0101, Lh_0301 and Lh_0204, which have the biggest relative errors in 2D methods

	V Fakir (nL)	V Fiji sphere (nL)	V Fiji ellipse (nL)	V man. Sphere (nL)	V man. Ellipse (nL)	V Ricordi table (nL)
Lh_101	152	263	209	309	241	28
Lh_301	56	102	89	112	95	28
Lh_207	11	6	4	8	5	6
Lh_204	6	13	10	16	11	11
% error Lh_0101		74%	38%	104%	59%	-82%
% error Lh_0301		82%	60%	100%	70%	-50%
% error Lh_0207		-43%	-60%	-26%	-57%	-45%
% error Lh_0204		107%	64%	159%	86%	81%

Figure 4.11: Comparison of volumes (nL) estimated by 2D methods with 3D Fakir method in Lh_0101, 0301, 0207, 0204, percentage error.

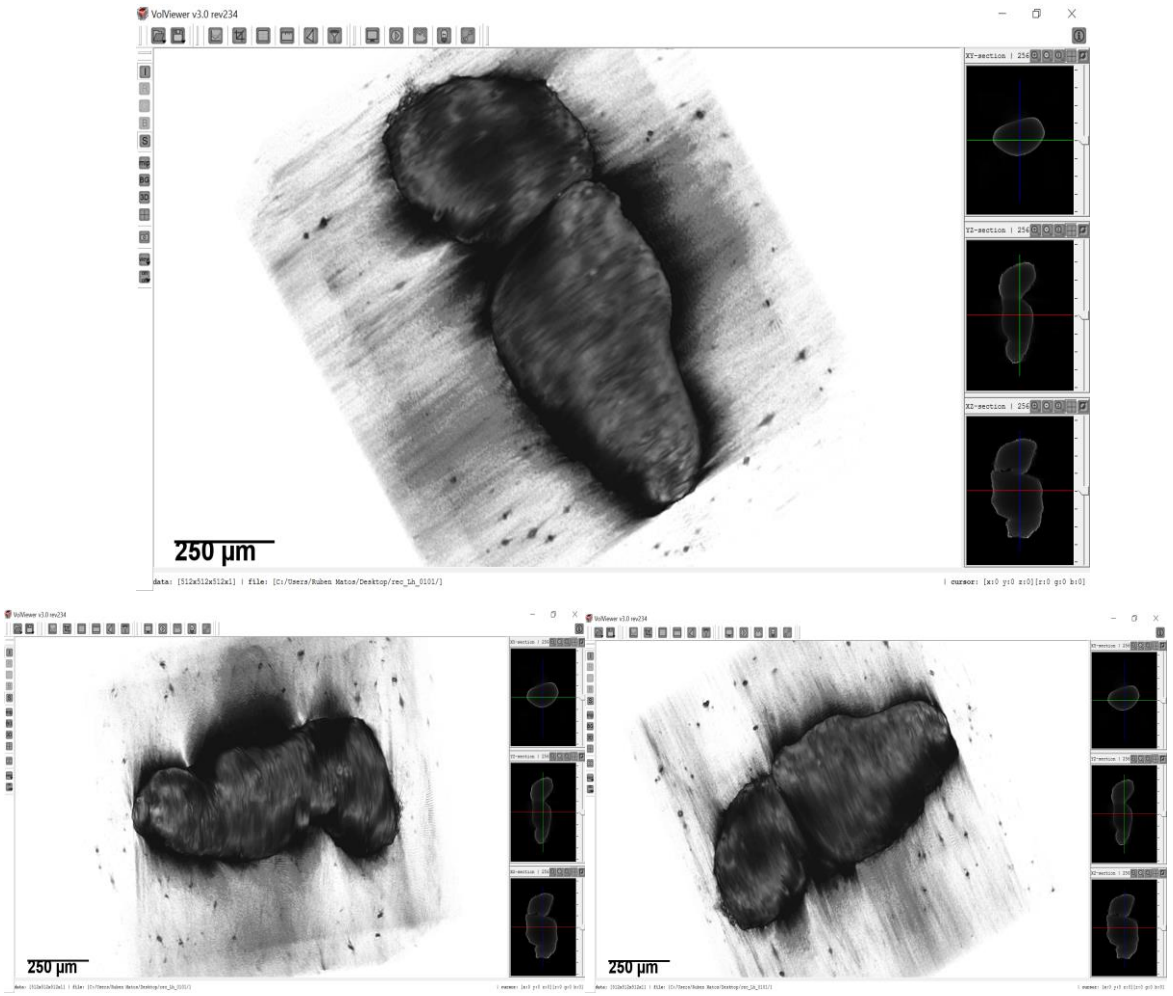
Table 4.3: Mean relative error of all 2D methods according to Fakir results.

	Ricordi	Manual ellipsoid	Manual sphere	Fiji ellipsoid	Fiji Sphere
Mean error	0.396	0.335	0.509	0.211	0.314
Trimmed mean error	0.387	0.317	0.487	0.191	0.292

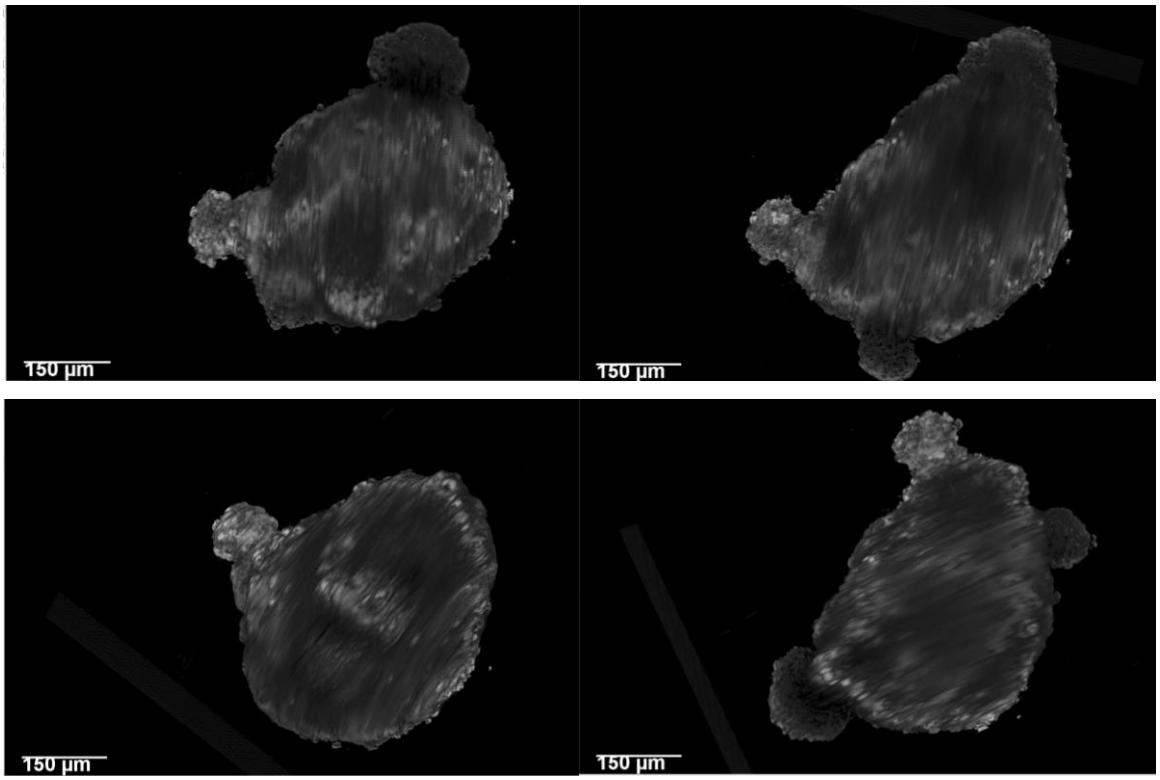
In Table 4.3 we illustrated mean and trimmed relative errors (absolute values) of islet volume estimation for all 2D methods. Trimmed mean represents the statistics excluding the worst cases like: Lh_0101, 0301, 0207, 0204.

The islets Lh_0101, 0301, 0204 were overestimated and 0207 underestimated by 2D methods islets, on Figure 4.12 we can see the islet, casted in gel, 3D reconstructed model, visualized in white and black background.

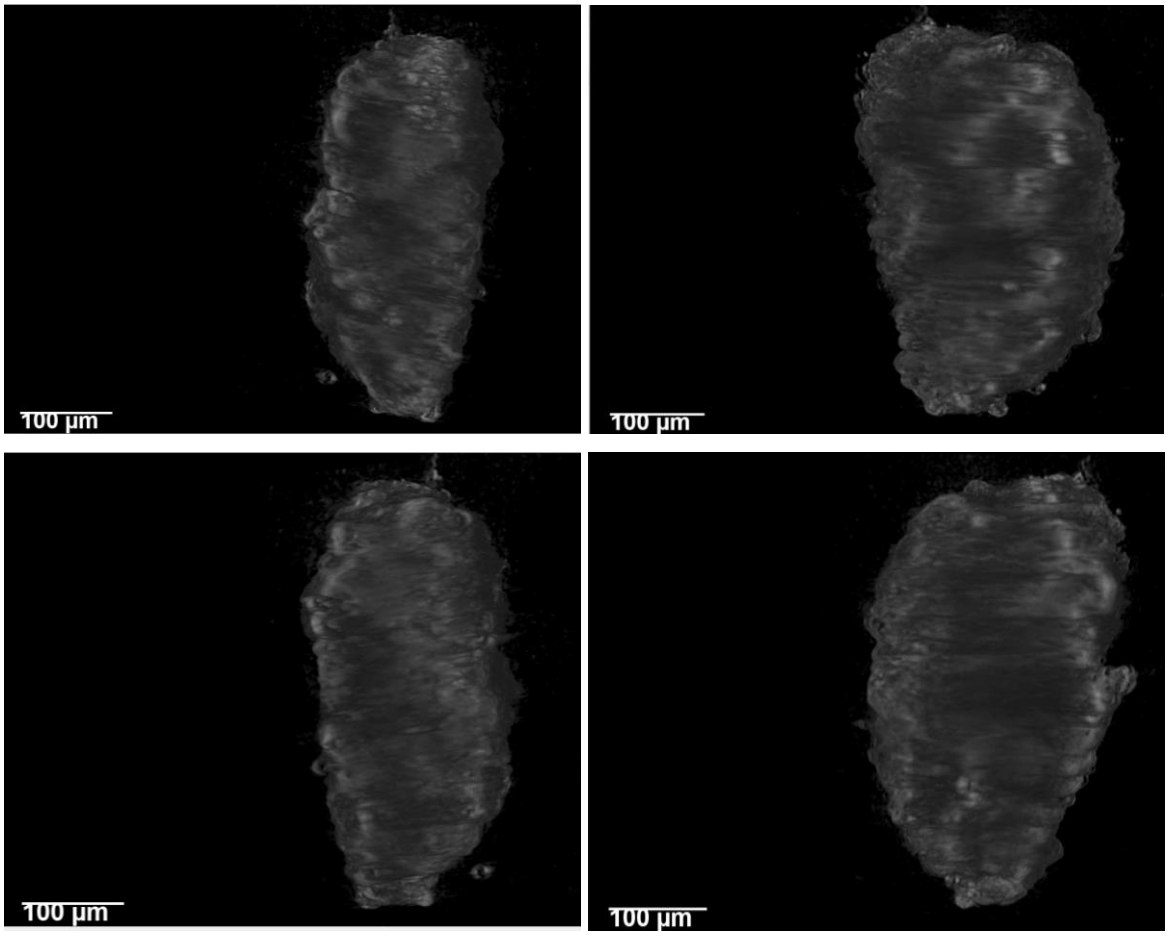
(a)



(b)



(c)



(d)

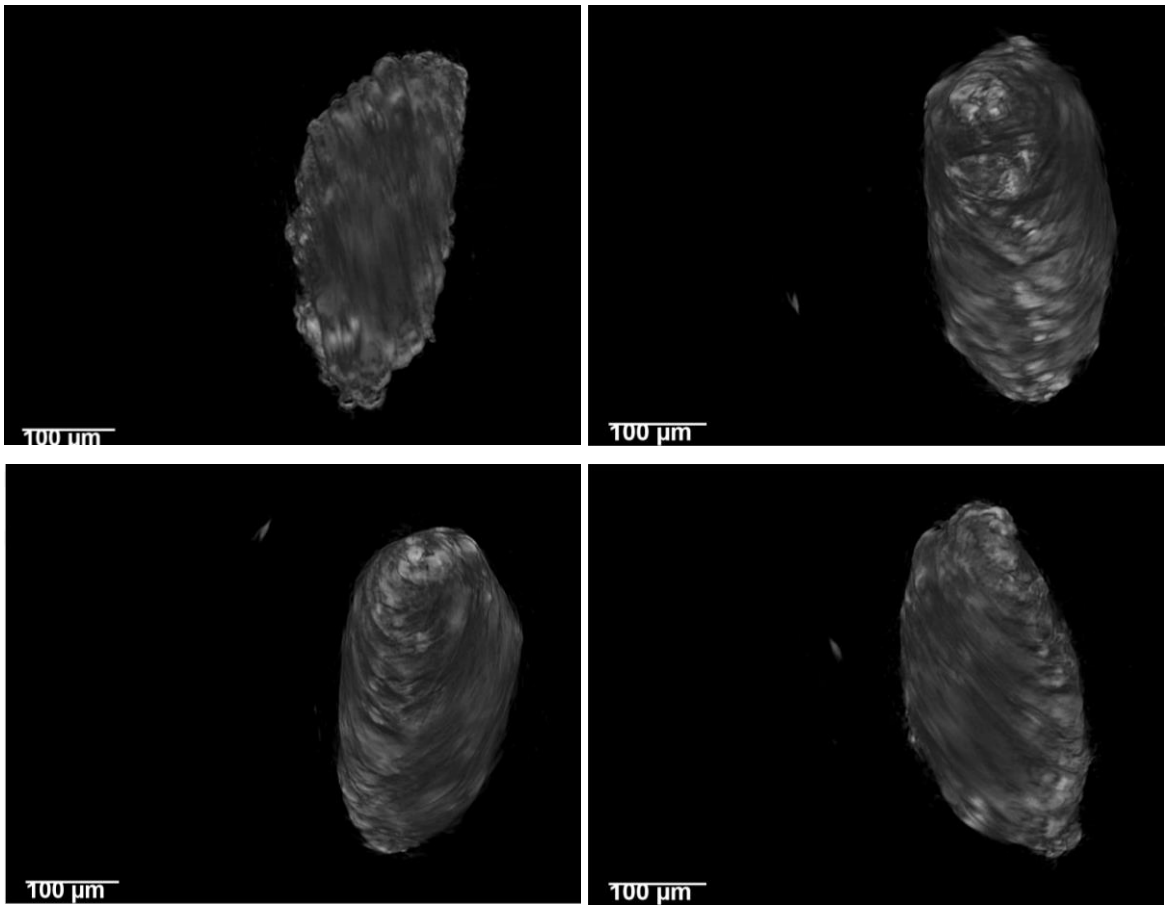


Figure 4.12: 3D reconstructed islet models, visualized in VolViewer, rotational view, Lh_0101 (a), Lh_0301 (b), Lh_0207 (c), Lh_0204 (d).

4.3.2. Synthetic Islets Volume Data Analysis

Synthetic data was measured by methods of spheres and ellipsoids in Fiji and was compared to known 3D volumes of synthetically generated data. Synthetic data was presented by known 3D volume (measured by randomly chosen axes, also could be measured by voxel counting) and 2D projection of the islet. The projections of each of 35 synthetic islets were estimated in Fiji and we received table of values (Table 2 on CD).

We measured relative, absolute errors of the methods. On Figure 4.12 we can see difference in volume estimations of ellipsoid and sphere, Fiji sphere is sorted from smaller to larger volume.

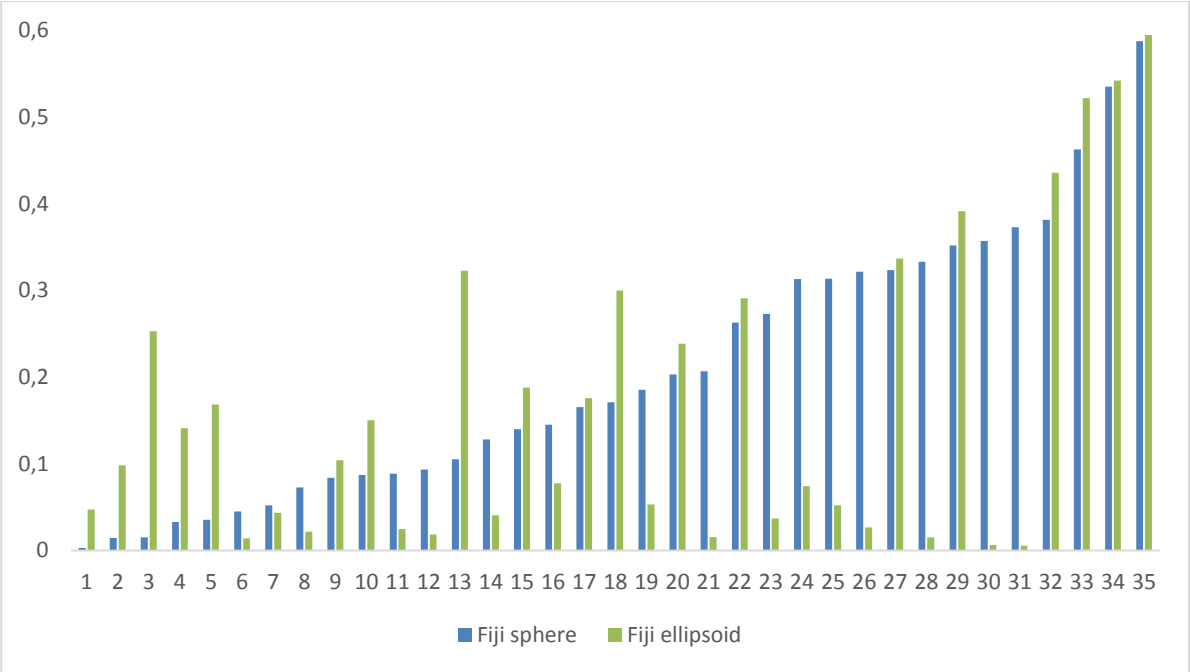
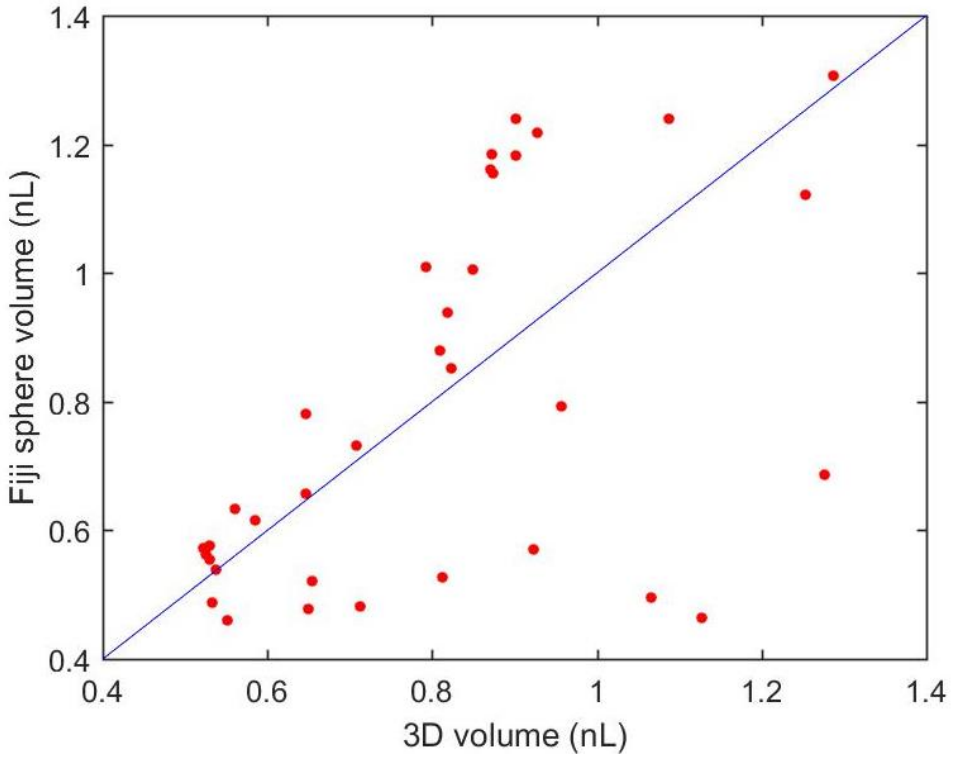


Figure 4.13: Comparison of relative errors of volumes estimated in Fiji (sphere and ellipsoid), absolute values, for synthetic islets.

(a)



(b)

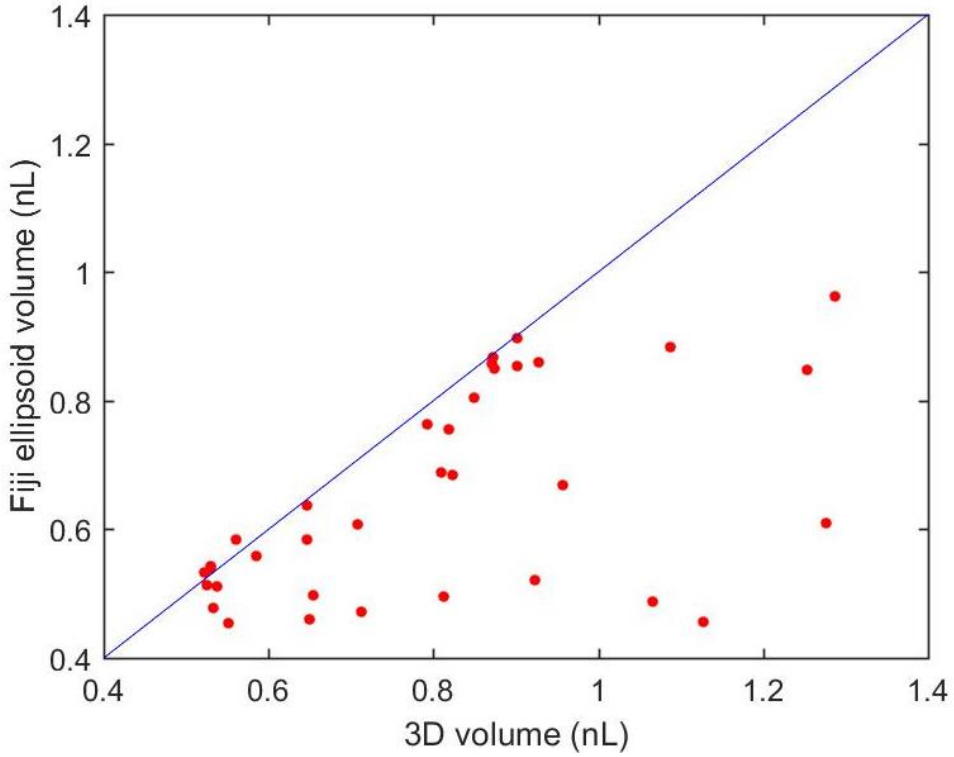


Figure 4.14: Scattered plot of volume distribution of synthetic islets estimated in Fiji sphere (a) and Fiji ellipsoid (b), data presented in nL.

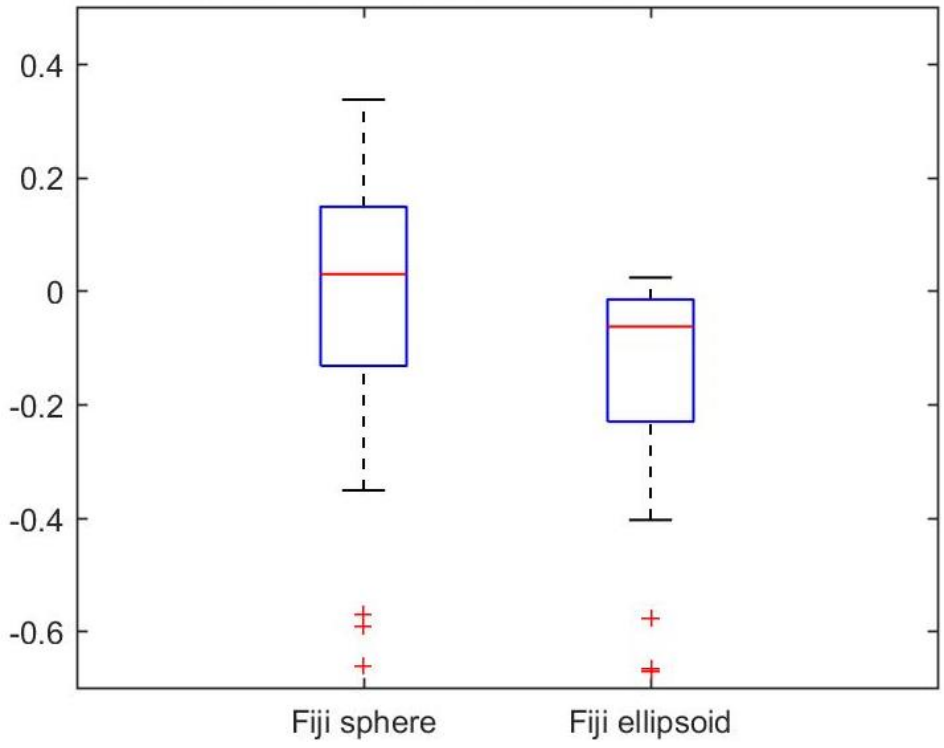


Figure 4.15: Box plot of an absolute error (in nL) of Fiji (sphere and ellipsoid) according to 3D volumes.

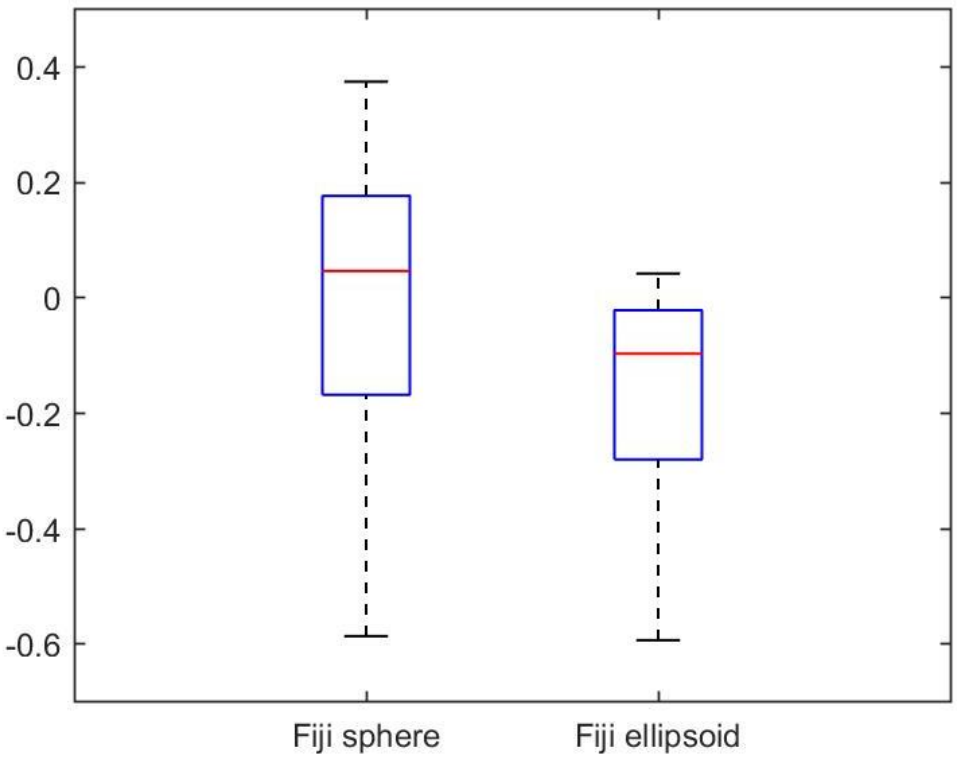


Figure 4.16: Box plot of relative error of Fiji (sphere and ellipsoid) according to 3D volumes.

5. Discussion

The aim of this work was to evaluate accuracy of 2D volume estimating methods for 2D microscopic images. We performed 3D volume estimation of Langerhans islets by OPT projection images and Fakir probes method for volume evaluating of small objects from reconstructed images. We used Fakir method as a reference to volume estimating 2D methods, such as Ricordi table, spherical and ellipsoidal methods by manual counting and using Fiji software plugins.

We obtained volumes of 65 real islets estimated by 3D Fakir method and 2D methods. We used 35 synthetically generated islets with known 3D volumes to make better comparison of methods. In spite of such limitations as number of donors and suitability of sampled islets for estimation purposes, we were able to obtain 65 live islets and perform analysis of methods for volume evaluating of 100 islets in total. In the perspective, the project is going to continue, we plan to obtain more donor islets and proceed with volume estimation experiments.

5.1. 3D Fakir Volume Evaluating Method

The obtained volumes we comprised into a table (Table 1 on CD). The program estimates volumes in μm^3, absolute error is counted by the program (Figure 4.3). The volumes of all live islets and the absolute error, estimated percentage error, were comprised into Excel table available on CD.

Fakir method is a reliable tool for measuring the volume, since it takes projections of an islet and then the reconstruction of it in 3 dimensions. The Fakir method was tested by calibration spheres ordered from “Distrilab” Netherlands (Table 3 on CD), which showed the method to give an error of 4.58%. For this reason, Fakir method was used as a reference in order to evaluate approximation currently used 2D approximations.

The 3D Fakir method for islet volume estimation has limitations and cannot be used in every day routine. Because of the process of islet casting (3.2.1), fitting the jellified islet on OPT Milano handle (3.3.1), centering and aligning the islets in the Tomos software in the live mode (3.3.2) and volume estimation by probes (3.3.5) this method is very time consuming and expensive, can be used only as a reference method for evaluating accuracy of 2D volume estimating methods.

5.2. Fiji (Method of Spheres and Ellipsoids)

We applied Fiji plugins (Appendix 1, Software) for binarizing and volume estimating of islets images. We evaluated all 100 islets by the method of spheres and ellipsoids in Fiji (Tables 1, 2 on CD). This way of volume estimation of islets is the fastest among all the others (Fakir, manual counting, Ricordi), the evaluation took about 30 seconds, that is the reason why Fiji approximation is a convenient method for the users in daily routine because it does not consume a lot of time .

On Figure 4.8 (a), the scatter plots show the correspondence of Fiji 2D volume approximations to Fakir volumes, the correspondence is shown as a red dot and the straight line is the reference used for the comparison. From the same Figure 4.8 (a), we can see that the ellipsoid approximation shows smaller deviation of the values from the blue line (reference), and we can see that it keeps the approximation near to the straight line, unlike the sphere approximation where we can see a bigger deviation from the straight line and specially for bigger islets (bigger diameter. It be explained as bigger islets tend to have more flat shape, where smaller islets are more spherical, all the estimated volumes can be found in the Table 1 on CD.

From the boxplots of absolute error on Figure 4.9, we can see that the method which keeps an error near to 0, and the absolute error population is less spread and close to 0 in Fiji ellipsoid, unlike others which have more spread data. Also from the boxplot is visible that the maximum and minimum error for Fiji ellipsoid is around ± 14 nL, error that is more likely from bigger islets, unlike others that have peaks of about ± 40 nL (in Ricordi table and manual sphere volume).

On Figure 4.10, boxplots of relative error are shown for all methods, and we can see that the relative error of Fiji ellipsoid is smaller because the median (red line) is closer to 0, also that the relative error population is less spread than others and that the difference between the maximum and minimum limits (according to boxplot) is the smallest. From the same Figure 4.10, we can see that Fiji sphere volume approximation is also a good approximation since the data are not so spread and difference of the maximum and minimum limits is not so big compared to others, except Fiji ellipsoid.

The mean percentage error for Fiji ellipsoid is 21.1%, for Fiji sphere it is 31.4% (from Table 4), hence basing on our observations we can deduce that Fiji ellipsoid approximation shows the best results among others.

5.3. Manually Estimated (Method of Spheres and Ellipsoids)

The results of the manual estimations (3.4.2) show that this estimation is one of the least accurate estimation, the reasons are following.

From Figure 4.6, we can see that the cumulative relative error is bigger compare to other methods, also from Figure 4.8 (b) we can see that both sphere and ellipsoid approximation have a big deviation from the straight line (reference), which means that the approximation is not so desired.

On the other hand from the boxplot of the Figure 4.9, we can see that the absolute error does have the median close to 0, also that it does have an absolute error of about ±30 nL, and that the population is not so spread as in the case of manual sphere approximation, because the difference between the maximum and minimum limits is less than in the sphere manual approximation,

From the boxplot of the Figure 4.10, we can see that the relative error between the manual sphere approximation and the ellipsoid manual approximation is different, in this case the ellipsoid approximation does have a median closer to 0 than the sphere approximation and also that the data of the ellipsoid is less spread than the sphere, with smaller difference between the maximum and minimum limit. According to (Table 3.2) mean percentage error of manual sphere is about 50.9% and manual ellipsoid has about 33.5%, hence the relative error of ellipsoid in Fiji is three times more precise than manual counting for ellipsoid.

5.4. Ricordi Method

From the Figure 4-4, we can clearly see that the Ricordi table is limited by the size of the diameter of the islets. Hence, the Ricordi table works only for islets of a smaller volume (with diameter $< 400\mu m$). Previous studies showed, smaller islets might be more effective for transplantation purposes (Lehmann et al. 2007).

Another disadvantage of the method is a rounding of volumes, hence it is impossible to obtain the precise value of an individual islet using Ricordi table: from the Figure 4.8 (c), we can see

that the performance of the Ricordi table is limited by the rounding of the volumes and sorted in several groups, where the highest value is 27 nL.

In the Figure 4.9 we can see that the absolute error for the islets is close to 0, but as illustrated on the Figure 4.10 we can see that the relative error is wide spread, where overestimation reaches 80% and underestimation is over 100% (only for islets with diameter <400 μm), maximum and minimum limits in this case are higher than in all the rest methods.

The calculation of the mean percentage error makes 39.6%, number which is not desired in the process of estimating the islet volume.

5.5. The Islets Lh_0101, 0301, 0204, 0207

We analyze the islets Lh_0101, 0204, 0207 0301 because of the errors presented in Table 4.2 (Figure 4-11), where overestimation or underestimation is too big to consider the measurements as sufficient and accurate.

The reason of islet Lh_0301 volume estimation consists in very irregular shape. The islets has bulges on its surface (Figure 4.12 b). 2D methods approximated the islet according to sphere and ellipsoid, while the real islet did not possess such shape, hence the error is justified by irregularity of Lh_301 islet's shape.

Islet Lh_0101 was estimated by Fakir method as the islet with the biggest volume. We believe, the islet is composed of two islets closely stuck together, hence. On Figure 4.12 (a) the 3D model of the islet shows, that it possess irregular shape, which in case of 2D volume approximating gives overestimation. From Figure 4.4 (distribution of volumes) and Figure 4.8 (scatter plots) we can see, that more essential difference from Fakir results have islets of bigger volumes. The percentage error for the islet Lh_0101 was 74% for Fiji sphere method and 38% for ellipsoid, in manually counted volumes the error was bigger (Figure 4.11). Hence, the volume difference between Fakir and Fiji estimation can be explained by the size and irregularity of shape of the islet.

The islet Lh 0204 is illustrated on Figure 3.1 (a, b). In the case of this islet an experiment was made, where the islet was put on the dish multiple times by the pipette and 2D images were made by stereomicroscope, the islet fall on its 'back' (flipped on the other side), hence the 2D profile of the islet 0204 changed. For volume estimation by 2D methods we used image (b), the volume was estimated from the 2D image, where the islet was of a bigger area. From 3D

reconstruction (Figure 4.12 d), we can see the islet is flat, hence, we obtained overestimation of volume from 2D methods, especially from sphere, (Table 4.2), and ellipsoid results were overestimated as well, because of deriving volume from the image (b) on Figure 3.1, where the minor axis of an ellipse is about twice bigger than image (a).

2D methods showed underestimation of the islet Lh_0207. The reason of the overestimation consists in the 2D image of the islet, where the islet was pictured from the side of smaller area and the 3D reconstructed model (Figure 4.12 c) showed that the orthogonal axis to the 2D projection is longer than minor diameter of the islet. Hence, the underestimation by ellipsoid and sphere is caused by the 2D profile of the islet.

5.6. Synthetic Islets

On Figure 4.13 we can see histogram with percentage error of volume estimation by sphere and ellipsoid in Fiji. A mean percentage error for sphere method is 20.7%, for ellipsoid it is 16.6%.

Scatter plots on Figure 4.14 (a), (b) show that values of volumes measured by ellipse fitting method are less scattered around the line of reference, than in method of spheres. Box plot of relative and absolute errors indicate, that method of ellipsoids tends to be underestimating (b). From the percentage error we can see, method of ellipsoids measured by Fiji shows better results in volume estimation for synthetic islets (Table 2 on CD).

Based on our experiments results, synthetic data was estimated with smaller mean error than real data. It is caused by the fact, that the islets were generated with 3 known radiuses and the shape of 3D volume was consistent (ellipsoid with different axis), whereas real islets are not perfectly ellipsoidal, 2D profile of real islets was not symmetrical and more irregular.

6. Conclusions

In the study we performed volume estimation of pancreatic islets by method of Fakir (used as a reference), method of spheres and ellipsoids (in Fiji and manually) and method of Ricordi table. Based on our experiments and analysis of volume data of pancreatic islets we can make following conclusions:

The currently used 2D methods for volume evaluation, which use sphere and ellipsoid approximation of islets cannot be as precise as 3D Fakir method, due to irregular islet shapes of pancreatic islets, they might overestimate (more probable in case of spherical method) real volumes of pancreatic islets . However, based on the results we obtained, we can say that the best approximation in general as well as for real and synthetic data, is the ellipsoid method, because the error presented in both Fiji and manual counting is lower comparably to the sphere approximation. Out of the two methods for ellipsoid approximation, the one which is more precise is the Fiji ellipsoid method, since it presented the least error in the results.

In the case of the manual approximation, it is true that the tendency is the same as in Fiji approximation (ellipsoid has less overestimation than sphere), but we cannot take it as sufficient option for volume estimation of islets, since the time consumption for the calculation is considerably bigger than for Fiji and because manual counting is influenced by a subjective factor, since the calculated radius can vary dependently on a subject, which does the evaluation. In the case of Ricordi table, it is visible that the performance is limited by the ranges of the Ricordi table, the performance of the Ricordi table for small islet is relatively good, in the contrast with islets of a big diameter where error increases, for getting a better performance of the method, the extension of the Ricordi table for islets with bigger diameters is needed.

To summarize the study, Fiji ellipsoid approximation showed the sufficient result for estimation of islet volumes among all the analyzed methods, which is suitable, fast and sufficient method for approximation of islets of all sizes.

List of Literature

Alanentalo, Tomas et al. 2007. “Tomographic Molecular Imaging and 3D Quantification within Adult Mouse Organs.” *Nature methods* 4(1): 31–33.

Buchwald, Peter et al. 2009. “Quantitative Assessment of Islet Cell Products: Estimating the Accuracy of the Existing Protocol and Accounting for Islet Size Distribution.” *Cell Transplantation* 18(10-11): 1223–35.

Dvořák, Jiří, Jan Švihlík, David Habart, and Jan Kybic. 2016. “Comparison of Volume Estimation Methods for Pancreatic Islet Cells.” 9788: 97881K.

Fiorina, P, A M Shapiro, C Ricordi, and A Secchi. 2008. “The Clinical Impact of Islet Transplantation.” *Am J Transplant* 8(10): 1990–97.

Girman, Peter. 2003. “Digital Imaging as a Possible Aproach in Evaluation of Islet Yield.” *Cell transplantation*.

Guide, Documentation, and Politecnico di Milano Andrea Bassi. 2012. “Documentation Guide.” (02): 0–12.

Janacek, J. 1999. “ERRORS OF SPATIAL GRIDS ESTIMATORS OF VOLUME AND SURFACE.” *Acta Stereologica*.

Kubinova, L, J Janacek, J Albrechtova, and P Karen. 2005. “Stereological and Digital Methods for Estimating Geometrical Characteristics of Biological Structures Using Confocal Microscopy.” *From Cells to Proteins: Imaging Nature across Dimensions* 3: 271–321.

Kubínová, L, J Janácek, Farshid Guilak, and Z Opatrný. 1999. “Comparison of Several Digital and Stereological Methods for Estimating Surface Area and Volume of Cells Studied by Confocal Microscopy.” *Cytometry* 36(2): 85–95.

Kubínová, L., and J. Janáček. 1998. “Estimating Surface Area by the Isotropic Fakir Method from Thick Slices Cut in an Arbitrary Direction.” *Journal of Microscopy* 191(2): 201–11.

Lehmann, Roger et al. 2007. “Superiority of Small Islets in Human Islet Transplantation.”

Diabetes 56(3): 594–603.

Niclauss, Nadja et al. 2008. "Computer-Assisted Digital Image Analysis to Quantify the Mass and Purity of Isolated Human Islets before Transplantation." *Transplantation* 86(11): 1603–9.

Olsson, Richard, and Per Ola Carlsson. 2011. "A Low-Oxygenated Subpopulation of Pancreatic Islets Constitutes a Functional Reserve of Endocrine Cells." *Diabetes* 60(8): 2068–75.

Ramachandran, Karthik, Han-Hung Huang, and Lisa Stehno-Bittel. 2014. "A Simple Method to Replace Islet Equivalents for Volume Quantification of Human Islets." *Cell transplantation* 24: 1–45.

Ricordi C., Gray D.W. et al. 1990. "Islet Isolation Assessment in Man and Large Animals." *Acta Diabetologica Latina 27(3)* XXVII(3): 185–95.

Schindelin, Johannes et al. 2012. "Fiji: An Open Source Platform for Biological Image Analysis." *Nature Methods* 9(7)

Shapiro, M James et al. 2006. "International Trial of the Edmonton Protocol for Islet Transplantation." *The New England journal of medicine* 355(13): 1318–30.

Sharpe J. 2004. "Optical Projection Tomography."

Sharpe, James. 2003. "Optical Projection Tomography as a New Tool for Studying Embryo Anatomy." *Journal of Anatomy* 202(2)

Skyscan. "Bruker microCT(skyscan.be/products/downloads.htm)."

Skyscan NV. 2011. "NRecon User Manual."

Švihlík, Jan et al. 2014. "Classification of Microscopy Images of Langerhans Islets."

Swoger, Jim, and James Sharpe. 2002. "OPTICAL PROJECTION TOMOGRAPHY: 3D MICROSCOPY OF EMBRYOS & ORGANS." : 2002.

List of Appendixes

Appendix 1: Materials, equipment and software

Materials and supplies

Langerhans islets

10 - 20 islets in 100 μL culture media just removed from incubator (per one session), 100 islets in general.

Dithizone

50mg DTZ, 800 μL 70% EthOH, 400 μL 1M NaOH, 15 mL HBSS (Hank's Buffered Salt Solution), 0.2% (wt. /vol.) Albumin

Agarose gel

2% (wt. /vol.) Low Gelling Agarose (Sigma) in HBSS (Sigma)

Melted at 70ºC

Tempered at 37ºC

Syringe (diameter 0.5 cm)

-sharp cut off the outlet;

Pipettes, tips and dishes

Equipment

Microscopes

Stereomicroscope SZ60, Olympus:

- objective magnifications: 10, 12, 20, 30, 63x

Inverted microscope CKX41, Olympus:

- objective magnifications: 12.5, 20, 40, 100x

Camera

MicroCam 1/2 CMOS, 3MPxl, Bresser

Images: dimensions 2048x 1536 pxl, format *.bmp*

Optical Projection Tomography microscope (at the laboratory of Czech Academy of Science, at the Institute of Physiology Biomathematics Department)

OPT Milano, by Dr. Andrea Bassi from Polytechnic University in Milan

Objective: Edmund Optics, PLAN APO ELWD 10x/0.28 (infinity corrected, long working distance - 33.5 mm, numerical aperture 0.28)

Edmund Optics, PLAN APO ELWD 5x/0.14 (infinity corrected, long working distance - 34 mm, numerical aperture 0.14).

Source of excitation are laser diodes:

1. GFP, excitation 405/10 nm, emission 447/60 nm (bandpass) or emission from 450 nm high pass (HP).
2. GFP+, excitation 472/30 nm, emission 520/35 nm (bandpass) or emission from 550 nm high pass (HP).
3. Cy5, excitation 624/40 nm, emission 692/40 nm (bandpass).

Software

Tomos (visualizing of the sample in live mode, its rotation and acquisition of a projection stack, available on OPT PC);

IrfanView (basic processing and visualization of taken projections and reconstructed slices) free at http://www.irfanview.com

NRecon (basic reconstruction of 3D models from obtained with OPT microscope) free at http://www.skyscanner.be/products/downloads.htm;

Zoner Photostudio Pro (for basic data processing and visualization of acquired OPT projections and reconstructed slices) free at http://www.zoner.cz

DataViewer (used for visualization of 3D reconstructed data in the forms of orthogonal projections) free at http://www.skyscanner.be/products/downloads.htm;

Fakir (volume estimation by the parallel lines fakir probes, which are folded in the triple orthogonal grid) free at http://www2.biomed.cas.cz/~janacek/fakir/3dtools.htm;.

VolViewer (creating a visual 3D model of a Langerhans islet with functions of 'volume rendering' and maximal intensity projections (MIP) and possibility of turning, zoom, more channels view, virtual slices, 3D measurements and more); free for download at:
http://cmpdartsvr1.cmp.uea.ac.uk/wiki/BanghamLab/index.php/VolViewer

FIJI (download free http://imagej.net/Fiji/Downloads)

2M_Binary_140430.py and Islet_Analyzer_Ellipse_MultiDiam4C_v0_exp.py plugins (available on demand by contacting Professor Jan Kybic kybic@fel.cvut.cz **)**

Gimps (segmentation of islets) at https://www.gimp.org/downloads/ free;

Excel (data storing and analyzing

Appendix 2: Working principle of OPT Milano

OPT projection microscope 'OPT Milano' was designed for imaging of small objects, in cooperation with Polytechnic University in Milan (Polytechnico di Milano) in 2012 by Andrea Bassi, the information is taken from 'Documentary guide'.

The sample is attached on a holder and put in a quartz cell, the device firstly visualizes an adjusted specimen, afterwards the specimen is rotated along the vertical axis for obtaining a number of its projection during rotation with changes of an angle. In order to subside scattering of light and reduce refractive index through the specimen, it must be casted or suspended in liquid, in this case the light will go through the object in almost straight lines and the good quality images can be obtained(Sharpe J. 2004)
Figure A illustrates the layouts of the system:

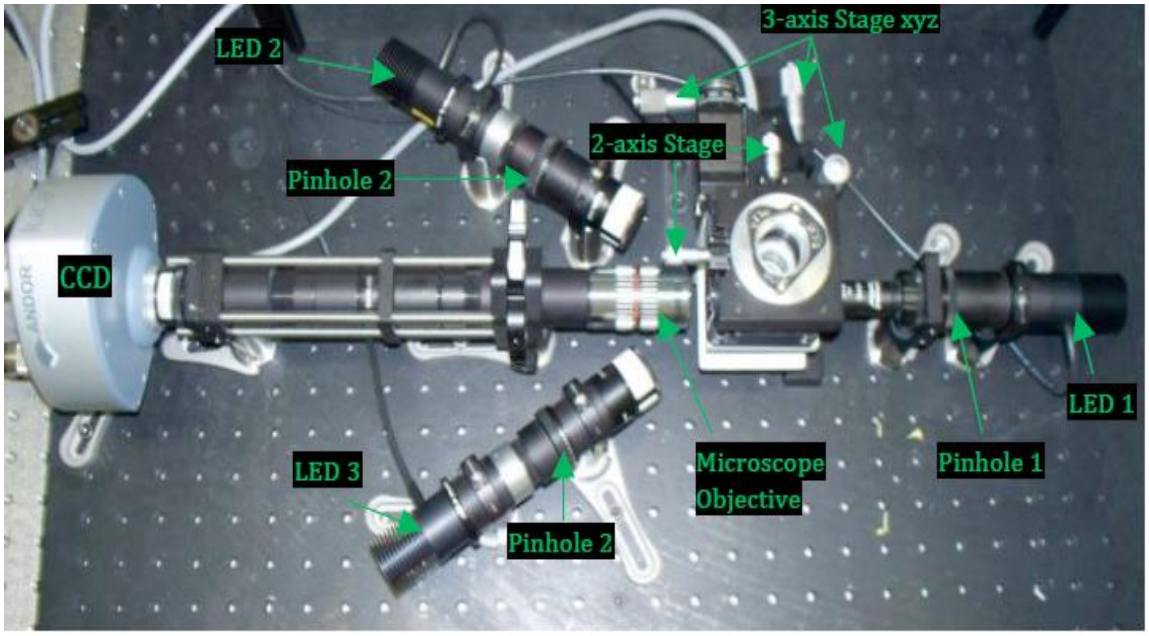


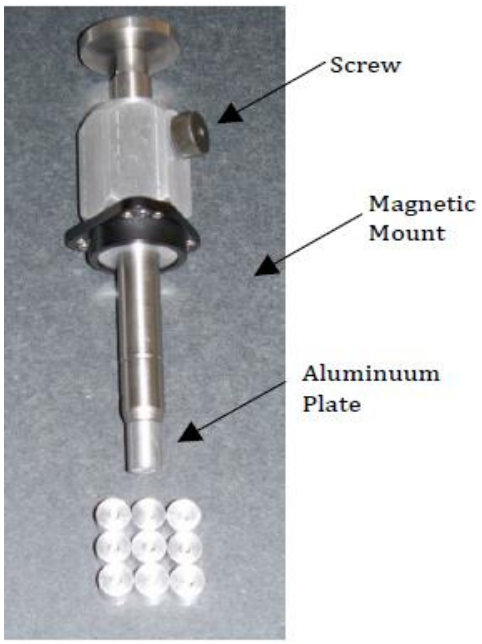
Figure A: Functional parts of OPT device.

Main units of the system are:
2-axis Stage is a transfer stage through which a holder is set on the rotation stage, which provides rotation of the sample for 360° and the sample can be centered. **3-axis Stage xyz** enables the whole sample holder assembly to move along 3 axes in the imaging plane, it is joined with rotation stage and provides centering of the sample in the field of view of the camera.

Illumination unit, which uses LED (Light Emitting Diodes) with the control of intensity in order to provide Brighfield and Fluorescence illumination .On the Figure 2.1, **LED 1** emits light, which is conducted through a diffuser (a glass rod) projected on the specimen by a telecentric lens, hence, Brightfield illumination is created, which is can to be regulated with the help of **Pinhole 1,** whereas Pinhole 2 and Pinhole 3 allow to control Fluorescence mode illumination**.** The next **2** and **3 LED** illuminators are set on the optical system (can be changed, or new LEDs can be added) are used for managing Fluorescence. LEDs are sources of excitation with available central wavelengths 405nm, 470nm and 625nm.

Detection unit Microscope Objective with long working distance (2x or 5x) gathers the passed through a sample or emitted light, which further proceeds to a filter wheel. On the Electron Multiplied CCD camera of 1004x1002 pixels resolution lenses produce and image of the specimen. Whereas about 6mm field of view is created in 2x objective by achromatic lens, about 2.5mm is in 5x objective.

There Figures A and B illustrates 2 types of holders in the OPT Milano:



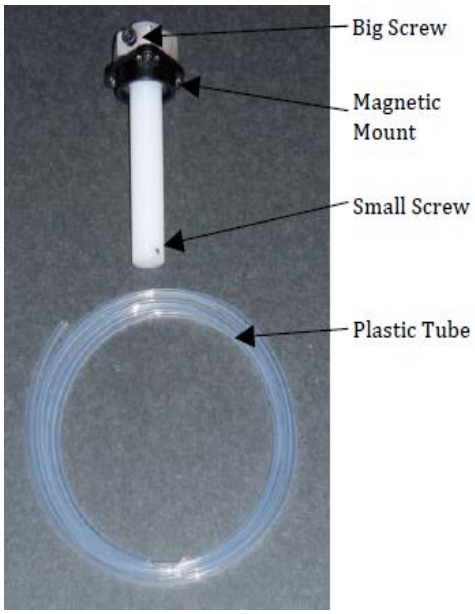


Figure B: Holder 1, fits samples, embedded to agarose, which can be attached to the aluminum base. Consists of screw, magnetic mount and 10 aluminum bases.

Figure C: Holder 2, works for setting 3mm diameter tubes, with samples put in a fluid to reduce refractive index, its set includes: a big screw, magnetic mount, small screw and a plastic tube.