Image Analysis System for Quantitative Immunofluorescence Measurement

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BIOGRAPHY

Yury Kolesnik PhD, MD is a professor and chief of pathophysiology department of Zhaporozhye State Medical University. He received his PhD degree for developing method of diabetes mellitus treatment by interval hypoxic trainings. In 1995 he obtained "Soros professor" degree. The current investigations of pathophysiology department is focused on new approaches of diabetes mellitus treatment, including administrations of neuropeptide. For the works in this area, Yury Kolesnik, Andrew Abramov and Maxim Orlovsky received award form Academy of Medical Science of Ukraine.

ABSTRACT

Immunofluorescence methods for hormone-containing cells exposure are widely used but the quantitative nature of the immunostaining is frequently ignored. In this study, quantitative measurements of fluorescent pancreatic beta-cells before and after glucose stimulation were used to determine parameters characterizing insulin synthesis and secretory activity. Beta-cells in Wistar rats pancreatic islets were stained using indirect immunohistochemistry, with subsequent processing on image analysis systems. The presence of three independent beta-cell classes with different insulin synthesis and secretion activity was shown by subsequent cluster analysis and confirmed by changes in insulin blood level.

KEYWORDS

insulin, beta-cells, immunofluorescence microscopy, image analysis

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INTRODUCTION

Immunofluorescence methods to demonstrate hormone-containing cells are widely used in many areas of application [1], but the quantitative nature of the immunostaining is frequently left out of these reports.

Image processing techniques devoted to the study of biological specimens have made many valuable contributions to science by providing new and high accuracy information about the objects being studied [2]. A wide range of software applications developed for image analysis can be used for automatic high-performance quantitative immunofluorescence analysis including cell enumeration, as well as measurements of staining intensities and positive areas (that is, morphometry) [3]. Besides, they give additional improvement of the analysis precision by allowing improvement of image quality (particularly reduction of out-of-focus glare) and automatic artifact exclusion [3]. Although modern image analysis techniques make it possible to perform complex and precise morphometry and densitometry measurements they are rarely used for quantitative synthetic and secretory process estimation. Thus, our goal was to prove the possibility of high-precision quantitative measurements of the secretion and synthetic process activity using immunofluorescence combined with subsequent quantitative image analysis.

The majority of recent works devoted to pancreatic beta-cells usually estimate their state by measuring insulin levels in extracellular medium or blood using radioimmunoassy techniques. However, such approaches do not allow one to perform complex evaluation of insulin synthesis and secretion activity because only the amount of secreted hormone can be measured and insulin synthesis activity can't even be estimated. The mRNA *in situ* hybridization, being another widely used method for endocrine cell investigation, should be exercised with caution in the case of beta-cells because the pre-pro-insulin mRNA content in them does not reflect insulin synthesis activity [4]. However, immunofluorescence methods coupled with computerized image analysis provide a perfect solution of this problem.

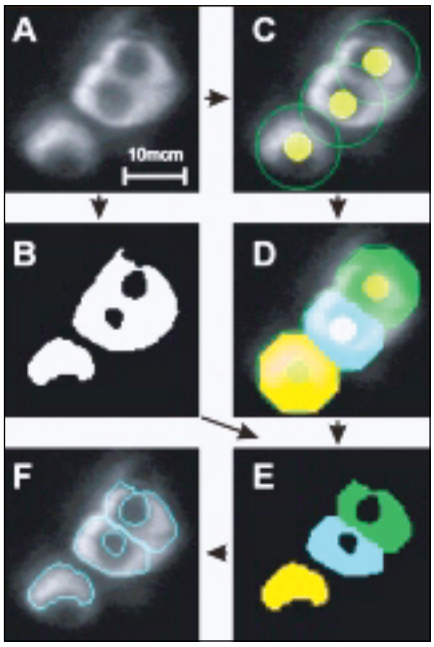

Figure 1:
Image analysis scheme. The description is given in the text.

MATERIALS AND METHODS

Experimental design

18 male Wistar line rats were divided into control and two experimental groups. 40% glucose solution i.p. (5 ml per kg) was given to the second and the third group of animals, which were killed after 5 mins (2nd group) and 30 mins (3rd group) respectively. These terms were selected according to the well-known insulin synthesis and secretion patterns after acute glucose loading: the maximum insulin secretion is observed from the third to the fifth minute [5, 6] and maximum insulin synthesis in beta-cells is observed after 30 mins [5, 7]. Immediately after killing the pancreatic gland was extracted, fixed in Bouin's solution and embedded in paraffin by standard techniques [8].

Immunofluorescence and image capture

Five-micrometre serial sections of pancreas were prepared on a rotation microtome. Beta-cells in pancreatic islets were stained for insulin using immunofluorescence kit IFK-7303G (Peninsula Laboratories Inc., USA) as described elsewhere [8]. Slices were viewed and captured using an Axioscop fluorescence microscope (Zeiss, Germany) with a 40x objective. To prevent signal fading all images in a slice were captured in the time interval of 2-3 mins [3] and were saved on a hard drive. According to recommendations in [3], a high-sensitivity CCD monochrome video camera (COHU-4722, COHU Inc, USA) with automatic gain control turned off was mounted on the microscope for image capture. All images were captured by a digital board PCB 965 with a video module PCB 966 framegrabber (Kontron Elenktronik, Germany) and automatically digitized to 386x256 pixels with 256 grey levels (8 bits per pixel).

Quantitative image analysis

Only clear images without artifacts were analyzed. The analysis was performed in several stages (Fig 1). In the first stage a noise mask obtained with the camera shutter closed was subtracted from the original image (Fig 1A) and areas with a non-zero grey level were selected for further processing (Fig. 1B). In the second stage, manual dot-marking of all beta-cells in the image was performed (Fig. 1C). Next, a new image with small circles (7 pixels in diameter) in place of the marker dots was created; circles were coloured in different colours and the background was eroded (Fig. 1D). This gave a discrete mask of beta-cells, which was logically XORed with the original image without noise mask (Fig 1E). After the described procedures a set of beta-cells, each of which was represented by a set of pixels with fixed grey level, was obtained (Fig 1F). For further calculations, instead of the original values of pixel grey levels we used a decimal logarithm of pixel grey level divided by a mean background grey level. The obtained value here and in future will be called an adjusted pixel grey level – APGL.

$$p'_n = \lg\left[cp_n\left(\sum_{i=1}^{c} b_i\right)^{-1}\right] \qquad (1)$$

where p'_n is the sought APGL, p_n – an initial pixel grey level, b_i –grey level of i^{th} background pixel, c – a number of pixels in background area (an area not belonging to any cell).

Within each identified beta-cell the following parameters were measured:

1. IR Area (S_{ir}, μm) – the area of a specific immunofluorescence within a single cell. (Area occupied by cell nucleus had been always excluded from immunofluorescence area.)

The area was calibrated in mm^2 by multiplying the number of image pixels occupied by specific fluorescence on X- and Y-axis metric coefficients.

2. IR Density (D_{ir}, fluorescence units – FU) – the mean fluorescence flux density within specific IR area of a single cell. D_{ir} was calculated as a mean AGPL value in cell boundaries:

$$D_{ir} = \frac{1}{n}\sum_{i=1}^{n} p'_i \qquad (2)$$

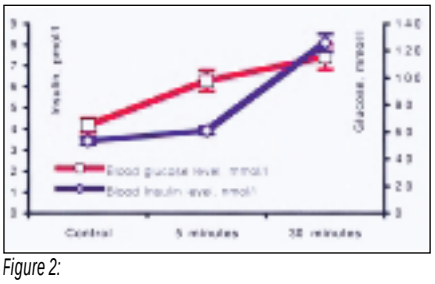

Figure 2:
Blood glucose and insulin levels prior to and following acute glucose loading.

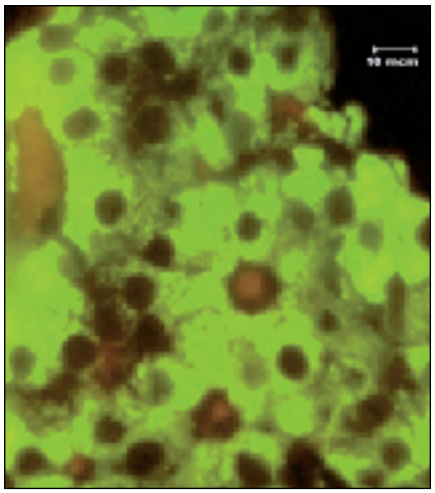

Figure 3:
Beta-cells of the pancreatic islet 5 mins after glucose loading. Indirect insulin immunostaining.

where n – the number of pixels in a cell, p_i – APGL of i^{th} pixel within a cell, b – mean background grey level. As described elsewhere [3], antigens during immunofluorescence reaction bind an equivalent antibody amount with an equivalent FITC content, so IR density can be considered to be dependent on the hormone concentration in a cell [9].

3. IR Content (C_{ir}, FU) – the integral fluorescence flux value within specific IR area of a single beta-cell was measured as a sum of APGL:

$$C_{ir} = \sum_{i=1}^{n} p'_i = nD_{ir} \qquad (3)$$

4. IR Heterogeneity (H_{ir}, FU) – the standard deviation of APGL values within single cell boundaries:

$$H_{ir} = \sqrt{\frac{\sum_{i=1}^{n}(p'_i - D_{ir})}{n-1}} \qquad (4)$$

This parameter shows the degree of hormone content heterogeneity in different compartments of a cell.

5. IR fragmentation. Hormone if being contained in a small amount in a cell often is not presented in all cytosol regions, so cell IR area has a fragmented look. The lesser content of hormone causes the increase of IR fragments number. We have measured IR fragmentation level as a percent of a cells containing more then one IR fragment.

Statistical analysis

Subsequent correlation analysis was exploited to answer the question if the chosen parameters were independent from each other. The analysis has shown no significant correlation between all parameters except C_{ir}, thus S_{ir}, D_{ir}, H_{ir} and F_{ir} were considered as independent variables.

The primary data in couple with our observations and literature evidence [10] suggest the heterogeneity of the islet beta-cell population in synthetic and secretory responses. The given plot (Fig 4) illustrates this suggestion showing abnormality of beta-cell distribution by the D_{ir} parameter with three modes. For proper separating of different classes of beta-cells we used the cluster analysis algorithm *"Partitioning around medoids"*, thoroughly described in [11]. This algorithm closely relates to another widely used cluster method *"K-means clustering"*, but exceeds it in precision of obtained results [11]. Cluster analysis was performed using three independent parameters (S_{ir}, D_{ir}, H_{ir}), which were normalized as follows:

$$S_N = \frac{1}{2}\lg S_{IR},\quad D_N = D_{IR},\quad H_N = \frac{1}{2}H_{IR} \qquad (5)$$

Inter- and intra-class statistical comparisons were done using Student's *t*-test.

Insulin and glucose blood levels

Additionally, blood insulin and glucose levels measurements were performed. Insulin blood levels were determined using radioimmunoassay techniques and a One-Touch II Glucometer (Life Scan, Johnson and Johnson, Milpitas, CA, USA) was used to measure the blood glucose for each individual animal [12]. The insulin and glucose levels were measured at the beginning of the experiment and 5 and 30 mins after intraperitoneal glucose loading immediately before killing.

Computer analysis

In all stages of image analysis the certified software image analysis system VIDAS–2.5 (Kontron Elektronik, Germany) was used. For the purpose of automatic image processing an original macro program was developed. All other calculations were performed on a Pen-

Table 1: Beta-cell parameters prior to and after 5 and 30 mins from acute glucose loading. All values are given as mean ± S.E.M. Statistical significance: 1 – to starting values, 2 – to the 5th minute values; a – p<0.05, b – p<0.01, c – p<0.01.

	IR area, ìm^2	IR density, FU	IR heterogeneity FU	IR fragmentation
Class 1				
Starting values	118.73 ± 1.08	3.13 ± 0.01	0.340 ± 0.003	1.1%
5th minute	114.70` ± 1.24 [1a]	3.03 ± 0.01 [1c]	0.319 ± 0.003 [1c]	0.9%
30th minute	118.55 ± 1.24 [2a]	3.04 ± 0.01 [1c]	0.346 ± 0.003 [2c]	1.7%
Class 2				
Starting values	91.46 ± 1.33	2.35 ± 0.01	0.304 ± 0.002	10.7%
5th minute	102.35 ± 1.20 [1c]	2.36 ± 0.01	0.293 ± 0.002 [1c]	5.8%
30th minute	103.93 ± 1.30 [1c]	2.34 ± 0.01 [2a]	0.297 ± 0.002 [1a]	4.2%
Class 3				
Starting values	56.19 ± 6.04	1.91 ± 0.02	0.284 ± 0.006	23.9%
5th minute	59.61 ± 1.42	1.67 ± 0.01 [1c]	0.276 ± 0.001	21.9%
30th minute	69.04 ± 2.53 [1b]	1.91 ± 0.01 [2c]	0.275 ± 0.002	25.1%

tium III computer (512 MB RAM, 10 GB HD). For statistic analysis the S-PLUS 2000 Professional system (MathSoft, Inc.) was used. Photos were printed on an Olympus Camedia 330NE digital color printer.

RESULTS AND DISCUSSION

Effects of acute glucose loading

5 mins after acute glucose loading, when the first phase of insulin secretion took place [5, 6], a significant rise of blood insulin and glucose level was observed (Fig 2). At 30 mins, when the second phase of insulin secretion and a rise in insulin synthesis was expected [5, 7], we observed further significant elevation of the insulin plasma level. Like others [5], we did not observe any significant decrease in blood glucose level as compared to the 5th minute.

In beta-cells significant decrease in IR area, concentration and heterogeneity were observed at 5 mins. Visually, beta-cells became smaller and darker and looked like 'empty' ones because of intensive secretion processes. Insulin coming to blood after exocytosis produced a significant rise in insulin blood level. At 30 mins IR area and heterogeneity in beta-cells significantly rose up to the control levels, while the rise in IR concentration took place to lesser extent. The IR parameters restoring indicates activation of synthetic process and renewal of the insulin store. To summarize discussion up to this point, we can conclude that increase of the IR area, concentration and heterogeneity values should be interpreted as the activation of beta-cell secretory activity, while decrease in these parameters should be suggested as activation of insulin synthesis.

Beta-cell population heterogeneity

However, caution should be exercised in the interpretation of the mean IR parameters, measured on the whole beta-cell population, because the convergence of evidence suggests a secretory response heterogeneity within the beta-cell population in pancreatic islets, which is thought to be due to different sensitivity to secretory stimuli, such as glucose [10]. From the current point of view, every beta-cell has a so-called set point for glucose. When the glucose concentration in the intercellular medium reaches the set point value, the beta-cell becomes depolarized and insulin-containing vesicles are secreted. The glucose set point lies under the humoral control, and a lot of currently known hormones and neuropeptides can affect insulin secretion by changing it [13]. The multiple-factor control of the glucose set point could be the main reason leading to the above-mentioned beta-cell population secretory heterogeneity. Results being obtained in our laboratory support this assumption (unpublished data).

In our microphotography (Fig 3), obtained at 5 mins following glucose loading, one can see cells which differ in their area, fluorescence density and distribution of the IR material. The histogram for C_{ir} (Fig 4) clearly shows three distribution modes, which in our

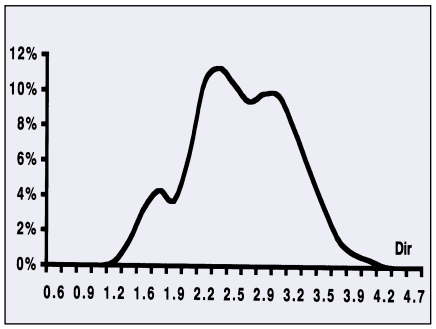

Figure 4:
Histogram of beta-cells three-modal distribution by the D_{ir} parameter.

assumption corresponds to three beta-cell classes with different insulin synthesis and secretion activity. To prove this assumption, all beta-cells in all experimental groups were clustered as described above. Cluster analysis was performed on the basis of three parameters, which were revealed as independent during previous correlation analysis.

Beta-cell classification

The cluster analysis resulted in the following data set (Table 1). As it can be seen from the table, the first class of beta-cells has the highest IR area, concentration, heterogeneity and lowest IR fragmentation value. As concluded above, it can take place in the case of the insulin synthesis predomination upon its secretion in the account of high synthetic activity or little secretion. Beta-cells in the third class show the lowest S_{ir}, C_{ir}, H_{ir} and highest F_{ir} values. This could be due to a predominance of

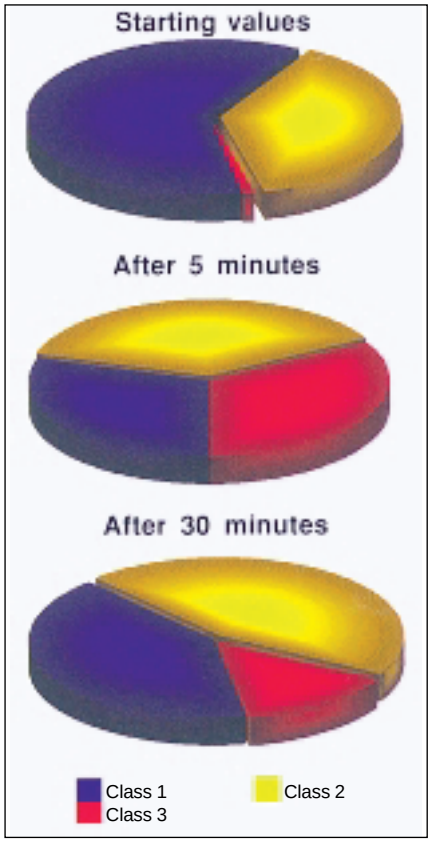

Figure 5:
Classes proportion prior to and following acute glucose loading.

insulin secretion under synthetic processes, which can be observed after acute beta-cell stimulation. The second class mean values lie between the first and the third class values. The cells in this class most probably begin to secrete or start to renew hormone storage after secretion completion. All these considerations will be confirmed if we look at the proportions of classes presented in different terms following glucose loading (Fig 5). Initially there were no beta-cells in the third class and the whole population was distributed between the first two classes. The activation of insulin secretion at 5 mins caused a great rise in the third class quantity, while at 30 mins it decreased and the cells amount in the second class increased.

It is noteworthy that the IR fragmentation parameter showing no correlation with other parameters and being not used by us in the classification varies considerably across different classes of beta-cells. We suggest this is additional evidence for the existence of three independent classes of beta-cells with different insulin secretory and synthetic activity.

CONCLUSIONS

To summarize the discussion given above we can conclude that quantitative measurements of immunofluorescence images by using image-analysis software are very useful and give a powerful tool not only for morphology, but also for cell physiology studies. Exploiting this tool in this study together with the cluster analysis we have obtained strong evidence for the existence in the pancreatic islets of at least three beta-cells groups with different insulin secretory activity. We hope that our results can help in a deeper understanding of endocrine and exocrine cell physiology.

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