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Optimization of endocrine pancreas fluorescence analysis using machine methods

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The study aims to establish the appropriate parameters of UV excitation intensity and permanent excitation time on the pancreatic islets photobleaching, the ratio of the intensity of the useful signal in the region of interest to the intensity of nonspecific background fluorescence.

Materials and methods. The pancreas of three adult Wistar rats was fixed in Bouin solution (20 hours) and poured into paraplast after standard histological processing. The study was carried out on paraffin sections of the pancreas. The islets' insulin and glucagon were determined by immunofluorescence method using monoclonal antibodies (Santa Cruz Biotechnology). The immunofluorescence reaction was studied using an AxioImager-M2 fluorescent microscope. AxioVision digital image analysis system was used for fluorescence imaging, and ImageJ 64-bit image analysis system was used for image quantification. 30 pancreatic islets with an area from 3000 μm^2 to 5000 μm^2 (8–13 % of the frame area) were analyzed.

Results. Measurements carried out at constant values of hormone concentration in endocrinocytes showed a different estimate of the average fluorescence intensity for insulin and glucagon, which depended on the intensity of UV radiation. As the intensity of UV radiation increased, the average fluorescence intensity in the region of interest for insulin and glucagon increased, but when the camera exposure was corrected, it became almost the same. Regardless of this, the intensity of nonspecific background fluorescence increased monotonically. The use of the ratio of the logarithms of the background fluorescence of the drug and the fluorescence of endocrinocytes in the calculations gives a stable estimate of the relative concentration of hormones, which does not depend on the intensity of the selected UV radiation regime, as well as on the duration of UV irradiation of the drug. This makes it possible to neutralize the effect of photodynamic discoloration of the preparation caused by continuous irradiation. Methods for machine selection of the region of interest by various algorithms of the ImageJ program lead to different estimates of its area, integral, and average fluorescence values. At the same time, the result closest to the "ideal" interactive method of highlighting the area of interest for insulin and glucagon was shown by Otsu's algorithm.

Conclusions. In immunofluorescent examination of the pancreas, a moderate UV radiation mode should be selected, exposure correction of the CCD camera before taking each frame, and the total time for examining the visual field of the sample should be limited to 1–2 minutes. To highlight the area of interest for insulin and glucagon in automatic analysis, it is recommended to use the Otsu algorithm. To obtain a quantitative estimate of the average fluorescence intensity in the region of interest, it is recommended to use the ratio of the logarithms of the background fluorescence of the drug and endocrinocytes in the calculations.

Ключові слова: флуоресцентна мікроскопія, система цифрового аналізу зображень, підшлункова залоза.

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Оптимізація флуоресцентного дослідження ендокринної частини підшлункової залози з використанням методів машинного аналізу

Т. В. Іваненко, А. В. Абрамов

Мета роботи – встановити оптимальні параметри інтенсивності, тривалості безперервного УФ-випромінювання на фотодинамічне знебарвлення досліджуваних тканин підшлункової залози, а також на співвідношення інтенсивності корисного сигналу в зоні дослідження флуоресценції та інтенсивності неспецифічної флуоресценції фону.

Матеріали та методи. Підшлункову залозу трьох дорослих щурів лінії Wistar фіксували в розчині Буена (20 годин) і заливали в парапласт після стандартної гістологічної обробки. Дослідження здійснили на парафінових зрізах підшлункової залози, в яких імунофлуоресцентним методом визначали інсулін і глюкагон у панкреатичних острівцях за допомогою антитіл виробництва Santa Cruz Biotechnology. Реакцію імунофлуоресценції вивчали на мікроскопі AxioImager-M2. Для зйомки флуоресценції використали систему аналізу цифрових зображень AxioVision, а для кількісного аналізу зображення – систему аналізу зображень ImageJ. Проаналізували 30 панкреатичних острівців площею 3000–5000 мкм^2 (8–13 % площі кадра).

Результати. Вимірювання, здійснені при постійних величинах концентрації гормонів в ендокриноцитах, показали різну оцінку середньої інтенсивності флуоресценції для інсуліну та глюкагону, що залежала від інтенсивності УФ радіації. З посиленням дії УФ радіації середня інтенсивність флуоресценції для інсуліну та глюкагону збільшувалась, але внаслідок корекції експозиції камери ставала майже однаковою. Незалежно від цього інтенсивність неспецифічної флуоресценції фону монотонно зростала. Використання в розрахунках відношення логарифмів флуоресценції фону препарату та флуоресценції ендокриноцитів дає стійку оцінку відносної концентрації гормонів, що не залежить від інтенсивності обраного режиму УФ радіації, а також від тривалості УФ опромінення препарату. Це дає змогу нівелювати ефект фотодинамічного знебарвлення препарату, спричиненого безперервним УФ опроміненням. Методи машинного

виділення ділянки інтересу різними алгоритмами програми ImageJ дають різну оцінку її площі, інтегральної та середньої величини флуоресценції. Результат, наближений до «ідеального» інтерактивного способу виділення ділянки інтересу для інсуліну та глюкагону, показав метод сегментації Оцу.

Висновки. Під час імунофлуоресцентного дослідження підшлункової залози слід обирати помірний режим УФ радіації, корегувати експозицію CCD камери перед кожним кадром, а загальний час дослідження поля зору зразка обмежити 1–2 хвилинами. Для виділення ділянки дослідження інсуліну та глюкагону під час автоматичного аналізу рекомендовано застосовувати алгоритм Оцу. Для отримання кількісної оцінки середньої інтенсивності флуоресценції у сфері дослідження рекомендоване використання в розрахунках відношення логарифмів флуоресценції фону препарату та ендокриноцитів.

The analysis of optical signals in microscopy plays an important role in scientific research in biology and medicine and in the diagnosis of human diseases. Among the many modern optical microscopy techniques, immunofluorescence is a standard approach among researchers (microscopists). It is used to detect and assess expression levels and localization of cellular proteins and other antigens in cells, tissue, and extracellular matrix. The use of some fluorophores in microscopy makes it possible to obtain photoemission in different spectral ranges, and, therefore, can be used to identify various cellular proteins synthesized within the cell or for the differentiation of intracellular organelles. To obtain accurate information in fluorescence imaging of macromolecules, a thorough understanding of the functional properties of fluorophores, optical imaging systems, and the effects of light-fluorophore interaction is required. The principles of fluorescence microscopy and the current development of this technology can be found in the review by S. Dunst & P. Tomancak [1], and various systems for obtaining optical data and approaches to their machine analysis are analyzed in the review by J. Fang et al. [2]. However, there are many different reasons why fluorescence imaging is sometimes not good enough to get true data. Some of the most common factors that reduce the quality of fluorescence imaging, as well as ways to improve the quality of fluorescence images, are reviewed by B. Herman [3], and J. W. Lichtman & J.-A. Conchello [4].

In the present study, we will analyze the factors set by the microscopist and affecting the quality of fluorescence imaging: the level of UV excitation intensity, the duration of permanent UV radiation of the tissue during microscopy (permanent excitation time), the role of photodynamic bleaching on the intensity of the useful fluorescence signal in the region of interest (ROI) and non-specific background fluorescence (BG), and methods of machine-defined ROI. As an object for research, we chose a pancreatic islet (islet of Langerhans), which is characterized by the heterogeneity of the cell population in both humans and rodents, and therefore is interesting for the study of multifluorescence. The number of pancreatic islets in the pancreas, the assessment of the pool of beta- and alpha-cells, as well as the quantitative assessment of the insulin and glucagon stores in pancreatic endocrinocytes, has an important practical meaning for understanding the mechanisms of pancreas endocrine function regulation, studying the mechanisms of immunological autoaggression against the islets, and their destruction in T1D (type 1 diabetes), the formation of amyloid, fatty infiltration, fibrosis, and inflammation (insulitis) in type 2 diabetes mellitus, as well as to assess the functional reserve of endocrinocytes for the creation of a bank of pancreatic organs and for their subsequent transplantation [5,6].

Aim

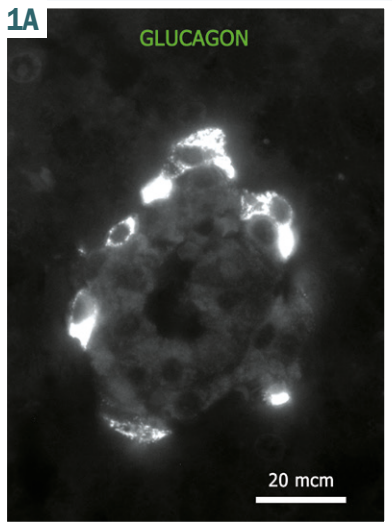
The study aims to establish the appropriate parameters of UV excitation intensity and permanent excitation time on the pancreatic islets photobleaching, the ratio of the intensity of the useful signal in the region of interest to the intensity of nonspecific background fluorescence.

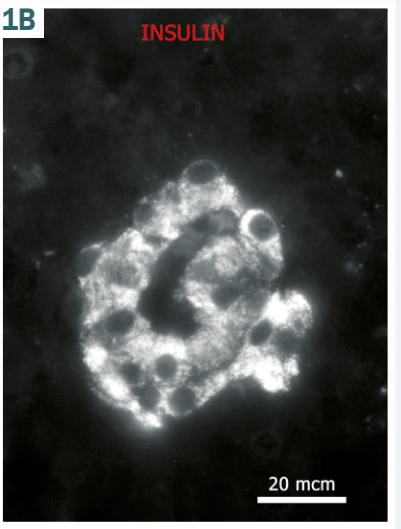
Materials and methods

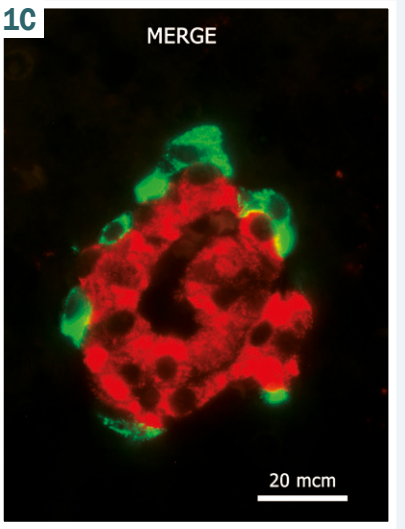
The pancreas of three adult Wistar rats was fixed in Bouin solution (20 hours) and poured into paraplast (McCormick, USA) after standard histological processing. Serial histological slices of the pancreas 5 μm thick were mounted on Superfrost™ Plus Microscope Slides (Menzel-Glaser, Germany), dewaxed, and unmasked in a citrate buffer solution (pH = 9.0) in the PT-module (Thermo Scientific, USA). Insulin and glucagon in pancreatic islets were detected by immunofluorescence techniques using antibodies produced by Santa Cruz Biotechnology (USA). Insulin Antibody (2D11-H5) AlexaFluor® 546-conjugated with Glucagon Antibody (K79bB10) FITC-conjugated were incubated in dilution 1:200 (wet chamber, T = +4 °C, 24 hours). Then slices were rinsed three times for 5 min in phosphate buffer solution (pH = 7.4) and enclosed in UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, USA) with cover glass (Menzel-Glaser, Germany).

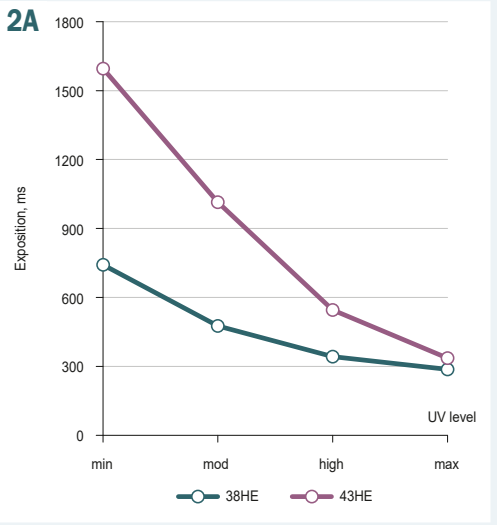
Immunofluorescence was studied using the AxioImager-M2 fluorescence microscope (Carl Zeiss, Germany) with mercury vapor lamp UV-light source HXP-120C (Leistungselektronik JENA GmbH, Germany), objective EC Plan-Neofluar 40x/1.3 Oil (Carl Zeiss, Germany), and the 14-bit digital monochrome camera AxioCam-HRm3 (Carl Zeiss, Germany). The high-emission 38HE filter (excitation 470/40 nm, beamsplitter 495 nm, emission 525/50 nm; Carl Zeiss, Germany) were used for glucagon detection, and the high-emission 43HE filter (excitation 550/25 nm, beamsplitter 570 nm, emission 605/70 nm; Carl Zeiss, Germany) were used for insulin detection. For fluorescent microscopy, we used Carl Zeiss™ Immersol™ immersion oil 518F (Fisher Scientific, Sweden). AxioVision Rel. 4.8.2 software (Carl Zeiss, Germany) was used for fluorescence frames shooting, and 64-bit image analysis system ImageJ Version 2.1.0/1.53c (an open-source under the GNU General Public License) was used for quantitative analysis of the immunofluorescence reaction.

We used grayscale images (1388 × 1040 pix) for quantitative analysis, on which the region of interest (ROI) corresponds to insulin or glucagon fluorescence, as well as the area of non-specific background fluores-









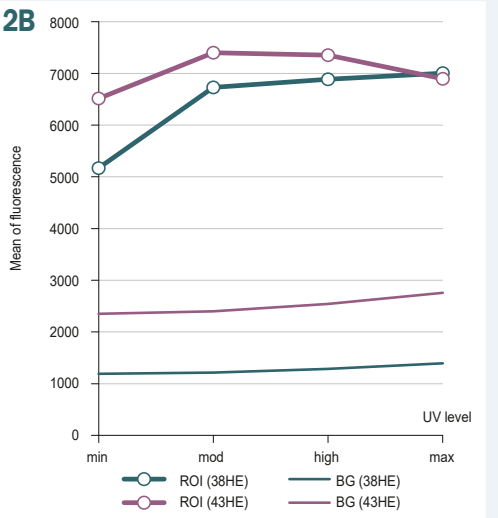


Fig. 1. A pancreatic islet (islet of Langerhans) of the adult Wistar rat.

Fig. 2. Influence of excitation intensity on the CCD camera exposure duration during shooting (**A**) and the mean of fluorescence intensity (**B**) in ROI and nonspecific BG fluorescence for FITC (filter set 38HE) and AlexaFluor®546 (filter set 43HE) fluorochromes.

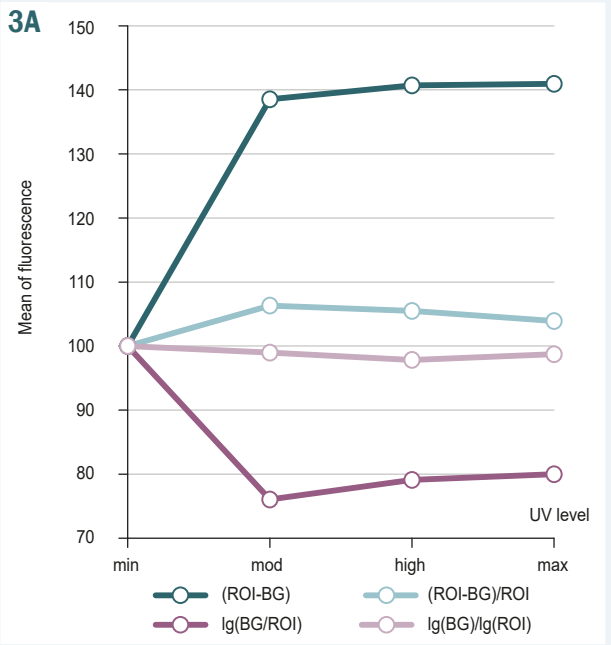
cence (BG) of the pancreatic slice, was selected by machine methods. Ten pancreatic islets from each animal (n = 30) with an area from 3000 to 5000 μm^2 (8–13 % of the frame area) were analyzed. Quantitative descriptors of fluorescence such as Total Area, Mean, Mode, Median, and Integrated density were measured using ImageJ software. The data are presented as average values of the measurement parameters of fluorescence in *Figs. 2–6* and *Table 1*.

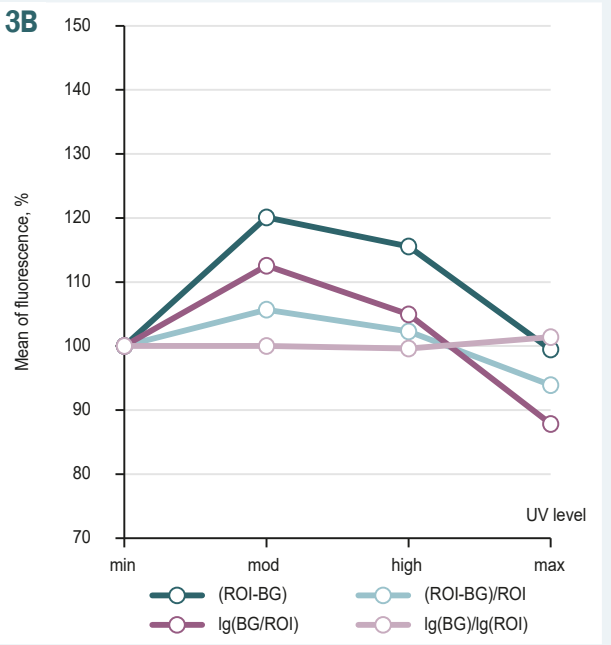
Results

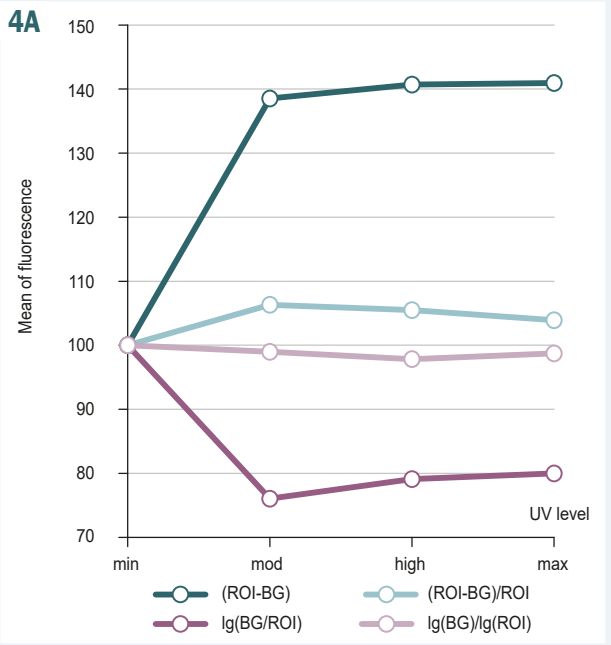
An example of a rat pancreatic islet is demonstrated in *Fig. 1*. We studied the effects of the intensity of UV radiation (excitation intensity), and the duration of radiation on the parameters of shooting a fluorescent image and on the quantitative characteristics of the fluorescence in the selected region of interest (ROI).

Excitation intensity influence. Modern digital CCD cameras have an auto-exposure mode for both light microscopy and fluorescence studies. For a better study of fluorescence, ultraviolet sources can adjust the mode of radiation. So, the HXP-120C UV source used by us allows using 4 modes, which visually differ in the intensity of fluorescence in the field of view, and which we conditionally called “minimum”, “moderate”, “high” and “maximum”. When choosing the mode of UV radiation intensity, the researcher must deal with two peculiarities of fluorescence microscopy: an increase of radiation intensity improves visualization in ROI but accelerates photodynamic bleaching, which affects the quantitative characteristics of fluorescence in ROI (*Fig. 2*).

The measurements were performed on the same pancreatic islet at constant values of hormones concentration (insulin and glucagon) in endocrinocytes. The obtained results showed different levels of average fluorescence intensity in ROI for insulin and glucagon. At the “min” mode of the excitation intensity, the frame exposure duration for FITC and AlexaFluor®546 fluorophores differed by more than 2 times. It is expected that as the UV radiation intensity increased, the camera exposure was shortened and became almost the same in the maximum mode for both fluorophores (*Fig. 2A*). The value of the average fluorescence intensity in ROI for insulin (filter 43HE) in the “minimum” mode of UV







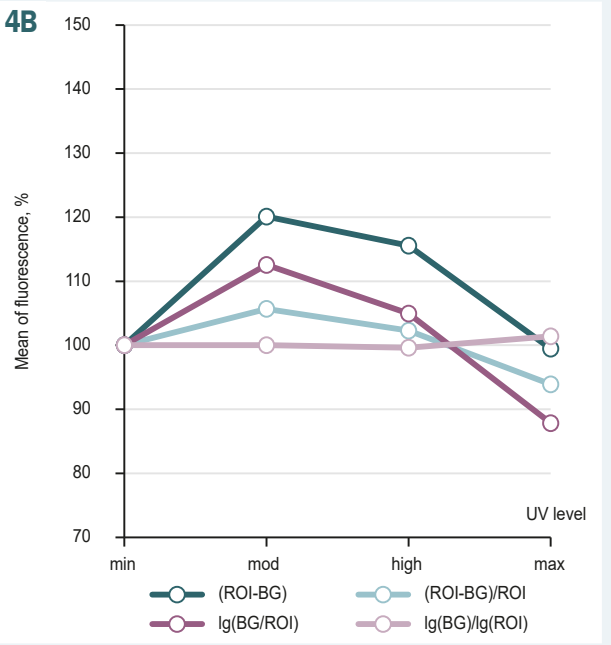


Fig. 3. Estimation of the average fluorescence intensity in ROI and nonspecific BG fluorescence for FITC (A) and AlexaFluor®546 (B) fluorophores at different excitation intensities.

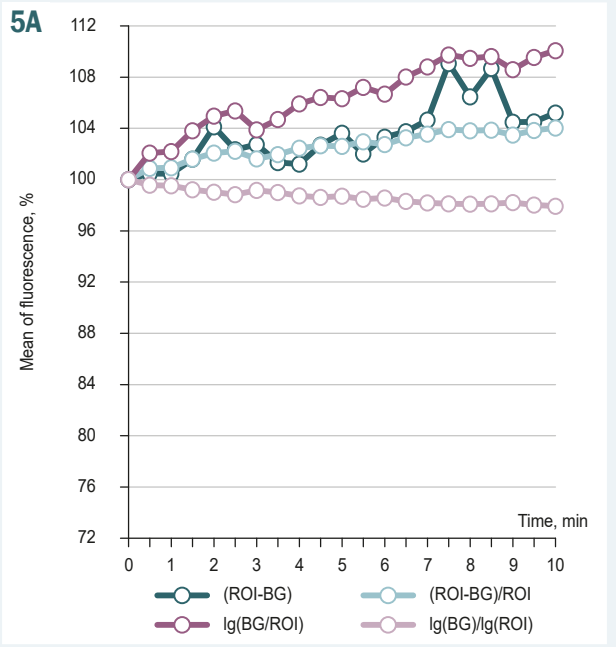
Fig. 4. Effect of permanent excitation time on CCD exposure time (A) and the average fluorescence intensity index (B) in ROI and nonspecific BG fluorescence for glucagon (FITC, filter set 38HE) and insulin (AlexaFluor®546, filter set 43HE).

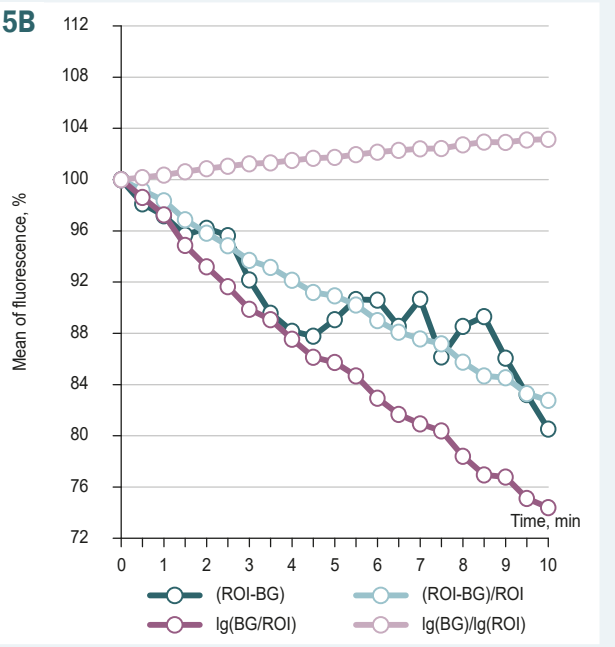
irradiation intensity was 26 % higher than for glucagon (filter 38HE), differed by 7–10 % in “moderate” and “high” modes, and was almost the same in “maximum” mode (*Fig. 2B*). At the same time, the intensity of nonspecific BG fluorescence increased predictably with increasing excitation intensity. Considering the fact that maximum parameters of average fluorescence intensity in ROI were obtained already at the moderate mode of excitation intensity, we consider this mode to be appropriate for use in practical fluorescence microscopy of the pancreas.

To estimate the average fluorescence intensity C_i in ROI, the following mathematical formulas were applied:

$$C_i = C_{ROI} - C_{BG} \quad (1);$$
$$C_i = (C_{ROI} - C_{BG}) / C_{ROI} \quad (2);$$
$$C_i = -lg\,(C_{BG} / C_{ROI}) \quad (3);$$
$$C_i = lg\,(C_{BG}) / lg\,(C_{ROI}) \quad (4).$$

where CROI is the average fluorescence intensity in ROI, and C_{BG} is the nonspecific BG fluorescence.





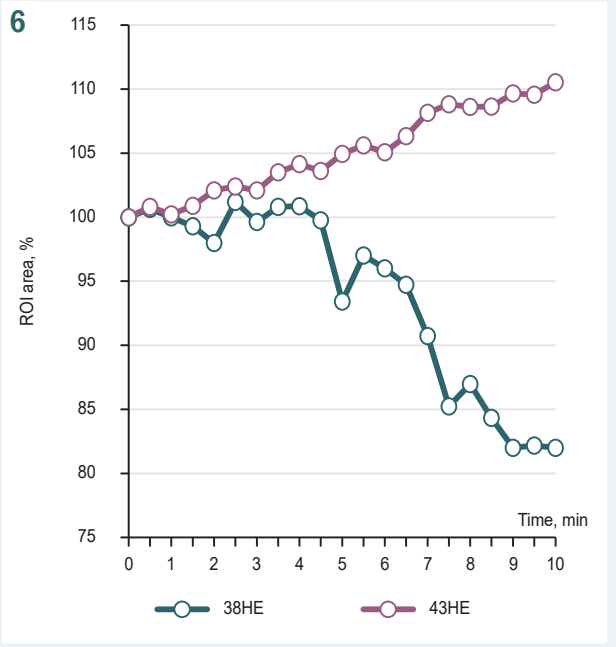


Fig. 5. Estimation of the average fluorescence intensity in ROI and nonspecific BG fluorescence for FITC, filter set 38HE (glucagon) **(5A)** and AlexaFluor®546, filter set 43HE (insulin) **(5B)** luorophores under continuous UV irradiation.

Fig. 6. Effect of permanent excitation time on the area of ROI for glucagon (FITC, filter set 38HE) and insulin (AlexaFluor®546, filter set 43HE). Otsu thresholding method was used to determine the boundaries of ROI.

The results shown in *Fig. 3* demonstrate that the ratio of the logarithms of the BG fluorescence of the pancreatic slice and the fluorescence of endocrinocytes (ROI) (formula 4) used in the calculation gives a stable estimate, independent of the intensity of the chosen excitation intensity mode.

Permanent excitation time influence. In immunofluorescence microscopy, the researcher constantly faces the effect of photodynamic bleaching of histological preparations with increasing duration of permanent excitation time, as well as with the impossibility of long-term storage of histological sections for analysis. To correct the effect of photobleaching and to adjust the fluorescent image in practice, the software for the microscopic camera uses automatic correction of exposure time and white balance setting. *Fig. 4* shows the analysis of the effect of the time of permanent UV radiation on camera exposure (Exposure Time) before imaging the same pancreatic islet. Exposure of the camera was adjusted by AxioVision program every 30 sec using the built-in algorithm for the emission length of each fluorophore separately, and UV radiation intensity was set to moderate mode.

As it can be seen from *Fig. 4*, the fluorescence of glucagon (FITC fluorophore) is stable and requires virtually no correction (-0.75 ± 1.60 ms every 30 sec), whereas insulin fluorescence (AlexaFluor®546 fluorophore) intensity decreases with prolonged excitation time and requires a correction with increased exposure (+79.35 ± 14.59 ms every 30 sec). Continuous 10-min UV irradiation slightly changes the average fluorescence intensity for glucagon

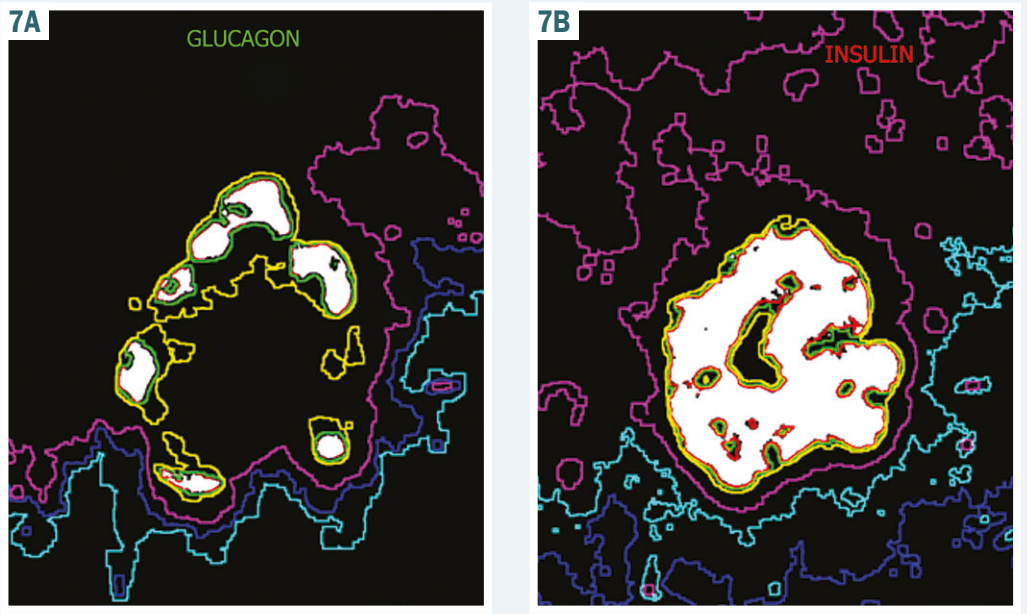


Fig. 7. Interactive selection of ROI (white), and ROI contours resulting from thresholding methods: Otsu (red), "Moments" (green), "MinError" (blue), "Huang" (cyan), "Mean" (magenta), "Li" (yellow).

Table 1. Thresholding method of the machine ROI selection and calculated area of ROI, mean and integrated fluorescence in ROI

Thresholding method of ROI selection	Glucagon (FITC, filter set 38HE)			Insulin (AlexaFluor546, filter set 43HE)		
	Area of ROI, %	Fluorescence		Area of ROI, %	Fluorescence	
		Mean, %	Integrated, %		Mean, %	Integrated, %
Interactive selection	100.00	100.00	100.00	100.00	100.00	100.00
Default	3653.83	26.87	981.75	**100.99**	**99.62**	**100.61**
Huang	9621.43	21.52	2070.97	1329.15	38.21	507.82
Intermodes	24.47	153.17	37.48	138.91	87.73	121.86
IsoData	**101.72**	**101.55**	**103.30**	99.65	100.08	99.74
IJ_IsoData	6791.62	23.51	1596.92	**100.99**	**99.62**	**100.61**
Li	432.45	54.05	233.73	124.24	92.03	114.35
MaxEntropy	**99.07**	**101.75**	**100.80**	129.07	90.51	116.81
Mean	6552.12	23.70	1553.18	580.42	48.39	280.89
MinError	8145.39	22.52	1833.94	1850.33	34.47	637.80
Minimum	0.94	226.45	2.13	114.59	95.10	108.98
Moments	165.74	82.15	136.15	106.80	97.64	104.28
Otsu	**99.30**	**101.62**	**100.90**	**100.24**	**99.85**	**100.09**
Percentile	7093.69	23.30	1652.94	1045.55	40.86	427.25
RenyiEntropy	84.46	108.78	91.88	133.28	89.16	118.83
Shangbhad	1168.40	36.92	431.41	2.69	167.71	4.51
Triangle	402.34	19.71	79.28	138.91	87.73	121.86
Yen	64.00	119.82	76.69	131.64	89.69	118.07

and insulin in ROI (statistical coefficient of variation corresponding to 1.91 % and 2.74 %, respectively). Nevertheless, there was a significant increase in nonspecific BG fluorescence for AlexaFluor®546 fluorophore (by +31.8 %, coefficient of variation 8.72 %), and a moderate decrease in this index for FITC (by -15.7 %, coefficient of variation 3.85 %). These changes occur due to the photodynamic bleaching phenomenon as the duration of permanent UV radiation increases.

Considering the peculiarities of the changes in the fluorescence pattern under permanent UV radiation, the issue of a reliable assessment of the average fluorescence intensity and the integral fluorescence in ROI arises. Solving this problem, we can estimate the average concentration of the hormone in the endocrinocyte dependent on fluorescence intensity, and the total content of the hormone in the pancreatic islet obtained by analysis of the integral fluorescence. The use of the ratio of the logarithms of the BG fluorescence and ROI fluorescence (formula 4) gives a stable assessment independent both on the intensity of UV radiation and permanent excitation time and, thus, decreases the effect of photodynamic bleaching of the pancreatic slice (*Fig. 5*). At the same time, it should be borne in mind that formula 4 gives a minimal negative time trend when assessing the concentration of glucagon ($y = -0.0889x + 99.664$) and a slow positive trend when assessing the concentration of insulin ($y = 0.1583x + 99.998$).

It is important to note another important fact of the fluorescence pattern change, related to the affection

of ROI determined by standard methods of machine ROI selection under the permanent excitation time influence (*Fig. 6*). In this case, the fluorescent pattern remains stable for the correct selection of ROI only for the first minute of UV irradiation.

Machine segmentation methods. Different thresholding methods of machine segmentation lead to different estimations of ROI area, integral and average fluorescence value in ROI (*Table 1*). Using Otsu method allows to obtain result closest to the "perfect" interactive method of ROI selection for insulin and glucagon. Fine results were also obtained using segmentation methods "MaxEntropy" and "IsoData" for glucagon, and methods "IJ_IsoData" and "Default" for insulin.

Some results of the machine thresholding methods of ROI selection are shown in *Fig. 7*.

Discussion

Pancreatic islets (islets of Langerhans) occupy about 1–2 % of the total volume of the pancreas, and analysis of the heterogeneity of its endocrine structure is the key to estimating both the mass of islet endocrinocytes in healthy people and the islet beta-cell reserve in patients with type 1 and type 2 diabetes [5,7]. Immunofluorescence technique is applied since the 1990s to estimate the number of pancreatic islets [8] and analyze the pattern of their distribution in pancreatic tissue [9]. Methods of digital analysis of fluorescent images were applied to count the mass of beta-endocrinocytes in the intact pancreas [10], the number and quality of isolated islets [11], and pancreatic transplant material [12].

The main steps in the digital analysis of any images, including fluorescence images, are the following: 1) image acquisition and preprocessing; 2) segmentation; 3) extraction of features (quantitative descriptors) which make possible the further classification of the different cell types [13].

1) When recording fluorescence it should be taken into account that fluorescence intensity behaves according to Planck's law: $\mathbf{E} = \mathbf{h}\nu = hc/\lambda$, where E is energy, h is Planck's constant, ν is light frequency, c equals light velocity, and λ equals light wavelength. Thus, energy is linearly proportional to the light frequency and inversely proportional to its wavelength. The quantum of energy (E) is greater for radiations of shorter wavelengths, such as UV radiation than for radiations of longer wavelength, such as IR radiation. Consequently, the quantum of energy emitted in the green part of the spectrum (FITC, filter set 38HE) is greater than the quantum emitted in the red spectral region (AlexaFluor®546, filter set 43HE). As a result of such energy difference of quanta, the effective exposure time required by the CCD camera during image capture for FITC fluorophore is less than for AlexaFluor®546 at all gradations of UV radiation intensity (*Fig. 2A*).

It should be considered that fluorophores are characterized by different fluorescence efficiency, or quantum yield (QY). A common way to compare the performance of fluorophores is to calculate the brightness index (BI): $\mathbf{BI} = \varepsilon \times \mathbf{QY} / 1000$, where ε is the extinction coefficient of the excitation irradiation. So, for the green fluorophore FITC $\varepsilon = 90 \times 10^3\ M^{-1}cm^{-1}$, $QY = 0.92$ and $\mathbf{BI} = 83$, and for the red fluorophore AlexaFluor®546 $\varepsilon = 104 \times 10^3\ M^{-1}cm^{-1}$, $\mathbf{QY} = 0.96$ and $\mathbf{BI} = 100$ [14]. Despite the difference in the value of the brightness index of fluorophores, due to the software correction of the CCD exposure time, approximately the same value of the useful fluorescence intensity in ROI is achieved (*Fig. 4*).

2) Segmentation is aimed to isolate ROI of objects: pancreatic islets or single endocrinocytes. This step is crucial for the subsequent feature extraction procedure [González2018]. Morphological studies of the pancreas necessary for reconstruction its natural 3-D configuration are labor-intensive, requiring high-quality immunofluorescence microscopy technology and analysis of a large array of serial sections using 2-D microscopy with subsequent mounting [5]. This method needs machine analysis of a large array of fluorescence images using machine learning techniques [2], and hence the need to determine the rules by which ROI is allocated.

Nobuyuki Otsu in 1979 proposed the thresholding method of ROI selection [15], which later became one of the most used binarization methods for a grey-level image [13]. It is a clustering-based thresholding method and it refers to a bimodal image histogram that separates foreground and background pixels. Otsu's algorithm proposes to minimize the weighted sum of intraclass variations in the foreground and background pixels to establish an optimal threshold. The optimal threshold value separating the two classes is selected by applying intraclass variance minimization and interclass variance. The mathematics of another thresholding methods of ROI selection can be found in M. Sezgin & B. Sankur [16].

Our analysis demonstrated the excellent performance of the Otsu's algorithm for the allocation of insulin and glucagon fluorescence in pancreatic multifluorescence studies, and also demonstrated the best results in solving the problem of differentiation and counting of microbial cells [17].

To obtain a quantitative assessment of fluorescence in ROI, it is also necessary to determine the boundaries of nonspecific BG fluorescence region. BG segmentation of the entire area of the fluorescent image beyond the islet zone, or ROI, is not appropriate. In the case of small islets, artifacts can be found in BG region, that increases the average fluorescence in the BG region. We recommend allocation BG region by the result of subtracting ROI for insulin and glucagon from the zone of the oval circumscribed around the islet. As a result, the BG region will include nuclei of alpha- and beta-cells, other islet endocrinocytes (delta-, epsilon-, and PP-cells), as well as intraislet vessels.

3) In a fluorescent examination, the microscopist must spend some time searching for the required object of study. This increases the permanent excitation time on the tissue sample and causes photodynamic bleaching, due to the interaction of the fluorophore with light and oxygen. Photobleaching reduces the fluorescence signal and hence the signal-to-noise ratio of an immunofluorescent image. This directly reduces the intensity of the useful fluorescence signal and, conversely, increases the intensity of the nonspecific BG fluorescence in practice. Thus, photobleaching affects the definition of quantitative descriptors of the region of interest.

To improve ROI quantification, we analyzed various approaches and found that the formula $Ci = lg(C_{BG})/lg(C_{ROI})$ provides the most constant relative fluorescence in ROI independently of UV intensity, and also eliminates the photobleaching effect which occurs with an increase in the duration of UV radiation. In practical fluorescence microscopy, the C_i parameter can be related to the concentration of the studied biomolecule (hormone, receptor) in the object (cell, islet), and its multiplication by ROI area ($Conti = Ci \times AREA_{ROI}$) corresponds to the total content of biomarker in an object of investigation (cell, islet, pancreas). We believe that such an approach to assessing the concentration and content of the biomolecules can be useful when comparing different experiments, including those conducted at a time distance from each other.

Conclusions

1. We propose to select the moderate mode of UV radiation for pancreatic immunofluorescence investigation with the necessary adjustment of the CCD camera exposure time before acquisition of frame and to limit the permanent excitation time within 1–2 minutes.

2. Otsu's algorithm is recommended as an optimal threshold selection method for the machine segmentation of the region of interest for insulin and glucagon.

3. We established that the ratio of the logarithms of the background and the region of interest gives stable fluorescence quantification even in photobleaching associated with a high excitation intensity and prolonged excitation time.

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