

Fluorescence microscopy as a source of automatic annotations for training neural network models for cell segmentation in bright-field microscopy

© A.I. Shvetsov¹, N.A. Kolotyeva^{1,2}

¹ Bauman Moscow State Technical University, Moscow, Russia

² Russian Center of Neurology and Neurosciences, Moscow, Russia

e-mail: shvetsov.anton@mail.ru

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The study presents a software-based approach for automated segmentation of cerebral endothelial cells in bright-field images using the YOLOv11 deep-learning architecture. The training dataset annotations are generated from fluorescent (Hoechst) images processed with classical computer-vision methods. The developed algorithm enables the extraction of endothelial cell nuclei in brain microvessels and the exclusion of regions corresponding to macrophages, which reduces the number of false-positive detections. The resulting binary masks are used for automatic generation of annotations required for training the neural-network model. The proposed method is potentially applicable to all types of cells amenable to fluorescent staining and enables automation of data preparation and analysis of cell images obtained via bright-field microscopy, thereby expanding the possibilities for non-invasive research in optical biophotonics.

Keywords: fluorescence microscopy, bright-field microscopy, machine learning, computer vision, image segmentation, endothelial cells, optical biophotonics.

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Introduction

Cerebral endothelium cells represent a universal *in vitro* model for investigating structural and functional features of blood-brain barrier, transendothelial transport, angiogenesis, neurovascular interaction, and pathogenic processes underlying the neurodegenerative and vascular central nervous diseases [1–4]. Due to its capability to form tight cell junctions and express specific transport and receptor systems typical of cerebrovascular endothelium, these cells make it possible to reproduce key properties of a neurovascular unit in controlled cell culture conditions, which makes them an indispensable tool both for fundamental studies and applied tasks of evaluating drug permeability through the blood-brain barrier and developing treatment strategies for cerebral pathologies [3–5]. For the purpose of biomedical studies, it is especially important to ensure continuous, non-contact and noninvasive monitoring of morphofunctional parameters of endothelial cells during their growth, migration and interaction with microenvironment.

Regardless of high specificity, traditional fluorescent imaging methods often have a set of constraints associated with introduction of exogenous pigments that can initiate cell stress, phototoxicity and metabolic disturbance, which distorts both cell morphology and behavior [6,7]. Fluorescent probes combining high specificity and minimized cytotoxicity become increasingly important in current research. Hoechst 33342, a vital DNA-intercalating dye containing ethyl group that provides effective penetration through a cell membrane, is one of such reagents. Due to its structure,

Hoechst 33342 is selectively accumulated in nuclei without staining cytoplasmatic components, which provides prerequisites for high-accuracy nucleus segmentation and real-time nucleus morphodynamics analysis. However, even vital fluorescent dyes offer exogenous action on a cell and require fluorescent irradiation, which can affect physiological state of cells during long-term observations. Therefore, optical imaging methods that don't infringe cell viability become especially important. Bright-field microscopy provides images of living cells without labeling, thus retaining their natural state and allowing long-term observation of a cell culture.

Regardless of the bright-field microscopy advantages, automatic segmentation of endothelial cells on the images is still a complicated task due to low contrast and cell boundary blurring, structural discontinuity of the cell layer and the presence of artifacts. Manual annotation is labor-consuming and poorly reproducible, which stimulates extensive employment of deep learning methods. One of early successful solutions is the use of U-Net for corneal endothelium segmentation where a model was able to achieve quite high quality scores during pixel classification and cell boundary detection [8]. A study devoted to performance evaluation of modern deep convolution networks for cell nucleus segmentation on bright-field images compared several architectures, including U-Net++, DeepLabv3+, Tiramisu, and a new light PPU-Net [9]. The authors have shown that deeper and advanced models are much better than the basic U-Net in terms of cell boundary detection accuracy and achieve a pixel accuracy

(F1 score, pixel-wise) of about 86%. A U-Net version with addition of residual and attention blocks was proposed in [10]. Such architecture provided much higher quality of semantic segmentation having reached mean-IoU of about 0.95. To separate adjoining cells, a watershed post-processing method was used to ensure correct segmentation of separate cells. Mobile-CellNet architecture proposed in [11] where several segmentation submodels and post-processing algorithms were combined provided cell density evaluation accuracy with an error less than 5% with a much lower computational cost than that of classical U-Net. The authors used 612 marked images with a resolution of 675×359 pixels, which emphasizes the need for a relatively larger amount of annotated data for effective training of neural network segmentation models.

To solve the problem of annotated data limitation and reduction of labor efforts for annotation, active learning approaches have been used in recent years, for example, in [12], an automated segmentation system combined with manual correction and fine-tuning of the U-Net and DeepLabV3+ models. In this work, segmentation generated by the neural network was subjected to manual correction using an interactive graphical interface allowing cell boundaries to be added, removed and corrected. Corrected masks were used for neural network re-training to improve segmentation quality on new images and reduce the annotation time for one image from 15–30 min to approximately 5 min.

Regardless of the effectiveness of active learning methods with manual correction, the annotation process is still dependent on the participation of an expert and requires considerable time input. Therefore, this work proposes fully automated approach to training data generation and endothelial cell segmentation that involves training dataset annotation on the basis of fluorescent image processing by classical computer vision algorithms. The proposed method removes the dependence of data preparation process on the manual annotation and has a high degree of versatility because it can be adapted to any type of cells susceptible to specific staining, which makes it a promising tool for automation of bright-field macroscopic image analysis and facilitates enhancement of noninvasive examination capabilities in the field of optical biophotonics.

Materials and methods

Primary endothelial cell culture production and incubation

Primary cerebral endothelium cell cultures were taken from 14-day old Wistar rats according to the standard procedure [13] with modifications [14]. Animals were pre-anesthetized, decapitated, brain was extracted, meninges and major vessels were removed by rolling the material on filter paper. Cortical substance was separated, large vessels were additionally removed in cold Hanks' balanced salt solution (PanEco, Russia). Cortex was mechanically ground

and centrifuged (1000 rpm, 3 min, room temperature). A doubled volume of 25% bovine serum albumin solution (BSA, Sigma, USA) was added to the obtained precipitate and the suspension was re-dissociated by pipetting. Centrifuging procedure (2000 rpm, 10 min, room temperature) followed by separation of the lower cell layer was repeated three times. Then the precipitate underwent enzymatic treatment in 0.1% collagenase II solution (Pan Eco, Russia) during 35 min at 37 °C in a roller. Enzymatic treatment was stopped by adding pre-heated Hanks' solution, then the sample was centrifuged (1000 rpm, 5 min). The precipitate was resuspended in DMEM/F12 (GIBCO Invitrogen Corporation, USA) supplemented by 20% fetal calf serum (HyClone, Austria), 2 mM glutamine (glutaMAX, Gibco, UK) and 16.6 mM glucose. Cells were put into 40 mm plastic Petri dishes (Medpolimer, Russia) pre-coated with matrigel, incubated at 5% CO₂, 37 °C and relative humidity of 98% (RWD Life Science CO₂ incubator, China). The medium was changed twice a week. After formation of confluent monolayer, cells were moved into 96-well culture plates (Servicebio, China), 100 µL per well containing $2 \cdot 10^5$ cells/mL. The Countess automatic meter (Invitrogen, USA) was used for cell viability evaluation and cell counting. Morphologic control of culture state during the growth process was performed by the phase-contrast microscopy method using the Olympus CKX41 (Japan) microscope.

Microscopy and data acquisition

Bright-field microscopy (Figure 1, *a*) and fluorescent microscopy (Figure 1, *b*) were used for cerebral endothelium cell examination. For nuclei imaging, cerebral endothelium cells were incubated with Hoechst 33342 (PanEco, Russia). For long-term growth observation, the JuLI Stage automated cell culture imaging system (Figure 1, *c*) (NanoEntek, South Korea) was used to provide simultaneous bright-field and fluorescent imaging. Fluorescent images were recorded using an optical channel with an excitation wavelength of 390/40 nm and radiation wavelength of 452/45 nm. A 5×5 frame imaging area was recorded in each well with 20x magnification. A frame resolution was 1936×1456 pixels, which corresponded to a $440 \times 330 \mu\text{m}$ field of view.

Results

For the purpose of experimental study, a cell biologist performed a preliminary morphological evaluation of confluent cell monolayer of the primary cerebral endothelium culture containing immune macrophages. In physiological conditions during angiogenesis, endothelial cells interact with resident immune cells of the central nervous system, in particular, with microglia that serves as tissue macrophages [15]. Consideration of this heterogeneous cell composition is critical for development of automated segmentation algorithms.

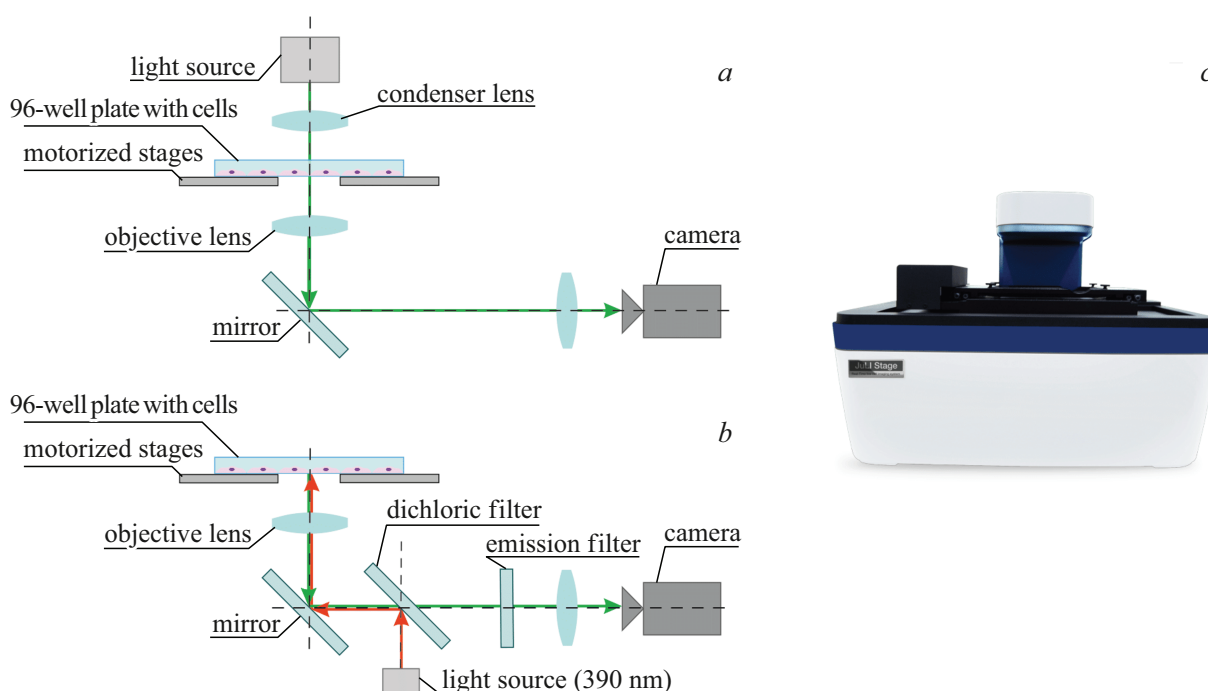


Figure 1. Experimental setup for bright-field and fluorescent microscopy of living cells: (a) optical circuit diagram of a bright-field microscope, (b) optical circuit diagram of a fluorescent microscope, (c) view of the JuLI Stage automatic microscope used in the work.

A software package on the basis of classical computer vision methods was developed for the task of automated fluorescent image annotation. The proposed algorithm performs endothelial cell nuclei segmentation followed by exclusion of objects localized in immediate proximity to macrophages, which reduces the number of false-positive objects induced by high fluorescent intensity.

Pre-processing stage involves adaptive histogram equalization by the CLAHE method for brightness distribution normalization, median filtration (3×3 nucleus) for granular noise suppression, and smoothing by the total variation method with a regularization coefficient of 0.1 to maintain object boundaries when removing high-frequency artifacts. Then, local threshold binarization with adaptation to inhomogeneous background (71×71 window, threshold coefficient of 1.05) and morphological treatment of the binary mask with removal of minor noise components (less than 1000 pixels) were performed.

Differences in the nature of dye accumulation in cerebral endothelium cell and macrophage nuclei cause pixel intensity range variations. Macrophages feature more compact nucleus morphology and, consequently, increased local fluorescent probe concentration, leading to a twice as high fluorescent signal intensity compared with endothelial cells. When macrophages are detected, exclusion zones with a radius of 5 pixels are formed around them. At the next stage, only objects at a distance more than 15 pixels from exclusion zones are maintained, which ensures correct separation of cell cultures.

At the final stage, morphological filtration by area is used to remove artifacts and aggregates, and adjoining nuclei are watershed separated with a minimum distance between 20 pixel peaks that ensures formation of topologically correct masks of isolated cells. Processing parameter values were selected using grid search on the basis of visual expert evaluation of segmentation results.

Obtained segmentation masks can be used for training neural network models built on the U-Net architecture. To form annotations compatible with annotation formats used in the YOLO models, masks are additionally transformed into a corresponding representation (polygonal object edges). This transformation is performed via a boundary detection (edge search) operation followed by smoothing the obtained edges to improve annotation correctness and stability. Fluorescent image processing visualization is illustrated in Figure 2.

To solve the task of automated segmentation of endothelial cell nuclei on bright-field images, the YOLOv11 deep learning architecture (Ultralytics) was selected [16], that consisted of an advanced real-time object detection and segmentation model encompassing localization and masking tasks within a single neural network architecture. This architecture is used because of its high computational efficiency, stability to object scale variations and high resolution image processing capability without significant loss of performance.

Training was performed on 5600 annotated images (80 % of sample) with an initial resolution of 1936×1456 pixels; validation was performed on remaining 20 % of data,

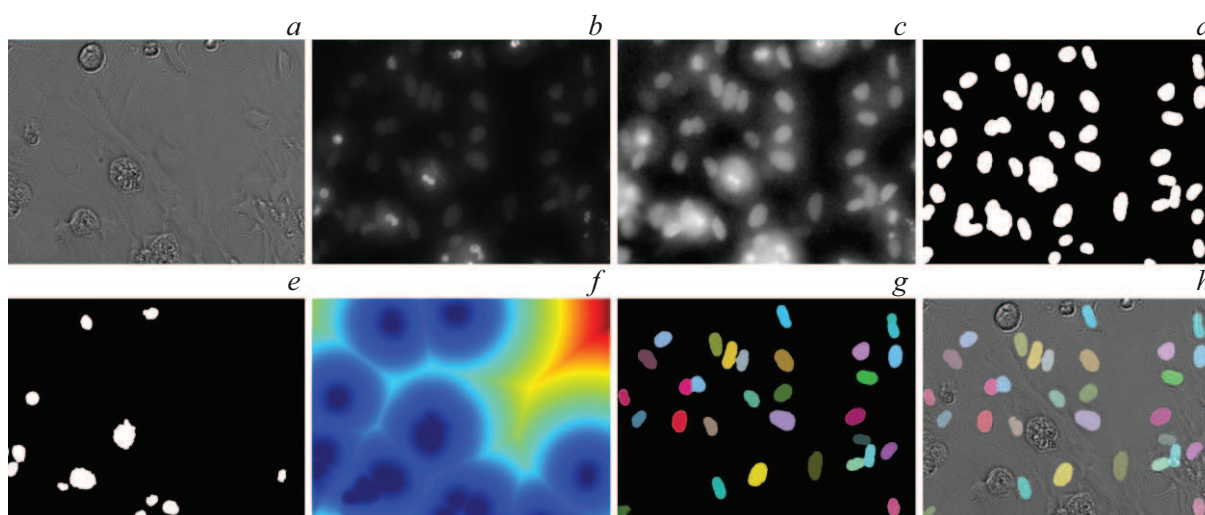


Figure 2. Example of fluorescent image processing: (a) initial bright-field image of endothelial cell nuclei, (b) initial fluorescent image of endothelial cell nuclei, (c) pre-processed fluorescent image of endothelial cell nuclei, (d) binary mask of all cells, (e) binary mask of third-party cells, (f) third-party cell distance map, (g) endothelial cell nuclei mask, (h) bright-field image with superimposed endothelial cell nuclei mask.

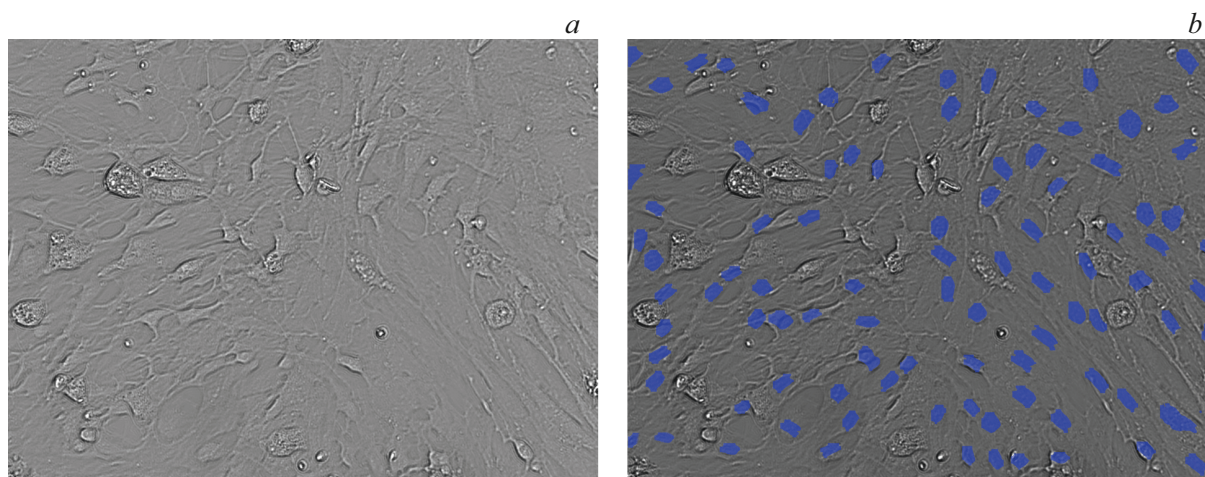


Figure 3. Example of endothelial cell segmentation: (a) initial bright-field image of endothelial cell nuclei, (b) processed bright-field image (predicted endothelial cell nuclei masks are marked in blue).

and testing was performed on an independent set of 50 images annotated by experts. The learning process used standard data augmentation methods aimed at improvement of generalization ability of the model, including random rotations, scaling, reflections and brightness and contrast variations. These transformations facilitated an increase in model resistance to a change of lighting conditions and morphological cell variations. Endothelial cell nuclei segmentation is illustrated in Figure 3.

Conclusion

Combination of the fluorescent microscopy and classical computer vision methods made it possible to computerize the bright-field image annotation process and form an ex-

tended dataset consisting of 7000 annotated images, which is much better than the manual annotation capabilities and ensures high data repeatability. The obtained dataset ensures training of a model that can perform cell segmentation on bright-field images in fully noninvasive mode without the need for using fluorescent imaging at the analysis stage.

Due to differences in dye accumulation in various types of cell nuclei and, consequently, to a difference in fluorescent signal intensity ranges, the developed approach can be adapted not only for endothelial cells, but also for any cell cultures susceptible to fluorescent staining, which considerably expands the scope of application and makes the method a virtually universal tool for analysis of homogenous and heterogenous cell cultures. At the same time, the method has a set of limitations caused by morphological

and functional heterogeneity of cell populations. It is primarily oriented to cells for which the nucleus is a dominating and structurally stable morphological feature. Moreover, in heterogeneous cultures where various types of cells accumulate a fluorescent probe with similar efficiency, leading to similar signal intensity, the algorithm is not able to perform reliable differentiation and classifies cells as a single cluster.

Thus, the proposed method forms a basis for the scalable and fully noninvasive cell structure analysis, being a significant process contribution to the development of optical biophotonics. Automatic annotation generation and subsequent cell detection on bright-field images significantly expand the capabilities of modern quantitative cell analysis methods. The obtained segmentation masks provide various morphometric parameters, including cell area and perimeter, degree of roundness and elongation, cell packing density, size distribution, and cell mosaic topology metrics such as the number of neighbours and polygonal structure shape variability. Definition of these characteristics is of high practical importance for functional state quantification and response of cell cultures to a change of microenvironment or occurrence of disease condition. In particular, cell area is a sensitive indicator of cell's metabolic activity, cytoskeletal organization and differentiation. Cell monolayer density reflects proliferative activity, cell interactions and barrier structure consistency, which is especially critical for dealing with blood-brain barrier models where confluency disturbance directly correlates with an increase in permeability. Such approach not only increases reproducibility and objectivity of biological experiments, but also provides the basis for development of label-free methods for early detection of cell disfunction.

Compliance with ethical standards

All experimental procedures involving laboratory animals were performed in accordance with the applicable law of the Russian Federation, internationally recognized principles of the Basel Declaration, and rules of humane care and use of laboratory animals. The study protocol was approved by the local ethics committee of the Russian Center of Neurology and Neurosciences (protocol № 10-9/23 as of 20.12.2023).

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Conflict of interest

The authors declare no conflict of interest.

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