

Phase-contrast microscopy for automated morphology analysis of astrocytes *in vitro* using deep learning-based method

© I.V. Simkin¹, T.E. Shcheveleva¹, N.A. Kolotyeva^{1,2}, S.V. Novikova^{1,2}, A.A. Simkina¹, E.V. Yakovlev¹, S.O. Yurchenko¹, A.B. Salmina^{1,2}, N.P. Kryuchkov¹

¹ Bauman Moscow State Technical University, Moscow, Russia

² Russian Center of Neurology and Neurosciences, Moscow, Russia

e-mail: vanyasimkin@gmail.com

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The study of astrocyte morphology *in vitro* is of fundamental importance for neuroscience. However, traditional analysis methods, which require cell fixation and staining, preclude the observation of dynamic structural changes in living cells. Phase-contrast microscopy is a non-invasive optical technique that can overcome this limitation, but the automatic segmentation and quantitative analysis of the resulting images remain challenging due to the variable and complex structure of astrocytes. This paper presents an advanced method for the automatic segmentation and morphometric analysis of phase-contrast images of astrocytes, based on a convolutional neural network architecture. The method was optimized through automatic hyperparameter tuning and achieved a Dice coefficient of 0.73 on the test dataset. The developed approach enables the automatic delineation of the astrocyte cell body and its processes, as well as precise measurements of key morphological parameters (area, length, and branching complexity) over time. Phase-contrast microscopy, as a non-invasive optical method, forms the basis of the developed approach, enabling real-time analysis of astrocyte morphology.

Keywords: phase-contrast microscopy, machine learning, image segmentation, YOLOv11, astrocytes, morphometric analysis, optical biophotonics.

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Introduction

Astrocytes are a distinct type of branched glial cell and play important roles in vital processes such as maintaining the structural and functional integrity of neurons, neuroplasticity, synapse formation, and regulation of blood flow, energy supply, and metabolism. As glycolytically active cells in the CNS, astrocytes mediate neuron–astrocyte metabolic coupling, meet the metabolic demands of stimulated neurons, and regulate local microcirculation [1]. In addition, astrocytes act as effector cells during neuroinflammation. During inflammatory processes, astrocytes become activated, which alters their functions. This functional change is associated with their immune-surveillance capacity, including antigen presentation and cytokine production, which enables them to modulate blood–brain barrier (BBB) permeability [2]. Interactions between astrocytes and extracellular matrix proteins are critical for maintaining BBB integrity [3]. In addition, astrocyte morphology reflects their metabolic state. Impaired astrocyte metabolic plasticity and changes in astrocyte structure and functional activity are observed in traumatic brain injury, stroke, neurodegeneration, and neuroinflammation [4, 5]. Thus, *in vitro* analysis of astrocyte morphology is essential for a better understanding of the contribution of astroglia to the regulation of brain function and the pathogenesis of CNS disorders.

Traditional methods of morphological analysis based on cell fixation and immunocytochemical staining are invasive

and preclude repeated observation of the same living cells during growth and functional activity. Moreover, these procedures can themselves affect subtle morphological features of the cells [6]. This limitation can be overcome by using noninvasive optical microscopy methods, among which phase-contrast imaging is one of the most effective tools for real-time observation of living cells without staining [7].

Phase-contrast microscopy remains one of the most accessible and widely used noninvasive methods for imaging living cells *in vitro*. However, advances in optical technologies and biophotonics over recent decades have led to the development of several alternative approaches for examining the morphological and metabolic state of cells without fixation or staining. One such method is digital holographic microscopy (DHIM), which is a form of quantitative interference microscopy. This method provides real-time data on the three-dimensional distribution of an object's refractive index, which reflects not only its morphological features but also its functional state [8–10].

Another promising approach is fluorescence lifetime imaging microscopy (FLIM), which enables assessment of the spatial distribution of the endogenous fluorescence of the cellular metabolic coenzymes NAD(P)H and FAD. The ratio of free to protein-bound NADH, estimated from fluorescence lifetimes, is a sensitive indicator of cellular metabolic status and enables noninvasive differentiation between glycolytic and oxidative energy-metabolism pathways without exogenous probes. This approach is particularly relevant to analyzing the metabolic plasticity of

neurovascular-unit cells under neuroinflammatory, hypoxic, and neurodegenerative conditions [11,12].

Surface-enhanced Raman spectroscopy (SERS) is used to assess mitochondrial redox state and respiratory-chain activity. This method enables real-time monitoring of redox transitions and conformational changes in cytochromes in living mitochondria, providing a unique opportunity to investigate mitochondrial dysfunction under cellular stress [11,13].

Raman microscopy provides information on the biochemical composition of cells based on inelastic light scattering. Owing to its high sensitivity, this method can detect subtle changes in the lipid, protein, and nucleic acid composition of cells associated with oxidative stress, inflammation, or differentiation [14].

Despite the rapid development of these imaging methods, phase-contrast microscopy was selected for the purposes of this study. This choice was motivated by its simplicity, accessibility, and minimal effect on living cells, which are critical for the experimental objectives. Nevertheless, automatic quantitative analysis of phase-contrast images is a serious computational task. The complex, low-contrast morphology of astrocytes renders many conventional image-processing algorithms unsuitable. Therefore, machine-learning methods, particularly convolutional neural networks (CNNs), are a promising approach and have demonstrated high performance in semantic segmentation of biological objects [15,16]. In [17], a Mask R-CNN (Mask Region-Based Convolutional Neural Network)-based approach to astrocyte segmentation was presented, demonstrating the feasibility of using CNNs to automatically segment cell bodies and processes in phase-contrast images, with a Dice coefficient of 0.72. In the present study, this approach was substantially improved by using the more recent YOLOv11 (You Only Look Once version 11) architecture [18] and optimizing its hyperparameters, which increased segmentation accuracy and method efficiency. This study presents an improved YOLOv11-based method for automated segmentation and morphological analysis of astrocytes. The Dice coefficient on the test set was 0.73. The developed approach automatically segments the cell body and its processes and enables accurate measurement of key morphological parameters, thereby creating new opportunities for nondestructive analysis of cell cultures.

Materials and methods

Isolation and culture of primary astrocytes

Primary astrocyte cultures were obtained from Wistar rats on postnatal days 1–2 (PD 1–2) by enzymatic–mechanical dissociation [19]. The isolated structures were washed with calcium- and magnesium-free phosphate-buffered saline (PBS; pH 7.4; Gibco Life Technologies, USA), incubated for 15 min at 37°C in a solution containing 0.05% trypsin and 0.02% EDTA (Gibco Life Technologies, USA), and the trypsin was then inactivated with culture medium.

The material was then dissociated by stepwise mechanical pipetting in culture medium. The culture medium consisted of 90% Eagle's minimum essential medium with Earle's salts (MEM; Gibco, UK), 10% fetal calf serum (HyClone, UK), 2 mM GlutaMAX (Gibco, UK), and 10 mM HEPES buffer (Sigma, USA), at pH 7.2–7.4. The cell suspension was seeded into two T-25 culture flasks. The cultures were maintained in an CO₂ incubator (RWD Life Science, China) at 37°C, 5% CO₂, and a relative humidity of 98%. After monolayer formation, the astrocyte cultures were transferred to 96-well plastic plates (Servicebio, China) at 100 µL of suspension per well, with a cell concentration of $2 \cdot 10^6$ cells/mL. Cell number and viability were determined using a Countess automated cell counter (Invitrogen).

Phase-contrast microscopy and data acquisition

The morphology of living astrocytes under native conditions was examined by phase-contrast microscopy (Fig. 1, *a*). For long-term monitoring of growth dynamics, an EVOS M7000 imaging system (Fig. 1, *b*; Thermo Fisher Scientific, USA) equipped with a built-in incubator was used to maintain the conditions required for cell viability (temperature, (37°C, CO₂, 5.0%; humidity, $\geq 80\%$). In each well, an imaging area comprising 5×5 frames was selected at random using $40\times$ magnification. Each frame had a resolution of 2048×1536 pixels, corresponding to a field of view of $320 \times 240 \mu\text{m}$. The experimental series included daily imaging of the cultures for 10 days.

Data preparation and annotation for model training

Annotating unstained astrocytes is complicated by the possible presence of other types of neuroglial cells in the images. To address this issue, representative examples of cells classified as astrocytes and non-astrocytes were collected. Among all images selected for neural-network training, only cells classified as astrocytes were annotated. Only astrocyte images in which the entire cell body was visible within the frame and was not obscured by other objects were used for training. The images were annotated using LabelMe software [20]. All annotated images (357 images) were divided into three datasets: 75% (268 images) were assigned to the training set, 15% (54 images) to the validation set, and 10% (35 images) to the test set.

To prevent overfitting, automated data augmentation was applied, including the following transformations: random image rotations (up to $\pm 15^\circ$), translations along the x and y axes (up to $\pm 10\%$ of the image size), scaling ($0.5\text{--}2.0\times$), noise addition, random changes in brightness and contrast, and horizontal flipping. These transformations were applied randomly during each training epoch, substantially increasing the variability of the training dataset without requiring manual annotation of additional images.

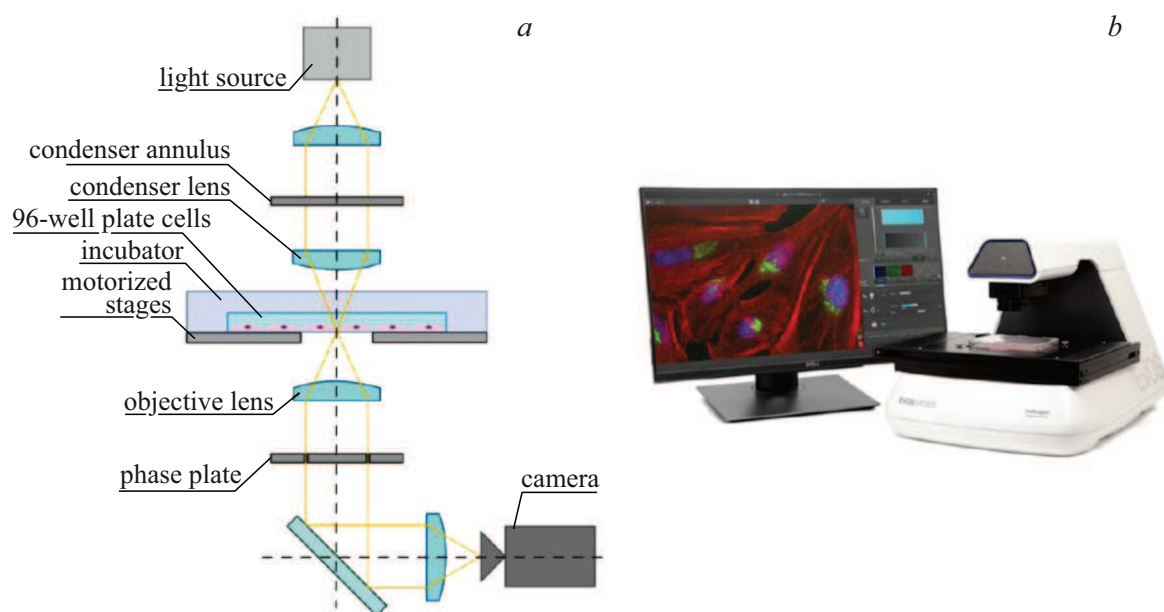


Figure 1. Experimental setup for phase-contrast microscopy of living cells. (a) Schematic optical layout of the phase-contrast microscope: light from the source passes through the condenser annulus and condenser lens, illuminates the cells in a 96-well plate, and is then collected by the objective. (b) Photograph of the automated EVOS M7000 microscope used in this study.

Results

A pre-trained Mask R-CNN model [21] with an Inception ResNet v2 backbone [22] was initially used to segment native astrocytes in phase-contrast images. Mask R-CNN is a well-established two-stage detector. The main advantage of Mask R-CNN is its high segmentation quality, particularly for objects with complex and variable morphology. However, its computational complexity and relatively low inference speed, resulting from the two-stage process, are major disadvantages. After training and hyperparameter tuning, the model achieved a Dice coefficient of 0.72 [18].

To improve the accuracy and efficiency of the algorithm, an alternative approach based on the YOLOv11 architecture (YOLOv11s-seg configuration) was used. YOLOv11 is a more recent single-stage detector than Mask R-CNN. The YOLO architecture performs detection and segmentation „in a single pass“. The main advantages of YOLOv11 are its substantially higher processing speed and lower computational-resource requirements while providing segmentation quality comparable to that of Mask R-CNN.

The YOLOv11 model was trained on the same dataset as Mask R-CNN. After hyperparameter optimization using the Optuna library and 150 training epochs, the final model showed a substantial improvement in segmentation performance. The Dice coefficient on the test set was 0.73. Comparative metrics of the models are shown in the table.

For a visual comparison, Figure 2 shows segmentation results obtained with Mask R-CNN and YOLOv11 for the same regions of astrocyte cultures. The YOLOv11-based method provides a substantial advantage in astrocyte detection while maintaining comparable segmentation qual-

ity: despite only a small change in the Dice coefficient, which characterizes contour accuracy, detection recall more than doubled. This improvement is important for the overall quantitative assessment of astrocyte cultures, because missed detections cause systematic bias in estimates of astrocyte morphological characteristics.

Metric	Mask R-CNN	YOLOv11 (current model)
Dice coefficient	0.72	0.73
Accuracy	0.29	0.60
Precision	0.71	0.76
Recall	0.33	0.73

ity: despite only a small change in the Dice coefficient, which characterizes contour accuracy, detection recall more than doubled. This improvement is important for the overall quantitative assessment of astrocyte cultures, because missed detections cause systematic bias in estimates of astrocyte morphological characteristics.

Conclusion

In this study, an improved approach for automated segmentation and morphological analysis of phase-contrast images of astrocytes was developed and validated. The key finding is that replacing Mask R-CNN with the modern single-stage YOLOv11 architecture, followed by hyperparameter optimization, substantially improved all key metrics of astrocyte detection and segmentation. As shown in the table, improvements were observed in the cell-



Figure 2. Comparison of segmentation results. (a) Original phase-contrast image of an astrocyte. (b) Mask R-CNN segmentation result. (c) YOLOv11 segmentation result.

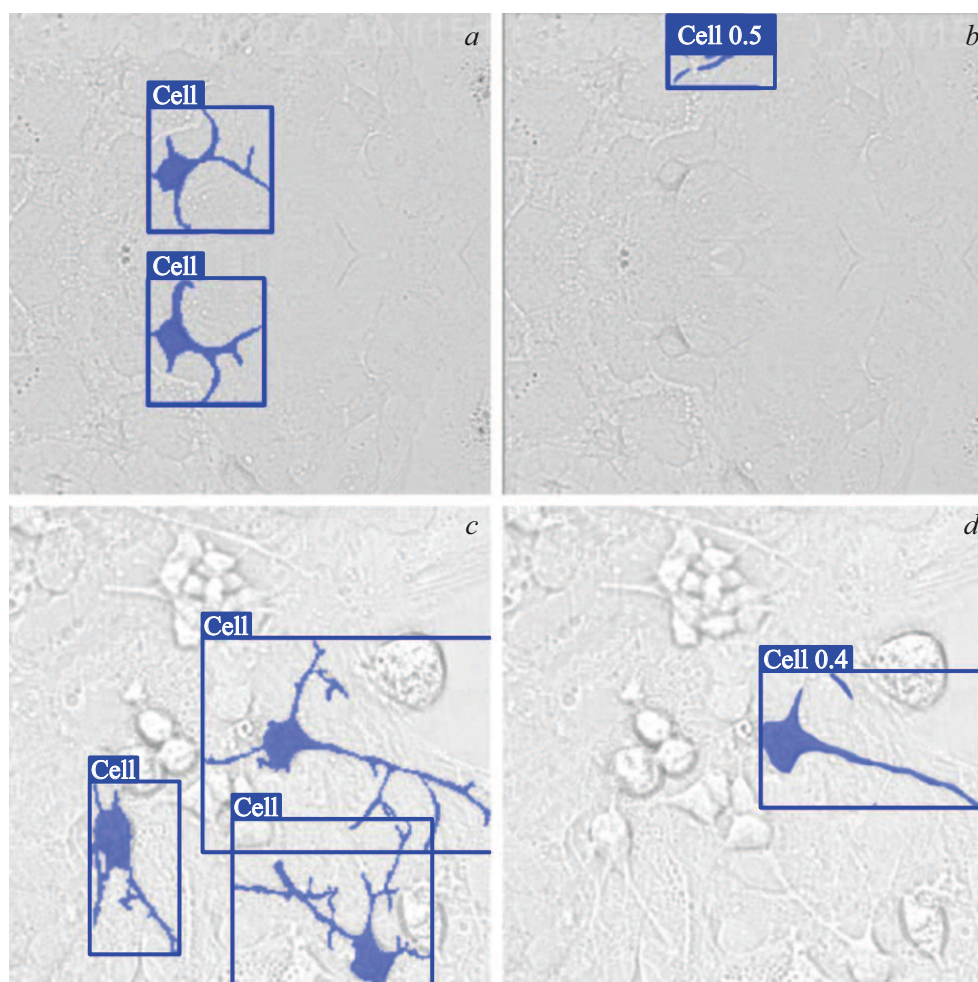


Figure 3. Examples of astrocyte segmentation errors: (a) ground-truth annotation (manual delineation of cells in the image); (b) false-positive prediction (the model segments objects absent from the annotation in (a)); (c) ground-truth annotation (manual delineation of cells in the image); (d) false-negative prediction (the model fails to segment objects present in the annotation in (c)).

detection metrics: accuracy increased from 0.29 to 0.60, and recall increased from 0.33 to 0.73. At the same time, segmentation accuracy (Dice coefficient, from 0.72 to 0.73) and detection precision (from 0.71 to 0.76) also increased. This consistent improvement across all metrics indicates that the developed model not only detects astrocytes more reliably in images but also delineates their contours more

accurately (Figure 3), which is particularly important for subsequent morphometric analysis. Thus, the developed method addresses a fundamental challenge in biophotonics and cell biology: the need for real-time, noninvasive observation and objective quantitative analysis using a non-destructive technique. As shown in our experiments [18], the method is sufficiently sensitive to detect statistically

significant morphological changes (cell area, total process length, and number of process branch points) induced by a modulator of NAD⁺ production and catalytic conversion, specifically nicotinamide [23]. This demonstrates its applicability to screening bioactive compounds and studying cellular responses to various stimuli. Thus, the proposed software approach is an effective tool for applications in optical biophotonics and enables automated processing of large image datasets acquired by phase-contrast microscopy.

The proposed YOLOv11-based machine-learning method is promising for studies of living cell cultures. Automated morphometric analysis can be performed without cell fixation or staining, enabling continuous monitoring of morphological changes in cells over extended periods. In addition, the method is suitable for high-throughput screening of bioactive compounds and for assessing cellular responses to dynamic changes in the microenvironment, particularly redox imbalance caused by reductive or oxidative stress, as well as in models of neuroinflammation and neurodegeneration. The high speed and efficiency of the single-stage YOLOv11 architecture make the method suitable for automated analysis of large datasets. This approach can be adapted to analyze other cell types of the brain neurovascular unit, opening new opportunities in neurobiology, pharmacology, and biomedical research. Integrating phase-contrast microscopy with other noninvasive optical methods provides opportunities for comprehensive analysis of cellular function. In this context, the development of high-accuracy machine-learning algorithms, such as the YOLOv11-based method proposed here, is particularly important because such algorithms may serve as a universal platform for automating the analysis of data acquired using different optical modalities.

Compliance with ethical standards

All experiments involving laboratory animals were carried out in accordance with the ethical standards established by the law of the Russian Federation and principles of the Basel Declaration, and were 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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