DOI: https://doi.org/10.17650/2313-805X-2026-13-1-93-102

Enzyme-enhanced immunocytochemical detection of cytoplasmic proteins in human leukocytes sorted by surface antigens using anti-CD antibody microarray

O.S. Fedyanina[1–3], A.O. Zakirova[1, 2], A.I. Khamidullina[4, 5], A.N. Khvastunova[1, 2], I.V. Grebennik[1, 2], A.V. Filatov[6], S.A. Kuznetsova[1, 2]

[1]Dmitry Rogachev National Medical Research Centre for Pediatric Hematology, Oncology and Immunology, Ministry of Health of Russia; 1 Samory Mashela St., 117198 Moscow, Russia;
[2]Centre for Theoretical Problems of Physicochemical Pharmacology, Russian Academy of Sciences; 30 Srednyaya Kalitnikovskaya St., Moscow 109029, Russia;
[3]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences; 47 Leninsky Prospekt, Moscow 119991, Russia;
[4]Laboratory of Molecular Oncobiology, Institute of Gene Biology, Russian Academy of Sciences; 34/5 Vavilova St., Moscow 119334, Russia;
[5]Ufa Institute of Chemistry, Ufa Federal Research Center, Russian Academy of Sciences; 71 Oktyabrya Prospekt, Ufa 450054, Russia;
[6]Institute of Immunology, Federal Medical and Biological Agency of Russia; Bld. 2, 24 Kashirskoe Shosse, Moscow 115478, Russia

Contacts: Sofya Alekseevna Kuznetsova *kuznetsova.sonya@gmail.com*

Anti-cluster-of-differentiation (anti-CD) antibody microarray is an attractive multiplex analogue to leukocyte immunocytochemistry for surface markers. The transparent plastic microarray support permits to sort leukocytes by their surface lineage-specific markers making them available for subsequent high-resolution morphology examination. The combined data on the pathologic cells' immunophenotype, morphology and cytochemistry from the microarray is sufficient to suggest preliminary diagnosis in many leukemia types. However, in some cases additional staining for internal markers is required, i.e. in T cell acute leukemia the T cell origin of the blasts can only be proved by the presence of cytoplasmic CD3 (cytCD3). Here we describe a new protocol of immunocytochemical detection of internal proteins in mononuclear cells captured by mouse anti-CD antibodies on the microarray surface. The protocol uses primary mouse FITC-conjugated (FITC – fluorescein isothiocyanate) antibodies against the target, secondary anti-FITC antibodies conjugated with alkaline phosphatase and colorimetric alkaline phosphatase substrate, BCIP/NBT. We show on normal leukocytes and leukemic cell lines that the protocol is sensitive and specific. The percentages of cytCD3-positive cells determined by this method in bone marrow aspirates of two patients with T cell acute lymphoblastic leukemia are in excellent agreement with flow cytometry results. This method expands the diagnostic capabilities of anti-CD antibody microarray.

Keywords: anti-CD antibody microarray, T cell acute lymphoblastic leukemia, Jurkat cell line, CD3

For citation: Fedyanina O.S., Zakirova A.O., Khamidullina A.I. et al. Enzyme-enhanced immunocytochemical detection of cytoplasmic proteins in human leukocytes sorted by surface antigens using anti-CD antibody microarray. Uspekhi molekulyarnoy onkologii = Advances in Molecular Oncology 2026;13(1):93–102.
DOI: https://doi.org/10.17650/2313-805X-2026-13-1-93-102

Иммуноцитохимическая детекция внутриклеточных белков в лейкоцитах человека, рассортированных по поверхностным антигенам с помощью клеточного биочипа

О.С. Федянина[1–3], А.О. Закирова[1, 2], А.И. Хамидуллина[4, 5], А.Н. Хвастунова[1, 2], И.В. Гребенник[1, 2], А.В. Филатов[6], С.А. Кузнецова[1, 2]

[1]ФГБУ «Национальный медицинский исследовательский центр детской гематологии, онкологии и иммунологии им. Дмитрия Рогачева» Минздрава России; Россия, 117198 Москва, ул. Саморы Машела, 1;
[2]ФГБУН «Центр теоретических проблем физико-химической фармакологии Российской академии наук»; Россия, 109029 Москва, ул. Средняя Калитниковская, 30;
[3]ФГБУН «Институт органической химии им. Н.Д. Зелинского Российской академии наук»; Россия, 119991 Москва, Ленинский пр-кт, 47;
[4]Лаборатория молекулярной онкобиологии ФГБУН «Институт биологии гена Российской академии наук»; Россия, 119334 Москва, ул. Вавилова, 34/5;

[5]Уфимский институт химии, ФГБНУ «Уфимский федеральный исследовательский центр Российской академии наук»; Россия, 450054 Уфа, пр-кт Октября, 71;
[6]ФГБУ «Государственный научный центр "Институт иммунологии" Федерального медико-биологического агентства России»; Россия, 115478 Москва, Каширское шоссе, 24, корп. 2

Контакты: Софья Алексеевна Кузнецова *kuznetsova.sonya@gmail.com*

Клеточный биочип – привлекательный аналог многоцветной иммуноцитохимии для определения поверхностных CD-антигенов лейкоцитов. Прозрачный пластиковый биочип позволяет сортировать лейкоциты по их поверхностным детерминантам для последующего морфологического исследования с высоким разрешением. Комбинации данных об иммунофенотипе, морфологических и цитохимических особенностях патологических клеток, полученных с помощью клеточного биочипа, достаточно для установления предварительного диагноза при многих типах лейкозов. Однако в некоторых случаях для этого необходимо определение цитоплазматических маркеров. Например, в случаях Т-клеточных лейкозов Т-клеточная природа бластов должна быть подтверждена наличием цитоплазматического CD3 (cytCD3).

В статье представлен новый протокол иммуноцитохимической детекции внутриклеточных белков в мононуклеарных клетках, связавшихся с мышиными anti-CD-антителами на клеточном биочипе. В нем использованы первичные мышиные FITC-конъюгированные (FITC – флуоресцеин изотиоцианат) антитела к цитоплазматическим белкам-мишеням, вторичные anti-FITC-антитела, конъюгированные с щелочной фосфатазой, и колометрический субстрат BCIP/NBT для щелочной фосфатазы. На лейкоцитах здоровых доноров и лейкозных клеточных линиях мы показали, что протокол является довольно чувствительным и специфичным. Процент cytCD3-положительных клеток, определенный описанным методом в аспиратах костного мозга 2 пациентов с Т-клеточным острым лимфобластным лейкозом, подтверждается результатами проточной цитометрии. Данный метод расширяет диагностические возможности клеточного биочипа.

Ключевые слова: клеточный биочип, Т-клеточный острый лимфобластный лейкоз, клеточная линия Jurkat, CD3

Для цитирования: Федянина О.С., Закирова А.О., Хамидуллина А.И. и др. Иммуноцитохимическая детекция внутриклеточных белков в лейкоцитах человека, рассортированных по поверхностным антигенам с помощью клеточного биочипа. Успехи молекулярной онкологии 2026;13(1):93–102. (На англ.).
DOI: https://doi.org/10.17650/2313-805X-2026-13-1-93-102

INTRODUCTION

Acute leukemia diagnosis is based on the detection of malignant leukocyte precursors (blast cells) in patient's bone marrow and definition of their lineage. The blast presence is usually independently verified by morphology in bone marrow aspirate films and by flow cytometry, while their lineage is usually determined either by cytochemistry [1] or by flow cytometry based on the expression of lineage-specific surface of cytoplasmic cluster-of-differentiation (CD) antigens and other markers [2]. Blast cell lineage determination in acute leukemias is not always straightforward as leukemic cells often aberrantly express surface markers normally restricted to another lineage [3, 4]. Current World Health Organization classification of tumours of haematopoietic and lymphoid tissues [2] suggests the presence of myeloperoxidase or at least two monocyte-specific markers (CD11c, CD14, CD64, non-specific esterase or lysozyme) as a proof of myeloid blast origin, the presence of CD19 and at least one B cell marker, CD79a, CD10 and cytoplasmic CD22 (at least two if CD19 expression is weak), as a proof of B cell blast origin while only cytoplasmic CD3 (cytCD3) can be used as a reliable T cell marker [2].

Earlier we have developed a new diagnostic method for leukemias and lymphomas with leukemization using an anti-CD antibody microarray on a transparent support for leukocyte sorting and subsequent high-resolution morphology analysis [5–10]. The mononuclear fraction of peripheral blood or bone marrow aspirate is incubated with monoclonal mouse immunoglobulins G (IgG) against main lineage-specific diagnostically relevant CD antigens including positive and negative controls (anti-CD45 and isotype control) immobilized on the microarray. After incubation and washing the antibody spots on the microarray are filled with specifically bound cells. The microarray with captured cells is then dried and stained after May–Grünwald–Giemsa. The microarray permits to determine the set of CD antigens present on the surface of cells with certain morphology (i. e. leukemic blasts) by analysing the percentage of these cells among the leukocytes captured by different anti-CD antibodies [6–10]; in this way, it combines the analysis of the pathologic cells' immunophenotype, morphology and cytochemistry. This makes the anti-CD antibody microarray a unique diagnostic instrument for diagnosis of many leukemia types, including acute leukemia, in resource-poor setting [6–10]. However, while myeloid or B-cell origin of the blasts can be verified for the anti-CD microarray-bound cells by the fact of the blast capture by a certain anti-CD antibody or by cytochemical reaction for myeloperoxidase or nonspecific esterase [6–8], the confirmation of acute T-lymphoblastic leukemia (T-ALL) cannot be performed without immunocytochemical staining of microarray-bound cells for intracellular CD3.

Here we describe a method for enzymatic immunocytochemical method for staining microarray-bound cells for cytoplasmic markers. As the widest variety of commercially available anti-CD antibodies are produced

in mouse, both the capturing antibodies on the microarray and primary antibody for immunocytochemistry come from the same species and enzyme-linked anti-species secondary antibodies cannot be used for signal enhancement. Using FITC-labeled (FITC – fluorescein isothiocyanate) mouse anti-human primary antibodies and secondary anti-FITC antibodies conjugated with alkaline phosphatase (AP) we demonstrate stable and specific enzyme-linked immunocytochemical detection of cytoplasmic markers in leukocytes, captured on anti-CD antibody microarray. The results can be visualized with simple bright field microscopy and the microarray with stained cells can be stored for reexamination without signal loss.

MATERIALS AND METHODS

Patients. This study used a sample of venous blood from three healthy volunteers (two females, 37 and 42 y.o. and one 27 y.o. male) and bone marrow aspirate from two patients of Dmitry Rogachev National Medical Research Centre for Pediatric Hematology, Oncology and Immunology, one with suspected T-ALL and one with suspected T-cell lymphoblastic lymphoma. Informed consent was obtained from all participants or their legal representatives after the nature and possible consequences of the study had been fully explained.

Cell line. Jurkat cell line (clone J.RT3-T3.5) was grown in RPMI 1640 medium (300 µg/mL L-glutamine, 25 mM HEPES, 25 mM $NaHCO_3$, 20 µg/mL gentamicin, 10 % fetal calf serum (FCS) at 37 °C at 5 % CO_2. The cell lines were reseeded every 3 days at the concentration of 5×10^5 cells per 25 cm^2 area culture flask. A log-phase growing culture was fixed in 1 % formaldehyde for 1 h at room temperature, washed with phosphate buffered saline (PBS), treated with 100 mM glycine for 1 min, washed with PBS and stained with anti-CD3-FITC (BD Biosciences, San Jose, CA, USA).

Manufacturing of anti-CD antibody microarrays. Microarrays were made according to the protocol described before [6, 10]. Antibodies to CD2, CD3, CD5, CD7, CD8, CD10, CD16, CD19, CD38, CD45, HLA-DR (Sorbent LTD, Moscow, Russia) [11] and to CD4, CD11b, CD11c, CD13, CD14, CD15, CD33, CD41a, CD61, CD45RA, CD45RO, CD64, CD117, CD123 (eBioscience, Waltham, MA, USA) were spotted on the microarrays.

Leukocyte sorting and analysis using anti-CD antibody microarray. Mononuclear cell fractions from venous blood (peripheral blood mononuclear cells, PBMC) or bone marrow aspirate taken on EDTA were isolated by density gradient centrifugation on Histopaque-1077 (Sigma, St. Louis, MO, USA) according to manufacturers' instructions. The mononuclear leukocyte fraction or harvested cells of the leukemic cell line was then incubated with anti-CD antibody microarray at 4°C for 1 hour as described by A.N. Khvastunova et al. [6] with minor modifications (isolated mononuclear cells were incubated with anti-CD antibody microarray in 100 % FCS (Sigma, St. Louis, MO, USA)). The microarray was then washed in PBS containing 1 % bovine serum albumin (BSA) to eliminate the unbound cells, covered with a small volume of FCS and dried in a cytocentrifuge by rotating around the axis normal to its surface at 5000–6000 rpm. The microarray-captured cells were then either stained after May–Grünwald–Giemsa for morphology examination and studied at ×1000 magnification using Nikon Eclipse Ni microscope equipped with Nikon DS-Ri1 camera (Nikon, Amstelveen, Netherlands) or permeabilised and stained for intracellular proteins as described below.

Staining microarray-captured leukocytes for cytoplasmic and intranuclear proteins. Microarray-captured leukocytes after the drying step were fixed and permeabilised in 10 % formalin in ethanol for 10, 30 s or 5 min. Longer permeabilisation time was not necessary as the staining results for 10, 30 s and 5 min fixation did not differ (table 1). To stain for intranuclear protein Ki-67 microarray-captured Jurkat cells were fixed and permeabilised in 70 % ethanol at –20 °C for at least 2 h. After washing in PBS, the microarray-captured cells were incubated for 30 min at room temperature in 1 % BSA on PBS to reduce non-specific binding, then stained for 30 min at room temperature with anti-CD3-FITC, anti-CD22-FITC or anti-Ki-67-FITC (UCHT1, Dako, Glostrup, Denmark) diluted 80 times in PBS with 1 % BSA, washed and analysed using confocal Axio Observer.Z1 microscope (Carl Zeiss, Jena, Germany). To enhance the signal, microarray with bound cells was further incubated for 30 min at room temperature with F(ab') fragment of rabbit anti-FITC-AP conjugated secondary antibodies (PNA ISH Detection Kit, Dako, Glostrup, Denmark), washed with PBS, and then incubated for 30–45 min with the BCIP/NBT substrate (PNA ISH Detection Kit, Dako, Glostrup, Denmark) of AP, washed in running water, and imaged under

Table 1. *The percentage of anti-CD45RA-captured peripheral blood mononuclear cells from the same donor staining positive for CD3 according to the protocol with enzyme-linked enhancement for different fixation time, average ± standard deviation, %*

Таблица 1. *Доля CD3+-клеток среди мононуклеарных клеток периферической крови одного донора, связавшихся на биочипе с анти-CD45RA в зависимости от времени фиксации, среднее ± стандартное отклонение, %*

Fixation time, s Время фиксации, с	Number of CD3+ cells Количество CD3+-клеток
10	66
15	78
30	84
300	67

Note. *Average ± standard deviation for different fixation time is 74 ± 9 %.*
Примечание. *Среднее ± стандартное отклонение при разном времени фиксации составило 74 ± 9 %.*

Nikon Eclipse Ni microscope using 100× and 40× objectives and Nikon DS-Ri1 camera. For negative controls the primary anti-CD3-FITC, anti-CD22-FITC or anti-Ki-67-FITC antibodies were replaced by isotype control mouse IgG1-FITC (Invitrogen, Camarillo, CA, USA). Cell staining intensity was estimated from inverted 32-bit greyscale photographs using ImageJ software.

Flow cytometry. Immunophenotypic study was carried out by 6-color laser flow cytometry according to the standard protocol (Navios, Beckman Coulter, Miami, FL, USA).

RESULTS

Confirmation of intracellular localization of anti-CD3-FITC staining. The protocol was tested by performing anti-CD3-FITC staining of Jurkat cell line, clone J.RT3-T3.5, a surface T cell receptor deficient mutant derived from Jurkat cell line originating from a pediatric patient with T-ALL [12] (fig. 1, *a–в*). Flow cytometry confirmed that less than 1 % of cells in this line are positive for surface CD3 (see fig. 1, *c*). The results of J.RT3-T3.5 staining with primary anti-CD3-FITC according to the described procedure are shown in fig. 1, *d*. The percentage of cells staining positive for internal CD3 was 100 %. The data shown in fig. 1, *d* demonstrate the results of anti-CD3 staining with enhancement by AP-labeled F(ab') fragment of secondary anti-FITC antibody and colorimetric AP substrate as described in Materials and methods (enzyme-enhanced immunocytochemical detection, EEID). The data presented in fig. 1 show that the suggested method allows sensitive and specific immunocytochemical staining for cytoplasmic CD3.

CytCD3$^+$ and cytCD22$^+$ microarray-captured lymphocytes had more intense coloration after enzyme-enhanced immunocytochemical detection than lymphocytes negative for these markers. The enzyme-enhanced immunocytochemical detection of cytoplasmic proteins CD3 and CD22 was performed on lymphocytes isolated from the blood of healthy donors incubated with an anti-CD antibody microarray as described in Materials and Methods. The results are shown in fig. 2 and 3. The intensity of anti-CD3-FITC staining is higher in anti-CD7 captured T cells than in anti-CD19-captured B cells and in cells stained with mouse-IgG-FITC (isotype control) (see fig. 2, *a–c*). The intensity threshold for determining CD3$^+$ was 60 a.u., cells with the staining intensity above 60 a.u. are CD3$^+$ (see fig. 2, *d*). Consistent results were obtained for anti-CD22-FITC, the intensity of anti-CD22-FITC staining is higher in anti-CD22 captured B cells than in anti-CD7-captured T cells and in cells stained with mouse-IgG-FITC (isotype control) (see fig. 3, *a–c*). The intensity threshold for determining CD22$^+$ was 30 a.u., cells with the staining intensity above 30 a.u. are CD22$^+$ (see fig. 3, *d*). We also qualitatively showed the possibility of using the enzyme-enhanced immunocytochemical method for detection of intranuclear protein Ki-67 in microarray-captured T-lymphocytes (fig. 4). The percentage of Jurkat cells staining positive for Ki-67 was 100 %, this result was confirmed by a parallel study of the Jurkat cell suspension by flow cytometry (data not shown). These results demonstrated the ability to reliably detect cytCD3$^+$, cytCD22$^+$ and Ki-67$^+$ cells among microarray-captured lymphocytes using enzyme-enhanced immunocytochemical detection.

The dried microarray-captured lymphocytes can be stored for 7 days before staining. The dried microarrays with captured normal PBMC were stored at room temperature in the dark for 1–7 days prior to fixation and staining according to the above-described protocol. Figure 5 shows the intensity diagram of anti-CD7- and anti-CD19-captured PBMC from normal blood stained for internal CD3 as described above after 0 and 7 days storage respectively. There is a clear difference between CD3-positive cells (CD7$^+$ cells) and CD3-negative cells (CD19$^+$ cells) while no drop in staining intensity is observed upon storage (fig. 5). Thus storage of a dry sample at room temperature for 7 days before staining for intracellular markers does not affect the results of the staining procedure.

The protocol application to T-ALL diagnosis. The cytoplasmic protein detection method was tested on two patients with suspected T cell lymphoproliferative diseases. For each patient the mononuclear fraction of the bone marrow aspirate was incubated with 2 identical microarrays, one of which was stained after May–Grünwald–Giemsa for morphology examination and another – with anti-CD3-FITC primary antibody with enhancement by enzyme-linked secondary antibody.

Patient M., a 17-y.o. boy, was admitted with suspected T cell lymphoblastic lymphoma. The flow cytometry of bone marrow aspirate showed a blast region of 53 % with blasts positive for CD2, CD5, CD7, CD15, CD19 and CD38. The anti-CD antibody microarray analysis of the mononuclear fraction of the patient's bone marrow aspirate showed 75 % of blast cells of average size with no granulation, large nuclei and thin rims of cytoplasm positive for CD2, CD7, CD15, CD19, CD38 and CD45 (fig. 6, *a*, panel 1). As the criteria for blast attribution to myeloid or B cell lineage were not fulfilled, the microarray-captured blasts were stained for internal CD3 according to the protocol with enzyme-linked enhancement described above. Among the anti-CD45-captured blasts 90 % were positive for cytoplasmic CD3 (see fig. 6, *a*, panel 2) in consistence with the flow cytometry data (88 % of blasts were positive for cytoplasmic CD3). Based on the presence of cytoplasmic CD3 and surface CD2 and CD7 on the blast cells, the microarray-based data suggests the diagnosis of pre-T-ALL (T-II) [13].

Patient R., a 23-y.o. man, was referred to the flow cytometry laboratory of Rogachev National Research Centre for Pediatric Hematology, Oncology and Immunology for diagnosis. The flow cytometry of bone marrow aspirate showed 96 % of blasts positive for CD2, CD7, CD11b (low expression), CD13, CD33, CD38, CD117 and HLA-DR. 9 % of blasts were positive for myeloperoxidase and 40% of blasts were positive for cytoplasmic CD3 by flow

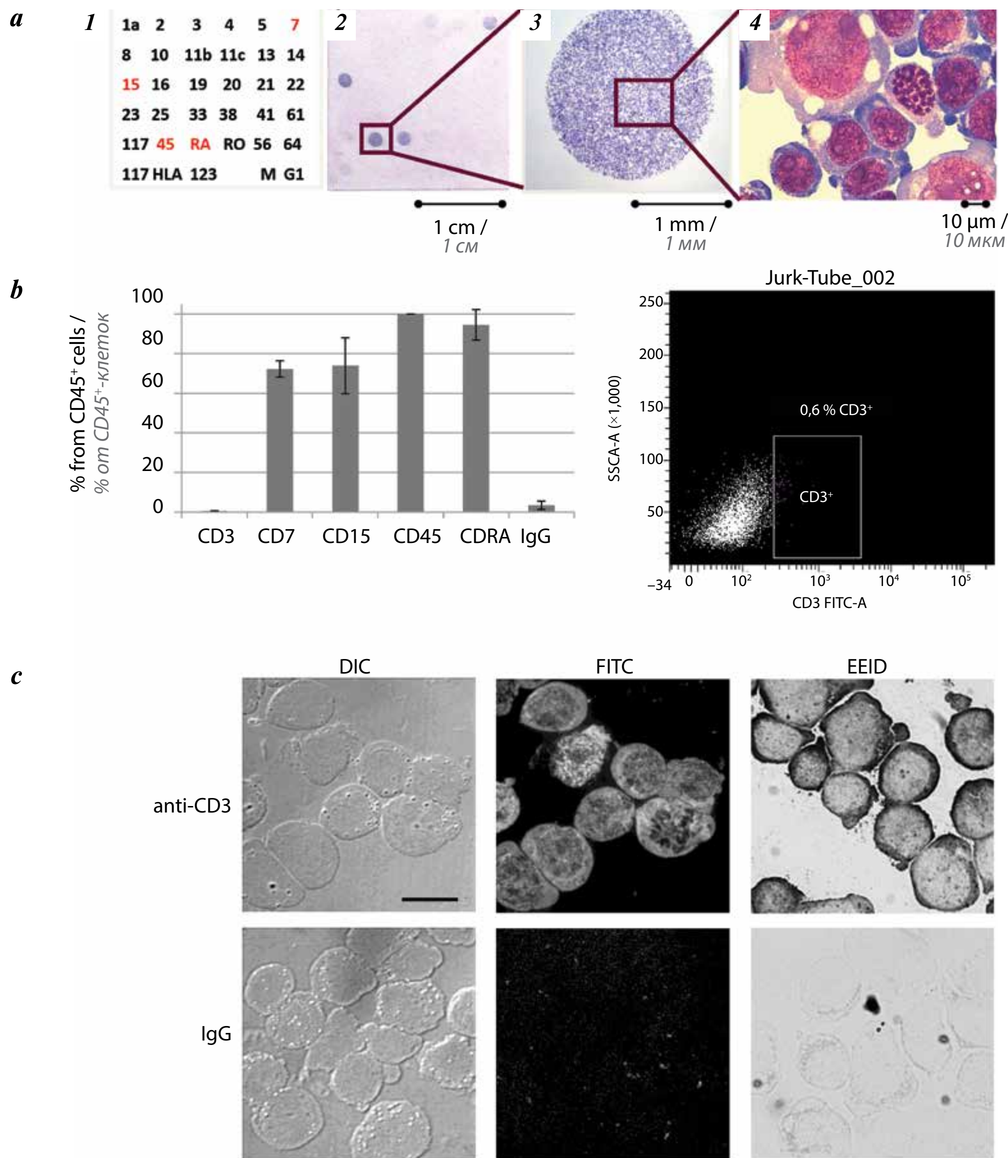

Fig. 1. *Confirmation of intracellular localization of anti-CD3-FITC (FITC – fluorescein isothiocyanate) staining in Jurkat cells (Jurkat cells were concentrated and incubated with anti-CD antibody microarray, after washing, drying and staining according to May–Grünwald–Giemsa): a – the "map" of the microarray with numbers indicating the spots of mouse immunoglobulin G (IgG) against corresponding CD antigens. Numbers of anti-CDx captured Jurkat cells are highlighted in red, mouse IgG1 (isotype control) indicates the negative control (panel 1); the whole microarray with captured cells specifically bound to anti-CDx (panel 2). Anti-CD45-bound Jurkat cells after May–Grünwald–Giemsa staining at different magnifications, ×40 (panel 3) and ×1000 (panel 4); b – percentage of Jurkat cells bound to anti-CDx; c – percentage of CD3+ Jurkat cells determined by flow cytometry; d – control of permeabilization quality using anti-CD3-FITC staining of surface CD3-negative clone of Jurkat cell line with enzyme-enhanced immunocytochemical detection (EEID) or without (FITC) enhancement. Enzyme-enhanced detection includes anti-CD3 staining of surface CD3-negative clone of Jurkat cell line with enhancement using mouse anti-CD3-FITC primary antibody (replaced with FITC-labeled isotype control for negative control), F(ab') fragment of rabbit anti-FITC-alkaline phosphatase secondary antibody and BCIP/NBT colorimetric substrate; the signal without enhancement (FITC) corresponds to primary antibody staining only. Original magnification ×1000, the scale bar represents 10 mm. DIC – differential interference contrast; SSCA-A – side scattering; FITC-A – anti-CD3 signal intensity*

Рис. 1. *Подтверждение внутриклеточной локализации anti-CD3-FITC-окрашивания (FITC – флуоресцеин изотиоцианат) в клеточной линии Jurkat (клетки Jurkat концентрировали и инкубировали с биочипом с anti-CDx-антителами, промывали, сушили и окрашивали по методу Мая–Грюнвальда–Гимзы): a – схема биочипа с номерами, указывающими расположение пятен мышиного иммуноглобулина G (IgG) против соответствующих CD-антигенов. Номера anti-CD-антител, связавших клетки Jurkat, выделены красным цветом, mIgG обозначает смесь мышиных IgG1 (изотипический отрицательный контроль, панель 1). Весь биочип с клетками, специфически связавшимися с anti-CDx-антителами, представлен на панели 2, клетки Jurkat, связанные с CD45, после окрашивания по методу Мая–Грюнвальда–Гимзы при ×40 – на панели 3, при ×1000 – на панели 4; b – доля клеток Jurkat, связанных с anti-CDx-антителами; c – процентное содержание CD3+-клеток Jurkat, определенное методом проточной цитометрии; d – контроль за качеством пермеабилизации, выполненный с помощью окрашивания отрицательной по поверхностному CD3 клеточной линии Jurkat anti-CD3-FITC-антителами, с иммуноферментным усилением (EEID) и без него (FITC). Иммуноферментное усиление включает окрашивание первичными мышиными анти-CD3-FITC-антителами (в отрицательном контроле заменялись на FITC-меченный изотипический контроль), вторичными антителами (F(ab')-фрагментом кроличьих анти-FITC-антител, конъюгированных с щелочной фосфатазой, и использование колориметрического субстрата щелочной фосфатазы BCIP/NBT. Сигнал без усиления предполагает окрашивание только первичными антителами. ×1000, масштабный отрезок соответствует 10 мкм. DIC – дифференциальный интерференционный контраст; SSCA-A – боковое светорассеяние; FITC-A – интенсивность окрашивания анти-CD3*

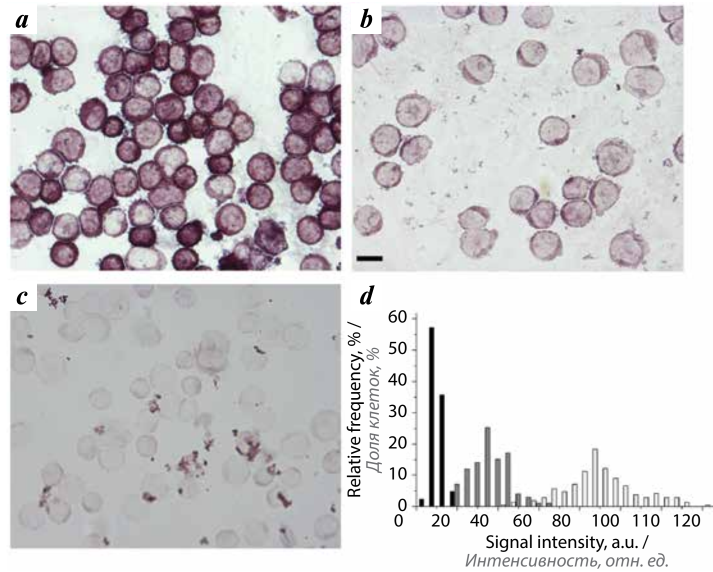

Fig. 2. *Normal peripheral blood mononuclear cells (PBMC) staining for cytoplasmic CD3 using protocol with enzyme-enhanced immunocytochemical detection (EEID). Normal PBMC captured by anti-CD7 (a, c) and anti-CD19 (b) stained for cytoplasmic CD3 (a, b). In fig. c represents a negative control (anti-CD3-FITC (FITC – fluorescein isothiocyanate) replaced by FITC-labeled isotypic control). Original magnification ×1000, the scale bar represents 10 μm. Staining intensity for cytoplasmic CD3 and negative controls using EEID protocol is shown in fig. d. The black color indicates IgG1-FITC staining of anti-CD7-captured PBMC, grey – anti-CD3-FITC staining of anti-CD19-captured PBMC, white – anti-CD3-FITC staining of anti-CD7-captured PBMC*

Рис. 2. *Окрашивание мононуклеаров, выделенных из периферической крови, на цитоплазматический CD3 с использованием протокола с иммуноферментным усилением (EEID). Мононуклеары, связавшиеся с антителами к CD7 (a, c) и к CD19 (b) на биочипе, окрашивались на цитоплазматический CD3 (a, b). На рис. c представлен отрицательный контроль (анти-CD3-FITC заменен на FITC-меченный (FITC – флуоресцеин изотиоцианат) изотипический контроль). ×1000, масштабный отрезок соответствует 10 мкм. Интенсивность окрашивания мононуклеаров анти-CD3-FITC с использованием протокола EEID представлена на рис. d. Черным цветом обозначены мононуклеары, связавшиеся с антителами к CD7 на биочипе и окрашенные IgG1-FITC (IgG – иммуноглобулин G1), серым – мононуклеары, связавшиеся с антителами к CD19 на биочипе и окрашенные anti-CD3-FITC, белым – мононуклеары, связавшиеся с антителами к CD7 антителами на биочипе и окрашенные анти-CD3-FITC-антителами*

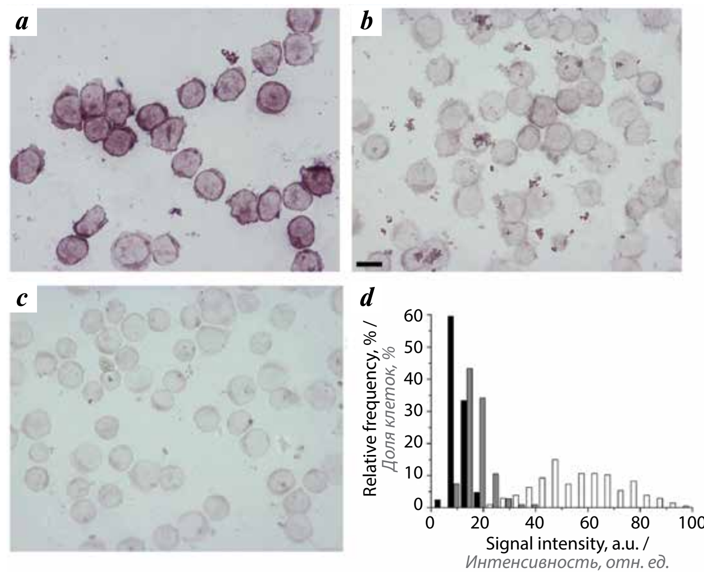

Fig. 3. *Normal peripheral blood mononuclear cells (PBMC) staining for cytoplasmic CD22. Normal PBMC captured by anti-CD19 (a, c) and anti-CD7 (b) stained for cytoplasmic CD22 (a, b). In fig. c represents a negative control (anti-CD3-FITC (FITC – fluorescein isothiocyanate) replaced by FITC-labeled isotypic control). Original magnification ×1000, the scale bar represents 10 mm. The staining intensity for cytoplasmic CD22 and negative controls using protocol with enzyme-enhanced immunocytochemical detection (EEID) is shown in fig. d. The black color indicates IgG1-FITC (IgG1 – immunoglobulin G1) staining of anti-CD19-captured PBMC, grey – anti-CD22-FITC staining of anti-CD7-captured PBMC, white– anti-CD22-FITC staining of anti-CD19-captured PBMC*

Рис. 3. *Окрашивание мононуклеаров, выделенных из периферической крови, на цитоплазматический CD22. Мононуклеары, связавшиеся с антителами к CD19 (a, c) и CD7 (b) на биочипе, окрашивали на цитоплазматический CD22 (a, b). На рис. c представлен отрицательный контроль (анти-CD3-FITC заменен на FITC-меченный (FITC – флуоресцеин изотиоцианат) изотипический контроль). ×1000, масштабный отрезок соответствует 10 мкм. На рис. d представлена интенсивность окрашивания мононуклеаров anti-CD22-FITC с использованием протокола с иммуноферментным усилением. Черным цветом обозначена интенсивность мононуклеаров, связавшихся с антителами к CD19 на биочипе и окрашенных IgG1-FITC (IgG – иммуноглобулин G1), серым – мононуклеаров, связавшихся с антителами к CD7 на биочипе и окрашенных anti-CD22-FITC, белым – мононуклеаров, связавшихся с антителами к CD19 на биочипе и окрашенных anti-CD22-FITC*

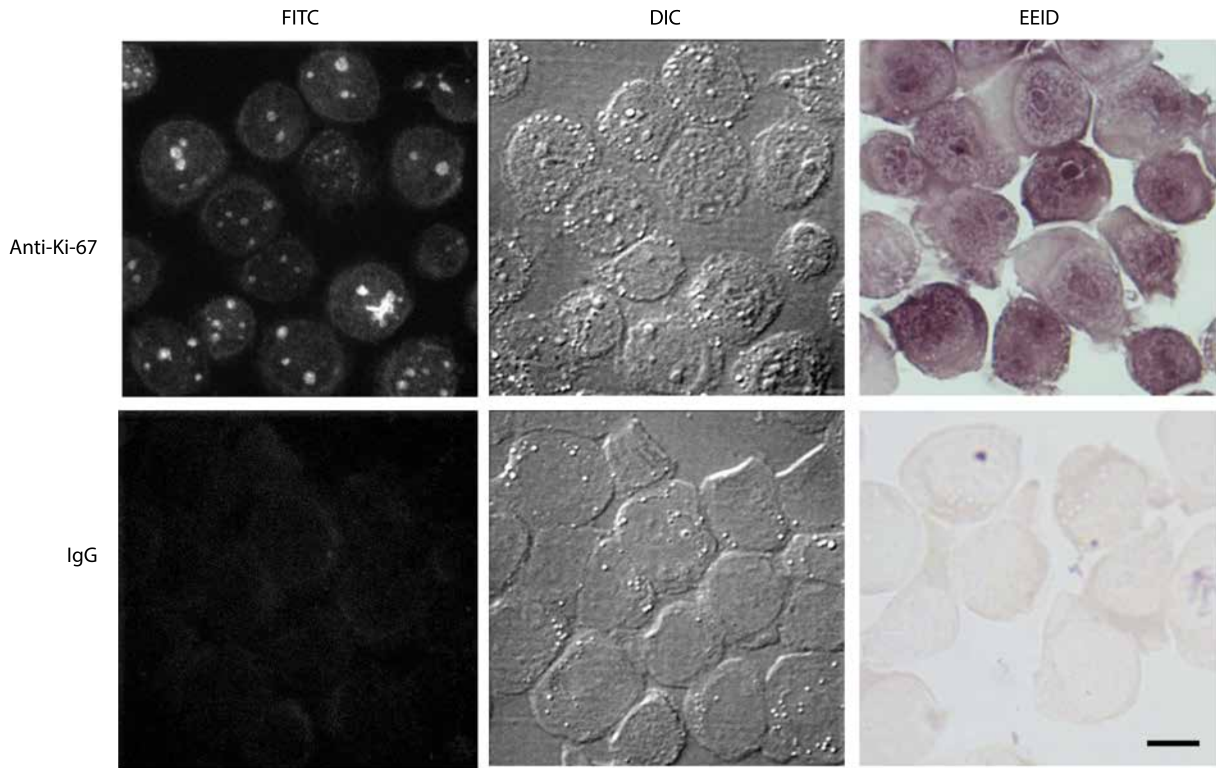

Fig. 4. *Anti-Ki-67-FITC (FITC – fluorescein isothiocyanate) staining according to protocol with enzyme-enhanced immunocytochemical detection (EEID) of anti-CD45-captured Jurkat cells. ×1000, the scale 10 μm. IgG – immunoglobulin G*

Рис. 4. *Окрашивание anti-Ki-67-FITC (FITC – флуоресцеин изотиоцианат) по протоколу с иммуноферментным усилением клеток Jurkat, связавшихся с антителами к CD45 на клеточном биочипе. ×1000, масштаб 10 мкм. IgG – иммуноглобулин G*

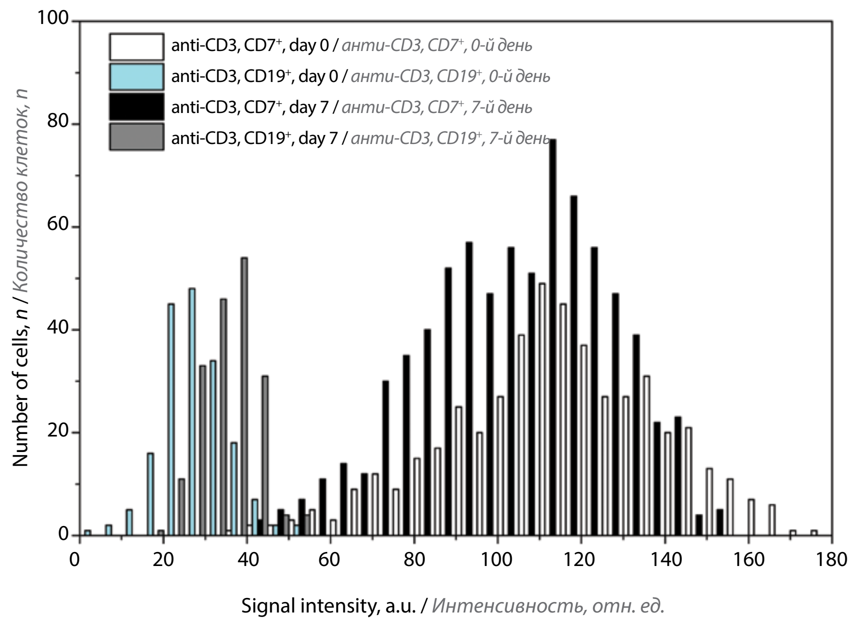

Fig. 5. *Intensity of anti-CD3-FITC (FITC – fluorescein isothiocyanate) staining of anti-CD7- and anti-CD19-captured normal peripheral blood mononuclear cells for cytoplasmic CD3 according to protocol with enhancement after storage 0 and 7 days. ×1000, the scale 10 mm*

Рис. 5. *Интенсивность окрашивания анти-CD3-FITC (FITC – флуоресцеин изотиоцианат) по протоколу усиления, мононуклеаров, выделенных из периферической крови и связавшихся на биочипе с антителами к CD7- или CD19, после 0 и 7 дней хранения. ×1000, масштаб 10 мкм*

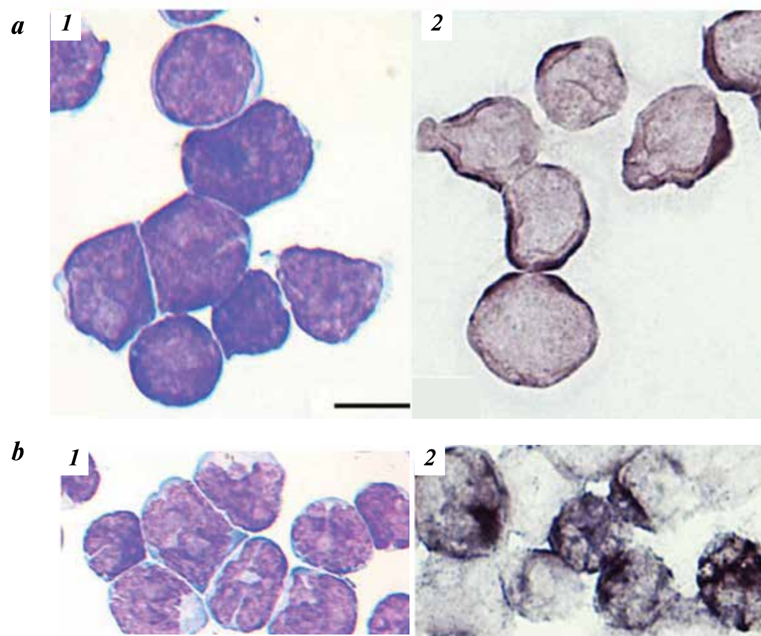

Fig. 6. *The protocol with enzyme-enhanced immunocytochemical detection application to T-lymphoblastic leukemia diagnosis: a – anti-CD19-captured mononuclear cells from bone marrow aspirate of patient M.; b – anti-CD2-captured mononuclear cells from bone marrow aspirate of patient R. Panels 1, 3 – morphology; panels 2, 4 – anti-CD3 staining with enzyme-linked enhancement. ×1000, the scale 10 mm*

Рис. 6. *Применение протокола с иммуноферментным усилением для диагностики Т-клеточного лимфобластного лейкоза: a – мононуклеары, выделенные из аспирата костного мозга пациента М. и связавшиеся с анти-CD19 на клеточном биочипе; b – мононуклеары, выделенные из аспирата костного мозга пациента Р. и связавшиеся с анти-CD2 на клеточном биочипе. Панели 1, 3 – морфология опухолевых клеток, панели 2, 4 – окрашивание анти-CD3-антителами с усилением. ×1000, масштаб 10 мкм*

cytometry data. The microarray analysis of the mononuclear fraction of bone marrow aspirate showed immature cells of average size, with large nuclei of irregular shape containing one or two nucleoli and scarce cytoplasm without granulation captured by anti-CD2, anti-CD7, anti-CD13, anti-CD33, anti-CD45RA, anti-CD117 and anti-HLA-DR (see fig. 6, *b*, panel 3). The presence of the earliest T cell marker CD7 together with myeloid markers, CD13, CD33, CD117 resulted in differential diagnosis between acute myeloid leukemia and early T cell precursor leukemia, a recently suggested diagnostic entity separated from pro-T-ALL [14]. Cytoplasmic CD3 staining of the microarray-captured cells showed the 45 % of anti-CD45RA-captured blasts stained positive for CD3 (see fig. 6, *b*, panel 4). According to the current guidelines, this data led to the diagnosis of early T cell precursor acute lymphoblastic leukemia [15].

DISCUSSION

The application of enzyme-linked enhancement increases the detection sensitivity, permits to use light microscopy for detection and to store the samples for later reexamination. Our data shows that the dry microarray with captured cells can be stored for up to 5 days before staining for intracellular markers, which is an advantage for a diagnostic laboratory. The application of mouse FITC-labeled primary and anti-FITC enzyme-linked secondary antibodies widens the choice for primary antibodies that can be applied to the cells captured by the immobilized mouse IgG on the microarray.

From the two enzymes commonly used in enzyme-linked enhancement, AP is a better choice for the analysis of mononuclear fraction of peripheral blood and bone marrow aspirate than the horseradish peroxidase (HRP). HRP colorimetric substrates also react with myeloperoxidase present in all myeloid cells [16]. Thus the myeloperoxidase present in normal or leukemic mononuclear cells would interfere with the detection of HRP introduced for signal enhancement. AP is present only in polymorphonuclear neutrophils [17] most of which are eliminated during the purification of the mononuclear cell fraction. However, the AP signal from a few mature neutrophils present in the sample should be taken into account during analysis.

Anti-CD antibody microarray on a transparent support is an attractive analogue to multicolour leukocyte immunocytochemistry for surface markers. Its main advantage lies in the possibility of high-resolution morphology examination of microarray-sorted cells. The diagnostic potential for this method for most chronic B-cell leukemias as well as for acute myeloid leukemia and acute B cell lymphoblastic leukemia has been demonstrated before [6]. However, the differential diagnosis of acute leukemias of T cell origin requires detection of intracellular CD3. The method described here eliminates the limitations for diagnostic applications of anti-CD antibody microarray

in leukemia diagnosis. It can also be used to test for coexpression of any surface with any intracellular marker on the same cells if the antibody specific for the surface marker is spotted on the microarray and used for cell sorting.

CONCLUSION

We described a new protocol of immunocytochemical detection of internal proteins in mononuclear cells captured by mouse anti-CD antibodies on the microarray surface. The protocol uses primary mouse (FITC)-conjugated antibodies against the target, secondary anti-FITC antibodies conjugated with AP and colorimetric AP substrate, BCIP/NBT. We show on normal leukocytes and leukemic cell lines that the protocol is sensitive and specific. The percentages of cytCD3-positive cells determined by this method in bone marrow aspirates of two patients with T-cell acute lymphoblastic leukemia are in excellent agreement with flow cytometry results.

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Acknowledgement. We thank the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Institute of Gene Biology Russian Academy of Sciences for providing the research facility and also S. Nikulin for kindly provided Jurkat cells, N.M. Kapranov for help with flow cytometry experiments and to P.A. Yadgarova for her help with anti-CD antibody microarrays manufacturing.

Благодарность. Мы благодарим Центр высокоточного редактирования генома и генетических технологий для биомедицины ФГБУН «Институт биологии гена Российской академии наук» за предоставление исследовательской базы, а также С. Никулину за предоставление клеток Jurkat, Н.М. Капранову за помощь в проведении экспериментов по проточной цитометрии и П.А. Ядгарову за ее помощь в изготовлении микрочипов антител к CD.

Authors' contributions

O.S. Fedyanina, S.A. Kuznetsova: development of research design, conducting experiments, data analysis, article writing;
A.O. Zakirova: conducting experiments, data analysis;
A.I. Khamidullina: cell cultivation, conducting experiments, data analysis;
A.N. Khvastunova: conducting experiments and data analysis;
I.V. Grebennik: data analysis;
A.V. Filatov: research design development.

Вклад авторов

О.С. Федянина, С.А. Кузнецова: разработка дизайна исследования, проведение экспериментов, анализ данных, написание текста статьи;
А.О. Закирова: проведение экспериментов, анализ данных;
А.И. Хамидуллина: культивирование клеток, проведение экспериментов, анализ данных;

А.Н. Хвастунова: проведение экспериментов и анализ данных;
И.В. Гребенник: анализ данных;
А.В. Филатов: разработка дизайна исследования.

ORCID of authors / ORCID авторов
O.S. Fedyanina / О.С. Федянина: https://orcid.org/0000-0001-7131-8006
A.O. Zakirova / А.О. Закирова: https://orcid.org/0000-0001-9475-3318
A.I. Khamidullina / А.И. Хамидуллина: https://orcid.org/0000-0001-8137-0625
A.N. Khvastunova / А.Н. Хвастунова: https://orcid.org/0000-0002-7117-0168
I.V. Grebennik / И.В. Гребенник: https://orcid.org/0009-0008-9607-101X
A.V. Filatov / А.В. Филатов: https://orcid.org/0000-0002-6460-9427
S.A. Kuznetsova / С.А. Кузнецова: https://orcid.org/0000-0001-5946-0026

Conflict of interest. The authors declare no conflict of interest.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Funding. The study was supported by the Russian Science Foundation grant (grant No. 25-25-20193).
Финансирование. Исследование выполнено при поддержке гранта Российского научного фонда (грант № 25-25-20193).

Compliance with patient rights and principles of bioethics
The study protocol was approved by the biomedical ethics committee of Centre for Theoretical Problems of Physicochemical Pharmacology, Russian Academy of Sciences (protocol No. 1/1-21(НЭК) dated 25.01.2021).
Соблюдение прав пациентов и правил биоэтики
Протокол исследования одобрен этическим комитетом ФГБУН «Центр теоретических проблем физико-химической фармакологии Российской академии наук» (протокол № 1/1-21(НЭК) от 25.01.2021).

Article submitted: 14.08.2025. **Accepted for publication:** 17.11.2025. **Published online:** 16.03.2026.
Статья поступила: 14.08.2025. **Принята к публикации:** 17.11.2025. **Опубликована онлайн:** 16.03.2026.