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PHENOTYPING AND SORTING OF MURINE BONE MARROW HAEMATOPOIETIC STEM CELLS USING FLOW CYTOMETRY

Kyryk V. M.

State Institute of Genetic and Regenerative Medicine of the National Academy of Medical Sciences of Ukraine, Kyiv

E-mail: biomedpost@gmail.com

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To develop a protocol of multiparametric phenotyping and sorting of LSK-subpopulations of hematopoietic stem cells and to determine their relative numbers in the bone marrow of mice was the goal of this research. The modified protocol of multiparametric phenotyping of murine hematopoietic stem cells enable to determine the content of $Lin^-Sca\text{-}1^+c\text{-}kit^+$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$ and $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ subpopulations in bone marrow of FVB mice. It was shown that the dominant population among LSK-cells represents the phenotype $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ (57.2 ± 6.8%), which characterizes the short-term hematopoietic stem cells responsible for myelopoiesis. Exclusion of cells with high intensity of forward and side light scattering allows reducing of false-positive results in measurements.

Also the protocol of sorting of murine bone marrow LSK-cells was proposed and its effectiveness for subsequent transplantation in experiments was demonstrated. At repeated phenotyping of sorted cells the purity of $Lin^-Sca\text{-}1^+c\text{-}kit^+$ cell fraction was 96.6 ± 1.8% with viability up to 89.6 ± 4.6%.

Key words: haematopoietic stem cells, multiparametric flow cytometry, cell sorting.

The fundamental stem cell research is actual not only for cell biology, but also for regenerative medicine, because modern cell technologies acquire much wider practical use. In particular, the transplantation of bone marrow hematopoietic stem cells (HSCs) has been used for several decades to restore hematopoiesis after radiotherapy and chemotherapy in the treatment of many hematological diseases [1]. The experimental studies on animals were a background for clinical application of bone marrow HSCs.

Given this, detailed characteristics of many types of stem and progenitor cells, including phenotype profile for surface and intracellular markers, are very important. One method that allows you to characterize quickly and accurately the phenotype of different cell populations is laser flow cytometry. Modern cytometers enable phenotyping for many cell markers simultaneously that opens up opportunities for researchers [2, 3].

Nowadays the total HSCs population is not considered homogeneous, and data concerning their immunophenotype is very controversial [4–6]. All currently known markers don't allow to allocate the true stem cells among their transient forms. Therefore, an active search for new combinations of many HSCs markers may be important in new studies.

In mice, HSCs that do not express markers of linearity Lin^- (CD3, CD11b, CD45R/220, Ly6C/G, TER-119), and express the stem cell antigen ($Sca\text{-}1^+$) and stem cell growth factor receptor ($c\text{-}kit^+$) make a special group LSK-cells [7, 8]. This population is heterogeneous by a number of additional markers and includes long-term HSCs expressing CD150, which can maintain hematopoiesis for a long time; and HSCs expressing CD34, which have a limited ability to self-renewal. The function of short-term CD150-negative HSCs is the replacement for the loss of progenitor cells and their rapid repopulation of differentiated cells, while there is a question of CD34-negative LSK-cells pluripotency [9, 10]. However, among the short-term HSCs there are isolated cells, characterized by expression of the type III receptor tyrosine kinase ($flt3^+$ or $CD135^+$, or $Flk2^+$) and providing mostly the recovery of lymphopoiesis; and cells without expression of this marker ($flt3^-$), which is responsible mainly for myelopoiesis [11, 12].

It is not found yet, whether the presence of these subpopulations show the initial stages of

differentiation, or the HSCs exist at different phases of the cell cycle. However, determination of relative content of these HSCs types by flow cytometry in experimental intact animals and under different types of injuries or therapeutic agents allows to obtain information about the reaction of hematopoiesis to relevant impacts.

Among many options of murine hematopoietic cells phenotyping there are not enough standardized multiparametric protocols available to determine the relative content of subpopulations, which are responsible for various components of hematopoiesis recovery. This is due to the difficulty of fluorochromes combinations selecting associated with some technical limitations of flow cytometer and with the need to develop a personal analysis algorithms, based on the cells morphology, discrimination of false positives, specific markers co-expression etc.

In addition, to relevant researches an actual is an opportunity to not only detailed phenotyping, but also sorting of a target cell population for further cultivation or transplantation. Protocols of murine HSCs sorting by flow cytometry are not always described in details. Therefore researchers for a long time have to choose sorting conditions that ensure the required sorting purity and high cell viability. Laser flow cytometry allows to characterize quickly and accurately the phenotype of different populations of stem and progenitor cells for solving relevant researches in modern regenerative medicine.

The aim of this study was develop the protocol of multiparametric phenotyping and sorting of LSK haematopoietic stem cell's subpopulations and determine their relative numbers in bone marrow of mice.

Materials and Methods

Experiments were performed on FVB-wt (wild type) mice from the vivarium of State Institute of Genetic and Regenerative Medicine NAMS Ukraine. All studies with the experimental animals were conducted in the compliance with Ukrainian and international laws and in compliance with all principles of bioethics.

The animals were euthanized by cervical dislocation under ether anesthesia. Bone marrow of FVB-wt males aged 3 months was isolated under sterile conditions by flushing of femurs with 1 ml RPMI-1640 medium (Sigma, USA). The fraction of bone marrow mononuclear cells was isolated using the Ficoll-Paque (Sigma, USA) gradient density method (d = 1.077 g/ml) by centrifugation for 15 min at 380 × g and washed in 5 ml RPMI-1640 medium.

Subpopulation of Lin^-, LSK ($Lin^-Sca\text{-}1^+$ $c\text{-}kit^+$), $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$ and $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ was identified by flow cytometry using rat anti-mouse monoclonal antibodies (Becton Dickinson, USA). We used Lineage Panel (Lin) for 5 antibodies (CD3e, CD11b, CD45R/B220, Gr1, Ly-76) conjugated with PerCP-Cy5.5; anti-Sca-1 PE-Cy7, anti-c-kit APC-Cy7, anti-flt3 PE, and anti-CD150 APC antibodies. Rat IgG_{2b}-kappa PerCP-Cy5.5 was used for isotype control.

For phenotyping of $1.5 \cdot 10^6$ mononuclear cells in 100 µl of BS (pH 7.2) we input into polystyrene tube and added monoclonal antibodies or isotype control in appropriate concentration (0.5 µg per sample). Samples were incubated for 30 min at +4 °C in the dark. After incubation cells were washed twice in 1 ml of CellWash (Becton Dickinson, USA), supplemented with 1% bovine serum adult — BSA (Sigma, CIIIA), by centrifugation at 380 × g and +4 °C for 10 min. After second centrifugation supernatant was removed and cells were resuspended in 300 µl PBS supplemented with 1% BSA. Samples were filtered through 70 µm cell strainer just before the measurement.

The relative number of viable cells was determined by added 5 µl of 7-aminoactinomycin D (7-AAD), which penetrates into injured cell's membrane and is detected on appropriate channel of fluorescence (wavelength 648 nm).

For sorting of HSCs subpopulations the concentration of mononuclear cell was adjusted to $2 \cdot 10^7$ cells/ml, added to the antibodies in a working concentration of 0.5 µg per 10^6 cells. Samples were incubated for 30 min at +4 °C in the dark. After washing in a RPMI-1640 medium by centrifugation at 380 × g and +4 °C for 10 min, cells were passed through 70 µm cell strainer in 2 ml of RPMI-1640 supplemented with 1% BSA, and adjusted to a concentration of $5 \cdot 10^6$ cells/ml.

Immunophenotyping and sorting of cells was performed with BD FACSAria cell sorter (Becton Dickinson, USA) using FACSDiva 6.1.2 software. We analyzed the forward and side scattering (FCS and SSC), and 5 parameters of fluorescence corresponding to fluorochrome detectors: 695 ± 40 nm filter for PerCP-Cy5.5, 780 ± 60 nm for PE-Cy7, 780 ± 60 for APC-Cy7, 585 ± 42 for PE, and 660 ± 20 nm for APC.

Aseptic conditions for sorting were provided using "Prepare for aseptic sort" mode of BD FACSAria, which includes alternate washing of entire sheath path and sample line with FACSRinse (Becton Dickinson, USA), ethanol and sterile DI water. After this procedure the fluidics lines were primed with sterile FACSFlow solution (Becton Dickinson, USA). The sorting chamber was isolated by air filter.

Sorted LSK cells were collected at +4 °C into 15-ml polypropylene tubes with 3 ml of RPMI-1640 supplemented with 20% BSA. Cells in 8–10 ml of medium were centrifuged for 10 min at 380 x g under stepwise increase of temperature from +4 °C to +15 °C. Supernatant was removed and added with 1 ml RPMI-1640 supplemented with 1% BSA.

The data were statistically analyzed using the Microsoft Excel 2010 software. Data were expressed as mean values and standard deviations (*mean ± SD*).

Results and Discussion

For reliable detection of a positive signal on each channel and correct compensation of fluorescence spectra overlap by multiparametric analysis it is important to use appropriate control samples. In our studies we used control samples of cells without antibodies (unstained control), samples with the isotypic control (isotype control), samples with each antibody alone (single-stained control) and samples with a combination of several antibodies without one (fluorescence minus one — FMO control). Thus, at set compensation for 5 fluorochromes the control panel with 12 samples was used (Table 1).

Table 1. **Control samples for compensation of spectral overlap for multiparametric phenotyping of HSCs**

№	Control samples	Description
1	Unstained control	w/o monoclonal antibody or isotype control
2	Isotype control	rat IgG_{2b}-kappa PerCP-Cy5.5
3	Single-stained control	Lin PerCP-Cy5.5
4		Sca-1 PE-Cy7
5		c-kit APC-Cy7
6		flt3 PE
7		CD150 APC
8	FMO control	Lin PerCP-Cy5.5 + Sca-1 PE-Cy7 + c-kit APC-Cy7 + flt3 PE
9		Lin PerCP-Cy5.5 + Sca-1 PE-Cy7 + c-kit APC-Cy7 + CD150 APC
10		Lin PerCP-Cy5.5 + Sca-1 PE-Cy7 + flt3 PE + CD150 APC
11		Lin PerCP-Cy5.5 + c-kit APC-Cy7 + flt3 PE + CD150 APC
12		Sca-1 PE-Cy7 + c-kit APC-Cy7 + CD150 APC + flt3 PE

Notes: FMO — fluorescence minus one.

The compensation matrix was created in manual or automatic mode based on the control samples data using BD FACSDiva software.

When developing the protocol of murine HSCs subpopulations phenotyping for Lin (CD3, CD11b, CD45R / 220, Ly6C / G, TER-119), Sca-1, c-kit, flt3 and CD150 markers a resulted algorithm was proposed:

1. On two-parameter dot-plot histogram of forward vs. side scattering (FCS vs. SSC) was created a polygonal gate, selecting events by size and morphological characteristics as mononuclear cells (Fig., *A*).

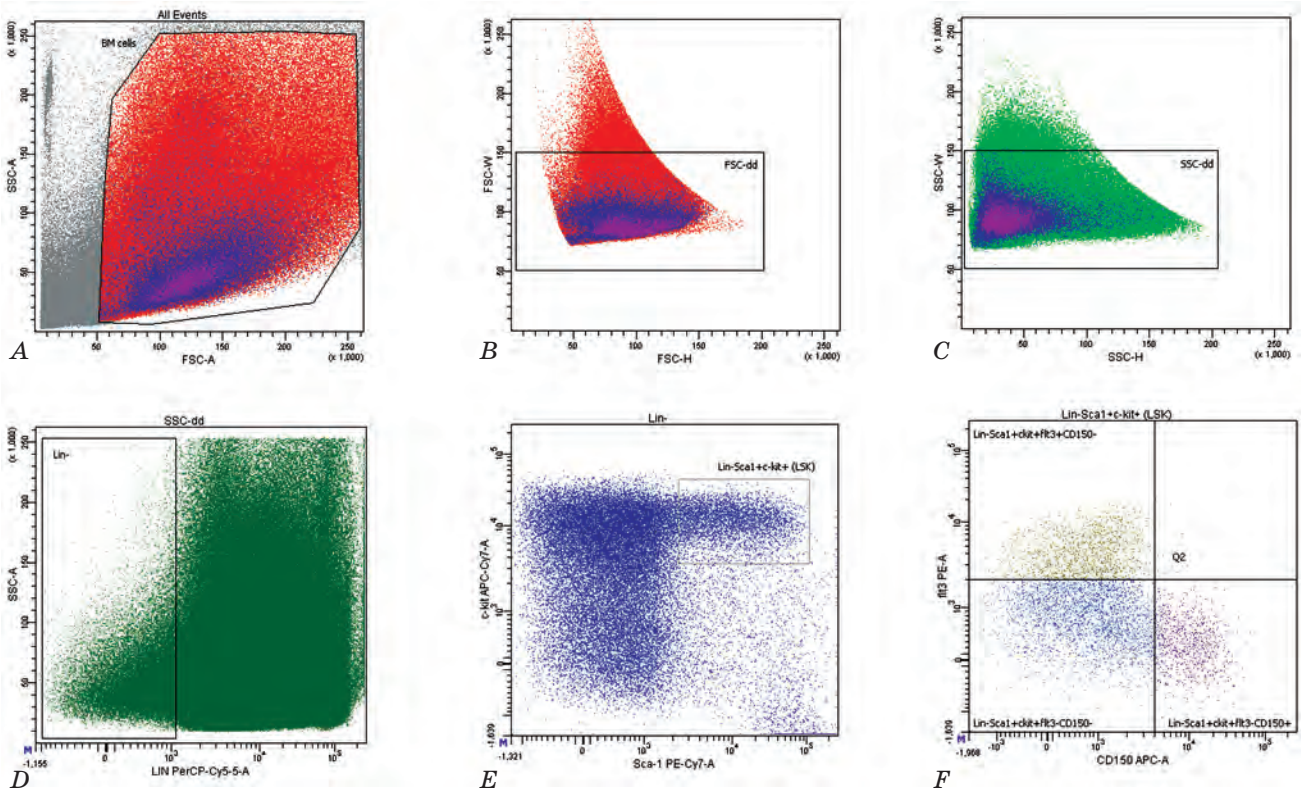
2. On two following two-parameter histogram of forward and side scattering by the signal width and height (FSC-W vs FCS-H and SSC-W vs SCS-H) among selected cells there were sequentially isolated rectangular gates, excepting the cells that have large pulse width of fluorescence signal. So we excluded events from the analysis that can match aggregates of cells and give false-positive results (Fig., *B, C*).

3. On the two-parameter histogram of SSC vs Lin panel in the population we isolated a gate of cells mentioned above with the lack of expression of linearity markers (Lin^-) (Fig., *D*).

4. On the two-parameter histogram Sca-1 vs c-kit we isolated a gate of Lin^- cells with simultaneous expression of both markers, selecting LSK population (Fig., *E*).

5. Among LSK-cells on two-parameter histogram CD150 vs flt3 we created a quadrant gate, dividing the population $flt3^+CD150^-$, $flt3^-CD150^-$ and $flt3^-CD150^+$ LSK-cells in the respective quadrants. In quadrant, corresponding $flt3^+CD150^+$ phenotype (Q2 — Fig., *F*), there can be single events that do not form clear cell population.

Considering that investigated HSCs subpopulations are minor compared to the total mononuclear cells the record file must include at least 1 million events to reduce measurement error. To improve the select and visualization of low-populations there should be used a biexponential scale on two-parameter histograms.



Dot-plot histograms of murine bone marrow HSCs subpopulation phenotyping (description in the text), *BD FACSDiva 6.1.2 software*

Using this protocol the relative content of hematopoietic stem LSK-cells and their subpopulations in the bone marrow of FVB mice was defined (Table 2). There was established that the dominant population of LSK-cells are cells with the phenotype $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$, characterizing short-term HSCs for myelopoiesis.

Based on the phenotyping protocol, the protocol of aseptic sorting of LSK-cells and their subpopulations for further studies in vivo and in vitro was developed. The optimal condition for sorting of these types of cells was found: cells concentration in suspension — $5 \cdot 10^6$ cells/ml, flow rate — 3 000 cells per second, flow pressure — 70 psi. We selected the sorting conditions under which the percentage of viable cells determined by the 7-AAD staining, was $89.6 \pm 4.6\%$ ($n = 8$). The percentage of viable bone marrow mononuclear cells immediately after preparation was $95.2 \pm 3.1\%$. By repeated phenotyping of sorted cell the purity of obtained fraction with phenotype $Lin^-Sca\text{-}1^+c\text{-}kit^+$ reached $96.6 \pm 1.8\%$.

Compliance of aseptic sorting conditions provides the ability to cultivation or transplantation of certain cell types to evaluate their proliferation, differentiation or migration potential. In particular, our study demonstrated that during cultivation of murine bone marrow after transplantation of sorted GFP-positive LSK-cells from FVB-Cg-Tg(GFPU)5Nagy/J mice (GFP-transgenic), form only granulocyte-macrophage GFP-positive colonies; and there were not detected GFP-positive colonies of stromal cells [13]. This confirms the literature data that LSK-cells are true hematopoietic cells without the properties of stromal stem cells and generate only myeloid and lymphoid hematopoietic progenitors [12].

The developed protocol is comparable to algorithms proposed by other authors [10–12]. Additionally it provides the phenotyping and sorting of murine HSCs in consideration of five key markers expression that defines belonging to subpopulations of cells responsible for maintaining myelo- and lymphopoiesis. This may be important in the research of various parts hematopoietic damage by chemicals, irradiation and other impacts and thus to evaluate the efficiency and possible targets for appropriate therapeutic agents.

Differences in the relative numbers of certain subpopulations are due to different methods of mononuclear cells suspension collection, the strain of mice, etc. that should be considered during the scheduling of

Table 2. **Relative numbers of bone marrow HSCs subpopulation in *FVB* mice (n = 11)**

HSCs subpopulation	Of total MNCs, %	Of LSK-cells,%
$Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK)	0.39 ± 0.09	–
$Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$	0.11 ± 0.03	27.1 ± 3.3
$Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$	0.22 ± 0.05	57.2 ± 6.8
$Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$	0.06 ± 0.03	15.7 ± 4.7

experiments design. For example, Adolfsson et al. [11] showed that relative content of $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+$ cells in the bone marrow of C57Bl/6 mice was 0.06%, while by our proposed protocol corresponding population of $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$ in FVB mice was 0.11 ± 0.03%.

There should also be noted the need to record some additional parameters (FSC-W, FCS-H, SSC-W, SCS-H), which can be important when creating of algorithm for data analysis and minimizing errors.

Selection of the optimal parameters for sorting of different cell types remains an actual problem for flow cytometry since stem and progenitor cells are particularly sensitive to exogenous impacts, which is a high pressure fluid flow, temperature, voltage on sorting plates etc. In many experiments double and triple sort are used that could affect the cell's viability [11–12]. Clarification of the sorter technical parameters, medium composition for cells collection, temperature and other factors made in this study allow for a one-time sorting to achieve a high purity of a target population, reduce cell damage, reduce the time of procedures and preserve the viability of cells which can subsequently used for cultivation or transplantation.

Thus, a protocol of multiparametric phenotyping of murine hematopoietic stem cells subpopulation was developed, allowing identification of the relative content of $Lin^-Sca\text{-}1^+c\text{-}kit^+$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$ and $Lin^-Sca\text{-}1^-c\text{-}kit^+flt3^-CD150^-$ subpopulations in the bone marrow of FVB mice. Exclusion from the analysis of cells that have a large width signal for forward and side scattering can reduce the doublets and false positive results. The effectiveness of aseptic sorting of murine bone marrow LSK-cells fractions for subsequent transplantation experiment was shown.

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ФЕНОТИПУВАННЯ ТА СОРТУВАННЯ ГЕМОПОЕТИЧНИХ СТОВБУРОВИХ КЛІТИН КІСТКОВОГО МОЗКУ МИШЕЙ ПРОТОЧНОЮ ЦИТОМЕТРІЄЮ

В. М. Кирик

ДУ «Інститут генетичної та регенеративної медицини НАМН», Київ, Україна

E-mail: biomedpost@gmail.com

Метою роботи було розробити протокол мультипараметричного фенотипування і сортування субпопуляцій гемопоетичних стовбурових LSK-клітин і визначити їх відносний вміст у кістковому мозку мишей.

Досліджено клітини кісткового мозку мишей ліній FVB та розроблено протокол їх мультипараметричного фенотипування, що дало змогу визначити відносний вміст $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK), $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$ і $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ -субпопуляцій. Встановлено, що домінуюча популяція LSK-клітин має фенотип $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ (57,2 ± 6,8%), який характеризує короткоіснуючі гемопоетичні стовбурові клітини, відповідальні за мієлопоез. Вилучення з аналізу клітин, що мають високу ширину сигналу за детекторами прямого і бічного світлорозсіювання, дає можливість зменшити хибнопозитивні результати вимірювань. Запропоновано також протокол і продемонстровано ефективність сортування фракції LSK-клітин кісткового мозку мишей для подальшої трансплантації в експерименті. За повторного фенотипування сортованих клітин чистота фракції $Lin^-Sca\text{-}1^+c\text{-}kit^+$ становила 96,6 ± 1,8% зі вмістом життєздатних клітин 89,6 ± 4,6%.

Ключові слова: гемопоетичні стовбурові клітини, мультипараметрична проточна цитометрія, сортування клітин.

ФЕНОТИПИРОВАНИЕ И СОРТИРОВКА ГЕМОПОЭТИЧЕСКИХ СТВОЛОВЫХ КЛЕТОК КОСТНОГО МОЗГА МЫШЕЙ ПРОТОЧНОЙ ЦИТОМЕТРИЕЙ

В. М. Кирик

ГУ «Институт генетической и регенеративной медицины НАМН», Киев, Украина

E-mail: biomedpost@gmail.com

Целью работы было разработать протокол мультипараметрического фенотипирования и сортировки субпопуляций гемопоэтических стволовых LSK-клеток, а также определить их относительное содержание в костном мозге мышей.

Исследованы клетки костного мозга мышей линий FVB и разработан протокол их мультипараметрического фенотипирования, что дало возможность определить относительное содержание $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK), $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^+CD150^-$, $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^+$ и $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$-субпопуляций. Установлено, что доминирующая популяция среди LSK-клеток имеет фенотип $Lin^-Sca\text{-}1^+c\text{-}kit^+flt3^-CD150^-$ (57,2 ± 6,8%), который характеризует короткоживущие гемопоэтические стволовые клетки, ответственные за миелопоэз. Исключение из анализа клеток, имеющих высокую ширину сигнала по детекторам прямого и бокового светорассеивания, позволяет снизить ложноположительные результаты измерений. Предложен также протокол и продемонстрирована эффективность сортировки фракции LSK-клеток костного мозга мышей для последующей трансплантации в эксперименте. При повторном фенотипировании сортированных клеток чистота полученной фракции $Lin^-Sca\text{-}1^+c\text{-}kit^+$ составляла 96,6 ± 1,8% с содержанием жизнеспособных клеток 89,6 ± 4,6%.

Ключевые слова: гемопоэтические стволовые клетки, мультипараметрическая проточная цитометрия, сортировка клеток.