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OMIP-059: Identification of Mouse Hematopoietic Stem and Progenitor Cells with Simultaneous Detection of CD45.1/2 and Controllable Green Fluorescent Protein Expression by a Single Staining Panel

Marcus Eich,[1] Andreas Trumpp,[1,2,3†] Steffen Schmitt[4†*]

[1]Heidelberg Institute for Stem Cell Technology and Experimental Medicine (HI-STEM gGmbH), Im Neuenheimer Feld 280, D-69120, Heidelberg, Germany

[2]Division of Stem Cells and Cancer, German Cancer Research Center (DKFZ), and DKFZ-ZMBH Alliance, Im Neuenheimer Feld 280, D-69120, Heidelberg, Germany

[3]German Cancer Consortium (DKTK), D-69120, Heidelberg, Germany

[4]German Cancer Research Center (DKFZ), Core Facility Imaging and Cytometry, Im Neuenheimer Feld 280, D-69120, Heidelberg, Germany

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*Correspondence to: Steffen Schmitt, German Cancer Research Center (DKFZ), Core Facility Imaging and Cytometry, Flow Cytometry Unit, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Email: steffen.schmitt@dkfz-heidelberg.de

†Joint last authors.

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Purpose and Appropriate Sample Types

THE panel was developed and optimized to identify mouse bone marrow hematopoietic stem cells (HSCs) and five multipotent progenitors (MPPs) along with controllable green fluorescent protein (GFP) expression as well as CD45.1 and CD45.2. HSCs and MPPs can be identified by analyzing the cell surface proteins Sca1, cKit, CD150, CD48, CD34, and CD135. The common myeloid progenitor (CMP), the granulocyte macrophage progenitor (GMP), the megakaryocyte erythrocyte progenitor (MEP), and the common lymphoid progenitor (CLP) are defined by specific expression patterns of Sca1 and cKit plus CD16/32 and CD34, CD135, and CD127. Additionally, the membrane integrity is determined with a life/dead discriminator to differentiate living from dead cells. The staining is performed in freshly isolated living cells without fixation obtained from transgenic mice (SCL-tTA H2B-GFP or ISRE-EGFP) expressing the GFP and the genetic alleles CD45.1 or CD45.2. The analysis of the status of these markers is included in the reported 16 parameter staining panel.

Background

HSCs are sitting on top of the hierarchically organized mouse hematopoietic system and progenitor cell types arising from them can be described using flow cytometry by a set of different cell surface markers (1–3). The number of surface markers is constantly increasing, leading to a steadily more detailed description and discrimination of the different cell populations. In parallel, functional and single cell data suggest a wide spectrum of molecular and functional heterogeneity (4,5). During the last years in particular HSCs and immediate downstream progenitors achieved high attention, as the hematopoietic system also serves as a model for other hierarchically organized stem cell systems. However, an optimized multicolor immune fluorescence panel (OMIP) characterizing these cell populations has not yet been reported (Tables 1 and 2). The optimized panel presented here was designed to differentiate HSCs, MPPs, and committed progenitor cells in the mouse bone marrow in conjunction with CD45.1 and CD45.2 as well as the GFP in one single 16 parameter staining panel.

Both alleles of CD45 differ by five amino acids and are frequently used in HSC transplantation experiments to differentiate between host and transplanted cells and its progeny (6,7). The intracellular reporter protein GFP and its derivatives such as enhanced GFP (EGFP) are used to label cells in many different applications (8). As models in which all HSC-progenitors as well as CD45 and GFP are present, we used two mouse strains. First, SCL-tTA H2B-GFP transgenic mice were crossed to express

Table 1. Summary table for the application of OMIP-059

PURPOSE	DIFFERENTIATION OF MURINE HEMATOPOIETIC CELL POPULATIONS DERIVED FROM BONE MARROW
Species	Mouse
Cell types	Hematopoietic stem and precursor cells
Cross-references	None

Table 2. Reagents used for OMIP-059

ANTIGEN	CLONE	FLUOROCHROME	PURPOSE
CD4	GK1.5	PE-Cy7	Lineage- cells
CD8a	53–6.7	PE-Cy7	Lineage- cells
CD11b	M1/70	PE-Cy7	Lineage- cells
B220	RA3-6B2	PE-Cy7	Lineage- cells
Gr1	RB6-8C5	PE-Cy7	Lineage- cells
Ter119	TER119	PE-Cy7	Lineage- cells
Sca1	D7	BV421	LSK-, LS-K cells
cKit	2B8	PE	LSK-, LS-K cells
CD150	TC15-12F12.2	BV785	HSCs, MPPs
CD48	HM48-1	BUV395	HSCs, MPPs
CD34	RAM34	Alexa-Fluor 700	LT-, ST-HSCs, CMP, GMP, MEP
CD135	A2F10	APC	MPP3, MPP4, CLP
CD16/32	2.4G2	BV480	CMP, GMP, MEP
CD127	A7R34	BV650	CLP
CD45.1	A20	PE-CF594	Internal reporter
CD45.2	104	BUV737	Internal reporter
Fluorescent protein			Purpose
GFP			Inducible internal reporter
Fluorescent dye			Purpose
Fixable NIR Dead Cell Stain Kit			Viability

CD45.1 (2,9). These mice express the H2B-GFP fusion protein under the HSC-myeloid progenitor specific SCL promoter, which can be repressed by the tetracycline-responsive regulatory element in the presence of doxycycline (2). Second, CD45.2 positive ISRE-EGFP transgenic mice express EGFP controlled by an interferon responsive element (10,11). To perform the staining, murine bone marrow was isolated by crushing the femurs, tibias, and the spine of the animals. Then erythrocytes were lysed using ACK lysing buffer (Lonza, Basel, Switzerland) and subsequently the resulting cells of both mouse strains were pooled to obtain a single sample, containing both CD45.1 and CD45.2 expressing cells. These were subsequently stained with a commercially available life/dead discriminator and the antibodies detecting the indicated surface markers. Before staining, all antibodies as well as the life/dead discriminator were titrated with the aim to maximize the resolution of negative and positive cells with the lowest necessary antibody concentration. The used dilutions range from 1:2000 to 1:125 (data are presented in the Supporting Information Fig. S1); however, this needs to be performed for each antibody lot supplied by the companies individually each time. The data were obtained at a BD FACS Aria Fusion cell sorter equipped with five lasers (355, 405, 488, 561, and 640 nm) and the DIVA v8.0.2 software (Beckton Dickinson, Franklin Lakes, NJ) using a nozzle with 100 μm diameter. Daily calibration of the cell sorter was performed manually using BD Calibrite beads (Becton Dickinson) in combination with Cell Sorting Set-up Beads for UV Lasers (Thermo Fisher Scientific, Waltham, MA). In line with other bead-based calibration protocols, the following values were tracked over time and daily analyzed for optimization: PMT sensitivity (voltage) and respective target values, laser delay (with window extension 0) and area scaling factors. Data analysis was performed with Flowjo v10.5 (Flowjo, LLC, Ashland, OR). Before the experiment, compensation was done manually with the DIVA software using single-stained OneComp eBeads (Thermo Fisher Scientific) by comparing median values of positive and negative single beads.

After exclusion of cellular debris and doublets using forward and side scatter parameters, cells are tested for their membrane integrity using the Fixable NIR Dead Cell Stain Kit (Thermo Fisher Scientific) and gated on the live cell fraction (Fig. 1A). During the optimization of the panel the Zombie NIR Fixable Viability Kit (Biolegend, San Diego, CA) was also evaluated for this purpose. Although it is also suitable for the panel, it seemed to have a higher spectral overlap especially in the RL730/45 detector, which is used to detect AF700. Both stains are detected by the RL-780/60 detector and are preferable to DNA intercalators like 4′,6-diamidin-2-phenylindol (DAPI) or propidium iodide (PI), because the spillover of these life/dead markers in nonspecific detectors is in general lower. In a subsequent step, cells expressing one or more of the indicated lineage markers (CD4, CD8a, CD11b, B220, Gr1, Ter119) are excluded from the analysis using one "dump channel" (Fig. 1A). Determination of the Sca1 and cKit expression reveals the well described LSK (Lineage-, Sca1+, cKit+) cell compartment containing progenitors and HSCs (Fig. 1A) (12). Within the LSK cells HSCs and the MPP 1–4 populations are discriminated as published (2,3,13). They are defined as indicated: HSCs (LSK, CD150+, CD48−, CD34−, CD135−), MPP1 (LSK, CD150+, CD48−, CD34+, CD135−), MPP2 (LSK, CD150+, CD48+), MPP3 (LSK, CD150-, CD48+, CD135−), and MPP4 (LSK, CD150−, CD48+, CD135+) (Fig. 1B). Another population within LSKs (LSK, CD150−, CD48−) was termed MPP5 (Fig. 1B). Additionally, the LS-K

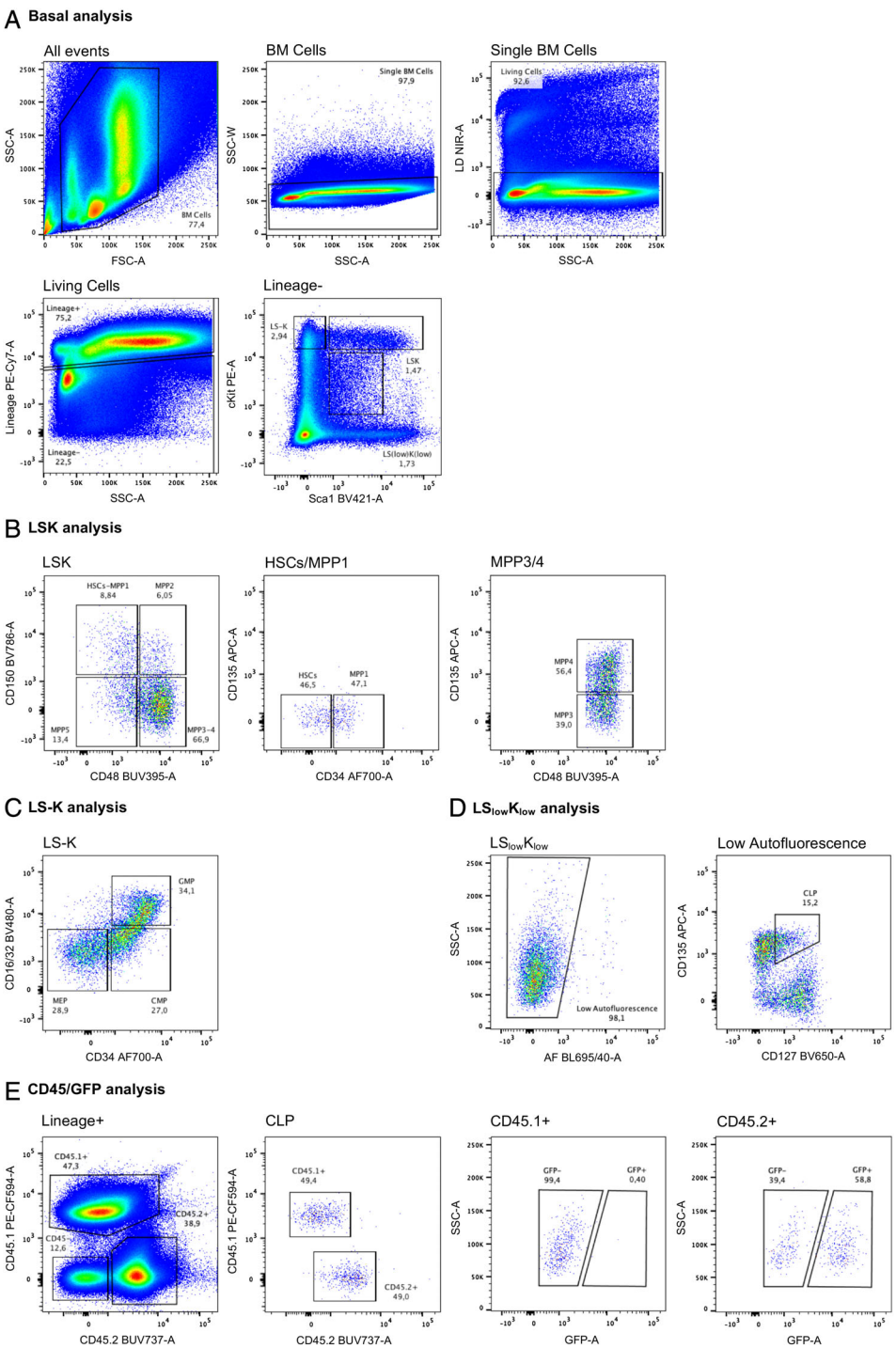

Figure 1. Single staining 16 parameter analysis of mouse bone marrow cells. After lysis of the erythrocytes, bone marrow cells were first stained with the life/dead discriminator and then with the indicated fluorochrome conjugated antibodies. (**A**) Basal analysis. First, bone marrow cells were plotted for forward and side scatter. After exclusion of cellular debris, doublets were discriminated from single cells by analyzing the side scatter area and width signals. Subsequently, cells were investigated for the viability using the Fixable NIR Dead Cell Stain Kit (upper panel). Alive single cells are then plotted for lineage markers (CD4, CD8a, CD11b, B220, Gr1, and Ter119). Lineage negative cells are analyzed for cKit and Sca1 to define the LSK and LS-K cell population as well as cells expressing low levels of both markers (lower panel). (**B**). LSK analysis. LSK cells are discriminated by expression of CD150 and CD48 to identify MPP2 and MPP5 as well as the mixed populations containing HSCs/MPP1 or MPP3/4. HSCs can be separated from MPP1s by lack of CD34 expression. The separation of MPP 3 versus 4 is achieved by differential CD135 expression. (**C**) LS-K analysis. To analyze the LS-K fraction, cells are separated based on CD16/32 and CD34 expression, revealing MEP, CMP, and GMP populations as indicated. (**D**) $LS_{low}KI_{ow}$ analysis. Cells expressing cKit and Sca1 at a low level are analyzed for CD135 and CD127 to define the CLP population after exclusion of highly autofluorescent cells by displaying the cells for the side scatter versus the BL695/40 detector. (**E**) CD45/GFP analysis. Expression of the isoforms CD45.1 and CD45.2 was analyzed exemplary in the lineage positive cell fraction and in the CLP population. In a subsequent analysis, the GFP expression is shown in the CD45.1 and the CD45.2 positive cells within CLPs. Since the lymphoid cell populations of the SCL-tTA H2B-GFP mouse do not express GFP, the CD45.1 expressing CLP cells are negative for GFP expression. In contrast, the CD45.2 expressing CLP population of the ISRE-EGFP mouse contains GFP negative and positive cells.

(Lineage−, Sca1−, cKit+) cell fraction is analyzed to identify the following progenitors (14): CMP (LS-K, CD34+, CD16/32−), GMP (LS-K, CD34+, CD16/32+) and MEP (LS-K, CD34−, CD16/32-) (Fig. 1C). In a first approach, a CD16/32 BUV737 and a CD34 AF 700 antibody was used for the staining. However, that fluorochrome combination resulted in a spreading of the MEP population. Therefore, the CD16/32 BUV737 antibody was replaced by a CD16/32 BV480 antibody resulting in an optimized resolution of the MEP population (data are shown in the Supporting Information Fig. S2). Furthermore, the CLP population, which was initially described in 1997 (15), is defined by the following surface markers: Lineage−, $Sca1_{low}$, $cKit_{low}$, CD135+, CD127+. The analysis of this particular population is impeded by highly autofluorescent cells, which are located inside the gate of the cells expressing Sca1 and cKit at a low level (Fig. 1D). The high autofluorescence of these cells can be seen also in other detectors. Therefore, they are excluded from the analysis using the BL695/40 detector after gating on the cell population with low Sca1 and cKit expression. Further explanation of the exclusion of these cells is described in detail in the supplement. Plots of different staining controls are displayed verifying that the high signal intensity is due to the autofluorescence of the cells itself (data are shown in the Supporting Information Fig. S3). The resulting cell population expressing Sca1 and cKit at a low level is then analyzed for CD135 and CD127 in order to determine the CLP population (Fig. 1D). Fluorescence minus one (FMO) controls are used to verify the gating for the expression of CD16/32, CD34, CD127, and CD135 (data for the gating on FMO controls are shown in the Supporting Information Fig. S4). The expression of CD45.1 and CD45.2 is shown for the lineage positive cells and for the CLP population (Fig. 1E). The GFP expression is displayed for the CD45.1 and CD45.2 positive CLPs. In the CD45.1 expressing SCL-tTA H2B-GFP mice, as expected the CLP population remains negative for GFP expression despite the mice were not treated with doxycycline (Fig. 1E). In the CD45.2 positive CLP population of the ISRE-EGFP mouse GFP positive and negative cells are visible. It should be noted that the presented analysis of CD45.1/2 and GFP is only selected to show exemplarily the variable potential of the multicolor panel. Additionally, all other cell populations, which are defined in the multicolor panel, can be analyzed for CD45.1/2 and GFP (detailed data of various populations are shown in the Supporting Information Fig. S5).

In summary, with the here reported panel, it is possible to discriminate and quantify all major populations of the mouse hematopoietic system from the earliest HSCs, via the MPPs to the lineage committed progenitor populations with high resolution. To increase the range of applications additional analysis of the internal reporter GFP as well as the genetic alleles, CD45.1 and CD45.2 are also included in the panel to study these cells also in a transplantation setting.

Similarities to Published OMIPs

There are no similarities to other published OMIPs.

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