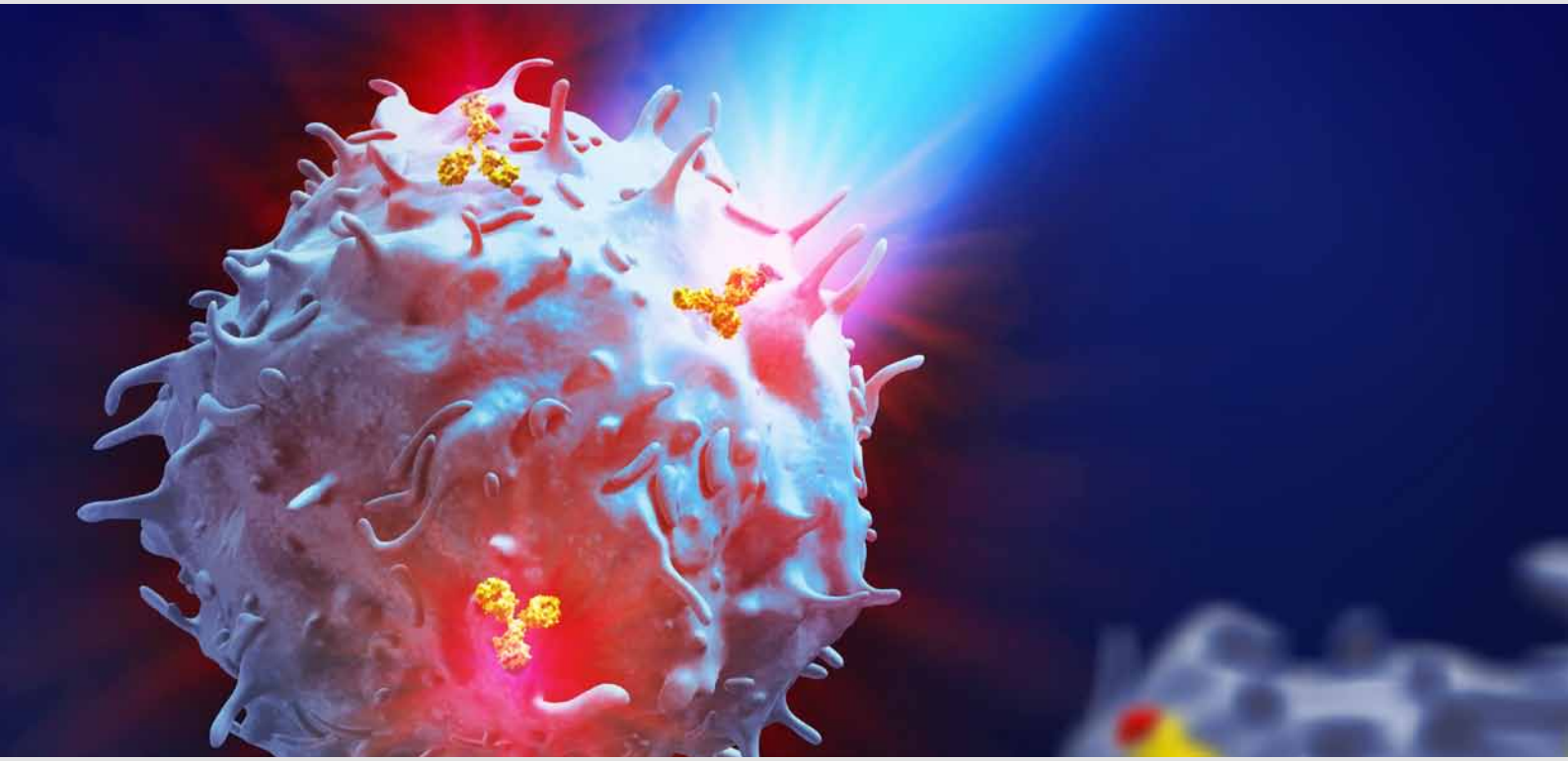
MACS&more

Excerpt from MACS&more Vol 14 – 1/2012

Analysis of hematopoietic cell differentiation using fluorescent protein reporters and multicolor flow cytometry

Hao Yuan Kueh and Rochelle Diamond

Division of Biology, California Institute of Technology (Caltech), Pasadena, CA, USA.



Analysis of hematopoietic cell differentiation using fluorescent protein reporters and multicolor flow cytometry

Hao Yuan Kueh* and Rochelle Diamond
Division of Biology, California Institute of Technology (Caltech), Pasadena, CA, USA.
*not shown. Rochelle Diamond is the Director of the Caltech Flow Cytometry and Cell Sorting Facility.

Introduction

Hematopoietic stem and progenitor cells have the ability to differentiate into different types of mature blood cells. The process by which stem cells choose between different mature cell fates is controlled by circuits of regulatory genes, which act to detect external developmental signals, choose between alternate fates, and irreversibly commit once a fate has been chosen (for a review see references 1 and 2). PU.1 is an Ets family transcription factor that works in regulatory circuits to control the lymphoid/myeloid fate decision. PU.1 is initially expressed at intermediate levels in stem cells, and is up-regulated in myeloid cells but down-regulated in lymphoid cells.[3,4] To understand how these regulatory gene circuits control cell fate choice, we need to measure the levels of different regulatory genes in single cells, and determine how these levels change as cells make fate choices and undergo differentiation. Recent developments of fluorescent protein reporter mouse strains have allowed us to accurately measure the levels of these regulatory genes, such as PU.1, in single cells using flow cytometry. By combining fluorescent proteins of different colors together with antibody staining of different developmental cell surface markers, it is now possible to use flow cytometry to determine the developmental fate of the cell and, at the same time, measure the state of the underlying gene regulatory circuitry controlling this cell fate decision. Such measurements will allow us to gain important insight into how regulatory gene circuits control stem cell fate choice.

Our current studies aim to understand how hematopoietic progenitor cells choose between two competing mature cell fates – the lymphoid (B cell) and the myeloid fates using the PU.1^{gfp} reporter mouse system[3]. To investigate the regulatory genes influencing this fate decision, we intend to introduce specific gene over-expression using transduction of various MSCV-IRES-mCherry retroviral constructs into purified hematopoietic progenitor cells isolated from the fetal livers of the PU.1^{gfp} mice. After various culture conditions, development of B cells and myeloid cells will be further analyzed by multiparameter flow cytometry as well as other downstream applications. To set forth on this investigation, it was necessary to determine that introduction of the retroviral construct would not alter the development of these cell types as well as determine that this type of flow cytometric analysis, multiple fluorescent proteins combined with immunophenotyping antibodies, can be performed.

The MACSQuant® VYB is a benchtop flow cytometer that has an optical bench configured with three lasers (405, 488, and 561 nm) and eight fluorescence detection channels. This instrument is optimized for simultaneous analysis of several fluorescent proteins, while still maintaining the ability to analyze common fluorochromes utilized in immunophenotyping (table 1). In this report, we validate the ability of the MACSQuant VYB to detect the simultaneous expression of two fluorescent proteins (GFP and mCherry) in addition to antibodies conjugated to APC, APC-eFluor® 780, or eFluor 450.

Materials and methods

Isolation of hematopoietic stem cells from PU.1^{gfp} mice and transduction with MSCV-IRES-mCherry

Fetal livers of E14.5 embryos from PU.1^{gfp} mice[3] were harvested and multipotent hematopoietic progenitors were isolated by depletion of lin$^+$ cells. Briefly, the harvested cells were stained with Lin$^+$ biotin-antibody cocktail (Ter119, CD19, Gr1, DX5 and F4/80). After the incubation, the cells were washed and labeled with Streptavidin MicroBeads (Miltenyi Biotec) and finally depleted using an LS Column and VarioMACS™ Separator (both from Miltenyi Biotec).

Isolated progenitor cells were transduced with the 'empty' retroviral vector construct MSCV-IRES-mCherry utilizing a retronectin® (Takara)-based plate system for 48 hours. The cultured cells were harvested and stained with Lin$^+$ biotin-antibody cocktail (Ter119, CD19, Gr1, DX5, F4/80) followed by Streptavidin-eFluor450, CD27-APC, and CD117-APC-eFluor780. The cells were sorted for lin$^-$CD117$^+$CD27$^+$ cells using an iCyt® Reflection® cell sorter (Sony iCyt Mission Technology).

In vitro stem cell differentiation and multiparameter cell analysis

MSCV-IRES-mCherry–transduced cells were cultured in alpha MEM + 20% FCS in the presence of 5 ng/mL SCF, Flt3L, and IL-7 to promote both B cell and myeloid development. After five days, cells were harvested and labeled with CD19-eFluor 450, CD11c-APC, and Mac1-APC-eFluor 780. Lymphoid and myeloid cell subsets were analyzed on the MACSQuant VYB for the expression of mCherry and GFP.

Results and conclusion

Flow cytometric analysis of the transduced cultured cells revealed that simultaneous detection of mCherry- and GFP-expressing cells was possible utilizing the Y2 and B1 channels of the MACSQuant VYB, with no compensation required between these two channels (fig. 1). Further analysis of these cultured progenitor cells revealed clearly separable infected (mCherry$^+$) and uninfected (mCherry$^-$) populations (fig. 2A).

Gating on these two populations (mCherry-positive and –negative, fig. 2A), we could determine the presence of distinct populations of myeloid precursors (Mac1$^+$), which were PU.1-GFP^{high}, and non-myeloid precursors (Mac1$^-$), which were PU.1-GFP^{low} (fig. 2B). In both infected and uninfected populations (top and bottom), over half of the myeloid precursors were CD11c$^+$CD19$^-$ (fig. 2C), suggesting that they were myeloid dendritic cell precursors. On the other hand, the majority of non-myeloid precursors were CD19$^+$CD11c$^-$, indicating that they were pro-B

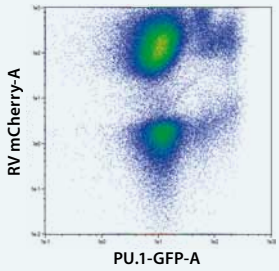


Figure 1: The fluorescent proteins GFP and mCherry are easily detected in the B1 and Y2 channels, respectively, on the MACSQuant VYB.

Laser	Channel	Filter (nm)	Fluorescent protein, dye, or parameter
Violet 405 nm	V1	450/50	CFP, AmCyan, VioBlue®, eFluor® 450
	V2	525/50	VioGreen™, Pacific Orange™
Blue 488 nm	B1	525/50	GFP, YFP, ZsGreen, FITC
	B2	614/50	PI, Lss-mKate
Yellow 561 nm	Y1	586/15	PE, tdTomato
	Y2	615/20	mCherry, dsRed, TexasRed®
	Y3	661/20	PE-Cy™5, APC, mKate, mPlum
	Y4	750 LP	PE-Vio770, APC-Vio770, PE-Cy7, APC-Cy7, APC-eFluor® 780
Yellow 561 nm	FSC	561/10	Size
	SSC	561/10	Granularity

Table 1: Optical configuration of the MACSQuant VYB.

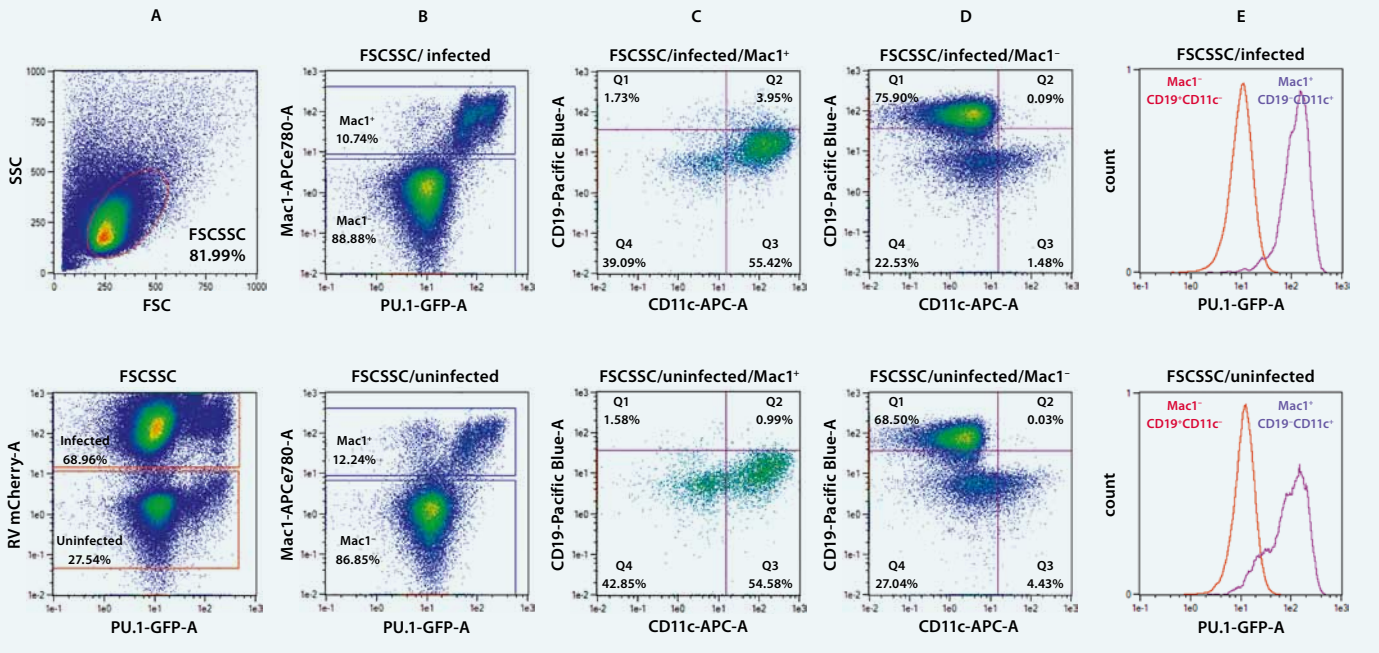


Figure 2: Infected mCherry$^+$ and uninfected mCherry$^-$ cells (A) were analyzed for progression to myeloid (Mac1$^+$) or non-myeloid (Mac1$^-$) precursors (B). Mac1$^+$ and Mac1$^-$ cells were further analyzed for the presence of CD11c$^+$CD19$^-$ myeloid dendritic cell precursors and CD11c$^-$CD19$^+$ pro-B cells (C and D). Myeloid dendritic cell precursors and pro-B cells were analyzed for PU-1-GFP expression (2E).

cells. (fig. 2D). Similar percentages of all the cell types were found in the infected and uninfected populations. Consistent with previous findings, myeloid dendritic cell precursors expressed high-levels of PU.1-GFP, whereas as pro-B cells expressed low levels of PU.1-GFP (fig. 2E). This confirms that introduction of the retroviral construct did not skew development toward one cell fate over the other.

These results highlight the utility of combining fluorescent protein reporters and antibody surface staining in studying hematopoietic stem cell differentiation, and also demonstrate the ability of the MACSQuant VYB to resolve multiple fluorescent proteins (GFP, mCherry) and organic dyes (eFluor 450, APC, APC-eFluor 780) at the same time.

The next steps will be to introduce specific influential genes via retroviral constructs to further investigate their effects on the differentiation of these cell lineages. As the MACSQuant VYB has the ability to visualize more fluorescent proteins in addition to GFP and mCherry, we hope to utilize this technology to add multiple reporter constructs to help further investigate these regulatory circuits.

Acknowledgments

We thank Ellen Rothenberg for advice and support in this project, and also for a critical reading of this manuscript. We also thank Stephen Nutt (Walter & Eliza Hall Institute, Melbourne, Australia) for generously providing the PU.1^{gfp} mice used in this experiment. We thank Josh Verceles for care and maintenance of the MACSQuant VYB and Michael Elowitz (Howard Hughes Medical Institute) for kindly sharing the use of the MACSQuant VYB with the users of the Caltech Flow Cytometry Cell Sorting Facility.

References

1. Rothenberg, E.V. *et al.* (2010) Immunol. Rev. 23: 150–168.
2. Singh, H. *et al.* (2005) Proc. Natl. Acad. Sci. USA. 102: 4949–4953.
3. Nutt, S.L. *et al.* (2005) J. Exp. Med. 201: 221–231.
4. Back, J. *et al.* (2005) Exp. Hematol. 33: 395–402.

MACS Product	Order no.
MACSQuant VYB	130-096-116
Streptavidin MicroBeads	130-048-101
VarioMACS Separator	130-090-282
LS Columns	130-042-401