High-Throughput Multiplexed Assay for Analysis of Hematopoietic Stem Cells Differentiation and Hematopoietic Toxicity

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Introduction

Hematopoietic stem cells (HSCs) give rise to all the blood cell types and are important for cell therapy and drug development. During the development of lymphoid and myeloid lineages, HSC differentiate into committed hematopoietic progenitors. Monitoring the expansion and differentiation of HSCs into lineage-committed hematopoietic progenitors is important for research of hematopoiesis and developing therapeutic processes with HSCs.

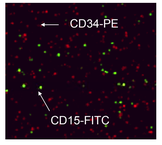
Effects of different growth factors on hematopoiesis have been studied by monitoring human CD34+ cells. Hematopoietic progenitors can also be used for hematopoietic toxicity and carcinogenesis screening. Differentiation of hematopoietic cells evaluated by expression of cell-surface antigens has been traditionally studied by flow cytometry, however, this technique has number of limitations.

Here we demonstrate the ability of IsoCyte laser scanning system (Molecular Devices) used with FCS Express® software (De Novo Software) to quantify expression of lineage-specific, and stem cell markers of hematopoietic cells. The assay uses a simplified protocol that allows real-time monitoring of HSC differentiation in a high-throughput compatible assay.

Method

Fluorescence Imaging of Hematopoietic Markers Measured by IsoCyte

The **IsoCyte-DL laser scanning platform** allows fast and simple cellular analysis in a multi-well plate format. Whole well images are captured using 488nm or 640nm laser excitation and 4 channels of detection. Entire 96 or 384-well plates can be scanned in < 5 minutes. Simultaneous image processing provides multiplexed analysis of expression levels of markers on single cells.



Differentiation of CD34+ cells: CD34+ cells from human bone marrow (Lonza) were plated 3K/well in 96w format in the presence of indicated cytokine combinations. Cell analysis was done by IsoCyte after 3 and 10 days of culture.

Hematopoietic Toxicity Assay:
CD34+ cells were prompted toward myeloid differentiation by stimulation with SCF and GM-CSF. Then cells were plated 10K/well and cultured for 3-7 days in the presence of anti-cancer drugs

Expression of specific markers was monitored by IsoCyte after staining cells with fluorophor-conjugated antibodies from Becton Dickinson:

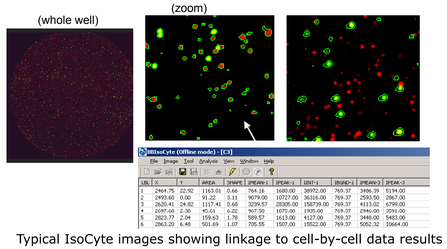
- Anti-CD34-PE
- Anti-CD15-FITC
- Anti-GlycophorinA-FITC

Data analysis done by using FCS Express®

- % of cells expressing lineage-specific markers
- Expansion of cells with different phenotype
- Mean intensity of fluorescence, total intensity of fluorescence

Fast Image Processing

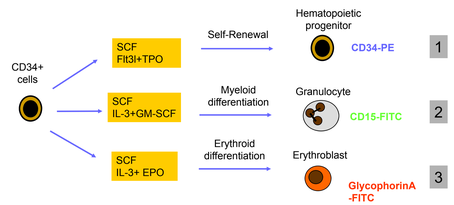
- Images acquired at 5 μm x 5 μm sampling
- Single cells are identified in a specific ROI in each image (Ch1 & Ch3)
- Cells are filtered by region of interest, area, intensity, etc.
- Cell-by-cell results are generated for each well and exported to FCS Express®



Typical IsoCyte images showing linkage to cell-by-cell data results

Results and discussion

Application I: Analysis of expansion and differentiation of hematopoietic stem cells

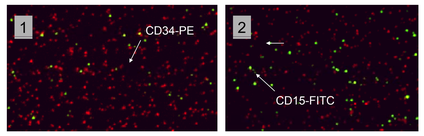


Human BM CD34+ cells were cultured in 96w format with hematopoietic cytokine combinations

- SCF, Flk3, TPO - promotes expansion of CD34+ cells
- SCF, IL-3, GM-CSF - promotes myeloid differentiation
- SCF, IL-3, EPO - promotes erythroid differentiation

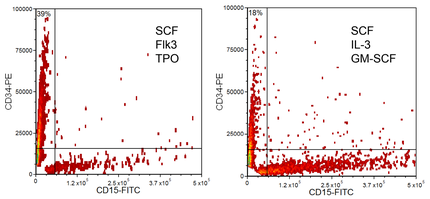
Monitoring the expansion of CD34+ cells in culture: SCF and TPO enhance expansion of CD34+ cells

Multicolor analysis of cell phenotype by IsoCyte



Day3 analysis

Data analysis done by FCS Express® software



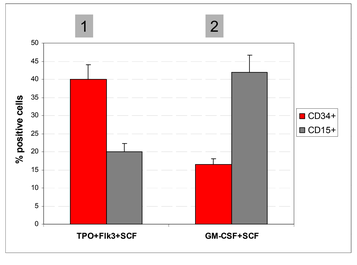
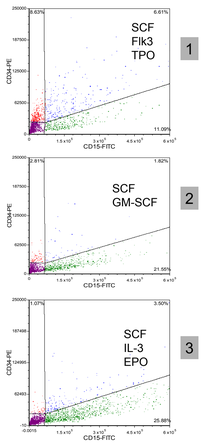


Figure 1. Cells were cultured in 96w format for 3 days in the presence of different cytokine combinations. The combination SCF, Flk3, TPO resulted in greater expansion of CD34+ cells (hematopoietic progenitors). GM-CSF promoted differentiation of cells toward myeloid cell phenotype (CD15+). % and numbers of CD34+ or CD15+ cells were measured by IsoCyte.

Monitoring differentiation of CD34+ cells in culture: SCF and TPO enhance expansion of CD34+ cells while GM-CSF or EPO promote differentiation of cells toward mature phenotype



Day10 analysis

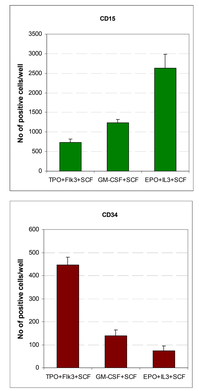


Figure 2. We have tested the effect of hematopoietic cytokines on differentiation of CD34+ cells. Cells were cultured for 10 days in the presence of different cytokine combinations. % and numbers of CD34+ and CD15+ cells were measured by IsoCyte.

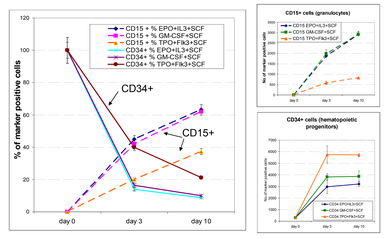


Figure 3. Effect of hematopoietic cytokines on differentiation of CD34+ cells. % and numbers of CD34+ and CD15+ cells were measured by IsoCyte.

EPO Promotes Differentiation Toward Erythroid Lineage

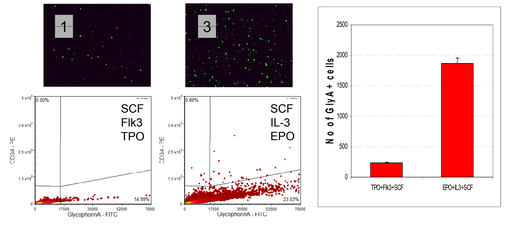


Figure 4. We have tested the effect of different hematopoietic cytokines on differentiation of CD34+ cells toward erythroid progenitors. % and numbers of CD34+ and GlycophorinA+ cells were measured by IsoCyte. Data analysis done by FCS Express® software.

Application II: Analysis of cytotoxic effect of anti-cancer drugs on expansion and differentiation of CD34+ cells

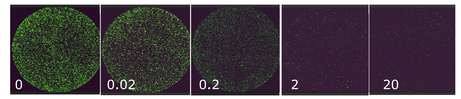
Many potential pharmaceuticals fail in clinical trials due to unacceptable toxicity. One way to improve information on toxicity is to introduce more biologically relevant assays early in drug discovery process. Hematopoietic stem cells or bone marrow is an important target for toxicity effects (anemia, neutropenia) and as such is a useful tool for obtaining toxicity information.

Hematopoietic Toxicity Assay:

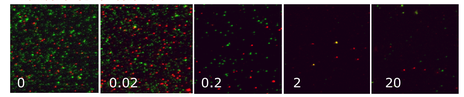
- CD34+ cells were prompted toward myeloid differentiation by stimulation with SCF and GM-CSF.
- Cells were incubated in the presence of 3 anti-cancer drugs (Imatinib, Fluoroadenin, & Cyclophosphamide) for 3-7 days. (Data shown below is from Day 5)
- Numbers of total viable cells as well as numbers of CD15+ cells (granulocytes) or GlycophorinA+ cells (erythroid progenitors) were measured by IsoCyte instrument and analyzed with De Novo Software.

Multicolor analysis: cell phenotype and toxicity

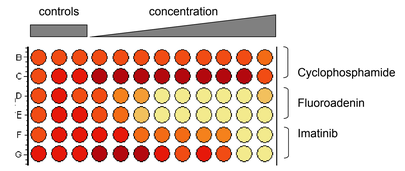
Viability test: CalceinAM, number of viable cells/well

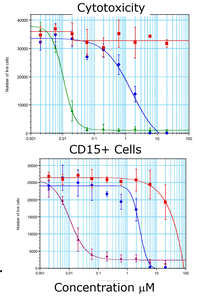


Impact on myeloid differentiation: CD15-FITC, propidium iodide exclusion; number of CD15+ cells/well



High-throughput assay for hematopoietic toxicity





IC50 imatinib 2 uM
IC50 fluoroadenin 0.1uM

Figure 5. We have tested the impact of cytotoxic drugs on expansion of hematopoietic progenitors and differentiation of CD34+ cells toward myeloid cells. CalceinAM used as tracker of total cells. Cells were incubated for 5 days. Percent and numbers of CD15+ cells were measured by IsoCyte after cell staining with FITC conjugated antibodies.

Summary

- We report the use of IsoCyte scanning cytometer (Molecular Devices) and a new, image-based version of FCS Express® (De Novo Software) to automate the study of cell differentiation & cytotoxicity in a high throughput format
- The IsoCyte is used to quantify expression of lineage-specific or stem cell markers in a multiplexed assay by using appropriate fluorophore-conjugated antibodies in either 96 or 384 plates with read times of 3-5 minutes per plate
- The new FCS Express® for Image Cytometry allows for rapid quantitation of image data and identification of populations of interest by applying flow cytometry data analysis techniques to the IsoCyte data
- We have demonstrated the ability to monitor expansion and differentiation of hematopoietic cells and the utility of IsoCyte for analysis of cytotoxic effect of anti-cancer drugs on expansion and differentiation of CD34+ cells
- In comparison to traditional FACS analysis, this assay method utilizes a simplified time-saving protocol and allows real-time monitoring of HSC differentiation in a high-throughput multi-well plate based format providing new tools to researchers in this area

Molecular Devices