Differential Counting of Bone Marrow by Flow Cytometry (13-Nov-2004)

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Introduction
Bone marrow examination is essential procedure for evaluation of a variety of clinical hematologic disorders and for preclinical toxicity studies of anticancer compounds and immunosuppressive agents [1,2]. This evaluation has been classically performed by morphologic assessment of bone marrow aspiration smears and core biopsy specimens by use of light microscopy. Differential cell counts have not been used routinely for evaluation of clinical specimens because of the complexity of the task and the time required. Differential cell counts have been used more frequently in preclinical toxicity studies. More recently flow cytometric methods for differential cell counting and immunophenotyping have also been described [3-5]. Initially described methods stained bone marrow cells with fluorescent stains. These methods were applied to several species. Subsequent studies evaluated bone marrow subpopulations by use of forward-angle vs. side-angle light scatter plots [6]. This technique was later combined with monoclonal antibodies for detection of lymphocytes and monocyte/macrophages [7,8]. Finally, CD45 labeling vs. side scatter plots have been described [9,10]. Each of these techniques have been compared to light microscopic differential cell counts and to a lesser extent to each other. In this presentation, I will review the various flow cytometric procedures for bone marrow differential cell counting and discuss the relative accuracy of each technique.

Collection and Processing of Clinical Bone Marrow Specimens
The marrow specimens are aspirated into syringes that have been rinsed with 2% EDTA solution [6,7]. Marrow can be aspirated repeatedly through 20 gauge needles to disperse units particles and cell clusters. Thereafter, smears are prepared and 10 ul of marrow specimen is placed in a 6 ml sterile plastic tubes for flow cytometric analysis. To remove contaminating erythrocytes from samples to be analyzed, 2 ml of erythrocyte lysis buffer (Erythrolyse solution, Serotec Inc, Washington, DC, USA) containing 0.5% paraformaldehyde is added. After 10 minutes, tubes are centrifuged and the cell pellet washed and resuspended in FACS buffer (Dulbecco's phosphate buffered saline solution containing 2% sheep serum, 1 mM sodium azide, 0.1% glycine). Fixed cells can be stored in at 4°C for a week or more. Unfixed samples can be stored at 4°C for up to 24 hours.

2′7′ dichlorofluorescin-diacetate (DCF) Technique
2′7′ dichlorofluorescin-diacetate is oxidized within cells to the fluorescent compound, 2′7′ dichlorofluorescin, therefore, the intensity of fluorescent staining of marrow cells is dependent on peroxidase activity [6,11,12]. Because of their high peroxidase activity, granulocytes have the greatest fluorescence intensity. Dog bone marrow specimens were incubated with 100 ul of 0.2 mM DCF (Aldrich, Milwaukee WI, USA) for 15 minutes at 37°C in the dark. Thereafter, 5 ul of 6.5 mM hydrogen peroxide was added as a substrate for the peroxidase reaction, and samples were incubated for an additional 15 minutes at 37°C in the dark. Samples were stored at 4°C and analyzed within 2 hours. Bone marrow cells were evaluated as scatter plots with fluorescence intensity on the x-axis and forward-angle light scatter on the y-axis. The DCF method resulted in one large discrete population with low fluorescence intensity and a second population with greater fluorescence intensity. However, distinct populations of mature and immature erythroid cells, lymphocytes, or megakaryocytes could not be identified [6].
3,3'-dihexyloxacarbocyanine (DiOC<sub>6</sub>)

Rat, mouse, dog, cat, and monkey bone marrow cells have been stained with 3,3'-dihexyloxacarbocyanine [6,13]. This compound is a lipophilic cationic dye whose binding to cells varies with cell membrane potential. In this procedure, bone marrow specimens, suspended in 100 ul of Dulbecco's phosphate-buffered saline solution containing 2% albumin, are incubated with 300 ul of 133 nM DiOC<sub>6</sub> (Aldrich, Milwaukee WI, USA) for 30 minutes at 37°C in the dark. DiOC<sub>6</sub> is dissolved in dimethyl sulfoxide, at a concentration of 2.5 mM, and is diluted to 133 nM with Dulbecco's phosphate buffered saline just before use. After incubation, bone marrow specimens are stored at 4°C and analyzed within 2 hours. Bone marrow cells are displayed as scatter plots with log green fluorescence intensity on the y-axis and forward-angle light scatter on the x-axis. A template has been developed to detect populations of immature and mature myeloid cells, immature and mature erythroid cells and lymphocytes based on previously described locations of these cell populations [6].

When results for the DiOC<sub>6</sub> method were compared to results for manual cell counts, statistically significant differences were not detected. Correlation coefficients for bands and segmenters and immature erythroid cells were high but correlation coefficients for immature myeloid cells, mature erythroid cells, and lymphocytes were lower. Compared to the manual method, the DiOC<sub>6</sub> method had lower numbers of band and segmenters, and higher numbers of immature erythroid cells. For cats, correlation coefficients varied between 0.61 and 0.96 [7]. The correlation coefficients for immature myeloid cells and lymphocytes exceeded 0.90 but correlation coefficients for mature myeloid cell, immature erythroid cells, and mature erythroid cells were less than 0.80. Data for immature and mature erythroid cells was not statistically significant indicating that data from the 2 methods were dissimilar.

**Forward-angle vs. Side-angle Light Scatter Plots Method (Scatter Plot Method)**

In the scatter plot method, bone marrow cells are displayed as forward-angle vs. side-angle scatter plots. A template has been developed to identified mature myeloid cells (i.e. bands and segmenters), immature myeloid cells (myeloblasts, promyelocytes), metamyelocytes, mature erythroid cells (rubricytes and metarubricytes), and immature erythroid cells (rubriblasts and prorubricytes; Fig 1) [6]. The template was initially constructed using subpopulations of marrow cells isolated by use of Percoll density gradient centrifugation. Because a distinct populations of lymphocytes and monocyte/macrophages could not be identified in scatter plots, these cell populations were not quantified. Additionally, megakaryocytes are difficult to differentiate from cell clusters. Results for the scatter plot method were compared to results for manual cells counts. The correlation coefficient for immature myeloid cells, metamyelocytes, immature erythroid cells, and mature erythroid cells was greater than 0.9 indicating a high degree of correlation. However, the correlation coefficient for band and segmenters was 0.76, indicating relatively poor correlation. When mean values were examined, means were similar except for mature erythroid cells, where the mean for the scatter plot method was higher than the mean for the manual count. When individual values were examined, only 4 of 50 total values differed by greater than 5%.

When the DiOC<sub>6</sub> method and the scatter plot method were compared to manual bone marrow differential cell counts, the scatter plot method had more similar mean values and had higher correlation coefficients [6]. Therefore, the scatter plot method has the potential of providing rapid semiquantitative assessment of bone marrow differential cell counts in dogs for specimens that contain low numbers of lymphocytes. However, it is less useful in rats and mice that have higher lymphocyte numbers in bone marrow.

![Figure 1. Flow cytometric forward-angle vs. side-angle light scatter plots (scatter plot) of canine bone marrow. A template was developed to detect mature (R2) and immature (R3) erythroid cells, immature myeloid cells (R5), metamyelocytes (R4), and mature myeloid cells (R1). - To view this image in full size go to the IVIS website at www.ivis.org . -](image)

**Scatter plot Method Combined with Monoclonal Antibodies**

Monoclonal antibodies have been used to quantify lymphocytes and monocytes/macrophages in the scatter plot method of bone marrow differential cell counting in dogs [8]. When cells in the mature erythroid gate were labeled with anti-CD18 or anti-MHC class-II, distinct fluorescent and nonfluorescent populations were identified. The percentages of fluorescent cells in the immature and mature erythroid gates varied directly with the number of lymphocytes as determined by manual differential cell counting. Labeling of CD18-positive cells with anti-CD21 or anti-CD3 identified these cells as lymphocytes. CD14-positive cells were concentrated in the metamyelocyte gate. Other gates contained less than 1% CD14-positive cells.

Ten clinical bone marrow specimens were analyzed by use the scatter plot method combined with anti-MHC class-II and anti
CD14 antibodies [8]. Lymphocytes were defined as the number of MHC class-II-positive cells in the erythroid gates and monocyte/macrophages were defined as the number of CD14-positive cells in the metamyelocyte gate. The number of lymphocytes and monocytes were subtracted from the number of erythroid cells and metamyelocytes respectively. Correlation coefficients for the revised method were greater than those for the scatter plot method for all cell categories. The correlation coefficients for lymphocytes and monocytes/macrophages was good.

**CD45 Expression and Intracytoplasmic Complexity**

Canine bone marrow cells can labeled anti-CD45 and labeling was evaluated as CD45 labeling intensity vs. side-angle light scatter plots (CD45 scatter plots; Fig 2) [9,10]. The CD45 scatter plot technique identify discrete populations of granulocytes, erythroid cells, lymphocytes, and monocyte/macrophages in bone marrow from healthy dogs. With the exception of monocytes, correlation with manual counts were 0.9 or greater. The CD45 scatter plot method overestimated monocyte numbers perhaps because monocytes are counted as myeloid cells in manual counts. When the CD45 scatter plot technique was compared to the scatter plot technique, correlation coefficients for granulocytes and erythroid cells were better. Other advantages included the capacity to quantify lymphocytes and monocytes.

The CD45 scatter plot technique also identifies a central discrete populations of blast cells in dogs [10,14]. In normal bone marrow, this area is poorly cellular. However, in dogs with leukemias and lymphomas a large blast cell population is present. In acute myeloid leukemia a large discrete population of blast cells was found in the central blast cell area [10]. The percentage of cells in this gate was similar to the percentage of blast cells determined by manual differential cell counting ($r = 0.93$). Dogs with myelodysplastic syndrome with excess, acute lymphocytic leukemia, lymphosarcoma metastatic to bone marrow, multiple myeloma, megakaryocytic leukemia, and mast cell tumor also had increased cell numbers of cells in the central blast area that correlated with the number of blasts cells observed in manual cell counts [10]. Therefore, this technique may provide a sensitive method for early detection of leukemias and lymphosarcoma in bone marrow, detection of residual malignant cells after chemotherapy, and early detection of recurrence of leukemias.

**Summary**

Flow cytometry provides an accurate and clinical applicable method for bone marrow differential cell counting. The scatter plot method, CD45 scatter plot method, and DiOC6 methods appear to provide suitable accuracy and precision for clinical application. The CD45 scatter plot technique provides an accurate differential cell count has the advantage of accurately quantifying blast cells. Many different types of canine leukemias and lymphomas, as well as myelodysplastic syndromes, result in an increase in cells in the blast gate. The simplicity of these methods, the short processing and analysis time, and the ease with which batches of samples can be performed, makes the technique potentially applicable as a routine test in clinical laboratories.
References


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