Application of Laser Scanning Cytometry to Clinical and Laboratory Hematology

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1. Introduction

Morphological diagnosis by May-Gruenwald-Giemsa (MGG) staining is an essential method in clinical and laboratory hematology. In addition, the detection of chromosomal aberration by G-band methods, the phenotyping of surface markers by Flowcytometry (FCM) or genetic diagnosis by reverse transcriptase-polymerase chain reaction (RT-PCR), Fluorescence in situ hybridization (FISH) and Southern blotting are important approaches to facilitate the establishment of a definitive diagnosis. These methods are found to be much more useful for the diagnosis of specific diseases showing characteristic abnormalities on cytogenetics including acute myeloid leukemia (AML) with *RUNX1-RUNX1T1* or *PML-RARA* and chronic myelogenous leukemia (CML), *BCR-ABL1* positivity and other such diseases etc (Arber et al, 2008, Vardiman et al, 2008). However, there are few methods other than morphological diagnosis that are described as effective for heterogenous populations comprising the majority of myelodysplastic syndrome (MDS), the detection of minimal residual diseases and the discrimination of cases showing slight dysplasia. Therefore, it is necessary to establish an effective technique of collecting other information.

2. The diagnosis of blood pathological cell using flow cytometry

The measurement of DNA content in the hematopoietic cells by flow cytometry (FCM) has been useful for the characterization of pathological blood cells, detection of residual diseases and prediction of disease prognosis in the hematological disorders. Trueworthy et al. (Trueworthy et al., 1992) reported that ploidy of lymphoblasts was the strongest predictor of treatment outcome in B-progenitor cell acute lymphoblastic leukemia of childhood. Almeida et al. (Almeida et al., 1999) showed that the combined use of immunophenotyping and DNA ploidy studies by FCM is a suitable approach for minimal residural disease (MRD) investigation in multiple myeloma patients, based on their applicability and sensitivity. Though FCM is an objective and reliable method to identify DNA ploidy of hematological cells, it is difficult to make inquiries about morphology and DNA ploidy in each cell (Sasaki et al., 1996).

3. The structure and advantage of laser-scanning cytometry

A laser-scanning cytometry (LSC) was developed by Kamentsky et al. (Kamentsky, LA & Kamentsky,LD, 1991) in 1991. The LSC (CompuCyte Corporation, Cambridge, MA) is an

instrument for being integrated image processing, flow cytometry, and automated digital microscopy, a rapidly high content analysis of cellular, tissue and other specimens. As cells pass through the scanning beams, the fluorescent light returns along the same path as the laser beams and is collected in the wavelength-specific photomultiplier. A photodiode scatter sensor detects scattered laser light for segmenting or brightfield imaging with Nomarski-like three-dimensional details. Specimens are observed with the microscope that is a component of the whole instrument and the morphological data are captured through the microscopic light path, and the LSC excites fluorescent-stained samples with up to 3 different wavelength, and acquires stoichiometric fluorescent and morphological data in as many as 5 detectors per laser.

LSC enables us to measure the cell population fixed on the slide glass by rapid scanning and acquire the fluorescent information of each cell like as FCM, and still better, LSC makes it possible to recall the cells of interest and evaluate their morphology with the microscope by switching the status between routine morphology observation and fluorescence condition (Martin-reay et al. 1994). LSC has recently been used for immunophenotyping of hematological malignancies in smear specimens or needle aspiration biopsy. Clatch et al. analyzed a total of 71 specimens including peripheral blood, tissue biopsy, bone marrow aspirates and lymphoid tissue, and concluded that LSC analysis provides advantages over FCM in point of using cells on the slide, examining microscopically at any time, low cost, easy for preparation (Clatch, 2001, Clatch & Walloch, 1997, Clatch & Foreman, 1998).

Whereas FCM yields useful information on the expression pattern and the amount of cellular molecules, LSC provides the morphologic information on the precise location of the molecules in the cell and makes it possible to retrieve the data file to examine the fluorescent features and morphology from a small number of the cells fixed onto the slide glass (Sasaki et al., 1996, Martin-Reay et al., 1994).

4. Comparative cell cycle analysis with LSC and FCM

A human myelocytic leukemia cell line, HL60 cells were fixed in 5ml cold ethanol (70%) for 30 minutes at 4°C, and after washing with PBS, the cells were resuspended in 500μl PBS and incubated with 2 mg/ml ribonuclease A (Sigma) at 37°C for 30 minutes, followed by the addition of 50μg/ml propidium iodide (PI, Sigma, ST. Louis, Missouri, USA) at room temperature for 20 minutes for DNA staining. HL60 cells were measured with FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA). PI fluorescence was measured at FL2 channel. Up to 10,000 events for each sample was measured by Cellquest software (BD Bioscience, Mansfield, MA, USA) and the cell cycle was analyzed with ModFit LT™ (Verity Software House, Inc, Topsham, ME, USA).

LSC is the apparatus for analyzing the fluorescence of the cells fixed onto the slide glass and is capable of evaluating the cell cycle by integrating the data of the cells whose nuclei are stained with PI. As the cell cycle analysis has been established and standardized by FCM, we examined the consistency of the data by LSC with that of FCM using HL60 cells in the proliferating phase. Figure 1 shows the comparative cell cycle analysis with FCM and LSC. The percentages of G0/G1, S, G2/M and subG1 phases of HL60 cell population measured with FCM were 35.7%, 58.2%, 6.0% and 0.5%, respectively (Fig. 1a.), whereas those analyzed with LSC were 55.7%, 25.8%, 20.2% and 1.0%, respectively (Fig. 1b.) (Tsujioka et al.,2008). Collectively, cell cycle analysis by the use of LSC did not always coincide with that of FCM because of different analytic methods, but both histogram patterns seemed similar to each

other. The measurement of DNA content of blood cells by FCM has been widely established and its accuracy control is excellently maintained. In contrast, the accuracy control of LSC method might be inferior to that of FCM. As shown in Figure 1, the peak pattern of the histogram demonstrating G0/G1 and G2/M phases is rather broad in LSC than that of FCM. It was reported that the coefficient of variation (CV) is almost in the range of 5-6% by LSC as compared with 3-7% in the case of FCM, but structural changes in the cells and nuclei associated with cell deposition on the solid surface of the slide glass may result in a broadening of the CV (Martin-Reay et al., 1994, Tarnok & Gerstner AOH, 2002, Kamada et al., 1997). In LSC, adjacent cells are often falsely recognized as a single giant cell with multiple nuclei, and the background fluorescence is not negligible particularly in the case of bone marrow specimens. To avoid such disadvantages, a relatively small number of the cells (less than five thousand cells per acquisition area) should be treated for cytospin preparation and the fluorescence-stained slide glass should be sufficiently washed (Pozarowski et al., 2006). Despite of some adverse points mentioned above, LSC exerts an excellent function that it enables us to evaluate both the morphology and the fluorescence information on each cell, and still better, it enables us to retrieve the individual cell and its information at any time as long as the fluorescence is sustained (Rodenburg et al., 1987, Chen TL et al., 1995, Rew et al., 1998, Kamiya et al., 1998).
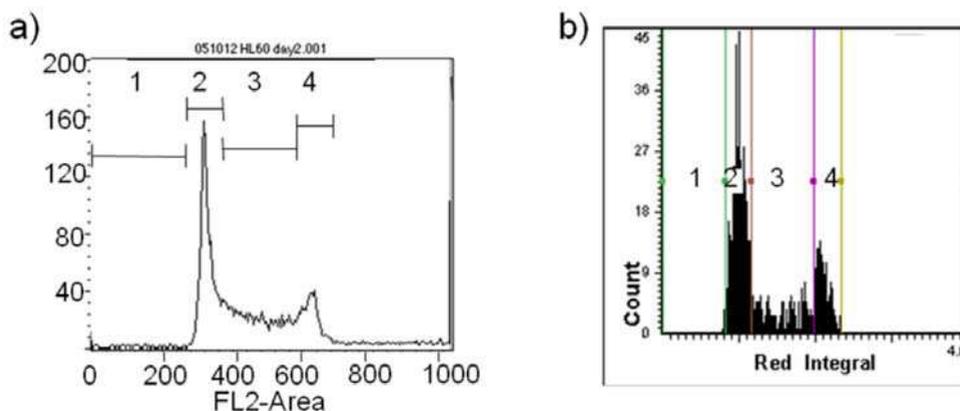

Fig. 1. Comparative cell cycle analysis with FCM and LSC (Tsujioka et al.,2008)
a) HL-60 cells were stained by PI as described in Materials and methods, then the cell cycle analysis was performed using FCM. 1:subG1 phase, 2: G0/G1 phase, 3: S phase, 4: G2/M phase
b) HL-60 cells were stained by PI as described in Materials and methods, then the cell cycle analysis was performed using LSC. 1: subG1 phase, 2: G0/G1 phase, 3: S phase, 4: G2/M phase

5. The model study on diagnosis of cases with megaloblastic anemia using LSC

Megaloblastic anemia is a common hematological disorder which is characterized by ineffective hematopoiesis due to impaired DNA synthesis of the bone marrow cells. This disease is usually caused by the disturbance of vitamin B12 absorption in pernicious anemia or

after gastrectomy, folic acid deficiency, or the adverse effect of some cytotoxic drugs. The prominent morphological features of megaloblastic anemia are megaloblasts in the erythroid lineage and neutrophils with hypersegmented nucleus or with larger cell size such as giant metamyelocytes and giant band cells in the myeloid lineage (Shojania, 1980, Parry, 1980, Beck, 1991). In the present study, we assumed that the dysplastic bone marrow cells found in megaloblastic anemia are the adequate candidates for DNA ploidy analysis. We performed the measurement of DNA ploidy with morphological analysis of a single cell by LSC.

6. DNA ploidy analysis on megaloblastic anemia using LSC

Bone marrow smears were obtained by bone marrow aspirations of 6 patients with megaloblastic anemia including 5 cases with pernicious anemia and 1 case with after gastrectomy. Normal bone marrow as a control was obtained from 3 volunteers with non-hematological diseases for routine hematological examination after informed consent. Normal peripheral blood cells were obtained from 9 healthy volunteers. Alternatively, we used MDS92 cell line which was established from the bone marrow of an MDS patient (Tohyama et al., 1994).

After the sample smears were stained with May-Gruenwald-Giemsa, the cells were morphologically assessed, captured visually with a CCD camera and their coordinates (x-and y-) on the slide were recorded (Fig.2a-g, upper stand.) (Tsujioka et al., 2008). Next the sample smears were destained with ethanol/acetate, treated with 2mg/ml of ribonuclease A for 1 hour at 37°C, and 100μg/ml of PI was added to the specimen slides (Fig. 2a-g, lower
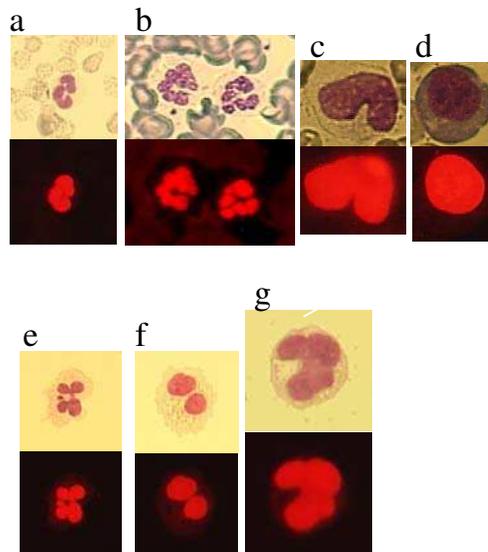

Fig. 2. Morphologic features of the cells with megaloblastic anemia and MDS92 cells (MDS cell line). Upper stand: May-Gruenwald-Giemsa staining, Lower stand: PI staining
a) normal neutrophil, b) hypersegmented neutrophil, c) giant metamyelocyte,
d) polychromatic megaloblast, e) neutrophil in MDS92 cell line, f) hyposegmented mature neutrophil in MDS92 cell line, g) giant metamyelocyte in MDS92 cell line (Original magnification 400X)

stand.) (Tsujioka et al.,2008). The slides were washed, scanned with the objective mode of LSC and the fluorescence data of more than 5,000 cells per slide were acquired. The nuclear stain PI was excited by a 488 nm wave-length argon-ion laser, and the red fluorescence emission was measured by appropriately filtered photomultiplier tubes. The mode value of DNA content was adjusted to 2.0 (this value implies a diploid cell at the G0/G1 phase). The data of each cell were retrieved corresponding to the identical cells formerly stained by May-Gruenwald-Giemsa according to their coordinates.

7. DNA ploidy analysis in abnormal neutrophils of the patients with megaloblastic anemia

Hypersegmented neutrophils, giant metamyelocytes, giant band cells or giant neutrophils are often detected in the peripheral blood or in the bone marrow of the patients with megaloblastic anemia. It has been one of the simple questions whether or not these morphologically abnormal neutrophils are ordinary diploid cells or perhaps hyperploid cells. Hence we selected these cells in May-Gruenwald-Giemsa-stained smears and subsequently determined their DNA ploidy after PI staining. We compared the DNA content of abnormal neutrophils found in the bone marrow of the patients with megaloblastic anemia with normal counterparts in the bone marrow of non-hematological disease. The average values±SD of DNA ploidy in normal neutrophils in the peripheral blood of healthy volunteers and hypersegmented neutrophils found in megaloblastic anemia were 2.0±0.1 (n=181) and 2.0±0.2 (n=18), respectively (data not shown). The average values±SD of DNA ploidy in normal neutrophils and hypersegmented neutrophils in the bone marrow were 2.0±0.1 (n=38) and 1.9±0.1 (n=36), respectively(data not shown). These data represented no significant difference of DNA content of normal neutrophils and hypersegmented neutrophils in both the peripheral blood and the bone marrow. Therefore it was suggested that DNA ploidy of hypersegmented neutrophils is not significantly increased as compared with that of normal neutrophils.

Giant metamyelocytes, giant band cells and giant neutrophils are extraordinary huge ones often detected in the bone marrow of the patients with megaloblastic anemia. The average DNA ploidy in giant metamyelocytes and giant neutrophils were 2.9±0.1 (n=23, p<0.01) and 3.5±0.2 (n=20, p<0.01), respectively. These data demonstrated that giant neutrophilic series have significantly higher DNA ploidy as compared with that of normal bone marrow counterparts (data not shown) (Tsujioka et al.,2008).

8. DNA ploidy analysis in the erythroid cells representing the megaloblastic change

The erythroid cells in the megaloblastic anemia represent morphological features of the comparatively large size and the delay of chromatin condensation as compared with cytoplasmic maturation. We measured DNA content of the abnormal erythroblasts including basophilic and polychromatic megaloblasts of the patients with megaloblastic anemia and compared them with normal erythroblasts of the bone marrow of non-hematological diseases

Table 1 show the distribution of DNA content in normal erythroblasts and megaloblasts, respectively. The cell cycle was divided into three phases by the measured values of DNA content: G0/G1 phase (1.8-2.4), S phase (2.5-3.8) and G2/M phase (3.9-4.2), and the

distribution pattern of three phases was compared between normal erythroblasts and megaloblasts (Tsujioka et al.,2008). Table 1 shows that the cell fraction in S phase was increased in the case of megaloblasts (Tsujioka et al.,2008). Wickramasinghe et al. (Wickramasinghe et al., 1982) previously reported the cell cycle distribution of normal erythroblasts in the bone marrow using a technique of combined Feulgen microspectrophotometry and ^{3}H-thymidine autoradiography and showed that the distribution of cell cycle was 32% in G0/G1 phase, 62% in S phase, 6% in G2/M phase in basophilic erythroblasts, and 15% in G0/G1 phase, 77% in S phase, 7% in G2/M phase in polychromatic erythroblasts. Fibach E et al. (Fibach & Rachmilewitz EA, 1993) reported that peripheral blood mononuclear cells were cultured with erythropoietin (2.0U/ml) and the cell cycle distribution of the erythroblasts was 47% in G0/G1 phase, 40 % in S phase and 13 % in G2/M phase. Considering Fibach's study and our data, the report by Wickramasinghe et al. might include overestimated proportion of S phase in normal erythroblasts.

On the contrary, the average DNA content of the cells in S phase were 2.7±0.1 in normal erythroblasts (n=27, range: 2.2-3.8) and 2.9±0.1 in megaloblasts (n=37, range: 2.3~3.8), and these values showed no significant difference (Tsujioka et al., 2008).

	Percentage		
	G0/G1	S	G2M
Megaloblastic anemia (6cases)	32.3% (n=20)	59.7% (n=37)	8.1% (n=5)
Normal control (3 cases)	66.1% (n=72)	24.8% (n=27)	9.2% (n=10)

Table 1. Proportion of the cells in three phases of cell cycle in normal erythroblasts and megaloblasts

The data of normal erythroblasts (n=109) were obtained from the bone marrow of 3 volunteers and those of megaloblasts (n=62) from the bone marrow of 6 patients with megaloblastic anemia. The specimens were stained by PI and up to 5,000 events for each sample were collected and analyzed by LSC. Normal samples and those with megaloblastic anemia were divided to G0/G1 (1.8-2.4), S (2.5-3.8), G2/M (3.9-4.2) phase. The G0/G1, S, G2/M fractions of the normal control were 66.1% (n=72), 24.8% (n=27), 9.2% (n=10), respectively. The G0/G1, S, G2/M fractions of those with megaloblastic anemia were 32.3% (n=20), 59.7% (n=37), 8.1% (n=5), respectively.

9. DNA ploidy analysis in normal megakaryocytes

In this study, we could not exactly estimate the analysis of DNA ploidy in megakaryocytes (MGK) with dysplasia, because bone marrow with megaloblastic anemia frequently shows hypercellular marrow and there are many other cells around MGK. Figure 3 shows DNA ploidy of MGK in normal bone marrow. DNA ploidy of MGK is much greater than that of erythroid cells or myeloid cells. As hematological diseases often show the characteristics of

MGK, such as micro-MGK and mononuclear-MGK in MDS or small-MGK in chronic myelogenous leukemia (Brunning et al, 2008, Vardiman et al, 2008), it would be interesting to measure the DNA ploidy of these cells using LSC in the future.
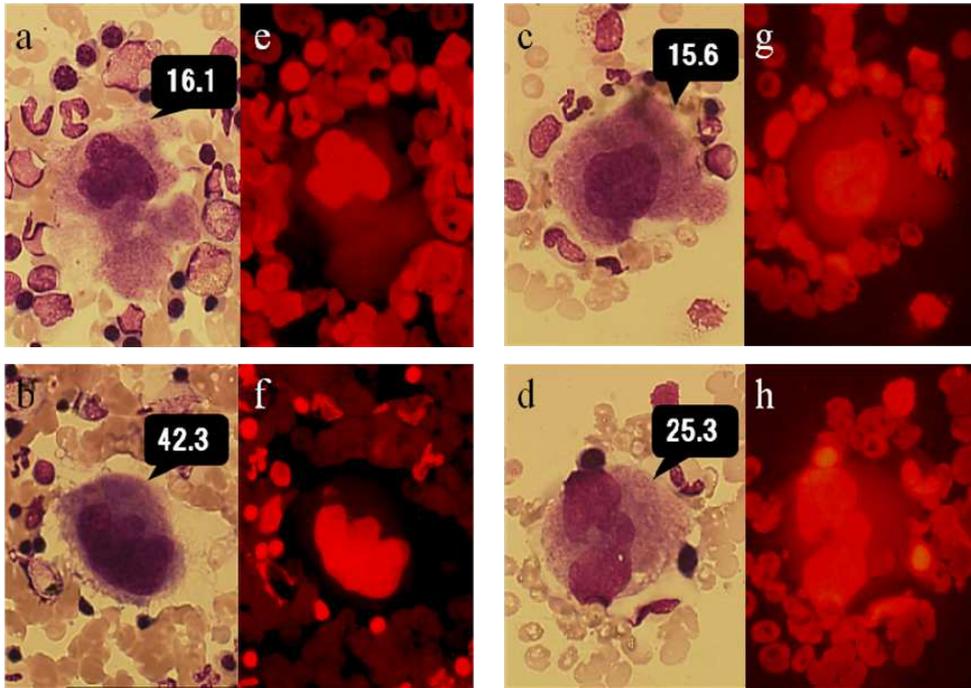

Fig. 3. DNA ploidy of megakaryocyte in normal bone marrow
a-d: May-Gruenwald-Giemsa staining
e-h: PI staining
a-h: megakaryocyte (Original magnification 400X)
The numerical value in the figure indicates DNA ploidy.

10. Overview of DNA ploidy analysis on megaloblastic anemia using laser scanning cytometry

In the present study, we have investigated the DNA ploidy of the erythroid /myeloid cells of the patients with megaloblastic anemia, because this anemia is characterized by dysplastic and often extraordinarily large erythroid/myeloid cells whose DNA ploidy is worthy to be examined. We actually confirmed for the first time that the DNA ploidy of giant metamyelocytes, giant band cells and giant neutrophils often found in the specimens of megaloblastic anemia is significantly increased over the diploid pattern of normal myeloid counterparts. As such maturing myeloid cells are considered to lose a capacity of cell division, the DNA ploidy of giant myeloid cells is obviously abnormal. On the contrary, the DNA ploidy of hypersegmented neutrophils is not significantly increased over normal diploid pattern.

As the studies dealing with clinical samples, DNA staining of the cells from normal or pathological tissues will be the best application for LSC. Our data about the DNA ploidy of the erythroid /myeloid cells of the patients with megaloblastic anemia demonstrate the impaired nuclear division although the underlying molecular mechanisms leading to the dysplasia remain unknown. From our data, we suspect that a larger number of megaloblasts are in the way of S-phase as a result of impaired DNA synthesis although it is not determined whether cell cycle process is retarded or arrested.

## 11. The future of LSC in clinical and laboratory hematology

The advantage of the 4th edition of the WHO classification in 2008 is the classification by genetic criteria newly defined for some diseases (eg: *JAK2/*V617F, *MPL*W515, *c-Kit* D816V) particularly among the myeloproliferative neoplasms (Vardiman et al, 2008). Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell diseases characterized by cytopenia, dysplasia, ineffective hematopoiesis and increased risk of developing acute myeloid leukemia. These are highly heterogenous populations except in MDS associated with isolated del (5q) defined by genetic criterion only (Hasserjian et al, 2008). Therefore, morphological diagnosis of MDS by MGG staining has been the main method of diagnosis lagging behind the progress of genetic analysis made in other fields. However it is very difficult for us to distinguish between hypoplastic MDS without chromosomal abnormality and aplastic anemia with slight dysplasia by morphological diagnosis alone. DNA ploidy analysis using LSC is useful as an adjunct to the diagnosis of these confusing cells. In this study, though we performed the measurement of DNA ploidy in dysplastic cells with megaloblastic anemia as a previous step, we further intend to measure the DNA ploidy of several other hematopoietic cells in the near future in order to clarify the relation of DNA ploidy to cell dysplasia in bone marrow disorders such as neutrophils with nuclear hypolobation and micro-megakaryocyte in MDS using LSC.

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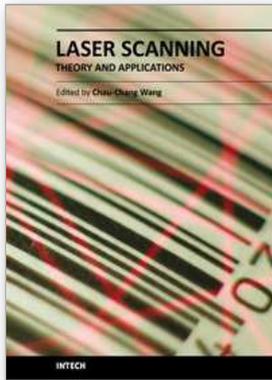
Laser Scanning, Theory and Applications
Edited by Prof. Chau-Chang Wang

Ever since the invention of laser by Schawlow and Townes in 1958, various innovative ideas of laser-based applications emerge very year. At the same time, scientists and engineers keep on improving laser's power density, size, and cost which patch up the gap between theories and implementations. More importantly, our everyday life is changed and influenced by lasers even though we may not be fully aware of its existence. For example, it is there in cross-continent phone calls, price tag scanning in supermarkets, pointers in the classrooms, printers in the offices, accurate metal cutting in machine shops, etc. In this volume, we focus the recent developments related to laser scanning, a very powerful technique used in features detection and measurement. We invited researchers who do fundamental works in laser scanning theories or apply the principles of laser scanning to tackle problems encountered in medicine, geodesic survey, biology and archaeology. Twenty-eight chapters contributed by authors around the world to constitute this comprehensive book.

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