

Technical Advance

The Development of a Cell Array and Its Combination with Laser-Scanning Cytometry Allows a High-Throughput Analysis of Nuclear DNA Content

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We developed a cell array device for analyzing cellular characteristics such as DNA ploidy, numerical chromosomal aberrations, and antigen expression in multiple specimens in a single experiment. Fifty (10 × 5) spots, 2 mm in diameter, were arrayed in an area of 30 × 16 mm on a glass slide, and ~1,000 cells were placed on each spot. To demonstrate the usefulness of the cell array, we measured nuclear DNA content using laser-scanning cytometry for DNA ploidy analysis in nine human tumor cell lines and normal lymphocytes. Combining the cell array with laser-scanning cytometry allows not only measurement of nuclear DNA content for 50 samples but also easy comparison of DNA ploidy among the samples in a single experiment. In addition, we used the cell array for fluorescence *in situ* hybridization using a DNA probe specific for the pericentromeric region of chromosome 11. (Am J Pathol 2000, 157:723–728)

Examination of cellular properties has become increasingly important in the diagnosis and treatment of malignant tumors, and various *in situ* methods are currently available. Immunohistochemistry is a routine procedure for histopathological examination of tumors. Fluorescence *in situ* hybridization (FISH), which is applicable to interphase cells and metaphase spreads, is widely used in both research and clinical analyses. FISH provides direct information for the diagnosis of chromosomal aberrations. In both immunohistochemistry and FISH, each marker is examined individually for each sample at a time. When many samples have to be examined, such a one-by-one procedure is laborious, cumbersome, and

time-consuming. Conventional methods require considerable amounts of antibody or DNA probe for each experiment. A novel method that allows efficient analysis of a large number of specimens would reduce both cost and time of analysis. To address this issue, a tissue microarray system that permits immunohistochemical and FISH analysis of hundreds of specimens in a single experiment was developed.^{1–4} However, this method does not always permit precise analysis of gene (chromosome) copy number aberrations because nuclei are frequently truncated in tissue sections. Although DNA ploidy is one of the useful parameters for estimating the biological characteristics of tumors,^{5–10} truncated nuclei in tissue microarrays hamper the precise measurement of nuclear DNA content. Alternative technology is necessary for a multiparameter analysis including DNA ploidy determination in a number of specimens. Usually, at least two experiments are made to determine DNA ploidy for each specimen. After the initial experiment, the addition of an internal standard, such as lymphocytes, is required to determine which peak represents diploidy in a DNA histogram. More convenient methods are necessary for clinical application of DNA ploidy.

In this brief communication, we present a novel but simple device that facilitates not only immunohistochemistry and FISH but also measurement of DNA ploidy across multiple samples in a single experiment. This cell array device makes it possible to analyze 50 tumor specimens on a single slide. We describe the potential impact of the cell array device for measuring nuclear DNA content by laser scanning cytometry (LSC). To further demonstrate the uses of the cell array device, we perform FISH using DNA centromere.

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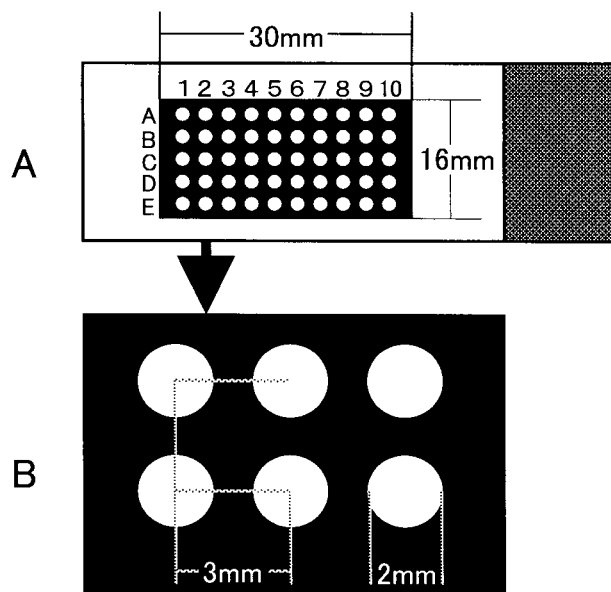


Figure 1. A specially designed glass slide on which 50 spots are encompassed by black printing ink in a 30 × 16 mm area. In this area, 50 (10 × 5) 2-mm diameter spots were arranged at intervals of 1 mm. Numbers (1 to 10) and letters (A to E) were printed along the margins of the black area to identify each spot.

Materials and Methods

Cell Array

A glass slide was specially designed for analysis of multiple samples as shown in Figure 1. A 30 × 16-mm square area on a glass slide was covered with black printing ink without significant autofluorescence. In the area, 50 (10 × 5) 2-mm diameter spots were arranged at 1-mm intervals. The surface of each spot was coated with triethoxyaminopropylsilane to prevent desquamation of cells from the slide glass. (Detailed information concerning the glass slide with spots was obtained from Matsunami Glass Industries Ltd., Kishiwada, Japan). The numbers (1 to 10) and letters (A to E) were printed along the margins of the black area to identify the position of each spot.

Cells

We used human peripheral blood lymphocytes and nine human tumor cell lines; U87MG, U105G, DBTRG65MG, U373MG, GI-1, SW1083, NB3911, HS683, and U251. The tumor cell lines were grown in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% calf serum at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were harvested with 0.25% trypsin in phosphate-buffered saline (PBS), then washed and resuspended in PBS, and fixed in 70% ethanol followed by 100% ethanol. Lymphocytes from peripheral blood were isolated by Ficoll-Hypaque, washed in PBS and fixed in 70% ethanol followed by 100% ethanol.

Cell Array Preparation

Drop cell suspensions were put as five spots on the glass slide with a micropipette and then air-dried to investigate the difference in DNA ploidy among spots of cells of the same type. The density of cells in a spot was adjusted manually using a microscope. Each spot contained 800 to 1,000 cells because more than 1,000 cells per spot decreased the number of countable cells. Peripheral lymphocytes were spotted in five positions as controls for the cultured cells. A total of 10 cell types was examined in the same glass slide. It took ~1 hour to prepare one array slide.

DNA Ploidy Analysis by LSC

The slides were treated with RNase (1 mg/ml; Sigma) for 30 minutes at room temperature and were then dipped in a solution of propidium iodide (25 μg/ml; Sigma). Coverslips were placed on the slides and sealed with nail polish. DNA content was measured with a laser-scanning cytometer (LSC 101; Olympus Co., Tokyo, Japan) as described previously.¹¹⁻¹⁴ At first, nuclear DNA content was measured for the area covered with black printing ink containing all spots. Namely, DNA content and cellular position data were stored for all cells in list mode. Overlapping nuclei were automatically excluded from the counting by special statistical filters.¹⁵ Subsequently, a DNA histogram was generated for each spot or group of spots and DNA ploidy was determined. Our device allows any combination of spots to be grouped and evaluated. The DNA index (DI) was calculated according to principles recommended by consensus.¹⁶ A DI equal to 1.0 indicates DNA diploidy.

FISH

To verify the reliability of our cell array system, we performed FISH using a Spectrum Green-labeled alphoid satellite DNA probe specific for the pericentromeric region of human chromosome 11 (D11Z1; Vysis, Inc., Downers Grove, IL), as previously described.¹⁷ Briefly, 10 μl of a hybridization mixture containing 1 μg/ml of salmon sperm DNA (Sigma Chemical Co.), 55% formamide, 2× standard saline citrate (SSC) (1× SSC is 0.15 mol/L NaCl and 15 mol/L Na citrate) and 10% dextran sulfate was heated in a water bath at 70°C for 5 minutes. The DNA mixture was applied to the slides, which were denatured at 70°C for 2 minutes, and they were then incubated overnight at 37°C in a moist chamber. The slides were transferred to a washing solution containing 50% formamide and 2× SSC at 45°C, and then the nuclei were counterstained by adding glycerol with propidium iodide (2 μg/ml; Sigma) and p-phenylenediamine dihydrochloride (1 μg/μl; Sigma Chemical Co.).

Scoring of Hybridization Signals

The number of hybridization signals in each nucleus was determined by observing more than 200 nuclei in each

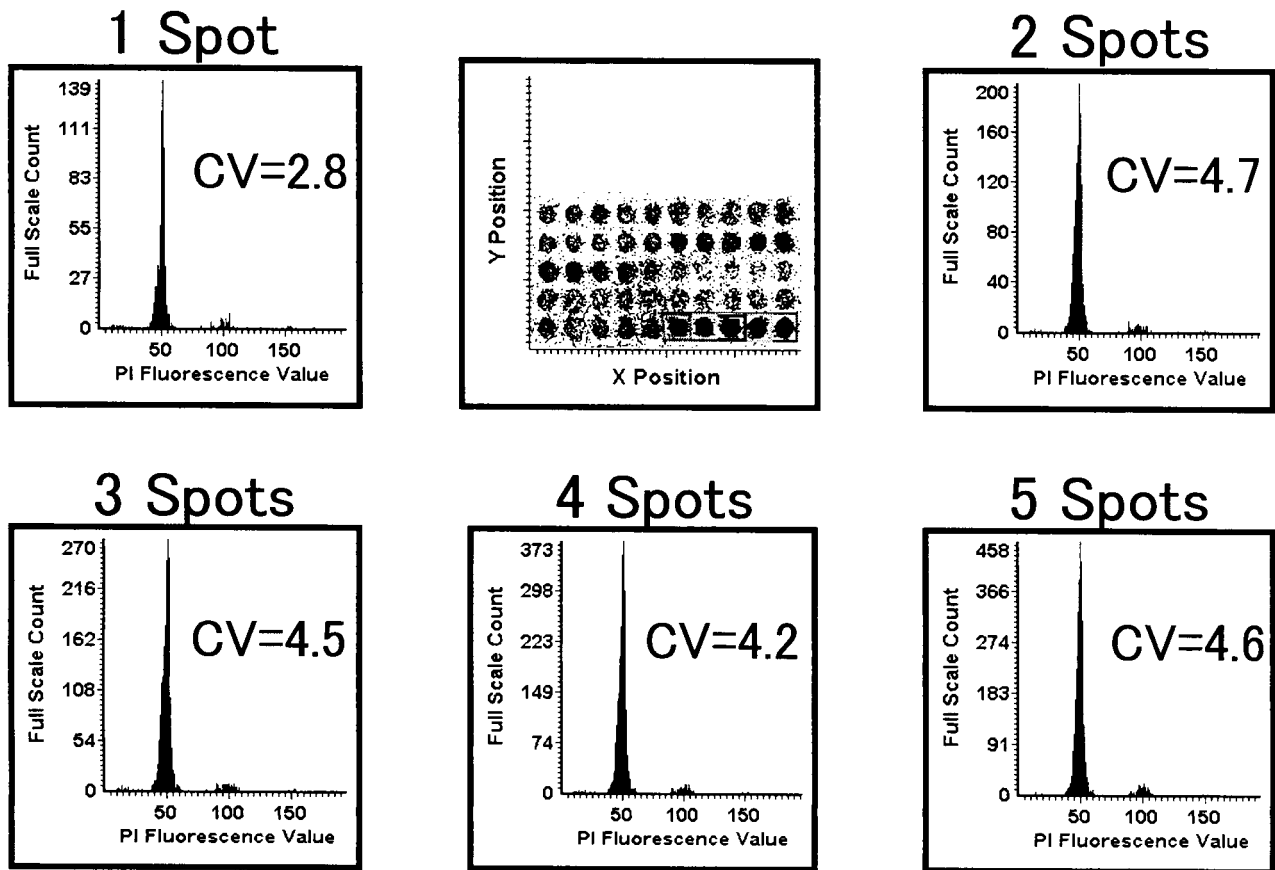


Figure 2. Cytograms and DNA histograms. A LSC cytogram of the X-Y position represents the distribution of both spots on a slide and cells in spots simultaneously (**upper center**). Each tiny dot indicates each cell, and a round shaped cluster of dots corresponds to a spot on a glass slide. DNA histograms of lymphocytes can be produced from each spot (**upper left**) or two or more spots together (two to five spots) by setting a gate window as shown here. A DNA histogram can be constructed from all cells on the slide glass.

spot with an epifluorescence microscope equipped with a 100 \times oil immersion objective (Olympus Co.). The percentage of cells with different signal counts was determined for each spot. The modal number of individual chromosomes represented the number of chromosomes in the tumor cells for each spot and grouped spots.

Results

Cell Array

We were typically able to count \sim 500 cells (range, 350 to 800 per spot) when aggregated cells were excluded from counting. Increasing the cell density in a spot resulted in overlapping cells, which reduced the number of cells that could be counted. Repeated experiments revealed that the optimal cell density was \sim 800 to 1,000 cells per spot.

Measurement of Nuclear DNA Content

Nuclear DNA content was measured by LSC for propidium iodide-stained cells. A total of 35,000 to 40,000 cells was counted for 50 spots in LSC DNA measurements. A DNA histogram for each spot was constructed

by setting a gate window in the cytogram, an X-Y scattergram, that represents the distribution of both spots on the glass slide and cells in each spot (Figure 2). Approximately 1 hour was necessary for scanning the 30 \times 16-mm area where 50 spots were arranged. The number of countable cells was \sim 500 per spot. The DI of the DNA histogram was determined easily without the addition of an internal standard because spots containing normal lymphocytes were included on the same slide. No difference in the position of the G₁/G₀ peak was found among DNA histograms for five spots containing normal lymphocytes (Figure 2). DI was the same for spots containing the same cell line. The positions of the G₁/G₀ peak in DNA histograms from lymphocytes and tumor cells differed distinctly (Figure 3). The DI of each cell line is summarized in Table 1. DI was easily compared among cell lines by delineating a gate window so as to encompass the spots of interest. It was also possible to combine two or more spots into one using the gating procedure, and accordingly spots consisting of the same type of cells grouped to form a single DNA histogram. The increase in the number of spots containing the same type of cells made each DNA histogram distinct (Figure 2). In this study, the coefficient of variation was \sim 5%.

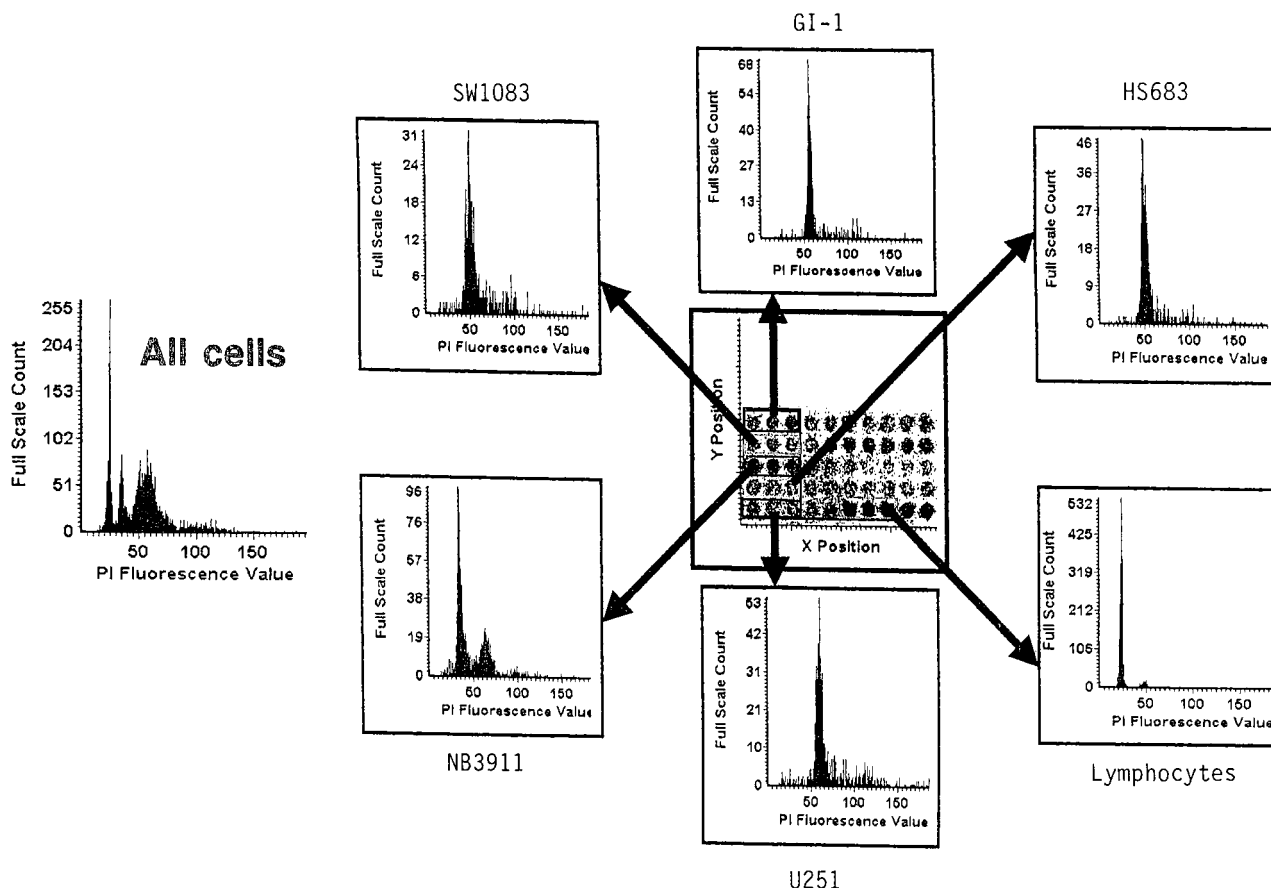


Figure 3. Comparison of DNA ploidy among different cell lines (GI-1, SW1083, NB3911, HS683, and U251) can be presented together as a histogram by using the gating procedure. Multiple peaks illustrate the differences in DNA ploidy among cell lines, and in this histogram, the left peak represents the G₀ peak of lymphocytes (left). A DNA histogram is also obtained from three spots that are defined by a gating window, and DNA index is 2.26, 2.96, 1.38, 2.02, and 2.40 in GI-1, SW1083, NB3911, HS683, and U251, respectively.

FISH

FISH using a DNA probe specific for the chromosome 11 centromere revealed the chromosome copy number in tumor cells and control lymphocytes. On average, 88%

Table 1. DNA Indices Determined by LSC and the Number of FISH Signals in Cell Lines and Lymphocytes

Cell line	DNA index*	No. of signals†
U87MG	1.02	4
U105G	1.36	4
DBTRG65MG	2.10	4
U373MG	1.86	4
GI-1	2.26	3
SW1083	1.96	4
NB3911	1.38	2
HS683	2.02	3
U251	2.40	4
Lymphocyte‡	1.0	2

*DNA index (DI) was calculated using the formula; propidium iodide fluorescence value at the G₁/G₀ peak position of tumor cells/propidium iodide fluorescence value at the G₁/G₀ peak position of normal lymphocytes¹⁶. Accordingly, DIs = 1 and 2 indicate DNA diploidy and DNA tetraploidy, respectively.

†The modal number of signals for chromosome 11 detected by FISH using a chromosome 11-specific DNA probe.

‡Peripheral blood lymphocytes obtained from a normal healthy donor.

(range, 86 to 90%) of lymphocytes had two signals in every spot, and lymphocytes with >3 signals were virtually not detected. In tumor cell lines, the number of signals from chromosome 11 was cell-dependent. The modal chromosome number was 4 with great intercellular variation in most of cell lines (Table 1). There was no significant difference in modal chromosome number among spots of the same cell lines including the lymphocyte controls.

Discussion

From the clinical point of view an efficient method is required for analysis of a large number of samples in a single experiment. The DNA microarray makes it possible to analyze the expression of thousands of genes in a single hybridization,¹⁸⁻²¹ and this technology becomes to be widely used because of its high analytic efficiency. The function of all human genes will be elucidated in the near future when the human genome project is completed. Accordingly, another high-throughput method that allows analysis of phenotype coupled with each gene in a given tissue and cell will be valuable. A tissue microarray which has recently been developed enables analysis of antigen expression in hundreds of tissue

specimens in a single experiment.¹⁻⁴ This is greatly advantageous to histopathological examination.

The cell array device presented here addresses the requirements mentioned above as well as functioning as a tissue microarray. However, our cell array system has advantages and disadvantages when compared to the tissue microarray. The cell array data provides no information about histological structures, which are available from the tissue microarray, but the cell array allows rapid analysis of DNA ploidy in multiple specimens. The cell array also permits reliable interphase cytogenetic analysis using chromosome-specific DNA probes. In contrast, a tissue microarray may lead to erroneous interpretation of chromosomal aberrations because of truncated nuclei in the tissue sections. This could be a serious problem because it is difficult to judge whether intercellular variations in chromosome number are accurate or artifactual. Cell array can compensate the disadvantages of tissue microarray, and *vice versa*.

Usually, more cells yield more reliable cytometric analysis. There is an inverse relationship between the size of the spots and the number of spots on a glass slide. There are fewer samples on a cell array than on a tissue microarray. Hundreds of cells are required for analysis of DNA ploidy and the cell cycle. This is why the cell array device is designed to have only 50 spots, and this is a difference between our cell array and other systems. It may be possible to increase the number of spots per area. For example, an array with 200 spots (spot size, 1-mm diameter; interspot distance, 1 mm) can be prepared. However, this is impractical because the number of cells within a smaller spot may be insufficient for DNA ploidy analysis (data not shown). A 2-mm spot diameter is a compromise between the density of spots and the number of cells for DNA ploidy analysis. In this study, the maximum number of countable cells was 1,000 per spot. However, an increase in cell density in a spot is inevitably accompanied by an increase in the number of overlapping cells, which reduces the number of countable cells. Empirically, the optimum number of applied cells per spot is ~1,000. However, the size of spots depends on analysis. In experiments that require only a small number of cells for analysis, the spot size can be reduced and the number of spots on a glass slide can be increased as mentioned above. As a result, more samples are examined in a single experiment.

The cell array device becomes more powerful when combined with LSC, which is a microscope-based cytometer with diverse analytical capabilities comparable to flow cytometry.¹⁵ In this study, LSC measurement of nuclear DNA content was done for nine cell lines and lymphocytes arrayed on a glass slide. This experiment proved that the combination of the cell array device with LSC is sophisticated and convenient for DNA ploidy analysis in many samples. Furthermore, use of the cell array device allows easy comparison of DNA ploidy among samples without inclusion of an internal standard because all samples stained equally with propidium iodide are on the same glass slide. In fact, no difference in the positions of the G₁/G₀ peaks was seen between DNA histograms generated from spots containing the same

type of cells. This combination method permits reliable comparative studies of cellular characteristics among multiple samples and it is applicable to all samples measurable by LSC, such as for surface markers²² and intracellular substances.¹² Cell array combined with LSC facilitates analysis of biomarkers in malignant tumors.

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