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Comparative DNA Analysis by Image Cytometry and Flow Cytometry in Non-small Cell Lung Cancer

Tatsuo Yamamoto,[1,2,4] Hisashi Horiguchi,[1] Hiroshi Kamma,[1] Masahiro Noro,[1] Takesaburo Ogata,[1] Yoshihisa Inage,[1,2] Eiichi Akaogi,[2] Kiyofumi Mitsui,[2] Motokazu Hori[2] and Masaya Isobe[3]

Departments of [1]Pathology and [2]Thoracic Surgery, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305, and [3]Ratoc System Engineering Co., Kirin First Bldg., Waseda-Tsurumaki-cho 540, Shinjuku-ku, Tokyo 160

To determine whether image cytometry (ICM) is advantageous for clinical DNA analyses of tumor cells, nuclear DNA contents measured by ICM were compared with those by flow cytometry (FCM), using 46 samples of non-small cell lung cancers. ICM was performed on smear specimens of fresh materials (f-ICM) and cell suspensions obtained from paraffin-embedded tumors (p-ICM). The same cell suspensions were also analyzed by FCM (p-FCM). Aneuploid rates/coefficient of variation (CV) of f-ICM, p-ICM, and p-FCM were 76.1/4.90, 71.7/5.01 and 60.9/5.31%, respectively. There was a high correlation in the DNA indices between p-ICM and p-FCM ($r=0.80$). In the comparative DNA analysis, there were seven discordant samples. Six of them were estimated as aneuploid by p-ICM, but they were miscounted as diploid or undefinable (impossible) by p-FCM. This was caused by measuring condensed nuclei or debris. All "impossible" samples in p-FCM were squamous cell carcinoma with necrosis. In cell cycle analysis, the S and S+G2/M phase fractions in diploid samples were higher in p-ICM than those in p-FCM ($P<0.005$), because the G0/G1 phase (2N) fraction presented by FCM was composed of cancer and non-malignant cells in diploid cancers. In ICM, they can be separately measured by means of morphological selection. These findings indicated that ICM is superior to FCM, especially for the practical DNA measurement of a few cancer cells and in the evaluation of the proliferation rates.

Key words: DNA analysis — Cell cycle analysis — Flow cytometry — Image cytometry — Lung cancer

DNA cytometry is a useful method to detect the presence of one or more cell clones as well as to predict their malignant potential.[1-4] DNA cytometry has been applied as an additional means of cytological diagnosis, because of the high frequency of aneuploid cell clones in various human cancers.[5-12] Cell cycle analysis by DNA cytometry also provides information about the proliferative activity of the tumors.[1, 13, 14]

There are two methods of DNA cytometry; image cytometry (ICM) and flow cytometry (FCM). FCM is now more commonly used than ICM, because of its rapidity.[7, 15-21] This advantage of FCM, however, has become less important since computer image analysis was developed. The image analyzer can automatically measure the DNA-dependent nuclear density on smear specimens stained by Feulgen's method.[22] We developed an ICM system, NASCA (nucleic acid speedy color analyser), using a color image analyzer.[23, 24]

The purpose of this study was to define the advantages of this ICM system for the clinical DNA analysis of tumor cells. We compared the accuracy of the DNA measurement in this system with that in the ordinary FCM. The two methods were compared using fresh and paraffin-embedded tissue of non-small cell lung cancer (NSCLC). The advantages of ICM in cell cycle analysis of cancer cells were also examined.

MATERIALS AND METHODS

Forty-six patients with NSCLC who were treated by surgery from August 1986 to August 1991 were accepted for this study. Their profiles are summarized in Table I.

Cell preparation Fresh smear specimens from the cut surface of resected lung tumors were prepared for ICM (f-ICM). Ordinary paraffin-embedded tumors were cut and deparaffinized in a xylene-ethanol series to make cell suspensions for FCM (p-FCM) and ICM (p-ICM).

Samples were prepared for FCM according to the conventional method with some modifications.[25] Briefly, two or three 50-μm sections were cut if it was histologically clear that at least one-fifth of the total area was occupied by cancer cells. Then they were deparaffinized, and rehydrated according to the following sequence: xylene (twice), 100% ethanol (twice), 95% ethanol, 90% ethanol, 80% ethanol, 50% ethanol and phosphate-buffered saline pH=7.4 (PBS)(twice). The solutions

[4] To whom correspondence should be addressed.

Table I. Characteristics of the Patients

Gender	male	34
	female	12
Age	mean±SD	64.1±9.9
	range	40–86
Histologic type	(case no.)	
	adenocarcinoma	22
	squamous cell carcinoma	17
	large cell carcinoma	4
	carcinoid	3
Pathologic stage	(case no.)	
	I	18
	II	9
	IIIA	7
	IIIB	2
	IV	10

were changed by intermediate centrifugation steps. The sections were incubated for 30 min at 37°C in PBS with 0.05% pepsin (Sigma Chemical Co., St. Louis, MO). After 0.2 mg/ml RNase (Sigma Chemical Co.) digestion, the nuclei were stained with propidium iodide.

Smear specimens of fresh tumors and the cell suspensions obtained from paraffin-embedded tumors were made for ICM on poly-L-lysine-coated microscope slides. These specimens were immediately fixed in a cytological fixative (20% formalin, 5% acetic acid and 75% of 95% ethanol) for over 60 min, then stained by Feulgen's method. Briefly, after rinsing for 10 min in deionized water, the slides were immersed for 45 min in 5 *N* hydrochloric acid to hydrolyze the nuclear DNA, then stained for 90 min with acriflavine-Feulgen. Finally, they were dehydrated three times with absolute ethanol, cleared in xylene, and mounted with coverslips.

FCM The nuclei stained by propidium iodide were measured with a flow cytometer (FACScan, Beckton Dickinson, Sunnyvale, CA). The data based on a minimum of 10,000 nuclei per sample were analyzed on a Hewlett Packard personal computer using the data analysis software program (CellFIT, Beckton Dickinson). The DNA content (proportional to the propidium iodide fluorescence) was measured on a linear scale at wavelengths greater than 590 nm. The coefficient of variation (CV) and cell cycle proportion, calculated using CellFIT, were used to determine the accuracy of the DNA measurement and to evaluate the proliferative activity of the tumors.

ICM Nuclear DNA was measured using a NASCA image analyzer system (Ratoc, Tokyo), which is a video-based, interactive image cytometer that uses the summed optical density of each Feulgen-stained nucleus to calculate the amount of DNA based on the Lambert-Beer law.[26] Nuclei were automatically selected on the basis of absorbance level and size criteria. The settings for these criteria could be adapted to eliminate erythrocytes, overlapping nuclei and other artificial materials. Thereafter, tumor or normal cells such as bronchial epithelial cells, lymphocytes, plasma cells, etc. were selected by visual elimination. At first, 50 nuclei of lymphocytes in the samples were measured, and their mean values were estimated as a diploid (2N) control. At the following step, more than 100 nuclei of tumor cells (mean, 480; range 102 to 1090) were measured. The CV and cell cycle values were also obtained using the NASCA system.

Classification of histograms In FCM, the histograms were basically classified as diploid and aneuploid. Histograms with only a single G0/G1 peak, i.e. DNA index (DI) = 1.0, were regarded as diploid. Those with more than one peak were regarded as aneuploid. The DI in aneuploid samples represented the ratio of the mean channel number of the second G0/G1 peak to that of the first G0/G1 peak.

In ICM, the histograms were also classified as diploid or aneuploid according to the DI. The DI represented the ratio of the mean channel number of the tumor G0/G1 peak to that of the control G0/G1 peak of the lymphocytes in the same sample. Samples of DI < 1.25 and DI > 1.25 were regarded as diploid and aneuploid, respectively.

Aneuploid in this study included tetraploid and polyploid.[13] Some histograms could not be appropriately classified because of the absence of distinct peaks. These were considered non-diagnostic, and named "impossible" in both FCM and ICM. Both diploid and aneuploid histograms of f-ICM, p-ICM, and p-FCM are shown in Fig. 1.

Statistics The DNA indices and proliferative fractions measured by each method were compared by use of the paired *t* test. Correlation was analyzed by using Spearman's rank test.

RESULTS

Accuracy of measurements To compare the accuracy of measurements between ICM and FCM, the CV values obtained by f-ICM, p-ICM, and p-FCM were analyzed. These were 4.90±1.75, 5.31±1.86, and 5.06±2.66 in f-ICM, p-ICM, and p-FCM, respectively. There were no significant differences among them, although a few samples with a CV value of 10% were found in p-ICM and p-FCM.

Aneuploid frequency The results of DNA ploidy analysis by f-ICM, p-ICM and p-FCM are summarized in Table II. Aneuploid rates varied from 60.9 to 76.1%, though the samples for each measurement were obtained from the same tumors. There were no significant differences in aneuploid rates. The variance in the frequency was due to the presence of a few low-quality samples, which were

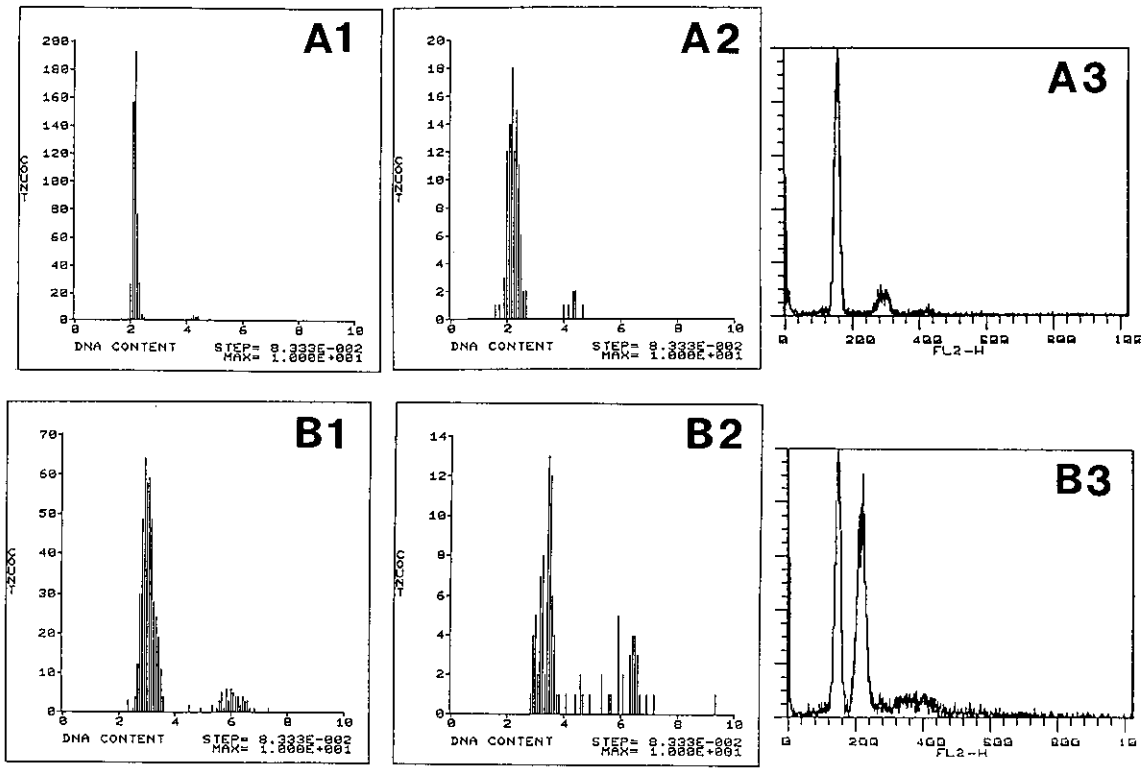

Fig. 1. Representive histograms of diploid (A1–3) and aneuploid (B1–3) samples by f-ICM, p-ICM, and p-FCM. 2N on the X axis of image cytometric histograms (A1–2, B1–2) shows the mean DNA content value of control lymphocytes. A: 40-year-old male, carcinoid, DNA index=1.0; CV=2.4, 6.1, 6.3% (f-ICM, p-ICM, p-FCM), respectively. B: 63-year-old male, well differentiated adenocarcinoma, DNA index=1.52, 1.76, 1.96; CV=4.9, 6.2, 5.8%, respectively.

Table II. A Comparative DNA Analysis by f-ICM, p-ICM and p-FCM in 46 Non-small Cell Lung Cancers

	f-ICM	p-ICM	p-FCM
Diploid	11 (23.9%)	11 (23.9%)	14 (30.4%)
Aneuploid	35 (76.1%)	33 (71.1%)	28 (60.9%)
Impossible	0	2 (4.4%)	4 (8.7%)
Discordant	6 (13.0%)		7 (15.2%)

f-ICM, image cytometry of fresh imprint; p-ICM, image cytometry of paraffin-embedded tissue; p-FCM, flow cytometry of paraffin-embedded tissue.

obtained from paraffin-embedded tumors with variable amounts of nuclear debris. There were no "impossible" samples when the DNA ploidy was estimated by f-ICM.

Discordance analysis in DNA ploidy The DNA ploidy of the tumors was determined in terms of the DI as described above. Fig. 2 shows the correlations of the DI values analyzed by ICM and FCM. There were good correlations between f-ICM and p-ICM and between p-ICM and p-FCM. However, a few samples had apparently different DIs and DNA ploidies (Fig. 2, Table II), so the discordance was analyzed.

1) Material factor analysis: The discordance of DNA ploidy between f-ICM and p-ICM depended on the difference of the type of sample (material factor) measured. The samples could be influenced by not only artefacts of the cell preparation procedures, including cutting, dilution, condensation, and loss of nuclei, but also the sites from which they were obtained. The background of the six discordant samples between f-ICM and p-ICM is shown in Table III. Three of them were aneuploid in f-ICM and diploid in p-ICM, and the others were diploid in f-ICM and aneuploid in p-ICM. These results might depend on the intratumoral heterogeneity in DNA ploidy[27–29] rather than artificial effects, because distinct peaks on the histograms were obtained by means of morphological selection in ICM. The discordant samples were histologically various types of NSCLC, which did not influence the results.

2) Method factor analysis: The discordance between p-ICM and p-FCM depended on the measurement

method (method factor), because the same cell suspension was used for both DNA measurements. The background of DNA ploidy discordance is also shown in Table III. All discordant samples except one were aneuploid in p-ICM. In p-FCM three of them were counted as diploid, and the peak could not be detected in the other three. These samples contained considerable nuclear

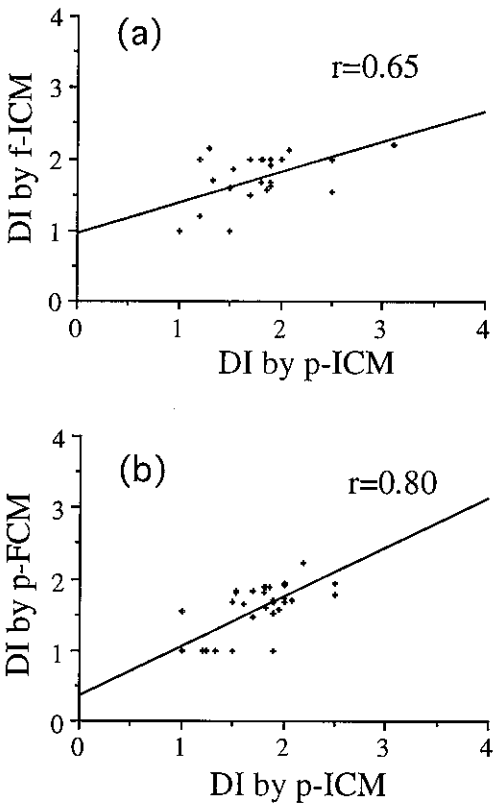

Fig. 2. (a) Correlation of DIs measured by p-ICM and p-FCM. A high correlation was found. (b) Correlation of DIs by f-ICM and p-ICM. The dogree of correlation was lower than the above, suggesting intratumoral heterogeneity in the DNA ploidy.

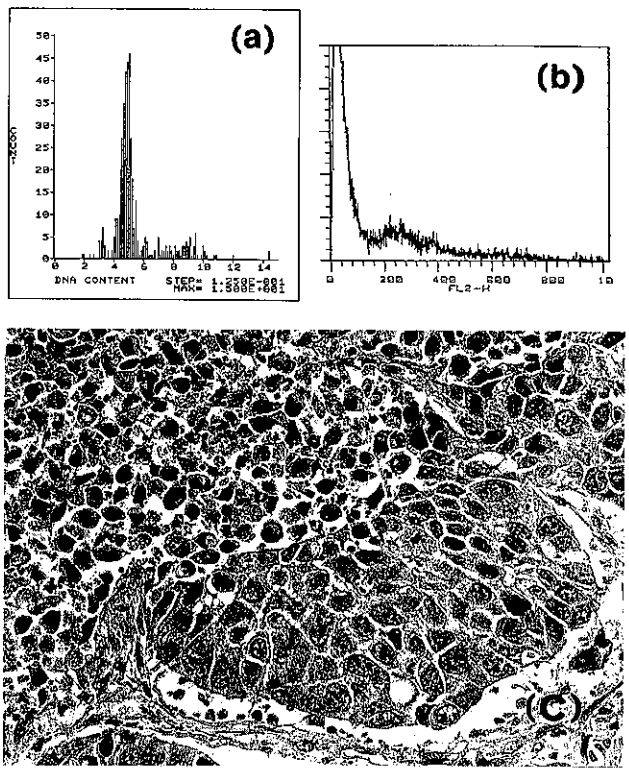

Fig. 3. Representive histograms by ICM and FCM from a patient with squamous cell carcinoma. (a) The ICM histogram shows an aneuploid pattern. DI=1.98, CV=6.5%. (b) Histogram by FCM. There were no significant peaks. It was impossible to determine the DNA ploidy. (c) Histologic appearance of a sample from this patient (68 year-old male). Squamous cell carcinoma with numerous necrotic or keratinizing cells.

Table III. Background of Ploidy Discordance among f-ICM, p-ICM and p-FCM

Case no.	f-ICM	p-ICM	p-FCM	Case no.	f-ICM	p-ICM	p-FCM
1 (SQ)	D	A	A	7 (SQ)	A	A	D
2 (SQ)	D	A	A	8 (SQ)	A	A	D
3 (AD)	D	A	A	9 (AD)	A	A	D
4 (LCC)	A	D	D	10 (SQ)	A	A	I[a]
5 (LCC)	A	D	D	11 (SQ)	A	A	I[a]
6 (AD)	A	D	D	12 (SQ)	A	A	I[a]
				13 (SQ)	D	I[b]	D

Cases no. 1–6; discordance between f-ICM and p-ICM, 7–13; discordance between p-ICM and p-FCM. AD, adenocarcinoma; SQ, squamous cell carcinoma; LCC, large cell carcinoma; A, aneuploid; D, diploid; I, impossible.
a) Undetectable peak.
b) Loss of cells.

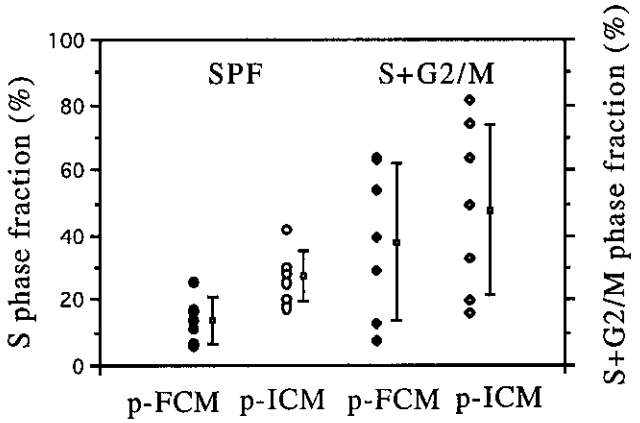

Fig. 4. Comparison of S and S+G2/M phase fractions found by p-ICM and p-FCM. The values of both S and S+G2/M phase fractions by p-FCM were less than those by p-ICM ($P<0.05$).

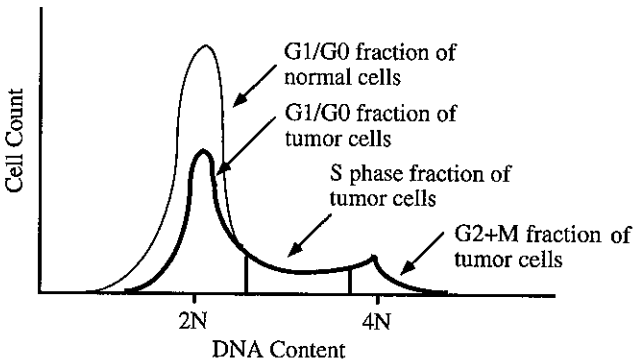

Fig. 5. A schema of the diploid histogram obtained by FCM. The first peak (G0/G1) includes normal and tumor cells. As a result, the fractions of S and S+G2/M phase of the tumor cells are calculated at less than the true values. The lower rates of S and S+G2/M phase fractions measured by FCM appear to be a characteristic weakness of flow cytometric DNA analysis.

debris, which made the DNA histograms equivocal in FCM. All "impossible" samples were obtained from squamous cell carcinoma, in which a large amount of necrosis was histologically detected (Fig. 3). Only ICM detected a distinct peak and allowed determination of the DNA ploidy by morphological selection. There was only one "impossible" sample in p-ICM, resulting from a loss of cells during preparation.

Cell cycle analysis To define further the advantages of ICM over FCM, cell cycle analysis was performed. The S phase and S+G2/M phase fractions practically indicate the proliferative activity of the tumors.[1, 13, 14] Fig. 4 shows these fractions of diploid samples analyzed by p-ICM and p-FCM. Both fractions found in p-ICM were significantly larger than those in p-FCM ($P<0.05$). This was because the G0/G1 phase (2N) presented by FCM was composed of both cancer cells and non-malignant cells in the diploid or near-diploid cancers.

DISCUSSION

The ICM used in this study is an improved method developed by our laboratory and Ratoc Co.[23, 24] The most characteristic feature of ICM is that a color image analyzer allows simultaneous analysis of the optimal density of Feulgen-stained nuclear DNA and the nuclear morphometry in the same specimen.

The accuracy of ICM using NASCA was almost equivalent to that of FCM, as indicated by the CV values. In the present DNA analysis, ICM was especially reliable with fresh materials. When the DNA ploidy of NSCLC was estimated by f-ICM, there were no "impossible" samples. The high correlation of DIs and the similarity of CV values in f-ICM, p-ICM and p-FCM indicated that retrospective study was possible by ICM as well as FCM.[20] Although DNA measurement using FCM is rapid and accurate, image cytometric DNA analysis has become common because very small samples can be analyzed and rare high ploidy cells can be detected. Furthermore, samples can be stored for long periods and the preparation procedures are simple.[30-39]

The discordant DNA ploidy revealed by f-ICM and p-ICM was due to intratumoral heterogeneity, because the samples were taken from different sites of the tumors. The discordance analysis between p-ICM and p-FCM demonstrated the superiority of ICM over FCM because of morphological selection at the step of DNA measurement. The condensation of nuclei or the formation of nuclear debris occurs during formalin-fixation, paraffin-embedding, and preparation of the cell suspension. They were frequently found in squamous cell carcinomas with necrosis. These artificial nuclear components affect the judgement of DNA ploidy especially in FCM, because it is impossible in FCM to separate these components from nuclei. On the contrary, ICM can measure the DNA content only in nuclei by separating them from the artificial components by means of morphological selection. In our ICM system, the observer could visually discriminate cancer cells from normal cells, although the subjectivity of this selection of the cells for measurement by the observer may also be one of the demerits of ICM. Nevertheless, measurement by skillful cytopathologists can afford reliable results.

Other investigators have also proposed that there is a high correlation between ICM and FCM in DNA analysis,[30, 33] and that ICM is more reliable than FCM because

only ICM allows visual elimination.[15, 31, 32, 40] Our comparative studies of the cell cycle revealed the superiority of ICM, especially in diploid samples. FCM cannot separate the G0/G1 fraction of diploid or near-diploid cancer cells from the fraction of non-malignant cells, such as lymphocytes, usually present in the samples. As a result, large numbers of non-malignant cells may mask the G0/G1 peak of diploid or near-diploid aneuploid cancer cells, as shown in Fig. 5. Elsheikh also reported that aneuploid cells were diluted by normal diploid cells in FCM.[41] The proliferative activity, represented by S or S+G2/M phase fraction, is one of the most reliable indicators of biological aggressiveness of cancer cells,[1, 13, 14] but Frierson mentioned that the S phase fraction could not be evaluated in multiploid samples.[42] Our results show for the first time that ICM is a more reliable method than FCM in the evaluation of the cell cycle in diploid samples.

One of the most important points in nuclear DNA analysis is to avoid errors in histogram classification, which may be caused by a blind measurement in FCM.[42-45] For example, one peak in a DNA histogram can mean at least diploid normal cells without aneuploid tumor cells, normal cells with diploid tumor cells, or only diploid or aneuploid tumor cells without normal cells. ICM can avoid such confusion by selectively measuring individual tumor and normal cells.

In conclusion, ICM using the NASCA system is a useful method of DNA analysis, especially for measuring the proliferation rates of tumors.

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