APPLICATION OF CONFOCAL LASER SCANNING MICROSCOPE ON FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

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Fluorescence *in situ* hybridization (FISH) with chromosome-specific probes enables several new areas of cytogenetic investigation by allowing visual determination of the presence and normality of specific genetic sequences in single metaphase or interphase cells. However, it is difficult to obtain the characteristic or three-dimensional information of individual DNA sequence in nuclei using the conventional fluorescent microscope. Confocal Laser Scanning Microscope (CLSM) was applied on the touch preparations of ovarian neoplasia and tissue sections of melanoma, which were hybridized with centromeric or c-erb B 2 (HER-2/neu) oncogene-specific probes. It enabled us to observe clear optical sections in vertical or horizontal directions at any depth from the top to bottom of samples and also to reveal the difference of distribution of specific DNA sequences in nuclei using image-analytical devices.

Copies of chromosome 6 centromeres showed an eccentric localization on optical sections, although those of c-erb B 2 distributed throughout the plans. Furthermore, the reconstructed three-dimensional images revealed that the centromere of chromosome 6 localized close to the nuclear membranes and c-erb B 2 oncogene spread sparsely, or in a dendrometric shape, throughout the nuclei. Since an application of CLSM on the samples which hybridized with DNA sequences specific probes clarified the individual localization of sequences in the nuclei of interphase, this is an especially useful method for biological dosimetry and cancer biology.

Fluorescence *In situ* Hybridization (FISH) with chromosome-specific probes is one of the non-isotopic *in situ* hybridization methods, and enables several new areas of cytogenetic investigation by allowing visual determination of the presence and normality of specific genetic sequences in single metaphase or interphase cells (2, 3). By this approach the genetic loci to be analyzed are made microscopically visible in single cells using *in situ* hybridization with nucleic acid probes specific to these loci. The DNA in target cells is made as a single strand and incubated with single-stranded fluorochrome-directly-labeled, or chemically

modified probe under conditions where the probe will anneal only with DNA sequences to which it has high homology. The bound, chemically modified probe is made visible by immunofluorescent detection methods.

Since nuclei control all of the biological phenomena in cells, much information could be obtained by analysis of intranuclear distribution of DNA sequences, i.e. genes. However, many fluorescent dots were out of focus and unable to be visualized on hybridized samples using a conventional fluorescent microscope. Confocal Laser Scanning Microscope (CLSM) is a device fully utilizing characteristics of the laser, and enables us to observe only the plans in focus. The horizontal and vertical plans of nuclei or cells can be obtained by CLSM without preparation of

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sections, and allows us to understand the internal structures three-dimensionally (1, 4, 6), the same as observing the human body by computed tomography (CT) or magnetic resonance imaging (MRI). Moreover, CLSM can analyze various information by using image-analytical devices. We observed the samples hybridized with centromeric or locus-specific probes by CLSM, and clarified their differences of distributions in nuclei.

MATERIALS AND METHODS

Cells, tissues and their preparation

Primary ovarian neoplasia (serous cystadenocarcinoma and dysgerminoma, histologically) and melanoma tissues were obtained at surgery. Ovarian tumor samples were prepared by touching a freshly cut tissue surface to a microscope slide (touch preparation) and fixed in methanol/acetic acid (3 : 1) for FISH, or fixed in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.2 for immunofluorescence. A small piece of melanoma tissue was fixed in 20% formalin solution and embedded in paraffin in a conventional way, cut 6 μ m in thickness and attached to silan-coated glass slides (7).

DNA probes and probe labeling

A specific DNA probe to a pericentrometric repeat sequence on chromosome 6 was made by polymerase chain reaction (PCR) as described elsewhere (10) with slight modification. About 2,000 of chromosome 6, which were flow-sorted from human X hamster cell line R342-A4, were used as the template. The pericentrometric repeat sequence was amplified and incorporated with digoxigenin using the two oligonucleotides as primers by PCR. Oligonucleotide primers were designed as 5'-GAAGCTTA(A/T)(C/G)T(C/A)ACAGAGTT(G/T)AA-3' and 5'-GCTGCAGATC(A/C)C(A/C)AAG(A/T/C)AGTTTC-3'. They were synthesized using phosphoramidite chemistry on a DNA-synthesizer (Applied Biophysics, model 380B, USA). The buffer containing 5 units of *Thermus aquaticus* (Taq) DNA polymerase (BRL, USA) was mixed in 200 μ l of the buffer (10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM of four deoxynucleoside triphosphates, 1.2 μ M oligonucleotide primers. All chemicals were purchased from Sigma, USA) and processed using a Thermal Cycler (Perkin Elmer Cetus, USA). A first denaturation was performed at 94°C for 180 sec and the following denaturation was done at 94°C for 120 sec, primer annealing at 53°C for 60 sec and extension at 72°C for 120 sec. There was a reaction at a total of 45 PCR cycles. Amplification of alpha satellite DNA was con-

firmed by gel electrophoresis of 10 μ l aliquots of the PCR reactions in 4% agarose (BRL) in 40 mM Tris-acetate, 1 mM EDTA buffer, pH 8.0, containing 0.5 μ g/ml ethidium bromide. Probe DNA was labeled with digoxigenin by resuspending a 4 μ l aliquot of the PCR reaction in 200 μ l of a mixture containing 8 units of Taq DNA polymerase, 0.16 mM digoxigenin-11-dUTP (Boehringer Mannheim) and 0.04 mM dTTP (10). Simultaneous amplification and DNA digoxigenin-labeling were performed during 20 additional PCR cycles with primer annealing at 53°C. Specificity of the PCR products was verified by FISH on lymphocytic metaphase chromosome spreads after the digoxigenin-labeling procedure.

A cK 17.10 cosmid probe (from A. Kallioeinen) hybridizing to the pericentrometric repeat sequence on chromosome 17 was labeled with digoxigenin-11-dUTP by nick-translation.

Two linearized c-erb B 2 (HER-2/neu) DNA probes were purchased from Oncogene Science (New York) and digoxigenin-labeling was performed by random primer labeling.

In situ Hybridization

Cells of serous cystadenocarcinoma tissue were treated with 0.01% pepsin (Boehringer Mannheim)/0.01 N HCl at 37°C for 10 min, dehydrated in an ethanol series and air-dried before thermal denaturation. Cells of dysgerminoma tissue were denatured without proteolytic digestion. Sections of melanoma tissue were dewaxed, rehydrated, digested in 0.5% pepsin/0.1 N HCl at 37°C for 15 min., fixed in 0.1% paraformaldehyde/0.01 M phosphate buffered saline, pH 7.2, followed by preservation in 50% formamide/2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7), and denatured (5). Cells and sections were denatured in 70% formamide/2 $\times$ SSC for 5 min (cells) or 10 min (sections) at 74°C, dehydrated in ethanol and air-dried.

A probe mixture was prepared as follows: digoxigenin-labeled probe (final concentration at 1 μ g/ml) and sonicated salmon sperm DNA (50 μ g/ml; Sigma) for c-erb B 2 probes or CotI DNA (BRL) for centrometric probe were mixed in 55% formamide/10% dextran sulfate/2 $\times$ SSC. The probe mixture was denatured for 5 min at 74°C, placed on ice immediately, and finally applied to denatured cells or tissue sections on glass slides. Hybridization was done overnight at 37°C under a coverslip in a moist chamber.

Immunofluorescent probe detection

The slides were washed twice in 50% formamide/2 $\times$ SSC, twice in 2 $\times$ SSC for 10 min at 45°C. They were washed in 4 $\times$ SSC at room temperature (RT), covered with 1% bovine serum

albumin/4 × SSC for 5 min and incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) for 30 min at RT. After washing with 0.01% CHAPS* (Pierce, USA)/4 × SSC three times for 5 min each, the slides were covered with FITC-anti-sheep IgG antibody (E-Y Lab, USA) for 30 min at RT, followed by three 0.01% CHAPS/4 × SSC washes. Nuclei were counterstained with propidium iodide (PI) in antifade solution (2).

Immunofluorescence for c-erb B 2 product

Touch preparations of the serous cystadenocarcinoma tissue, fixed in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.2, were washed in 0.01 M phosphate buffered saline, pH 7.2 (PBS). Then the cells were covered with 1% bovine serum albumin/PBS and incubated with anti c-erb B 2 oncoprotein (DAKO, USA) for 30 min. After washing in PBS, they were covered with biotinated anti-rabbit IgG (Nitirei, Tokyo) and finally with avidin-FITC (BioMakor, Israel). Nuclei were also counterstained with PI in antifade solution.

Confocal laser scanning microscope and image analysis

Two-channel scanning using a LSM-GB 200 type CLSM (Olympus, Tokyo) with laser light source (argon laser) was carried out at 488 and 514.5 nm of wavelength. Confocal plans were obtained at directions of X-Y, X-Z and X-Y-Z. The sample stage rose and fell to 0.3 or 0.5 micrometer at the acquisition of the confocal X-Y-Z-plans. OZ (Olympus, Tokyo) or VIDAS (Carl Zeiss, Germany) softwares were applied

for three-dimensional reconstruction using confocal X-Y-Z-plans.

RESULTS

All observation was done on interphase nuclei

1. Observation of FISH-samples using a conventional fluorescent microscope

Photographs using a conventional fluorescent microscope were shown in Figs. 1a and 1b. Since centromeric copies of chromosome 6 were relatively large and clear, it was easy to observe and evaluate them by low power view (Fig. 1a). On the other hand, we had to evaluate the copies of c-erb B 2 gene by high power view using a micromovable device because they were smaller and not as bright. Furthermore, many copies were often distributed through a whole nucleus (Fig. 1b).

2. Observation of FISH-samples using a CLSM

a. X-Y-plans

Optical sections with none or many copies of chromosome 6 and 17 specific centromeres, or c-erb B 2, differing in brightness were observed at any depth from the top to bottom of samples in touch preparations and tissue sections. In these sections, each nuclear contour was discriminated, and intranuclear copies were extremely clear (Fig. 2a). Each X-Y-plan revealed three-dimensional information of intranuclear distribution of the DNA sequences. The centromeric copies generally showed eccentric distribution, although those of c-erb B 2 gene spread through-

FIGS. 1a, b. Photographs using a conventional fluorescent microscope. Hybridized with chromosome 6 specific centromeric probe (Fig. 1a) or c-erb B 2 probe (Fig. 1b).

a. Dysgerminoma. Touch preparation. Propidium iodide (PI) staining. ×500 Note six copies in some nuclei. b. Serous cystadenocarcinoma. Touch preparation. PI staining. ×1200 Note many small copies.

* CHAPS; 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate.

out a nucleus. The shapes of copies on each plan were oval, round or dot-like. This variation of shape was prominent especially in samples hybridized with centrometric probes.

The fluorescence became much clearer by accumulation of copies with relatively high density-value of each optical section in one memory, although discrimination of each signal and nucleus was often difficult because of overlapping (Fig. 2b).

The existence of c-erb B 2 oncoprotein in the cellular membrane was demonstrated by the immunofluorescence method with anti-c-erb B 2 oncoprotein antibody in a X-Y-plan (Fig. 2c).

b. X-Z-plans

Samples on glass slides were observed vertically; Round or oval nuclei on X-Y-plans became mound-shape on X-Z-plans. Plans with none or many copies differing in brightness were observed in the touch preparations, similar to those shown on X-Y-plans. Spots in the deepest region were observed in the connecting area of the nucleus on the glass slide (Fig. 2d).

3. Reconstruction of three-dimensional images of FISH samples

Three-dimensional reconstruction of interphase nuclei revealed the localization of specific DNA sequences, i.e., a pericentrometric repeat sequence on chromosome 6 and c-erb B 2 oncogene.

Three-dimensional reconstruction of nuclei hybridized with chromosome 6 specific centrometric probe: Three-dimensionally reconstructed images of

nuclei which hybridized with chromosome 6 specific centrometric probe are shown in Fig. 3a and c. Intranuclear location of copies was visualized through the semi-transparent nuclear margin. The copies shown in yellow seemed to be adjacent, and partly come in contact with the nuclear membrane. Interestingly, each copy varied in shape and size (Fig. 3a).

Although nuclei of melanoma tissue were cut in paraffin sections and would not show the whole contour, copies were also considered to be in contact with nuclear membranes (Fig. 3c).

Three-dimensional reconstruction of nuclei hybridized with c-erb B 2 (HER-2/neu) specific probe: In three-dimensional pictures of FISH samples using c-erb B 2 probe, the copies distributed in a dendriform pattern in the upper part of the nuclei, although they were pressured in the lower portion because of the artifact of preparing touch preparation (Figs. 3e, f). In some nuclei a three-dimensional structure was shown with clear contour lines, and the intranuclear structure was not observed often (Fig. 3f). About one-third of the copies were smaller than the others (Figs. 3e, f).

DISCUSSION

Three-dimensional recognition of the intranuclear distribution of specific DNA sequences was available by CLSM, because CLSM provided serial optical sections of samples in horizontal or vertical

FIGS. 2a-d. Photographs by confocal laser scanning microscopy. The sample stage rose and fell to 0.3 micrometer at the acquisition of the confocal X-Y-Z-plans.

a. A X-Y optical plan of nucleus hybridized with c-erb B 2 probe. Clear copies are shown. Serous cystadenocarcinoma. PI staining. Objective lens $\times 60$ Zoom $\times 2$ b. A photograph of nucleus hybridized with chromosome 17 centrometric probe. Accumulation of copies with relatively high density-value of each optical section in one memory. Some nuclei showed one copy (arrows). Serous cystadenocarcinoma. PI staining. Objective lens $\times 60$ Zoom $\times 4$ c. A photograph of immunofluorescence showing c-erb B 2 protein on cellular membrane. Objective lens $\times 60$ Zoom $\times 2$ d. An optical section at X-Z direction. Hybridized with c-erb B 2 probe. Serous cystadenocarcinoma. PI staining. Objective lens $\times 60$ Zoom $\times 4$

directions against glass slides without preparation of tissue sections (1, 4, 6, 9). Since chromosomal aberration has been almost the main theme of FISH, and intranuclear distribution of chromosomes was rarely studied (4), three-dimensional information was most attractive when we observed FISH-samples by CLSM. The recognition of the internal structure was available by showing the optical sectional area in two planes crossing vertically. However, it was considered that this information could be obtained easily and sensitively by observation of samples on some parallel optical plans provided by CLSM against glass slides.

Furthermore, pseudo-three-dimensional images were obtained by an assembly of these optical serial plans. Samples of ovarian neoplasia and melanoma, which hybridized with the chromosome 6 specific centromeric probe or c-erb B 2 gene probe, were selected for reconstruction of three-dimensional images by OZ and/or VIDAS softs. It was clarified that one or more copies of the chromosome 6 centromere existed extremely adjacent to the nuclear membranes in cases of dysgerminoma (touch preparations) and melanoma (sections). Analysis of locations of chromosomes in nucleus has become easy by CLSM and would offer much genetic information, because intranuclear

FIGS. 3a-f. Reconstruction of three-dimensional images of FISH samples. The sample stage rose and fell to 0.3 micrometer at the acquisition of the confocal X-Y-Z-plans.

a, b. Touch preparation of dysgerminoma tissue, hybridized with chromosome 6 specific centromeric probe. PI staining.

a. Reconstructed by OZ. Intranuclear location of copies was visualized through the semi-transparent nuclear margin (arrows). Objective lens $\times 60$ Zoom $\times 2$ b. An optical plan of the same case. Objective lens $\times 60$ Zoom $\times 1$

c, d. A tissue section of melanoma, hybridized with chromosome 6 centromeric probe. PI staining. Objective lens $\times 60$ Zoom $\times 1$

c. Copies are shown in blue. Nuclei are brown and contour of them are indicated by arrow. Because of enzymic and chemical pretreatments, a boundary of each nucleus became slightly unclear, however, copies were adjacent to the nuclear membrane. Reconstructed by OZ. d. An optical plan of the same case.

e, f. Touch preparation of serous cystadenocarcinoma, hybridized with the c-erb B 2 probe. Reconstructed by OZ (Fig. 3e), and the other nucleus was reconstructed by VIDAS (Fig. 3f). Objective lens $\times 60$ Zoom $\times 2$

e. Copies are shown in blue and are visualized through the semi-transparent nuclear image (yellow). f. Copies are shown in red and the nucleus is seen in different directions.

distribution of chromosomes may closely relate to biological phenomena being engaged in cells. It is also interesting that the shape of copies varied on samples hybridized with chromosome 6 centromeric probe in each tumor nuclei. We did not focus on distribution of chromosomes in normal cells, however, it was suggested that chromosome 1 centromeres also existed close to the nuclear membrane in a normal lymphocytic nucleus (4). It was interesting that hereditary inactive centromeric copies localized close to the nuclear membrane of normal and/or tumor cells in interphase.

It is well-known that c-erb B 2/neu protein is one of the growth factor receptors and exists in cellular membrane (11). Its over-expression suggested that this is a poor prognosis of breast cancer or ovarian cancer (8). Amplification of this gene develops with increasing of copy number (3), and we confirmed that the ovarian cancer nuclei with many copies were detected by FISH using c-erb B 2 DNA probe. Reconstruction of a three-dimensional image revealed that the copies distributed sparsely or in a dendriform, and one-third of them were smaller than the others, although they were packed in the lower parts of nuclei because of the artifact of preparing touch preparation.

Difference of intranuclear distributions of these DNA sequences was clarified by FISH using centromeric and locus-specific probes, and application of CLSM on hybridized samples. It was expected that further findings of distribution of biologically or medically interesting DNA sequences on chromosomes might be shown by multicolor FISH using samples stained with diaminophenyl indole (DAPI) and use of UV laser. It was also expected that the samples less artificially prepared, i.e. free cells (1) or tissue sections in thickness of more than 50 μm , should be used. Application of CLSM on FISH would provide much information concerning the gene, namely, copy number, shape, localization and possible function.

Two different softwares were applied for reconstruction of pseudo-three-dimensional images in this study. The accurate position of subjects was considered to be obtained by the reconstruction of an image using contour lines by VIDAS. However, observation of intranuclear structures was often disturbed by the clear lines. It became easier to evaluate the many intranuclear copies by semi-transparency of the nuclear contour by OZ. The highly resolvable optical sections in the voluntary vertical or horizontal directions of nuclei at any depth from the top to bottom of samples were obtained by CLSM. Therefore, CLSM was considered to be extremely useful for observation

of thick materials, which had not been observed by conventional fluorescent microscope. Moreover, observation of the internal structure of cytoplasm or nucleus and each accumulating fluorescence was easy. Consequently, application of CLSM on FISH depended upon these characteristics. With increase in the number of copies per nucleus, it was difficult to focus on these nuclei at the same time by conventional fluorescent microscope. Therefore, fluorescence was observed by the micromovable device moving up and down, when the copy number increased remarkably, as shown in the result of c-erb B 2. When the increased copies were observed by CLSM, clear optical sections with a highly analytical degree showing characteristics of each copy were obtained. The shapes of copies on each plan of hybridized samples with centromeric probes were oval, round or dot-like, possibly a characteristic for malignant tumors.

We also demonstrated the localization of c-erb B 2 (HER-2/neu) protein on the cellular membrane which was over-expressed in ovarian cancer cells. The protein revealed diffuse or focal distribution on cellular membranes on the cells. CLSM should be frequently used in detection of fluorescence in the cellular membrane as well as the nucleus or cytoplasm.

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REFERENCES

1. Bradl, J., Hausmann, M., Ehemann, V., Komitowski, D. and Cremer, C.: A titling device for three-dimensional microscopy; application to *in situ* imaging of interphase nuclei. *J. Microsc.* 168; 47-57, 1992.
2. Gray, J. W., Kuo, W-L., Lucas, J., Pinkel, D., Weier, H-UI. and Yu, L-C.: Molecular cytogenetics using fluorescence *in situ* hybridization. In "Flow Cytometry and Image Analysis for Clinical Applications" ed. by I. Nishiya, L. S. Cram and J. W. Gray, Excerpta Medica, Amsterdam, New York, Oxford, Elsevier Science Publishers B.V., Amsterdam, 1991, pp. 73-82.
3. Kallioniemi, O. P., Kallioniemi, A., Kurisu, W., Thor, A., Yu, L-C., Smith, H. S., Waldman, F. M., Pinkel, Dan. and Gray, J. W.: ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization. *Proc. Natl. Acad. Sci. USA* 89; 5321-5325, 1992.
4. Kett, P., Geiger, B., Ehemann, V. and Komitowski, D.: Three-dimensional analysis of cell nucleus structures visualized by confocal scanning laser microscopy. *J.*

- Microsc.* 167; 169–179, 1992.
5. Matsuta, M., Matsuta, M., Weier, H.-Ul. and Gray, J. W.: Interphase cytogenetics by fluorescence *in situ* hybridization. *Oncologia* 7; 469–472, 1992 (in Japanese).
 6. Minamikawa, T., Takamatsu, T. and Fujita, S.: Application of laser micrography to *in situ* three-dimensional subcellular morphology. *Acta Histochem. Cytochem.* 24; 55–60, 1991.
 7. Rentrop, M., Knapp, B., Winter, H. and Schweizwer, J.: Aminoalkylsilan-treated glass slides as support for *in situ* hybridization of keratin cDNA to frozen sections under varying fixation and pretreatment conditions. *Histochem. J.* 18; 271–276, 1986.
 8. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. and Press, M. F.: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244; 707–712, 1989.
 9. Van Dekken, H., Van Rotterdam, A., Jonker, R. R., Van der Voort, H. T. M., Brakenhoff, G. J. and Bauman, J. G. J.: Spatial topography of a pericentrometric region (1Q12) in hemopoietic cells studied by *in situ* hybridization and confocal microscopy. *Cytometry* 11; 570–578, 1990.
 10. Weier, H.-Ul., Segreaves, R., Pinkel, D. and Gray, J. W.: Synthesis of Y-chromosome specific, labeled DNA probes by *in vitro* DNA amplification. *J. Histochem. Cytochem.* 38; 421–426, 1990.
 11. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K.: Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* 319; 230–234, 1986.